PhD-FSTM-2021-070
The Faculty of Sciences, Technology and Medicine

DISSERTATION

Defence held on 09/09/2021 in Luxembourg

to obtain the degree of

DOCTEUR DE L'UNIVERSITÉ DU LUXEMBOURG

EN BIOLOGIE

by

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TUMOUR IMMUNE EVASION IS PROMOTED BY ACTIN CYTOSKELETON-DRIVEN POLARIZATION OF INHIBITORY SIGNALS TO THE IMMUNOLOGICAL SYNAPSE

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Affidavit

I hereby confirm that the PhD thesis entitled “Tumour immune evasion is promoted by actin cytoskeleton-driven polarization of inhibitory signals to the immunological synapse” has been written independently and without any other sources than cited. All necessary ethical approvals have been obtained in accordance with the law on the use of clinical samples and on the Care and Use of laboratory animals.

Luxembourg, 06.08.2021

X

Hannah Wurzer

Acknowledgments

Writing these acknowledgements feels almost impossible – I don´t know where to begin in thanking the many people I have come to realise it takes to finish a PhD thesis. I will start by fervently apologising to anyone I have missed! It turns out that despite having scripted my thesis acknowledgments in a million daydreams, when it actually comes to writing them it is hard to find the words that will come close to expressing my gratitude.

I would like to express my gratitude to all the members of my jury, Prof. Michel Mittelbronn, Dr. Lotte Wieten, Dr. Emily Mace, and Dr. Fulvia Vascotto for accepting to evaluate my thesis. Thank you for your precious time and effort and I am looking forward to an exciting discussion about science and the actin response. I would also like to thank Dr. Elisabeth Letellier and Dr. Jacques Zimmer for being members of my "Comité d'encadrement de these" and their thoughtful comments and suggestions during the past years.

My thesis work would not have been possible without the funding from the "Fond National de la Recherche" PRIDE program and the financial support from Think Pink Luxembourg. Being part of the CANBIO doctoral research and training program was a great opportunity and I would like to thank Dr. Simone Niclou for providing me with the chance to start my PhD journey in her department, and thank you, Malou, for supporting and helping all PhD candidates.

I will start my acknowledgments by thanking my incredible and compassionate supervisor, Clément Thomas. You saw potential in my sometimes crazy and often idealistic ideas, you gave me a vision and the tools to transform my ideas. You kept me on track when I wanted to rush the project or got lost in detail and in working together, we were able to build this amazing research project. It is not an exaggeration to say that you helped transform my life as well, because doing a PhD is never an easy thing but I am incredibly lucky to have you helping me through the ups and downs. I am proud of what we did, and I wish you and your family all the best.

I am also grateful to my wonderful team, the Cytoskeleton and Cancer Progression group – or Les Fleurs – at the Luxembourg Institute of Health. I am so privileged to work with such dedicated people who are so passionate about research and who allowed themselves to be infected by my "madness for research". Thank you, Céline, for your calm demeanour, your dedication and patience (that I am sometimes lacking) and for your impressive and beautiful pictures. Without your help this thesis would only be half as exciting to look at as it is now. Merci, Flora, for your never-ending dedication, for guiding and helping me in the lab, for sharing your knowledge with me and of course for the countless hours of virus work. I know we both hate this tedious work, but that did not stop you from trying again and again until we finally had our working models ready. Takouhie, thank you for not only being a colleague, but also a friend, a good listener, and being the role model of an integer and motivated researcher. Giving up is no option for us! Thank you, Liza, for supporting my project and being on-board with starting new and exciting projects. I learned a lot from you, and I admire you for your focus. motivation and success-promising commitment. Hopefully I will be as cool as you one day. Of course, great thanks to Odile, who shared my need for organization and is the greatest help in the lab. You devoted yourself to your work, helped wherever and whenever

it was needed, and I know that without you it would have been so much harder to get to this point of my journey. And now to my successors as PhD candidates, Andrea, Elena, and Max. Thank you all for your help in the past year(s). Thank you for following my down the rabbit hole of actin response research and sharing your ideas with me. You have been great colleagues and friends and I feel so lucky to have such talented people championing my research. You all have a bright future ahead of you and I am excited to see where your ideas are taking you. Good luck to you on all your endeavours. I also want to thank all the past members of this team, Tony, Josh, and Jérôme. I enjoyed working with you and learning from you.

One very big shout out to Julia "Jules" Michalska who has been with me since Stockholm and now from across the Alps for every step of my PhD journey. I would not have been able to pull through with the past four years without your support. Whenever I was struggling, you were there for me, our vacations helped me re-focus, slow down and make me question other life decisions that put me in the middle of the Zamser Hölle, and no one understands better than you that doing nothing during our days off is relaxing. You taught me that it´s okay to fall, even if it´s just half a meter down the wall. You just also have to get up again, look sicking and make them eat it. You´re the best and I hope I can one day repay you for everything. Rockin', rockin' and rollin', girl.

Ernesto, thank you for your (emotional) support and our gossip/lunch/coffee meetings. They were the highlights of some of my weeks. Soon you will also get your level up and hit that sweet ASI to finally get a positive charisma score. And wherever your future takes you, we will meet again Friday evening in Barovia.

Susanne, thank you for every single funny moment we spent together, the car ride discussions, your understanding, committing to jogging with me (I still don´t like it though), and just for being a friend. This PhD would have been so much more difficult without you.

Carina, my favourite Austrian! Thank you for our office door discussions, your honest opinions and steadfast support. You care (and rock ;-)) and I am so happy to have met you. Keep it up!

Thank you, Lia, Yolanda, Mohamad, Giulia, Iria, Malina, Rédouane and all the other PhD candidates, for all the support you gave me, for listening, for spending weekends in the lab together, for sharing the opportunity to unburden our hearts, and for the fun during our retreats. I will miss you.

Maira, I cannot thank you enough for your support with our diva. We both know she´s a sometimes moody lady. Thank you for your trust and your help with my project, and I am so proud of you for pulling off that MBA!

To my close friends, thank you from the bottom of my heart. Jenny, Jan, Izzy, Gianna, Marton, Phil, Klaudi, Marina, and Leigh, you have believed in me and supported me for years. I have learned so much from you all and am so glad to have you in my life. Thank you for the hope, support, and fun. I finally have time for you all again!

My gratitude also goes out to my other colleagues at LIH. It was a pleasure to get to know you and work with you.

Finally, I want to thank my lovely family for their support through the ups and downs, for their encouragement and support even though they do not really understand what I am doing in the lab. Danke, Papa und Mama, ich weiß, dass die letzten Jahre schwierig waren, aber ich konnte mich immer auf eure Unterstützung verlassen und ich wäre heute nicht hier ohne eure Liebe und Hilfe. Ich habe euch sicher manchmal Sorgen bereitet, aber ihr habt immer an mich und meinen Sturkopf geglaubt und ich hoffe ich konnte euch stolz machen und eure Erwartungen erfüllen. Lukas, Felix, und Anel, danke dass ihr die besten Brüder und die allerbeste Schwägerin seid. Eure Ermutigungen, Unterstützung, Motivations-Kalender, Telefonate und denkwürdigen Grimassen haben wirklich geholfen und ich bin euch so unendlich dankbar. Andrea, Claudia, und Ursula, ohne euch und eure stete Unterstützung wäre ich nicht so weit gekommen. Lieber Päter, danke dass du immer für mich da warst, mir ein offenes Ohr geschenkt hast und versucht hast mein kryptisches Wissenschaftler-Deutsch zu entschlüsseln und Oma zu erklären. Oma, danke dass du immer für mich da warst. Du musst inzwischen zahllose Kerzen für mich angezündet haben, aber Opa scheint beim Herrgott ein gutes Wort für mich eingelegt zu haben, denn ich habe es endlich geschafft!

"The question is not what you look at, but what you see."

Henry David Thoreau

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List of abbreviations

ACT	Adoptive cell transfer
ABP	Actin binding protein
ADCC	Antibody dependent cell cytotoxicity
ADF	Actin depolymerizing factor
ALL	Acute lymphoblastic leukaemia
Arp2/3 complex	Actin Related Protein 2/3 complex
AR	Actin response
ATP	Adenosine triphosphate
B2M	Beta-2 microglobulin
Bak	Bcl-2 homologous antagonist/killer
Bat3	HLA-B-associated transcript 3
Bax	Bcl-2 associated X protein
Bid	BH3 interacting-domain death agonist
BiTEs	Bispecific T cell engagers
CAR	Chimeric antigen receptor
Cc	Critical concentration
CCD	Charge-coupled device
CCL3/4	Chemokine ligand 3/4
CDC	Complement-dependent cytotoxicity
CDC42	Cell division control protein 42 homolog
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
CH	Calponin homology
CLL	Chronic lymphocytic leukaemia
COX	Cyclooxygenase
CR	Complete remission
CRC	Colorectal cancer
CRR	Complete response rate
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAD	Diaphanous auto-inhibitory domain
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DISC	death-inducing signalling complex
DNA	Deoxyribonucleic acid
DNAM-1	DNAX Accessory Molecule-1
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-regulated kinase
ERM	Ezrin/radixin/moesin
F-actin	Filamentous actin
FADD	Fas-associated protein with death domain
FAK	Focal adhesion kinase

FasL	Fas ligand
FH1	Formin homology 1
GAP	GTPase activating protein
GBD	GTPase binding domain
GDI	Guanosine dissociation inhibitor
GEF	GTPase exchange factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft-versus-host-disease
HBV	Hepatitis B virus
HER2/neu	Receptor tyrosine-protein kinase erbB-2
HIF	Hypoxia inducible factor
HIV	Human immunodeficiency virus
HL	Hodgkin lymphoma
HLA	Human leukocyte antigen
HMGB-1	High-mobility group protein 1
HPV	Human papillomavirus
IC	Immune checkpoint
ICAM-1	Intercellular adhesion molecule 1
ICB	Immune checkpoint blockade
iIC	Inhibitory immune checkpoint inhibitor
ICOS	Inducible T cell co-stimulator
IDO	Idoleamine 2,3-dioxygenase
IFC	Imaging Flow Cytometry
IFN-γ	Interferon gamma
IL	Interleukin
ILK	Integrin-linked kinase
ILV	Intraluminal vesicles
IQGAP	IQ motif containing GTPase activating protein
IS	Immune/Immunological synapse
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
KIR	Killer-cell immunoglobulin-like receptor
Kyn	Kynurenine
LAG-3	Lymphocyte activation gene 3
LAMP-1/2	Lysosomal-associated membrane protein-1/-2
LAT	Linker for activation of T cells
LFA-1	Leukocyte function associate antigen-1
LYST	Lysosomal trafficking regulator gene
mAb	monoclonal antibody
MCT	Monocarboxylate transporter
mDia1	Diaphanous-related formin-1
MDSC	Myeloid derived suppressor cells
MEK	Mitogen-activated protein kinase kinase
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
MIC-A/-B	MHC class I polypeptide–related sequence A/B
MRM	Membrane repair mechanism
MTOC	Microtubule organizing centre, centrosome

NCR	Natural cytotoxicity receptor
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NK cell	Natural killer cell
NKG2A	Natural killer group 2A
NKG2D	Natural killer group 2D
NO	Nitric oxide
NPF	Nucleation promoting factor
NSCLC	Non-small cell lung cancer
N-WASp	Neural Wiskott-Aldrich syndrome protein
ORR	Overall response rate
PAK	p21-activated kinase
PAP	Prostatic acid phosphatase
PD-1	Programmed cell death protein 1
PD-L1	Programmed death ligand 1
PI3K	Phosphatidylinositol 3-kinase
PID	Primary immunodeficiency disorder
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP5K	Phosphatidylinositol-4-phosphate 5-kinase
PKCθ	Protein kinase C-theta
PLC γ	Phospholipase C γ
pMHC	peptide-loaded MHC-I complex
PTK	Protein tyrosine kinase
Rac1	Ras-related C3 botulinum toxin substrate 1
Raf	Rapidly accelerated fibrosarcoma
Rag2	Recombination activating gene 2
RCC	Renal cell carcinoma
RhoA	Ras homolog family member A
ROCK1	Rho-associated, coiled-coil containing protein kinase 1
ROS	Reactive oxygen species
SAP	SLAM-associated protein
SCAR	Suppressor of cAMP activator
SHP-1/SHP-2	Src homology 2-containing protein tyrosine phosphatase 1 and 2
SLAM	Signalling lymphocyte activation molecule
SMAC	Supramolecular activation cluster
SRF	Serum response factor
STAT3	Signal transducer and activator of transcription 3
TAM	Tumour associated macrophages
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TDI	Time-delay integration
tdLN	Tumour draining lymph node
TGF-β	Transforming growth factor beta
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIL	Tumour-infiltrating lymphocyte
Tim-3	T cell immunoglobulin and mucin-domain containing-3
TIRF	Total Internal Reflection Fluorescence
TMB	Tumour mutational burden
TME	Tumour microenvironment
TNBC	Triple negative breast cancer

TNF-α	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing ligand
TReg	Regulatory T cell
ULBP	UL-16 binding protein
US FDA	United States Food and Drug Administration
Vav1	Vav guanine nucleotide exchange factor 1
VCA domain	Verprolin, central, acidic domain
VCAM-1	Vascular cell adhesion protein 1
VEGF-A	Vascular endothelial growth factor
WASH	WASp and SCAR homologue
WASp	Wiskott-Aldrich syndrome protein
WAVE	WASp-family verprolin-homologous protein
WH2	Wiskott-Aldrich syndrome homology region 2
XLP	X-linked lymphoproliferative disease

List of publications

I. Al Absi A, **Wurzer H**, Guerin C, Hoffmann C, Moreau F, Mao X, Brown-Clay J, Petrolli R, Pou Casellas C, Dieterle M, Thiery JP, Chouaib S, Berchem G, Janji B, Thomas C. Actin cytoskeleton remodeling drives breast cancer escape from natural killer-mediated cytotoxicity. *Cancer Research.* 2018;78 (19):5631-5643
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V. **Wurzer H**, Krecke M, Filali L, Mgrditchian T, Hoffmann C, Ockfen E, Biolato AM, Kleine-Borgmann F, Girardello R, Perez Hernandez D, Dittmar G, Mittelbronn M, Thomas C. Actin cytoskeleton-dependent polarization of immune checkpoint ligands correlates with suppression of cytotoxic NK cell functions and tumor immune evasion *in vitro* and *in vivo*. *Manuscript in preparation*

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II. **Wurzer H**, Al Absi A, Hoffmann C, Thomas C. Do tumor cells escape from natural killer cell cytotoxicity by mimicking dendritic cells? *Oncotarget*. 2019;10(25):2419-2420
III. Biolato AM, Filali L, **Wurzer H**, Hoffmann C, Gargiulo E, Valitutti S, Thomas C. Actin remodeling and vesicular trafficking at the tumor cell side of the immunological synapse direct evasion from cytotoxic lymphocytes. *International review of cell and molecular biology*. 2020;356:99-130
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Thesis summary

Natural killer (NK) cells are innate immune cells that are the first line of defence against infection and malignant transformation. They have the ability to recognize and destroy virally infected or cancerous cells without the need for priming or activation and therefore represent a promising target for new immunotherapeutic approaches against cancer. For their anti-tumour function, NK cells rely on actin cytoskeleton remodelling, in particular during the formation of the lytic immunological synapse (IS) with prospective target cells. The IS is characterized by an extensive assembly of filamentous actin (F-actin) and polarization of the NK cell for directed delivery of lytic granules. However, the IS allows bi-directional exchange of information, and anti-tumour effector functions of NK cells are often impaired through inhibitory signals that are transmitted through killer immunoglobulin-like receptors (KIRs) or the CD94/NK group 2 member A (NKG2A) heterodimeric receptor. Moreover, resistant tumour cells can polarize their own actin cytoskeleton to the IS in a process called actin response, enforcing the formation of an evasion IS or actin response-IS (AR-IS).

This PhD thesis focuses on the evaluation of associated resistance mechanisms that occur at the AR-IS, the conservation of these processes and their translation to *in vivo* models of cancer. For this purpose, individual cancer-NK cell conjugates were analysed by high-throughput imaging flow cytometry (IFC) to investigate the accumulation of F-actin at the IS and the distribution of inhibitory and activating ligands in relation to the IS. The AR was associated with a wider synaptic cleft, prominent recruitment of predominantly inhibitory ligands and inhibition of NK cell-induced target cell death. Further, the AR could also be applied to cytotoxic T lymphocyte (CTL) attack during antigen specific IS formation. To explore the *in vivo* relevance of the AR, modified murine cancer cell lines were established that allowed for the evaluation of impact of the AR on tumour progression, the tumour immune landscape, immune cell activation and exhaustion. Reduction of the actin response was associated with a reduction of tumour volume, enhanced infiltration of CTLs and NK cells and higher numbers of effector T cells.

In summary, this study reports a novel, highly conserved immune-escape mechanism that exploits fast remodelling of the actin cytoskeleton of cancer cells to induce clustering of inhibitory ligands at the AR-IS and prevent NK cell activation. This AR is characterized by fine synaptic filopodia-like protrusions (SFPs) that are decorated with inhibitory ligands and probe the surface of the NK cell in addition to providing a steric hindrance for NK cell attachment to target cells. In our pre-clinical mouse model, we could demonstrate that a reduction of the AR results in the restoration of anti-tumour immunity. Understanding the mechanism that enables or initiates the AR and finding new ways to target this mechanism has the potential to improve cancer immunotherapy, especially for NK cell-based approaches.

Introduction

I. Tumour immunology

Based on his "side-chain" theory from 1897[1,2] and Emil Fischer´s lock-and-key hypothesis from 1894[3], Paul Ehrlich proposed the first receptor ligand concept in 1901[4,5]. His idea of antibody-antigen interactions and the concept of humoral immunity were based on the hypothesis that an antitoxin (antibodies) would bind to cellular receptors (antigens) "as lock and key"[6]. Paul Ehrlich's basic assumption of receptor-controlled immune reactions is still valid today - despite some improvements - and is one of the cornerstones of modern immunology. In 1909, Paul Ehrlich suggested that the immune system must also play a role in the control against malignant cells and tumours that would otherwise occur with great frequency[7], starting a century of controversy over immunologic control of malignancies[8]. Almost five decades later Lewis Thomas and Frank Macfarlane Burnet published their hypothesis of **cancer immunosurveillance**[9–11], a concept that was based on immune tolerance and the idea that tumour neo-antigens must result in the elimination of malignant cells, similar to homograft rejection observed in mice[12].

Deficiencies in the assembly of a functional immune response convey increased susceptibility to bacterial and viral infections but are also associated with a predisposition to the development of cancer. Primary immunodeficiencies (PIDs) are a heterogeneous group of genetic disorders that are commonly characterized by poor or missing humoral and/or cell-mediated immunity in the absence of a secondary cause such as medical treatment, malnutrition, or another disease (e.g., acquired immunodeficiency syndrome (AIDS) resulting from untreated human immunodeficiency virus (HIV) infection). In patients with severe PIDs, malignancies are the second-most common cause of death after infections. First indications for an increased predisposition for malignancies in PID patients date back to 1959[13–15] and support Burnet`s prediction of an increased risk of cancer development in the absence of immunosurveillance.

The cells of the immune system continuously check the body's tissues. In humans, immune effector cells are natural killer (NK) cells and cytotoxic T lymphocytes (CTLs). NK cells sense stress-associated molecules on damaged or cancerous cells. Dendritic cells (DC) activate CTLs which can recognize tumour-associated antigens, using their T cell receptor (TCR) and other receptors. Once activated, NK cells and CTLs form a tight connection with the (pre)malignant cell in the form of an immunological synapse (IS). In this confined space they release lytic molecules such as perforin and granzymes. These molecules perforate the membrane of tumour cells and induce apoptosis. As the tumour evolves, genetic changes occur that can give some tumour cells a survival advantage. This results in morphological and phenotypic differences between tumour cells and a heterogeneous mix of distinct clonal tumour subpopulations within the tumour mass. For instance, tumour cells can reduce or lose the expression of exactly those molecules that are recognized by the killer immune cells. As the immune system continuous to kill the tumour cells it can recognize, the cells it cannot sense become more prevalent. This process is called **cancer immunoediting**[16]. It leads to the emergence of a tumour that cannot be detected by the immune system.

With the understanding that the immune system plays a very complex role in its interaction with a tumour, the following chapters will discuss the "Three E´s" of cancer immunoediting, **elimination**, **equilibrium**, and **escape**[17], associated immune evasion strategies[18] and immune escape strategies that occur primarily at the immunological synapse in more detail.

1. Elimination (Cancer immunosurveillance)

The elimination phase is the first part of the cancer immunoediting process and is characterized by the successful immunosurveillance of cancer cells. During this phase, a complex interplay between innate and adaptive immune cells ensures that pre-malignant and malignant cells are recognized and destroyed before developing into clinically apparent tumours. During oncogenesis, several innate immune effector cell populations (NK cells, NK-T cells, and γδ T cells), but also DCs and macrophages (Mϕ) recognize and destroy transformed cells[19]. NK cells are the main inflammatory anti-tumour lymphocytes of the innate immune system and gained their name by their spontaneous activation upon detection of triggering ligands on malignant cells. This first line of defence is targeting cancer cells, thereby promoting the release of tumour neo-antigens that are taken up and processed by innate dendritic cells (iDCs) for cross-presentation to initiate an adaptive immune response in the frame of the "Cancer-Immunity Cycle"[20]. Mature DCs migrate from the tumour bed to the tumour draining lymph nodes (tdLNs) where they proceed to cross-present captured tumour antigens to naive $CD4^+$ and $CD8^+$ T cells for priming and activation. This initiates the tumour-specific adaptive immune response through activated $CD8^+$ CTLs that infiltrate the tumour site where they engage in cancer cell killing.

1.1 NK cell immunosurveillance

Many genetic and environmental factors play a role in the formation of a tumour. Chronic damage to cells by extrinsic factors such as ultraviolet (UV) radiation or chemical carcinogens leads to their malignant transformation and uncontrolled cell division. As a precaution against malignancies, stressed and/or damaged cells upregulate stress-associated germ-line encoded ligands during early cellular transformation. These ligands can be recognized by NK cells which express a repertoire of germ-line encoded receptors that sense ligands associated with DNA damage, an altered cell cycle or malignant transformation. NK cell function is controlled by strong inhibitory receptors, such as killer-cell immunoglobulin-like receptors (KIRs) and CD94/NKG2A that bind to classical major histocompatibility complex class I (MHC-I) or non-classical HLA class I histocompatibility antigen, alpha chain E (HLA-E), respectively. MHC-I are expressed on all nucleated cells, thus preventing NK cells from attacking healthy cells through recognition of "self".

Phenotypically and functionally, NK cells can be divided into two major subpopulations based on the relative expression of CD56. $CD56^{dim}$ NK cells represent the majority of peripheral NK cells and show high cytotoxicity, supported by expression of the type III Fcγ receptor CD16 that mediates antibody-dependent cellular cytotoxicity (ADCC)[21]. The second population are immunomodulatory $CD56^{bright}$ NK cells that show low expression of CD16, KIRs and the effector molecules perforin and granzymes. While their cytotoxic potential is relatively low, peripheral $CD56^{bright}CD16^{dim/-}$ NK cells represents potent cytokine and chemokine producers and are the predominant subset of NK cells in the lymph node (LN)[22]. Some authors consider the peripheral $CD56^{bright}CD16^{dim/-}$ subpopulation to be immature precursors, however they could play an important role in the crosstalk with DCs and both, DC and NK cell priming[23]. Generally, $CD56^{dim}CD16^{bright}$ NK cells are considered to be the most effective anti-tumour innate lymphocytes due to their quality to spontaneously detect and destroy stressed or (pre-) malignant cells.

Historically, scientists Kärre and Ljunggren first proposed a "missing-self" hypothesis for NK cell activity regulation[24]. This was based on observations with $MHC\text{-}I^{null}$ lymphoma cell lines that showed a high susceptibility to NK cell-mediated cytotoxicity while the original $MHC\text{-}I^{pos}$ cancer cell

line was resistant. This was attributed to newly described MHC class I specific inhibitory receptors in humans[25] and mice[26]. The "missing-self" hypothesis initially predicted that NK cells initiate target cell lysis in the absence of MHC class I inhibition. Following studies however demonstrated that NK cells also require an activating signal. This activating signal can be provided by cellular ligands or by viral or stress-induced proteins that can be recognized by an array of germ-line encoded receptors and co-receptors. The antigen-independent direct cytotoxicity of NK cells towards cancer cells relies on different recognition and killing pathways that will be described in the upcoming chapters. Engagement of adhesion receptors and activating receptors with their corresponding ligands induces the formation of a lytic immunological synapse and a cytotoxic response because of "altered-self" recognition. This initiates a cancer-NK cell immunity cycle that results in destruction of early transformed or stressed cells expressing a high NK cell activating-to-inhibitory ligand ratio that clearly marks them different from healthy cells. In the presence of both inhibiting and activating signals the outcome is decided by quantitative differences in signal strength between the two. Through differences in signal strength of activating and inhibitory receptors, "self" tolerance of NK cells is ensured.

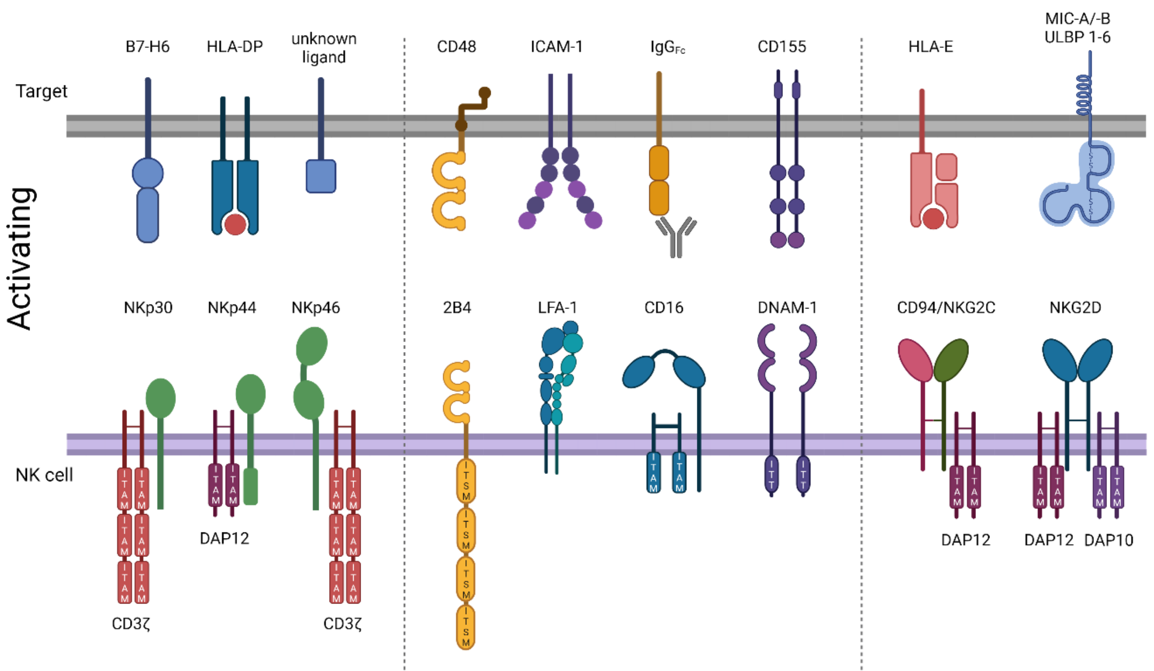
1.1.1 NK cell recognition of tumour cells

As the main effector cells of the innate immune system, NK cells contribute to tumour immunosurveillance by rapid killing of tumour cells, but they also display secretory functions. These include the production of chemokines (CCL3, CCL4) as well as pro-inflammatory cytokines (e.g., IFN-γ and TNF-α), that further shape the innate and adaptive anti-tumour response.

The induction of NK cell cytotoxic functions requires strong activating signals by both adhesion receptors such as leukocyte function associate antigen-1 (LFA-1) and activating receptors, including NKG2D, 2B4 and natural cytotoxicity receptors (NCRs) such as NKp30, NKp44, NKp46, and NKp80[27]. Activating and inhibitory receptors can signal through conserved cytoplasmic domains carrying either immunoreceptor tyrosine-based inhibitory motifs (ITIM), immunoreceptor tyrosine-based switch motifs (ITSMs), or immunoreceptor tyrosine-based activation motifs (ITAM). These motifs are either part of their own cytoplasmic domain (e.g. 2B4) or can be found in the intracellular domain of adaptor proteins[28]. An example for signalling through adaptor proteins are NCRs. These receptors do not signal through ITAMs in their cytoplasmic domain, but rather through association of a positively charged residue in their hydrophobic transmembrane domain (TM) with a corresponding negatively charged residue in the TMs of ITAM adaptors. These adaptors are CD3ζ or the Fc ε receptor subunit γ (FcεRIγ), in the case of NKp46, and NKp30 or DAP12 for NKp44[29] (Fig. 1.1).

KIRs generate very strong inhibitory signals to suppress NK cell activation and induce "self" recognition in response to high affinity-binding to classical MHC-I molecules that include the human leukocyte antigens A (HLA-A), HLA-B, and HLA-C. The inhibition of NK cell activation is mediated through signals transduced by their cytoplasmic ITIMs that recruit the Src homology 2-containing protein tyrosine phosphatase 1 and 2 (SHP-1, SHP-2) upon phosphorylation[30–32]. Interestingly, activating KIRs, that can promote NK cell activation upon interaction with HLA-C, also exist[33]. These KIRs cannot signal through motifs in their cytoplasmic tail but recruit ITAM-carrying signal transduction proteins such as DAP12[34,35]. The KIR repertoire shows a high degree of variety, that is summarized in 2 haplotypes termed as "A" and "B"[36]. The "A" haplotype contains the genes of the main inhibitory KIRs (iKIR) for recognition of HLA-A (KIR3DL2), HLA-B (KIR3DL1), HLA-C (KIR2DL1 and KIR2DL3), and HLA-G (KIR2DL4). Another member of this group is the activating KIR2DS4 that

recognizes a highly conserved bacterial recombinase A peptide sequence motif for HLA-C*05:01[37], but has also been proposed to have an affinity for HLA-Cw4[38] and to bind to non-class I MHC proteins on melanoma cells[39]. However, these are rare and specific ligands indicating a minor role for KIR2DS4 as activator of NK cell functions. Moreover, 70% of Caucasians that show homozygous expression of group "A" KIRs also show non-functional deletion variants of KIR2DS4[40]. Haplotype "A" KIRs differ as a result of allelic polymorphisms[41,42]. All remaining KIRs are collectively



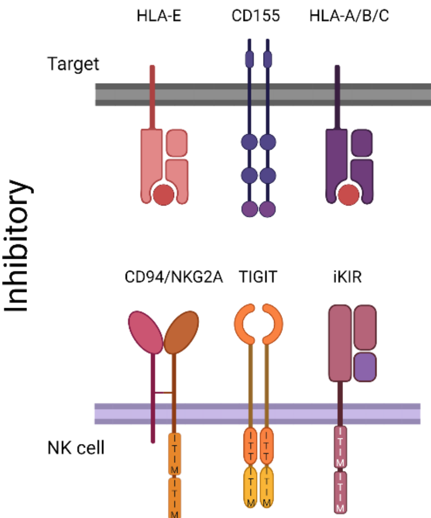


Figure 1.1 Activating and inhibitory NK cell receptors and their ligands. Cognate ligands for germ-line encoded receptors are expressed on malignant or infected cells or on healthy cells. Activating receptors transmit signal through their own cytoplasmic ITAM, ITSM or ITT domains or recruit adaptor proteins (DAP10, DAP12, CD3ζ) that carry these domains. Activating signalling is associated with the recruitment of adapter proteins involved in actin cytoskeleton remodelling, but also leads to an increase of intracellular Ca^{2+} levels via PLCγ and PI3K activation. ITIM signalling of inhibitory receptors is associated with recruitment of SHP-1 and SHP-2 that block phosphorylation of adaptor proteins and intervene with actin cytoskeleton rearrangement. Adapted from Saeed et al.[144] Created with BioRender.com

summarized as haplotype "B" and gain their variability as a consequence of differences in both presence and/or absence of KIR genes and KIR gene copy number[43,44]. This results in a combination of activating and inhibitory KIR genes for this group[45]. All nucleated cells express classical MHC-I that inhibit NK cell activation through KIR signalling. The KIR ligand HLA-A is further divided into three groups dependent on the amino acid sequence of the KIR-binding epitope. The same differentiation can be made for HLA-B, although only the HLA-Bw4 motif serves as a ligand for KIRs. For HLA-C two groups have been defined (group C1 and group C2) that differ at position 80 of their amino acid sequence, with HLA-C1 presenting an asparagine and HLA-C2 carrying a lysine. This polymorphism defines the specificity of HLA-C1 for KIR2DL2/3 and HLA-C2 for KIR2DL1[46]. Most tumour cells display a downregulation of MHC-I, presumably to escape from CTLs, but ultimately triggering NK cell activation and consequently target cell killing.

NKG2A and **NKG2C** are both receptors that heterodimerize with **CD94** upon binding to the non-classical HLA class I histocompatibility antigen, alpha chain E (HLA-E). Recognition of HLA-E by CD94/NKG2C leads to signalling activation in NK cells through the ITAMs of DAP12 transmembrane proteins[47]. On the other hand, most NK cells and some T cell subsets express the inhibitory receptor NKG2A that shows a strong inhibition of IS maturation by interfering with the activation of both NK cells and $CD8^{+}$ CTLs. NKG2A mediates this inhibition through ITIM domains in its cytoplasmic tail, and it has been shown that NKG2A has a higher affinity for HLA-E than NKG2C[48], an observation that follows the hypothesis of a dominance of inhibitory signals over activating signals in modulation of NK cell functions[49,50]. Under physiological conditions, HLA-E is expressed at low levels in healthy tissue, but upregulated on trophoblasts to prevent embryo rejection by inhibiting uterine decidual NK cells[51]. Its assembly and surface expression is stabilized through binding of a restricted subset of peptides derived from HLA-A, -B, -C, and –G[52] and therefore dependents on classical MHC-I expression and provides an important "self"-signal to NKG2A-expressing immune cells under normal conditions. Since HLA-E is a non-classical HLA, its function has not been thoroughly investigated, and its role in anti-tumour immunity might be underappreciated. This can be attributed to the higher degree of polymorphism of classical HLA molecules and their dominant role in transplant rejection[53]. HLA-E on the other hand shows low nucleotide polymorphism and is considered to be a conserved gene due to its potential key role in in the modulation of immune responses and presentation of leading peptides for "self" recognition and semi-allograft immunotolerance during pregnancy[54]. Interestingly, HLA-E cannot be detected on the surface of most tumour cell lines used for *in vitro* experiments but is frequently found to be upregulated in primary neoplastic tissue[55–57]. This makes it more difficult to investigate the interaction of CD94/NKG2A and HLA-E in regulating the anti-tumour immune response of NK cells and CTLs. But it also raises the question how in tumours with low or absent MHC-I expression HLA-E surface expression is stabilized, especially if loss of classical MHC-I is the result of inhibition of transporter associated with antigen processing (TAP) or other parts of the antigen presentation pathway components, because these protein complexes are also required for peptide-loading of HLA-E[58,59]. Studies have reported that in malignancies the HLA-E peptide repertoire can change, but also that these irregular peptides do not mediate protection from NK cell-mediated lysis[60]. This is in line with contradicting reports about the clinical relevance and the prognostic value of HLA-E expression in different types of tumours that has been suggested to depend on cooperative upregulation of the non-classical HLA-G to facilitate tumour immune evasion[55,57,61–67].

NKG2D is an activating receptor of the NKG2 family of C-type lectin-like receptors[68] that can be found on all cytolytic cells of the immune system[69]. The activating signal can be transmitted by two

different adaptor proteins, DAP10 and DAP12, that mediate activation via different signalling pathways[70]. Ligands for NKG2D, proteins from the MHC-I polypeptide-related sequence (MIC) and the cytomegalovirus UL-16 binding protein (ULBP in human, RAET1 in mice) family, are so called "induced-self" antigens, and are not or only poorly expressed on healthy cells. These ligands are only upregulated as a response to a stressor such as senescence or cellular transformation[71], DNA damage[72], oxidative stress[73,74] and viral infection[75]. Transplantation of NKG2D-ligand expressing tumours in mice results in fast tumour rejection as a result of NK cell activation through NKG2D[76,77]. Direct evidence of the important role of NKG2D for tumour immunosurveillance comes from studies using NKG2D^{null} mice showing that while NKG2D is not required for NK cell development, it plays a critical role for the immunosurveillance of spontaneous malignancies[78]. Recognition of "induced-self" is accordingly an important surveillance strategy of infection and cellular transformation as it results in the destruction of affected cells by activated NK cells, but also γδ T cells and CD8^{+} αβ T cells. It has since been suggested that one of the early events of immunoediting is the downregulation of NKG2D ligand MIC-A/-B expression, e.g., by shedding of the protein from the tumour cell surface.

CD16 is the Fc receptor (FcγRIII) that enables NK cells to mount ADCC against antibody-coated (opsonized) target cells. Expression of CD16 is predominantly found on CD56^{dim} NK cells with strong cytotoxic functions[79,80]. CD16 is a unique receptor, as it is the only activating receptor that is self-sufficient to induce activation of NK cells[81]. Activating signalling follows homo- or heterodimerization of CD16 with TCR ζ and/or the FcR γ chains[82]. Accordingly, CD16 engagement initiates the classical ITAM pathway *via* activation of Src-family kinases, Syk recruitment and subsequent activation of phosphatidylinositol 3-kinase (PI3K), phospholipase C γ (PLC γ), and Vav1. Downstream messengers, such as phosphatidylinositol-4,5-bisphosphate (PIP2), are produced and accumulate in the NK cell membrane at the IS by activation of phosphatidylinositol-4-phosphate 5-kinase (PIP5K)[83,84]. Through PI5K activity, secretion of lytic granules can be induced in resting NK cells, although granule polarization is regulated independent of CD16 signalling[85]. Additionally, these enzymes have been suggested to be crucial for the serial killing trait of NK cells, possibly as a result of PIP5Kγ-mediated regulation of Munc13-4 that is involved in lytic granule recycling[86].

LFA-1 is an integrin and mediates cell-cell adhesion through binding to its ligand, the intercellular adhesion molecule 1 (ICAM-1). Formation of any NK cell IS is primarily dependent on adhesion. Firm adhesion of NK cells to target cells is enhanced by integrin inside-out signalling that increases the affinity of LFA-1 to its ligand[87,83,88,89]. Engagement of LFA-1 with its ligand has been shown to result in an increase in NK cell functions, such as TNF-α and IFN-γ production, but is also crucial for polarization of the microtubule organizing centre (MTOC) and cytotoxic granules to the centre of the lytic IS. Polarization of the cytolytic machinery is mediated by LFA-1 signalling alone in NK cells[90]. *In vitro* experiments have shown that downregulation of ICAM-1 on tumour target cells is associated with improper NK cell target cell recognition and target cell lysis, and that ICAM-1 loss can be the result of NANOG overexpression. This transcription factor is involved in the self-renewal of embryonic stem cells by suppressing cell determination factors, and it also regulates ICAM-1 expression[91,92]. Likewise, loss of LFA-1 results in an impairment of conjugate formation and consequently NK cell cytotoxicity against target cells[93]. In cancer patients, low ICAM-1 expression on tumour cells has been shown to be linked to a higher rate of recurrence[94].

2B4, or CD224, is a NK cell receptor of the immunoglobulin (Ig) superfamily signalling lymphocyte activation molecule (SLAM) receptor family that recognizes CD48. This ligand is constitutively

expressed on cells of hematopoietic lineage and is increased on B cells during Epstein-Barr virus (EBV) infection[95], but lost on HIV-infected T lymphoblasts[96]. CD48 has been reported to be highly expressed in renal cancer, but also in malignant glioma[97], and it has been demonstrated that CD48 expression correlates with a malignant phenotype and a generally unfavourable prognosis. In other types of cancer, such as breast cancer[98] and colorectal cancer, a high CD48 expression has been linked to a favourable prognosis, probably due to high tumour infiltrating lymphocytes (TILs) that indicate an active immune and inflammatory response. Interestingly, the cytoplasmic domain of 2B4 includes four ITSMs that allow interaction with adaptor proteins involved in both inhibitory and activating signal transduction[99,100]. Furthermore, 2B4 is expressed as two isoforms due to differential splicing. This results in a difference of five amino acids between the immunoglobulin V and C2 domains, with the shorter isoform showing a higher affinity for CD48 and consequently increasing NK cell cytotoxicity and secretion of IFN-γ[101]. 2B4-induced activation of NK cells can be dampened by inhibitory receptor signalling through activity of Src kinases allowing fine-tuned regulation of NK cell cytotoxic functions[102]. SLAM-associated protein (SAP) is one of the adaptor proteins that is required for the initiation of activation signalling, and in its absence SHP-1 and SHP-2 are recruited to generate inhibitory signals[103]. In patients with X-linked lymphoproliferative disease (XLP) SAP expression is disrupted, and studies have shown that SAP-deficient NK cells fail to induce natural cytotoxicity while ADCC triggered by the Fc receptor CD16 is functional[103,104]. The dual role of 2B4 as an activating and inhibitory receptor is dependent on the expression level of 2B4, but also on adaptor molecule expression levels, availability and competitive binding[105,106].

NKp30 (encoded by *NCR3*) is an activating receptor of the Ig-like superfamily of natural cytotoxicity receptors and enables NK cell activity and cytotoxicity through binding to B7-H6[107]. Three alternative splicing variants have been described for NKp30, and while NKp30a and NKp30b are both involved in NK cell activation, the third isoform NKp30c has been associated with decreased degranulation and production of IFN-γ and IL-12 in an IL-10-dependent manner[108]. In healthy tissue B7-H6 expression is absent, but its expression is induced by inflammatory conditions. Several independent studies have shown a high expression of B7-H6 in different cancer tissue, including lymphoma[109], ovarian cancer[110], hepatocellular cancer[111], cervical cancer[112], breast cancer[113] and glioma[114,115]. High B7-H6 expression has been positively associated with tumour progression and invasiveness, as well as distant metastasis status[116].

NKp44 and **NKp46** are two additional members of the NCR family. NKp44 (*NCR2*) is exclusively expressed on activated NK cells and has been initially suggested to be involved in non-MHC-restricted lysis[117]. Recently, HLA class II molecules, specifically a subset of HLA-DP molecules, have been identified as ligands for NKp44 that trigger NK cell activation[118]. Other NKp44 ligands have been proposed to be involved in NKp44-mediated recognition of tumour cells. These include proliferating cell nuclear antigen (PCNA) that upon overexpression positively influences tumour cell proliferation[119] and mediates resistance against NK cell cytotoxicity through high-affinity interaction with the ITIM-bearing NKp44-1 isoform[120]. Another proposed ligand is 21spe-MLL5, a shorter splicing variant of mixed-lineage leukaemia protein-5 (MLL5) that in contrast to the original nuclear protein can be found in the cytoplasm and at the cell surface of hematopoietic and non-hematopoietic tumour cells[121]. NKp46 (*NCR1*) on the other hand is a highly conserved NK cell lineage defining activating receptor that can trigger NK-mediated lysis of various tumour cells through direct engagement of unknown tumour ligands. Crosslinking of NKp46 has been shown to induce calcium influx and cytokine release and enhance signalling of other activating receptors in response to binding of viral glycoproteins, such as the hemagglutinin of influenza viruses[122]. And

while tumour-associated ligands of NKp46 remain elusive, experiments have shown that NKp46-Fc is only bound to primary tumour tissue, but not healthy tissue[123]. This binding was further dependent on the presence of NKp46-bearing NK cells in the tumour environment[124]. Additionally, depletion of NKp46 in preclinical *in vivo* experiments resulted in an increase in distant metastasis[125], and in clinical settings *NCR1* expression is used as a strong prognostic marker for solid tumours associated with a favourable outcome[126].

DNAM-1 (DNAX Accessory Molecule-1) and **TIGIT** (T cell immunoreceptor with Ig and ITIM domains) are two receptors on NK cells that both recognize two stress-induced ligands from the poliovirus receptor (PVR) family, CD155 or PVR, and CD112 or nectin-2. DNAM-1 is an adhesion molecule that synergizes with activating receptors to mediate NK cell activation upon binding of its ligands[127]. The intracellular signalling motif of DNAM-1 has been proposed to be an immunoreceptor tyrosine tail (ITT)–like motif that recruits Grb2, resulting in the activation of Vav1 and other downstream effectors[128]. On NK cell progenitors, DNAM-1 expression is common, but some NK cells downregulate its expression upon maturation, leading to DNAM-1^{pos} and DNAM-1^{neg} peripheral NK cell populations. DNAM-1^{neg} NK cells have been described as a terminally differentiated NK cell subpopulation with limited killing activity and poor IFN-γ, IL-6, CCL5 and GM-CSF production upon stimulation with IL-12 and IL-18[129,130]. There is increasing evidence that chronic exposure of DNAM-1 to its ligands ultimately results in downregulation of this receptor, as seen in several tumour types[131,132]. The other receptor for CD155 and CD112 is TIGIT, an inhibitory receptor that signals through an ITT-like motif and an ITIM. Via its downstream effectors Grb2 and SHIP1, it inhibits NK cell polarization and degranulation[133–135], as well as IFN-γ production[136]. Consequently, TIGIT signalling results in impaired NK cell functions such as cytotoxicity. In the context of NK immunosurveillance, CD112 has been described to possess stronger inhibitory or immune evasion capacity through its interaction with TIGIT than by binding to DNAM-1. This is supported by studies that combined trastuzumab-induced ADCC with blockade of TIGIT, resulting in an increased susceptibility of HER2/neu^{+} breast cancer cells to NK cell-mediated cytotoxicity[137]. Furthermore, TIGIT blockade in combination with IL-15 stimulation of tumour-infiltrating NK cells leads to increased cytotoxicity *in vitro* and successful tumour control *in vivo*[138,139].

1.1.2 NK cell immunological synapses

To recognize and kill tumour cells, NK cells need to form a tight, physical contact with their target cells. The immunological synapse has been defined as the interface between a CTL or NK cell and a cell that they are in the process of recognizing[140]. But the IS has also been described between CTLs and B cells, and CTLs or NK cells and APCs. NK cells have been described to form different types of IS with different target cells (Fig. 1.2)[141]. These cell-cell contact sites can phenotypically be differentiated by the localization of the MTOC, the accumulation of filamentous actin (F-actin) in both target and effector cell, and the positioning of inhibitory and activating receptors and ligands[141,142].

Recognition of a stressed or (pre-) malignant cells induces the formation of a **lytic IS** between NK cell and its target cell. The lytic IS can be divided into three phases that all show further division into serial events. This type of synapse requires a strong activating signal, either through downmodulation of inhibitory ligands and/or upregulation of activating ligands that subsequently initiate a firm adhesion of the NK cell through integrins and induction of activating signals. LFA-1 signalling further induces dynein-dependent lytic granule convergence to the MTOC[143]. This defines the “recognition” phase of the lytic IS. In the following “effector” phase, activating receptor

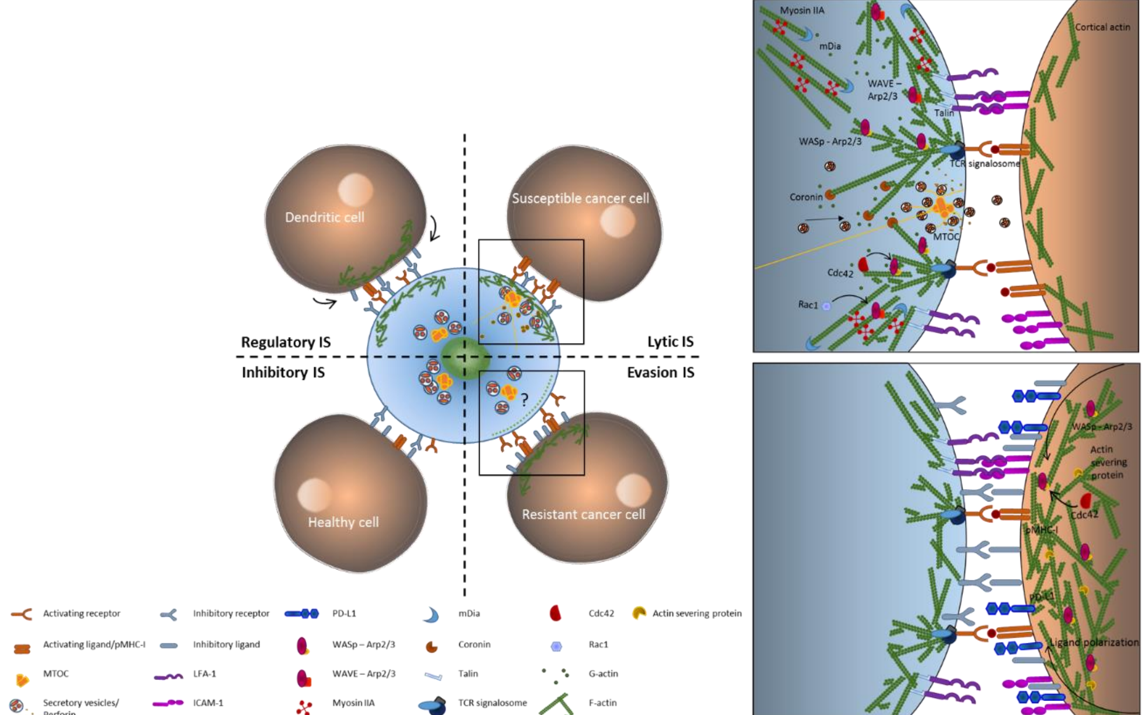


Figure 1.2 Types of NK cell immunological synapses. The type of IS formed between NK cells and a prospective target cell depends on several different factors, that include activating and inhibitory ligand presentation, F-actin polymerization on both sides of the IS and susceptibility of the target cell. Healthy cells do not express activating ligands and thus induce the formation of an inhibitory IS (bottom left). DCs have been reported to recruit and stabilize inhibitory MHC-I at the regulatory IS with NK cells in an actin-dependent manner, resulting in F-actin accumulation on both sides of the IS and inhibition of NK cell effector functions (top left). Susceptible target cells express high levels of activating ligands and induce the formation of a lytic IS with NK cells, inducing effector functions through engagement of multiple activating receptors (top right). Recently a new type of NK cell IS has been described, that shows polarization of inhibitory ligands on resistant cancer cell, the evasion IS (bottom right). It has been suggested that this mechanism leads to inhibition of NK cell effector function and is associated with immune evasion of cancer. From: Wurzer et al., 2019.

signalling induces an accumulation of F-actin through recruitment of adaptor proteins such as the kinases Src, Lck and Lyn that induce actin cytoskeleton reorganization through activation of e.g., SYK, ZAP-70 and Vav1/2, as well as inhibition of SHP-1 and SHP-2 through SAP[27,141]. Actin reorganization and accumulation is especially incisive in the peripheral supramolecular activation cluster (pSMAC). This phase is also accompanied by clustering of receptors at the IS as well as cell membrane reorganization by clustering of lipid rafts[144,145]. This is followed by the polarization of the MTOC, surrounded by lytic granules, towards the central SMAC (cSMAC). MTOC polarization is dependent on LFA-1 signalling[146], but also requires clearance of F-actin from the cSMAC[144,147] and is initiated by actin-microtubule cytoskeleton linkers such as IQGAP[148]. Docking of the MTOC with the presynaptic membrane precedes the fusion of the lytic granules with the plasma membrane, resulting in the directional release of perforin, granzymes, as well as TNF-α and IFN-γ into the synaptic cleft[149], and an accumulation of death receptor ligands at the presynaptic NK cell membrane[150]. Maintenance of the lytic IS requires constant activating signal through receptors and is energy dependent. Eventually, it results in clear signs of NK cell activation, including CD69 upregulation, downregulation of activating receptors and loss of intracellular TNF-α, IFN-γ, and granzymes through degranulation[151]. Lysis of the target cells initiates the "termination" phase of the lytic IS and detachment of the NK cells. This process can be facilitated through NK cell contact with a new target cell[152].

When healthy cells are approached by NK cells, the engagement of MHC-I and KIRs results in the formation of an **inhibitory IS**. Through the strong inhibitory signalling, actin cytoskeleton reorganization is blocked and MTOC and granule polarization do not occur[27,89,153].

Although NK cells do not require activation by DCs, their cytolytic capacity, especially the translation of IFN-γ, can be significantly increased by priming through DC-derived IL-15 and IL-18[154–156]. This cross-talk not only promotes NK cell functions, but can also induce DC maturation through release of strictly regulated amounts of TNF-α and IFN-γ[157–159]. The kinetics of this interaction need to ensure that DCs are protected from NK cell-mediated cytotoxicity. Barreira da Silva and colleagues have shown, that NK cells form a **regulatory IS** with DCs during which the actin cytoskeleton of the DC needs to accumulate at the site of the IS where it leads to stabilization of exclusively inhibitory MHC-I but not MHC-II[160]. This ensured a strong inhibitory signalling in NK cells through interaction with KIRs, but also priming of resting NK cells and maturation of DCs at the same time.

Recently, a fourth type of IS between NK cells and target cells has been described[141]. This so-called "**evasion IS**" is characterized by the contact between a NK cells and a malignant cell that shows an accumulation of F-actin at the postsynaptic membrane[161]. This actin accumulation has been associated with resistance against NK cell-mediated cytotoxicity through low levels of intracellular granzyme B in target cells and has been termed "actin response". The further investigation of this type of IS, as well as the associated resistance mechanisms, are the main topic of this thesis. Further projects, which aim to explore the role of the actin response as a platform for different intrinsic immune escape strategies of cancer are being followed, including the definition of triggering events and identification of markers and target pathways.

1.1.3 NK cell cytotoxicity

The cytotoxicity of NK cells can be exerted via different pathways: lytic granule exocytosis-mediated apoptosis, pyroptosis, and death receptor (DR)-induced apoptosis. All require the formation of an IS. Granules in the NK cells cytoplasm are packed with proteins such as perforin and serine proteases, with granzyme A and granzyme B being the most abundant ones[162]. These lytic or cytotoxic granules are transported along microtubules and converge around the MTOC and polarize to the centre of the lytic IS upon NK cell activation. Fusion of lytic granules with the NK cell membrane occurs in the actin hypodense region of the cSMAC[149] and allows for polarized secretion into the nanometre-scale synaptic cleft between NK cell and tumour cell. This degranulation process has been used as an indirect measurement of NK cell cytotoxic activity by detection of the transient appearance of lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) and -2 (LAMP-2 or CD107b)[163]. Upon release, perforin and granzymes initiate target cell death cooperatively. Perforin binds to the target cell membrane and oligomerises in a Ca^{2+} dependent manner to form pores[164]. These pores allow for the passive diffusion of pro-apoptotic granzymes into the target cell, but also trigger a transient calcium influx into the target cell and initiate membrane repair mechanisms (MRM)[165]. As a consequence of MRMs, perforin and granzymes are both endocytosed in a clathrin- and dynamin-dependent manner in enlarged early endosomes, so called "gigantosomes"[166–168]. Perforin has been reported to form holes in the membrane of these gigantosomes, mediating a gradual release of granzymes into the cytoplasm of tumour cells until the gigantosomes rupture[168]. In perforin-deficient mice, NK cell-mediated immunosurveillance of cancer and metastasis control are severely impacted[169–171], underlining the crucial role of perforin for both T cell and NK cell-mediated target cell lysis. Granzymes themselves enact their function through direct or indirect activation of the target cell's intrinsic caspases, triggering several

apoptosis pathways simultaneously (Fig. 1.3). They have also been shown to induce target cell death by mediating detachment of cells *in vitro* by cleavage of extracellular proteins, thus triggering cell death by anoikis[172,173]. Granzyme B is the most extensively studied granzyme. Its intracellular substrates include the BH3-only pro-apoptotic protein Bid, that mediates mitochondrial permeabilization and cytochrome c release together with Bax and/or Bak, as well as the effector caspases 3 and 7 that initiate a caspase activation cascade to promote apoptosis upon proteolytic

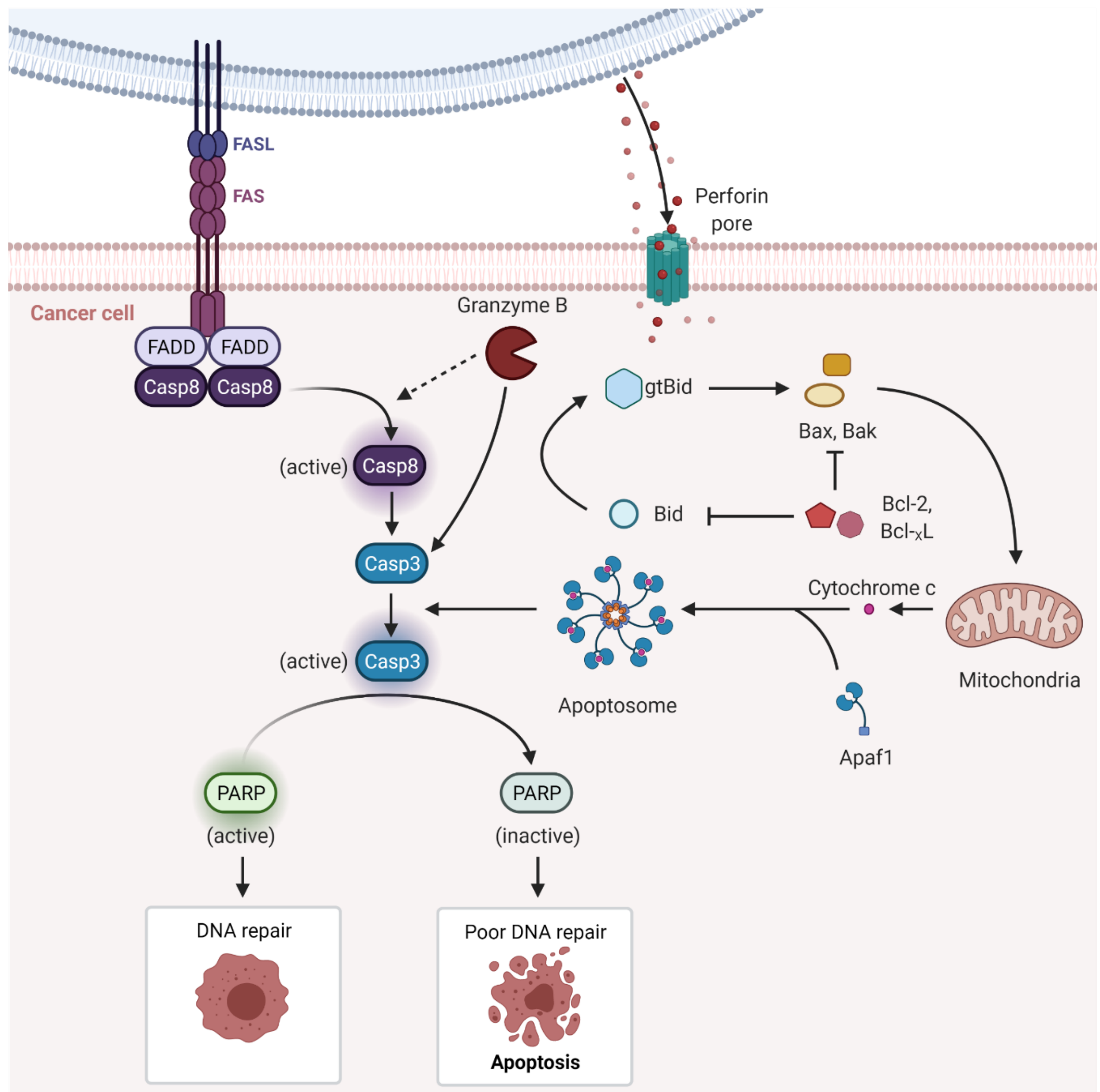


Figure 1.3 Induction of apoptosis through Fas and granzyme B. NK cells can induce target cell death through different pathways that all have in common the cleavage of pro-caspases into their active forms. Binding of Fas to FasL on effector lymphocytes induces recruitment of the Fas-associated protein with death domain (FADD) adaptor and initiates cleavage of pro-caspase 8 and caspase 10. Caspase 8 is responsible for activation of the pro-caspase 3 as well as the proteolytic cleavage of Bid. Similarly, granzyme B is involved in generation of granzyme B-truncated Bid (gtBid) and cleavage of pro-caspase 3 into its active form. Translocation of gtBid to mitochondria results in recruitment of pro-apoptotic Bax and Bak and their integration into the mitochondrial membrane and subsequent cytochrome c release. Binding of apoptotic protease activating factor 1 (Apaf1) to cytochrome c results in oligomerization of the apoptosome that is involved in cleavage of pro-caspase-9 and pro-caspase-3. Caspase-3 is involved in execution of apoptotic pathways and the main responsible protein for cleavage and inactivation of poly(ADP-ribose)polymerase-1 (PARP). Additionally, perforin pores allow diffusion of ions and water, destroying the integrity of the target cell membrane. Created with BioRender.com

cleavage [162,174,175] (Fig. 1.3). Both NK cell activation through “altered-self” recognition and ADCC can trigger lytic granule degranulation, although an important prerequisite for this pathway is the engagement of LFA-1 with its ligand ICAM1 that plays a critical role in mediating granule convergence and MTOC polarization in synergy with F-actin depletion from the cSMAC[27,147,176].

As a complementary pathway, innate effector cells can also kill target cells by pyroptosis, a form of cell death that is triggered by proinflammatory signals and is strongly associated with inflammation. This pathway is characterized by activation of caspases 1/4/5 in humans (and caspase 1/11 in mice) through the inflammasome[177] and gasdermin D-dependent pore formation in the target cell membrane[178]. Recently is has been shown that IFN-γ leads to an upregulation of gasdermins and that gasdermin D cleavage can also be mediated by granzyme A[179]. As an inflammation-associated programmed cell death, pyroptosis can be associated with cancer cell killing and tumour control in the presence of endogenous damage-associated molecular pattern (DAMPs) molecules such as HMGB-1, adenosine triphosphate (ATP) and cell-free DNA[180]. However, the release of inflammatory cytokines can also create an immunosuppressive TME[181] and result in downregulation of gasdermins in cancer cells through methylation of promoter regions, as shown for breast cancer and gastric cancer[182–185].

Another pathway of target cell killing is a receptor-based system to induce apoptosis of target cells. This pathway depends on the expression of proteins of the tumour necrosis factor receptor (TNFR) superfamily that carry intracellular death domain signalling motifs. NK cells express the TNF receptor ligands Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL) that bind to Fas receptor or DR4 and DR5, respectively. Binding of its ligand induces the oligomerization of Fas in a death-inducing signalling complex (DISC)[186] with the adaptor protein Fas-associated protein with death domain (FADD) and initiator caspases 8 and 10[187,188]. The downstream signalling of DISC initiates target cell apoptosis through cleavage of Bid and caspase 3[189,190,145] (Fig. 1.3). DR4 and DR5 have been suggested to signal through the same pathways, requiring FADD to mediate apoptosis[191]. Recent research has shown that FasL cannot only be found membrane-bound on the surface of CTLs and NK cells, but is also located on the membrane of CD63$^+$ intraluminal vesicles (ILVs)[150]. Target cell recognition results in a re-localization of these vesicles, like that of lytic granules, and in an increased surface density of FasL toward the IS. Further, ILVs give rise to exosomes, supporting earlier findings of FasL on exosomes of CTLs and NK cells that upon release could potentially induce cell death over a greater distance[192,193].

1.2 CTL immunosurveillance

Immunogenic or necrotic cell death and DAMPs produced by dying cells create a pro-inflammatory environment that attracts other immune cells, including iDCs that take up tumour-neoantigens for processing and cross-presentation on MHC-I and MHC-II molecules. Cross-presentation in secondary lymphoid organs, such as lymph nodes, is critical for activation and priming of neo-antigen-specific effector CD8$^+$ T lymphocytes. The strength of the adaptive immune response against transformed cells is established at this step of the cancer-immunity cycle and is dependent on the ratio between effector to regulatory T cells. A defining feature of adaptive immunity is its high adaptability due to somatic hypermutation and irreversible genetic recombination of antigen receptor gene segments (V(D)J recombination) during early lymphocyte development. Rag2 knockout mice are deficient for the recombination activating gene 2 (Rag2) and accordingly lack the ability to initiate V(D)J rearrangement and fail to generate mature adaptive immune cells, namely αβ T lymphocytes, γδ T lymphocytes, NK-T cells, and B lymphocytes. This results in the loss of the

highly diverse repertoire of antibodies and TCRs. Using this mouse model, Shinkai et al.[194] and Shankaran et al.[195] could show that TCR variety is necessary for cancer immunosurveillance. Mice deficient for V(D)J recombination were more prone to develop spontaneous and carcinogenic-induced tumours, emphasizing the importance of a strong adaptive immune response against tumours.

Activated and mature CTLs exit the lymph node and traffic to the tumour site where they infiltrate the tumour bed. Through the recognition of tumour-associated neo-antigens on MHC-I by the TCR, CTLs also engage in an immunological synapse and kill their target cancer cells either by directed degranulation of perforin and granzymes or caspase-dependent apoptosis through interaction of death receptors with their ligands. As described for NK cells, this immunogenic cell death results in the release of more neo-antigens and an acceleration of the cancer immunity cycle[20]. Additionally, CTLs can shape the immune response through secretion of cytokines.

1.2.1 CTL immunological synapse

As with NK cells, the initial contact of a CTL with a prospective target cell occurs through LFA-1 and adhesion molecules on the target cell membrane (ICAM-1, ICAM-2). But only binding of the TCR to a specific peptide:MHC-I complex generates a sufficient activating signal for the CTL to advance to the formation of a lytic IS (Fig. 1.4).

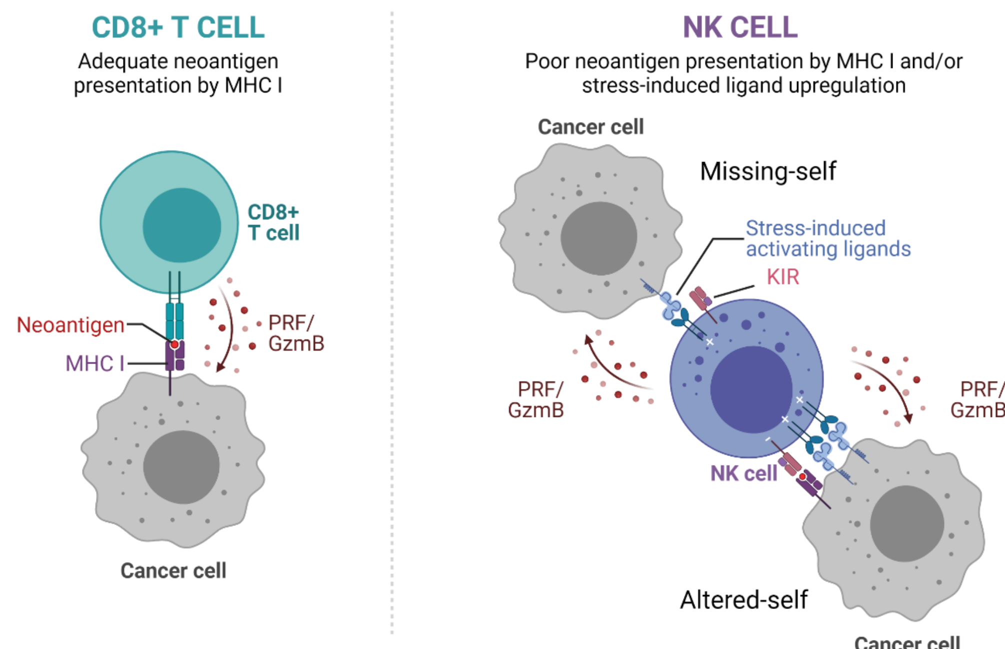


Figure 1.4 The immunological synapse of CTLs and NK cells. The formation of a lytic IS in CTLs is dependent on sufficient surface expression of peptide-loaded MHC-I (pMHC-I) molecules by target cells and specificity of their TCR for the presented peptide. Binding of the TCR to pMHC-I and interaction of co-receptors with their corresponding ligands results in initiation of target lysis. The lytic NK cell IS is formed in response to "missing-self" or "altered-self" recognition, so the loss of inhibitory ligands in combination with induction of activating ligands or a high expression of inhibitory ligands resulting in a sufficiently strong activating signal, respectively. Through this complementary mechanism, CTLs can eliminate a variety of cancer cell clones via specific interaction of tumour neo-antigens with their TCR, and NK cells can destroy tumour clones that try to escape CTLs by downregulation or loss of MHC-I. Created with BioRender.com

Unlike the NK cell IS, the IS formed between a CTL and its target consists of three SMACs. The distal SMAC (dSMAC) is enriched for sialophorin (CD43), CD44, and CD45 and is characterized by an extensive network of branched actin filaments, similar to the lamellipodium of migrating cells. The ligand for CD43 has not been identified yet, but this transmembrane protein has been characterized to be involved in intracellular activating signalling of T cells and loss of its expression is associated with the PID Wiskott-Aldrich syndrome[196,197]. The protein tyrosine phosphatase CD45 has also been shown to be involved in the modulation of NK and T cell functions, probably through direct interactions with protein tyrosine kinases (PTKs) of the Src family, Lck[198,199] and Lyn[200]. The adjoining part of the IS is the pSMAC which contains clusters of LFA-1 and CD2 and shows extensive acto-myosin arcs. TCR and co-receptor containing signalling microclusters are formed in this peripheral part of the IS and are transported along actin filaments towards the central region of the IS, the cSMAC, under sustained activating signal. Transport of these signalling clusters is supported by a symmetric retrograde flow of the actin-myosin arcs in the pSMAC that is driven by continuous F-actin nucleation and extension in the dSMAC. The architecture of the IS is further stabilized by the contractility of the actin-associated non-muscle myosin IIA[201]. The cSMAC on the other hand is characterized as an actin-hypodense region and activating signals of microclusters have been shown to stop at its borders.

Further actin structures, that can be found at the IS, are the so-called actin foci, which colocalize with TCR microclusters. A great fraction of activating receptors is associated with the activation of actin-regulatory proteins. TCR/CD3 engagement, for example, results in the recruitment of Lck and ZAP70, that through phosphorylation of linker for activation of T cells (LAT), recruit Rho-family GTPase exchange factors (GEF) Vav1, PIX, and SLAT, as well as the adapter Nck and the IL-2-inducible T cell kinase (Itk). GEF activation then results in the activation of the Rho GTPases Rac1 and cell division control protein 42 homolog (CDC42). An extensively branched actin cytoskeleton network is generated by nucleation of new F-actin through Cdc42/WASp-dependent activation of the Arp2/3 complex[201]. These actin foci result in the formation of TCR-enriched protrusions that have been shown to facilitate cytotoxic functions of CTLs[202,203]. LFA-1 “outside-in” signalling parallels actin reorganization induced by TCR/CD3 activation and its adaptor protein talin further promotes the recruitment of Arp2/3 to engaged integrins. This ensures a self-sustained feed-forward loop whereby activating integrin signal is dependent on F-actin generated forces and leads to enhanced actin reorganization through the recruitment of nucleating factors and actin polymerizers[87,204,205]. Other costimulatory receptors such as CD28 also contribute to F-actin remodelling[206,207]. CD28 facilitates activating signal transduction by recruiting protein kinase C-theta (PKCθ) to the IS[208]. The intracellular part of CD28 also includes binding domains for filamin-A, an actin filament crosslinker, the adapter protein Grb-2, and the PTKs Lck and Fyn, that connect CD28 with modulating actin cytoskeleton dynamics through Vav1-mediated Cdc42 activation[201,209].

In CTLs, lytic granule convergence and MTOC polarization are dependent on the strength of the TCR signal. While a weak TCR signal can induce centrosome polarization, it is not sufficient to stimulate trafficking of the lytic granules[210]. This independent control of MTOC and granule polarization ensures fine tuning of CTL-mediating target cell killing. As in NK cells, the formation of a lytic IS and sufficient TCR signal strength triggers degranulation of CTLs and target cell killing by secretion of perforin, granzymes and other cytolytic effector proteins.

2. Equilibrium

During the elimination phase tumour cells are effectively destroyed by both CTLs and NK cells. This applies Darwinian pressure on the tumour cells to avoid immunosurveillance and leads to the so-called equilibrium phase. This phase is characterized by the co-existences of effector lymphocytes and tumour cells and a balance between immunosurveillance of immunogenic tumour clones and the escape of poorly immunogenic tumour cells (**immunoselection** and **immunoediting**). Tumour immunogenicity is constantly edited during this phase, but disease progression is still controlled by the immune response[8,211] (Fig. 1.5). Both adaptive and innate effector lymphocytes recognize and destroy pre-malignant and malignant cells and thereby shape the ligand expression profile of tumour cells. Poorly immunogenic tumour clones survive, and ultimately, this gives rise to sub-populations of tumour cells that can escape from CTLs and NK cells.

Figure 1.5 Cancer immunity cycle and immunoediting. The cancer immunity cycle is initiated by the release of tumour neo-antigens either by destruction of cancer cells through NK cells or spontaneous cell death. Neo-antigens are phagocytosed and processed by antigen-presenting cells for presentation on MHC-I molecules (pMHC-I). Priming of antigen-specific CTLs occurs in lymph nodes through cross-presentation. Activated CTLs migrate from secondary lymphoid organs to the tumour bed and infiltrate the mass of malignant cells. Recognition of cancer cells by CTLs is mediated by TCR binding to neo-antigens on pMHC-I. The elimination of cancer cells releases more tumour neo-antigens and provides a pro-inflammatory environment. Through repeated cycles of this process, the immune system selects for resistant cancer cell clones that can develop into a tumour surrounding itself with an immunosuppressive tumour microenvironment for further protection. Created with BioRender.com

3. Escape

Immunoselection ultimately results in the emergence of new tumour clones that are invisible to both innate and adaptive effector lymphocytes or employ other mechanisms to avoid destruction by the immune system. In this final escape phase, a complex of resistant tumour cells and associated immune and stromal cells forms the **tumour microenvironment** (TME). Cancer cells interact with the TME and can shape it to their needs, allowing for their survival and outgrowth into clinically detectable tumours. Active **immune evasion**, the intrinsic capacity of tumour cells to

escape from the immune system, is one of the core hallmarks of cancer[212] and tumours exploit different escape strategies to weaken effective anti-tumour immunity.

3.1 Avoidance of immune recognition

As the main effector lymphocytes of the adaptive immune system, CTLs depend on recognition of specific antigens in the context of MHC-I presentation to exert their anti-tumour functions. Escape from CTLs can accordingly be achieved directly, through antigenic loss by silencing or mutating immunogenic epitopes, or indirectly, by deficient antigen presentation due an altered antigen processing machinery or MHC-I downregulation. In fact, low MHC-I expression can be found on various types of tumours, including melanoma, lung cancer, and breast cancer, and is a prognostic factor for poor patient outcome. TAP2 has been shown to be involved in the dysregulation of the antigen-processing machinery in colorectal cancer (CRC)[213], and B2M deletion has been associated with immune escape in melanoma[213,214]. Loss of MHC-I facilitates tumour escape from CTLs but enables NK cell-mediated cytotoxicity through the removal of KIR inhibition. However, malignant cells can also downregulate activating ligands for NK cells and/or upregulate inhibitory ligands, such as HLA-E and HLA-G[215], or immune checkpoint (IC) ligands such as PD-L1[18]. Furthermore, these non-classical MHC-I molecules are released as soluble factors into the TME to prevent NK cell infiltration and induce exhaustion. Thus, tumour cells also avoid recognition by NK cells and escape the surveillance of the innate immunity.

3.2 Establishment of an immunosuppressive tumour microenvironment

The TME is shaped by different tumour-intrinsic and -extrinsic mechanisms. A prominent intrinsic mechanism to generate an immunosuppressive TME is the upregulation of inhibitory ligands and surface molecules, including the induction of IC ligands. Their engagement to their corresponding receptors on effector lymphocytes suppresses the anti-tumour response of CTLs and NK cells that infiltrated the TME. In this context, the immune response is a double-edged sword, as in particular IFN-γ induces the upregulation of programmed cell death protein 1 (PD-1) ligands PD-L1 and PD-L2 on tumour cells[216,217]. Another tumour-intrinsic evasion mechanism is the **exclusion of effector lymphocytes** from the TME. One of the first pathways to be associated with a low T cell infiltration in melanoma was the activation of the WNT/β-catenin pathway[218]. This immunophenotype has been attributed to a failure of T cell priming in tdLNs because of inefficient infiltration of CD103$^+$ iDCs into the TME due to low expression of the chemokine CCL4 of tumour cells. Furthermore, the lack of CD103$^+$ DCs and in the following low levels of CXCL10 in the TME was also associated with low CTL recruitment after adoptive transfer[219]. Other oncogenic signalling pathways, such as KRAS in combination with activation of c-Myc signalling mediate immune suppression through induction of CCL9-mediated recruitment of macrophages and angiogenesis, as well as IL-23-dependent exclusion of CTLs, NK cells, and B cells[220].

The often **hypoxic environment** of a tumour is a consequence of fast proliferation and inadequate angiogenesis. Hypoxia-inducible factors (HIFs) are transcription factors that are stabilized under hypoxic conditions and induce the expression of several genes involved in oxygen-independent ATP synthesis, as well as vascular endothelial growth factor (VEGF-A), which mediates angiogenesis, and cyclooxygenase (COX). In endothelial cells, VEGF-A has been associated with low ICAM-1 and VCAM-1 expression[221]. This negatively impacts the leukocyte adhesion cascade and consequently effector lymphocyte trafficking to the tumour bed due to poor infiltration from the blood stream[222]. Tumour-derived COX is one of the main suppressors of type I IFN- and effector lymphocyte-

mediated tumour eradication[223] and induces a type II inflammatory signature that has been associated with tumour progression and exclusion of anti-tumour iDCs, NK cells, and CTLs[224,225].

As the hypoxic environment is a consequence of insufficient perfusion, so is the lack of nutrients and an accumulation of by-products of cellular metabolism in the TME. Many cancer cells adopt an aerobic glycolytic metabolism to maintain their high proliferation rate and meet their energy consumption. The high uptake of glucose by tumour cells, known as the "Warburg effect", is followed by **lactic acid fermentation** even in the presence of abundant oxygen that should usually facilitate energy production through mitochondrial citric acid cycle and oxidative phosphorylation[226,227]. Anaerobic glycolysis is less efficient with regards to ATP production, but favours anabolism by providing additional metabolites for cellular proliferation. The production of lactic acid leads to an acidification of the TME, ranging between pH 6.0 and 6.5, as cancer cells adapt to their altered metabolism with an increased transfer of lactate and protons into the extracellular space through monocarboxylate transporters MCT1 and MCT4 that are upregulated as a response to hypoxia[228,229]. While the tumour-associated acidosis facilitates angiogenesis and metastasis, it also generates an immunosuppressive environment[230]. It has been shown that the increased presence of lactic acid in the TME leads to a low intracellular pH and consequently mitochondrial dysfunction and apoptosis of tumour-infiltrating NK cells[231]. Blockade of tumour-derived lactic acid production increases the cytolytic function of NK cells and has been shown to be associated with tumour reduction in mouse models[232]. Lactic acid in the TME additionally interferes with IFN-γ production and survival of NK-T cells[233] and furthermore leads to a suppression of the PI3K/Akt/mTORC1 pathway in CTLs and NK cells[234,235]. This metabolic restriction inhibits effector functions and reduces cytokine production, as well as intracellular ATP levels, ultimately promoting apoptosis.

Another key metabolic pathway, that is dysregulated in cancer cells, is the tryptophan metabolism. The first and rate-limiting step of the tryptophan catabolism is regulated by the enzyme **idoleamine 2,3-dioxygenase (IDO)** that converts tryptophan into kynurenine (Kyn)[236]. While hypoxia on the one hand has been shown to reduce IDO expression[237], the same conditions enhance IFN-γ production from $CD4^+$ T cells and NK cells that in turn increase IDO mRNA expression through NF-$_\kappa$B regulatory pathways[238]. While the immunosuppressive functions of increased Kyn levels in the TME have not been fully understood, studies have shown that Kyn can modulate the expression of activating NK cell receptors and lower granzyme A expression, resulting in decreased cytolytic functions[239,240]. Furthermore, increased IDO expression in cancer cells has been associated with an inhibition of the T cell response[241], and adaption of a tolerogenic DC phenotype[242]. IDO expression is a consequence of immunoediting, and is not only produced by cancer cells, but also by myeloid-derived suppressor cells (MDSCs) in the phase of immune escape[243]. Its presence in the TME favours expansion of regulatory T cell (T_{Reg})[244] and recruitment and activation of MDSCs[245], reinforcing the immunosuppressive TME that inhibits effective anti-tumour immunity and instead supports tumour growth.

3.2.1 Immunosuppressive cell populations

Cancers can also implement immune suppression through more subtle mechanisms, including the recruitment of immunosuppressive immune cell populations, such as **T_{Regs},** or the accumulation of **MDSCs** in the TME. Both types of cells can suppress effector lymphocyte functions.

T_{Regs} are a specialized subset of $CD4^+$ T cells that play an important role in maintaining self-tolerance and preventing autoimmunity[246]. Activation of T_{Regs} leads to an inhibition of DCs and CTL maturation through cellular (cell-cell contact-dependent) or humoral (cell-cell contact-independent) mechanisms. Expression of co-inhibitory immune checkpoint ligands, such as CTLA-4, can suppress DC maturation by binding to CD80/CD86, thereby affecting T cell priming, but also inducing IDO expression in DCs[247]. Cellular immune suppression is further enhanced by membrane-bound TGF-β, inducing Notch1 signalling in target cells and modulating the CTL immune response[248,249]. Moreover, it has been suggested that T_{Regs} possess cytolytic capacities and can induce caspase-dependent cell death in NK cells and CTLs through the perforin pathway[250]. Humoral immunosuppressive mechanisms include the enhanced consumption of IL-2, depriving the TME of this pro-survival cytokine and inducing CTL and NK cell apoptosis[251]. Furthermore, T_{Regs} release high levels of immunosuppressive cytokines, including IL-10, IL-35, and TGF-β, that induce tolerogenic DCs, as well as expression of immune checkpoints, and inhibit effector lymphocyte functions[252,253], while sustaining T_{Regs} functions[254].
Cancer-associated myeloid cells are the most important "protectors" of the TME and consists of MDSCs and tumour-associated macrophages (TAMs). Upon pathogenic signalling, they differentiate from the same progenitor cells into immunosuppressive subsets. MDSCs expand as a reaction to tumour-derived factors that stimulate myelopoiesis while inhibiting the differentiation of mature myeloid cells. But also factors released by activated effector lymphocytes can result in the activation of MDSC[255]. Several mechanisms of how MDSCs exert their suppressive functions have been described[256]. These involve direct cell-cell contact and signalling through receptors and/or release of short-lived mediators, but also generation of nitric oxide (NO) and reactive oxygen species (ROS) that interfere with lymphocyte functions and induce apoptosis. TAMs often present a pro-tumour M2-like phenotype, that is induced by cytokines such as IL-4, IL-10, and IL-13, that are secreted by T_{Regs}[257] and by tumour-derived lactic acid in a HIF1α-mediated manner[258]. In general, both myeloid populations contribute strongly to the generation of an immunosuppressive TME. Their exact functions differ dependent on their localization and are a result of their high phenotypic heterogeneity[259–261].

3.2.2 The tumour cell-NK cell interactome

NK cell activation is greatly influenced by the balance of activating and inhibiting (co-) receptor signalling, but NK cell activation is also regulated by receptors for soluble factors in the TME. Pro-inflammatory cytokines play an important role in the induction of NK cell activation and effector functions. Especially potent production of immunostimulatory cytokines, including TNF-α, macrophage inflammatory protein-1α (MIP-1α), and IFN-γ, by NK cells is enhanced in the presence of IL-2 and IL-12[262–264]. Studies have shown that IL-15, as well as IL-18 play a critical role for survival and proliferation of NK cells, in particular in the context of DC-induced NK cell maturation[156,265] and IFN-γ production[156]. IL-21 is involved in the maturation of NK cells too[266], but has also been shown to act synergistically with NKG2D to enhance the anti-tumour effects of NK cells[267]. This has been partially attributed to the reversal of exhausted NK cells by induction of the PI3K-AKT-Foxo1 and STAT1 signalling pathways by IL-21[268] and an increased expression of the effector molecules perforin and granzyme A and B at both mRNA and protein levels[269].

Besides these activating cytokines, NK cell function is also directly influenced by receptors for other soluble factors, including **TGF-β**, **activin-A**, and **adenosine**. Binding of these molecules to their corresponding receptors TGF-β receptor 1 (TGFβR1) and TGFβR2, activin A receptor type I (ACVR1),

or adenosine A_{2A} receptor (A2AR) impairs NK cell function and metabolism. Receptors for activin-A, a dimeric growth and differentiation factor of the superfamily of TGF-β, and TGF-β transduce signals through recruitment and phosphorylation of receptor-regulated R-SMADs SMAD2 and SMAD3[270]. Activated SMAD complexes translocate to the nucleus and act as transcription factors resulting in downregulation of IFN-γ[271], NKG2D and NKp30[272], chemokine receptors CXCR3, CXCR4, and CX3CR1[273], and EOMES[274]. Furthermore, the NK cell metabolism, especially glycolysis and mitochondrial metabolic pathways, is negatively influenced by TGFβR signalling, resulting in impaired NK cell fitness and survival[275,276]. Anti-inflammatory adenosines in the TME are the result of ectonucleotidase CD39-induced ATP conversion into adenosine diphosphate (ADP) and adenosine monophosphate (AMP) and subsequent hydrolysis by the 5'-nucleotidase CD73[277,278]. In healthy tissue adenosine levels are low, but cellular stress and hypoxia result in an increase of extracellular adenosine concentrations[279,280]. In response to hypoxia, CD39 is upregulated on a variety of cells, including endothelial cells, stroma cells, tumour cells and immune cells[281,282]. Also, CD73 can be found on both tumour cells and immune cells. Interestingly, while >99% circulating NK cells express no CD73, tumour-infiltrating NK cells and NK cells exposed to mesenchymal stem cells gain significant CD73 expression[283,284], contributing to increased levels of adenosine in the TME. Binding of adenosine to its high-affinity receptor A2AR results in a G_s-protein coupled response and an accumulation of intracellular cAMP in immune cells[285] that, through protein kinase A, mediates increased expression of inhibitory cytokines[286], downregulation of pro-inflammatory cytokines (e.g., INF-γ, TNF-α, IL-6)[285] and upregulation of inhibitory receptors, including immune checkpoint receptors PD-1 and lymphocyte-activation gene 3 (LAG-3)[287,288].

Through constant exposure of NK cells to activating signals in the context of chronic inflammation or tumours, as well as through the microenvironment with its plethora of immune-suppressive soluble factors and pro-tumorigenic cells, NK cells become unresponsive and alter their phenotype. This NK cell exhaustion is often characterized by a downregulation of activating receptors, such as NKG2D, 2B4, and CD16, accompanied by reduced expression of cytolytic molecules including FasL, TRAIL, granzymes, perforin, and effector cytokines (e.g., IFN- γ)[289,290]. This hinders their activation and cytotoxic capacity, and is observed in a variety of malignancies, including CRC[291], breast cancer[292], and chronic lymphocytic leukaemia (CLL)[293]. The constant perturbation of activating signalling and associated transcriptional programs is worsened by the absence of activating cytokines, such as IL-15, that also has an impact on peripheral NK cells, resulting ultimately in an arrest of NK cell maturation in the bone marrow[294]. The unresponsive phenotype and intratumoral NK cell dysfunction are further augmented by upregulation of inhibitory ligands, including NKG2A, and co-inhibitory **immune checkpoints (ICs)** that can include Tim-3 (T cell immunoglobulin and mucin-domain containing-3), LAG-3, and PD-1.

3.3 Inhibitory immune checkpoints

ICs are co-stimulatory and -inhibitory molecules that modulate the immune response, regulate T cell priming, and are crucial for self-tolerance. They can tip the balance between activating and inhibitory signals for both CTLs and NK cells and thus fine-tune their effector functions. Co-stimulatory ICs play an important role in the induction and expansion of antigen-specific T lymphocytes. These include amongst others CD27 that is involved in the generation and maintenance of T cell memory. As another activating IC, CD28 binding to its B7 ligands CD80 (B7-1) and CD86 (B7-2) on DCs supports T cell proliferation and induces expression of interleukins in synergy with TCR signalling. An inducible co-stimulatory IC is Inducible T cell Costimulator (ICOS or

CD278) that is essential for T cell priming und upregulated upon antigen specific TCR engagement. Through binding to its ligand ICOS-L (B7-H2), ICOS controls polarization of $CD4^+$ T helper (Th) cells[295] and regulation of the Th2 immunity[296]. As a result of a certain degree of redundancy with CD28 signalling, ICOS can also influence T effector cell fate decisions, T_{Reg} induction[297] and promotion of humoral immunity[298].

The term IC is mainly used for co-inhibitory surface molecules that show a high potential to control the cytotoxic potential of NK cells and CTLs. Co-inhibitory ICs provide a physiological brake of immune activation and enact their full potential during the recognition phase of IS formation. For NK cells, these include KIRs, NKG2A, and TIGIT, all of which prevent autoimmunity and regulate activation of cytotoxic effector functions. Importantly, dysregulation of checkpoint receptor signalling, for example by upregulation of these surface molecules on non-DCs, contributes to NK cell dysfunction (Fig. 1.6)[299]. However, inhibitory ICs (iIC) also play an important role during T cell priming. During this process, DCs induce cytotoxic T cell functions and Th1 immune responses, while upregulation of ligands for iIC protects them from being killed by CTLs[300]. However, tumour cells show aberrant expression of iIC as part of their immune escape strategy, which will be discussed in the following chapter.

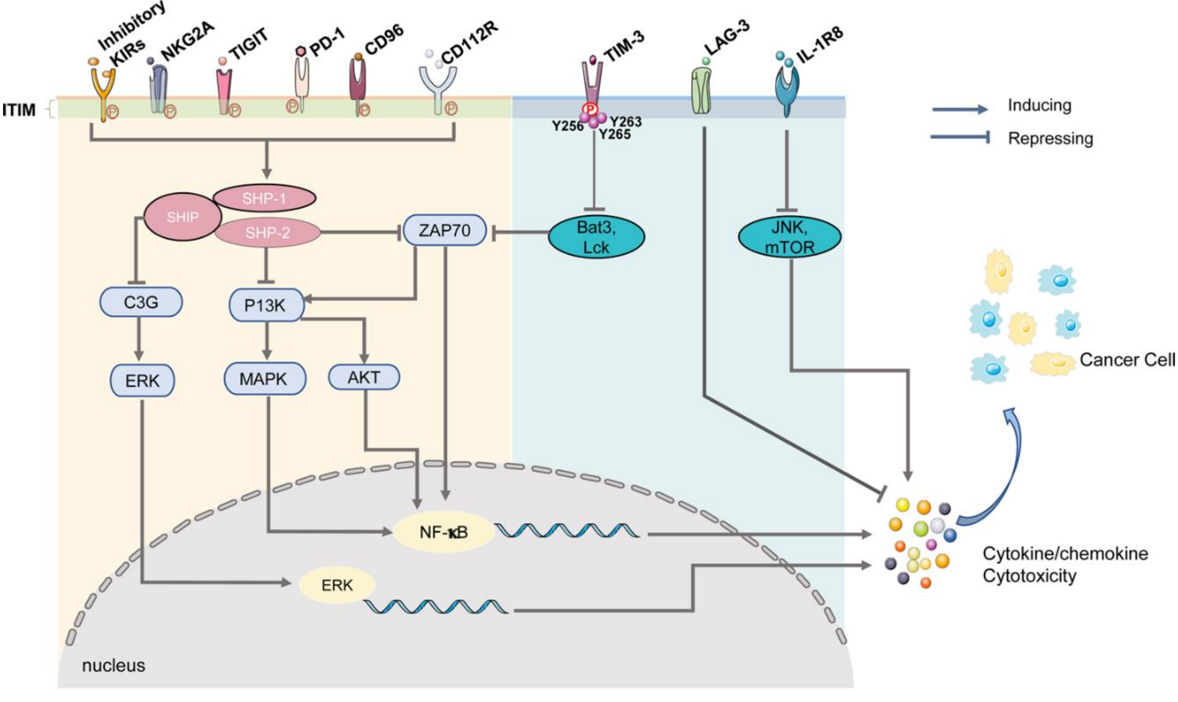


Figure 1.6 Immune checkpoint signalling in NK cells. Binding of inhibitory immune checkpoints (iIC) to their ligands prevents NK cells from executing their cytotoxic functions. Most iIC signal through their intracellular ITIM or ITT domain that upon phosphorylation recruit SHP-1, SHP-2, and SHP and SH2 domain-containing inositol-5-phosphatase (SHIP). These phosphatases intervene with activating receptor signalling at several points and supress NK cell activation and target cell lysis. From: Cao et al., 2020

High affinity binding of **TIGIT** to CD155 and CD112, induces an inhibitory signalling pathway that suppresses NK cell and CTL effector functions by preventing NF-κB and PI3K activation[301]. In T_{Regs} however, TIGIT signalling induces an immunosuppressive phenotype[302]. Indeed, it has been suggested that TIGIT predominantly inhibits anti-tumour immune response through induction of T_{Regs} activation rather than inhibition of effector lymphocytes[303]. The signalling pathway of TIGIT

and how it transduces inhibitory signals to inhibit NK cell function have been described previously in chapter 1.1.1. "*NK cell recognition of tumour cells*".

Like ICOS, **CTLA-4** shares structural similarities with CD28, but shows a higher affinity for the same ligands CD80 and CD86. Antigen-specific ligation of the TCR leads to stabilization of intracellular CTLA-4 and an increased surface expression, where it outcompetes CD28 for binding to its B7 ligands. Upon binding, CTLA-4 transmits an inhibitory signal that interferes with IL-2 production and induces T cell cycle arrest. CTLA-4 expression can also be detected on activated NK cells and tumour-infiltrating NK cells in different mouse models of solid tumours, where it prevents the release of IFN-γ and could play a role in shaping the NK cell anti-tumour response[304,305]. It has been proposed that CTLA-4 expression is induced by IL-2 and that tumour-infiltrating NK cells are more sensitive to this IL-2 dependent IC upregulation due to a high expression of IL-2Rα (CD25)[304]. CTLA-4 activation is associated with a recruitment of SHP-2 that inhibits ZAP70 microclusters formation, as well as lipid raft rearrangement and cytoskeletal changes that are necessary for the formation of a lytic IS[306]. In general, CTLA-4 negatively regulates CTL and NK cell functions during the early phase of lymphocyte activation.

The probably most prominent iIC is the **PD-L1/PD-1** axis with its pleiotropic functions in the regulation of T cell and probably NK cell anti-tumour response. TCR engagement during priming induces the expression of PD-1 on different T cell subsets with distinct functions depending on the differential stage and the environment. On initially activated T cells, PD-1 binds to PD-L1 or PD-L2 on DCs to prevent autoimmunity and ensure survival of the antigen presenting cells. This binding results in the recruitment and phosphorylation of SHP-2 which interferes with TCR and CD28 signalling via inhibition of ZAP70 and PI3K, respectively. This leads to the suppression of several transcription factors that are involved in the regulation of T cell proliferation, cytokine production, and survival. It is therefore not surprising that PD-1 is highly expressed on exhausted T cells and is used in combination with other surface proteins as a marker of late stage and even terminal effector lymphocyte exhaustion. In contrast to that PD-1 signalling in T_{Regs} is associated with their proliferation and survival, as well as expansion of their immunosuppressive functions. PD-1 expression on NK cells is a rather new observation that is still under debate. Several groups have reported PD-1^{+} NK subpopulations, especially in the context of cancer or infection[307–310], while other groups claim to have found little to no evidence for PD-1 expressing NK cells[311,312]. If PD-1^{+} NK cells were found, they belonged exclusively to the CD56^{dim} or CD56^{neg} phenotype of fully mature NK cells, and in healthy humans their presence was associated with an acute or chronic HCMV infection[313]. In cancer patients, CD56^{dim}PD-1^{+} NK cells were found with a higher frequency in the periphery, but especially among tumour-infiltrating NK cells, and they demonstrated poor secretory functions and impaired NK cell cytotoxicity[310,313]. SHP-2 recruitment to the IS negatively impacts direct cytotoxicity of NK cells by interfering with IS formation and stabilization, and further modulates expression of granzymes, perforin, and pro-inflammatory cytokines (Fig. 1.6). In mouse models, PD-1/PD-L1 interaction mediated tumour escape from NK cell immunosurveillance. PD-1^{+} NK cells could be detected in several types of both, primary and metastatic cancer[309,314], and their presence was associated with a poor prognosis[315]. The pro-inflammatory TME induces PD-L1 expression on tumour cells through different mechanisms. IFN-γ released by NK cells and CTLs can induce PD-L1 upregulation via JAK/STAT signalling[316]. Alternatively, TNF-α can modulate PD-L1 expression via NF-$_{\kappa}$B signalling[317]. This also explains why PD-L1 expression is most often seen on tumour cells in the invasive margin. Moreover, PD-L1 is stabilized by the transcription factor HIF1α[318] and through oncogene activation such as the AKT-mTOR pathway[319], MEK-ERK

signalling[320], and MYC[321,322] or KRAS[323] activity. In summary, overexpression of both, PD-1 and PD-L1 in the TME are a significant factor for immune suppression and tumour immune escape. PD-L1/PD-1 interaction inhibits NK cell and CTL activation and results in their dysfunction or exhaustion, while augmenting the development and function of T_{Regs} further contributing to immune suppression in the TME. Moreover, Azuma *et al.* have proposed that in addition to providing tumour cells with a protective shield against effector lymphocyte attack, interfering with the activating signal in NK cells and CTLs and IS formation, PD-L1 also confers resistance to apoptosis[324]. In this hypothesis, PD-L1 acts as a receptor for tumour cells that by transducing an anti-apoptotic signal providing protection against both, granule-mediated cytolysis, and death receptor-mediated apoptosis.

Similar to PD-1, **LAG-3** is induced upon NK cell and T cell activation and is associated with inhibition of the anti-tumour response[325]. It is constitutively expressed on T_{Regs} and confers a suppressive phenotype[326]. LAG-3 shares structural homology with CD4, but it binds to MHC class II (MHC-II) molecules with a higher affinity than CD4. Other suggested ligands include liver sinusoidal endothelial cell lectin (LSECtin) and fibrinogen-related protein 1 (FGL-1), which are overexpressed in some cancer types, including melanoma[327]. Their cytoplasmic domain contains a conserved KIEELE motif[328] that has been shown to be essential for the negative regulation of CD4+ T cell functions. Cancer is characterized by chronic exposure to neo-antigens and the development of dysfunctional or exhausted CTLs and NK cells. For tumour-infiltrating T cells, the exhausted phenotype has been associated with the co-expression of both, LAG-3 and PD-1, indicating a cooperation in inhibitory signalling to reduce CTL anti-tumour response[329–331]. In NK cells it has been shown that high LAG-3 expression is associated with poor cytokine production[332]. However, the exact pathways of how LAG-3 signalling modulates CTL, NK cell and T_{Reg} functions remain elusive.

The inhibitory IC **Tim-3** is expressed on all mature $CD56^{dim}CD16^{+}$ NK cells and has been suggested as a marker rather for maturation and activation of NK cells, than for exhaustion[333]. In fact, Tim-3 expression and signalling has been associated with inhibition of NK cell-mediated cytotoxicity and poor prognosis[334–337], but also increased IFN-γ production by binding to its cognate ligand galectin-9[338,339]. It has been suggested, that Tim-3 might influence NK cell sensitivity to other stimuli and that $Tim-3^{+}$ NK cells are functionally diverse, depending on the stimulus responsible for Tim-3 expression[333]. Amongst the known inducers of Tim-3 expression on NK cells are Fc: FcR interactions, certain cytokines (IL-2, IL-12, and IL-15) and NK cell susceptible tumours. In CTLs, on the other hand, Tim-3 plays a critical role in suppressing the anti-tumour response and in the induction of T cell exhaustion. Interestingly, and in contrast to other iIC, the cytoplasmic domain of Tim-3 does not contain an ITIM or ITSM. During CTL activation Tim-3 can be found in lipid rafts and is recruited to the IS. Studies have shown that HLA-B-associated transcript 3 (Bat3)[340] and the Src family PTK Fyn[341] interact with the cytoplasmic tail of Tim-3 in the absence of its cognate ligands. This results in the recruitment of Lck and the augmentation of ZAP70/LAT/PLCγ1/Ca^{2+} TCR signalling at the IS. However, in the presence of its ligand, the cytoplasmic tail of Tim-3 becomes phosphorylated[342,343]. This leads to the release of Bat3 and the dephosphorylation of Lck through recruitment of phosphatases, which negatively impacts CTL proliferation and survival[344]. A similar mechanism to modulate NK cell function is possible, because NK cell cytotoxicity is also dependent on recruitment and phosphorylation of Fyn and Lck[345].

Taken together, ICs are important modulators of the adaptive and innate immune response and affect several different immune cell subpopulations. Their effects differ depending on their expression pattern and the context of the cell-cell interaction. In the elimination phase of cancer immunoediting, the balance between co-stimulatory and co-inhibitory ICs ensures optimal CTL and NK cell priming and induction of an efficient anti-tumour immune response while preventing autoimmunity. However, during the escape phase, expression of inhibitory ICs becomes predominant and induces immune suppression and a dysfunctional anti-tumour immune response.

The targeted interaction between IC ligands and their corresponding receptors on effector lymphocytes facilitates tumour escape. In CTLs clonal expansion, cytokine production and survival can be negatively impacted, resulting in T cell exhaustion and apoptosis. In NK cells this receptor-ligand interaction can result in a modification of effector functions through differential expression of their numerous inhibitory and activating receptors, as well as expression of effector cytokines, granzymes and perforin. While ICs have a permanent effect on effector lymphocytes, their immediate potential for immune escape lies within the prevention of recognition. Through the strong inhibitory signals that are mediated by each of the IC receptors the multistep process of IS formation and function can be interrupted on several occasions. Presentation of inhibitory ICs in the context of an IS negatively impacts activating receptor signalling and accordingly the formation and maturation of the lytic IS. This is achieved either by dampening the inside-out signalling of integrins, especially LFA-1, that is required for firm adhesion of NK cells and CTLs to their target cells, or by preventing actin cytoskeleton rearrangement that is a prerequisite for IS maintenance, as well as centrosome and lytic granule polarization. Especially in NK cells, synergistic activating receptor signalling is required for induction of degranulation. And in CTLs, lytic granule polarization is dependent on TCR signalling strength. Recruitment and stabilization of inhibitory ligands on the cancer side of the IS could thus provide a potent immune escape mechanism against both adaptive and innate effector lymphocytes.

In summary, exploitation of co-inhibitory immune checkpoint expression by tumour cells enables them to escape immune surveillance and induce immune suppression. Accordingly, iICs are an interesting target for immunotherapy, especially by blocking antibodies but also through transfer of genetically modified effector lymphocytes, that aim to restore the anti-tumour immune response by releasing the brake of inhibitory ICs.

II. TUMOUR IMMUNOTHERAPY

As already described in the previous chapter, the immune system has a dual role in tumour immunity. One the one hand, effector lymphocytes are crucial for the detection and elimination of tumour cells, on the other hand this selective pressure drives the expansion of resistant tumour clones and the creation of a microenvironment that favours tumour immune escape. Studies have identified a variety of different immune escape strategies that have been described in section I. The maintenance of the cancer-NK cell immunity cycle for example requires the continuous recruitment of activated NK cells into the tumour bed that drive intrinsic inflammation, recruitment, and support cDC1s to induce an adaptive immune response, and to ultimately form a "hot" tumour. In the absence of NK cells or their inactivation in the TME, "cold" tumours arise that are characterized by the additional absence of an adaptive immune response and often the presence of immunosuppressive cell populations. To convert these cold tumours, chemotherapy, radiation, targeted therapy, or a combination of all three aimed at increasing the immunogenicity of tumours. Studies have shown that chemotherapeutics such as cisplatin or 5-fluoriuracil trigger the upregulation of activating NK cell ligands on tumour cells[346]. DNA damaging reagents have been associated with an increased expression of NKG2D ligands[71]. And in *in vivo* experiments, the cytostatic combination of MEK and cyclin-dependent kinase 4 (CDK4) and CDK6, resulted in a significant NK cell-dependent reduction of lung cancer growth[347]. However, efficient restoration of anti-tumour immunity requires additional strategies that help overcome the immune escape mechanisms that can be found in the TME and during direct cancer cell-lymphocyte contact.

Immunotherapy presents a therapeutic approach that artificially stimulates anti-tumour immunity, targeting the immune system rather than the tumour cells. Immunotherapeutic interventions exploit the presence of tumour neo-antigens and have as a goal to induce a new and/or re-activate an existing anti-tumour immune response. As discussed previously, tumours employ different strategies to escape the immune system and accordingly different types of immunotherapies have been developed. Immunotherapy can be classified as either **passive or active**, dependent on whether the therapy possesses intrinsic anti-tumour activity (passive) or engages the host's immune system (active). However, also "passive" immunotherapies spark an anti-tumour immune response from the host, making a clear distinction difficult[348]. Passive immunotherapy requires the a priori knowledge about neo-antigens that can serve as targets for monoclonal antibodies (mAbs), or *ex vivo* expanded cytotoxic lymphocytes after adoptive cell transfer (ACT). In contrast, active immunotherapies include cancer vaccinations, immunostimulatory cytokine therapy, and inhibitors of immunosuppressive metabolism. Yet, the most representative active immunotherapy are immunomodulatory mAbs, especially **immune checkpoint blockade (ICB)** that can release the brakes of tumour exerted negative immune regulation.

1. Passive immunotherapy

As mentioned before, passive anticancer immunotherapy is based on antigen-specificity, and they directly target tumour cells. As such, naked **neo-antigen targeting mAbs** where one of the first immunotherapies developed, and the first generation included the HER2/neu-targeting mAb trastuzumab, the EGFR-targeting mAbs cetuximab and panitumumab, and a mouse-human chimeric mAb targeting CD20, rituximab. These antibodies can disrupt signalling pathways that allow cancer cell to proliferate uncontrollably by neutralizing trophic signals[349–351], but also enable complement-dependent cytotoxicity (CDC)[352] and ADCC by NK cells[353,354] by opsonization, and

have immunostimulatory functions[355,356] (Fig. 2.1). Recently, it has also been shown that mAbs can initiate a potent tumour-specific CD8$^+$ T cell response that is required for tumour regression[357–359]. This secondary immune cell-mediated killing challenges the categorization of tumour-targeting mAbs as passive immunotherapy, especially since the development of so-called **"bispecific T cell engagers" (BiTEs)**, that are considered active immunotherapy[360] (Fig. 2.1). These chimeric proteins consist of two variable antibody fragments, one targeting tumour neo-antigens, and the other engaging activating receptors on effector lymphocytes. The first BiTE was developed for therapy of Philadelphia chromosome-negative precursor B-cell acute lymphoblastic leukaemia, simultaneously targeting CD19 and CD3 to harness polyclonal cytotoxic T cell potential[361,362]. Irrespective of the disputed categorization of mAbs, this tumour-targeting immunotherapy demonstrates an extraordinary success, with so far over 20 distinct tumour-targeting mAbs approved by the US FDA and the EMA[363,364].

A cell-based passive immunotherapy is **adoptive cell transfer**, that involves the (re)infusion of patient-derived and *ex vivo* selected, modified, expanded, and/or activated effector lymphocytes (Fig. 2.1). In order to improve the anti-neoplastic cytotoxicity of transferred immune cells, these cells are often genetically modified to show enhanced and unique antigen specificity[365,366], improved cytokine secretion[367], an extended lifespan of tumour-reactive lymphocytes[368], and/or increased resistance to inhibitory factors of the TME and tumour cells themselves[369]. A variant of ACT are effector lymphocytes modified to express a **"chimeric antigen receptor" (CAR)**, that combine the antigen-specific domain of a receptor with one or more activating intracellular receptor domains. This allows the generation of effector lymphocytes that are reactive to virtually any tumour neo-antigen. The most efficient intracellular signalling domain is the ITAM of CD3ζ in combination with one or more chimeric endodomains of co-stimulatory receptors or proteins, including CD28, CD27, CD134, and CD137[370,371]. The first CAR-T cell trials were focused on hematologic malignancies and designed to target CD19 on relapsed or refractory B-cell derived leukaemia[372,373]. These trials showed remarkable results with a majority of acute lymphoblastic leukaemia (ALL) patients until the age of 25 achieving complete remission (CR)[374], and a CR rate of 40-54% in adults with relapsed or refractory large B-cell lymphoma[375,376]. However, despite the elegant approach, autologous CAR-T cell therapy has its limitations. Especially chronic leukaemia is associated with T cell lymphopenia and significant CTL exhaustion that can hinder the collection of sufficient numbers of peripheral CD8$^+$ T cells. Furthermore, the production of CAR-T cells is a costly and time consuming method, making patients with fast progressing diseases ineligible, while also raising the question of cost-effectiveness especially for older patients, with the long-term clinical outcome being a critical but so far still unclear determinant[377,378]. Since CAR-T cells harbour the risk of a graft-versus-host disease (GVHD), off-the-shelf therapy of allogenic CAR-T cells would require further genetic modification, including TCR deletion. Another reported challenge is the loss of the target antigen, for example through selection of CD19-negative clones and/or downregulation of CD19 expression, a phenomenon known as antigen escape, or lineage switch of leukaemia that can collectively contribute to disease relapse under CAR-T cell therapy[379,380]. Antigen escape has been tried to circumvent by bispecific targeting, which in a first clinical trial with CD20/CD19-targeting CAR-T cells in B cell non-Hodgkin lymphoma (HL) or chronic lymphocytic leukaemia (CLL) patients showed an impressive outcome with an overall response rate of 100% and a CR rate of 92%[381]. However, in a rare but fatal case, a single leukemic cell can be harvested during leukapheresis and contaminate the CAR-T cell product, making the transduced leukaemia cell resistant to the therapy[382]. As an alternative approach and to circumvent these

challenges, recent trials used **CAR-NK cells** that do not cause GVHD in the allogeneic setting and showed promising results as an off-the-shelf ACT therapy[383] (Fig. 2.1). Allogeneic NK cells for CAR therapy can be isolated from a variety of sources, including immature NK cells with a high proliferative capacity from umbilical cord blood[384], phenotypically and functional mature peripheral blood NK cells[385], as well as induced pluripotent stem cell-derived immature, CD16^{dim}, highly proliferative NK cells[386]. Another advantage of CAR-NK cells is that through HLA mismatching, an anti-tumour immune response can still occur even after antigen loss through alloreactivity with simultaneous low GVHD[387,388]. Yet, a limitation of CAR-NK cells is their relatively short lifespan in the absence of pro-survival cytokines, and both, CAR-T and CAR-NK cells must face the immunosuppressive TME. Recently, new genetic engineering strategies to improve persistence and activity of CAR-NK cells, such as autocrine IL-2 and IL-15 stimulation, and silencing of NKG2A, have been tried and are showing promising results in preclinical settings[389,390]. Since the survival, expansion, and anti-neoplastic activity of CAR-T and CAR-NK cells largely depends on cytokines, some protocols include the administration of exogenous interleukins[391], while others studies transfer genetically modify CAR effector cells that secrete pro-survival cytokines. In both cases, ACT and especially CAR cell therapy also stimulate the host´s immune system, challenging again the

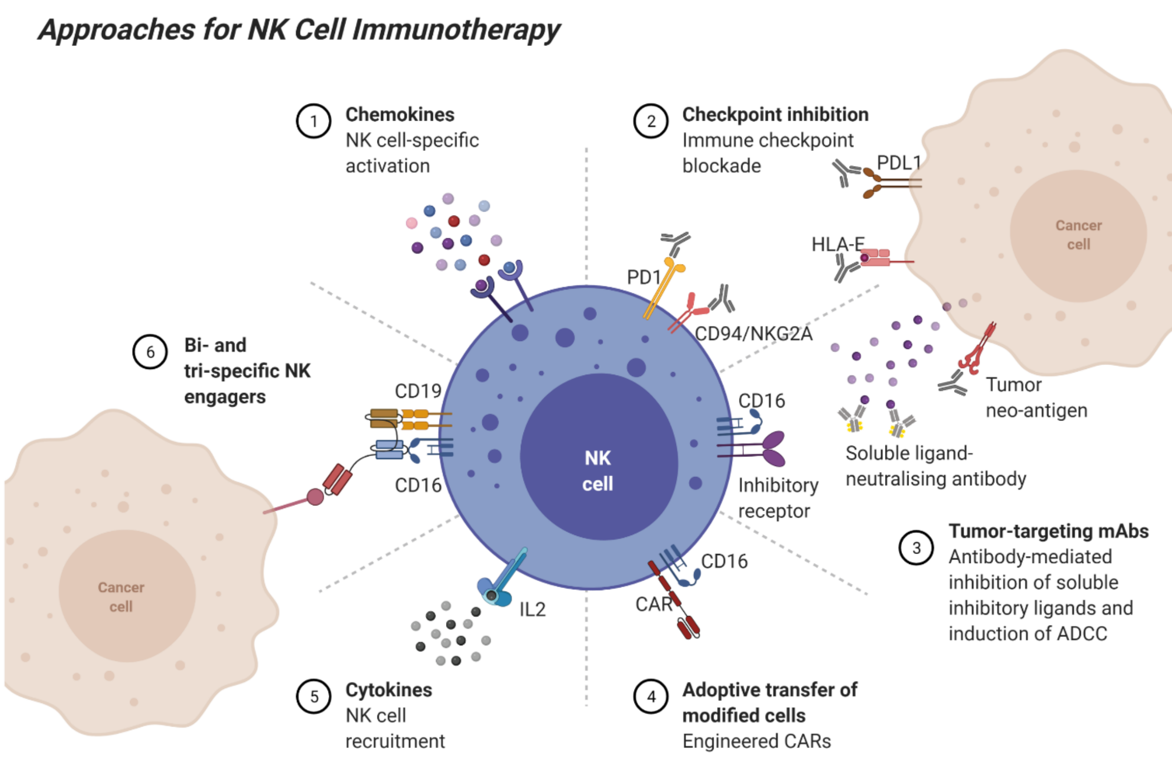


Figure 2.1 Approaches for NK cell immunotherapy. (1) Stimulatory chemokines can trigger invasion, activation and expansion of the autologous NK cells and enhance their cytotoxicity. (2) mAbs against inhibitory immune checkpoints can facilitate NK cell cytotoxicity either by preventing receptor/ligand interaction and/or by inducing ADCC against opsonized target cells. (3) Tumour-targeting mAbs can mediate ADCC through binding of CD16 to the Fc domain but can also prevent soluble ligands from interacting with inhibitory receptors on NK cells. (4) Adoptive transfer of engineered NK cells. NK cells can be obtained from PBMCs, NK cell lines, or cord blood and are designed to express chimeric antigen receptors (CAR), which allow *ex vivo* expansion and antigen-specific recognition of tumour cells. (5) Cytokines can induce maturation and recruitment of cytotoxic NK cells to the tumour bed and sustain their effector phenotype. (6) Application of BiKE or TriKE target to CD16 or NKG2D (on NK cells) and tumour antigens promotes the formation of immune synapses between NK cells and tumour cells. Created with BioRender.com

categorization of passive immunotherapy. And while CAR-ACT therapy shows remarkable results in hematologic malignancies, solid cancers are a more difficult target, not least because of difficult target antigen identification. Tumour cells show a high degree of heterogeneity and the selected antigen(s) need to be exclusively expressed on malignant cells, requiring polyfunctional CAR effector cells for successful ACT therapy[392]. In solid cancer, CAR-NK cell therapy is more prevalent, making up for about half of the currently ongoing clinical trials[393]. Other limitations are inefficient CAR-T and -NK cell trafficking to the tumour bed and the hostile and immunosuppressive TME, that currently still limit the clinical use of these therapies despite its many advantages.

2. Active immunotherapy

Through different targeted approaches, DCs and other APCs can be used in active immunotherapy. Cancer vaccines can be peptide- or DNA-based, as well as preventive or therapeutic. **Preventive cancer vaccines** have been proven to be especially successful against types of cancer that are caused by oncoviruses, such as cervical cancer and liver cancer. In fact, most cervical cancers are associated with an infection with human papillomavirus (HPV), and today it has been over a decade since the first HPV vaccine was introduced. Long-term follow up studies show not only a decline in HPV infection rates in teenagers by over 80%, but also a striking decrease in cervical precancerous lesions in young women[394–396]. Another virus infection that is associated with a higher risk for cancer is hepatitis B and prevention of HBV infection can help prevent liver cancer. Because preventive cancer vaccines target the oncovirus but do not induce an immune response against cancer cells, they are not categorized as immunotherapy. **Therapeutic cancer vaccines** on the other hand are used to treat existing cancer and stimulate anti-tumour immunity and are therefore considered immunotherapy. The principle of therapeutic cancer vaccination is based on the cross-presentation ability of DCs and their capability to induce a neo-antigen specific immune response and promote the polarization of $CD4^+$ T cells to a T_H1 phenotype to establish and maintain an anti-tumour CTL response[397]. Peptide-based cancer vaccines work by administering both full-length tumour-associated antigens and shorter peptides thereof in combination with an adjuvant to induce antigen uptake and processing as well as DC maturation. On the other hand, DNA-based vaccines utilize neo-antigen coding constructs that are either administered naked or vectored in the form of viral particles or non-pathogenic bacteria. In the case of bacterial vectors, the bacteria produce the tumour-associated antigens and serve as a source for APCs, while naked constructs or viral vectors can transform APCs or other cells. For both approaches, the selection of neo-antigens is crucial. A distinction can be made between *unique* tumour antigens, that are a result of mutations and can therefore only be found in individual tumours, and *shared* tumour antigens that are found on many tumours. Consequently, therapeutic cancer vaccines can be either individualized or an "off the shelf" therapeutic with personalized components. An example for a personalized therapeutic cancer vaccine targeting a shared tumour antigen is Sipuleucel-T or Provenge that has been approved for the treatment of hormone-refractory prostate cancer in 2010. For this treatment, patient-derived DCs are isolated during leukapheresis, loaded *ex vivo* with a fusion protein consisting of the tumour-associated antigen prostatic acid phosphatase (PAP) and the DC-maturation stimulant granulocyte-macrophage colony stimulating factor (GM-CSF), and activated DCs are then reinfused into the patient. PAP is not only a serum biomarker of prostate cancer but is also expressed on over 90% of prostate cancer cells allowing for the desired specificity in targeting tumour-specific antigens. Overall, the selection of targeted antigens defines the success of therapeutic cancer vaccines, but also presents a potential shortcoming. To reduce autoimmunity,

systemic cancer vaccines are designed to target exclusively highly immunogenic tumour neo-antigens to induce an antigen-specific anti-tumour immune response. However, by focussing on a too narrow selection of epitopes, immune selection can lead to the development of resistant cancer cells through antigen loss. Furthermore, many tumour-associated antigens are self-antigens, meaning that they are also expressed on healthy cells and that self-tolerance of T cells limits the clinical success. A way to circumvent this problem is *in situ* vaccination. In this approach, the vaccine is injected intratumoral, with the consequence of inducing cancer cell death and the release of tumour neo-antigens. Recently, also RNA-based vaccines have been developed, that introduced the concept of individualized mutanome and shared tumour-associated self-antigens vaccination in combination with injection into inguinal lymph nodes. In first clinical trials, this approach led to a robust immune response against neo-epitopes and accordingly a reduction of metastatic events and an increase in progression-free survival in metastatic melanoma patients[214]. Further clinical trials using mRNA-based vaccines are being evaluated in different types of cancer, such as triple-negative breast cancer (NCT02316457), advanced melanoma (NCT02410733), ovarian cancer (NCT04163094), and metastatic castration resistant prostate cancer (NCT04382898).

In contrast to tumour neo-antigen targeting antibodies, **immunomodulatory monoclonal antibodies** are design to interact with components of the immune system and initiate or reinstate an anti-tumour immune response. This can be achieved either through blockade of inhibitory receptors on CTLs or NK cells or their cognate ligands on cancer cells, by targeting activating receptors on effector lymphocytes, or through neutralization of immunosuppressive soluble factors in the TME. Combination of costimulatory mAbs targeting for example CD137 (4-1BB), a member of the TNF receptor family, with chemotherapy (NCT00351325), chemoradiation (NCT00461110) or either tumour-targeting (NCT01775631, NCT02420938, NCT02110082, NCT02252263) or other immunomodulatory mAbs (NCT00803374, NCT02253992) are still in clinical trials, however early studies showed promising results[398]. The success of these costimulatory mAbs has been partially attributed to the promotion of NK cell effector functions, such as IFN-γ production, improved ADCC[399,400], and facilitated NK cell-DC crosstalk[355]. Recently, costimulatory mAbs are combined with blocking antibodies, such as the bispecific antibody CDX-527, targeting CD27 as a complementary target to PD-1[401], that has been shown to enhance ADCC *in vitro*, boost vaccine efficiency, and promote direct anti-tumour immunity. While immunomodulatory mAbs are less specific than their neo-antigen targeting counterparts, their clinical efficiency most likely still relies on tumour neoantigens. This might be especially true for mAbs targeting ICs because these therapies are designed to (re)activate an anti-tumour immune response[402], while also presenting the largest and most representative group of immunomodulatory mAbs.

3. Immune checkpoint blockade

Interference of inhibitory receptor-ligand interaction through mAbs is commonly referred to as immune checkpoint inhibitor therapy or **immune checkpoint blockade (ICB)**. As mentioned in the first section, chapter 3 (“Escape”), tumour cells can exploit inhibitory immune checkpoints on effector lymphocytes to escape immune surveillance. ICB interrupts this interaction with the aim to reactivate effector lymphocytes and promote anti-tumour immunity. These therapies are considered a breakthrough in cancer immunotherapy and their relevance was further illustrated in the 2018 Nobel Prize in Physiology and Medicine that was awarded to James Allison and Tasuku Honjo for their work on CTLA-4[403] and PD-1[404] targeting mAbs. After initial studies demonstrated

the remarkable effect that CTLA-4 and PD-1 blockade had in cancer patients[405,406], fundamentally changing the outcome for patients with advanced cancer, clinical development of new ICBs accelerated drastically.

3.1 FDA/EMA-approved immune checkpoint blockade

The first ICB in clinical trials was an **anti-CTLA-4** antibody (ipilimumab; NCT00094653), that prevents its inhibitory interaction with CD28 on effector T lymphocytes and showed remarkable effects in patients with advanced melanoma [407]. In March 2011, ipilimumab was approved by the FDA for late stage (metastatic) skin cancer[408]. However, the mechanisms of action responsible for the clinical efficiency of anti-CTLA-4 blockade are diverse and not completely defined, although the understanding is that CTLA-4 signalling increases the activation threshold of T cells, and blockade of its binding to CD80 and CD86 promotes CD28-mediated signalling during antigen presentation. While it has been shown that blockade of CTLA-4 modulates T cell subpopulations, such as the expansion of tumour-specific CTLs, CTLA-4 is also constitutively expressed by T_{Reg} and is found to be strongly upregulated on $CD4^+$ T cells upon activation[409]. This suggests that targeting of CTLA-4 not only modulates $CD8^+$ T effector cell expansion and their functions, but that T_{Reg} present an additional target. Indeed, studies have shown that following anti-CTLA-4 treatment, CTLA-4 expressing T_{Regs} were selectively depleted through Fc receptor-mediated phagocytosis by macrophages[410,411]. Other studies found that anti-CTLA-4 therapy increased the population of $ICOS^{hi}$ effector $CD4^+$ T cells, resulting in a shift of the effector-to-regulatory T cell ratio in favour of effector cells and also further increasing IFN-γ production[412]. In the end, further studies are required to complete our understanding of the pleiotropic effects of anti-CTLA-4 blockade.

The second FDA-approved checkpoint inhibitor was an **anti-PD-1** mAb (pembrolizumab), that was tested in one of the largest clinical trials in oncology (NCT01295827) and approved for patients with metastatic melanoma in 2014[413], followed by nivolumab, another anti-PD-1 mAb[414], later that year. In the following years, numerous clinical trials have been initiated, testing anti-PD-1 blockade efficiency in a variety of solid tumour, including squamous cell lung cancer, renal-cell carcinoma, non-small cell lung cancer (NSCLC), colorectal cancer (CRC), and triple-negative breast cancer (TNBC)[415], but also hematologic malignancies such as HL. This (thriving) list of cancer types reflects the relevance of inhibitory immune signalling and its role in tumour escape. Constitutive PD-1 expression is only found on follicular T_{Reg}[416], but can be induced on effector T lymphocytes through stimulation of the TCR, exposure to pro-inflammatory cytokines, and TGF-β[417,418]. The PD-1 ligands PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273) are commonly found on APCs, but can also be found on non-lymphoid tissue following exposure to IFN-γ[419]. The inhibitory signalling following interaction of PD-1 with its ligands is best described in $CD8^+$ T cells and initiates through its ITIM motif the recruitment and activation of SHP-2. Accordingly, PD-1 signalling restricts simultaneous T cell activation through early TCR/CD28 signal transduction and consequently IS formation, as well as leading to reduced expression of cytokines and transcription factors involved in effector functions[420]. Like CTLA-4 blockade, anti-PD-1 treatment induces the expansion of specific $CD8^+$ T cell subpopulations[421], but further shows reactivation of exhausted effector CTLs[422,423]. It has been suggested that PD-1 checkpoint blockade works by inducing the expansion of $Tcf1^+$ $PD\text{-}1^+$ memory-like intratumoral $CD8^+$ T cells[424]. Moreover, studies have demonstrated that anti-PD-1 mAb therapy also impacts $PD\text{-}1^-$ T cell populations through induction of the transcription factor Tcf1 that is associated with enhanced proliferation and effector functions, including cytokine

production and secretion of IFN-γ, TNF-α and granzyme B[425]. For both, PD-1$^+$ and PD-1$^-$ effector T cells, Tcf1 has been identified as a critical factor for the self-renewal and memory-stimulating effects of PD-1 immune checkpoint blockade and effective immunotherapy.

Targeting the same signalling axis, **anti-PD-L1** mAbs have shown remarkable and durable responses in clinical trials. First approved by the FDA in 2016 for the treatment of bladder cancer (Atezolizumab)[426], the approval was extended to NSCLC in 2018. While the clinical results seem to mimic the effect of anti-PD-1 blockade[427], PD-L1 checkpoint blockade can further induce ADCC[428]. As mentioned before, PD-L1 expression is induced upon IFN-γ and may therefore be an indicator of an initially efficient anti-tumour immune response[429], while actively contributing to escape from immunosurveillance and local immune suppression.

3.2 Next generation immune checkpoints

Following the success of CTLA-4 and PD-1/PD-L1 checkpoint blockade, either alone or in combination, and with the understanding that these targets are limited to a subset of patients, the search for new targets has resulted in a portfolio of alternative immunomodulatory pathways eligible for ICB. Antibodies targeting inhibitory ICs are being investigated in numerous clinical trials, with encouraging results. **LAG-3** checkpoint blockade is currently being tested as monotherapy or in combination with anti-PD-1 in a variety of solid cancer types (NCT01968109), but also hematologic malignancies (NCT02061761). The physiological function of LAG-3 is the suppression of effector lymphocyte activation and cytokine production mainly in CTLs and NK cells[325,326], but it also negatively affects CD4$^+$ T cell proliferation[430]. Interestingly, *in vivo* studies suggest that LAG-3 checkpoint blockade in combination with cancer vaccination increases number and function of CD8$^+$ TILs independently of CD4$^+$ T cell activity, and results in a delay of tumour outgrowth and a reduction in tumour grade[329]. Moreover, LAG-3 can also be found on T_{Reg} in cancers such as NSCLC[431], and interaction of LAG-3$^+$ T_{Reg} with MHC-II$^+$ APC has been shown to lead to an increase in the production of immunosuppressive cytokines, including IL-10 and TGF-β[251,430,432]. Inhibition of LAG-3 is therefore another multifactorial approach to restore not only effector lymphocyte function but also modulate the immunosuppressive TME.

Another promising target is **Tim-3**, an inhibitory IC than is expressed on mature NK cells and CTLs after initial activation. Tim-3 checkpoint blockade is currently in clinical trials for solid and haematological cancer (NCT03489343) but is also being tested in combination with anti-PD-1/PD-L1 (NCT03680508, NCT03099109) or anti-PD-1 and anti-LAG-3 treatment (NCT04370704). Interestingly, LAG-3 has been identified as a central regulator of cytokine production in NK cells[433] and co-expression of PD-1 and Tim-3 on CTLs is considered a marker of severe dysfunction and terminal exhaustion[326]. Frequency of Tim-3$^+$ CTLs in cancer has been associated with a poor prognosis in NSCLC, even more than PD-1 expression, suggesting that the inhibitory signalling of PD-1 and Tim-3 is more likely to act synergistically than redundant in preventing effector lymphocyte activation. Further, high Tim-3 expression can be found on the majority of FoxP3$^+$ T_{Reg} in the tumour bed and it has been suggested that this cell population possesses superior immunosuppressive functions[434–436] and their presence in lung cancer is an indicator for metastasis and poor prognosis[437]. This supports the hypothesis that inhibition of Tim-3 could potentially increase effector function in both effector lymphocyte populations, while inhibiting the suppressive functions of Tim-3$^+$ T_{Reg}, especially in combination with other ICB treatments.

A third inhibitory IC being tested in clinical trials is **TIGIT** (NCT04150965, NCT04047862). This receptor binds with a high affinity to CD155, preventing its interaction with the activating receptor DNAM-1 (CD226)[326]. This signalling pathway consisting of an activating and inhibitory receptor for the same ligand is reminiscent to the interaction between B7 and CD28 or CTLA-4. In addition to suppressing effector functions in CTLs and NK cells through its intracellular ITIM motif and recruitment of SHIP1 to the IS that results in inhibition of IS formation[135,136], binding of TIGIT to CD155 on the surface of DCs promotes IL-10 production and adoption of a tolerogenic phenotype[438]. In cancer, TIGIT expression can be found on terminally exhausted PD-1$^+$ Tim-3$^+$ LAG-3$^+$ CTLs, but also on highly immunosuppressive tumour-resident T_{Reg} populations. Combination of anti-TIGIT mAbs with ICB targeting PD-1/PD-L1 has been shown to work synergistically in restoring CTL proliferation and anti-tumour functions in both, mouse models and patients[439,440]. It has been suggested that TIGIT enhances the immunomodulatory effect of T_{Reg}[303] and marks a specific subset of immunomodulatory cells that suppress the proinflammatory Th1 and Th17 immune response[302], and thus TIGIT checkpoint blockade could promote anti-tumour immune response by reversing CTL exhaustion while simultaneously inhibiting T_{Reg} functions.

Alternative approaches explore activating ICs as therapeutic targets for immunotherapy. Manipulation of one such immune regulatory pathway could potentiate immunotherapy efficiency by stimulating the anti-tumour immune response from different immune cell populations. An activating IC that is currently under clinical investigation is the **ICOS/ICOS-L** axis. This receptor shares homology with CD28 and CTLA-4 and its signalling has been mainly associated with the production and secretion of cytokines and development of effector and memory T cell subsets. Interestingly, in patients treated with anti-CTLA-4 mAbs or cancer vaccines, an increase in the CD4$^+$ ICOS$^+$ and CD8$^+$ ICOS$^+$ T cell populations was observed, leading to an increase in the effector-to-regulatory T cell ratio in the TME[441]. Moreover, expression of ICOS-L is not limited to professional APCs[442], but can be induced through TNF-α on somatic cells, including cancer cells[443–446]. However, ICOS expression is not exclusive to CD4$^+$ T helper cells and CD8$^+$ CTLs, but the highest expression can be found on tumour-resident FoxP3$^+$ T_{Reg} on which binding to its ligand induces production of the immunosuppressive cytokines IL-10 and TGF-β and that have been shown to promote conversion of CD4$^+$ T cells to a immunosuppressive regulatory phenotype[447,448]. Studies have suggested an important role of this FoxP3$^+$ ICOS$^+$ T_{Reg} subpopulation for sustained immunosuppression in the TME of different cancer types[449,450]. Accordingly, and in dependence on the target population, induction of the ICOS/ICOS-L pathway shows either promotion of anti-tumour functions (effector CTLs and Th1 T cells), or an enhanced immunosuppressive function and proliferation of pro-tumour T_{Reg}. Because of these observations, both agonistic and antagonistic mAbs are currently being tested in clinical trials, either alone (NCT02520791), or in combination with CTLA-4 or PD-1/PD-L1 targeting therapies (NCT02904226, NCT03989362, NCT04128696, NCT03693612). The rationale behind combinational therapy is that *in vitro* experiments and preclinical studies demonstrated an additive effect of agonistic anti-ICOS mAbs with CTLA-4 checkpoint blockade that has been attributed to the expansion of CD4$^+$ FoxP3$^-$ ICOS^{hi} T cells and ICOS^{hi} CD8$^+$ CTLs[451,452]. However, clinical trials will have to determine if targeting of the ICOS/ICOS-L pathway with agonistic or antagonistic mAbs holds more promise as a variety of concerns regarding the signalling pathway and its impact on the anti-tumour immune response remain unanswered.

3.3 Immune checkpoint blockade and the role of NK cells

First generation IC inhibitors and other novel targets for ICB were designed with the understanding that CTLs are responsible for the strongest and most specific anti-tumour immune response and with the aim to reactivate effector T cell, considering this cell population the most important for anti-tumour immunity and successful immunotherapy. However, this narrative underestimate the role of NK cells during anti-tumour immunity. In general, intratumoral NK cell frequency is a critical determinant for durable response to ICB, partially through recruitment of DCs, but also through initiation and maintenance of the cancer-immunity cycle[453] (Fig. 1.5). At the same time is the role of canonical ICs, including CTLA-4, PD-1, and LAG-3 for the modulation of NK cell function less understood, and their expression – except for PD-1 on a subset of mature CD56^{dim} NK cells[308] – low even under pathophysiological conditions. PD-1$^+$ NK cells have been shown to possess weaker secretory function as well as reduced degranulation efficiency[310] and consequently PD-1 checkpoint blockade could also restore NK cell anti-tumour functions and increase the overall immune response, as shown in *in vivo* studies[309]. But the role of PD-1 on NK cells is still a subject of debate and while some studies have shown the presence of PD-1$^+$ NK cells[454] and clear clinical implications for NK cells in PD-1/PD-L1 checkpoint blockade[309], other groups claim that NK cells lack PD-1 presentation under physiological and pathophysiological conditions[312]. It is possible that the NK cell-dependent clinical effect during PD-L1 checkpoint blockade is the results of ADCC rather than release of inhibitory signalling during NK cell activation or IS formation, or that activating signalling through CD16 tips the balance between inhibitory-to-activating signal just enough to induce NK cell-mediated cytotoxicity by promoting ADCC[455–458]. In general, it could be speculated that the canonical CTL-associated ICs play a less prominent role in the control of NK cell tolerance.

Notably, Tim-3 and TIGIT expression has been reported for mature NK cells and these receptors can be considered major ICs for NK cells[133,334,134,459]. TIGIT can be considered a marker of NK cell exhaustion and restoration of T cell anti-tumour immunity in response to TIGIT checkpoint blockade has been suggested to be partially NK cell dependent[138]. The role of Tim-3 on NK cells on the other hand seems to be dependent on the initial activation stimulus, with a functional diversity of Tim-3$^+$ NK cells making it difficult to categorize it as an inhibitory IC[333]. While studies connect Tim-3 expression on NK cells with reduced anti-tumour activity, exhaustion and a modulation of receptiveness to stimuli[335,336,460,339], other groups have demonstrated an increased IFN-γ production from Tim-3$^+$ NK cells[338]. So, while the role of these ICs in regulating NK cell functions is only partially understood, NK cells are likely to contribute to the success of ICBs targeting Tim-3 or TIGIT.

For cancer cells to grow into a clinically detectable tumour, immune escape is indispensable[17]. Induction of NK cell tolerance can be achieved by engagement of major NK cell-specific inhibitory ICs. As a result, new ICBs are designed to target inhibitory NK cell receptors, such as the **KIR**-targeting mAb lirilumab. First clinical studies in patients gave no indication that KIR blockade would lower the NK cell activation threshold against healthy cells or trigger fraternal killing of NK cells[461]. However, a recently completed clinical trial in patients with AML showed no clinical benefit of anti-KIR blockade over placebo (NCT01687387). Despite this, further clinical trials are testing lirilumab in combination with PD-1 blockade in different types of cancer (NCT03203876, NCT01592370, NCT03532451). Still, tumour progression is often associated with a downregulation or loss of classical MHC-I molecules on cancer cells that serve as ligands for KIRs and treatments such as

lirilumab could fail to show the expected clinical success due to a reduced relevance of KIR signalling especially in the late stage of tumour development.

Considering the different relevance of canonical ICs in regulating CTL and NK cell anti-tumour functions, ICB blockade aimed at NK cells plays mainly a role in immunotherapy of low immunogenic tumours that do not invoke a strong antigen-specific T cell response[462], and hematologic malignancies, that occupy the same biological niches as NK cells[463]. Targeting of NK cell receptors does not necessarily have to act on inhibitory ICs, but can include tumour-targeting antibodies, such as rituximab or trastuzumab that have been described to enhance NK cell recognition and lysis of tumour cells through ADCC. Other tumour targeting antibodies can block NK cell-relevant iICs. In this context, targeting of the non-classical MHC-I **HLA-G** on tumour cells can prevent its interaction with the inhibitory receptors ILT-2 and KIR2DL4 and stabilize lytic IS formation in NK cells[464,465], while further inducing ADCC. HLA-G is physiologically expressed during foetal development and in immune-privileged tissues, but an increasing amount of studies find HLA-G upregulated in tumour tissue[466,467] as a reaction to a hypoxic TME[468], where it even at low expression rates mediates efficient immune evasion[469,470]. The key role of HLA-G for immune escape cannot simply be explained by inhibition of cytolytic NK cell functions. Indeed, ILT-2 can be found on antigen-specific CTLs, $CD4^+$ T cells, B cells, DCs, and MDSCs. In these immune cell populations, HLA-G/ILT-2 interactions leads to an inhibition of cytolytic functions, proliferation and IFN-γ production (CTLs), adaption of the immunosuppressive $FoxP3^+$ T_{Reg} phenotype with increased production of IL-10 and TGF-β ($CD4^+$ T cells), switch to a DC-10 phenotype that is associated with high IL-10 production (DCs), CTL and NK cell anergy and together with MDSCs mediates immune cell immaturity through CD80, CD86, and MHC-II, as well as inhibition of antibody production from B cells[471]. The inhibitory effect of HLA-G can be further enhanced by trogocytosis, through which $CD4^+$ T cells and T_{Reg}, but also CTLs can acquire HLA-G, reversing their function from effector to regulatory cell[472–474]. Especially in haematopoietic cancer, HLA-G expression is described as a biomarker for shorter progression-free survival[475,476] and a high risk for cancer relapse or transformation[477]. Further studies showed that HLA-G expression prevents NK cell cytotoxicity, even in natural cytotoxicity models with a KIR^- $NKG2A^-$ $NKG2D^+$ phenotype, such as the NK-92 cell line[465,478,479]. Indeed, HLA-G is the rare case of a major inhibitory IC, while also acting as a tumour neo-antigen, making it an ideal candidate for targeted immunotherapy. Considering this, targeting of HLA-G is currently being investigated in clinical trials (NCT04300088, NCT04485013).

Alternatively, tumour cells can up-regulate non-classical MHC-I molecules that engage iICs, such as **HLA-E** the ligand for CD94/NKG2A. More than any other MHC-I, HLA-E can be found to be upregulated on cancer cells and therapeutic strategies to down-modulate or block HLA-E are expected to show great potential in reactivating an efficient anti-tumour immune response by reactivating the anti-tumour activity of NK and NKT cells, but also CTLs. As other non-classical MHC-I molecules, HLA-E cannot only be found on the surface of tumour cells, but is also shed and present as a soluble factor in the TME and the circulation where it can serve as a biomarker for fast disease progression[480–482]. A limitation of HLA-E targeting commercial mAbs was that they were not specific for peptide-loaded HLA-E but also recognized shared epitopes with classical MHC-I (HLA-A, -B, -C), as demonstrated by a recent throughout study[483]. *In vitro*, treatment with monospecific mAbs for HLA-E results in an increased proliferation of activated $NKG2A^+$ $CD8^+$ CTLs, while also preventing interaction of HLA-E with its receptors, possibly restoring tumour cell recognition and lytic IS formation. However, *in vivo* NKG2A expression on TILs is restricted to $PD\text{-}1^{hi}$ CTLs[484], indicating the

need for combinational immunotherapy, and since HLA-E can further serve as a ligand for the activating receptor NKG2C, alternative targeting of its inhibitory receptors NKG2A and NKG2B on NK cells and CTLs might be more beneficial.

Accordingly, research has also explored to role of **anti-NKG2A** mAb such as monalizumab that prevents the interaction of this major inhibitory IC found on NK cells and subsets of activated CD8⁺ T cells[485]. Expression of HLA-E is increased in cancer as a consequence of immuno-editing and high IFN-γ levels in the TME[486,487], and a high expression has been associated with a poor post-operative prognosis in lung, breast, colorectal, gastric cancer and glioblastoma[62,488,489]. This indicates a critical role of the HLA-E/NKG2A signalling axis for NK cell-mediated immunosurveillance, but also CD8⁺ T cell functions[55,490], and a rationale for the development of anti-NKG2A checkpoint blockade, because HLA-E itself is difficult to target and has, as mentioned above, also activating functions. Several clinical trials are currently underway, testing this mAb in combination with other immunomodulatory mAbs and ICBs (NCT03794544, NCT02643550, NCT02671435, NCT04307329). In mouse models, anti-NKG2A blockade in combination with a PD-L1-targeting mAb or neo-antigen targeting mAb showed additive effects and promoted effector functions in both, NKG2A⁺ NK cells and NKG2A⁺ PD-1⁺ CD8⁺ T cell, besides enhancing IFN-γ production by NK cells and ADCC[485,491]. In another study, a combination of small kinase inhibitors targeting CDK4, CDK6 and MAPK increased susceptibility of lung cancer cells to NK cell cytotoxicity through the induction of ICAM-1 and the NKG2D ligands ULBP2 and MIC-A as a consequence of induced senescence[347]. Interestingly, senescent cells have been shown to evade destruction by NK cells and CD8⁺ T cells through the upregulation of HLA-E, an escape mechanism that could be reverted through NKG2A blockade[492]. In summary, these study results imply a plethora of possibilities to not only recruit NK cells to the tumour bed, but also harvest their full potential for anti-tumour immunity using mAb.

3.4 Limitations of immune checkpoint blockade therapy

ICB revolutionized (and maybe even revived) the field of solid tumour immunotherapy and shows remarkable results in some patients, while also changing the way clinicians and researchers evaluate treatment and new therapeutic approaches. While preclinical parameter such as tumour mutational burden (TMB) or expression of ICs such as PD-L1 were historically mostly associated with a poor prognosis and resistance to standard or targeted therapy, they are now used as indicators for a favourable response to immunotherapy[415,493]. Even more, FDA-approval of PD-1 ICB was given for tumours with a high TMB due to microsatellite instability, without specification of the type of tumour, because this oncologic mechanism of immune evasion was found to be conserved regardless of the tumour type[415]. The combination of chemoradiation, to increase the neo-antigen expression in tumours, and immunotherapy has been a step forward in further accelerating the efficiency of ICB. One of the most remarkable successes of PD-1 checkpoint blockade is the long-term remission observed in about 20% of late-stage melanoma patients[494].

However, immunotherapy became a victim of its own success, due to an easy-to-understand concept and coverage in mainstream media that raised hopes in cancer patients, but responses differ greatly between cancer types and even between patients diagnosed with the same type of cancer. While the overall response rate (ORR) in treatment-naive melanoma patients to PD-1 checkpoint blockade has been reported to vary between 33 and 36%[495,496], and patients with NSCLC or renal cell carcinoma (RCC) showed ORR of 41 and 21%[497,498], respectively, patients with HL had ORR rates of over 60%[499]. And in a multiple tumour type clinical study, the ORR to anti-PD-1

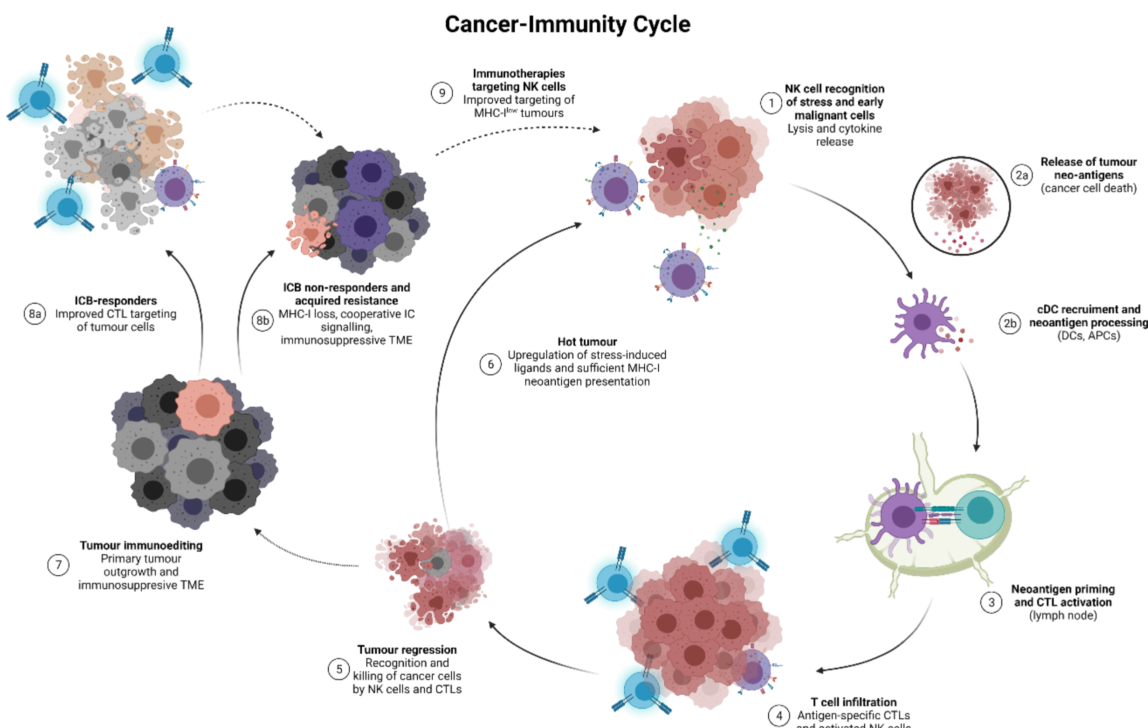


Figure 2.2 Cancer immunity cycle and the impact of immune checkpoint blockade. The administration of ICB can result in restoration of anti-tumour immune response (8a), leading to clearance of the tumour or reconstitution of tumour immunosurveillance. But most patients are non-responders or show acquired resistance to ICB (8b) either due to inefficient MHC-I expression, cooperative iIC signalling that is not completely prevented by mono-ICB, and the challenges that are imposed by the immunosuppressive TME. NK cell-based immunotherapies could help convert cold tumour or improve targeting of MHC-I^{low} tumours by ICB (9). Created with BioRender.com

monotherapy was reported to be 29%[500]. And numbers for monotherapy with ipilimumab (anti-CTLA-4 mAb) are even lower, as shown by clinical studies in previously untreated melanoma patients and NSCLC patients that reported ORR of 13 and 24%[495,501], respectively. Furthermore, the duration of the clinical response to ICB monotherapy can differ greatly. Overall, non-responders or relapse after an initial response are still the majority, with about two-third of patients not responding at all (**primary resistance**) or developing resistance (**acquired resistance**) to ICB[502] (Fig. 2.2). A good example for this is TNBC, a type of cancer that is defined by the lack of hormone receptors (oestrogen and progesterone receptor) and the growth factor receptor HER2/neu. This type of cancer accounts for about 15 to 20% of all breast cancer diagnosis and is characterized by an aggressive growth and fast development of resistance to the standard treatment[503]. Several key characteristics of TNBC suggest that this type of cancer would be an ideal candidate for ICB, including high number of TILs[504], expression of PD-L1 on tumour cells and immune cells[505], and a high TMB[506]. Still, initial response rates of TNBC to ICB monotherapy are low and also combination of PD-1 blockade with chemotherapy as a first line of treatment demonstrated efficiency only in about 20% of TNBC patients[507]. Further clinical trials are ongoing, with preliminary results indicating an increase in the complete response rate (CRR) of about 40%, but only in certain subgroups of patients.

Besides the identification and confirmation of new targets for ICB, combinational immunotherapy seems to be a promising approach to increase ORR rates. Especially **dual ICB**, the combination of mAbs targeting two different immunomodulatory targets, is an interesting option with more than

1000 clinical trials currently testing different combinations of ICB[508]. *In vivo* studies have demonstrated that tumour growth can be significantly reduced by dual IC treatment while monotherapy failed to demonstrate a response in murine models of melanoma and colon cancer[331]. A possible explanation for resistance to IC monotherapy could be a compensatory upregulation of other inhibitory ICs. For example, in murine models of ovarian cancer, compensatory signalling between PD-1, LAG-3, and CTLA-4 has been demonstrated to be responsible for resistance to ICB monotherapy[509], and compensatory upregulation of PD-1 and Tim-3 has been attributed to ICB resistance in a murine model of lung cancer[510]. Moreover, in patients with advanced melanoma, a similar compensatory mechanism has been discussed[511], while the exact mechanism of how inhibitory IC cooperation is involved in immunotherapy resistance remains unexplored so far. In addition to combinational ICB treatment, **bispecific antibodies** are tested in clinical trials (NCT03440437, NCT03708328). The best investigated group of this type of antibodies are designed to redirect nonspecific CTL cytotoxicity to malignant cells through crosslinking of CD3 and tumour neo-antigens, but bispecific antibodies engaging CD16 and tumour neo-antigens to trigger NK cell-mediated cytotoxicity are also in preclinical and clinical trials[512] (Fig. 2.1). Other concepts explore the potential of bispecific antibodies to activate immunomodulatory receptors of tumour-resident immune cells, such as APCs through CD40 or effector lymphocytes via 4-1BB, to induce an anti-tumour immune response[513]. The third group of bispecific antibodies combine the targeting of two IC receptors to achieve additive or synergistic effects, but preclinical trials are required to determine whether this treatment is superior to dual ICB[512].

It has been suggested that novel therapies are required to target intrinsic characteristics of cancer cells that enable immune evasion to further accelerate the clinical success of ICB[141,161,514]. Intrinsic immune evasion strategies of cancer that interfere with the very core of the anti-tumour immune response, the formation of the lytic IS between cancer cells and effector lymphocytes, can undermine the efficiency of immunotherapy. New biomarkers to predict efficiency of ICB or suggest new combinational targeted therapy are therefore required to optimize immunotherapy and meet the high expectations of cancer patients. But even then, cancer treatment will not be completely resolved, as other obstacles, such as tumour heterogeneity, the immunosuppressive TME, and immunoselection provide the next hurdle in the race against cancer.

III. ACTIN CYTOSKELETON

Together with microtubules and intermediate filaments, filamentous actin (F-actin) makes up the cell's cytoskeleton. All three components are capable of fast reorganization and create a dynamic network that is found to be conserved in plants, prokaryotes and eukaryotes[515]. While the primary function of the cytoskeleton are cell shape and stabilization of tissues, especially remodelling of the actin cytoskeleton facilitates and fine-tunes essential cellular functions, including cell division, migration, phagocytosis, but aberration of its dynamics are also involved in malignant transformation, invasion, and metastasis[516]. The assembly of actin filaments in bundles and networks is orchestrated by the coordinated recruitment and function of actin binding proteins (ABPs) in response to external signals. Moreover, the cortical actin cytoskeleton serves as a scaffold for signal transduction and tethering of signalling complexes to the peripheral cell membrane, facilitating fast and sustained signalling of transmembrane receptors.

The main objective of my PhD thesis is to investigate how the fast dynamics of the actin cytoskeleton in tumour cells can contribute to their intrinsic resistance against cytotoxic lymphocytes, especially NK cells. In the following, I will give a short introduction to the regulation of actin cytoskeleton dynamics and its implications for immune evasion.

1. Actin cytoskeleton assembly and function

Actin exists in three isoforms with distinct functions. While α-actin is found in cardiac, skeletal and smooth muscle cells as a major component of the contractile mechanism, γ-actin can be found in smooth muscle and non-muscle cells, and expression of cytoplasmic β-actin is restricted to non-muscle cells[517,518]. The existence of these isoforms is also one answer to the questions of how actin can fulfil such a large range of cellular functions.

1.1. Actin polymerization

Actin is also the most abundant protein in eukaryotic cells and exists in a monomeric globular form (G-actin) and as F-actin that is comprised of two strands of polymerized G-actin, with a pointed (-) end and a fast-growing, barbed (+) end. Along with the identification of these two forms[519–521], Straub and Laki and colleagues could demonstrate that monomeric G-actin exists in an ATP-bound state, that is hydrolysed during polymerization into F-actin[522,523]. Scanning electron microscopy and X-ray crystallography suggest that a single G-actin monomer consists of an α- and β-domain, also called small and large domain, that are separated by a cleft[524,525]. ATP with a bound bivalent cation such as Mg^{2+} or Ca^{2+} can bind to the cleft between the subdomains SD2 and SD4, that represents the so-called ATPase fold[526,527]. Also located between SD2 and SD4 exists a hydrophobic patch that is the primary binding site of ABPs[528]. Interaction between actin subunits is mediated by the hydrophobic loop between SD3 and SD4 that allows contact with the SD2 and SD1 of other F-actin subunits[529–531]. Dependent on its ATP- or ADP-bound state, G-actin exhibits different conformations that alter the binding strength between subunits and affinity of ABPs.

Polymerization of actin can occur spontaneous *in vitro* in the presence of ATP and bivalent cations and is a three-step process. The first and rate-limiting steps is the nucleation phase that is initiated through the formation of a G-actin dimer, followed by addition of a third G-actin monomer to form an unstable trimer[532]. During the following elongation phase, ATP-bound G-actin dimers and monomers bind to the barbed end, resulting in fast growth of actin filaments. Interestingly, ATP hydrolysis does not seem to be a requirement for F-actin assembly. Instead, conversion of ATP into ADP occurs within the assembled filaments, shortly after G-actin integrates into the growing end of

the filament. Currently there are two hypothesis trying to explain this thermodynamically unfavourable observation: the stochastic model suggests random ATP hydrolysis influenced by neighbouring molecules, and the vectorial hypothesis suggests that ATPase function of G-actin is initiated adjacent to ADP-bound molecules[533,534]. In either case, the hydrolysed P_i remains covalently bound to ADP-actin for some time, resulting in three actin forms within the double-stranded F-actin structure: ATP-actin at the barbed end, ADP+P_i-actin in the intermediate part of the filament, and ADP-actin at the pointed end. The third and final step is the stationary or equilibrium phase. During this phase, F-actin growth and therefore filament stability is dependent on the critical concentration (C_C) of freely available ATP-bound G-actin, so the ratio between addition and elimination of G-actin from the actin filament that does not result in a change in filament length. Because F-actin is a polar structure, the pointed end is enriched in ADP and disassembles at a C_C of approximately 0.7 µM, while the barbed end has a C_C of 0.1 µM[535]. Therefore, the rate of loss at the (-) end is balanced by growth of the (+) end and no net growth of F-actin occurs. This turnover of actin filaments is called tread milling and is the responsible microforce for cell migration, endo- and exocytosis, and cell division[536]. Importantly, ABPs can alter the C_C, resulting in changes in F-actin assembling dynamics and structure generation.

1.2. Actin cytoskeleton in biological processes

Besides its role in the contractile apparatus of muscles, F-actin structures play a role in a large list of cellular functions. One of these that is reminiscent of muscle contractility is **cytokinesis**. In eukaryotic cells, the last step of cell division, the physical separation of the cells, is dependent on the assembly of a contractile ring of actin and myosin. The core of this cytokinetic ring is comprised of linear β-actin filaments that are generated through the interplay of different ABPs, such as the Arp2/3 complex and formins in a Ras homolog family member A (RhoA)-dependent manner[537–539].

Another biological process, that exploits the contractility of the actin cytoskeleton through interaction with myosin, as well as the pushing force generated during F-actin elongation, is **cell motility**. Migrating cells are characterized by a high degree of diversity of their actin cytoskeletal organization. In reaction to external stimuli, motile cells form planar lamellipodia at their leading edge that consists of a branched network of F-actin, from which individual bundles of F-actin extend beyond the edge of the cell. These protrusions are filopodia, which consists of uniformly oriented F-actin bundles with their barbed end pushing against the cellular membrane. While in theory these structures could extend indefinitely, their elongation is a challenging task that requires the activity of certain ABPs, such as fascin and Ena/VASP, but also the consistent delivery of G-actin and ABPs to the tip of the filopodium where the elongation occurs. Filopodia are believed to guide the direction of cell motility through interaction with other cells or the extracellular matrix (ECM). The integrin-enriched tip of filopodia through which these protrusions can form focal adhesions with the ECM facilitates this probing. It has been shown in migrating leukocytes, that the strength of this adhesion is dependent on ezrin/radixin/moesin (ERM) adaptors that connect the cortical actin to the cytoplasmic domain of transmembrane proteins such as integrins[540]. The outside-in signalling of integrins is further mediated by linker proteins, such as vinculin and talin, but also integrin-linked kinase (ILK) and focal adhesion kinase (FAK) that increase both the size and the strength of the adhesive complex[541]. Through recruitment of ABPs these proteins initiate spreading of the actin meshwork into the direction of the adhesion and thus defining the direction of the crawling motion[542]. In the following step, contractile stress fibres, bundles of F-actin and non-muscle myosin that are attached to focal adhesions through integrin-associated proteins, induce the retraction of

the rear of the cell, allowing the cell to move forward[541,543]. While cells studied *in vitro* on solid 2D-substrates demonstrate exaggerated stress fibres and focal adhesions, the actin cytoskeletal organization of migrating cells in more physiological, complex 3D-matrices changes. Fibroblasts, that migrate through the ECM along collagen fibres and remodel the ECM, show especially strong stress fibres when exposed to a stiff surface, an observation that has been hypothesized to be linked to their attempt to remodel an unresponsive substrate[543,544]. Also in cancer cells, 3D matrixes induce the formation of special actin-enriched structures, so called invadopodia (or podosomes). These structures are associated with ECM degradation and invasion of cancer cells, facilitating metastasis[545].

The process of programmed cell death, **apoptosis**, is regulated by caspases, but studies have implicated a regulatory role of the actin cytoskeleton both preceding and after caspase activation[546–549]. The dynamic turnover of the actin cytoskeleton has been shown to be associated with longevity, while a decreased F-actin turnover can promote ageing and even cell death[550]. Stabilization of the actin cytoskeleton through drugs such as jasplakinolide results in a fast accumulation of F-actin and has been shown to induce or accelerated apoptosis upstream of caspase activation[551,552]. This has been associated with a reduced mitochondrial membrane potential as a consequence of open voltage-dependent anion channels and release of pro-apoptotic proteins, increasing the cells sensitivity to apoptotic stimuli. But also treatment with the actin depolymerizing drug cytochalasin D induces apoptosis[553,554], indicating that prevention of F-actin turnover rather than alteration of the state of the actin cytoskeleton results in induction of apoptosis. Accordingly, the actin cytoskeleton has been suggested to be both initiator and mediator of the apoptosis process[555]. While apoptosis can be triggered by a variety of external and internal signals, the associated morphological changes are very consistent, including cell rounding and membrane blebbing as a consequence of RhoA signalling but also through caspase-mediated activation of downstream targets of RhoA[548,549,556]. Interestingly, actin is also a target for cleavage by caspases. Cleavage of actin and its associated anti-apoptotic regulator gelsolin by caspases does not only results in production of 31 kDa and 15 kDa actin fragments, but also destruction of gelsolin, accelerating apoptosis-associated morphological changes and apoptosis progression through unregulated severing of the actin cytoskeleton[550]. Studies have shown that apoptosis induction through CD95 (FasL) is dependent on ezrin-mediated interaction with the actin cytoskeleton, with ezrin being associated with the intracellular FERM domain of CD95[557,558]. However, other studies have demonstrated an increase of CD95-mediated apoptosis as a consequence of ezrin knockdown, suggesting a negative regulatory role of ezrin in translating CD95 apoptosis induction, contradicting previous results[559]. Since the signalling of ezrin can be influenced by its phosphorylation status, its function in regulating apoptosis might also be phosphor-regulated. The de-regulation of apoptosis is a hallmark of cancer and enables their survival and invasion. Gelsolin expression has been suggested to be dysregulated in cancer cells, enabling their survival[560]. Cleavage of gelsolin into its pro-apoptotic N-gelsolin form can still occur in a caspase-3 dependent manner, but association of the ABP cofilin with actin abolishes the actin-DNase I complex severing function of N-gelsolin, retaining DNase I in the cytoplasm and thus preventing DNA fragmentation[561]. And dysregulated expression of cofilin, as well as other ABPs, has been reported for several types of solid cancer and is often associated with a poor prognosis and chemoresistance[562–566].

As suggested previously, aberrant actin cytoskeleton dynamics are commonly found in cancer cells and altered expression of actin isoforms and ABP have been suggested to play a role in metastasis

and resistance to chemotherapy. Viscoelastic properties of malignant cells differ from healthy cells and their mechanical properties, that allow metastatic invasion, are the consequence of fast adaption of their actin cytoskeleton to external stimuli[567]. A critical process enabling metastasis is **epithelial-to-mesenchymal transition (EMT)** during carcinogenesis. This leads to a loss of intercellular bonds, adaption of an elongated morphology and an enhanced cell motility through amoeboid migration. EMT has been associated with the abnormal expression of different ABPs, including the ERM protein moesin[568], Rho GTPases[569,570], and formins[571,572], as well as α-smooth muscle actin and the intermediate filament vimentin[573]. Interestingly, while hypoxia and cell density have been suggested to be drivers of EMT, a recent study found that EMT did not occur under these conditions unless actin cytoskeleton remodelling was induced in cancer cells, either through intrinsic or external stimuli[574]. Recently, the role of EMT in chemoresistance is being investigated, since research on breast and pancreatic cancer has suggested no increase in metastatic potential but acquired resistance to chemotherapeutic agents following EMT[575,576]. This is supported by the observation that the function of the EMT transcription factor Twist1 in single-cell dissemination is dependent on intact cell-cell adhesions[577], suggesting that the link between EMT and metastatic invasion and chemoresistance might be highly context dependent.

2. Actin-binding proteins

The most commonly found F-actin structures are bundles and networks that have functional similarities in providing the supportive framework that defines the cellular shape but differ structurally. By engaging ABPs, F-actin is assembled in these structures and organized into functional higher-order networks in response to specific signalling pathways. ABPs can be grouped by their function and in the following some of the best studied actin regulatory proteins will be introduced.

If *in vitro* observations were applied to cellular concentrations of G-actin only a fraction (< 1%) of ATP-G-actin would remain freely available while the vast majority would polymerize into a static network[578]. To maintain a sufficient pool of ATP-bound G-actin for fast actin cytoskeleton remodelling, **monomer-binding proteins** regulate cellular G-actin concentrations. Such a protein that is conserved in all eukaryotes is profilin. By binding to the barbed end groove of G-actin between SD1 and SD3, profilin prevents nucleation and elongation at the pointed end of F-actin, while it has a weak affinity to ATP-actin on the barbed end[579]. Further, profilin has been shown to induce nucleotide exchange by reducing the affinity of G-actin for ADP resulting in faster dissociation of ADP from depolymerized monomeric actin and thus increasing the pool of ATP-G-actin[580]. As explained below, profilin-actin complexed are integrated into growing F-actin by proteins such as formin and other proline-rich formin homology 1 (FH1) domain containing proteins, including WASP and VASP. Thymosin-β4 is one of the most abundant actin monomer binding proteins in many tissues and has functional overlaps with profilin. Through its high affinity to bivalently bound ATP-G-actin and steric hindrance that prevents polymerization, thymosin-β4 maintains a pool of non-polymerized ATP-G-actin[581].

As described before, nucleation can occur spontaneously, but is a thermodynamic unfavourable process until a G-actin trimer is formed. Spontaneous F-actin nucleation is a slow process, while F-actin assembly and turnover in cells occurs on timescales of tens of seconds. To substitute for this slow actin nucleation and overcome the competition with profilin and thymosin-β4 for monomeric ATP-G-actin, **actin nucleation proteins** ensure fast initiation of filaments either *de novo*, branching from the side of existing filaments, or through severing of F-actin. Different proteins promote each

of these modes of actin nucleation. In eukaryotic cells, the main nucleating proteins are formins and the actin related protein 2/3 complex (Arp2/3). Formins are a group of multidomain proteins that are characterized by the presence of at least one FH domain (FH1, FH2, and FH3), but can contain other domains such as Wiskott-Aldrich syndrome homology region 2 (WH2 motif/domain), GTPase binding domain (GBD)[582], and diaphanous auto-inhibitory domain (DAD)[583]. The proline-rich FH1 domain can interact with profilin-actin complexes at the barbed end of F-actin, indicating a role in actin filament elongation. The FH2 domain is not only required for homodimerization of formins but is also required for their actin nucleation-promoting activity[584], as well as interaction with and stabilization of microtubules[585]. Formin activity can be auto-inhibited through binding of DAD to the N-terminal GBD; an interaction that is released upon binding of small Rho GTPases to the GBD and subsequent protein activation. In mammalian cells, formins do not only nucleate actin filaments, but also possess elongation function and their activity initiates the formation of an unbranched F-actin network. A branched actin network is generated through the activity of the Arp2/3 complex. This highly conserved protein complex is composed of seven subunits, with Arp2 and Arp3 having structural similarity to monomeric actin. Through separation of the two Arp moieties the complex remains inactive until it binds to the side of an existing ("mother") actin filament[586]. This brings Arp2 and Arp3 in proximity and binding of ATP-G-actin generates a stable actin trimer. The Arp2/3 complex remains bound while the free barbed end of the daughter filament growths at a distinct 70° angle from the mother filament[587]. The binding of the Arp2/3 complex to actin filaments is a conformational unfavourable process and spontaneous stable binding slow[578,587]. Nevertheless, the actin nucleation activity of the Arp2/3 complex can be enhanced through **nucleation promoting factors (NPFs)**, including Wiskott-Aldrich syndrome protein (WASp) and neural WASp (N-WASp), WASp-family verprolin-homologous proteins (WAVE), and WASp and SCAR [suppressor of cAMP activator] homologue (WASH). Depending on the cellular function and localization, different NPFs associate with the Arp2/3 complex. WASp family proteins usually exist in an auto-inhibited form[588]. The cooperative binding of Rho GTPases and PIP_2 to the central GBD and PIP2-binding domain, respectively, relieves the locked, inactive conformation and opens the C- terminal VCA (verprolin, central, acidic) domain for interaction with the Arp2/3 complex and actin monomers. Binding of active WASp family proteins to the Arp2/3 complex does not only induce the conformational changes required for stable binding to mother filaments, but they also carry ATP-G-actin that are rapidly integrated into the daughter filament. Arp2/3 complex activity is usually found in cellular regions of dynamic actin turnover, such as the lamellipodium, in phagocytic cups, for internal membrane traffic, and at the immunological synapse. Accordingly, mutations in the WAS protein, that is exclusively expressed in cells of haematopoietic lineage, result in severe primary immunodeficiency, such as Wiskott-Aldrich syndrome (loss of function) and X-linked neutropenia (gain of function), that are characterized by immune dysregulation[589], altered to phagocytosis[590,591] or failure of immunological synapse assembly[592,593].

Dynamic F-actin turnover is required for many cellular processes, e.g., cytokinesis and motility, and this includes rapid depolymerisation of actin filaments. The actin depolymerizing factor (ADF)/cofilin family of **actin severing proteins** is associated with the characteristic instability of actin filaments and includes the proteins destrin (also known as ADF), cofilin-1 in non-muscle (hereafter called cofilin) and cofilin-2 in muscle cells[578]. Cofilin has a dual function, as it has binding affinity to both G- and F-actin. At high concentrations, cofilin protects the actin filament, and its binding changes the twist of F-actin[594]. However, low concentrations of F-actin-associated cofilin can induce severing of existing actin filaments and accelerated dissociation of ADP-G-actin from the

pointed end. Due to its severing function, cofilin provides free barbed ends for further elongation and in cooperation with the Arp2/3 complex, cofilin reorganizes the actin cytoskeleton and promotes vesicle transport[595,596]. Through its higher affinity for ADP-G-actin, ATP hydrolysis and phosphate dissociation along the actin filament set the timer for the binding of cofilin. Moreover, cofilin bound to ADP-G-actin can prevent nucleotide exchange[597], reducing the pool of freely available ATP-G-actin, though profilin has been shown to compete with cofilin for the binding to monomeric actin[598].

To generate an intact higher-order actin cytoskeleton, individual actin filaments need to be assembled in bundles or networks by **actin cross-linking and bundling proteins**. In structures such as filopodia, stress fibres and invadopodia, F-actin is arranged in parallel bundles to generate sufficient force to deform the cell membrane because a single actin filament would buckle under the cells surface[599]. The most abundant actin bundling protein is fascin that is localized to the leading edge of cells and enriched all along the filopodia structure. Fascin plays an important role in cancer biology, as it is found to be upregulated in invasive and metastatic cancer and be associated with invadopodia[600,601]. Interestingly, temporary T_{Reg} suppression of APCs has been shown to be linked with contact-dependent sequestration of fascin-1 and skewed F-actin polymerization towards the T_{Reg}-APC IS that hinders $CD8^+$ T cell priming[602]. Although this lethargic state was reversible upon detachment of the T_{Reg}, it indicates that fascin-dependent polarization of the APC's actin cytoskeleton can induce short-term immunosuppression. Another protein that is important for the assembly of filopodia is the actin cross-linking protein L-plastin (also known as plastin 1), that along with α-actinin, dystrophin, filamin, and others makes up the calponin homology (CH) domain superfamily of actin cross-linking proteins. The CH domain does not only allow integration of F-actin into bundles and networks, but also binds to intermediate filaments and other ABPs. The two CH domains of L-plastin are positioned in close proximity, allowing the generation of tight actin bundles that are required for cellular processes such as cytokinesis, but it is also associated with cellular structures such as microvilli[603] and focal adhesions[604]. Other actin cross-linkers, such as the FERM domain-containing proteins ezrin, radixin and moesin are required for cytoskeleton-membrane cross-linking. While radixin has been found to be enriched in focal adhesions were it connects the barbed end of F-actin to the membrane, ezrin and moesin have been suggested to be involved in facilitating binding of large actin cytoskeletal structures to sites of membranous protrusions that are involved in cell-cell signalling and cell motility[605–609].

Many aspects of intracellular actin cytoskeleton dynamics are under the control of a family of small G proteins, the **Rho GTPase family** of the Ras superfamily that act as molecular switches and transmit external signals into rearrangement and reorganization of the actin cytoskeleton. Rho GTPases are expressed in all eukaryotic cells[610] and the three best studies members of this family are cell division control protein 42 homologue (CDC42), Ras-related C3 botulinum toxin substrate 1 (Rac1) and RhoA. Rho GTPases themselves are under the regulation of three classes of regulatory proteins, guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanosine dissociation inhibitors (GDIs)[611]. Typically, small G proteins are inactive when bound to GDP. Activation of Rho GTPases is associated with the exchange of GDP for GTP that is catalysed by GEFs and results in conformational rearrangement of the small G proteins. GTP hydrolysis can occur spontaneously at a slow rate but is stimulated by GAPs that accordingly control the length of the signalling event. GDIs bind to ADP-bound Rho GTPases in large complexes and retain them in their inactive state, but furthermore prevent their translocation within the membrane and the cytoplasm, resulting in spatial control of Rho GTPase activation[611]. Rho GTPases have been

implicated to play a role in various cellular functions, including establishment of cell polarity, vesicle trafficking and cell motility. RhoA activity has mostly been associated with stress fibres and contractility of the actomyosin network. Interestingly, RhoA contributes to cell shape and cytoskeletal integrity, governing stem cell commitment and differentiation of mesenchymal cells[612]. TGF-β receptor signalling is a known direct activator of RhoA and in cancer cells this signalling pathway has been associated with adaption of a mesenchymal phenotype and increased invasiveness by inducing stress fibres[613]. Overexpression of RhoA is found in many types of cancer, including prostate cancer[614], gastro-intestinal cancer[615,616], and high-grade testicular cancer[617]. A role of RhoA, through overexpression or expression of a mutated form, can accordingly be hypothesized to play a role during EMT. RhoA has several downstream effectors including Rho-associated, coiled-coil containing protein kinase 1 (ROCK1) and diaphanous-related formin-1 (mDia1). ROCK1 is a main regulator of actomyosin contractility and interaction with its downstream substrates often results in actin cytoskeleton rearrangement and stabilization of F-actin[618,619]. The role of mDia1 is less defined, and studies have attributed a role for this protein in stress fibres and filopodia formation, formation of adherent junctions and phagocytic cups, a potential role as a transcription factor and accelerator of actin nucleation and elongation, and through its interaction with microtubules a regulatory role in the cytokinetic ring. Rac1 has been found to locate to lamellipodia and accordingly this protein has been suggested to play a role in adhesion and cell motility. Its downstream signalling includes activation of LIM kinase that in turn induces activation of ADF/cofilin through phosphorylation, activation of WAVE through the adapter molecules IRSp53 and Abl, as well as PI3K regulation and modulation of the ERK pathway[620]. The third prototypic member of the Rho GTPase family is CDC42, a homodimer that is involved in cell cycle regulation, but also migration, endocytosis, and cell morphology[621]. There are numerous downstream effectors of CDC42, including WASp and N-WASp, p21-activated kinases PAK1 and PAK2, and the transcription factors SRF, STAT3, and NF-KB[622]. Through these interactions and its role during G1-S phase progression and mitosis, CDC42 has been attributed a leading role in cancer progression. Overexpression of CDC42 has been associated with an increase in malignancy in cervical neoplastic tissue[623], but has also been documented in patient samples of NSCLC, CRC, skin cancer, breast cancer, and testicular cancer[622]. Furthermore, cancer cells expressing high levels of CDC42 demonstrate increased motility and metastatic potential, an observation that has been attributed to the ability of CDC42 to promote filopodia formation[623,624]. By interacting with PAKs, CDC42 can further modulate the expression of oncogenes such as Ras and EGFR that through induction of the Raf-MEK-ERK pathway can lead to tumour initiation and cancer progression. Through its multiple interactions and its role in tumorigeneses, CDC42 has been discussed as a therapeutic target, however its role in other physiological mechanisms, including formation and maintenance of the IS, makes it a difficult target.

3. Actin cytoskeleton and cytotoxicity

The synchronized function of ABPs for cellular process and dependency on sequential actin cytoskeleton-governed steps is best illustrated in cytotoxic lymphocytes. Cytotoxicity of CTLs and NK cells is a dynamic process that involves several steps that are fine-tuned and governed by actin cytoskeleton dynamics and remodelling. Recognition of infected or transformed cells requires extensive communication between target cells and effector lymphocytes that relies on cell-cell contact and receptor-ligand interactions. Failure to establish this contact is associated with an increase of malignant transformation, an observation that is particularly apparent in PID patients[516,589]. As mentioned in sections 1.1.2 and 1.2.1, the lytic IS is characterized by a fast and

drastic polarization of F-actin and the MTOC to the site of the contact. The characteristic bull´s eye shape that forms the different zones of the cytolytic IS, the SMACs, is generated through *de novo* F-actin polymerization and actin filament reorganization. The outer part of the IS, the dSMAC, is formed through activation of the Arp2/3 complex that creates a densely branched actin network, while the inner concentric pSMAC consists of linear formin-generated actin bundles. Dispersed throughout the IS, actin foci that are formed through local actin nucleation as a result of WASp activity have been associated with activating signalling and receptor microclusters[625,626]. While the architecture of the IS and the role of actin cytoskeletal changes for structural and functional purposes in CTLs and NK cells are similar, the lytic NK IS is formed through signalling of germ-line encoded receptors, while CTLs rely on their antigen receptor (TCR) in combination with co-receptors (CD28, CD3).

CTLs are extremely motile cells, covering a distance of up to 10-15 µm per minute, while forming multiple protrusions to probe for the right pMHC complex[627]. Their TCR is highly sensitive and initial contact with pMHC results in an increase in receptor sensitivity as well as actin cytoskeletal rearrangements at the site of the TCR-pMHC interaction. Sustained calcium influx from triggered TCRs is achieved through exclusion of the phosphatase CD45 from microclusters and continued actin polymerization, that is also required for stabilization of microclusters and progression of IS formation[628]. One the critical activation threshold is reached, the CTL stops migrating and instead spreads over the target cell in an actin cytoskeleton-dependent manner[516].

NK cells on the other hand recognize their target cells through presentation of self and non-self ligands. Activation of the lytic machinery is not a zero-sum game, as combinational signalling from activating receptors can lead to a greater activating signal than individual receptor signalling, but at the same time do inhibitory receptors not only signal faster than activating receptors, but their signal strength can also differ among each other[27,89,629]. Studies have suggested that receptor distribution on NK cells is also organized in microclusters containing both activating and inhibitory receptors[630,631] and their associated kinases or phosphatases, respectively[632,633]. As in CTLs, formation and maintenance of the IS in NK cells is dependent on sustained actin cytoskeleton polymerization that is associated with continuous activating receptor-induced calcium influx. The dynamics and the speed of the retrograde actin flow at the NK IS have been associated with differential signalling from inhibitory and activating receptors, with inhibitory receptors such as CD94/NKG2A or iKIR slowing the actin flow in addition to inhibiting *de novo* F-actin polymerization[516,633,634].

The further polarization of effector lymphocytes includes the convergence of the lytic granules, as well as positioning of the MTOC near the cSMAC of the IS through cooperation of the actin cytoskeleton and microtubule network. While the clustering of lytic granules around the MTOC is partly dependent on calcium influx, the signal strength of the TCR has been shown to define the number of granules that converge, while in NK cells this process is dependent on LFA-1 signalling and follows an all-or-nothing response[90,210]. Degranulation of lytic granule content is the final step of effector lymphocyte activation. While the cSMAC has historically been described as lacking actin filaments, super-resolution microscopy has revealed a fine, pervasive F-actin network[635]. Local actin remodelling allows passage of granules through widening of the actin meshwork[149,636]. Recovery of the cortical actin at the central portion of the IS terminates degranulation[637]. The importance of actin cytoskeleton remodelling for degranulation in NK cells is further supported by observations in patients lacking either coronin1a expression, an actin regulator responsible for the deconstruction of F-actin networks, or expression of the lysosomal trafficking regulator gene (LYST), causing Chediak-Higashi syndrome, who´s NK cells fail to perform degranulation events, either due

to insufficient F-actin clearance from the cSMAC or due to increased lytic granule size, respectively[638,639]. Further, inhibition of actin cytoskeletal dynamics in NK cells after IS formation through treatment with F-actin stabilizing drugs such as jasplakinolide or CK-666, an Arp2/3 complex inhibitor, also prevents degranulation, underlining the importance of granule permissive F-actin clearance at the cSMAC[516].

IV. IMAGING FLOW CYTOMETRY AS A TOOL TO EXPLORE LIGAND-RECEPTOR INTERACTIONS AT THE IS

The interaction of ligands on tumour cells with their corresponding receptors on immune cells is a critical determinant for the activation status of the effector lymphocyte. In recent years, advances in single-cell high-throughput technologies and data-processing algorithms allowed for the detailed analysis of heterogeneous cell populations. Among these techniques, imaging flow cytometry (IFC) represents a combination of flow cytometry and fluorescent microscopy that enables the evaluation of intensity, location, and co-localization of markers on single cell level simultaneously by bright field, dark field, and fluorescence. Multispectral imaging is achieved by a spectral decomposition element that directs distinct spectral bands to channels on a charge-coupled device (CCD) detector and the time-delay integration (TDI) technology. This technology optically decomposes an image into subsets of images that each correspond to a different colour component and are spatially separated from the other sub-images. This spectral decomposition system allows the acquisition of up to 10 fluorescent images per cell and gives information about the detailed localization of fluorescent probes.

The classical imaging modes to study the IS are confocal, TIRF, or super-resolution microscopy, but these are time-consuming techniques, the imaging of rare events is difficult, and the selection of conjugates may be influenced by observer bias. For quantitative studies and functional analysis, several hundreds of conjugates of target and effector cells need to be analysed for robust statistical analysis. By combining the statistical power of conventional flow cytometry with the advantages of fluorescence microscopy, IFC can help overcome these limitations. Additionally, the detection limit of IFC is as low as 0.2 µm, a consequence of the segmentation of objects by grouping of pixel intensities above the background pixel intensity, rather than relying on an intensity threshold for detection[640,641]. Besides its use as a diagnostic tool in the assessment of hematologic diseases[642,643], it has been suggested that differential diagnosis[644], but also *ex vivo* drug-susceptibility screening[645], could benefit from IFC. While conventional flow cytometry identifies an intrinsic set of features that is inherent to single objects, IFC allows for feature construction and analysis in defined areas of a single cell.

Using IFC, several groups have characterized the IS between APCs and T cells in the human system[646–648], but also the lytic IS between NK cells and tumour cells in human[649] and mouse[650]. Since the IS is the focal point of anti-tumour immunity executed by NK cells and CTLs, immune escape mechanisms that interfere with either IS formation or effector cell activation or functions provide an effective strategy for cancer cells to avoid destruction. Using IFC, even small subpopulations of tumour cells that show resistance against cytotoxic effector lymphocytes can be identified and characterized regarding protein expression. Moreover, spatial information about the localization of ligands and other markers, such as for example lytic granules and their content, the centrosome, transcription factors, and the autophagic machinery can be evaluated. Therefore, IFC provides the possibility of real-time verification of ligand-receptor interactions in the context of the IS and their consequences on immune cell activation and target cell fate.

The progress in machine learning and deep learning methods could soon allow the automation of IFC data processing for the detection of rare events to assess for example disease progression, drug resistance or cancer-immune cell interactions under different therapeutic conditions. In combination with other single-cell high-throughput technologies, such as (imaging) mass cytometry for dissection of the tumour immune landscape *in situ* and the monitoring of changes

during and after chemotherapy and immunotherapy, IFC displays a high prognostic potential to guide immunotherapeutic decisions.

PhD project

1. Scientific objectives

An effective anti-tumour response from CTLs and NK cells is mainly dependent on the formation and maintenance of a cytolytic IS. Inhibitory receptor signalling through transmembrane proteins such as PD-1 or CTLA-4 on CTLs or CD94/NKG2A and iKIR on NK cells can prevent IS formation altogether or interfere with crucial effector functions such as granule convergence, MTOC polarization or degranulation. However, also tumour cells can exploit their actin cytoskeleton, not only to increase their invasive potential and dysregulate anti-apoptotic signalling, but also to increase their resistance against cytolytic effector functions of CTLs[651,652] and NK cells[141,161,465]. While the initial observation that a rapid and sustained accumulation of F-actin at the cell-cell contact site between breast cancer cells and NK cells has led to the first description of an intrinsic resistance mechanism coined “actin response”, the objectives of my PhD thesis were:

I. to confirm its conservation in other types of solid and haematological cancers and in the CTL IS
II. the identification of associated resistance mechanisms and the potential impairment of NK cell activation
III. and the characterization of the role of the actin response in *in vivo* models of cancer.

2. REVIEW ARTICLE I: ACTIN CYTOSKELETON STRADDLING THE IMMUNE SYNAPSE BETWEEN CYTOTOXIC LYMPHOCYTES AND CANCER CELLS

In this review article we have introduced the immunological synapse between cytotoxic lymphocytes and cancer cells in great detail. We paid special attention to the actin cytoskeleton dynamics at the pre- and post-synapse and the architecture of the immunological synapse as a consequence of differential transmembrane receptor signalling. I invite the reader to focus their consideration to our description of the different types of immunological synapse that can be formed and their consequence for lymphocyte function and target cell fate as we extended the existing research by a fourth type of NK cell immunological synapse – the evasion synapse – that characterizes the interaction of NK cells with resistant cancer cells.

Review

Actin Cytoskeleton Straddling the Immunological Synapse between Cytotoxic Lymphocytes and Cancer Cells

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Received: 15 April 2019; Accepted: 13 May 2019; Published: 16 May 2019

Abstract: The immune system is a fundamental part of the tumor microenvironment. In particular, cytotoxic lymphocytes, such as cytolytic T cells and natural killer cells, control tumor growth and disease progression by interacting and eliminating tumor cells. The actin cytoskeleton of cytotoxic lymphocytes engaged in an immunological synapse has received considerable research attention. It has been recognized as a central mediator of the formation and maturation of the immunological synapse, and its signaling and cytolytic activities. In comparison, fewer studies have explored the organization and function of actin filaments on the target cancer cell side of the immunological synapse. However, there is growing evidence that the actin cytoskeleton of cancer cells also undergoes extensive remodeling upon cytotoxic lymphocyte attack, and that such remodeling can alter physical and functional interactions at the immunological synapse. In this article, we review the current knowledge of actin organization and functions at both sides of the immunological synapse between cytotoxic lymphocytes and cancer cells, with particular focus on synapse formation, signaling and cytolytic activity, and immune evasion.

Keywords: actin cytoskeleton; cytotoxic T lymphocytes; immune evasion; immune surveillance; immunological synapse; natural killer cells

1. Introduction

The tumor microenvironment (TME) plays multiple and central roles in cancer progression, e.g., by promoting tumor invasion, chemo and radiation-resistance, and by modulating the antitumor immune response. Ongoing research in the field of tumor immunobiology has identified immune escape as a classical hallmark of cancer and characterized different escape strategies elaborated by cancer cells [1–3]. The immune cell population with the best studied anti-tumor effector functions are cytotoxic lymphocytes cells, including cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. The concept of immunoediting is highly dependent on these key cells of the adaptive and innate immune systems. That the immune system and cancer cells exist in a delicate balance was already described more than a hundred years ago by Paul Ehrlich, when he suggested that host defense may prevent neoplastic cells from developing into tumors [4]. Sir Frank Mac Farlane Burnet hypothesized later that tumor cell neo-antigens induce an immunological reaction against cancer and subsequently formulated the immune surveillance theory [5]. The elimination phase of the immunoediting process is characterized by successful immunosurveillance of cancer cells that are recognized and eradicated by

cytotoxic lymphocytes [6]. Constant immune selection pressure allows tumor clones to emerge, which escape immune cell-mediated elimination in the so-called equilibrium phase. During this phase, tumor cells develop different stratagems to escape immune surveillance, such as altered expression of surface markers, immune cell inhibition and establishment of an immunosuppressive TME [7–10]. Finally, tumors progress into the third phase of the immunoediting process, the escape phase, which leads to faster disease progression and poorly immunogenic tumors. Tumor cells have the ability to change their surroundings to their benefit, a feature that is accentuated by the characteristics of the TME, such as hypoxia—as a result of poor vascularization and rapid proliferation of cancer cells [11–13]. Hypoxia creates a hostile environment for cytotoxic immune cells that hinders their activation and effectiveness, while promoting activity of immune suppressive cell populations [11,14–17].

The actin cytoskeleton mainly consists in a complex network of polarized actin filaments (AFs) that contributes to nearly all fundamental cellular processes, including morphogenesis, motility, differentiation, division, membrane trafficking and signaling and the reader is invited to read the following review suggestions referring to these fields [18–25]. The actin cytoskeleton is subject to the activity of over 100 actin-binding proteins (ABPs) that regulate the organization and dynamics of AFs [26,27]. Broadly, ABPs can be distinguished according to their functions as actin nucleators, AF severing and capping proteins, and AF crosslinkers [25,26]. Actin polymerization occurs by polymerization of globular actin monomers, a process facilitated by actin nucleators, such as the Arp2/3 complex and formins, which promote the assembly of branched and linear arrays of AFs, respectively [26,28–30]. Further organization of AFs into higher-order structures, such as parallel bundles and three-dimensional networks, is mediated by crosslinking proteins of differing structural properties [31]. Severing proteins, such as actin depolymerizing factors (ADFs) and cofilin, play important roles in regulating AF dynamics by either increasing the amount of fast-growing barbed ends available for polymerization or by accelerating depolymerization from AF pointed ends [26,32].

A fundamental process underlying cytotoxic lymphocyte-mediated cancer cell killing is the formation of a specialized cell-cell junction, referred to as the immunological synapse (IS), between the immune cell and its prospective target. Different types of ISs can be formed, postulated to execute several functions including but not limited to the directed secretion of cytokines or lytic granules in a lytic IS with the goal of target cell lysis. The formation of a lytic IS requires activation of CTLs and NK cells through interaction of the T cell receptor (TCR) with peptide-loaded major histocompatibility complex-I (pMHC-I) on the target cell surface, or recognition of increased stress-induced ligands or loss of inhibitory MHC-I on the target cell surface, respectively. Initiation, formation and maturation of a lytic IS between cytotoxic lymphocytes and targets is a delicate process associated with major rearrangements of the actin cytoskeleton in cytotoxic lymphocytes [33–35]. In addition, the directed secretion of cytolytic granules containing perforin and granzymes, which ultimately promotes osmotic cell death and caspase-mediated apoptosis, also critically rely on actin cytoskeleton remodeling on the immune cell side of the IS [36–40]. On healthy cells, self-antigens presented on the MHC-I are not recognized by the TCR and the amount of stress-induced ligands is below the threshold required for NK cell activation, resulting in the formation of an inhibitory IS that protects the target cell from lymphocyte-mediated cytotoxicity. Another function executed by the IS formed between CTLs and dendritic cells (DCs) is the regulation of lymphocyte activation and maturation. A similar type of IS has been reported for the cell-cell-contact between NK cells and mature DCs and this interaction was termed regulatory IS [35,41–43]. Intercellular communication in a regulatory synapse can induce CTL activation and priming, increase the capacity of NK cells to release cytokines, and promote DC maturation. The different types of ISs are depicted in Figure 1.

Specific changes in actin cytoskeleton organization and dynamics are critically required for the initiation and stabilization of an IS, as well as for the activation and efficacy of associated immune cell effector functions. Such changes are the subject of much ongoing research [35]. Beside the well-defined actin cytoskeleton organization and roles in cytotoxic lymphocytes cells during IS formation and killing activity, there is increasing evidence that the actin cytoskeleton of cancer cells is also quickly remodeled

in response to immune cell attack [44]. This review aims to discuss the role of the actin cytoskeleton in both, immune cells and cancer cells, during their interaction in an IS. The formation of an IS follows well-orchestrated spatiotemporal rearrangements of cytoskeletal components, that we will describe in detail. Particular attention will be paid to the organization and functions of the actin cytoskeleton during IS formation, signaling, and activity. In addition, we will discuss recent studies supporting that actin cytoskeleton remodeling in cancer cells can alter IS activity and lead to cancer immune evasion.

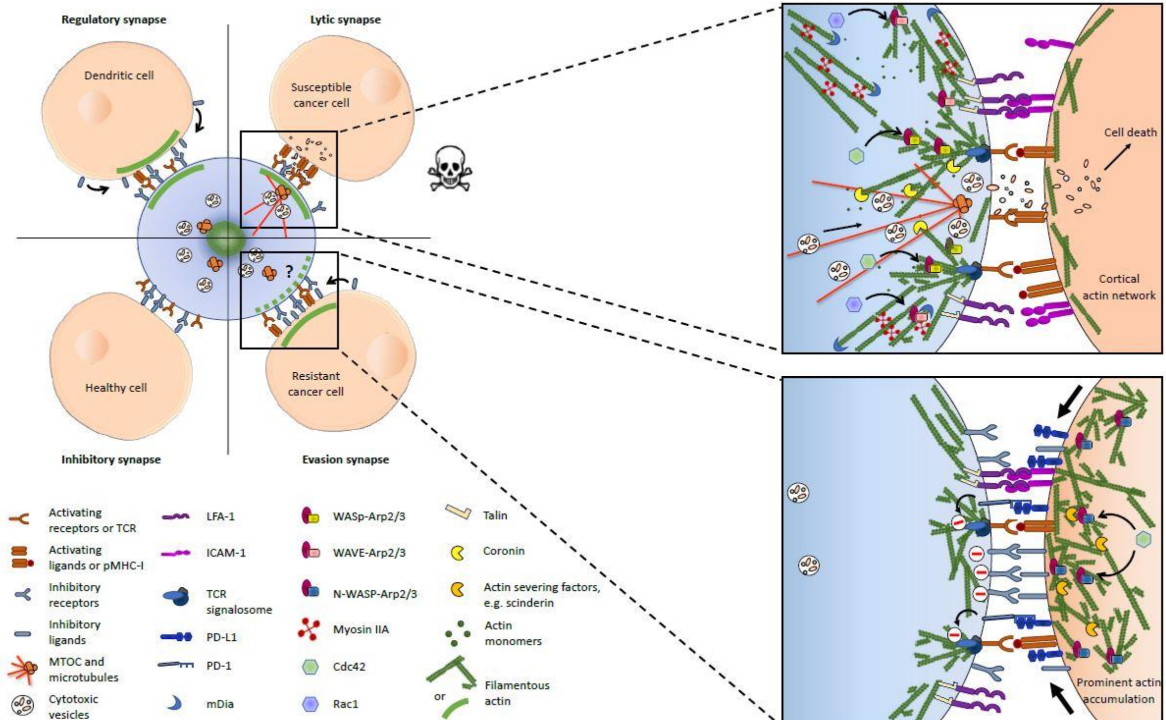

Figure 1. Actin cytoskeleton at the immunological synapses between a cytotoxic lymphocyte (blue) and its target cells. The left panel provides a general overview of the four types of immunological synapses (ISs) discussed in the article and shows the presence or absence of F-actin accumulation (large green line) on both sides of the IS (inspired from [45]). The specific actin filaments (AF) organization and some key upstream regulators, as well as the functions of AFs in intercellular signaling are depicted in the right panels for the lytic (upper panel) and evasion (lower panel) synapses. The reader should pay attention that the provided information sometimes originates exclusively from studies based on either natural killer (NK) cells or cytotoxic T lymphocytes (CTLs) and is invited to refer to the main text for more details.

2. Actin Cytoskeleton and Immunological Synapse Architecture

2.1. Actin Assemblies at the Immunological Synapse

In CTLs, immune cell activation is triggered by interaction of the T cell receptor (TCR) with a non-self-antigen presented on the major histocompatibility complex class I (MHC-I) and engagement of further costimulatory receptors trigger [46–49]. In contrast, NK cell activity is regulated by the balance between activating and inhibiting ligands presented on the target cells surface. A vast array of inhibitory and activating receptors are co-expressed on the surface of NK cells, and activation occurs when signals from activating receptors dominate over the counterbalancing signals from inhibitory receptors [50,51]. In both CTLs and NK cells, engagement of activating receptors induces phosphorylation of membrane proximal signaling molecules and formation of a signalosome that includes many signaling and adapter molecules. Sustained signaling stimulates actin polymerization and generation of a branched AF network at the synapse periphery. The architecture of an IS is

commonly divided in three regions defined by distinct actin assemblies and with specific functions in relation to signaling, adhesion, and cytolytic activities. The so-called supramolecular activation clusters (SMAC) are organized in a bulls-eye shape with a central SMAC (cSMAC), a peripheral SMAC (pSMAC), and a distal SMAC (dSMAC) from the inside out [35,52,53]. Live imaging of AF assembly and organization using fluorescent reporter, such as the small actin peptide Lifeact or GFP-actin, revealed a radially symmetric actin organization with differently assembled structures [54–58].

Initial engagement of the TCR–MHC-I complex activates actin polymerization in CTLs leading to a ring-shaped branched actin network in the dSMAC, shortly after contact with the target cell [48,59–61]. This Arp2/3-complex-mediated branched actin network, which exhibits high similarities to that of the lamellipodium of migrating cells, allows CTLs to spread across the surface of their targets [56,62], and provides support for the symmetric retrograde actin flow towards the cSMAC (see below). The pSMAC is composed of a lamella-like actin arc network that defines the radial symmetric contractile capability of this part of the IS [63]. Mechanistic studies have established that the assembly of the pSMAC actin arc is mediated by formins and organized by myosin II into antiparallel concentric arcs [63,64]. Indeed, selective depletion of the formin mDia prevents formation of the linear AF arrays required to generate the typical concentric myosin IIA-rich arcs [63,65,66]. The innermost part of the IS, the cSMAC, is referred to as an actin poor or actin hypodense region, which contains a fine AF network, which can only be visualized using super resolution imaging [53,66–69]. Moreover, the surface of CTLs it not flat, but is dominated by highly flexible and dynamic microvilli that show a high degree of AF polymerization at their core [70]. A recent study showed that using super resolution fluorescence imaging techniques and subsequent 3D reconstruction of the CTL membrane topography makes the tips of these membrane protrusions enriched for TCR clusters, which facilitate antigen screening. In addition, AF depolymerization not only inhibits microvilli formation, but also prevents the selective localization of TCR clusters [71]. Moreover, intercellular communication between CTLs and APCs is thought to be facilitated in parts by large, TCR-enriched membrane particles that are generated at the tip of these microvilli [72]. Delivery of these CTL-derived particles to cognate APCs was reported to increase DC activation status. Assembly of TCR microclusters was suggested to be a sequential process to which the actin cytoskeleton contributes by lowering the dissociation rates due to increased pulling forces at the IS, which maintain the different parts of the TCR signalosome together [73]. Intact actin dynamics may accordingly not only be involved in the retrograde movement of surface proteins towards the cSMAC but could also account for the observation that TCR microclusters are segregated from other protein clusters. Maintenance of the TCR signalosome composition during the lateral movement within the immune cell membrane is also subject to actin dynamics [74,75].

The integration of several different actin regulatory pathways underlying the formation of a functional IS makes this structure highly vulnerable to cytoskeletal alterations. The majority of actin cytoskeleton defects leading to primary immunodeficiencies (PIDs) are associated with a disruption of assembly and disassembly of AFs in multiple immune cell subtypes altering cell migration and adhesion, intercellular communication, intracellular signaling, and IS formation [76–81]. The architecture of the IS is subject to the spatiotemporal distinct activity of different actin polymerizing and regulatory proteins that result in topographical differences in AF organization. Engagement of activating receptors triggers Arp2/3 complex-dependent polymerization of branched AF, as illustrated in Figure 1. Polymerization activity of the Arp2/3 complex is regulated by different actin nucleation promoting factors (NPFs), such as WASp or WASp-verprolin homolog 2 (WAVE2). Actin regulation mediated by these NPFs does not occur on the effector immune cell side of the IS, but also plays an important role in DCs. Activity of WASp in target cells increases the overall IS area, and restricted knockdown of WASp in target DCs affects IS stability by inhibiting integrin polarization [82]. Actin cytoskeleton remodeling in target cells consequently represents a key component of normal IS formation through association of adhesive interaction and modulation of immunological synapse stability. These NPFs have to be associated with distinct functions corresponding to their localization within the IS. WAVE2 has been found primarily at the edges of the IS, and its activity has been associated with CTL adhesion and spreading over

the antigen-presenting surface [83,84]. In addition to its role in modulation of IS stability, WASp is involved in the formation of actin-rich membrane protrusions at the secretory area, which is consistent with its predominant localization at the center of the IS [85–87].

Gene-expression profiling of peripheral CTLs from chronic lymphocytic leukemia (CLL) patients revealed a set of dysregulated actin regulatory proteins that was upregulated after cell-cell contact with leukemia cells [88]. Interestingly, the same cytoskeletal defects could be reproduced with CTLs from healthy donors that were cocultured with patient-derived CLL cells, indicating that cancer cells can promote cytoskeletal changes in cytotoxic lymphocytes. Further evaluation of the underlying mechanism identified several upregulated inhibitory ligands on leukemic cells that compromised the activation of key actin regulators including the Ras homolog (Rho) GTPases RhoA and Rac1, while promoting Cdc42 activity [89,90]. The combination of reduced RhoA/Rac1 signaling and increased Cdc42 activation resulted in the negative regulation of integrin activity in CTLs and subsequent adhesion and motility defects. Interestingly, defective actin polymerization was not limited to the IS between CLL cells and CTLs, but was also observed between APCs and CTLs, indicating that the cytoskeletal modifications in CTLs preventing functional IS formation were sustained over an extended period of time. A possible explanation for these effects comes from an elegant study in which it was demonstrated that leukemia cell-mediated changes in Rho GTPase activation result in impaired integrin signaling in CTLs, and that these defects could be rescued by the administration of the immunomodulatory drug lenalidomide, which resulted in the normalization of Rho GTPase activation signaling [90,91].

The ability of tumor cells to have a lasting negative impact on actin cytoskeletal dynamics in CTLs by compromising regulation of Rho GTPases shows a potential Achilles´ heel of cytotoxic lymphocytes. A variety of actin cytoskeleton regulators are involved at different spatiotemporal points during the formation and activity of a lytic IS, some of them were mentioned above. Dysregulation of one or two of these proteins can have a significant impact on anti-tumor immunity by inhibiting IS formation or affecting related functions, such as directed degranulation.

2.2. Actin Cytoskeleton Organization and Integrin Signaling at the Immunological Synapse

Binding of cytotoxic lymphocytes to target cells and IS maturation are dependent on the interaction of integrins on both sides of the IS. Cytotoxicity and polarized degranulation rely on a tight connection between CTLs and their prospective target cell. The integrin lymphocyte function-associated antigen 1 (LFA-1) was recently reported to be organized in nanometer-scaled clusters around the secretory domain of cytotoxic CD8+ T lymphocytes to allow confined delivery of cytotoxic granules [64,92,93]. The pSMAC shows the highest density of integrins, with a further increased LFA-1 density at the pSMAC/cSMAC boundary [94–96]. This integrin-mediated adhesive ring around the cSMAC was also suggested to help to seal the directed degranulation of lytic vesicles in the direction of the target cell [97,98]. Additionally, the sheer forces generated by the retrograde actin flow support binding of LFA-1 to its ligand intercellular adhesion molecule 1 (ICAM-1) by inducing a conformational change to its high affinity form. Beside this adhesion function, ICAM-1-bound LFA-1 also serves as a costimulatory signal for CTL and NK cell activation [96,99,100]. Topographical positioning and clustering of LFA-1 at the IS was recently shown to be confined to interstices within the actin cytoskeleton meshwork and underlie the activity of the actin nucleator WASp in CTLs [101]. The mobility of ICAM-1 in turn influences the actin flow-dependent signaling and cytotoxic lymphocyte functions. The actin cytoskeleton of antigen-presenting cells (APCs) engaged in an IS with CTLs was reported to limit the mobility of ICAM-1 on the cell surface. Release of this restriction by actin depolymerization in APCs negatively influences LFA-1 activation on T cells and adhesion properties [102]. Resistance of non-small cell lung cancer cells to CTL-mediated lysis was associated with the overexpression of the actin cytoskeleton regulators scinderin and ephrin-1A [103]. Scinderin is an actin severing protein and its overexpression is associated with increased actin dynamics, while signals of the receptor tyrosine kinases ephrin-A1 are translated into increased actin dynamics via activation of the N-WASp–Arp2/3

complex-pathway [104,105]. Silencing these genes using RNA interference restores both actin dynamics and cancer cell susceptibility to CTL-mediated lysis, giving indirect evidence that altered actin dynamics in cancer cells promotes immune evasion [103]. Detailed analysis of the IS formed between resistant non-small cell lung cancer (NSCLC) cells and CTLs revealed significant structural changes with sparse areas of tight cell-cell junctions and a wide IS cleft, contrasting with the tight IS formed with susceptible NSCLC cells. In a follow up study, the focal adhesion kinase (FAK) signaling pathway and Rho GTPase signaling were found to play a role in NSCLC resistance to CTLs [106]. FAK is a non-receptor tyrosine kinase that is activated by phosphorylation in response to integrin signaling [107]. Activated FAK can be found where cells attach to extracellular matrix components and at cell-cell contact areas, where it plays an important role in regulating AF turnover [108]. Expression of FAK has been reported to be upregulated in certain types of cancer, including ovarian and breast cancer [109]. Experimental silencing of FAK in NSCLC induces both, adhesion deficiency and increased resistance to CTL-mediated lysis by impairing IS formation [103,106].

These studies highlight that adhesion of CTLs to their prospective target is a crucial prerequisite for effective target cell lysis. Actin cytoskeletal dynamics drive the segregation of transmembrane protein clusters, such as integrins and induce their maturation by exertion of sheer forces to further increase their ligand affinity. Cancer cells can exploit the dependence of integrins for immobilized ligands by increasing actin cytoskeletal dynamics and increased ligands motility to prevent formation of the sealing ring at the pSMAC/cSMAC border to reduce efficiency of cytotoxic granule delivery.

3. Actin Cytoskeleton and Immunological Synapse Signaling

3.1. Actin Cytoskeleton and Receptor Signaling

The majority of engaged TCRs assemble in microclusters with their associated costimulatory receptors in the so-called TCR signalosome and can be found within the pSMAC. Signalosomes can also be found in the NK cell IS, although their composition differs from the TCR signalosome [110,111]. From the periphery, signalosomes move following the retrograde actin flow until they reach the pSMAC/cSMAC boundary [56,64]. Treatment with actin depolymerizing drugs, such as latrunculin, prevents microcluster-formation and consequently CTL and NK cell activation, highlighting the importance of actin dynamics in signaling at the IS [58,95]. Robust signaling of TCR microclusters occurs during the transition from the dSMAC to the cSMAC, with an abrupt stop upon reaching of the cSMAC [58]. As part of the TCR signalosome, linker of activation of T cells (LAT) recruits other adaptor proteins such as Nck and Vav1 that integrate TCR signaling into the rearrangement of the actin cytoskeleton by modulating WASp activity [60,66,112]. In the actin hypodense cSMAC, the connection between the signalosome and the actin cytoskeleton is lost, and thus TCR signaling comes to an abrupt stop after transition of TCR microclusters into the cSMAC, where exhausted TCRs are internalized in a Rho-dependent manner [35,58,113]. The strength of TCR signaling is directly correlated to the time it takes TCR microclusters to transit through the dSMAC/pSMAC region of the IS and, in primary CTLs, this depends on the speed of the retrograde actin flow [63,65]. Integrin signaling slows the retrograde actin flow resulting in activating signal by the TCR over longer periods of time. The actin-based molecular motor myosin IIA is not only involved in CTL motility and spreading over an antigen-coated surface, but has also been suggested to take part in the formation of the pSMAC and cSMAC, maturation of the IS, and persistence of TCR signaling [114]. Formation of TCR microclusters and their retrograde flow were both associated with recruitment and sustained activity of myosin IIA [115]. However, the exact role and importance of myosin IIA in IS formation and TCR signaling remain a matter of debate [114,116].

Although the role of activating receptor microclusters in activation of cytotoxic lymphocytes has been well established, much less is known about the distribution and stabilization of activating and inhibitory ligands on target cells. Cytotoxic lymphocytes do not only form an IS with infected or cancer cells, but also with APCs, such as DCs, with the aim to promote immune functions on both sides of the

IS. In this regard, recent studies have shown that DCs also exhibit prominent actin polymerization in the region of the IS upon conjugation with CTLs and NK cells [43,82]. Such cytoskeletal remodeling was also shown to stabilize inhibitory ligands at the IS, and thereby prevent activation of immune cell effector functions. Preventing actin cytoskeleton polymerization in DCs was sufficient to increase the release of cytokines and cytotoxicity of NK cells against conjugates DCs. Such an IS was termed "regulatory synapse" [45] and was also reported between CTLs and DCs [82,117]. All in all, these works support that the actin cytoskeleton plays an important role in bridging TCR signaling to AF polymerization and that actin polymerization in DCs are the basis for the strong inhibitory signals in a regulatory IS.

Classification of functionally different ISs was proposed based on the status of actin polymerization on both sides of the IS, including the inhibitory synapse with a healthy target cell (AF accumulation on either side), the lytic synapse with a transformed target cell (AF accumulation in the immune cell only), and the regulatory synapse between DC and cytotoxic immune cells (AF accumulation in the APC only, as seen in Figure 1) [45]. Recently, we provided evidence that cancer cells use a similar mechanism as the regulatory synapse to prevent the activation of interacting NK cells [44,118]. Using high resolution confocal microscopy and high-throughput techniques, we found that resistant breast cancer cells in conjugation with cytotoxic NK cells accumulate AFs at their side of the IS, while susceptible cells do not [44]. As observed with DCs [43], the inhibition of actin polymerization using RNA interference was sufficient to abolish cancer cell resistance to NK cell-mediated lysis [44]. Moreover, actin accumulation in resistant cancer cells was also associated with increased levels of inhibitory ligands, including PD-L1 and MHC-I, at the IS. Both types of ligands induce negative receptor signaling in NK cells and can tip the balance between activating and inhibitory signaling in favor of cancer cells. This suggests that resistant cancer cells can mimic an increased expression of inhibitory ligands and thereby escape killing by NK cells, and possibly, by CTLs (through PD-L1) [118]. Accordingly, we propose a fourth type of IS that is formed between NK cells (possibly CTLs) and resistant cancer cells, which is characterized by actin polymerization on the target cell side (Figure 1). We named it "evasion synapse".

3.2. *Actin Cytoskeleton and Mechanopotentiation of Signaling and Killing at the Immunological Synapse*

Over the last years, it has become increasingly clear that the actin cytoskeleton is not only a platform, but actively involved in the integration of spatial, molecular, and biochemical signals, as well as mechanical features into the formation and maintenance of the IS [119–122]. The forces submitted in the context of the IS are the result of membrane dynamics, cell spreading, and rearrangement of the actomyosin cytoskeleton.

Recently, direct evidence was given that the TCR acts as a mechanoreceptor and that activation of TCR signaling only occurs during applied tangential force, indicating that TCR–MHC-I interaction alone is not sufficient for CTL activation [123,124]. Following its activation, the TCR induces force changes at the IS by triggering dynamic actin cytoskeleton reorganization that translates into biochemical signals. As a result of antigen recognition, integrin-mediated adhesion force increases, with a direct correlation between TCR specificity and the strength of adhesion forces [125–127]. Forces in the frame of the IS have been shown to influence affinity maturation of integrins and strengthening of LFA-1/ICAM-1 interaction at the IS [128,129]. In addition, it was demonstrated that the cytotoxicity of CTLs is directly correlated to the mechanical forces applied on the conjugated target cell [130]. Furthermore, the actin cytoskeleton plays a key role as a main driver of force generation at the IS with regard to the retrograde translocation of receptor clusters originating from pushing and pulling forces at the dSMAC and pSMAC, respectively. The previously mentioned integrin-mediated adhesive ring around the cSMAC region; helps to apply the actin-based pulling force that supports pore formation by perforin [64,130]. In turn, actin remodeling on the target cell side of the IS locally modifies membrane properties, e.g., by inducing a substantial increase in cellular stiffness that can alter CTL activation [120,121,126,131]. CTLs interact with a variety of different cells, including endothelial cells, APCs, and target cells. The viscoelastic properties of endothelial cells and APCs change in

response to inflammatory conditions and these changes are associated with myosin activity and actin polymerization dynamics [132,133]. Interestingly, mature APCs exhibit a higher degree of actin filament reorganization and a polarization of AFs towards the IS region [134,135]. This actin rearrangement in APCs was found to be critical for functional IS formation and T cell priming. Furthermore, it has been reported that ex vivo activation, expansion, and differentiation of CTLs is dependent on substrate rigidity [136]. CTLs interacting with immobilized stimulatory ligands show increased activation and proliferation under conditions of variable substrate stiffness as a result of increased signaling by the mechanosensitive TCR and other receptors [119,136]. The increased actin accumulation observed in APCs is thus sufficient to directly influence mechanotransduction by stabilizing inhibitory ligands at the IS, but also important for the maintenance of TCR signaling by providing a rigid substrate [122]. Additionally, mechanical forces at the IS can potentiate CTL-mediated killing of target cells. A recent study reported, that actin-enriched membrane protrusion in the dSMAC and cSMAC of the IS facilitate directed delivery of cytotoxic granules while also inducing the physical deformation of the target cell membrane [87]. These protrusions were dependent on WASp and Arp2/3 complex activity at the central parts of the IS and were a crucial requirement for force exertion in the context of the IS and potentiated target cell lysis.

4. Actin Cytoskeleton and Immunological Synapse Cytolytic Activity

In addition to signaling function, the IS is also the place of directed secretion of cytolytic granules toward cancer cells. Following interaction with a target in a lytic IS, CTLs and NK cells both show dynein-mediated polarization of the microtubule organizing center (MTOC) towards the IS [137–139]. The docking of the MTOC to the plasma membrane at the boundary between the actin hypodense cSMAC and the secretory domain of the IS is required for intracellular trafficking of lytic granules to the secretory domain of the IS [97] as seen in Figure 1. The content of lytic granules induces target cell death by osmotic cell lysis or via activation of caspases or pro-apoptotic proteins [140,141]. The mechanical force necessary for MTOC reorganization towards the membrane has been reported to involve different actin regulatory proteins, in particular formin family members that bind to microtubule plus ends. Accordingly, depletion of FMNL-1 and mDIA-1 has been shown to result in irregular MTOC positioning at the lytic IS [142,143]. It was initially suggested that the cytolytic granules can passively pass through the loose mesh of cortical AFs in the cSMAC [67,68]. However, recent high-resolution imaging-based studies established that, while AF dynamics at the cSMAC are reduced as compared to the directional actin flow in the dSMAC and the pSMAC, a fine network of AFs at the cSMAC shows dynamics at nanoscale level [69,144].

In vitro studies using cover slips or planar lipid bilayers coated with activating ligands or antibodies mimicking the target cell surface showed that in NK cells and CTLs in interaction with an activating surface, results in increased AF network mesh size at the cSMAC. The actin network mesh size reaches its peak at the same time the highest value of directed degranulation is observed. This granule-permissive clearance of actin is conserved in lytic ISs of both, CTLs and NK cells, although their actin clearance kinetics differ. A recent study found that in an IS formed by primary CTLs, actin clearance at the cSMAC appeared earlier, reached its peak faster and was short-lived compared to actin meshwork depolymerization observed in primary NK cells [69]. Using lattice light-sheet imaging, it could be shown that the MTOC and lytic granules clustered around it were positioned at the center of the cSMAC [53,139]. Based on the above results, it has been proposed that the actin network at the cSMAC serves as a barrier for lytic granules, and that transitory AF depletion decreases actin network density to initiate degranulation events [139]. Additionally, actin dynamics and myosin contractility have been shown to support the fusion of lytic granules with the plasma membrane by providing the required membrane tension. Furthermore, while myosin IIA activity was shown to be dispensable for NK cell adhesion to target cells, it was reported to interact directly with lytic granules, and facilitate the approach to the cell membrane at the secretory domain of the cSMAC [145,146]. Accordingly, mutations in the heavy chain of non-muscle myosin IIA were reported to be associated

with immunodeficiency syndromes characterized by decreased CTL-mediated cytotoxicity despite otherwise normal IS formation [147–149]. The approach of lytic granules to the CTL membrane is accompanied by coronin 1A-mediated deconstruction of AFs at the cSMAC [53]. Another recent study also showed that secretion of perforin was associated with the formation of actin-rich protrusions in the secretory domain of CTLs, additionally highlighting that the cSMAC is not simply an actin-poor region as previously suggested, but exhibits highly regulated actin dynamics [87]. The termination of degranulation events is also actin cytoskeleton-dependent, as replenishment of AFs at the cSMAC acts as barrier for further granule degranulation and results in retraction of the MTOC from its position close to the IS area [150]. Therefore, rapid actin cytoskeleton remodeling seems to be crucial for initiation, but also the termination of directed secretion. Treatment of CTLs with actin depolymerizing drugs, such as Latrunculin A, increases duration of lytic granule secretion, underlining the role of AFs in creating a physical barrier for granule release.

Although only a few studies have directly investigated the organization and functions of the actin cytoskeleton in cancer cells during cytotoxic lymphocyte attack, there is growing evidence that actin remodeling plays a central role in mediating tumor immune evasion by altering either IS formation and function [44,103,106,151]. As previously discussed, we recently reported a prominent accumulation of AFs on the tumor cell side of the IS between intrinsically resistant breast tumor cells and NK cells, and that such process was correlated to immune evasion [44]. Selective targeting of actin cytoskeleton remodeling in cancer cells by targeted downregulation of the ARP2/3 complex regulators Cdc42 or N-WASP was sufficient to substantially increase target cell susceptibility to NK cell-induced lysis, as well as to restore high levels of NK cell derived-granzyme B levels in target cells. Similar to the regulatory synapse between DCs and NK cells, key inhibitory ligands, including HLA-A,B,C and PD-L1, were observed to accumulate at the evasion synapse together with actin accumulation in resistant cancer cells (Figure 1). A central role for the actin cytoskeleton in tumor immune evasion is further supported by an immune escape screen that identified Cdc42 as a mediator of increased resistance to antigen-specific CTL-mediated cytotoxicity [152]. Importantly, the expression of a constitutively active form of Cdc42 in mouse fibrosarcoma and human colorectal cancer cells was sufficient to induce resistance against CTLs and NK cell cytotoxicity in vitro, and promoted tumor growth in different mouse models, while it did not affect cell proliferation in vitro. Finally, additional evidence that actin remodeling promotes tumor immune evasion comes from the multiple links between the epithelial-to-mesenchymal transition (EMT), a process involving extensive modification of actin cytoskeleton organization and dynamics [153], and acquisition of cancer cell resistance to cytotoxic lymphocytes [154–158]. Such links have been recently reviewed and the reader is invited to refer to the following article [3].

5. Conclusions

Actin remodeling plays central roles in the three main processes underlying cytotoxic lymphocyte-mediated tumor cell killing, including target cell recognition, immune cell activation, and cancer cell killing. Actin remodeling at the cancer cell side of IS is emerging as an important mechanism of tumor immune evasion; however, our knowledge regarding this aspect remains fragmented. Additional work is required to identify clinically relevant targets to selectively impair actin organization and/or the dynamics in tumor cells and thereby restore an effective anti-tumor immune response. If and how the actin cytoskeleton of cancer cells could serve as a drug target for immunotherapy should be further investigated.

Funding: A.A.A. and H.W. are recipients of PhD fellowships from the National Research Fund (FNR), Luxembourg (AFR7892325 and PRIDE15/10675146/CANBIO, respectively). Work in C. Thomas's group is supported by the Cancer Foundation Luxembourg (ACTIMMUNE).

Acknowledgments: The authors thank J. Michalska for her constructive criticism and proofreading of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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3. Review article II: Escape of tumor cells from the NK cell cytotoxic activity

Following the 18th Meeting of the Society for Natural Immunity (NK 2019), where I was part of the local organising, we were kindly invited by Dr Claudia Cantoni and Dr Massimo Vitale to participate in a review about cancer cell escape mechanism that occur at the NK cell immunological synapse. In this review we have described the receptor signalling in NK cells that result in formation and maturation of the immunological synapse (Section 2.2) and have addressed cancer intrinsic mechanisms that can interfere with formation of a lytic immunological synapse (Section 3.2). We further have provided the graphical summary and illustrations for this review. For better understanding of my scientific PhD project objectives, I especially recommend the chapters 3.2.1 “Receptor-ligand interaction” and 3.2.2 “Cytoskeletal rearrangement”.

DOI: 10.1002/JLB.2MR0820-652R

REVIEW

Escape of tumor cells from the NK cell cytotoxic activity

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Abstract

In recent years, NK cells, initially identified as potent cytotoxic effector cells, have revealed an unexpected complexity, both at phenotypic and functional levels. The discovery of different NK cell subsets, characterized by distinct gene expression and phenotypes, was combined with the characterization of the diverse functions NK cells can exert, not only as circulating cells, but also as cells localized or recruited in lymphoid organs and in multiple tissues. Besides the elimination of tumor and virus-infected cells, these functions include the production of cytokines and chemokines, the regulation of innate and adaptive immune cells, the influence on tissue homeostasis. In addition, NK cells display a remarkable functional plasticity, being able to adapt to the environment and to develop a kind of memory. Nevertheless, the powerful cytotoxic activity of NK cells remains one of their most relevant properties, particularly in the antitumor response. In this review, the process of tumor cell recognition and killing mediated by NK cells, starting from the generation of cytolytic granules and recognition of target cell, to the establishment of the NK cell immunological synapse, the release of cytotoxic molecules, and consequent tumor cell death is described. Next, the review focuses on the heterogeneous mechanisms, either intrinsic to tumors or induced by the tumor microenvironment, by which cancer cells can escape the NK cell-mediated attack.

KEYWORDS

cancer, cytoskeleton, cytotoxicity, immune evasion, immunological synapse, NK cells

1 | INTRODUCTION

Since their first discovery, Natural Killer (NK) cells have been clearly identified for their ability to exert powerful cytotoxic activity against various targets including tumor cells, providing important clues for their potential therapeutic implications. Hence, from the onset, NK cells were intensely studied and the mechanisms that regulate recognition and killing of tumor cells were characterized in 2 decades.[1] This achievement, however, turned out to be only the first step in the definition of the complex biology of these cells. Now it is well known

ABBREVIATIONS: ADAM, a disintegrin and metalloproteinase; ADCC, Ab-dependent cell-mediated cytotoxicity; AP-3, adaptor protein-3; Bid, BH-3 interacting domain death agonist; BLOC1, biogenesis of lysosome-related organelles complex 1; CAD/DFF45, caspase-activated DNAse; CHS, Chediak-Higashi syndrome; CIML, cytokine-induced memory-like NK cells; cSMAC, central supramolecular activating cluster; EMT, epithelial to mesenchymal transition; EOMES, eomesodermin; EV, extracellular vesicles; FADD, nFAS-associated death domain protein; FAS-L, FAS ligand; GEF, guanine nucleotide exchange factor; Grz, granzyme; HCC, hepatocellular carcinoma; HIF-1α, hypoxia-inducible factor 1α; hnRNP K, heterogeneous nuclear ribonucleoprotein K; ICAM-1, intercellular adhesion molecule-1; IDO, indoelamine-2,3-dioxygenase; ILK, integrin-linked kinase; IS, immunological synapse; ITAM, immunotyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; KIRs, killer-cell Ig-like receptors; LAMP-1, lysosomal associated membrane protein-1; LFA-1, lymphocyte function-associated Ag-1; lnc, long noncoding; Mac-1, Mϕ-1 Ag; MACPF/CDC, membrane attack complex/perforin/cholesterol dependent cytolysin; MDSC, myeloid-derived suppressor cells; MIF, migration inhibitory factor; miR, microRNA; MLL-5, mixed-lineage leukemia protein-5; MMPs, matrix metalloproteases; MOMP, mitochondrial outer membrane permeability; MT6/MMP25, membrane type 6 matrix metalloproteinase; MTOC, microtubule organizing center; NCRs, Natural Cytotoxicity Receptors; NID1, Nidogen-1; NKIS, NK cell immunological synapse; NPM, nucleolar phosphoprotein nucleophosmin; PACS, phosphofurin acidic-cluster sorting-proteins; PARP-1, poly(adenosine 5′-diphosphate-ribose) polymerase-1; PCNA, proliferating cell nuclear Ag; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; PGE2, prostaglandin E2; PI-9, proteinase inhibitor 9; pSMAC, peripheral supramolecular activation cluster; RUNX3, Runt-related transcription factor 3; SYK, Syk kinases; TAFs, tumor-associated fibroblasts; TAM, tumor-associated Mϕs; T-bet, T-box gene expressed in T cells; TCR, T cell receptor; TME, tumor microenvironment; WASp, Wiskott-Aldrich syndrome protein.; WIP, WASp-interacting protein;; αPIX, PAK-interacting exchange factor alpha.

Received: 16 June 2020 | Revised: 11 August 2020 | Accepted: 13 August 2020

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that the NK cell population displays considerable phenotypic and functional heterogeneity, which is in part related to the distribution of these cells in different body compartments, where, besides cytotoxicity, they can exert regulatory functions and even influence tissue homeostasis.[2-5] NK cells also show functional plasticity as they can adapt their function to the environment, sensing changes in the MHC-I expression levels (decreasing or increasing their cytolytic potential), in the cytokine milieu, or in O_2 tension levels.[6-9] Finally, NK cells can also undergo exhaustion after prolonged activation, increase the expression of different checkpoint inhibitory receptors, and lose their antitumor efficacy, but, in certain conditions, they can also acquire long-term memory-like properties.[10-14] All these aspects represent important key-points to be considered for an effective exploitation of NK cells in antitumor therapies, and, in this context, a number of strategies or means are under investigation at the preclinical and clinical level. Nevertheless, the detailed characterization of the process of tumor cell recognition and killing, along with the related tumor escape mechanisms, remains an ineludible central point for any reliable NK cell-based therapy.

Two major mechanisms account for the ability of NK cells to kill tumor cells: the recognition of tumor cells by a number of specific NK receptors and the subsequent release of cytotoxic granules (containing perforins and granzymes), and the engagement of death receptors on the tumor cell surface by TNF-α, FAS ligand (FAS-L), and TRAIL. This latter mechanism of killing is slower than the one mediated by granules and, although it proved important in certain conditions, such as the control of virus infections in the liver, generally it does not represent the prevalent effector arm of NK cells. Rather, it can supplement the cytotoxic action of perforins and granzymes.

In this review, we will mainly focus on the process that leads to the recognition and killing of the tumor cell via the cytotoxic granule release and on the related tumor escape strategies.

2 | THE PROCESS OF TUMOR CELL RECOGNITION AND KILLING VIA CYTOTOXIC GRANULE RELEASE

In order to exert cytolytic activity against susceptible target cells, NK cells have to manage a number of quite complex processes that include: (1) the generation and safe storage of readily accessible ammunitions (cytolytic granules and their cytotoxic content), and the modulation of their amount in response to environmental changes, (2) the generation and regulation of receptors involved in the target cell recognition and the control of receptor signaling, (3) the recognition of the target cell, the formation of a stable effector-target interaction, termed immunological synapse (IS),[15,16] and the release of the cytotoxic granule content in the synaptic cleft, and (4) the entrance in the target cells of the cytotoxic effector molecules of the granules and the execution of their cell death program within the target cell. With some differences in the granule expression regulation and (obviously) in target cell recognition, these processes are common to cytotoxic T lymphocytes (CTLs) and NK cells and are crucial for the control of tumor insurgence, growth, and progression. Indeed, essentially all of them are targets of tumor escape mechanisms (see Graphical Abstract).

2.1 | Cytotoxic effector molecules

NK cells produce and store in their cytoplasm a number of cytotoxic granules that can be used to kill specific targets, including tumor cells. These granules contain different cytolytic effector molecules, and accessory proteins that support their maintenance within the granules, their entrance in the target cell cytoplasm, and their activation. Cytolytic molecules are essentially represented by different proteolytic enzymes (granzymes) that, once released in the target cell, cleave various key proteins, including caspases, and trigger a cascade of events leading to the rapid death of the cell. Five types of granzymes can be stored within cytolytic granules of NK cells in humans. Granzymes B and A (GrzB and GrzA) are the most studied and perhaps play a major role in inducing tumor cell death, while granzymes K, M, and H (GrzK, GrzM, GrzH) may rescue or complement the activity of GrzB and GrzA by partly reproducing their signaling cascades or by cleaving unique targets.[17-19] Another cytotoxic molecule is represented by granulysin, a member of the saposin family, which comprises proteins involved in the metabolism of sphingolipids and endowed with membrane perturbing capabilities. Granulysin is produced as a 15 kDa molecule and processed in its active cytotoxic 9 kDa form within the cytolytic granules. This molecule can attack the membrane of intracellular pathogens causing their death through a process termed microptosis, but it also mediates alterations of mitochondria membrane, inducing cell death.[20,21] Interestingly, the 15 kDa form can be released as alarmin, contributing to the induction of adaptive response.[21]

Cytolytic granules also contain perforins. These molecules belong to the family of the Membrane Attack Complex/Perforin/Cholesterol Dependent Cytolysin (MACPF/CDC) proteins, which comprises elements, such as complement factors and different bacterial toxins, capable of forming pores in either eukaryotic or prokaryotic cells. In particular, perforins bind to cholesterol-rich membranes of eukaryotes, therefore specifically exerting their action on virally infected and tumor cells, and not on bacteria.[22] After target cell recognition and synapse formation, the cytotoxic granules converge, fuse to the killer cell membrane, and release their content in the synaptic cleft. Released perforins associate to the target cell surface membrane, polymerize, and form 19–24 mer pores that enable the entry of granzymes and other molecules released from granules.[23] Cathepsin B and Lysosomal Associated Membrane Protein-1 (LAMP-1/CD107a), which are exposed at the killer cell membrane following granule fusion, inhibit the pore formation at the NK cell side. Specifically, CD107a prevents perforins from binding to the membrane, while cathepsin B may proteolytically inactivate them.[24,25] Besides providing direct entryways, the pores also induce local membrane repair actions, which cause endocytosis of granule effector molecules and vesicle formation in the target cell. Such vesicles can then release granzymes and granulysin by their perforin-based pores.[26]

As perforins and granzymes are crucial for NK cell cytotoxicity, their synthesis and function are tightly regulated, both along the maturation steps of NK cells and in response to environmental stimuli. Eomesdermin (EOMES), a key factor for NK cell maturation, can bind the -1 kb enhancer of the *PRF1* gene, and its expression appears to be decisive for precursor commitment to cytotoxic cells.[27] Mature NK cells constitutively express perforins, although with differences depending of the final steps of differentiation or the acquisition of specific functional status. Thus, the relatively immature $CD56^{bright}$ cells express about 10-fold less perforins than the $CD56^{dim}$ mature NK cells, and within the $CD56^{dim}$ population, the terminally differentiated $CD57^{+}KIR^{+}$ cells express highest perforin and GrzB levels.[28,29] This latter cell population is also characterized by the expression of genes related to cytoskeletal remodeling, which are important to exert cytolytic function. In addition, mature NK cells interacting with self HLA class I by mean of their inhibitory Killer-cell Ig-like receptors (KIRs) are "licensed" or "educated" to express higher cytolytic activity, and show a structural change of the lysosomal compartment and increased accumulation of cytotoxic granules.[30] By contrast, the $CD56^{-}CD16^{+}SIGLEC7^{-}$ NK cells display reduced transcripts for perforins, GrzB, and other cytolytic markers. These cells characteristically express many type I IFN-responsive genes and release chemokines (CCL3, CCL4, CCL5), suggesting that they may exert an antiviral activity through interaction with plasmacytoid dendritic cells and T cells rather than through cytotoxicity.[29] Upon the exposure to infections or to an inflammatory milieu, NK cells can undergo epigenetic changes acquiring the ability to recognize and promptly respond to certain stimuli and to pass this capacity to the progeny. Such "memory" NK cells have been described in different contexts and, in humans, include the "adaptive" $NKG2C^{+}$ NK cells expanded in $HCMV^{+}$ individuals, the cytokine-induced memory-like NK cells (CIML) (which can be induced by the combined action of IL-12, IL-18, and IL-15), or memory-like NK cells that are induced either by CD16 engagement[31] or by interaction with tumor cells (tumor-induced memory-like, TIML, NK).[32] Remarkably, these different types of memory-like NK cells show high levels of perforins and GrzB gene expression, and, in the case of CIML, they can further increase GrzB expression in response to IL-2.[29] In general, cytokines, including IL-2, IL-6, IL-12, IL-15, IL-21, and IFN-β, play a crucial role in increasing perforin expression in NK cells.[27] Indeed, several members of the cytokine-activated STAT family (i.e., STAT1, 3, 4, 5) and NF-kB can bind the -1 kb enhancer of the *PRF1* gene. In addition, STAT4 has also been shown to enhance the transcription of the Runt-related transcription factor 3 (RUNX3), which is necessary for *PRF1* expression.[33] Nevertheless, it should be also considered that different activating receptors, including NKp30, NKG2D, DNAM-1, and 2B4, could contribute to NF-kB activation,[34,35] suggesting that also target cell recognition may stimulate *PRF1* gene transcription. A further control of perforin and granzyme protein levels is exerted post-transcriptionally. In particular, several microRNA (miR) have been shown to exert direct or indirect effects on perforin and/or GrzB translation. Thus, miR-150 and miR-30e directly inhibit perforin expression, miR-378 inhibits GrzB, and miR27a* inhibits both.[36-38] Moreover, miR-146a inhibits perforin expression by targeting STAT1,[39] while miR-362-5p sustains both perforin and GrzB expression by inhibiting CYLD, a deubiquitination enzyme that negatively regulates NF-kB.[40]

Once synthesized, perforins and granzymes need to be transported and safely stored in the granules. Granzymes are produced as zymogens, moved to the Golgi where they are added mannose-6-phosphate (M6P) moiety, and then sorted via M6P receptors (MPR) to the cytotoxic granules.[17,18] In the cytotoxic granules, granzymes are activated by the lysosomal protease Cathepsin C (and also Cathepsin H for GrzB) and associated to the anionic proteoglycan serglycin to form multimeric complexes.[17,18,41] Serglycin should ensure efficient accumulation of GrzB in the granules, as suggested by a recent study on serglycin gene-disrupted mice.[42] As secure mechanism, the proteinase inhibitor 9 (PI-9/serpin B9), belonging to the serine proteinase inhibitor (serpin) family, is present in the cytosol and can inactivate the granzymes that inadvertently exit the granules.[43] At variance with granzymes, perforins may be already active (and potentially dangerous) at the high Ca^{2+} concentration and neutral pH of the endoplasmic reticulum (ER), and need to be rapidly moved to the Golgi and then to the granules, where they can be maintained in an inactive status due to the local acidic pH. A highly efficient ER export motif at the C-terminal of the perforin molecule is crucial for its rapid release from the ER. In addition, the N-linked glycosylation of the C-terminal prevents perforin from polymerizing and forming unwanted pores in the ER.[44] In the Golgi, a complex system of protein sorting ensures the correct and selective delivery of perforins from the trans-Golgi network to the cytotoxic granules. In particular, the adaptor protein-3 (AP-3) complex and Pallidin, a component of the Biogenesis of Lysosome-related Organelles Complex 1 (BLOC1),[45-47] as well as AP-1 and LAMP-1 (CD107a)[25,48] seem to be crucial for the effective perforin accumulation in the granules.

2.2 | Synapse formation

During the encounter between an NK cell and a susceptible tumor cell, a stable interaction, termed lytic immunological synapse, is formed, ultimately leading to tumor cell death. This process involves specific receptor-ligand interactions, cytoskeletal rearrangement, and, finally, granule polarization and secretion (Fig. 1).

2.2.1 | Receptor-ligand interactions

The first steps of NK cell-mediated target cell killing are NK cell adhesion to the target cell and polarization of actin cytoskeleton. The β2-integrin LFA-1 (lymphocyte function-associated Ag -1 or CD11a/CD18) has been shown to play a central role in the early phase of NK cytotoxic synapse formation.[49] Interaction of LFA-1 with ICAM-1 (intercellular adhesion molecule-1) on target cells initiates an intracellular signaling cascade leading to tyrosine phosphorylation of different substrates, including CD3ζ chain, Pyk2, paxillin, the guanine nucleotide exchange factor Vav1, and resulting in actin cytoskeleton modifications.[50-53] Following interaction with target cells, LFA-1 molecules localize in an outer region of the IS, termed peripheral supramolecular activation cluster (pSMAC), mediating a tight adhesion and delivering activation signals.[54-56] In human NK cells, binding

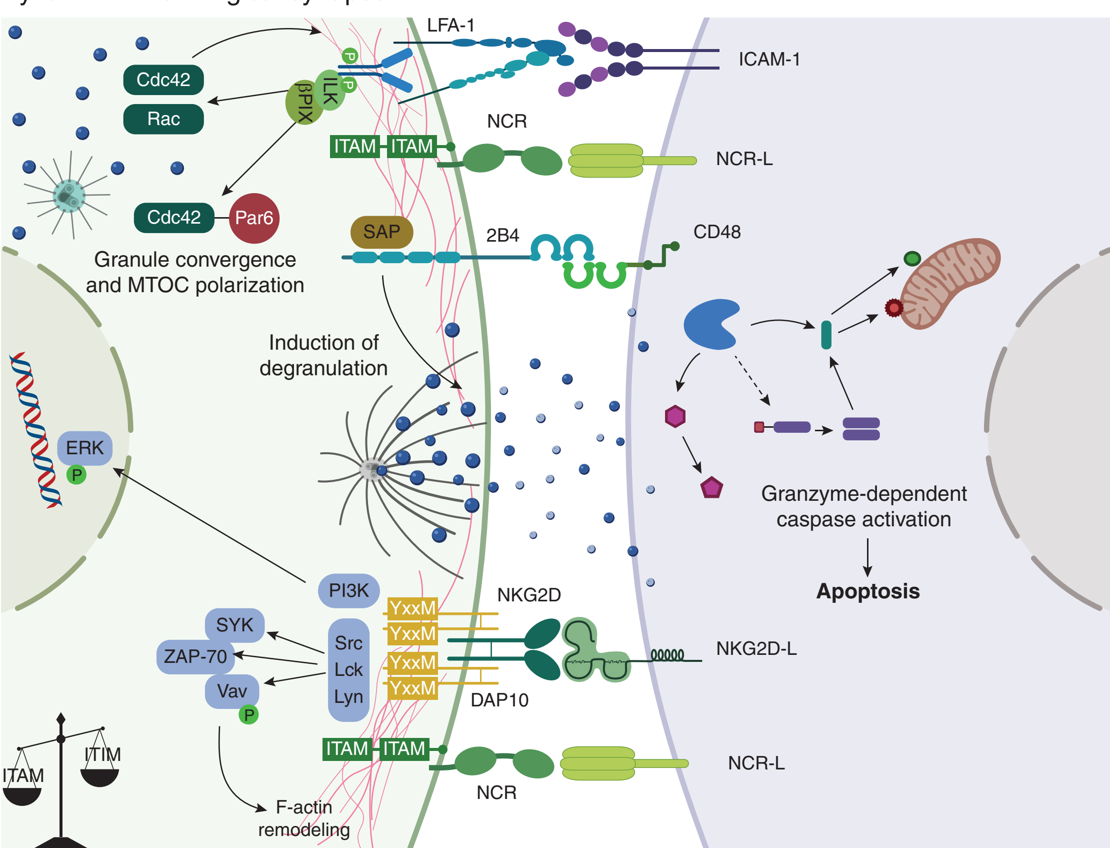

FIGURE 1 The lytic NK cell immunological synapse. The engagement of adhesion molecules, followed by the recognition of specific ligands by activating receptors, results in signaling cascades inducing cytoskeletal rearrangement, stable cell-to-cell interaction, and granule polarization and release into the synaptic cleft.

of LFA-1 to ICAM-1 on target cells promotes not only adhesion and cell conjugate formation but also polarization of cytotoxic granules and their accumulation at the site of NK cell contact with the target cell together with the microtubule organizing center (MTOC).[56-58]

In T cells, inside-out signals (from T cell receptor - TCR) are necessary for proper LFA-1 function, as such signaling induces an open conformation of LFA-1, resulting in an increased affinity for its ligands. Remarkably, NK cells can organize granule polarization upon the sole engagement of LFA-1.[57,58] Nevertheless, inside-out signals by activating receptors can enhance LFA-1 binding to ICAM-1 also in NK cells. In particular, triggering of DNAM-1 receptor on NK cells has been shown to provide signals for LFA-1, increasing the efficiency of conjugate formation.[59] Indeed, DNAM-1 is rapidly recruited with LFA-1 to the IS, especially in the presence of DNAM-1 ligands.[60,61] In fact, affinity maturation of LFA-1 may be induced by the ligand-mediated engagement or co-engagement of different receptors and co-receptors, including DNAM-1, NKG2D, CD2, 2B4, and NTB-A, although a definite consensus on the receptor type and possible synergies has not yet been achieved.[59,62] Moreover, NKG2D can synergize with 2B4 in case of 2B4 and NKG2D co-engagement. Cytokines able to increase the activity of NK cells, such as IL-12, IL-15, and IL-18, do not induce inside-out signals, but pre-incubation of NK cells with these cytokines can lower the threshold for subsequent activating receptor stimulation.[62]

The ability of NK cells to adhere to target cells has been addressed with respect to the process of the so-called licensing (or education), in which NK cells modulate their effector functions depending on the availability/recognition of self MHC class I molecules.[6,63] Licensing has been shown to increase the strength of NK-target cell interaction; although both licensed and unlicensed NK cells express perforin and granzymes,[6] unlicensed NK cells are less efficient in establishing stable conjugates with target cells due to a decreased inside-out signaling to LFA-1 by activating receptors and a consequent diminished adhesion to target cells.[64] Educated NK cells, displaying a high cytotoxic potential, generally exhibit a higher DNAM-1 expression, and several experimental observations suggest that the coordinated expression of LFA-1 and DNAM-1 could play a central role in NK cell education.[61] In addition, 2B4 and NKG2D have been shown to induce LFA-1 activation more efficiently in educated NK cells.[62] Analysis of LFA-1 activation in different NK cell subpopulations indicated that triggering of activating receptors induced the strongest LFA-1 activation in the most mature CD56^{dim}CD57^{+} NK cell subset, characterized by potent cytotoxic activity, whereas LFA-1 activation in CD56^{bright} NK cells was much less efficient.[62]

In NK cells, lytic granule polarization and degranulation are 2 uncoupled events. LFA-1 binding to ICAM-1 induces the polarization of granules but it is not sufficient to induce their effective release in

the synaptic cleft.[58,59] The engagement of activating receptors, recognizing specific ligands on tumor cells, represents the crucial event. The main activating receptors involved in tumor cell recognition and cytotoxicity triggering are represented by natural cytotoxicity receptors (NKp46, NKp30, and NKp44), NKG2D, and DNAM-1.[65-67] In addition, NK cells also express several co-receptors, including 2B4 and NTBA, belonging to the SLAM family,[65,68] and NKp80.[69] During tumor cell recognition, activating receptors are recruited to the NK cell immunological synapse (NKIS).[61,70-72] For example, NKp46 has been shown to cluster at high density at the IS and to mediate both F-actin accumulation, contributing to cytoskeletal rearrangement, and granule polarization.[71] A minimal requirement for natural cytotoxicity mediated by resting NK cells has been established as the co-engagement of LFA-1, NKG2D, and 2B4.[59] Engagement of NKG2D, DNAM-1, 2B4, or CD2 alone or in combination with LFA-1 is not sufficient to induce degranulation, while co-engagement of NKG2D and 2B4 through ligand recognition on target cells results in degranulation signals. Combinations of activating receptors acting synergistically are generally required to deliver degranulation signals, the only exception being represented by CD16 (or FcγRIII3a), whose interaction with IgG is sufficient to induce degranulation.[58]

Upon receptor engagement, several activating receptors have been shown to localize to membrane microdomains (or lipid rafts), cholesterol-rich membrane domains enriched at the NKIS.[73,74] Membrane microdomains are essential for NK cell activation, and their disruption affects NK cell-mediated cytotoxicity.[75] The engagement of inhibitory receptors by HLA class I molecules during NK-tumor cell interaction can result in the formation of an inhibitory IS.[15,16,76] Clustering of KIR and NKG2A occurs very rapidly and is an actin-independent process,[59,77] resulting in the inhibition of adhesion to target cells, LFA-1 activation, synaptic accumulation of F-actin, and activating receptor clustering at the IS.[78-80]

Surface density of activating receptors is one of the relevant factors predicting NK cell-mediated cytotoxic activity against tumor cells. Resting NK cells constitutively express most activating receptors, and further increase the expression of some of them upon exposure to different cytokines, such as IL-2, IL-12, IL-15, and IL-18.[2] Remarkably, IL-2, IL-15, or IL-1β further extends the NK cell ability to recognize and kill tumor target cells by inducing the *de novo* expression of the activating receptor NKp44.[81,82] It is of note, however, that activating receptor expression can be heterogeneous in different NK cell developmental stages and in distinct NK cell subsets. Also, miRNA can indirectly influence activating receptor function. In particular, mir-155, up-regulated by IL-2, IL-15, and IL-21,[83] can enhance NK cell-mediated cytotoxicity by regulating molecules involved in the control of NK cell activation, including SHP1 phosphatase, and by increasing IFN-γ production.[84,85] Tumor ligands bound by activating NK receptors are quite heterogeneous, but they mainly include molecules scarcely expressed on healthy cells and up-regulated following cellular stress or transformation.[65,86-89] The expression of multiple ligands for activating receptors, and the subsequent triggering of different signaling pathways, is fundamental in dictating the susceptibility of tumor cells to NK cell-mediated killing.

2.2.2 | Cytoskeletal rearrangement

Both pharmacological experiments and studies on immune cells with defects in actin cytoskeleton organization and dynamics have documented that actin cytoskeleton remodeling is critical for the effector functions of cytotoxic immune cells and correct function of the immune system altogether. Since the first description of the IS in T lymphocytes by Abraham Kupfer and the extension to NK cells by Daniel M. Davis and Jack Strominger, immunological synapses have been extensively studied.[15,90] Actin rearrangement in cytotoxic immune cells is traditionally analyzed using flow cytometry to measure total F-actin content over time and confocal microscopy after stimulation of activating receptors with chemokines or Ags. Actin dynamics at the IS can be studied in detail from initiation to termination using surfaces coated with activating Abs, or by employing hybrid techniques, such as imaging flow cytometry.[91-96] Since the first publications, the role of the actin cytoskeleton in shaping the architecture of the NKIS has been investigated with increasingly sophisticated techniques, providing us with a more detailed understanding of the process. The formation of a mature lytic NKIS requires several steps of cytoskeletal rearrangement. Although NKIS formation can be divided into different steps, a linear description of the underlying actin cytoskeleton rearrangements is difficult because several of these events take place simultaneously.[97,98]

The recognition phase is characterized by the assembly of a lamellopodial structure that is further stabilized by sufficient activating signals. In migratory cells, the lamellopodium is the region where focal adhesions are formed and the actin cytoskeleton is rearranged in a branched, protrusive network. Structural similarities between the leading region of migrating cells and the assembly of the T cell IS have been previously described.[91,99] An analogue structure allows NK cell spreading over the target cell surface and results in the formation of a symmetrical IS.[100] Far-reaching actin cytoskeletal rearrangements mark an important step toward the effector phase and target cell cytotoxicity. *De novo* actin filament polymerization and F-actin rearrangement lead to actin filament accumulation in a pSMAC that is characterized by an accumulation of cell-cell adhesion molecules, such as CD2, LFA-1, and Mϕ-1 Ag (Mac-1 or CD11b/CD18), but also activating receptors, such as NKp30 and NKp46.[71,72,101] Clustering of integrins allows for a firm adhesion of the NK cell to its target. Studies have shown that engagement of LFA-1 with its ligand ICAM-1 is involved in early activating signaling in NK cells.[57,102] Outside-in signaling of LFA-1 is mediated by the integrin-linked kinase ILK and its downstream target PAK-interacting exchange factor alpha (αPIX) that functions as a guanine nucleotide exchange factor (GEF) for small Rho GTPases, in particular Rac and Cdc42.[103-105] This promotes a rapid activation of actin cytoskeleton rearrangement and branch formation from existing actin filaments through both Rac and Cdc42.[103,106] Similar to the CTL IS, accumulation of integrins and activating receptors at the lytic NKIS results in an increasing activating signal that sustains the actin reorganization. The majority of NK cell activating receptors signal through homo- or heterodimerization of their cytosolic Immunereceptor Tyrosine-based Activation Motifs

(ITAMs).[107,108] ITAM-based signaling is also used by other activating immunoreceptors, as for example the TCR, and is therefore a well characterized activating pathway.[109-111] Engagement of activating receptors with their ligand induces phosphorylation of ITAMs by Src family kinases, which in turn initiates recruitment of further kinases to the NKIS.[112] High-resolution imaging revealed that clustering of signaling receptors at the NKIS is accompanied by recruitment of actin cytoskeleton regulatory proteins such as Vav-1, -2, -3, ZAP-70, and Syk kinases (SYK), but also adaptor proteins Grb2, SLP-76, and BLNK.[50,113-119] Actin cytoskeleton branching at the pSMAC of the NKIS is largely driven by Wiskott-Aldrich syndrome protein (WASp) signaling and Arp2/3 complex-induced actin filament nucleation and branching.[120] Defective WASp signaling as seen in Wiskott-Aldrich syndrome patients is characterized by severe immune deficiency due to dysfunctional F-actin network branching.[121,122] Remarkably, stimulation of NK cells with IL-2 can activate a parallel pathway to F-actin rearrangement at the NKIS via the WASp homologue WAVE2 and restore IS formation and cytotoxicity.[123] The upstream activator of WASp Cdc42 is itself regulated by DOCK8 GEF. Activation of DOCK8 has been reported to mediate NK cell cytotoxicity through Src kinase activation and talin interaction.[124,125] Consequently, ablation of DOCK8 activity in NK cells interferes with conjugate formation and was associated with a decrease of F-actin content and integrin accumulation at the NKIS without affecting overall F-actin content.

The recognition and initiation processes in NK cells that result in the formation of an IS are fast occurring events that rely on complementary actin cytoskeleton regulatory pathways. The actin rearrangement is induced by the engagement of ITAM-bearing receptors on the NK cell surface and results in the formation of a stable NKIS that forms a tight connection between effector and target cell. In contrast to cytotoxic T cells, where the engagement of a few TCRs results in coupling of several ITAMs and a potent activating signal, activating receptor signaling in NK cells can be overridden by inhibitory signaling as a safety mechanism. Signaling of inhibitory receptors has been suggested to prevent NKIS formation through interference with early actin cytoskeleton rearrangement steps.[78] Signaling of inhibitory receptors, such as KIRs and the HLA-E-specific receptor NKG2A, occurs through the Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM). Clustering of inhibitory receptors at the NKIS has been demonstrated to occur independent of actin cytoskeleton dynamics.[117,126] Phosphorylation of ITIMs after ligand binding leads to recruitment of the Src homology region 2 domain-containing phosphatases SHP-1 and -2 as well as the tyrosine kinase c-Abl.[127,128] Phosphatase activity of SHP-1 can lead to an inactivation of the GEF Vav-1, while signaling complexes involving c-Abl have been shown to control actin cytoskeleton rearrangement through inhibition of the adaptor molecule Crk. Activating and inhibitory receptors cluster together in NK cells, similar to what was described as TCR microclusters in CTLs.[117,129-132] Through their ITIM signaling pathways, inhibitory receptors block actin cytoskeleton rearrangement in close proximity to activating ligands.[120,133] This ensures regulation of NK cell-mediated cytotoxicity by prevention of NKIS formation in the presence of inhibitory ligands, and provides an elegant solution to prevent progression of the lytic NKIS as early as the initiation stage.

2.2.3 | Granule polarization and secretion

Proceeding of the lytic NKIS leads to entry of the effector phase and is accompanied by convergence of lytic granules to the MTOC.[134] While granule convergence was reported to rely on integrin signaling, polarization of lytic granules to the IS is dependent on synergistic signaling of at least 2 different activating receptors in NK cells.[59] Activating signaling by different receptors is accompanied by fine-tuned actin filament deconstruction at the central supramolecular activating cluster (cSMAC) of the NKIS.[134,135] This activation-induced clearance of the dense actin network is mediated by myosin IIA-induced contractility, as well as coronin 1A-induced actin filament reorganization.[48,113,135,136] Lytic granule polarization, the pre-requisite for localized NK cell cytotoxicity, is initiated by the increment in cortical actin meshwork size at the cSMAC of the NKIS and is dependent on colocalization of WASp-interacting protein (WIP), another actin regulatory protein, with lytic granules.[137-139]

RNAi-mediated knockdown of WIP has no impact on NKIS formation, but specifically prevents lytic granule polarization and therefore NK cell cytotoxicity. Degranulation itself is initiated by actin filament rearrangement that results in an increase in the actin mesh size that allows for lytic granule passage.[134,135] Inhibition of cortical actin remodeling either by introduction of specifically mutated actin regulatory proteins or interference with actin dynamics using molecular inhibitors prevents NK cell degranulation at the lytic IS.[135] High resolution imaging techniques have revealed that actin filaments are indeed not entirely cleared from the cSMAC of the NKIS, but that they are instead rearranged in a way to open up the dense actin mesh and allow for the passage of lytic granules, their docking to the NK cell membrane and, finally, release of their content into the synaptic cleft.[113,140]

The role of cortical actin as a barrier for the secretion of organelles is further highlighted by a recent study addressing the impairment of NK cell cytotoxicity in Chediak-Higashi syndrome (CHS) patients.[141] This primary immunodeficiency is caused by a mutation in the lysosomal trafficking regulator gene LYST that results in enlarged lytic granule formation in NK cells. While initiation of a lytic NKIS is normal and experiments confirmed the lytic granules to be functional, under normal circumstances sufficient actin clearance at the cSMAC is not increasing the mesh size enough to allow for passage of these giant lytic granules. Further disruption of the cortical actin network using actin destabilizers, such as Latrunculin B or swinholide, allowed for lytic granule degranulation and restored NK cell cytotoxicity.[141] In summary, MTOC and granule polarization to the NKIS depends on actin rearrangement, although the precise regulatory mechanisms are not described yet. Degranulation events at the lytic NKIS are initiated by clearance of the cortical actin network at the cSMAC and work through a size-exclusion mechanism that allows for passage of lytic granules into the synaptic cleft between effector NK and target cell.

While steps involved in NK cell activation and formation of NKIS have been deeply investigated, much less is known about

mechanisms allowing NK cells to detach from their targets. Dissociation of NK cells and interruption of cell conjugates are highly regulated processes, and can vary depending on the presence of other susceptible target cells. In particular, detachment from an initially bound target is accelerated if NK cell establishes a conjugate with new target cells.[142] Freshly detached NK cells express markers of activation and degranulation, that is, CD69 and CD107a, and a reduced expression of activating receptors because of ligand-induced receptor internalization. For instance, NKG2D is internalized by clathrin-mediated endocytosis upon ligand recognition[143]; on the other hand, CD16 modulation from NK cell surface can occur following NK cell activation through proteolytic cleavage of its extracellular portion by ADAM17 (a disintegrin and metalloproteinase-17) or by MT6/MMP25 (membrane type 6 matrix metalloproteinase).[144,145] MT6/MMP25 expression and translocation to the cell surface from intracellular compartments is induced by IL-2, and this protease is recruited to the IS during Ab-dependent cell-mediated cytotoxocity (ADCC).[145] Recently, this mechanism of CD16 shedding has revealed important in favoring the disassembly of NK-IS.[146] Detachment of NK cells from the killed target cell is necessary for the subsequent interaction with (and killing of) a new target cell. Thus, this sequence of events enables NK cells to sustain the serial killing of multiple targets, significantly enhancing the efficacy of the NK cell-mediated cytotoxic response.[147-149] The observation that about 10% of the available granules are released in a single killing event suggests that NK cells have enough ammunitions to sustain serial killing.[150] The molecular dissection of this process has recently indicated that, indeed, the early killing events during serial killing are very fast and are mediated by lytic granule release, while the last ones are slower and occur through Fas-FasL interactions.[151]

2.3 | Granule-mediated tumor cell death

As mentioned above, granzymes are the major effectors of the granule-mediated tumor cell death. In particular, GrzB exerts strongest effects. It acts through 2 main mechanisms: the cleavage and activation of Caspase 3 and the processing of Bid (BH-3 interacting domain death agonist) with the generation of a truncated form (tBid). Caspase 3 activation leads to cell death through the caspase activation cascade, whereas tBid is translocated to the mitochondria where it interacts with Bax and/or Bak increasing the mitochondrial outer membrane permeability (MOMP) and the subsequent release of Cytochrome C and Smac/Diablo from the intermembrane space. Cytochrome C and Smac/Diablo favor the formation of a complex, the apoptosome, which drives the caspase cascade. In addition, Smac/Diablo also removes the association of Inhibitor of Apoptosis Protein (IAP) with Caspase 3, increasing the proapoptotic effect of GrzB-Caspase 3 interaction. Remarkably, GrzB can further enhance its pro-apoptotic activity by processing the inhibitor of Caspase-activated DNAse (I-CAD/DFF45) resulting in the release of CAD and consequent DNA fragmentation.[152] GrzB-induced pathways can be regulated at different points. For example, the anti-apoptotic protein Bcl-2 interferes with the activity of Bak/Bax,[153] while the phosphofurin acidic-cluster sorting-proteins 2 and 1 (PACS2 and PACS1) favor Bid cleavage and Bak/Bax oligomerization, respectively. PACS2 is positively regulated at the transcriptional level by PCAF and ADA3 factors.[154,155]

GrzA activity is independent on caspase or Bid, as it is not inhibited by Bcl-2 or caspase inhibitors, and complements GrzB effects. GrzA rapidly induces mitochondria to generate reactive oxygen species (ROS) that contribute to the translocation to the nucleus of the ER-associated complex SET.[156] In the nucleus, GrzA cleaves SET inducing the release of 2 DNAses, NM23-H1 and TREX1, which contribute to the DNA damage.[157] In addition, GrzA also inactivates the DNA damage sensor poly(adenosine 5′-diphosphate-ribose) polymerase-1 (PARP-1)[158] and the DNA repair molecule Ku70,[159] further increasing DNA damage.

GrzK partially overlaps the activity and substrate specificity of GrzA, as it induces ROS production and SET translocation to the nucleus. GrzK also cleaves the microtubule network protein β-tubulin thus potentially influencing a number of cell functions including motility, intracellular trafficking, mitosis, and cell survival.[160] Finally, an additional target of GrzK is the heterogeneous nuclear ribonucleoprotein K (hnRNP K), whose expression has been shown to sustain tumor cell survival and epithelial to mesenchymal transition (EMT).[161,162] Interestingly, hnRNP K is targeted by all granzymes.

GrzM causes cell death in a caspase 3- and Bid-independent way. Although its mode of action is not yet completely understood, different targets have been identified. GrzM cleaves FAS-associated death domain protein (FADD) causing oligomerization of truncated FADD, which induces caspase 8 activation.[163] In addition, it can target α-tubulin and disorganize the microtubule network in tumor cells.[164] Finally, GrzM also inactivates the nucleolar phosphoprotein nucleophosmin (NPM), an inhibitor of apoptosis,[165] and, remarkably, it can also enhance GrzB activity by inhibiting the GrzB inhibitor, serpin B9.[166]

GrzH may induce cell death via a bcl-2-sensitive mitochondrial pathway, which, however, is Bid-independent.[167] In addition, it has also been proposed that GrzH could cleave I-CAD/DFF45. However, a consensus on the GrzH pathways has not yet been achieved.

3 | TUMOR ESCAPE STRATEGIES

Tumor cells and, more in general, the tumor microenvironment (TME) can inhibit NK cell-mediated antitumor response by interfering with virtually all the processes that enable NK cells to form an active lytic synapse, and also by inactivating lytic molecules released into target cells (Fig. 2).

3.1 | Modulation of cytotoxic effector molecules

In this latter decade, several studies have reported alterations of perforin and/or GrzB expression in NK cells from patients with different solid or hematologic tumor types, indicating these effector molecules among the targets of the tumor escape strategies (Fig. 2A). TGF-β, a key player of the tumor-mediated immunosuppression, appears to have a role in perforin/granzyme down-regulation. This cytokine

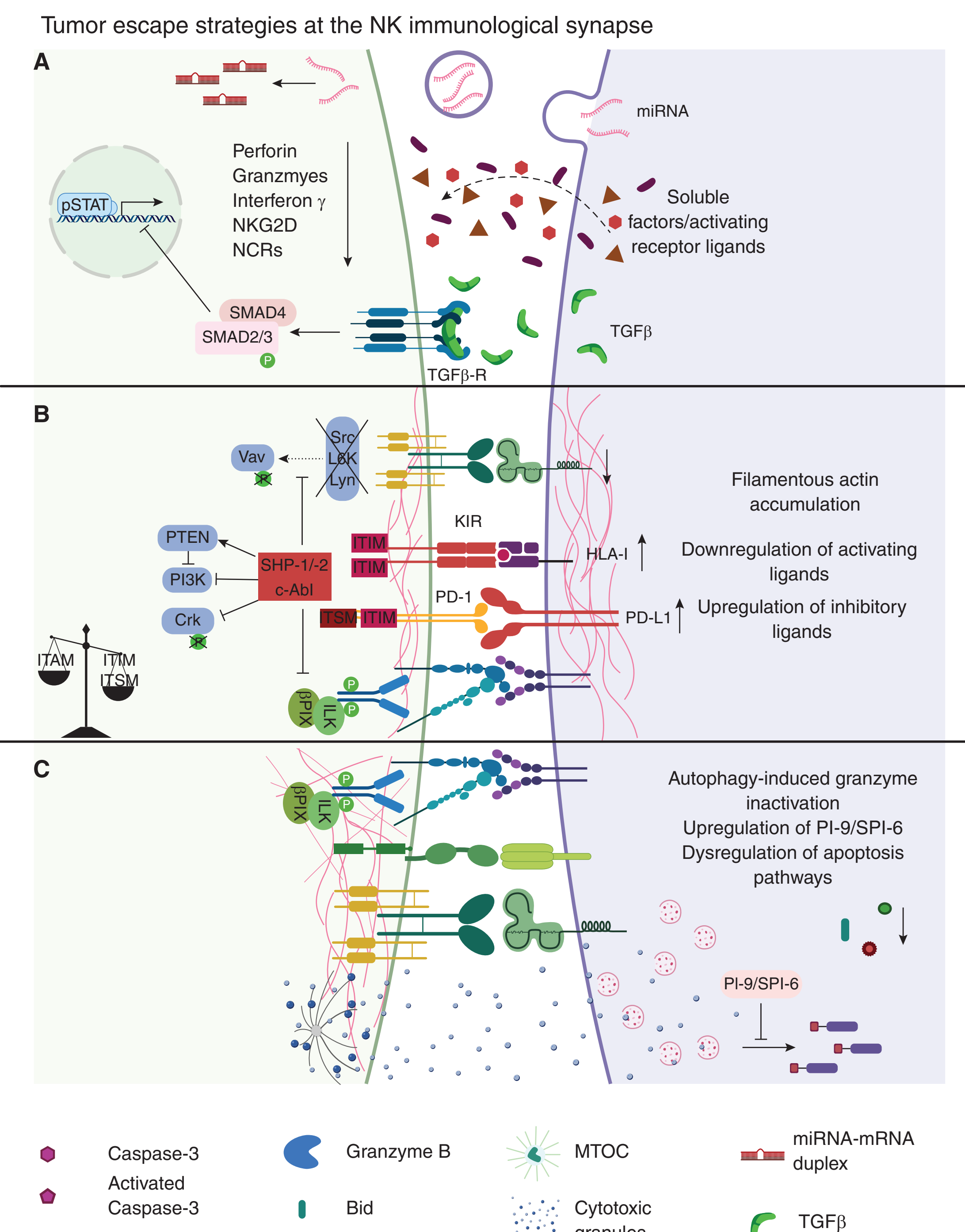

FIGURE 2 **Tumor escape strategies involving the process of tumor cell recognition and killing through cytotoxic granules.** Tumor cells can inhibit NK cell-mediated anti-tumor response at several levels: generation of cytotoxic granules and expression of triggering NK cell receptors (panel A); tumor cell recognition, immune synapse formation, and delivery of lytic effector molecules into the target cell (panel B); execution of the programmed cell death by the delivered lytic effectors (panel C).

can be produced by different cell types of the TME, including tumor cells, or presented at the surface of regulatory immune cells, and can also be stored within the ECM as inactive latent complex.[168,169] Different enzymes, including some matrix metalloproteases (i.e., MMP9 and MMP14) and the interaction with certain αv-based integrins expressed by DC and endothelial cells induce the release from the ECM and activation of TGF-β.[168,170] Along this line, tumor cells expressing MMP9 and indoelamine-2,3-dioxygenase (IDO) have been shown to induce down-regulation of GrzB, perforins, and activating NK receptors.[171] Active TGF-β1 acts on NK cells by inducing SMAD2/3 phosphorylation and subsequent formation of the SMAD4-SMAD2/3 complex, which inhibits GrzB and IFN-γ gene transcription. Actually, SMAD4 appears to play a key role in the orchestration of the anti-tumor response, acting on NK cell function and, specifically, on GrzB expression.[172,173] Indeed, in the absence of TGF-β signaling, SMAD4 can associate to JUNB to positively regulate GrzB transcription in NK cells.[172] TGF-β1 also affects NK cell function by acting on various miRNA targeting different effector molecules and receptors.[174] For example, TGF-β1 (and IL-10) induces expression of the STAT1/perforin inhibitor miR146a (see above).[39] TGF-β1 can also be stored in tumor cell extracellular vesicles (EV) or exosomes. EV from pancreatic ductal carcinoma carrying TGF-β1 have been recently reported to induce SMAD3 phosphorylation and inhibit NK cell cytotoxicity.[175]

Actually, tumor-derived exosomes are carriers of different immune-modulating molecules, including miR, capable of suppressing perforin or granzyme expression. For example, AML cells release exosomes enriched in different miR,[176] including miR-150, which targets, among others, perforin mRNA (see above).[36] It has also been recently shown that AML cells also deliver (likely by exosomes) miR-29b, which interferes with the expression of EOMES and T-box gene expressed in T cells (T-bet), 2 key transcription factors for the maturation of efficient cytotoxic and IFN-γ producing NK cells.[177] Along this line, EOMES can associate with the -1 Kb enhancer of the perforin locus.[27] NK cells from patients with hepatic cell carcinoma (or with HBV infection) showed increased expression of miR-146a, which correlated with low NK cell function. MiR-146a overexpression experiments demonstrated that miR-146a targets STAT-1 and induces down-regulation of IFN-γ, TNF-α, and perforin.[39] Interestingly, miR-146a has also been recently shown to be up-regulated in melanomas and to correlate with lower survival and higher metastasis formation in mice.[178] Another immunosuppressive miR is represented by miR-544, which is up-regulated in NK cells from patients with liver cancer. MiR-544 promotes immune escape by suppressing the expression of NKp46 and RUNX3.[179] RUNX3, which regulates the expression of different genes in T and NK cells,[180] has also been proposed to favor perforin gene transcription (see above).[27] Remarkably, NK cells from liver patients also displayed a reduced expression of the long noncoding (lnc)RNA GAS5, an inhibitor of miR-544 capable of recovering NK cell function.[181] Tumor cells may also inhibit expression of miR acting as tumor suppressors, as in the case of miR-186, which has been shown to target both different tumor-related genes and TGF-β pathway. MiR-186 is delivered in exosomes by NK cells, but its expression is inhibited in high risk neuroblastoma patients.[182]

The chronic exposure to the tumor microenvironment may also induce the so-called “exhausted” phenotype in NK cells.[183-187] Such exhausted NK cells are often characterized by the expression of one or more inhibitory checkpoint receptors, such as programmed cell death protein 1 (PD-1), TIM-3, or TIGIT, which are generally induced on activated T and NK cells to limit or finish their responses. Exhausted NK cells show reduced expression of one or more activating receptors and decreased ability to proliferate, release cytokines, and degranulate. Low responsiveness to cytokines, low activating receptor expression, and the action of inhibitory checkpoint receptors may account for their reduced function. Although perforins and granzymes are generally expressed at normal (or even higher) levels in exhausted NK cells,[184,186,187] a study on esophageal cancer has shown a correlation between expression of TIM-3 and the reduced mRNA expression of GrzB.[188] In addition, TIGIT signaling has also been shown to alter granule polarization resulting in NK cell-mediated killing inhibition.[189]

3.2 | Inhibition of synapse formation

3.2.1 | Receptor-ligand interaction

Dysregulation of the multiple receptor-ligand interactions occurring between tumor and NK cells probably represents one of the most effective escape strategies for the tumor cells to avoid NK cell cytotoxicity. In fact, the decision to kill is determined by the integration of both inhibitory and activating signals that the NK cell receives depending on which and how many receptors and adhesion molecules are engaged during target recognition. Therefore, diverse escape mechanisms may alter this balance, targeting either activating or inhibitory receptor-ligand interactions in favor of tumor cell survival. Importantly, TME plays a pivotal role in the process, by providing soluble factors and cellular contacts (involving tumor cells, stromal cells, and different immune cell types) capable of modulating expression/function of NK receptors or of their tumor-expressed ligands (Fig. 2A and B).[190-193]

Natural Cytotoxicity Receptors (NCRs), NKG2D, and DNAM-1 are the main receptors responsible for the recognition and killing of tumor cells[65]; their expression can be down-regulated due to several factors released in the TME. TGF-β, able to exert multiple immunosuppressive effects, has been shown to inhibit NKp30 and NKG2D expression.[194,195] The induction of IDO enzyme is associated with an immunosuppressive TME too, due to the production of the tryptophan catabolite L-kynurenine, which impairs IL-2-mediated increase of NKp46 and NKG2D receptors.[196,197] Different immune cells present in TME, including Treg cells, myeloid-derived suppressor cells (MDSC), and tumor-associated Mϕs (TAM), can negatively affect NK cell antitumor effector functions, mainly through the release of TGF-β. Notably, immunosuppressive factors in the TME can be also produced by non-immune cells. In different tumor types, such as melanoma, colorectal cancer, and hepatocellular carcinoma (HCC), tumor-associated fibroblasts (TAFs) can prevent IL-2 induced up-regulation of NKp30, NKp44, and DNAM-1 receptor expression, via the production of prostaglandin E2 (PGE2).[198-200] Tumor cells can also directly affect NK cell-mediated effector functions. For instance, melanoma cells can impair their

recognition and killing by NK cells through the inhibition of NKp30, NKp44, and NKG2D expression via IDO and PGE2.[197] Ovarian cancer cells can release Mϕ migration inhibitory factor (MIF) that induces NKG2D modulation,[201] or they can express the glycoprotein MUC16, able to inhibit the synapse formation between tumor and NK cells.[202] The modulation of activating NK receptors can also be induced by hypoxia, which often characterizes tumor tissues. In vitro experiments have shown that NK cells cultured under hypoxic conditions express hypoxia-inducible factor 1α (HIF-1α) and are unable to up-regulate the surface expression of NCRs and NKG2D in response to activating cytokines.[203] A recent study based on single-cell RNA sequencing, dissected the role of HIF-1α up-regulation in tumor-infiltrating NK cells, and demonstrated that HIF-1α acts as an immune checkpoint impairing NK cell effector functions.[204] Indeed, HIF-1α inhibition could restore the antitumor activity of NK cells in the tumor tissue. Hypoxia can also regulate miRNA expression, as shown in prostate cancer, where tumor infiltrating NK cells overexpress HIF1-α that induces miR-224, which, in turn, is responsible for the suppression of NKp46 signaling.[205] Hypoxia, together with other factors present in TME, can induce EMT, a process generally associated to increased malignancy. NK cells, also, can participate in EMT induction through cytokine secretion and cell-to-cell contact. Remarkably, tumor cells undergoing EMT show increased ability to down-regulate activating receptors on NK cells.[206]

Regulation of gene expression through miRNA can represent an alternative mechanism of tumor escape. In particular, several miRNAs have been shown to regulate NK receptor expression. Interestingly, some of them are induced by TGF-β, including miR-1245, involved in the modulation of NKG2D,[207] and miR-183, responsible for the down-regulation of DAP12, thus indirectly affecting NKp44 expression and function.[208] NK cells from patients with liver cancer overexpress miR-1544, which is responsible for the modulation of NKp46 through the targeting of RUNX3 transcription factor.[179]

As cited above, persistent stimulation of tumor-associated NK cells with ligands exposed by cancer cells can lead to a decreased receptor expression, resulting in tumor escape from NK cell-mediated attack. For example, DNAM-1 engagement by PVR and/or Nectin2 ligands can modulate DNAM-1 expression and NK cell function in patients with AML or ovarian cancer.[209,210] Similar findings have been reported in several tumor cell types in which modulation of NCRs and NKG2D was observed.[211-219]

Receptor endocytosis mediated by recognition of ligands expressed on tumor cells has been well characterized in the NKG2D system, both in humans and mice.[220] In humans, internalization of ligand-engaged NKG2D occurs upon DAP10 ubiquitination and results in the delivery of NKG2D-DAP10 complexes to lysosomes for degradation.[221] Ubiquitination processes are also involved in the down-regulation of CD16 receptor following recognition of Ab-coated tumor cells, through degradation of CD3ζ subunit.[222]

Another possible mechanism for decreasing cell surface receptors implicates receptor shedding through proteolytic cleavage. In particular, proteases belonging to the MMP (matrix metalloproteinases) and ADAM (a disintegrin and metallopeptidase domain) families play a role in this process. CD16 has been shown to undergo shedding from the membrane through the action of ADAM17 and MT6/MMP25 proteases.[144,145] Interestingly, MMP or ADAM17 inhibitors have been shown to increase ADCC activity and IFN-γ production in NK cells, and their use could improve the efficacy of therapeutic Abs.[144,223-225] Regarding DNAM-1, proteolytic cleavage as a mechanism for receptor down-regulation has not been clearly demonstrated so far, although high levels of soluble DNAM-1 have been detected in cancer patients and shown to correlate with decreased DNAM-1 surface expression on NK cells.[226,227] More in general, the molecular mechanisms leading to NCRs and DNAM-1 down-regulation have not been completely elucidated yet, and the relative contribution of ligand-induced endocytosis versus shedding by proteolytic cleavage is presently undefined. Overall, a reduced surface expression of activating NK receptors and a consequent impairment in NK cell function have been observed in patients affected by different types of both hematological and non-hematological malignancies.[211-213,216,228-231] These findings have to be considered in the context of therapeutic approaches utilizing NK cells, and suggest that strategies aimed at restoring activating receptor expression and function could synergize with and improve the efficacy of such therapies.

As mentioned in the previous chapter, NK cells that up-regulate the expression of inhibitory checkpoint receptors can become less efficient in the elimination of tumor cells. Notably, the expansion of a PD-1$^+$ NK cell population has been described in certain cancer patients. These patients showed reduced antitumor NK cell-mediated responses,[184,232] which could be related to the frequent expression of PD-1 ligands (i.e., PD-L1 and -L2) on tumor cells. Similarly, TIM-3-expressing NK cells[233] were found to be increased in patients with melanoma, gastric cancer, and lung adenocarcinoma.[185,234,235] Several ligands for TIM-3 have been identified, including Galectin-9, Ceacam1, high mobility group box 1 (HMGB1) protein, and phosphatidylserine,[236] and in a recent study the TIM-3/Galectin-9 pathway has been indicated as a possible tumor escape mechanism in AML.[237] TIGIT and CD96 represent other inhibitory checkpoint receptors; both molecules compete with DNAM-1 activating receptor for ligand recognition, since they can bind PVR and Nectin-2 with higher affinity as compared to DNAM-1.[238] In colon cancer patients, TIGIT expression on tumor-infiltrating NK cells correlated with tumor progression and was associated with functional exhaustion of NK cells.[186]

Given the plethora of activating and inhibitory receptors involved in the regulation of NK cell effector functions, the alteration of ligand expression on tumor cells represents an important tumor escape mechanism avoiding tumor cell recognition and killing (Fig. 2B). Ligands recognized by NKG2D have been extensively characterized both in humans and in mice[239-242]; human NKG2D can bind molecules whose expression is up-regulated upon cellular stress, neoplastic transformation, or viral infection, and include MICA/B and 6 ULBPs (ULBP1-6). Reduction of NKG2D ligands from tumor cell surface can take place by different mechanisms. The action of proteases (i.e., ADAMs and MMPs) found in the TME is frequently responsible for ligand shedding and decreased efficiency of tumor cell recognition by NK cells.[243-248]

Ligand shedding can also be influenced by posttranslational modifications; for instance, palmitoylation of MICA can favor its recruitment to membrane microdomains and subsequent shedding.[249]

Another way to generate soluble ligands is their release in exosomes. Indeed, all NKG2D ligands have been detected in exosomes derived from different tumor cells, such as prostate cancer, ovarian cancer, melanoma,[250-253] and have been shown to impair NK cell-mediated cytotoxicity. Disappearance of NKG2D ligands from the cell surface has also been observed in some tumors as a consequence of intracellular retention[254] or internalization and proteasomal degradation.[255] Finally, some ULBPs can be produced by tumor cells as soluble molecules, due to translation of alternatively spliced transcripts.[256]

DNAM-1 ligands include PVR (CD155) and Nectin-2 (CD112) molecules, belonging to the Nectin family and involved in cell-cell adhesion.[67,88,257] DNAM-1 ligand expression can be induced in cancer cells, and contributes to tumor recognition and killing.[88] PVR can be produced in soluble form as result of splicing isoforms lacking the transmembrane region.[258] Interestingly, increased levels of sCD155 have been detected in the serum of patients with lung, gastrointestinal, breast, and gynecologic cancers, and correlated with disease progression.[259] Recently, tumor-derived soluble CD155 has been demonstrated to inhibit DNAM-1-mediated cytotoxic activity in a murine model of melanoma.[260]

DNAM-1 ligands can undergo posttranslational modifications, which result in a rapid regulation of their surface expression and, consequently, in a reduced susceptibility of tumor cells to NK cell-mediated attack. For example, following ubiquitination, Nectin-2 is retained in intracellular compartments and degraded by proteasome.[261] PVR can undergo SUMOylation in cells derived from multiple myeloma patients, and this modification has been shown to inhibit PVR surface expression.[262] In HCC, the unfolded protein response (UPR) can decrease PVR expression by promoting its degradation, and a correlation exists between low PVR levels and poor prognosis in HCC patients.[263]

The picture of ligands recognized by NCRs is still incomplete, although recently several studies provided novel information on this field.[65,66,86,87,89,264-266] Several microbial ligands for NKp46 have been characterized, while the molecules recognized by this receptor on tumor cells are still elusive. Experimental data, however, highlight NKp46 as one of the major receptors responsible for natural cytotoxicity.[267,268] Evidence for the existence of tumor escape mechanisms from NKp46-mediated recognition has been obtained in mouse models, in which NKp46 contributes to tumor immunoediting, that is the selection of cancer cells with NK cell-resistant phenotypes; in this context, tumors induced in NKp46-deficient mice have been shown to display higher levels of NKp46 ligands as compared to those developing in wild-type mice.[269]

The first identified ligand for NKp30 was BAT3/BAG6, a ubiquitously expressed nuclear protein that can be released from tumor cells in response to stress signals and interact with NKp30 on NK cells.[270] Few years later, a transmembrane protein belonging to the B7 family, B7-H6, was described as an additional NKp30 ligand, broadly expressed on both hematopoietic and non-hematopoietic tumors, and up-regulated on myeloid cells in inflammatory conditions.[271,272] The release of NKp30 ligands as a possible immune evasion mechanism has been investigated; although several studies documented the presence of soluble NKp30 ligands in different neoplastic conditions, few of them directly addressed the mechanisms involved in their release. BAT3/BAG6 can undergo exosomal secretion but can also be released in a soluble form; soluble BAG6 has been shown to inhibit NK cell-mediated cytotoxicity, while exosomal BAG6 can promote NK cell activation following NKp30 engagement.[273,274] The presence of B7-H6 in exosomes has been demonstrated in the sera of patients with sepsis and associated with increased mortality,[272] and, more recently, in the sera of pregnant women.[275] An alternative escape mechanism involves proteases. B7-H6 ectodomain can be cleaved from the cell surface by tumor-derived ADAM-10 and -17, resulting in a decreased NKp30-mediated antitumor activity.[276]

Soluble forms of NKp30 ligands, either derived from proteolytic cleavage, freely released, or released in exosomes, has been demonstrated in sera derived from cancer patients, and often display immunosuppressive properties; their presence is generally associated with poor prognosis and advanced disease stages.[214,215,273,276-278] These findings, besides suggesting the involvement of these molecules in the modulation of their respective receptors following receptor-ligand interaction, encourage their use as possible predictive biomarkers and to monitor the course of the disease.

The third NCR is represented by NKp44, whose expression is induced on NK cells following cytokine stimulation.[81] Activating ligands for this receptor have been characterized. The first one is a peculiar isoform of mixed-lineage leukemia protein-5 (MLL-5), termed 21spe-MML5, frequently observed in different tumors.[279] More recently, platelet-derived growth factor (PDGF)-DD was shown to bind to NKp44 and to induce TNF-α and IFN-γ production by NK cells.[264] NKp44-mediated effector functions can be inhibited by tumor cells through the overexpression of proliferating cell nuclear Ag (PCNA), a ubiquitous nuclear protein that can be recruited to the cell surface and convey an inhibitory signal upon interaction with NKp44.[280] Another putative tumor escape mechanism related to NKp44 is represented by Nidogen-1 (NID1), an extracellular matrix glycoprotein, that can be released by tumor cells, bind to NKp44 and modulate NK cell function, possibly acting as a decoy ligand.[266] Notably, increased soluble NID1 levels are detected in the sera of cancer patients and proposed as possible biomarkers.[281,282]

The expression of NK receptor ligands can also be regulated at post-transcriptional level, through the action of several miRNAs.[174,283,284] In particular, tumor cells can overexpress miRNAs involved in the down-regulation of NKG2D ligands. For instance, MICA is targeted by different miRNAs, including miR-183 (up-regulated by TGF-β), miR-20a, miR-146b-5p, and miR-25/93/106b.[285-288]

Tumor cells can also become resistant to NK cell mediated-killing by increasing the expression of ligands for inhibitory receptors. Inhibitory signals can be delivered through KIRs or CD94/NKG2A receptors upon HLA class I recognition, resulting in the block of NK cytotoxicity. Up-regulation of both classical and non-classical HLA class I molecules

on tumor cells can be promoted by IFN-γ released by activated NK cells and represents a way to avoid NK cell-mediated killing.[289,290] In general, the expression of immune checkpoints on tumor cells is a possible target of immune evasion. In this context, PD-L1 expression can be increased on tumor cells by IFN-γ through the JAK/STAT pathway[291]; in addition, HIF-1α, frequently induced in tumors, up-regulates PD-L1 transcription.[292] Finally, B7-H3, a member of B7 family expressed by different tumor types, can also contribute to reduce antitumor NK response by delivering inhibitory signals following interaction with a still unknown receptor on NK cells.[293]

3.2.2 | Cytoskeletal rearrangement

Assembly of an IS between cancerous cells and NK cells can be interrupted at several stages of this multistep process. NK cells express a wide variety of germ-line encoded receptors and engagement of these receptors with their corresponding ligands influences actin cytoskeleton dynamics (Fig. 2B). Activating receptors can induce actin filament polymerization and rearrangement that are necessary for the formation of a lytic NKIS through their ITAM motif. Inhibitory receptors on the other hand can interfere with the formation of the IS by down-modulating the activity of actin cytoskeleton regulators, as mentioned before. Arrangement of inhibitory receptors in microclusters at the NKIS is also dependent on actin cytoskeleton dynamics, at least during the early stages of IS formation, suggesting that actin cytoskeleton dynamics are not only important for the formation of a lytic IS but also an inhibitory IS.[294] If both activating and inhibitory signals are present at the NKIS, the quantitative differences between the signal strength determine whether or not a lytic NKIS is formed, although inhibitory signals are assumed to have a stronger impact on actin dynamics and can cancel out activating signaling through interruption of activating receptor clusters.[295]

Sustained prevention of IS formation has been shown in experiments with immune cells derived from leukemia patients. After contact with leukemic cells, CTLs failed to establish an IS with other cancer cells, but also with APCs.[296] This was attributed to the presentation of inhibitory ligands at the IS that resulted in sustained down-regulation of Rho GTPases in CTLs. Drug treatment with the immune modulator Lenalidomide resulted in degradation of Ikaros family zinc finger proteins and the down-regulation of inhibitory ligands on leukemic cells, resulting in restoration of lytic IS formation between malignant target cells and T lymphocytes. In follow-up studies, positive effects of Lenalidomide treatment on NK cell proliferation, NKIS formation, and NK cell-mediated cytotoxicity against different hematologic malignancies was documented.[297,298] Reactivation of dysfunctional NK cells, and restoration of cytokine production and lytic NKIS formation after drug-induced down-modulation of inhibitory ligands indicates that inhibitory receptor signaling can have lasting effects on actin dynamics, not only in CTLs, but also NK cells.

Not only changes in the effector cell´s actin cytoskeleton can lead to successful immune evasion of cancerous cells. The importance of mechanotransduction for T lymphocyte signaling has been highlighted by several reports,[299-303] and a recent study suggested that NK cell activation also relies on mechanotransduction.[304] Interestingly, cells infected with human cytomegalovirus (HCMV) can avoid recognition by both NK cells and T lymphocytes. Their evasion is mediated by the viral protein UL135 that prevents actin cytoskeleton remodeling through interfering with WAVE2 localization and de novo actin polymerization.[305] Such defects in actin dynamics precludes establishment of a lytic IS and allows for persistence of infected cells. Other reports have shown that target cells' actin cytoskeleton can influence recognition by CTLs and lysis efficiency by NK cells. In these studies, changes in actin dynamics in cancer cells were associated with immune evasion either through inhibition of IS formation or by interference with effector functions.[306-309] Decreased cortical actin on the cancer cell side of the IS may not only prevent firm adhesion of effector immune cells, but also reduces the strength of mechanoreceptor signaling resulting in insufficient activating signal at the IS. Alternatively, increased amounts of polymerized actin could counteract the mechanical pulling forces exerted by effector immune cells to promote the pore-formation activity of perforin.[301] Enhanced actin dynamics in target cells at the NKIS have been recently associated with decreased transfer of cytotoxic enzymes, such as perforin and GrzB,[308] supporting the notion that actin cytoskeleton on both sides of the IS determines the outcome of cytotoxic lymphocyte attack on cancer cells (Fig. 2B).

3.2.3 | Granule polarization and release

Cytotoxic polarization and release are the hallmarks of a lytic NKIS. Following the characteristic rearrangement of the actin cytoskeleton, the MTOC is repositioned to the cSMAC as a requirement for directed degranulation with a minimum of collateral damage to healthy bystander cells. The related secretory machinery relies on microtubules, but the polarization of the MTOC only occurs if the NKIS is properly assembled and actin dynamics are sustained. Both insufficient activating signals and strong inhibitory signals can interfere with the assembly of the NKIS and prevent granule convergence and MTOC polarization (Fig. 2B). It is important to highlight that granule convergence and degranulation of lytic granules are uncoupled in NK cells and depend on different activating receptor signaling pathways.

Granule convergence has been reported to be strongly dependent on integrin signaling, namely LFA-1 outside-in signaling, and dynein activity, and occurs before MTOC polarization.[310-312] This step of the cytolytic cascade has been suggested to serve as preparation for rather than commitment to target cell lysis.[311] LFA-1 outside-in signaling has been demonstrated to be centered around the ILK-Pyk2-paxillin and Cdc42-Par6 signaling pathways that both control cell polarity and MTOC polarization.[312] Correlation between ICAM-1 expression on cancer cells and efficiency of NK cell-mediated lysis have been previously reported and sets an example of how tumor cells can evade NK cell cytotoxicity by affecting the later steps of NKIS assembly.[313,314] In addition, down-modulation of activating ligands and/or up-regulation of inhibitory ligands are sufficient to prevent degranulation due to insufficient Ca^{2+} mobilization and through interference with actin polymerization at the NKIS.[315-317] One example is

engagement of the receptor PD-1 with its ligand PD-L1. NK cells are increasingly recognized to contribute to successful immunotherapy and to play an important role in tumor surveillance of MHC-I-deficient tumors.[318,319] PD-1/PD-L1 blocking therapy shows beneficial outcomes in MHC-I^{low} PD-L1^{high} cancers, such as multiple myeloma and Hodgkin lymphoma.[183,320] One recently proposed mechanism by which PD-1 engagement could interfere with NK cell cytotoxicity is the impairment of the LFA-1 signaling pathway.[321] In this study, activation of PD-1 signaling was shown to prevent integrin-linked kinase (ILK) recruitment to the NKIS and was associated with a lack of granule polarization, highlighting again the importance of integrin signaling for granule polarization. Yet, no conclusions were drawn on the strength of the NK cell-target cell conjugate in this context.

Degranulation triggered by the binding of CD16 to opsonized target cells in the frame of ADCC is a particular case of NK cell-mediated cytotoxicity. Indeed, CD16 signaling in NK cells can lead to non-directed degranulation.[58] Serial engagement in lytic NKIS through ADCC results in less efficient granule release as a result of CD16 shedding from the NK cell membrane. Though counterintuitive, this was associated with NK cell survival and sequential NK cell cytotoxicity could be restored by activation through an alternative activating pathway, but ultimately also resulted in depletion of perforin storages.[146]

3.3 | Resistance to granule-mediated cell death

Besides affecting cytolytic granule formation, avoiding NK cell-mediated recognition, and influencing the establishment of productive synapse formation, tumor cells can also limit the efficacy of the effector molecules delivered by NK cells. This latter strategy of escape involves 2 major mechanisms: (1) the modulation of the granzyme-induced pro-apoptotic pathways inside the tumor cells; (2) the inactivation/elimination of perforins and granzymes (Fig. 2C).

Different molecules along the granzyme-induced death pathways may be altered in tumor cells.[322] For example, inactivating mutations of Caspase 8 have been identified in 10% of 122 samples of advanced gastric cancers[323] and 13% of 69 hepatocellular cell carcinomas (HCC).[324] Although rare, also mutations of Caspase 3 and 9 have been found in different tumor types.[325,326] In a recent study, it has also been shown that miR-519a-3p inhibits the expression of Caspase 8, 7, and TRAIL-R2, conferring resistance to GrzB- and TRAIL-induced apoptosis to breast cancer cells.[327] Tumor cells may also up-regulate the expression of anti-apoptotic molecules, such as Bcl-2[328] or members of the IAP family,[329] or inactivate apoptosis mediators, such as BAX[330] and PCAF.[331] To what extent these various alterations could modify tumor cell susceptibility to NK cell-mediated killing is not really known. The array of granzymes that can be delivered in the tumor cell and the multiplicity (and even redundancy) of the triggered pathways suggest that in most cases NK cell-mediated killing can be modulated and not completely blocked, as it occurs, for example, in the case of target cells expressing Bcl-2.[332]

Tumor cells can also directly act on granule-released effector molecules. For example, leukemic cells from 6 AML patients have been shown to prevent perforin binding to their surface, and impairment of perforin binding conferred resistance to NK cell-mediated killing to a leukemic cell line.[333] Many human cancers also express high levels of the perforin degrading molecule Cathepsin B,[334] and in a recent study, it has been shown that melanoma cells release Cathepsin B resulting in perforin inactivation during CTL-mediated synapse formation.[335] Tumor cells can also eliminate or inactivate granzymes. The GrzB inhibitor Serpin B9, which has been shown to inhibit GrzB-dependent CTL-mediated cell death, is up-regulated in different tumors, including lymphomas.[336,337] In addition, Serpin B4, which can bind GrzM and inhibit GrzM-mediated cell death, is highly expressed in squamous cell carcinomas.[338] Finally, tumor cells can eliminate granzymes by the process of autophagy, which can be induced by hypoxia, a condition frequently present in the tumor microenvironment. Remarkably, GrzB degradation by autophagy increases resistance of tumor cells to NK cell-mediated killing.[339]

4 | CONCLUDING REMARKS

As depicted in the present review, tumor cells and, more extensively, the TME rely on a wide and heterogenous range of strategies to avoid or block NK cell-mediated cytotoxicity and, eventually, tumor cell killing (Figure 2). The concrete final effects of the various escape mechanisms on the tumor progression are not always easily evaluable. Studies on animal models suggest that some of them may be effective in the patients; on the other hand, NK cells generally kill, to some extent, tumor cells, in spite of their possible mechanisms of resistance. On the whole, it is conceivable that the ability of the host to control the tumor development via NK cells is variably modulated, depending on type and number of active escape mechanisms. Their characterization in the different tumors will help to unmask the still unexploited antitumor potential of NK cells in different patient types, providing the premises for an effective NK cell-based personalized medicine.

AUTORSHIP

All authors contributed to the writing of the manuscript and approved it for publication.

ACKNOWLEDGMENTS

This work was supported by: Fondazione AIRC, grant number IG 2014 id. 15428 (M.V.); Ministero della Salute, grant RF-2018-12366714 (M.V.); Cancer Foundation Luxembourg (FC/2019/02) (C.T.), and the National Research Fund> (C19/BM/13579644) (C.T.). H.W. is recipient of a PhD fellowship from the National Research Fund (FNR), Luxembourg (PRIDE15/10675146/CANBIO).

DISCLOSURE

The authors declare no conflict on interest.

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How to cite this article: Cantoni C, Wurzer H, Thomas C, Vitale M. Escape of tumor cells from the NK cell cytotoxic activity. *J Leukoc Biol*. 2020;1-22. https://doi.org/10.1002/JLB.2MR0820-652R

Results

In a recent scientific publication, we have introduced the cancer cell intrinsic immune escape mechanism “actin response” as a strategy of breast cancer cells to avoid NK cell cytotoxic activity (Annex I). Through participating in this original research output, I was able to formulate the questions that I wanted to answer with my own PhD thesis project.

In the following published research article, I answered the question about the conservation of the actin response in hematologic cancers and documented its existence in patient-derived cancer cells. This article also illustrates the capability of leukemic cells to avoid NK cell cytotoxicity by upregulating non-canonical MHC-I molecule HLA-G that can interact with inhibitory receptors on NK cells. This is of interest also for cellular immunotherapy approaches as some CAR-NK cell therapies utilize the NK-92 model as a source (CAR-NK92 cell) that expresses two of the three described receptors for HLA-G, ILT-2 and ILT-4, but not KIR2DL4. Blocking of the inhibitory interaction restored conjugate formation between primary cancer cells and NK-92MI cells. In this article we could further demonstrate that reduction of the actin response through inhibition of CDC42 activation resulted in an increased susceptibility of primary leukaemia cells to NK cell cytotoxicity. However, not all patient samples showed a reduction of the actin response after CDC42 inhibition, suggesting an alternative signalling pathway in these cancer cells that enables the formation of an actin response.

As indicated in the author’s contributions of this article, designing, and carrying out experiments as well as manuscript writing was mainly performed by myself, with support from my research group and my supervisor. In the general discussion, this article is referred to as **article I**.

ORIGINAL RESEARCH
published: 24 May 2021
doi: 10.3389/fimmu.2021.619069

Intrinsic Resistance of Chronic Lymphocytic Leukemia Cells to NK Cell-Mediated Lysis Can Be Overcome *In Vitro* by Pharmacological Inhibition of Cdc42-Induced Actin Cytoskeleton Remodeling

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Natural killer (NK) cells are innate effector lymphocytes with strong antitumor effects against hematologic malignancies such as chronic lymphocytic leukemia (CLL). However, NK cells fail to control CLL progression on the long term. For effective lysis of their targets, NK cells use a specific cell-cell interface, known as the immunological synapse (IS), whose assembly and effector function critically rely on dynamic cytoskeletal changes in NK cells. Here we explored the role of CLL cell actin cytoskeleton during NK cell attack. We found that CLL cells can undergo fast actin cytoskeleton remodeling which is characterized by a NK cell contact-induced accumulation of actin filaments at the IS. Such polarization of the actin cytoskeleton was strongly associated with resistance against NK cell-mediated cytotoxicity and reduced amounts of the cell-death inducing molecule granzyme B in target CLL cells. Selective pharmacological targeting of the key actin regulator Cdc42 abrogated the capacity of CLL cells to reorganize their actin cytoskeleton during NK cell attack, increased levels of transferred granzyme B and restored CLL cell susceptibility to NK cell cytotoxicity. This resistance mechanism was confirmed in primary CLL cells from patients. In addition, pharmacological inhibition of actin dynamics in combination with blocking antibodies increased conjugation frequency and improved CLL cell elimination by

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Edited by:
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

***Received:** 19 October 2020*
***Accepted:** 23 April 2021*
***Published:** 24 May 2021*

Citation:
Wurzer H, Filali L, Hoffmann C, Krecke M, Biolato AM, Mastio J, De Wilde S, François JH, Largeot A, Berchem G, Paggetti J, Moussay E and Thomas C (2021) Intrinsic Resistance of Chronic Lymphocytic Leukemia Cells to NK Cell-Mediated Lysis Can Be Overcome In Vitro by Pharmacological Inhibition of Cdc42-Induced Actin Cytoskeleton Remodeling. Front. Immunol. 12:619069. doi: 10.3389/fimmu.2021.619069

NK cells. Together our results highlight the critical role of CLL cell actin cytoskeleton in driving resistance against NK cell cytotoxicity and provide new potential therapeutic point of intervention to target CLL immune escape.

Keywords: actin cytoskeleton, Cdc42, immune evasion, immunological synapse, tumor immunology, natural killer (NK), B cell neoplasms

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most prevalent lymphoproliferative disorder in the United States and Europe and is characterized by the clonal expansion of mature CD5$^+$ CD23$^+$ B cells (1, 2). As first reported in 1999, the mutational status of the immunoglobulin heavy chain variable region genes (*IGHV*) is associated with overall survival and is by now considered one of the most important molecular prognostic factors (3, 4). A higher degree of somatic mutations is considered a good prognostic marker, while patients with a non-mutated IgH_V region show shorter progression-free and overall survivals (5). An additional marker of prognostic and predictive value is the tumor protein 53 gene (*TP53*) status that can be affected by 17p13 deletion ((del(17p)) and/or somatic *TP53* mutations (6, 7). The double knockout of the *TP53* gene renders CLL cells resistant to most chemo-immunotherapies and has also been shown to be involved in resistance against monoclonal antibodies, such as rituximab (8).

A cardinal feature of CLL is an acquired immune system dysregulation and immune response dysfunction of both innate and adaptive immunity that gradually worsens over time even without disease progression (9–19). The immune response of cytotoxic lymphocytes, such as cytotoxic CD8$^+$ T and (natural killer) NK cells, is regulated by molecular interactions occurring in the context of immunological synapses (IS). The formation of a lytic IS between cytotoxic lymphocytes and their target cells is a tightly coordinated process that ensures that only infected or transformed cells are lysed (20–22). Because the IS is the point of convergence for cytolytic effector functions, it is susceptible to immune evasion strategies of cancer (23, 24). Interestingly, it has been shown that immune escape of CLL can be achieved by various means, notably by those interfering with the formation or the function of the lytic IS (25–29). Lytic IS formation of CD8$^+$ T cells and NK cells with CLL cells can be rescued in part by drug treatments, such as lenalidomide or blocking antibodies, or can even be bypassed by infusion of genetically modified cytotoxic lymphocytes which do not rely on MHC-mediated antigen presentation as they form a non-classical IS (30–36). Nevertheless, toxic side effects or acquired resistance against these new therapeutic options have been reported and result in disease progression (37, 38).

NK cells are commonly described to play an important role in the immunosurveillance of hematologic malignancies (23). NK cell effector function is regulated by the balance between inhibitory ligands, mainly canonical and non-canonical MHC-I, and activating ligands presented by the target cell at the IS. Down-modulation of MHC-I is a common feature on cancerous cells and, if accompanied by upregulation of stress-induced activating ligands, leads to activation of NK cells and subsequent target cell lysis (24). Sufficient activating signal results in the release of cytokines, such as IFN-γ and TNF-α, and the formation of a lytic IS that includes directed degranulation of cytotoxic molecules, such as perforin and granzyme B, towards the conjugated target cell (23). CLL immune evasion from NK cells has been described to occur mainly through the upregulation of non-canonical MHC-I isoforms HLA-G and HLA-E (39–41). The NK cell repertoire of an individual can be defined by the simultaneous expression of different receptors and is quite diverse with up to 30’000 different phenotypic populations. Interestingly, the expression of the HLA-G receptor KIR2DL4 is universally found on all NK cells (40, 42, 43). This indicates that overexpression of HLA-G on CLL cells can provide immune evasion from any NK cell subpopulation. Accordingly, monoclonal antibody blockade therapies targeting HLA-E or HLA-G overexpression successfully increased the natural cytotoxicity of NK cells from CLL patients *in vitro* (40, 44). However, commercial HLA-E monoclonal antibodies are not specific and show cross-reactivity with HLA-A/B/C (45) and HLA-G is characterized by the presence of several isoforms and a high intra- and interpatient heterogeneity, making it a difficult target (23). Alternative inhibition of the inhibitory HLA-E receptor NKG2A showed promising results *in vitro* (46) and is currently tested in several clinical trials, however a phase I/II study of Monalizumab in combination with Ibrutinib including CLL patients was terminated in 2018 (NCT02557516).

Even though NK cell expansion in CLL patients has been reported, these NK cells are described to be hyporesponsive due to a downregulation of activating receptors. They also show a reduced degranulation efficiency against malignant B lymphocytes, through both natural or antibody dependent cell cytotoxicity (ADCC) triggered by rituximab (23). The exhausted NK cell phenotype is enhanced in patients with a progressive disease and results in a loss of NK cell cytotoxicity against CLL target cells. However, CLL patients’ exhausted NK cells can be replaced by activated NK cells coming from a healthy donor. Such allogenic adoptive cell therapy studies showed that unmutated CLL cells are susceptible targets for activated NK cells (46, 47). As demonstrated for other hematologic malignancies, cytotoxic lymphocytes expressing chimeric antigen receptors (CARs) can efficiently lyse tumor cells, and in an attempt to circumvent toxic side effects of CAR-T cell therapy, anti-CD19 CAR-NK cells have been tried for B cell malignancies (48). While this new therapeutic approach holds promising results in first clinical trials (48), little is known about the CAR IS (49). Although some preliminaries studies suggest

that CAR IS are superior to conventional NK/T cells IS (50, 51), it remains unclear whether these IS can also be affected by resistant subpopulations of tumors that can modulate IS formation or functions.

These new treatment options set the focus on the lytic IS formed between CLL cells and NK cells and the underlying resistance mechanisms that could result in disease progression. Actin cytoskeleton remodeling has recently emerged as an important process underlying evasion of solid tumor cells, such as breast cancer cells, from NK cell cytotoxicity (23, 24, 52–55). However, the role of the actin cytoskeleton in CLL cells during NK cell attack has not been evaluated so far. Here, we show that a subset of CLL cells from four cell lines, but also patient-derived cells respond to NK cell attack by fast polarization of actin filaments at the IS. Live cell imaging and imaging flow cytometry analyses suggest that synaptic actin accumulation protects CLL cells against NK cell-mediated killing by reducing intracellular levels of granzyme B. Remarkably, pharmacological inhibition of an actin regulatory pathway in CLL cells was sufficient to prevent actin cytoskeleton remodeling, promote granzyme B accumulation, and restore high susceptibility to NK cell-mediated cytotoxicity. Similar results were obtained with patient-derived CLL cells that showed reduced resistance to NK cell-mediated cell death after inhibition of actin dynamics. In this context, blocking antibodies targeting HLA-G also demonstrated that release of the inhibitory interaction of HLA-G with its receptor in NK cells improves conjugate formation. Our data support that interfering with actin cytoskeleton remodeling in CLL cells in combination with antibody blockade provides an opportunity to restore a potent NK cell anti-tumor response in aggressive CLL.

METHODS

Cell Lines and Cell Culture Conditions

The CLL cell lines used in this study were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany). Cell lines were authenticated through STR profiling analysis (Microsynth, Switzerland) or purchased directly from DSMZ. HG-3, PGA-1, JVM-3 and MEC-1 cell lines were cultured in RPMI-1640 (ThermoFisher Scientific, cat. # 61870010) supplemented with 10% (v/v) fetal bovine serum (FBS, Life Technologies, cat. #10500-064), 100 U/mL penicillin and 0.1 mg/ml streptomycin (Westburg, cat. #LO DE17-602E). The NK-92MI cell line was kept in RPMI-1640 supplemented with 10% (v/v) FBS, 10% (v/v) horse serum (ATCC, cat. # 30-2040), 100U/ml penicillin and 0.1 mg/mL streptomycin. All cell lines were cultured under humidifying conditions at 37°C and 5% CO_2 and were checked routinely for mycoplasma contamination using the MycoAlert detection kit (Lonza, cat. # LT07-318).

Isolation of Human Primary NK Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy, anonymous donors provided by the Luxembourg Red Cross. Upon receipt, buffy coats were diluted ten times with Ca^{2+}/Mg^{2+} free phosphate buffered saline (PBS) and the low-density PBMC fraction was isolated by centrifugation over a Lymphoprep density gradient (Stemcell Technologies, cat. # 07861). After centrifugation, the PBMC layer was collected, washed several times with Ca^{2+}/Mg^{2+} free PBS and red blood cells were lysed with ACK buffer (ThermoFisher Scientific, cat. # A1049201). Following erythrocytes lysis, cells were washed once with Ca^{2+}/Mg^{2+} free PBS, counted with Trypan blue and cell concentration adjusted for NK cell isolation. NK cells were isolated with the MojoSort human NK cell isolation kit (BioLegend, cat. # 480054) combined with a LS column (Miltenyi Biotec, cat. # 130-042-401). Isolated NK cells were cultured overnight in RPMI 1640 supplemented with 10% FBS, 10 mM HEPES (ThermoFisher Scientific, cat. # 15630056), 100 U/mL penicillin, 0.1 mg/mL streptomycin, 100 U/mL recombinant human interleukin-2 (IL-2; Peprotech, cat. # 200-02) and 10 ng/mL recombinant human IL-15 (IL-2; Peprotech, cat. # 200-15).

Isolation of Human Primary CLL Cells

Peripheral blood samples were collected from anonymous CLL patients. All samples used in this study were obtained after informed consent in accordance with the Declaration of Helsinki and the Comité National d'Ethique de Recherche Luxembourg (CNER No. 201707/02 Version 1.2). CLL was diagnosed according to standard clinical criteria. PBMCs were isolated from fresh blood samples using standard density centrifugation over a Lymphoprep gradient. Isolated cells were washed twice in Ca^{2+}/Mg^{2+} free PBS and suspended in complete medium (RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin). PBMCs were either used immediately or were cryopreserved in FBS with 10% DMSO. After thawing, cells were allowed to recover overnight before being used for further experiments.

Cell Transduction and Cdc42 Inhibition

mEmerald-Lifeact-7 was a gift from Dr. M. Davidson (Addgene plasmid # 54148). For generation of stable cell lines, the mEmerald-Lifeact fragment was subcloned into the viral pCDH-EF1α-MCS-IRES-puro plasmid (System Biosciences, cat. # CD532A-2). Infectious particles were produced using HEK293 cells and used to infect HG-3, PGA-1, JVM-3, and MEC-1cell lines. Transduced cells were selected with puromycin (0.5 μg/ml, Sigma-Aldrich, cat. #P8833).

To inhibit Cdc42 activity in CLL cell lines, the cells were incubated for 1 h with 50 μM of the cell-permeable Cdc42 inhibitor ZCL278 (Sigma Aldrich Merck Calbiochem, cat. # 500503) (56). Cells were washed after treatment and allowed to recover for 1 h or 5 hrs in complete medium before stimulation with human recombinant EGF (0.1μg/mL; PeproTech, cat. # AF-100-15) for 15 min. Inhibition of Cdc42 activity upon stimulation was confirmed using a Cdc42 G-LISA Activation Assay following the manufacturer protocol (Cytoskeleton Inc., cat. # BK127-S).

Cytotoxicity Assay

For cytotoxicity assays, NK cells (effectors) were counted and stained with anti-human CD56-PE/Cy7 (BioLegend, cat. # 318318, clone HCD56). Effector cells were co-cultured with mEmerald-Lifeact$^+$ HG-3, PGA-1, JVM-3 or MEC-1 (target cells) at effector-target (E:T) ratios of 1:1 and 5:1 for 4 hrs at 37°C/5% CO_2. After incubation, the plate was placed on ice in the dark to stop the experiment until acquisition on the flow cytometer. Immediately before acquisition on a CytoFLEX (Beckman Coulter), TO-PRO-3 Iodide (ThermoFisher Scientific, cat. # T3605) was added to the samples (0.05 µM final concentration). Generated data were analyzed with FlowJo v10.6.2. software.

Flow Cytometry

To assess cell death in target cells, mEmerald-Lifeact$^+$ HG-3, PGA-1, JVM-3 or MEC-1 target cells were incubated for 45 min with CD56-PE/Cy7-labeled NK-92MI cells. Cells were washed with cold Annexin V binding buffer (Biolegend, cat. # 422201) twice. Afterwards, cells were resuspended in 100 µl Annexin V binding buffer with 5 µl Alexa Fluor® 647 Annexin V (Biolegend, cat. #640912) and 5 µl propidium iodide staining solution (Sigma-Aldrich, cat. #P4864) per million cells. Cells were incubated for 15 min at RT in the dark, before addition of 400 µl Annexin V binding buffer and analysis by flow cytometry on a CytoFLEX (Beckman Coulter). Generated data were analyzed with FlowJo v10.6.2. software.

Imaging Flow Cytometry

For conjugate formation, NK cells were counted and stained with anti-human CD56-PE/Cy7 (BioLegend, cat. # 318318, clone HCD56), before co-culture with mEmerald-Lifeact$^+$ target cells at an E:T ratio of 3:1 in the presence of Hoechst 33342 (0.5 µg/mL final; Miltenyi Biotec, cat. # 130-111-569). Conjugation was allowed for 40 min at 37°C before fixation with 2% paraformaldehyde (PFA; Agar scientific, cat. # R1026) for 15 min at 37°C, and permeabilization with 0.1% Triton X-100 (Sigma Aldrich, cat. # T9284) for 10 min at room temperature (RT). Prior to intracellular staining, samples were washed twice with PBS and then stained for anti-Granzyme B-APC (BioLegend, cat. # 372204, clone QA16A02).

To analyze apoptosis in target cells, cells were centrifuged after 30 min of co-incubation and stained with Zombie Red (BioLegend, cat. # 423110) in PBS for 10 min at RT. Cells were then washed with cold cell staining buffer (BioLegend, cat. # 420201), resuspended in 100 µl Annexin V binding buffer (BioLegend, cat. # 422201) with 5 µl Alexa Fluor® 647 Annexin V (BioLegend, cat. # 640943) and stained for 15 min at RT in the dark. Cells were then washed with Annexin V binding buffer and fixed in 2% v/v PFA diluted in Annexin V binding buffer. After fixation, cells were washed in Annexin V binding buffer and kept at 4°C in this buffer until acquisition. For acquisition, ImageStream®X Mark II (EMD Millipore) with four built-in lasers (405 nm, 488 nm, 561 nm, 642 nm) and the INSPIRE® software (EMD Millipore) were used. Analysis for AR, including the gating strategy, masks and features, were described previously (52) and are shown in **Supplementary Figures S1C, D**.

For analysis of patient-derived CLL cells, PBMCs were stained for 30 min with 0.2 µM of the cell permeable F-actin probe SiR-actin (Spirochrome AG, cat. #SC001). To inhibit Cdc42 activity, cells were then treated for 1 h with 50 µM of ZCL278 or vehicle control in complete medium. Before co-culture with NK-92MI cells, patient CLL cells were stained with anti-human CD19-FITC (BioLegend, cat. #302256, clone HIB19), anti-human CD5-BV605 (BioLegend, cat. #364019, clone L17F12), and 10 µg/mL anti-human HLA-G (BioLegend, cat. #335902, clone 87G) or control IgG (BioLegend, cat. #400201, clone MOPC-173) for 30 min at 4°C. Cells were allowed to conjugate with NK-92MI for 30 min in the presence of Hoechst 33342 (0.5 µg/mL final concentration), before staining with 0.1X Live-or-Dye NucFix™ Red for 15 min in PBS. Conjugates were washed in PBS containing Ca^{2+}/Mg^{2+} and fixed with 2% PFA for 15 min at 37°C. For analysis of AR and cell death, CLL cells were identified as CD19+/CD5+ cells in conjugation with CD56+ NK-92MI cells.

Confocal Microscopy

For labelling, mEmerald-Lifeact$^+$ target cells were settled on a Poly-L-Lysin (25 µg/mL, Sigma-Aldrich, cat. # P4707) coated µ-slide 8 well (Ibidi, cat. # 80826) for 10 min before fixation with 2% paraformaldehyde (PFA). Cells were permeabilized with 0.1% Triton X-100 and labelled with anti-α-tubulin antibody (Sigma-Aldrich, cat. # T5168, clone B-5-1-2), goat-anti-mouse Alexa Fluor 633 (Invitrogen, cat. # A-21126) and with acti-stain 555 phalloidin (100 nM, Cytoskeleton Inc., cat. # PHDH1) and DAPI (0.2 µg/mL, Sigma-Aldrich). For conjugate formation, NK cells were counted and stained with the CellTracker™ Orange CMRA dye (1 µM, Invitrogen, cat. # C34551), before co-culture with mEmerald-Lifeact target cells at an E:T ratio of 1:1 in the presence of Hoechst 33342 (0.5 µg/mL final). Conjugation was allowed for 40 min at 37°C, then cells were settled on a Poly-L-Lysin coated µ-slide 8 well for 10 min before fixation with 2% PFA. After 2 washings, PBS was replaced by mounting medium (Ibidi, cat. # 50001) before cell imaging. For acquisition, high-resolution pictures were acquired on a Zeiss LSM880 fastAiry confocal microscope, in the Airy mode. A multitrack configuration was used with laser 405 nm, 488 nm, 543 nm, and 633 nm for excitation. A stack of 50 slices with an interval of 0.2 µm was acquired. The fluorescence intensity was measured on the maximum intensity projection picture of the stack in a rectangle of 5 µm width set in the center of the IS, with the macro "GetProfileExample" in the Image J v1.53e software.

For live cell imaging, NK92MI cells were counted and stained with the CellTracker™ Orange CMRA dye (1 µM), before co-culture with mEmerald-Lifeact target cells at an E:T ratio of 1:1 in the presence of SYTOX™ Blue (2 µM, Invitrogen, cat. # S11348). For acquisition, cells were maintained under the microscope at 37°C and 5% CO_2. A single-track configuration was used with excitation at 405 nm, 488 nm, and 543 nm. The pinhole was open to acquire a 2 µm depth slice. A stack of 4 slices with an interval of 2 µm was acquired for 1 h at a rate of one picture every 4 min.

Statistical Analysis

The paired Student's t-test and 2-way ANOVA in Prism 9 (GraphPad) were used to determine the statistical significance of the results obtained. For apoptosis experiments, a Z-score test for two population proportions was used to determine the statistical significance between samples. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

RESULTS

CLL Cell Resistance to NK Cell-Mediated Cytotoxicity Correlates With Actin Cytoskeleton Polarization to the Immunological Synapse

To evaluate actin cytoskeleton organization and dynamics in aggressive forms of CLL, three IGHV mutated cell lines, namely PGA-1, JVM-3 and MEC-1, and one IGHV non-mutated cell line (HG-3) (**Supplemental Figure S1A**) were modified to stably express the mEmerald-tagged actin marker Lifeact (**Figure 1A**) (57). With this approach, labeling of the actin cytoskeleton became obsolete and a spill-over from the NK cell actin cytoskeleton could be avoided. Cytotoxicity assay found MEC-1 as a highly resistant CLL cell line compared to the other three CLL cell lines (**Figure 1B**). After 4 hrs of co-culture with an excess of NK-92MI cells at a 5:1 E:T ratio, MEC-1 cells were lysed with an average rate of 17%. In comparison, HG-3, PGA-1, and JVM-3 cells were significantly more susceptible with average NK cell-specific lysis rates of 54%, 47%, or 44%, respectively. Using confocal microscopy, we found that some NK cell-conjugated CLL cells showed a strong polarization of filamentous actin to the synaptic area (**Figure 1C** and **Supplementary Figure S1B**). We recently reported similar synaptic accumulation of filamentous actin during NK cell attack in breast cancer cells and termed this

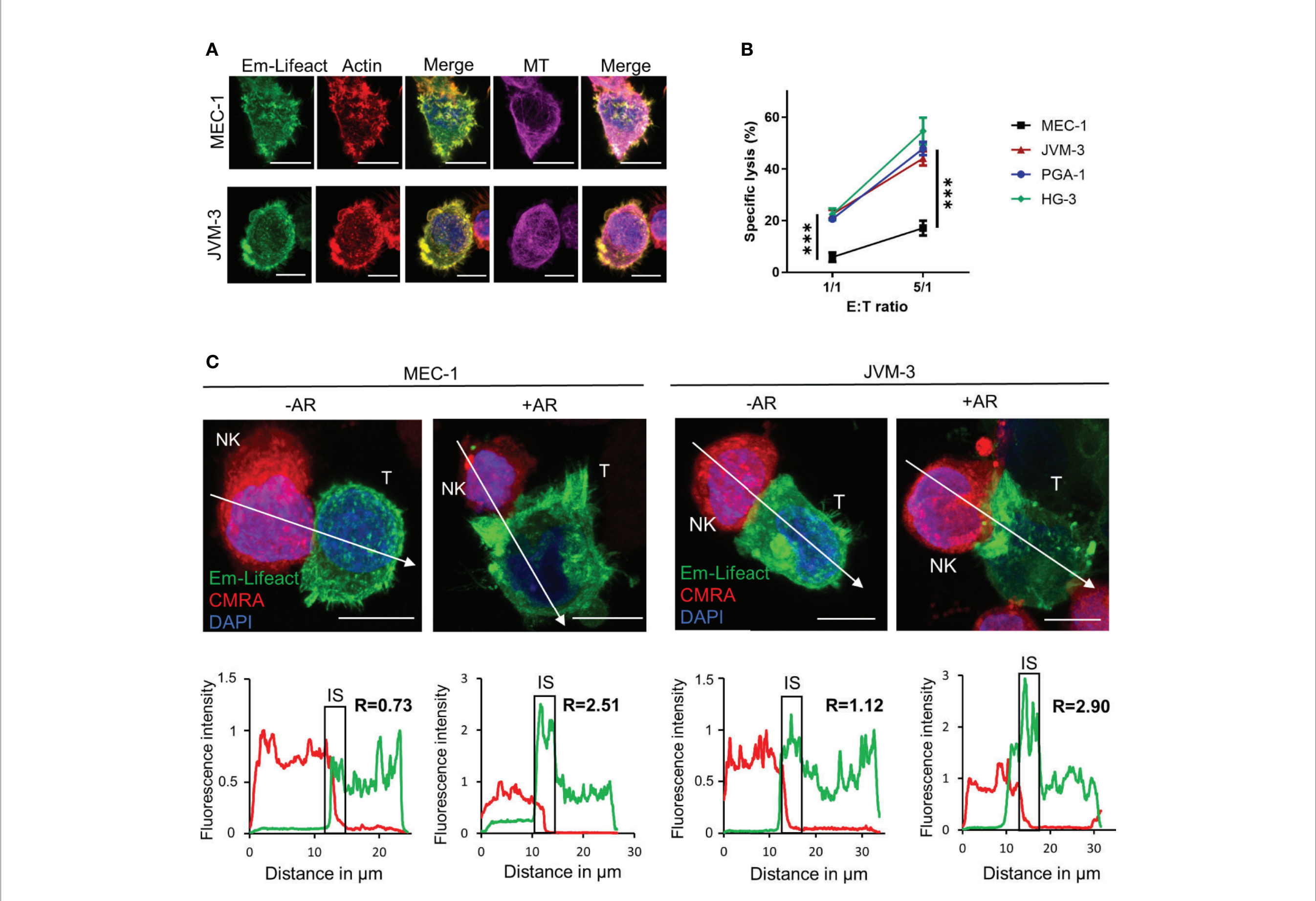

FIGURE 1 | CLL cells have the ability to respond to NK cell attack with an actin response associated to their resistance. **(A)** JVM-3 and MEC-1 cells were transduced to express the actin cytoskeleton marker Emerald-Lifeact (green). Stable cell lines were stained with Acti-stain 555 phalloidin (red) and anti-tubulin antibody (MT, violet). The yellow-green signal shows the co-localization of the two actin cytoskeleton probes. Bars: 10 µm. **(B)** Cytotoxicity assays with four CLL target cell lines and effector NK-92MI cells at 1:1 and 5:1 E:T ratios for 4 hrs. 2-way ANOVA was applied to determine statistical significance; *** denotes $p < 0.0001$. **(C)** Confocal microscopy pictures of MEC-1 (left) and JVM-3 (right) cells (T) in conjugation with NK-92MI cells (NK) with and without an actin response. The charts below show the relative fluorescent intensity of Emerald-Lifeact and CMRA along the trajectories (white arrow). The fluorescence was normalized to 1 at the opposite site of the synapse. The region of the immunological synapse is indicated with "IS". Compared to the opposing end, target cells with an actin response have a more than 2-fold higher fluorescent signal at the IS. Bars: 10µm.

phenomenon "actin response" (AR) (52). Analysis of confocal microscopy images revealed that CLL cells with an AR exhibit a more than 2-fold increase of F-actin at the IS as compared to CLL cells without an AR (**Figure 1C** and **Supplemental Figure S1B**). CLL cells without an AR showed a relatively homogenous distribution of F-actin.

Quantitative analysis of the relative number of NK cell-conjugated CLL cells with and without an AR was conducted using high-throughput imaging flow cytometry. For analysis of conjugates, $5x10^3$ double-positive events were acquired per experiment with the same settings. After quality control, over 1000 conjugates between CLL and NK cells from 3 independent experiments were evaluated for the presence or absence of an AR (**Supplementary Figures S1C, D**). Our data revealed that a majority (about 63%) of highly resistant MEC-1 cells exhibited an AR, while in more susceptible CLL cell lines only a small fraction exhibited this phenotype (**Figure 2A**). HG-3 cells showed the lowest rate of AR with an average of only 14% of conjugated CLL cells forming an AR, while PGA-1 and JVM-3 had an AR frequency of 21 and 23%, respectively. To better characterize and compare the AR in CLL cells, we analyzed both the total F-actin content in AR^- and AR^+ cells, as well as the

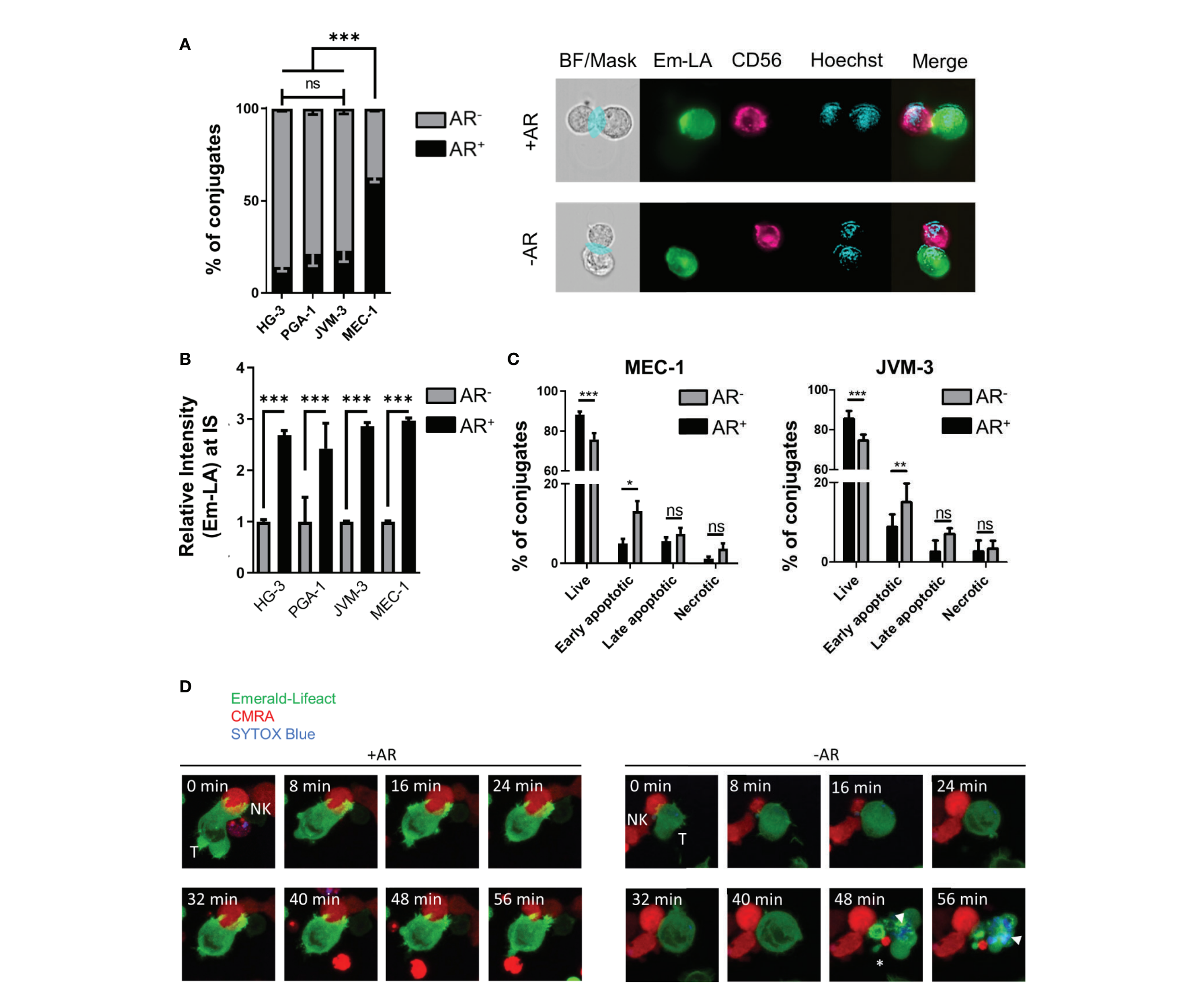

FIGURE 2 | Quantification and functional consequence of the actin response during NK cell attack. **(A)** Quantitative Imagestream analysis of CLL-NK cell conjugates. CLL cells HG-3, PGA-1, JVM-3 and MEC-1 were analyzed for their actin response frequency in conjugates with NK-92MI cells. Percentages of target cells in conjugation with NK-92MI cells with (black, AR^+) and without (grey, AR^-) an actin response. *** denotes $p < 0.0001$ **(B)** Relative intensity of Emerald-Lifeact at the IS in target cells conjugates with NK cells with respect to absence (grey, AR^-) to presence (black, AR^+). Data represents results of 6 different experiments and plots over 2000 conjugates per cell line. Data was normalized to conjugates without an AR. *** denotes $p < 0.0001$ **(C)** Imagestream analysis of target cell death in CLL target cells conjugated with NK cells in the presence (black, AR^+) or absence (grey, AR^-) of an actin response. Target cell death was assessed by Annexin V and propidium iodide staining. * denotes $p<0.05$, ** denotes $p< 0.001$, *** denotes $p < 0.0001$ **(D)** Time lapse imaging of actin dynamics in MEC-1 CLL cells upon NK cell attack. The AR^+ target cell can resist NK cell-induced cell death. Target cells not capable to produce an actin response are effectively lysed as seen by the SYTOX blue staining (white arrow head) and disappearance of normal cellular structures and membrane blebbing (asterisk). ns, non significant.

relative signal intensity of Emerald-Lifeact within the synaptic region of each type of cells. The results show that the total F-actin content is not significantly different in conjugated CLL cells with or without an AR (**Supplementary Figure S2A**). However, in CLL cells with an AR, the relative intensity of F-actin at the IS was increased almost 3-fold (**Figure 2B**), indicating a prominent polarization of the actin cytoskeleton toward NK cells. Additionally, these data suggest the accumulation of F-actin at the IS is compensated by equivalent depolymerization of actin filaments in other parts of the cells, resulting in no net increase of the overall F-actin content in target cells.

To further characterize the link between the AR and resistance to NK cell-mediated death, individual cell-cell conjugates were analyzed using imaging flow cytometry after tumor cells were labeled with the live/dead cell discrimination marker Zombie Red and Annexin V. Early apoptotic cells were characterized as Annexin V$^+$/Zombie Red$^-$, late apoptotic cells as Annexin V$^+$/Zombie Red$^+$, and Annexin V$^-$/Zombie Red$^+$ cells were classified as necrotic for quantitative analysis. In both MEC-1 and JVM-3 cell lines, AR$^+$ cells showed significantly less signs of apoptosis, especially early apoptosis, than AR$^-$ cells (**Figure 2C**). Similar results were obtained for the other two CLL cell lines HG-3 and PGA-1 (**Supplementary Figure S2B**). Since NK and target cells were allowed to conjugate for only 45 minutes, induction of primarily early apoptosis is within the time frame of normal NK cell cytotoxic activity (58, 59). The protective effect of the AR was similar in all cell lines. Yet, it is important to consider that the size of the AR$^+$ cell subpopulation greatly differs between the MEC-1 cell line and the HG-3, PGA-1, and JVM-3 cell lines, explaining the difference in their overall susceptibility.

Live cell imaging analysis revealed that the AR in CLL cells is induced immediately after their first physical contact with NK cells and persisted throughout the whole cell-to-cell interaction time (**Figure 2D** left and **Supplementary Movie 1**). In addition, it provides direct evidence that NK cell-conjugated CLL cells that successfully assembled an AR survived the immune cell attack, while those that failed to mount an AR were efficiently lysed, as shown by uptake of SYTOX blue viability dye (**Figure 2D** right, **Supplementary Movie 2** and **Supplementary Figure S2C**).

In conclusion, we identified a subpopulation of cells in four CLL cell lines that responds to NK cell attack with fast polarization of the actin cytoskeleton to the IS (or AR), a process that closely correlates with resistance to NK cell-mediated lysis. Thus, the overall susceptibility of a given cell line can be directly deduced from the relative size of this subpopulation within the cell, with a large subpopulation being predictive of a highly resistant phenotype.

Targeted Inhibition of Actin Remodeling in CLL Cells Restores High Susceptibility to NK Cell-Mediated Killing

The Rho GTPase cell division control protein 42 homolog (CDC42) is a key regulator of actin polymerization and cell polarity (60). It promotes F-actin polymerization in association with the neuronal Wiskott-Aldrich syndrome protein (N-WASP) and the Arp2/3 complex (61). In addition, CDC42 localizes the N-WASp-Arp2/3 complex close to the cell membrane through interaction with phosphatidylinositol (4, 5) bisphosphate (62). In an attempt to inhibit fast actin remodeling in CLL cells during NK cell attack and to confirm the causal relation between the AR and CLL cell-intrinsic resistance to NK cell-mediated lysis, CDC42 was pharmacologically inhibited using the cell-permeable CDC42-specific inhibitor ZCL278 (56). 50 µM of the inhibitor was found to achieve significant and sustained inhibition of CDC42 activity, without inducing significant toxicity (**Supplementary Figure S2D**). To avoid side effects on the actin cytoskeleton of NK cells, CLL cells were pre-treated with ZCL278 and the drug was washed out before co-culture with NK cells for following experimental assays (63).

Inhibition of CDC42 activity in the resistant MEC-1 cell line resulted in potently impaired AR formation with a more than four-fold decrease in the relative number of conjugated cells exhibiting an AR as compared to DMSO-treated control cells (16.9% and 73.3%, respectively) (**Figure 3A**, left). Remarkably, such an effect was paralleled by an almost five-fold increase in tumor cell susceptibility to NK cell-mediated lysis. Indeed, 52.2% of ZCL278-treated MEC-1 cells were lysed at a 5:1 E:T ratio, while only 10.6% of DMSO-treated cells were lysed in the same conditions (**Figure 3B**, left). Thus, inhibition of *de novo* F-actin polymerization and the AR in CLL cells was sufficient to turn the initially highly resistant MEC-1 cell line into a highly susceptible phenotype. Spontaneous cell death did not change in response to ZCL278 treatment (**Supplementary Figure S2E**), indicating indeed increased susceptibility of MEC-1 cells to NK cell-mediated lysis after CDC42 inhibition. Pharmacological inhibition of CDC42 in HG-3, PGA-1, or JVM-3 cells could not further reduce the small subpopulation of CLL cells with an AR in these cell lines (**Figure 3A**, right and **Supplementary Figure S3A**) and had accordingly no effect on their already highly susceptible phenotype (**Figure 3B**, right and **Supplementary Figure S3B**).

Then MEC-1 cells were pre-treated with DMSO or 50 µM ZCL278 prior to 45 min co-culture with NK cells (1:1 E:T ratio), subsequent labelling with AnnexinV and propidium iodide and quantification of target cell killing by standard flow cytometry. Inhibition of the AR using ZCL278 resulted in a significant increase in apoptotic MEC-1 cells, especially early apoptotic cells, compared to DMSO-treated control cells (**Figure 3C**). The percentage of early apoptotic cells increased from 5.8% to 15%, a value that parallels the imaging flow cytometry analysis of cell death in AR$^-$ MEC-1 conjugates with NK cells (**Figure 2C**), indicating restoration of a susceptible phenotype. Longer incubation time points did not change the distribution of the populations significantly, as late apoptotic and necrotic cells were removed during the washing steps (data not shown). Consistent with our previous results and the intrinsically low AR frequency in the other CLL cell lines, ZCL278 treatment did not significantly modify apoptosis in these cell lines (**Figure 3C** and **Supplementary Figure S3C**). Altogether, these results indicate that the AR is mediated by CDC42 dependent actin polymerization and that inhibition of CDC42 activity potently restores CLL cell susceptibility to NK cell-mediated cytotoxicity.

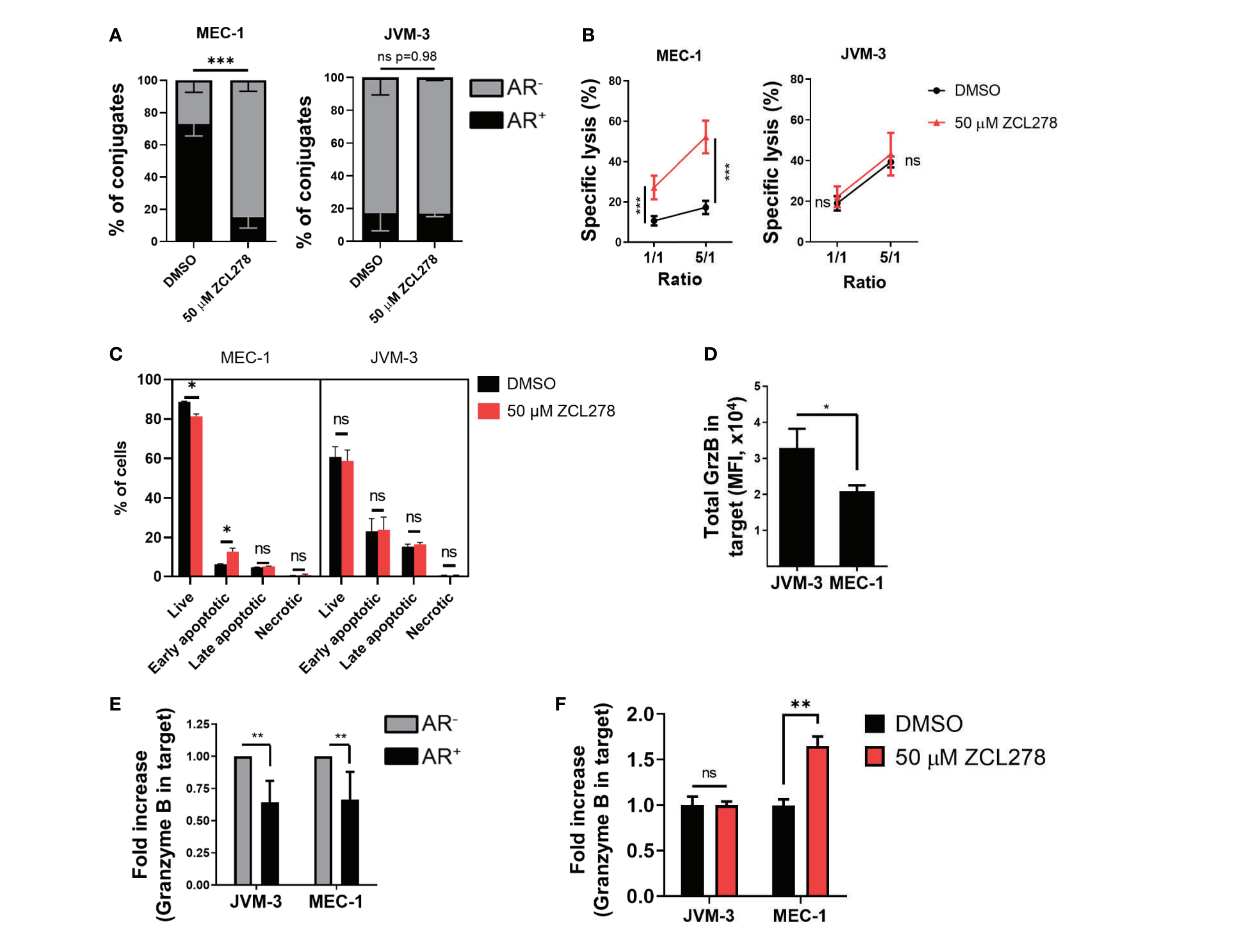

FIGURE 3 | Pharmacological inhibition of Cdc42 increases CLL cell susceptibility to NK cell attack by lowering actin response frequency. **(A)** Quantitative Imagestream analysis of CLL-NK cell conjugates. CLL cells were pre-treated with 50 μM ZCL278 and analyzed for their actin response frequency in conjugates with NK-92MI cells. *** denotes $p < 0.0001$ **(B)** NK cell-mediated cytotoxicity against DMSO- or ZCL278-treated CLL cells. Pre-treated JVM-3 and MEC-1 cells were co-cultured for 4 hrs with NK-92MI cells at E:T ratios of 1:1 and 5:1. Cell death was evaluated by To-Pro-3 staining and adjusted to NK cell-specific lysis. *** denotes $p < 0.0001$ **(C)** Flow cytometry analysis of DMSO- or ZCL278-pretreated CLL target cells after 45 minutes of co-culture with effector NK-92MI cells at a 1:1 E:T ratio. Apoptosis was evaluated by Annexin V and PI staining. * denotes $p < 0.05$, ** denotes $p < 0.001$, *** denotes $p < 0.0001$ **(D)** Imagestream analysis of total granzyme B load in target cells conjugated to NK-92MI cells after 45 minutes of co-culture. * denotes $p < 0.05$ **(E)** Target cells were categorized into AR^+ (black) and AR^- (grey) and granzyme B load in target cells evaluated. Data was normalized to AR^- conjugates. ** denotes $p < 0.001$ **(F)** Imagestream analysis of intracellular granzyme B in target cells after ZCL278-induced Cdc42 inhibition. ** denotes $p < 0.001$. ns, non significant.

Synaptic Actin Remodeling Leads to Reduced Granzyme B Levels in NK Cell-Conjugated CLL Cells

Direct cytotoxicity of NK cells occurs through the release of cytotoxic granules (23, 24). These granules contain among others granzymes and perforin that trigger cancer cell lysis through formation of membrane lesions and induction of caspase-3 and caspase-8 activation. We assessed the levels of one key granzyme, namely granzyme B, transferred into CLL cells. On average, JVM-3 cells in conjugation with NK cells showed a higher intracellular intensity of granzyme B compared to MEC-1 cells (**Figure 3D**), which is consistent with the respective cell line susceptibility to NK cell-mediated lysis and ability to remodel actin cytoskeleton following immune attack. Moreover, in all four cell lines, intracellular levels of granzyme B were considerably reduced (by approximately 35%) in the cell subpopulation exhibiting an AR as compared to the cell subpopulation without an AR, suggesting that the AR leads to reduced amounts of granzyme B transferred to target cells (**Figure 3E** and **Supplementary Figure S3D**).

To assess if inhibiting the AR could restore elevated levels of granzyme B in MEC-1 cells, MEC-1 cells were pre-treated with 50 μM ZCL278 to lower the cell subpopulation with an AR and granzyme B levels were quantified after 45 minutes incubation with effector cells (**Figure 3F** and **Supplementary Figure S3E**). The results show that AR inhibition increased the intracellular granzyme B intensity to levels comparable to JVM-3 cells. Altogether our data provide strong indication that the AR protects CLL cells from cytotoxicity mediated by NK cells through the granzyme B/perforin pathway.

Primary NK Cells Induce the Actin Response and Confirm CLL Cell Line Intrinsic Actin Response Frequency

The NK-92MI cell line used in this study is a CD16- effector cell lines that additionally lacks major inhibitory receptors such as Killer-cell immunoglobulin-like receptors (KIR) and NKG2A receptors (64, 65). These results in induction of natural cytotoxicity through recognition of activating ligands such as MHC class I homologues MIC-A (MIC-A), MIC-B, and UL-16 binding protein (ULBP) on target cells and interaction of lymphocyte function-associated antigen 1 (LFA-1) with its ligand intercellular adhesion molecule 1 (ICAM-1). According to its "hyperactive" phenotype, the NK cell line kills target cells in an unrestricted manner and with low specificity. Thus, we re-evaluated the AR in CLL cell lines challenged with primary NK cells isolated from healthy donors.

To this end, primary NK cells were isolated from PBMCs using a negative selection kit reaching a purity of >90% (**Supplementary Figure S3F**) and kept in culture overnight with IL-2 and IL-15 for activation. Cytotoxicity of primary NK cells against JVM-3 and MEC-1 target cells was lower compared to lysis rates achieved with the NK-92MI cell line. However, the previously established difference in intrinsic susceptibility between the two target cell lines was confirmed (**Figure 4A**). Indeed, despite inter-donor variability, JVM-3 cells were more effectively lysed by donor-derived NK cells than MEC-1 target cells.

Quantitative analysis of the AR frequency with primary NK cells using imaging flow cytometry resulted in remarkably comparable results as seen with the NK-92MI cell line (**Figure 4B**), with about 25% of AR+ conjugates with JVM-3 cells and 60-71% of conjugated MEC-1 cells showing an AR. Confocal microscopy provided direct evidence that primary NK cell attacks also invoked an AR in some individual CLL cells (**Figure 4C**).

In conclusion, activated donor-derived healthy NK cells induce an AR in CLL cells at a same frequency as the NK-92MI cell line, supporting that the AR is a process intrinsic to the CLL cells and is not dependent on the origin of the effector NK cells, being a cell line or isolated form a healthy donor.

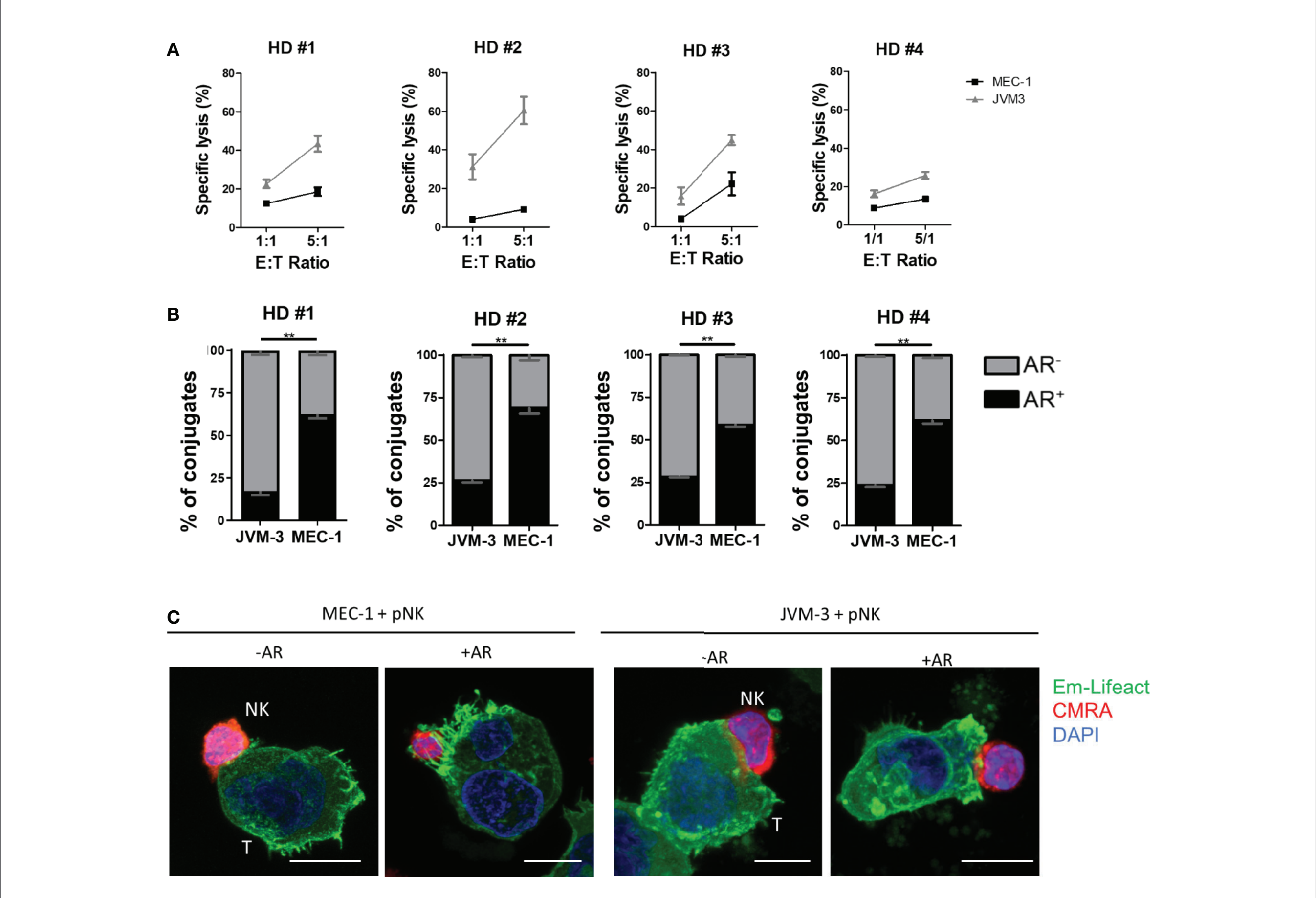

FIGURE 4 | Primary human NK cells can invoke the actin response in MEC-1 and JVM-3 cells. **(A)** Cytotoxicity assay with primary NK cells isolated from four healthy human donors and JVM-3 and MEC-1 CLL target cells at 1:1 and 5:1 E:T ratios. Target cells and primary NK cells were co-cultured for 4 hrs before analysis of NK cell-specific lysis by flow cytometry. **(B)** Quantitative analysis of primary NK-CLL conjugates with (black, AR+) and without (grey, AR-) actin response by imaging flow cytometry. For each donors a minimum of 200 conjugates were analyzed. ** denotes $p < 0.001$ **(C)** Representative confocal microscopy pictures of primary NK cells (CMRA, red) in conjugation with MEC-1 (left) and JVM-3 (right) cells with or without an actin response. Bars: 10 µm.

Inhibition of the Actin Response in Combination With HLA-G Blocking Antibody Restores Patient-Derived CLL Cell Susceptibility to NK Cell-Mediated Killing

To investigate if primary CLL cells mount an AR and if inhibition of the latter improves their susceptibility to NK cell-mediated cytotoxicity, CLL patient samples (n=10) were analyzed in a series of *ex vivo* assays. In these assays, PBMCs were isolated from peripheral blood and their actin cytoskeleton was stained with SiR-actin, a cell-permeable and F-actin specific probe. As illustrated in **Figure 5A**, ARs were observed in primary CLL cells conjugated with NK-92MI cells. These ARs were of slightly lower intensity compared to those seen with CLL cell lines, with a roughly 1.8-fold increase of fluorescence intensity for F-actin at the IS as compared to the opposing cell side. This could be explained by the smaller cell size of primary CLL cells. The AR was then quantified in CLL cells originating from six patients using imaging flow cytometry. Our results revealed a similar and relatively high rate of AR in all primary CLL cell samples with values ranging from 38.4% to 51.2% of analyzed primary CLL-NK-92MI cell conjugates (**Figure 5B**). We noticed that the conjugation rate of primary CLL cells with NK-92MI cells was particularly low (~7% of all $CD5^+/CD19^+$ cells; **Figure 5C**), which can be explained by a high surface expression of HLA-G on *ex vivo* CLL cells (40) and expression of the cognate inhibitory receptor immunoglobulin-like transcript 2 (ILT-2, LILRB1) on NK-92MI cells (66). Interaction of ILT-2 with HLA-G has been reported to negatively impact not only NK cell polarization, but also to interfere with F-actin assembly at the NK cell side of the IS (67) and can thereby prevent conjugate formation between patient-derived CLL and NK-92MI cells (24).

To improve conjugate formation, primary CLL cells were treated with an anti-HLA-G blocking antibody for 1 h prior to co-culture with NK-92MI cells. It is noteworthy that NK-92MI cells do not express the Fc receptor CD16 (64), excluding the risk of ADCC. As anticipated, HLA-G blocking antibody increased the frequency of target CLL cells in conjugation with NK-92MI cells compared to control IgG, indicating a release of the inhibitory ILT-2 signaling and restoration of IS formation (**Figure 6A**). Additionally, we aimed at evaluating the effect of ZCL278 on primary CLL cells. Inhibition of CDC42 activity with 50 µM ZCL278 in combination with blocking antibody was found to only minimally or not to alter the rate of conjugation in comparison to vehicle control (DMSO). Conversely, treatment with anti-HLA-G antibody did not alter the rate of AR in primary CLL cells, while ZCL278 treatment reduced the AR in three out of five patients (**Figure 6B**). Although this remains speculative, the lack of response in CLL cells originating from patient 8 and 9 could be explained by an initially low AR frequency (patient no. 9) or upregulation of alternative, CDC42-independent actin polymerization pathways (patient no. 8).

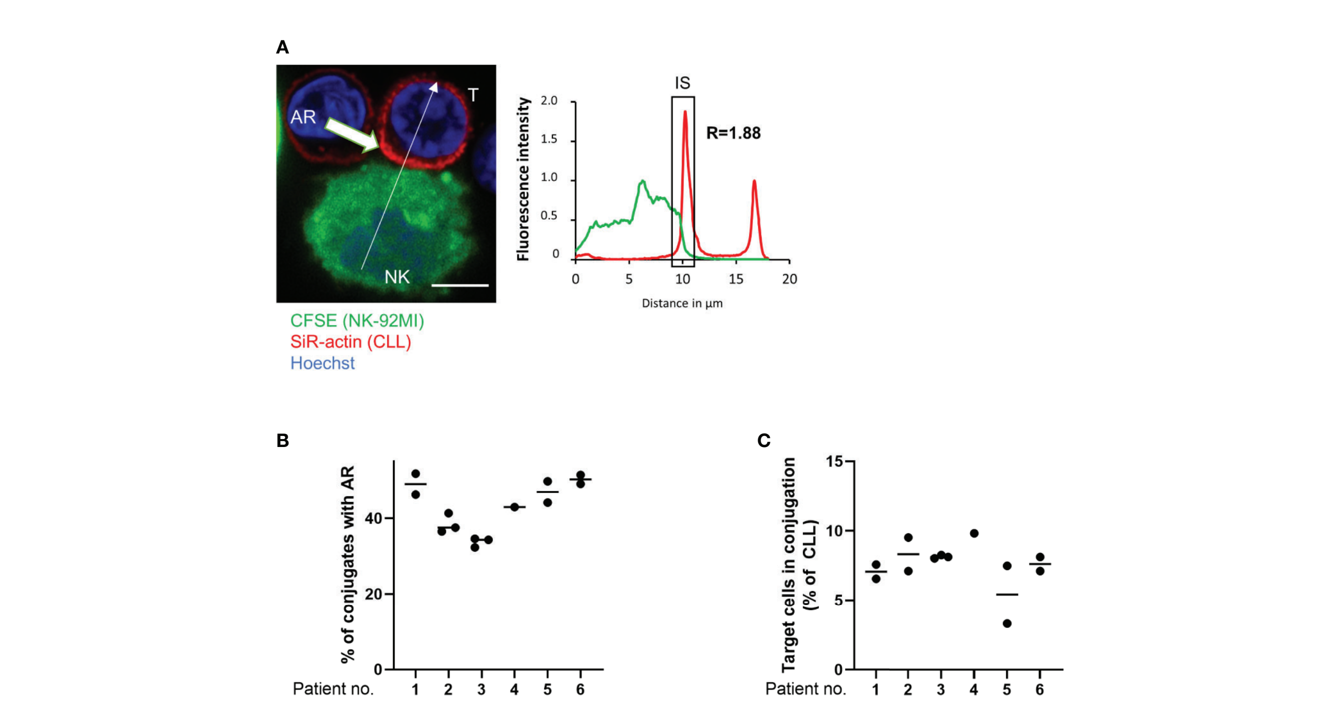

FIGURE 5 | The actin response in patient-derived primary CLL cells. **(A)** Representative confocal microscopy image of an immune synapse between a primary CLL cell (T) and NK-92MI cell (NK). The chart to the right shows the relative fluorescent intensity of SiR-actin along the trajectory (white arrow). The fluorescence was normalized to 1 at the opposite site of the synapse. The region of the immunological synapse is indicated with "IS". Bar: 10 µm **(B)** Quantitative imaging flow cytometry analysis of the AR in primary CLL cells in conjugation with NK-92MI effector cells. CLL cells were identified as $CD5^+/CD19^+$ cells. Actin staining was performed using spirochrome labelling. **(C)** Quantification of primary CLL cells in conjugation with NK-92MI cells after 45 minutes of co-culture as percentage of total target population.

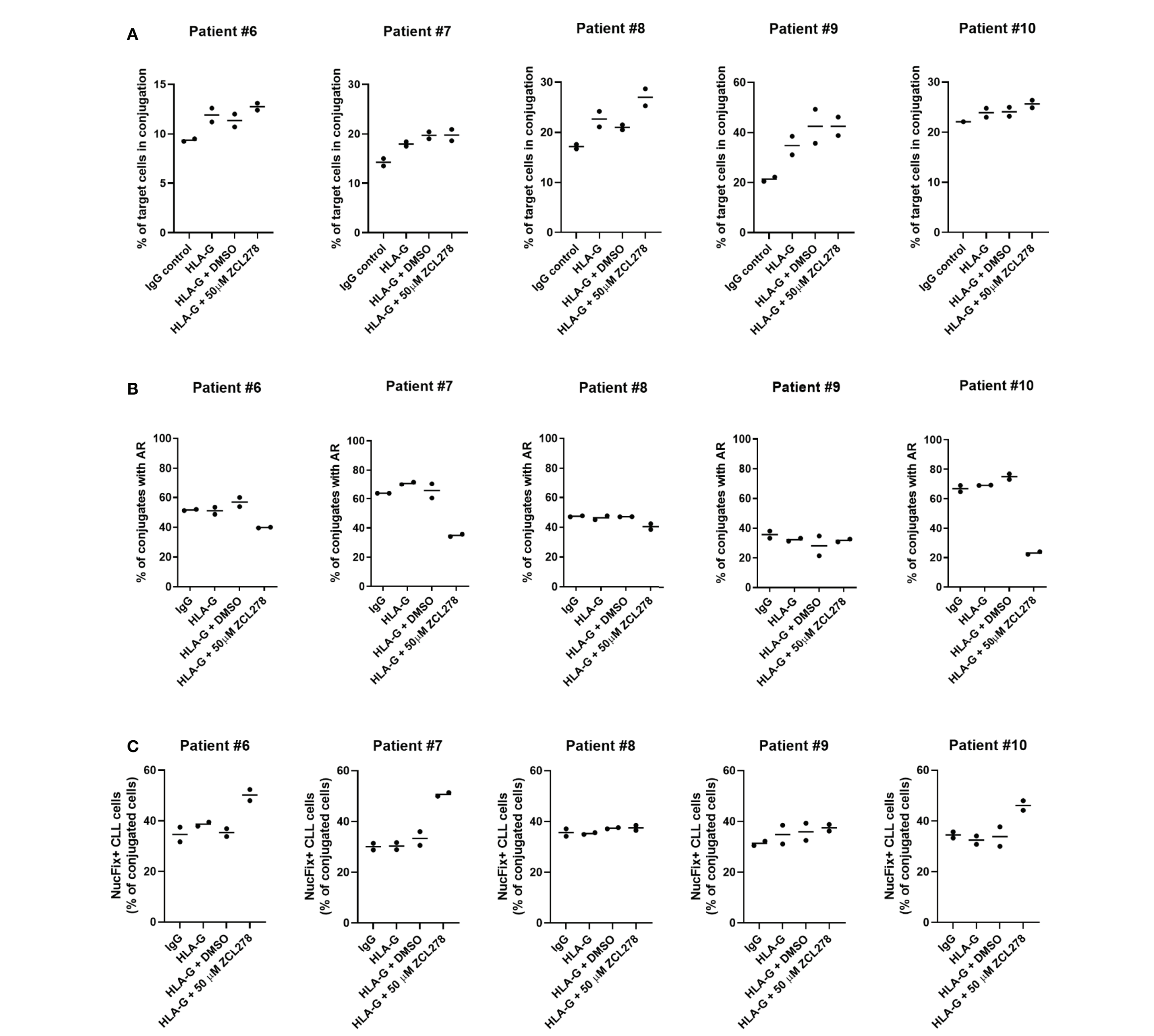

FIGURE 6 | Inhibition of the actin response in combination with anti-HLA-G blocking antibody substantially improves NK cell-mediated killing of CLL cells. **(A)** Conjugation frequency of primary CLL cells as quantified by imaging flow cytometry in the absence or presence of HLA-G blocking antibody. Target cells were incubated with 10 µg/mL control IgG or blocking antibody against HLA-G, and were used either untreated (IgG, HLA-G), DMSO treated, or after incubation with 50 µM ZCL278 for 1 h before conjugation. Effector and target cells were co-cultured at a 3:1 E:T ratio for 45 min at 37°C and fixed with 2 v/v% PFA. Target cells in conjugation are shown as % of total target cells. **(B)** Quantitative analysis of primary CLL-NK cell conjugates with an actin response in the presence of control IgG or anti-HLA-G blocking antibody by imaging flow cytometry. Target cell were either untreated (IgG, HLA-G) or conjugated after treatment with vehicle (DMSO) or 50 µM ZCL278. For each patient, a minimum of 100 conjugates were analyzed. **(C)** Average percentage of Live-or-Dye NucFix™ Red positive primary CLL cells in conjugation with NK-92MI cells in the presence of control IgG or anti-HLA-G blocking antibody. Target cells are either untreated, treated with vehicle control or 50 µM ZCL278.

Finally, apoptosis in primary CLL cells in conjugation with NK-92MI was evaluated with regards to anti-HLA-G antibody treatment and CDC42 inhibition. Samples treated with 50 µM ZCL278 that previously showed no changes in the AR frequency (**Figure 6B**) demonstrated similar levels of apoptosis as compared to IgG control, HLA-G, or HLA-G in combination with vehicle treated samples from the same patient (**Figure 6C**, patient no. 8 and 9). In contrast, with CLL samples that showed a reduction of the AR in response to CDC42 inhibition (**Figure 6B**), an increased percentage of conjugated primary CLL cells showed signs of apoptosis (**Figure 6C**, patient no. 6, 7, and 10). These results extend our analysis with CLL cell lines and indicate that the AR is a frequent process in primary CLL cells (with more than 50% of NK cell-conjugated CLL cells showing an AR for

most patients) and that targeting of this process can substantially increase CLL cell susceptibility to NK cell-mediated cell death.

In conclusion, we report here for the first time AR in patient-derived cancer cells and show that specific targeting of key actin regulators in combination with anti-HLA-G blocking antibody, increases conjugate formation and target cell susceptibility to NK cell-mediated cytotoxicity opening up the possibility of combinational targeting for CLL patients.

DISCUSSION

Despite substantial recent advances in the therapy of CLL, treatment options, especially for patients diagnosed with an aggressive disease, particularly with *TP53* deletion and/or mutation, are limited. Effectors of both, the adaptive and the innate immunity immune systems, show severe signs of dysfunction that allow for successful immune evasion of malignant B cells. This includes inhibition of cytotoxic CD8$^+$ and activated CD4$^+$ T lymphocytes and induction of the immune suppressive M2-like monocyte phenotype instead of pro-inflammatory immune sub-populations. Additionally, CLL induces expansion of regulatory T cells (T_{Reg}), overall resulting in the development of a tolerogenic environment and disease progression (11, 68). While special attention has been paid investigating the interaction between CLL and T lymphocytes, recent studies focused on NK cells as an alternative target of chemo-immunotherapy. NK cells derived from CLL patients were described as hyporesponsive due to a loss of the mature, CD56^{dim} NK cell population, possibly due to activation-induced apoptosis as a result of constant exposure to malignant B cells. This was accompanied by a downregulation of activating receptors such as NKG2D that affects the natural cytotoxicity of NK cells (23). However, upon sufficient activating signal through CD16, NK cell function can still be induced, showing that CLL-derived NK cells of the CMV-associated NKG2C$^+$/CD16$^+$ phenotype are fully functional (69). Total NK cell numbers have repeatedly been reported to be elevated in CLL patients compared to healthy controls, often with an emphasis on CMV-related NKG2C$^+$/CD56^{dim}/CD16$^+$ NK cells (23, 70, 71). These phenotypes accordingly cannot explain the lack of anti-tumor response or disease progression in CLL.

The NK cell line we used in the present study is negative for CD16 and a common model of natural cytotoxicity of NK cells. Although these NK cells are fully activated and effectively recognize their targets, a subpopulation of CLL cells was still resistant to NK cell-mediated cytotoxicity. This indicates an additional intrinsic resistance of CLL that allows escape from NK cells that can be activated either through activating ligands or possibly even through ADCC. Here we show that the actin cytoskeleton of CLL cells plays a critical role in the intrinsic capacity of these cells to avoid destruction by degranulating immune effector cells.

The fast synaptic actin remodeling we observed in CLL cells attacked by NK cells strongly resembled the "actin response" or AR previously described for breast cancer cells and was strongly associated with resistance to NK cell cytotoxicity. Independent of IGHV mutational status, CLL cell lines showed a resistant subpopulation that was characterized by the AR. While HG-3, PGA-1, and JVM-3 showed similar results in all experimental assays with a high susceptibility to NK cell-mediated lysis and high intracellular granzyme B load, MEC-1 cells demonstrated a high resistance to NK cell-mediated cytotoxicity and decreased uptake of NK cell-derived granzyme B. We attributed these differences to the relative size of the AR$^+$ subpopulation as we were able to show that *de novo* F-actin polymerization on the cancer side of the IS is strongly associated with survival and resistance during NK cell attack. Pharmacological inhibition of CDC42 activity drastically reduced the size of the AR$^+$ subpopulation and resulted in an increase of early apoptotic cells and overall cell death in MEC-1 that can be explained by increased amounts of granzyme B transferred into target cells. Although CDC42 is a central actin cytoskeleton regulator, other pathways might be involved in the process of the AR, as suggested by the remaining AR$^+$ subpopulation in HG-3, PGA-1, JVM-3 and MEC-1 cells that resisted treatment with the CDC42-specific inhibitor ZCL278 (56).

An interesting aspect that could be worth further investigation is the potential role of *TP53* in enabling the AR, as the four cell lines differ in their *TP53* mutational status. HG-3, PGA-1, and JVM-3 cells have all been reported to express wildtype *TP53*, while MEC-1 are identified as a *TP53*$^{del/mut}$ cell line, expressing a truncated 40kDa version of the p53 protein without transcriptional activity. In CLL patients, mutations of *TP53* or loss of one *TP53* allele are associated with a significant decrease in survival and are predictive for an impaired response to chemo-immunotherapy (72). Additionally *in vitro* experiments attributed expression of wildtype *TP53* or mutational *TP53* and/or loss of *TP53* to differential drug response in several CLL cell lines, including JVM-3 and MEC-1 (73–75). Other studies have shown, that rescue of mutational *TP53* function can restore granzyme B-mediated apoptosis in breast cancer through down-modulation of anti-apoptotic proteins (76). However, in these studies, *TP53* reactivation was achieved in cell lines expressing a missense mutational p53, while in many CLL cases with aberrant *TP53* expression, deletion of the short arm of chromosome 17 (del17p13) results in a complete loss of *TP53*, often in association with *TP53* mutations on the other allele (77). Whether *TP53* status is therefore a critical determinant of the AR frequency in CLL will need to be determined with a larger, better defined patient cohort in the future.

Most importantly, we were able to show that the AR is not an artefact of cell lines but can indeed be found in patient-derived CLL cells. In this context, the inhibitory interaction of surface molecules, such as the non-classical MHC-I isoforms HLA-E and HLA-G, with their corresponding ligands on NK cells might have been underestimated in their significance. Even the NK-92MI cell, known to not express key inhibitory receptors such as KIR or NKG2A, showed a deficiency in conjugate formation with patient-derived CLL cells. This dysfunction could be rescued by antibody blockade targeting HLA-G. However, antibody opsonization of target CLL cells had no impact on the

frequency of the AR in primary CLL cells. In combination with CDC42 inhibition, anti-HLA-G blocking antibody greatly increased NK cell-induced apoptosis in patient-derived CLL cells. We hypothesize that this is a consequence of the relative size of the AR^+ population that could be decreased by pharmacological CDC42 inhibition. This is further supported by the observation that in individual patient samples in which this treatment failed to reduce the size of the AR^+ population, the frequency of CLL cells showing signs of apoptosis was unchanged compared to untreated and DMSO-treated samples. Since NK-92MI cells are $CD16^-$, antibody blockade did not trigger ADCC that could explain the increase in target cell death. It is however worth speculating that NK cells capable of ADCC could be even more effective in inducing apoptosis in CLL cells that underwent dual blocking antibody and CDC42 inhibition therapy.

Yet, actin cytoskeleton targeting drugs, such as cytochalasins or latrunculins, show intolerable toxicity with particularly severe adverse effects on cardiac structure and function and are therefore unfit for clinical trials. Experimental drugs that target cancer-specific F-actin components and confirmation of their efficiency *in vivo* (78, 79) demonstrate however, that targeting of the actin cytoskeleton dynamics is a possibility in our search for new innovative cancer drugs. Importantly, targeting of intrinsic immune escape mechanisms such as the AR can only be effective in combination with other therapies, such as immune checkpoint inhibitor blockade and/or opsonization with tumor-targeting antibodies. Without these therapies, cytotoxic lymphocyte activation, but also IS formation that is fundamental to a functional anti-tumor immune response cannot take place.

Overall, NK cells are emerging as a valuable tool for the control of CLL disease progression and reactivation of their cytotoxic capabilities against cancer cells could potentially improve overall outcome. Selective targeting of intrinsic immune escape mechanisms, such as the here described AR, could provide a new line of therapy for the difficult to treat or relapsing CLL subtypes. It is important to highlight that irrespective of the expression status of poor prognostic markers, such as *TP53* and *IGHV*, all four CLL cell lines, as well as all ten patient-derived CLL samples demonstrated an AR^+ subpopulation that proved to be resistant against pharmacologic inhibition of CDC42 activity. This shows the presence of another signaling pathway allowing cancer cells to maintain resistance against NK cell cytotoxicity. Further studies employing patient cohorts will be needed to address and confirm the clinical importance of the AR in CLL and its therapeutic value. Further it needs to be evaluated whether the hyporesponsive phenotypical state of CLL-patient derived NK cells is revertible or if allogeneic NK cell therapy could benefit from selective targeting of the AR (47), possibly in combination with other immunomodulatory drugs (44, 80, 81).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité National d'Ethique de Recherche Luxembourg (CNER No. 201707/02 Version 1.2). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HW designed and performed most of the experimental work, analyzed results, and wrote the manuscript. LF, CH, MK, and AB performed experiments and analyzed results. JM performed NK cell isolation. HW, LF, and AL performed patient CLL cell isolation. Patient samples were provided by SD, JF, and GB. JP and EM organized CLL sample collection and contributed to supervise the study. CT supervised the study and wrote the final version of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING SUPPORT

Work in CT's group is supported by grants from the National Research Fund (FNR, Luxembourg, ACTIVASION, C19/BM/13579644), La Fondation Cancer (Luxembourg, ACTIMMUNE, FC/2019/02) and Think Pink Lux (Luxembourg). HW is recipient of a PhD fellowship from the National Research Fund (FNR, Luxembourg), Luxembourg (PRIDE15/10675146/CANBIO). AB and MK are recipient of PhD fellowships from Fonds National de la Recherche Scientifique (FNRS, Belgium, Televie #7.4536.19 ACTIPHAGY and Televie #7.4531.20 TRANSACTINg, respectively). AL is supported by a post-doctoral fellowship from the FNRS (Televie #7.4503.19). JP is supported by the National Research Fund (FNR), Luxembourg (INTER/DFG/16/11509946).

ACKNOWLEDGMENTS

We thank Flora Moreau and Odile Lecha for providing Emerald-Lifeact expressing cell lines and help with data collection.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.619069/full#supplementary-material

Supplementary Figure 1 | (A) List of CLL cell line characteristics. **(B)** Confocal microscopy pictures of HG-3 and PGA-1 Emerald-Lifeact cells (T) in conjugation with NK-92MI cells (NK) with and without an actin response. Charts below show the relative fluorescent intensity of Emerald-Lifeact and CMRA along the trajectories (white arrow). The fluorescence was normalized to 1 at the opposite site of the synapse. The immunological synapse is indicated with "IS". Cells with an actin response show a more than 2-fold higher fluorescent signal at the IS. Bars: 10μm.

(C) Analysis of imaging flow cytometry images. First, a scatter plot of aspect ratio vs. area was used to gate for cells and exclude debris and control beads (R1). Second, a gradient RMS (root mean square for image sharpness) histogram was used to gate for cells in focus (Focus). Third, the intensity of PE/Cy7 (CD56) was gated against the intensity of Emerald-Lifeact (EmLA). Double positive events were categorized as conjugates and used for subsequent image analysis. We defined AR^+ CLL cells as target cells with an increased relative intensity of EmLA fluorescence in the IS mask in relation to the total intensity of EmLA in target cells. For determination of granzyme B content in CLL cells, the NK cell part of the immune synapse is excluded from the analysis. **(D)** Immune synapse definition using IDEAS. Cell shape defined by surface labelling (CD56) or EmLA expression was extended by 3px in all dimensions using the dilate function. The overlapping region was defined as the immune synapse mask (IS mask) in NK cell-CLL cell conjugates. Created with BioRender.com

Supplementary Figure 2 | (A) Imaging flow cytometry analysis of total F-actin fluorescent intensity in AR^+ and AR^- HG-3, PGA-1, JVM-3, and MEC-1 cells. Differences between cell lines are the result of differences in transduction efficiency and transgene expression. Analysis was performed using a Wilcoxon paired t-test. **(B)** Apoptosis in HG-3 and PGA-1 conjugates with NK-92MI as evaluated by imaging flow cytometry. Cells were gated based on the absence (AR^-) or presence (AR^+) of an actin response. * denotes to $p < 0.05$, ** denotes to $p < 0.01$ **(C)** Measurement of SYTOX Blue fluorescence intensity over time in the MEC-1 cells with and without an actin response following conjugation with NK cells. Image J software was used to do the quantification of LSM880 acquired images.
(D) Evaluation of GTP-loaded Cdc42 by G-LISA colorimetric activation assay. MEC-1 cells were pre-treated for 1 h with 50 µM ZCL278 before drug was removed and cells resuspended in fresh complete medium. Cells were allowed to recover for 1 and 5 hrs after drug removal before stimulation with 0.1 µg/mL human recombinant EGF for 15 minutes to measure inducible CDC42 activity. *** denotes to $p < 0.0001$.
(E) Spontaneous cell death in HG-3, PGA-1, JVM-3, MEC-1 cells treated with either DMSO or 50 µM ZCL278 for 1 h. Drugs were removed and cells allowed to recover for 45 minutes before cell death analysis using Annexin V and propidium iodide.

Supplementary Figure 3 | (A) Imaging flow cytometry analysis of DMSO- or ZCL278-treated HG-3 and PGA-1 with regards to actin response frequency in conjugates. **(B)** Cytotoxicity assays of DMSO- or ZCL278-treated HG-3 and PGA-1 cells with NK-92MI at E:T ratio of 1:1 and 5:1. **(C)** Apoptosis assay of DMSO- or ZCL278-treated HG-3 and PGA-1 cells with NK-92MI effector cells. Cells were co-cultured for 45 minutes before live/dead staining with Annexin V and propidium iodide. **(D)** Quantitative imaging flow cytometry analysis of granzyme B load in HG-3 and PGA-1 CLL cells in conjugation with NK-92MI cells. **(E)** Imaging flow cytometry analysis of granzyme B load in DMSO- or ZCL278-treated HG-3 and PGA-1 CLL cells after 45 minutes of co-culture with NK-92MI cells. **(F)** Flow cytometry gating strategy for buffy coat-derived PBMC before and after negative selection for NK cells.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The second article presented in this thesis is a manuscript in progress that includes the main findings of my PhD research. This part of my work aims to address one of the potentially many underlying resistance mechanisms of the actin response: the polarization of inhibitory ligands to the post-synaptic target cell membrane. This observation was initially documented in our publication from 2018 (see Annex I) and the importance of iIC for tumour immune escape was further emphasised by the Nobel Prize for Physiology and Medicine in the same year that was awarded to Dr James P. Allison and Dr Tasuku Honjo.

In the following results I characterize the actin response immunological synapse (AR-IS) using comparative high resolution confocal microscopy and electron microscopy and addressed the functional consequence of inhibitory ligand polarization for NK cell activation. I have further investigated the actin response as a reaction to CTL attack and the influence of different sizes of actin response-competent cancer subpopulations on *in vivo* tumour growth and the tumour immune landscape.

Design of the research, execution of most experiments and data analysis has been done by me, while specialized methods, such as electron microscopy, single cell RNA analysis, and proteomic analysis has been done in collaboration with scientists from other research groups. In the general discussion, this article draft is referred to as **article II**.

Actin cytoskeleton-mediated polarization of immune checkpoint ligands correlates with suppression of cytotoxic NK cell functions and tumour immune evasion *in vivo*

Manuscript in progress

Introduction

Cancer immunotherapy have dramatically changed the therapeutic options and clinical outcome of multiple cancer types, but a remaining fundamental challenge are primary and acquired resistance. Natural killer (NK) cells are effector lymphocytes of the innate immune system that are best known for their ability to detect and kill virally infected or transformed cells without the need for priming. While the most common understanding is that cytotoxic T lymphocytes (CTLs) possess the strongest anti-tumour potential, they require intact and sufficient antigen presentation as well as priming by antigen presenting cells. To this end, the role of NK cells in initiating the cancer-immunity cycle, mediating a sustained anti-tumour response during tumour immuno-editing and their contribution to therapeutic success of immune checkpoint inhibitors (ICIs), have potentially been underestimated.

Like CTLs, NK cells interact with prospective target cells in dynamic cell-cell contacts, so called immunological synapses (IS). This interaction coordinates the bidirectional exchange of information through receptor-ligand interaction and determines the effector response. Formation of a cytolytic IS in NK cells is regulated by recognition of cognate stress-induced ligands on candidate target cells by germline encoded activating receptors. Inhibitory receptors, such as the killer cell immunoglobulin-like receptors (KIRs) and the NK cell immune checkpoint CD94/NK group 2 member A (NKG2A) heterodimeric receptor, counterbalance activating receptor signalling and act as a check on cytotoxic NK cell functions. Healthy cells express major histocompatibility complex class I (MHC-I) molecules on their surface that serve as display for peptide presentation to CTLs, but also function as ligands for KIRs. Reduction of physiological MHC-I levels is an immune evasion strategy employed by viruses to prevent the presentation of viral peptides[653], but that are also observed in tumours to evade CTL response against tumour neo-antigens. This loss of MHC-I leaves cancer cells vulnerable to NK cells by lowering the strength of the inhibitory signal at the NK-IS. In combination with recognition of stress-induced ligands, such as MHC class-I polypeptide-related sequence A/B (MIC-A/-B) and UL16 binding proteins 1-6 (ULBP1-6), by activating receptors on NK cells, such as NKG2D, cytolytic activity of NK cells is triggered and polarization and secretion of cytotoxic granules towards the conjugated target cells is initiated.

Communication at the IS is bidirectional and modulation of the balance of activating and inhibitory signals in NK cells can enable immune escape of cancer. If activating signal is strong enough, the formed cytolytic IS undergoes fast and sustained actin cytoskeleton remodelling and accumulation of filamentous actin (F-actin) at the NK cell side. Conversely, engagement of inhibitory NK cell receptors induces recruitment of protein tyrosine phosphatases SHP-1 and SHP-2 that interfere with actin cytoskeleton dynamics and activation of downstream effectors of activating receptors. Recently, we reported that resistant breast cancer cells and chronic lymphocytic leukaemia (CLL) cells engaged with NK cells exhibit a similar behaviour and polarize their own actin cytoskeleton toward the IS. This reaction to NK cell attack is the so-called “actin response” that is characteristic to a resistant subpopulation of cancer cells. The relative size of this subpopulation in cancer cell lines, but also patient derived tumour cells, is closely associated with their overall susceptibility to NK cell-mediated lysis. The presence of an actin response in a cancer cell-NK cell conjugate correlates with reduced amounts of granzyme B in target cells and lower rates of apoptosis in

individual cancer-NK cell conjugates. Reduction of actin dynamics in cancer cells through RNA interference or pharmacological inhibition of key actin cytoskeleton regulators inhibits the formation of the actin response and increases cancer cell susceptibility to NK cell cytotoxicity.

Here, we demonstrate that the actin response is a highly conserved cancer cell intrinsic mechanism that acts as a scaffold for polarization and stabilization of predominantly inhibitory ligands, namely MHC-I and PD-L1, to the NK-IS. This spatial ligand organization, rather than the F-actin accumulation itself, correlates with an inhibition of NK cell cytotoxic functions through inhibition of activating receptor signalling. This results in reduced lytic granule polarization and directed degranulation and facilitates immune evasion of actin response-competent cancer cells. Using single cell RNA sequencing and proximity-based biotinylation assays, we have identified ezrin and moesin, members of the ERM protein family, as important components of the actin response and associated ligand polarization. The frequency of the actin response can be manipulated in a cell line, e.g., through the expression of mutated versions of the small Rho GTPase CDC42 (cell division cycle 42), and modulation of the actin response frequency affects *in vivo* tumour growth in immune-competent mice drastically. Using an aggressive mouse melanoma model and a mouse breast cancer model, we could demonstrate that a low actin response rate was associated with reduced tumour growth and an increased number of activated tumour infiltrating lymphocytes (TILs). We suggest that the AR could be implicated in primary resistance against ICI therapy. Taking together, we propose that the actin response is a primary and widely conserved resistance mechanism against NK cell-mediated cytotoxicity and overexpression of ezrin and/or moesin in combination with the actin response-dependent polarization of inhibitory ligands to the IS is a novel immune evasion mechanism of cancer.

Material and Methods

Cell culture

Cell lines MDA-MB-231, MCF-7, A-375, A-549, 4T1, and B16-F10 cells were grown in DMEM supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (complete DMEM). Hs578T cells were cultured in completed DMEM supplemented with 0.01 mg/mL human recombinant insulin. SK-OV-3 cells were cultured in McCoy´s 5a medium containing 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. U87 were cultured in EMEM supplemented with 10% heat inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. NK-92MI cells were grown in complete RPMI-1640, supplemented with 10% FBS, 10% horse serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (RPMI 20%). All cell lines were periodically validated by STR profiling (Microsynth). Primary human NK cells were cultured in RPMI-1640 with 10% FBS, 1 mmol/L sodium pyruvate, 1 mmol/L HEPES, 0.1 mmol/L NEAA, 100 U/ml human recombinant IL-2 (PeproTech), 100 U/mL penicillin, and 100 µg/mL streptomycin. Primary mouse NK were cultured in DMEM with 10% FBS, 1 mmol/L sodium pyruvate, 1 mmol/L HEPES, and 55 mmol/L 2-mercaptoethanol (Gibco™), and 1000 U/mL human recombinant IL-2. All cells were cultured at 37°C with 5% CO_2 under humid conditions.

Stable cell line generation Viral particles were prepared by transfecting HEK-293 cells using a packaging, envelop, and the target plasmid, and Xtreme gene. Infectious particles were harvested and subsequently used to infect target cells. Target cancer cell lines were transduced to express the mEmerald-tagged filamentous actin marker Lifeact using a pCDH-CMV-MCS-EF1α-Puro cloning and expression lentivector. Expression of the dominant negative form (Cdc42(T17N)) or the constitutively active form of the Cdc42 protein (Cdc42(Q61L) in murine cancer cell lines was induced

using a pLV-EF1A-Flag-Cdc42(T17N)/Myc-Cdc42(Q61L)-Blasticidine gene expression vector (Vectorbuilder) with in-line expression of an antibiotic resistance gene. Human cancer cell lines were transduced with the same constructs. HLA-E transduced cells were generated using a pLV-EF1A-HLA-E-Neo lentiviral vector (Vectorbuilder). For stabilization of HLA-E surface expression, target cells transduced to express HLA-E were incubated with 200 µM of leader sequence peptides (VMAPRTLIL or VMAPRTLFL) in serum-free medium for 2.5 hours at 37°C prior to experiments. Control cells were transduced with an empty control vector under selection of the same antibiotic. Expression of transgenes was confirmed by western blot or flow cytometry. For proximity biotinylation experiments, target cells were transduced using a pLV-CMV-HLA-E/PD-L1-3xGGGGS-BioID2-myc-Bsd lentiviral expression vector (Vectorbuilder). Transduced cells were tested for expression of BioID-fusion proteins by western blot. Polyclonal cells expressing the desired transgene were enriched by antibiotic selection.

Proximity-based biotinylation assay HLA-E-BioID2 and PD-L1-BioID2 expressing target cells and control cells were cultured in complete medium supplemented with 50 µM excess biotin for 24 hours. For experiments with HLA-E-BioID2 expressing cells, target cells were peptide-pulsed as described before conjugation. Subsequent, target cells were exposed to NK-92MI effector cells for 60 minutes to induce IS formation. Medium and NK cells were completely removed, and plates washed with ice-cold PBS before lysis in 540 µl lysis buffer (50 mM ammonium bicarbonate with 1% SDC and protease inhibitors, pH=8) and samples snap frozen in liquid nitrogen and kept at -80°C until further use for immunoprecipitation of biotinylated proteins. To detect steady state biotinylated proteins, BioID-fusion samples without NK-92MI co-culture were used. Randomly biotinylated proteins and proteins that non-specifically bind to avidin/streptavidin beads used for biotin-affinity purification were detected using control cells lacking BioID ligase expression that were cultured with an excess of biotin. After protein extraction, biotinylated proteins were isolated using avidin/streptavidin agarose beads for affinity purification. Samples were incubated with beads for 16 hours at 4°C on a rotator. After incubation, samples were washed 5 times with 8 M urea in 50 mM Tris·Cl, pH 7.4 and split for analysis by western blot and mass spectrometry. Proteins enriched in samples that were in contact with NK-92MI cells were analysed for actin binding and actin regulatory proteins.

Transient infection Selective knockdown of target genes was achieved using RNA interference. Cells were grown to 70-80% confluence and transfected with 100 nM siRNA against target genes or a scramble control using DharmaFECT 1 transfection reagent (Horizon Discovery). 48 hours post-transfection, cells were collected for assessment of protein knockdown and subsequent experiments. Knockdown of target genes was confirmed by Western Blot or flow cytometry.

CTL and NK cell isolation and activation Buffy coats from healthy donors were collected from the Luxembourg Red Cross. Peripheral blood mononuclear cells (PBMC) were purified from buffy coats by Lymphoprep (Stemcell Technologies) density gradient centrifugation. NK cell enrichment was performed by negative selection using a NK cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's recommendations. Purified NK cells were activated overnight with 100 U/mL recombinant human IL-2. Purity of isolated NK cells and characterization of receptor expression profiles of effector NK cell populations were generated by flow cytometry.

Primary mouse NK cells were enriched from splenocyte culture from healthy wild type mice (BALB/c or C57BL/6) at age 8 – 12 weeks. In brief, spleens were dissociated, and single cell culture passed through a 40 µM cell restrainer. Erythrocytes were lysed with ACK Lysis buffer (Gibco™) and remaining splenocytes cultured at a density of $3x10^6$ cells/mL in the presence of 1000 U/mL recombinant human IL-2. At day 4 of culture, fresh IL-2 was supplemented. Enriched primary NK cells were used after 7 – 9 days.

Activated antigen-specific CTLs against gp33 were kindly provided by Dr. M. Grusdat and Prof. D. Brenner (Department of Infection and Immunology, Luxembourg Institute of Health, Esch-sur-Alzette).

Imaging flow cytometry

For analysis of ligand polarization to the IS, NK cells ($6x10^5$) and target cells ($2x10^5$) were stained for surface antigens for 20 min at 4°C in MACS buffer prior to conjugation. All antibodies used for staining were purchased from Biolegend, unless otherwise noted. NK cells were stained for CD56 (clone HCD56). Target cells were stained for MHC-I (clone W6/32), PD-L1 (clone 29E.2A3) and MIC-A/B (clone 6D4). Cells were mixed in 200 µl of RPMI 20% and allowed to conjugate at 37°C for 40 min. Culture medium was supplemented with 1000 U/mL IL-2 when primary NK cells were used. Conjugates were fixed with 2v/v% paraformaldehyde (PFA) in PBS for 15 min at 37°C. For evaluation of functional receptor-ligand interactions, surface staining was performed post-fixation. To analyse the effect of blocking antibody on actin response frequency and NK cell activation, conjugation was performed in the presence of anti-CD94 (clone #131412, bio-techne) antibody. For intracellular staining, fixed samples were permeabilized with 0.05v/v% Triton X-100 for 10 min at room temperature. Samples were then washed, stained for granzyme B (clone QA16AO2) for 30 min at 4°C in MACS buffer, washed twice and resuspended in 50 µl PBS.

Assessment of apoptosis markers has been described before [CLL paper ref]. In short, cell conjugates were allowed to form for 30 min before centrifugation and staining with Zombie Red ($1.5µl/10^6$ cells in PBS) for 10 min at 37°C. In a subsequent step, conjugates were stained for AnnexinV in AnnexinV Binding buffer (cat. # 422201, Biolegend) for 10min at RT, before fixation with 2%v/v PFA in AnnexinV Binding buffer.

All samples were acquired on an ImageStream®X Mark II (Luminex) equipped with four built in lasers (405 nm, 488 nm, 561 nm, 642 nm) and the INSPIRE® software (Luminex), and analysed using IDEAS™ software (Luminex), as described previously[147,474].

Cytotoxicity assay

$5x10^4$ target cells were incubated for 4 hours with effector NK cells at different effector/target (E:T) ratios in 96-well U-bottom plates. Prior to acquisition on a CytoFLEX (Beckman Coulter) flow cytometer, samples were stained with 0.05 µM TO-PRO-3 iodide (cat. # T3605, ThermoFisher Scientific). Collected data was analysed using FlowJo software (V10.6.2, Tree Star Inc.) Percentage of specific lysis was calculated for each well as follows:

% specific lysis = [Expected E:T Ratio*($GFP^+/ToPro\text{-}3^+_{experimental\ conditions}$ – $GFP^+/ToPro\text{-}3^+_{target\ alone}$)] / Measured E:T ratio

Confocal microscopy

Granule polarization To evaluate lytic granule polarization to the IS, MDA-MB-231 EmLA target cells were incubated with pNK for 30 min in suspension. Conjugates were carefully transferred to ply-L-lysine coated Ibidi chambers and allowed to attach for 10 min. Conjugates were fixed and permeabilized and stained for granzyme B.

Live cell imaging For live cell imaging of granule polarization, primary human NK cells were stained with 500 nM Lysotracker Red DND-99 in serum-free RPMI-1640 for 30 min at 37°C. After washing, effector immune cells were conjugated with cancer target cells at 37°C and 5% CO_2 and imaged using a confocal laser scanning microscope (LSM880 Airyscan, Carl Zeiss).

Mice and *in vivo* procedures

Mice WT BALB/cJ and C57BL/6J mice for primary NK cell isolation were bred in house. 8–12-week-old female BALB/c and C57BL/6 mice for *in vivo* tumour growth experiments were purchased from the Charles River France. All mice were housed under SPF conditions. Experiments involving laboratory animals were performed under protocols approved by the Animal Welfare Structure Luxembourg and the Ministry.

Tumour experiments For subcutaneous (s.c.) B16-F10 WT and CDC42 mutated tumour implantation, $3x10^5$ tumour cells were resuspended in 50 µl RPMI without FBS were injected into the right flank of syngeneic C57BL/6J mice. For orthotopic injection in the mammary fat pad, breast cancer cells were resuspended in 50µl of a 1:1 mix of PBS and Matrigel. Parental 4T1 breast cancer cells and derived Cdc42 mutant daughter cell lines were injected at a cell density of $5x10^4$. Tumour size was measured in three dimensions by calliper and is expressed as the product of three perpendicular diameters.

Tumours were excised from sacrificed mice, cut into pieces, and dissociated using the Tumour Dissociation Kit and a gentleMACS dissociator (Miltenyi Biotec) according to the manufacturer's recommendations. Tumour cell suspensions were stained with antibodies against surface molecules in PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA (MACS Buffer) for 30 min at 4°C. Specific antibodies against CD45, CD3, CD8, CD4, DX5, PD-1, Tim-3, LAG-3, IFN-γ, TNF-α, Granzyme B, and Zombie Aqua are purchased from Biolegend. Clones are listed in Sup. Table 1. Samples were subjected to flow cytometry analysis with a FACSAria™ III (BD). Data were analysed with FlowJo software (V10, Tree Star Inc.).

Statistical analysis

The results are expressed as mean ± SEM and percentages. GraphPad Prism software was sued for statistical analysis. Statistical significance ($P < 0.05$) was determined using a repeated measures two-way ANOVA, and a p-value ≤0.05 was considered statistically significant, where *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. To compare the effects of different AR rates on tumour volume, we used repeated measures two-way analysis of variance and Tukey's multiple comparisons test with individual variances computed for each comparison.

Results

The actin response is a conserved tumour cell intrinsic mechanism that correlates with immune escape

We analysed human tumour cell lines for their ability to produce an actin response (AR) when in contact with a cytotoxic NK cell. These target cell lines represent 6 types of malignancies, including skin cancer, non-small cell lung cancer, ovarian cancer, breast cancer, glioblastoma, and myelogenous leukaemia (Fig. 3.1A). In all cancer cell lines we observed AR-competent cells. We found that while all tumour cell lines were able to mount an AR at the IS, there was considerable intertumoural heterogeneity with regards to the frequency of AR^+ cancer cells. In agreement with our previous data on breast cancer cell lines[161] there was a direct correlation between cancer cell susceptibility to NK cell-mediated lysis and the AR frequency, supporting the hypothesis that the AR is involved in immune evasion from NK cell cytotoxicity. To further characterize the architecture and composition of the AR, we performed high-resolution fluorescent microscopy and examined the distribution of microtubules and vimentin filaments relative to F-actin. Interestingly, neither

microtubules nor intermediate filaments polarize to the IS, making the actin cytoskeleton the sole main, possibly unique, contributor to the AR. The AR proved to consist primarily of a dense array of actin-rich membrane protrusions projecting into the synaptic cleft toward conjugated NK cells (Fig. 3.1B).

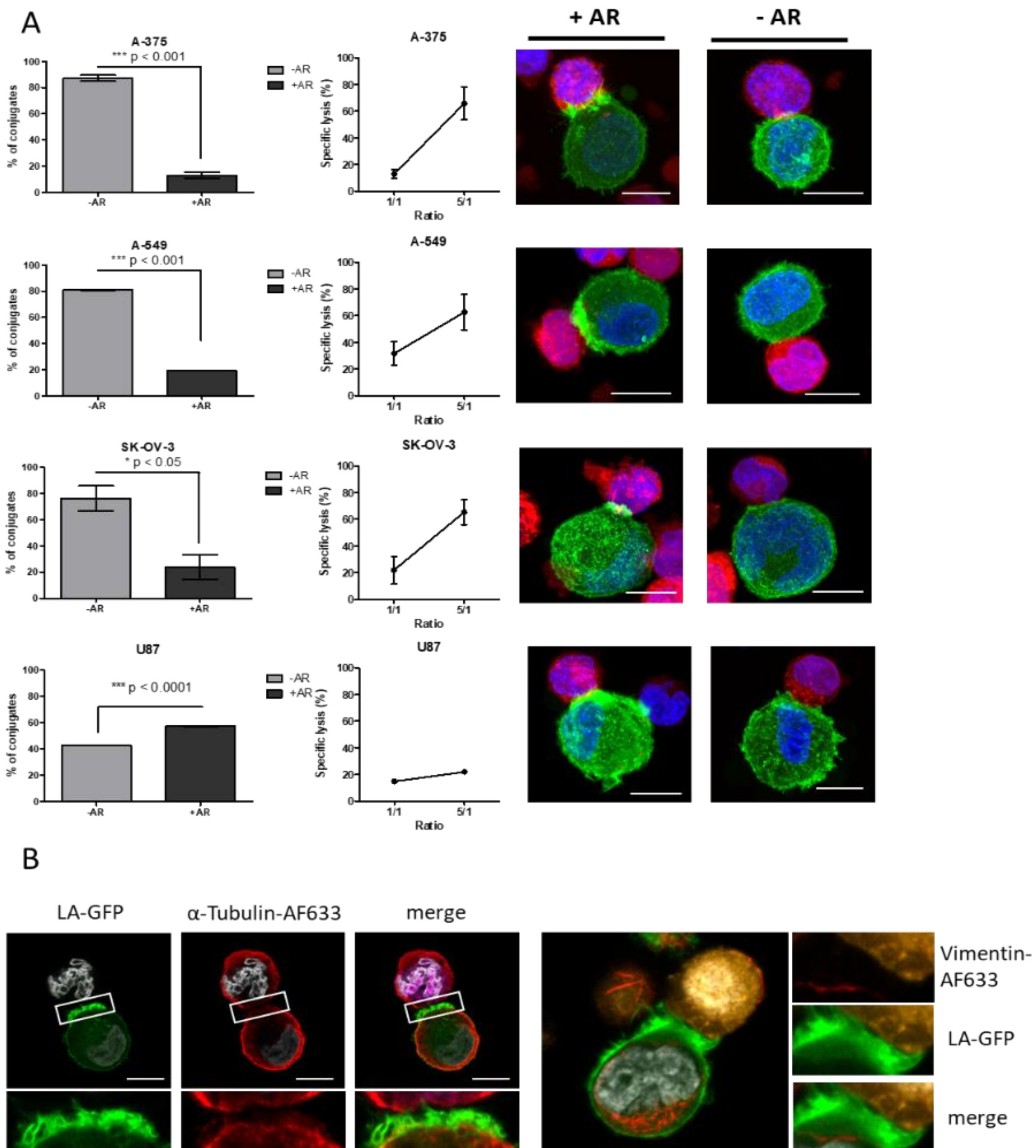


Figure 3.1 The actin response is a highly conserved mechanism of human cancer cells. (A) Human cancer cell lines were transduced to express the actin marker Emerald-Lifeact and their conjugates with NK-92MI cells analyzed for the absence or presence of the actin response by Imagestream and confocal microscopy. (B) High resolution imaging of the actin response indicates the presence of prominent actin-rich membrane protrusions while no significant involvement of the other compartments components of the cell's cytoskeleton, such as microtubules (left) or intermediate filaments (right).

Correlative light electron microscopy (CLEM) was used to further evaluate the nature of the above-described synaptic protrusions and the morphology of the synaptic cleft in the presence and absence of an AR (Fig. 3.2A). While we did not perform quantitative measurements of the synaptic cleft width, the distance between an AR-competent target cell and NK-92MI cells was obviously

much wider compared to the synaptic cleft between non-AR target cells and effector cells (Fig. 3.2B). Electron microscopy revealed that the membrane protrusions originating from target cells are filled with closely packed parallel arrays of long actin filaments and closely resemble filopodia at the leading edge of migrating cells. We accordingly termed them synaptic filopodium-like protrusions or SFPs (Fig. 3.2C). Importantly, these SFPs crossed the entire synaptic cleft and some of them bent against the lymphocyte membrane. In addition, high-resolution fluorescence microscopy revealed that SFPs are heavily decorated with programmed death-ligand 1 (PD-L1) also known as CD274 or B7-H1 (Fig. 3.2D). The wider synaptic cleft and projection of inhibitory ligand presentation beyond the target cell membrane suggest improper IS formation and function. As a result of the increased distance between the target cell and NK cell membrane and consequently limited interaction of activating receptor/ligand pairs, NK cell activation can be stalled and immune escape of AR-competent cancer cells from NK cells facilitated.

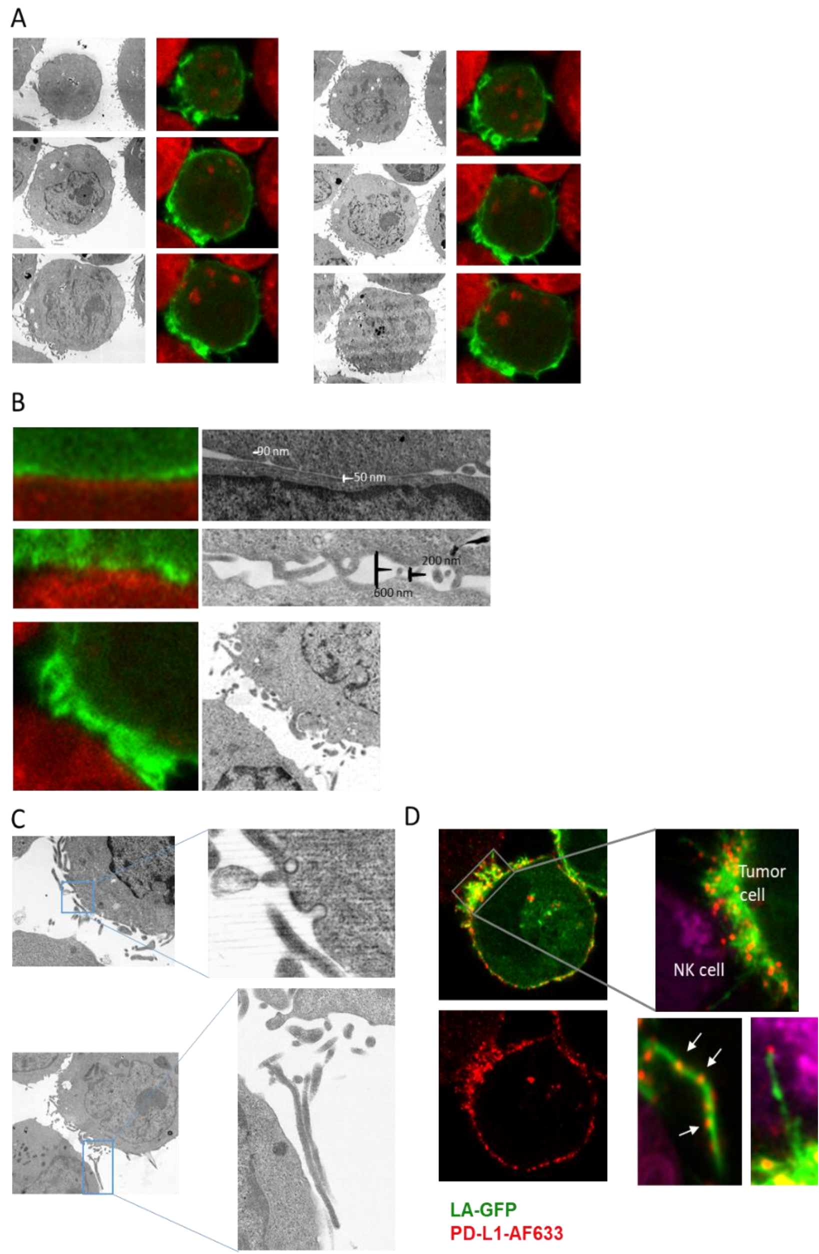


Figure 3.2 Correlative electron microscopy and confocal microscopy identify synaptic filopodia-like protrusions at the IS. (A-B) Correlation of confocal microscopy slides and the corresponding plane in electron microscopy allow of conjugates of MDA-MB-231 (green) and NK-92MI (red) with and without an AR. The synaptic cleft in cells without an AR covers a range range of about 50 nm, while at the AR-IS the synaptic cleft is 200-600 nm wide. Lysosomes identfied on the cancer cell (green) side of the IS were used for size comparison (90nm diameter). (C) At the AR-IS endo- or exocytotic processes can be observed, as well as the individual SFPs that form the AR. (D) High resolution confocal microscopy of PD-L1 (red) positioning on SFPs at the AR-IS with NK-92MI (purple). SFPs are decorated with PD-L1 that probe the cell surface of NK cells.

Receptor-independent polarization of inhibitory ligands is driven by the actin response and associated with a reduction in target cell death

To test our hypothesis, that the AR was associated with a lack of NK cell activation, we investigated activation of NK cells engaged in an AR-IS. Indeed, primary healthy donor NK cells (pNK) showed almost no polarization of lytic granules to the AR-IS (Fig. 3.3A), indicative of inhibition of NK cell activation and no commitment to target cell lysis. Quantitative analysis revealed that a majority (80 ± 9 %) of pNK in contact with tumour cells without an AR polarized the lytic machinery to the site

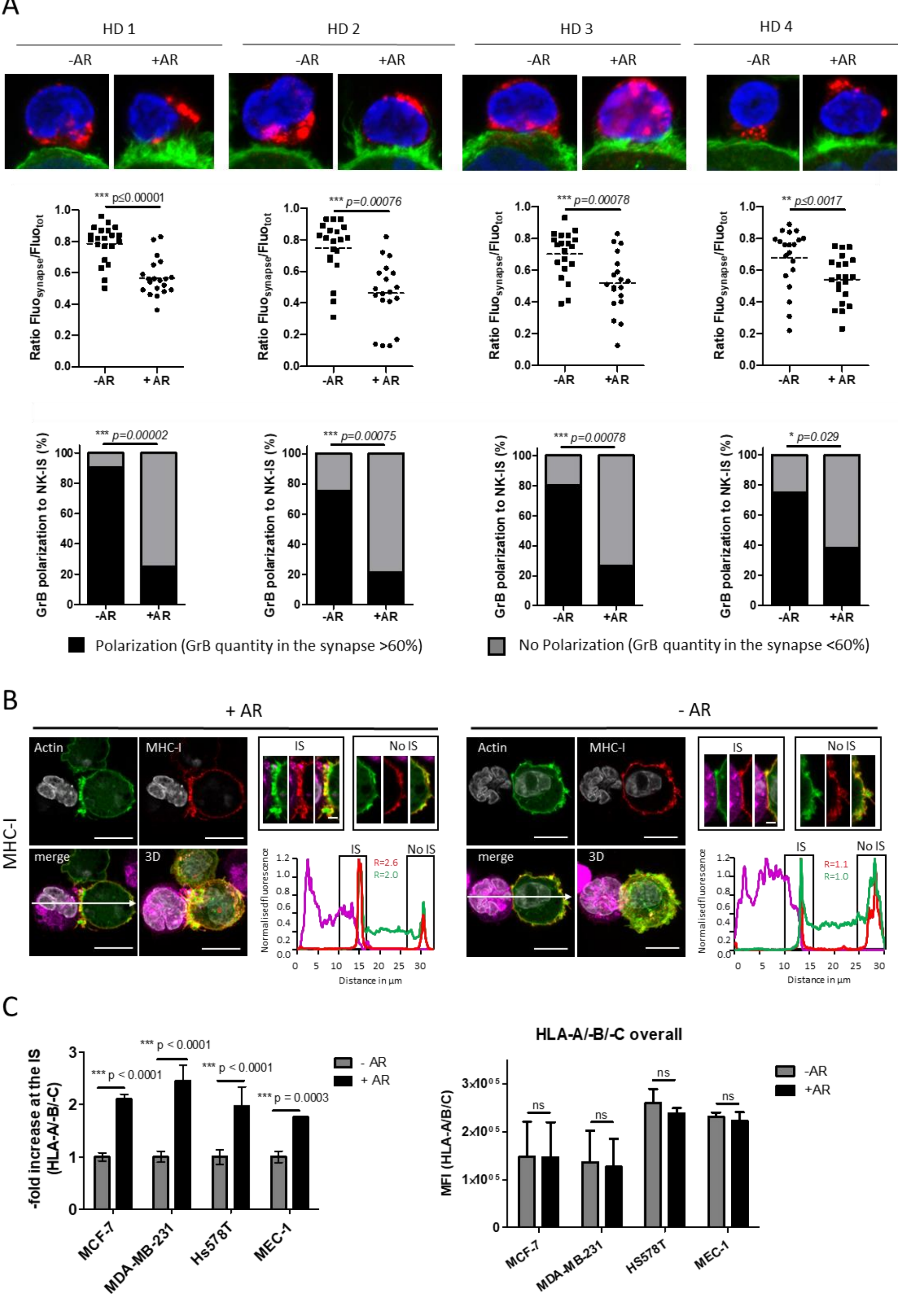


Figure 3.3 The actin response inhibits pNK cell activation and polarized MHC-I at the synapse of conjugated NK-92MI cells. (A) Peripheral pNK from healthy donor were purified by negative selection and cultured with 100U/ml human recombinant IL-2 for activation for 16 h. The pNK were allowed to conjugate with MDA-MB-231 (green) and stained for granzyme B (red). Lytic granule polarization was assumed when granzyme B staining was deteced in the 40% of the pNK cell area facing the target cell. (B) NK-92MI (purple) and MDA-MB-231 (green) cells were allowed to form conjugates and stained for HLA-A/B/C post-fixation (red). In the presence of an AR, HLA-A/B/C accumulates at the IS while it shows homogenous distribution in the absence of an AR. (C) Quantitative analyse of breast cancer cell-NK-92MI conjugates by Imagestream shows a 2- to 2.5-fold increase of HLA-A/B/C at the AR-IS. Overall expression of HLA-A/B/C is unchanged between conjugates with and without an AR, but cell lines show differences in mean fluorescence intensity.

of the cell-cell contact, while only a minor fraction (20 ± 17 %) of pNK in an AR-IS showed granule polarization (Fig. 3.3A). The correlation of the AR frequency, susceptibility to *in vitro* cytotoxicity assays, and lack of polarization of pNK pointed toward an early disruption of activating signalling in NK cells in contact with AR-competent cancer cells and disruption of lytic IS maintenance.

We reasoned that since the AR interfered with IS formation and lytic granule polarization that ligand positioning on target cells could affect NK cell activation. To this end, we investigated the distribution of major inhibitory and activating ligands with regards to the NK-IS. High resolution confocal microscopy revealed a polarization of inhibitory MHC-I to the site of tumour cell-NK cell contact in the AR-IS, with an almost 3-fold increase of ligand presentation at the IS, while AR^{-} conjugates showed a homogenous distribution of MHC-I on target cells (Fig. 3.3B). High-throughput analysis of cancer cell lines using multispectral imaging flow cytometry (Imagestream) confirmed this observation (Fig. 3.3C). Since the NK cell line used for these experiments lacks KIRs, we could rule out receptor-induced clustering of MHC-I to the IS. Using pNK, we found a similar induction of the AR and associated MHC-I clustering to the IS (Fig. 3.4A). These experiments further allowed us to investigate the functional impact of MHC-I polarization on NK cell capacity to induce target cell death. Clustering of MHC-I at the IS was associated with a significant decrease of conjugated target cells showing signs of target cell death in individual conjugates, underlining the implication of the AR as an immune evasion strategy during NK cell attack (Fig. 3.4B).

To test the capacity of the AR to polarize other transmembrane proteins, we analysed the recruitment of the inhibitory ligand PD-L1 (Fig, 3.4C and D), but also activating ligands (MIC-A/-B) to the IS (Fig. 3.4E). We observed that the AR was predominantly associated with a polarization of PD-L1, but not MIC-A/B. Moreover, pNK also induced polarization of PD-L1 to the IS in a receptor-independent manner. Similar observations have been made before in dendritic cell (DC)-NK cell contacts, that reported selective actin cytoskeleton-dependent stabilization of MHC-I at the regulatory IS that was linked to suppression of NK cell cytotoxic functions[128;146;510].

To determine whether the AR could also facilitate immune escape through other mechanisms, we used the human myelogenous leukaemia cell line K-562, that has an intrinsically low AR frequency and is characterized as an MHC-I^{null}/PD-L1^{null} cancer cell line expressing high levels of the activating ligands ULBP2/5/6, MIC-A/-B, and ICAM-1[654]. Transduction of this cell line with the constitutively active CDC42 mutant CDC42(Q61L) resulted in an increased AR frequency compared to wild-type control from 10% to almost 80% (Fig. 3.4F, left). Interestingly, no changes in *in vitro* cytotoxicity assays were observed between the cell lines (Fig. 3.4F, right). Despite the previous observation, that the AR is associated with a wider synaptic cleft, we hypothesis that a sufficiently strong activating signal can overcome the steric hindrance of the AR and SFPs. Moreover, as previously described NK-92MI cells lack KIRs, making them indifferent to AR-dependent inhibitory ligand polarization. These results indicate that the AR serves as a scaffold for polarization of inhibitory ligands, increasing the inhibitory effect of these ligands by clustering them at the AR-IS and like this

facilitating immune escape during NK cell attack, in addition to a potential role in generating a steric hindrance during IS formation.

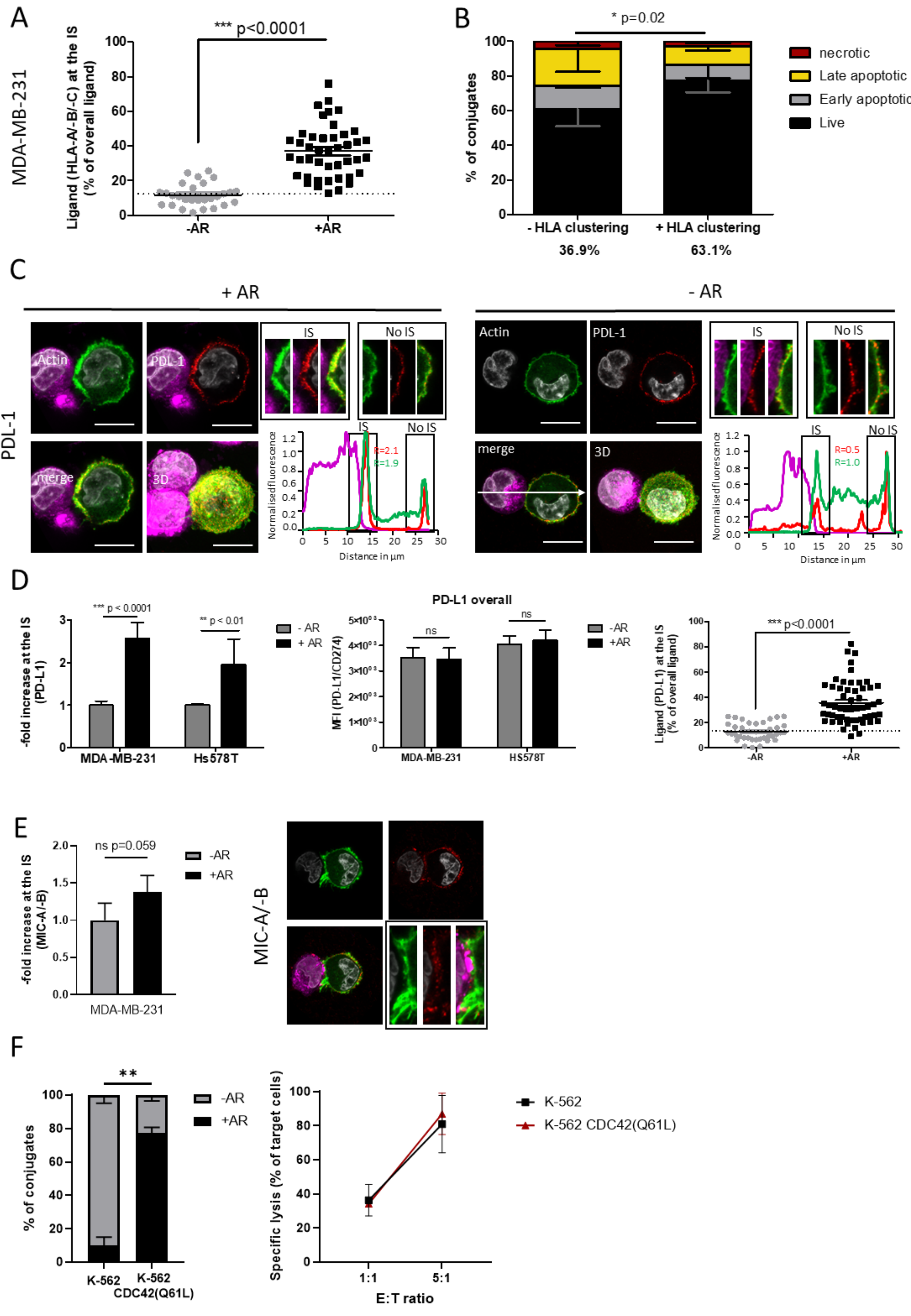


Figure 3.4 PD-L1 concentrated at the AR-IS of conjugates between breast cancer cells and NK-92MI cells. (A) Peripheral pNK were used to confirm MHC-I accumulation on MDA-MB-231 at the AR-IS with receptor-expressing effector cells. Target and effector cells were allowed to form conjugates for 40 min and ligand staining was performed post-fixation. (B) Evaluation of functional consequence of MHC-I clustering on execution of NK cell effector functions. Following conjugation, samples were stained for Annexin V and Zombie Red and target cells identified as live (Annexin V^-/Zombie Red^-), early apoptotic (Annexin V^+/Zombie Red^-), late apoptotic (Annexin V^+/Zombie Red^+), or necrotic (Annexin V^-/Zombie Red^+). Accumulation of MHC-I was associated with an increase in target cell survival. (C) Confocal microscopy analysis of PD-L1 (red) positioning during IS formation with and without an AR on MDA-MB-231 Emerald-Lifeact (green) cells inc ontact with NK-92MI (purple). The AR was associated with a 2.1-fold increase of PD-L1 at the IS. (D) Quantitative analysis of PD-L1 recruitment to the IS in breast cancer cells in conjugation with NK-92MI cells (right). Overall expression of PD-L1 on target cells was similar between target cells with and without an AR (middle) and PD-L1 clustering at the AR-IS could be confirmed with pNK effector cells (right). (E) Quantitative analysis of MIC-A/-B recruitment to the IS. The formation of an AR-IS was associated with a non-significant increase of MIC-A/-B clustering at the IS (left). Using confocal microscopy, only a weak clustering of MIC-A/-B (red) could be observed in conjugates with an AR-IS. (F) Quantification of the AR frequency in wild-type K-562 and K-562-CDC42(Q61L) target cells with NK-92MI cells by Imagestream (left). In a 4 h *in vitro* cytotoxicity assay using NK-92MI effector cells, NK cell-specific lysis of target cells was evaluated as percentage of GFP+/ToPro-3+ cells. The increase in AR in K-562-CDC42(Q61L) target cells did not alter their susceptibility to NK cell lysis.

Exposure to NK cells positively selects for resistant cancer clones

To test the hypothesis of selection for actin response-competent clones by NK cells, we exposed susceptible MCF-7 cells to NK-92MI cells in a 4-hour cytotoxicity assay. Surviving target cells were sorted by FACS and kept in culture. Analysis of susceptibility in cytotoxicity assays and evaluation of the rate of the AR in the pre-challenged MCF-7 cell line showed a drastic increase in resistance against NK-92MI cells and an almost 3-fold increase in conjugates showing an AR (Fig. 3.5A). Over time, pre-challenged MCF-7 cells adopted their susceptible phenotype again, suggesting that the AR does not provide a selection advantage during normal cell culture (Fig. 3.5B). Interestingly, pre-challenged MCF-7 cells reached the same susceptibility against NK-92MI as the parental MCF-7 cell line, indicating that the size of the AR-competent subpopulation is constant.

In order to identify possible markers of the AR, we submitted samples of mock-challenged and pre-challenged MCF-7 cells after co-culture with NK-92MI cells to single cell RNA sequencing (scRNA-Seq). The analysis revealed two distinct population clusters and differential expression between these clusters indicated upregulation of HLA-B and S100P in pre-challenged cells (Supp. Fig. 3.1). Upregulation of MHC-I in response to IFN-γ release by NK cells is an expected result that has been previously described. However, the calcium sensor S100P has been demonstrated to play a role in Ca^{2+}-dependent activation of the ERM protein ezrin, a membrane/F-actin cross-linker. Ligation of ICAM-1 to LFA-1 has been described to be one of the first steps during IS formation and has furthermore been associated with increased intracellular Ca^{2+} signalling[655].

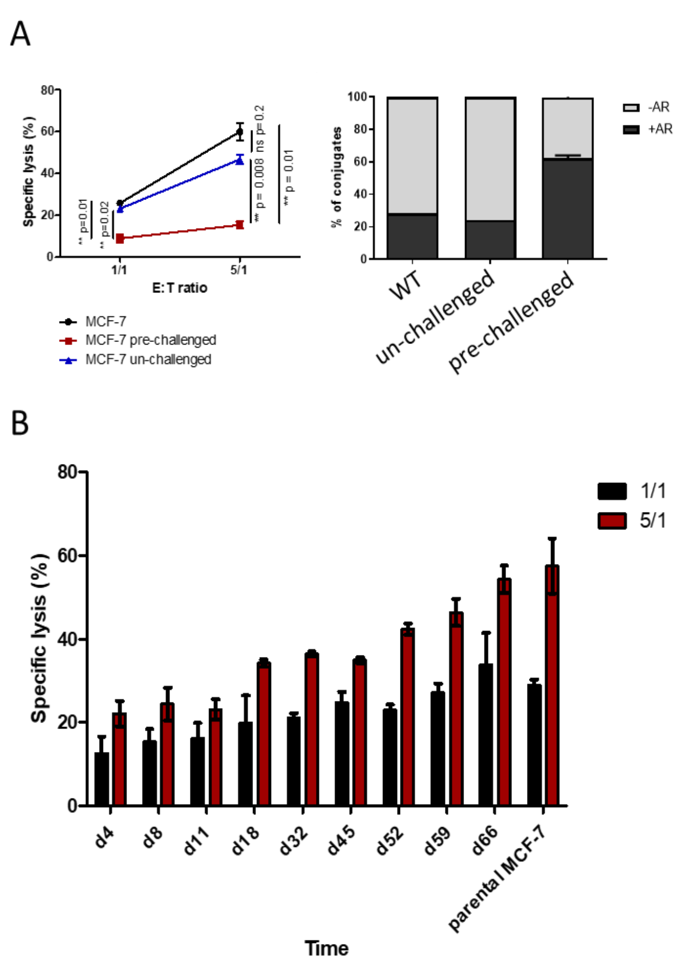


Figure 3.5 NK cells positively select for AR-compenent tumour cells *in vitro*. (A) Cytotoxicity assay with parental MCF-7, mock-challenged MCF-7, and pre-challenged MCF-7 target cells and NK-92MI effector cells (left). Pre-challenged target cells were sorted 48 hrs before the experiment as surviving cells from a 4 h killing assay and kept in culture. Evaluation of conjugates of the same target cell populations by Imagestream with NK-92MI target cell revealed an increased AR rate in pre-challenged MCF-7 (right). (B) Long-term culture of pre-challenged MCF-7 cells and repeated 4 h cytotoxicity assays with NK-92MI cells suggest gradual adaption of the original susceptible phenotype.

Identification of actin linkers associated with target ligand polarization

We used a proximity-based biotinylation assay to determine potential linkers between the AR and inhibitory ligand recruitment to the NK-IS. Biotinylated proteins enriched in target cells expressing PD-L1-BioID after contact with NK-92MI cells were analysed for their role in actin cytoskeleton regulation. Expression and function of the biotin-ligase fusion protein were confirmed prior to experiments and affinity purification of biotinylated proteins (Supp. Fig. 3.2).

Potential candidates include the actin-binding and crosslinking protein LCP1, Coronin-1A, myosin heavy chain-9, and the FERM domain-containing protein moesin (Fig. 3.6A). Because moesin is involved in the outside-in signalling of integrins and ICAM-1 and translation of this signalling into actin cytoskeleton remodelling, we examined the localization of this protein in target cells during IS formation with NK cells. Using high-resolution confocal microscopy, we could observe a strong accumulation of moesin, but also ezrin to the target site of the IS correlating with the AR (Fig. 7B, left). Signalling of both, moesin and ezrin is closely related to activation of small RhoGTPases and their downstream effectors and overexpression has been described for triple-negative and ER-positive breast cancer and been associated with cancer progression[656–659] (Fig. 3.6B, right). Recruitment of these linker proteins to the site of the AR-IS suggests their involvement in initiation or maintenance of the AR during cell-cell contact. Furthermore, activation of these pathways has been associated with EMT and cancer metastasis[660–662], that are both processes that modify actin cytoskeleton remodelling dynamics.

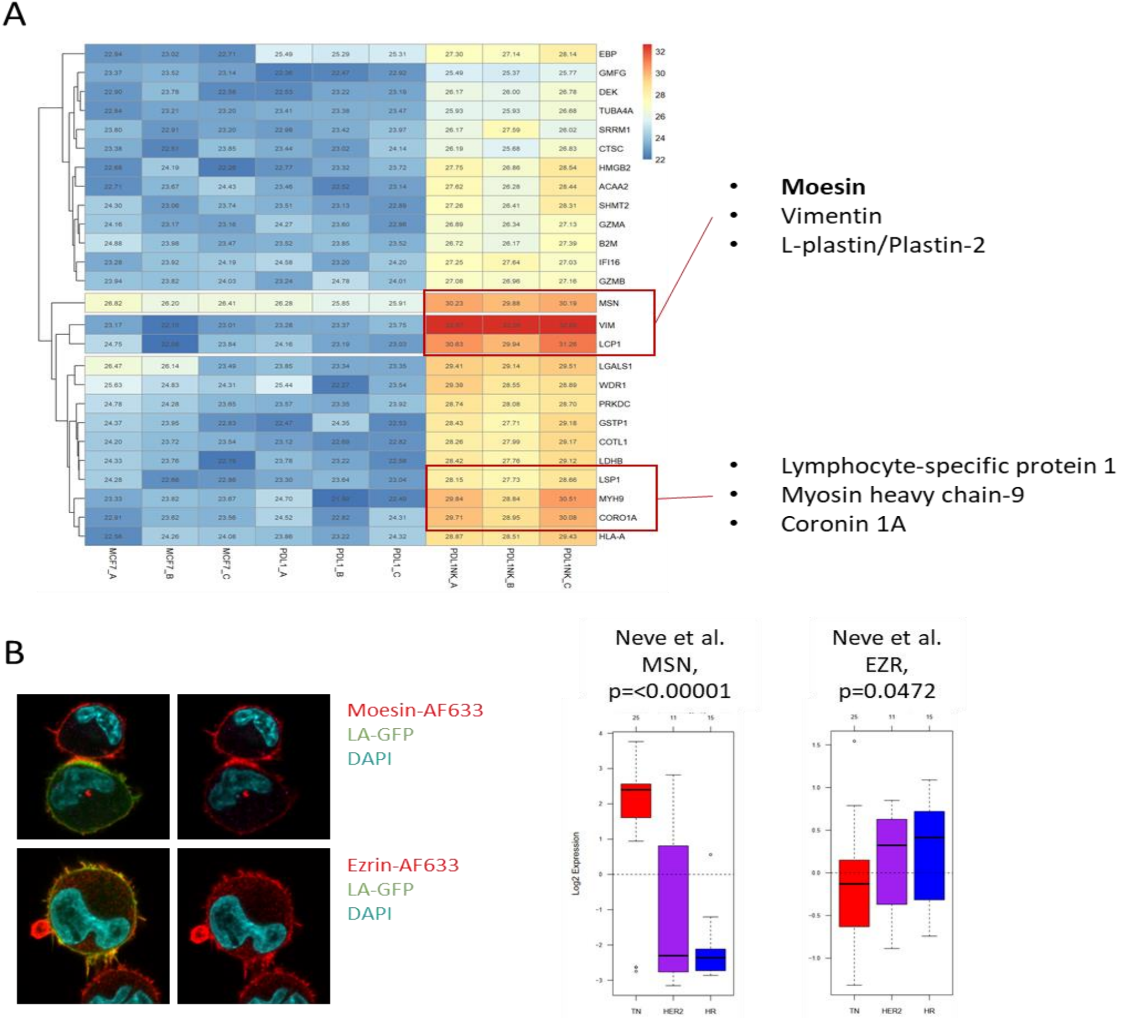
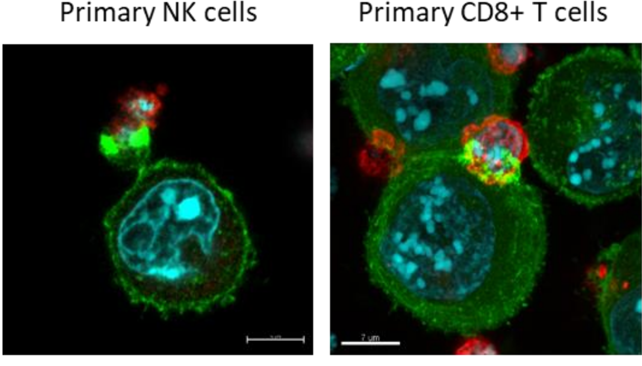


Figure 3.6 Proximity-based biotinylation assay identifies actin-linkers as interaction partners of PD-L1 during IS formation with NK-92MI cells. (A) MCF-7 cells expressing PD-L1-BioID2 or control cells were incubated for 24 h with an excess of 50 µM biotin. Target cells were exposed to NK-92MI cells for 1 h to induce IS formation, before effector cells and dead target cells were washed out and samples lysed. Mass spectrometry analysis of isolated biotinylated proteins identified several actin cytoskeleton-related proteins enriched in cells after contact with NK cells. (B) Confocal microscopy staining of moesin (red, top) and ezrin (red, bottom)) in MDA-MB-231 (green) cells in conjugation with NK-92MI cells. The AR-IS shows enrichment of both proteins on the target cell side of the IS (left). Dysregulated expression of moesin (MSN) and ezrin (EZR) are commenly observed in different types of breast cancer (right).

Manipulation of the actin response and its impact on tumour growth in vivo and TIL activation

Using a B16F10 cell line expressing the lymphocytic choriomeningitis virus (LCMV) glycoprotein GP33 (B16F10^{gp33}) and activated antigen specific CD8^{+} T cells from P14 transgenic mice, we could further confirm that the AR could be induced by CTLs with the same frequency as with primary NK cells (Fig. 3.7). This suggests that the AR could be an immune evasion mechanism from both, NK cells and CTLs.



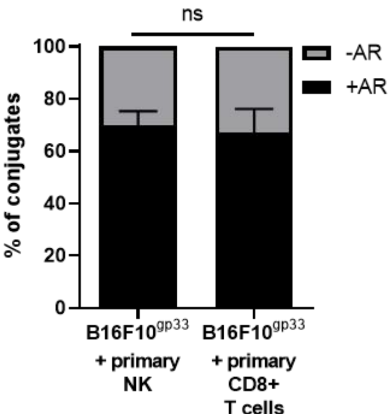


Figure 3.7 Induction of the AR by antigen-specific CTLs in contact with melanoma cells. Confocal microscopy analysis of conjugates between B16F10^{gp33} Emerald-Lifeact target cells (green) and primary C57BL/6-derived NK cells or P14-derived activated CD8+ T cells (red). CTLs were prepared using an adoptive transfer model, vaccination of host mice with LCMV, and subsequent negative selection of CD45.1 gp33-specific T cells from splenocytes. Quantitative analysis of B16F10^{gp33} target cell conjugates with primary NK and P14 CTLs showed similar rates of AR in conjugates with both types of effector cells.

To modify the actin remodelling capacity of cell lines, we transduced murine cancer cells to stably express mutated forms of the CDC42 protein. The cell lines were transduced with the constitutively active CDC42(Q61L) protein, expressing a mutation that prevents endogenous and GAP-stimulated GTPase activity of Cdc42, hence the protein is always in an active GTP-bound state. While expression of this mutant in 4T1 cancer cells increased the size of the AR-competent subpopulation to almost 60% compared to 20% in parental wild-type cells (Fig. 3.8A, left), B16F10-CDC42(Q61L) cells had the same high AR frequency as the parental cell line (Fig. 3.8B, left). The dominant negative CDC42(T17N) protein carries a mutation that prevents binding of GTP and reduces the proteins affinity for GDP resulting in a protein that is present in an inactive state either nucleotide-free or GDP-bound. This results in strong binding of CDC42 GEFs and reduction of endogenous Cdc42 activation through these GEFs. Expression of this protein did not affect AR rate in 4T1 epithelial mouse breast cancer cells (Fig. 3.8A, left), but resulted in a reduction of the AR in B16F10-CDC42(T17N) melanoma cells to 40% of conjugated target cells compared to wild-type B16F10 cells with an intrinsically large AR^{+} subpopulation of over 60% (Fig. 3.8B, left). *In vitro* cytotoxicity assays using primary NK cells from syngeneic mice indicated that a low AR frequency correlated with differences in susceptibility to NK cell lysis in both models. Resistance of 4T1-CDC42(Q61L) cells increased compared to wild-type 4T1 cells, and susceptibility of 4T1 cells to NK cell lysis was further enhanced in 4T1-CDC42(T17N) cells (Fig. 3.8A, middle), while B16F10-CDC42(T17N) cells were more susceptible than their parental wild-type cell line or B16F10-CDC42(Q61L) sibling cell line (Fig. 3.8B,

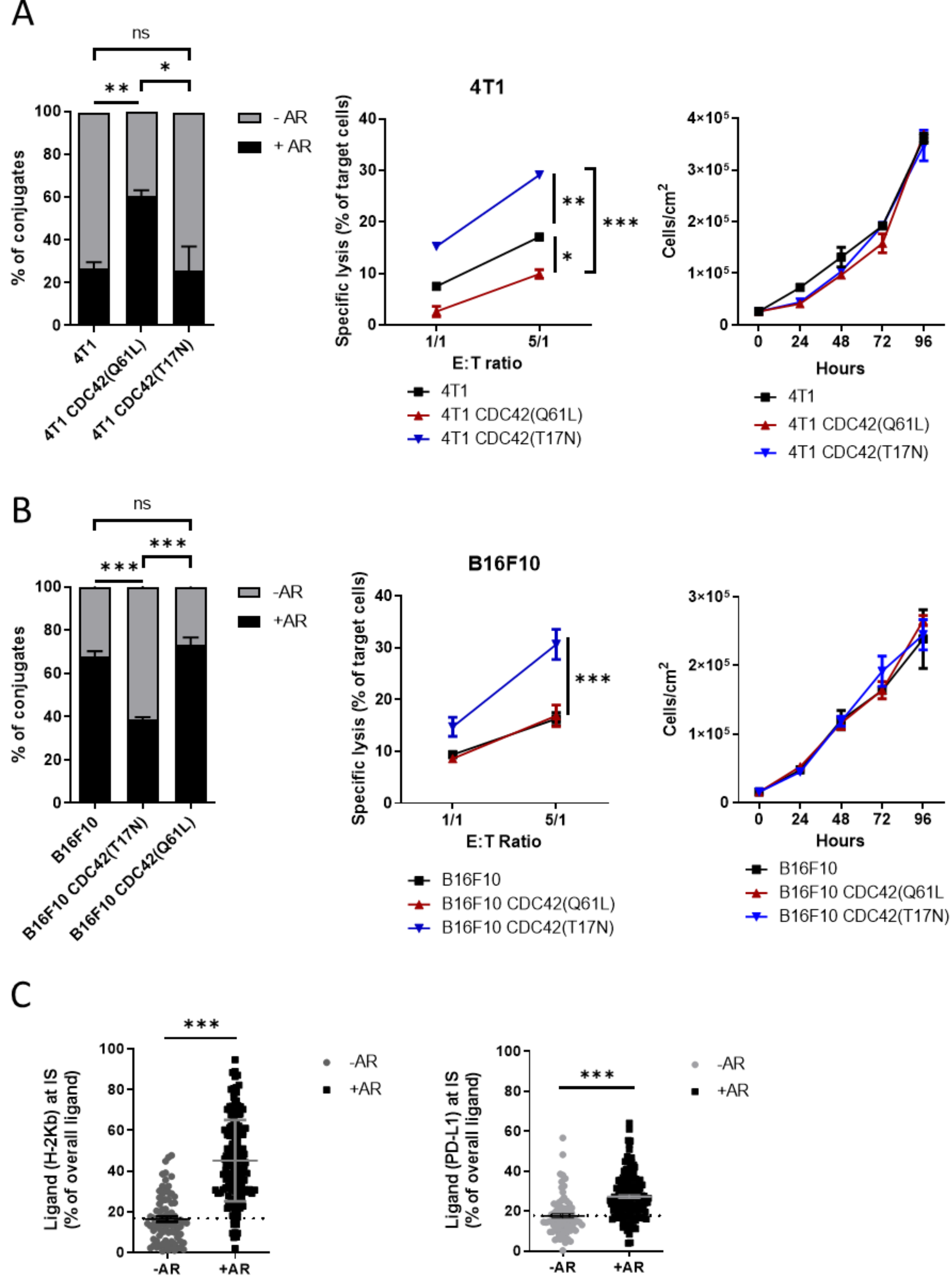


Figure 3.8 Generation of stable cell lines with altered AR frequency changes their susceptibility to NK cell cytotoxicity *ex vivo*. (A) and (B) 4T1 breast cancer cells or B16F10 melanoma cells transduced to express CDC42(T17N) or CDC42(Q61L) were conjugated with primary NK cells isolated from BALBc mice or C57BL/6 mice, respectively, for evaluation of their AR rate by Imagestream. Evaluation of target cell susceptibility to NK cell-mediated lysis in a 4 h cytotoxicity assay using ToPro-3 as cell death indicator. *In vitro* proliferation rate was evaluated by cell culture over 96 h and determination of cell count every 24 h. (C) Quantitative analysis of the murine MHC-I homologue H-2Kb (left) and murine PD-L1 (right) polarization on B16F10 cells in conjugation with primary NK cells from C57BL/6 mice.

middle). Because CDC42 is involved in cell cycle regulation, we tested *in vitro* growth of parental and CDC42 mutant daughter cell lines and could not find a significant difference in proliferation

between the three cell lines for 4T1 (Fig. 3.8A, right) and B16F10 (Fig. 3.8B, right). We could further confirm the polarization of inhibitory ligands to the IS between B16F10 and primary mouse NK cells for both, the murine MHC-I H-2Kb and PD-L1 (Fig, 3.8C, right and left, respectively), suggesting that AR-driven polarization of inhibitory ligands to the NK-IS is also a conserved mechanism.

In immune competent mice, implantation of parental and CDC42 mutant daughter cell lines resulted in significant changes of tumour growth. Growth of 4T1-CDC42(Q61L) tumours was significantly accelerated, while 4T1-CDC42(T17N) cells grew at rates similar to the wild-type cell

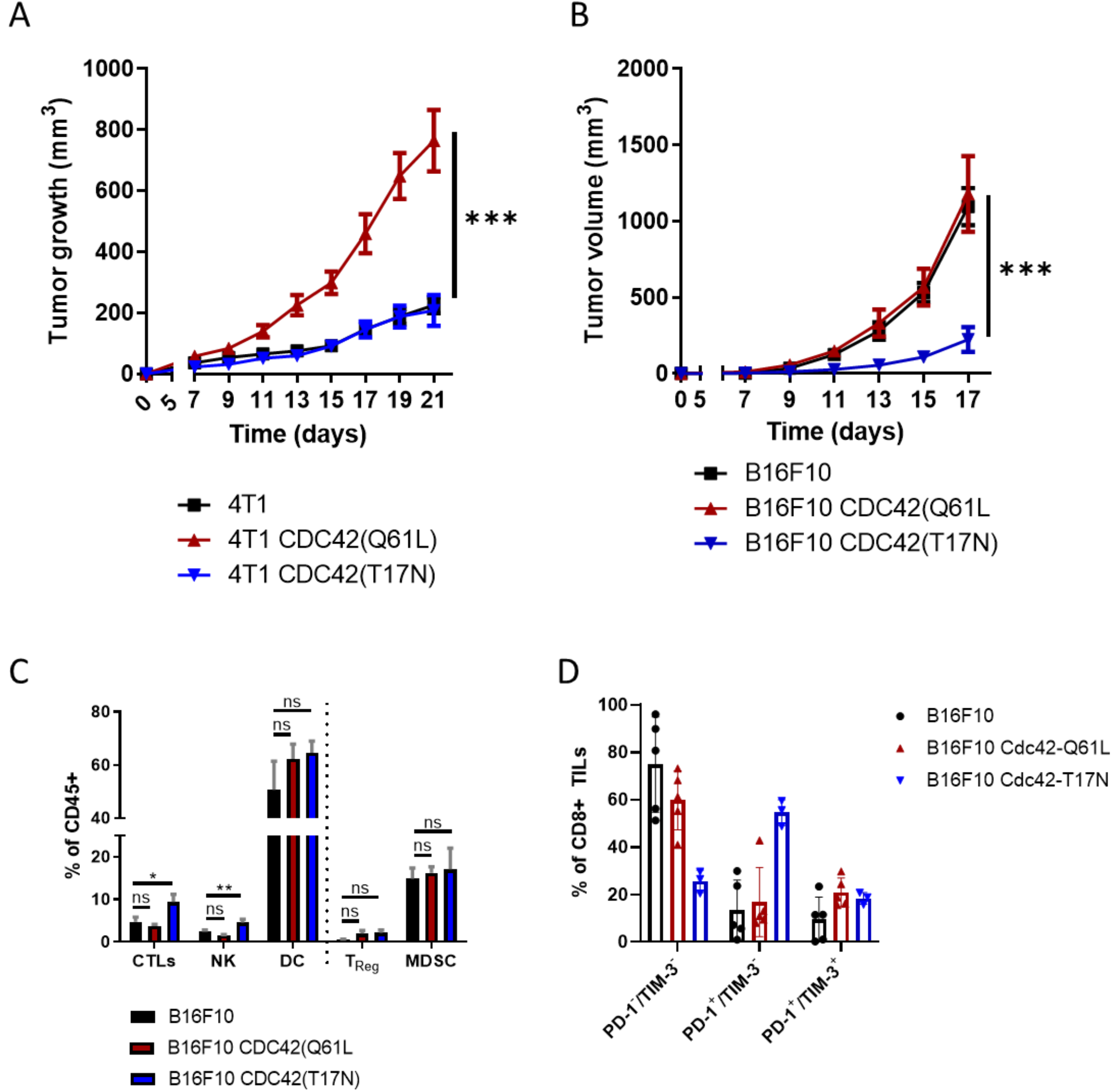


Figure 3.9 The AR impacts tumor growth in immunecompetent mice and is involved in immunosuppression of CTLs and NK cells. Implantation of $5x10^4$ 4T1 parental or CDC42 mutant tumor cell lines into the mammary fat pad of immune compentent BALBc results in palpable tumors 7 days post-injection. Tumor volume was measured in 3 dimensions every second day. Expression of CDC42(Q61L) accelerated tumor growth significantly, while 4T1-CDC42(T17N) tumors grew at similar rates as parental 4T1 cells. (B) Tumor growth of sub-cutanously injected B16F10 wild-type or CDC42 mutant cells in C57BL/6 mice. Stable expression of CDC42(T17N) was associated with a decrease in tumor volume. (C) Flow cytometry analysis of the tumor infiltrating immune populations in B16F10 tumors. Reduced tumor volume in B16F10-CDC42(T17N) tumors was linked to an increased infiltration of CTLs and NK cells. (D) Analysis of PD-1/Tim-3 expression on CTLs by flow cytometry in B16F10 tumors. Majority of CTLs in B16F10 wild-type and CDC42(Q61L) tumors were double negative, while the main population of CTLs isolated from B16F10-CDC42(T17N) tumors was PD-1^+/Tim-3^-.

line (Fig. 3.9A). In the mouse melanoma model, expression of CDC42(Q61L) did not impact tumour grow, but B16F10-CDC42(T17N)-derived tumours grew much slower than the parental cell line or the B16F10 CDC42(Q61L) cell line (Fig. 3.9B). To examine the potential role of the AR in shaping the tumour microenvironment, we analysed the tumour immune landscape of B16F10 tumours by flow cytometry. We observed an increased infiltration of cytotoxic T lymphocytes (CTLs) and NK cells in B16F10-CDC42(T17N) tumours compared to the other models, but no changes in the immunosuppressive populations of regulatory T cells (T_{Reg}) and myeloid-derived suppressor cells (MDSC) (Fig. 3.9C).

Co-expression of Tim-3 and PD-1 has been suggested to indicate CTL exhaustion and dysfunction in several models of murine cancer[663]. To characterize the impact of the AR on CTL and NK cell exhaustion, we examined the PD-1/Tim-3 expression pattern of these TILs. Within the NK cell population, we observed weak staining for both markers (data not shown), but in CTLs we could identify three main subpopulations (Fig. 3.9D). CTLs isolated from B16F10 CDC42(T17N) tumours showed almost equally distributed PD-1$^-$Tim-3$^-$, PD-1$^+$Tim-3$^-$, and PD-1$^+$Tim-3$^+$ populations, indicating an ongoing anti-tumour immune response, while the majority of CTLs from B16F10-WT or –CDC42(Q61L) tumours were double negative (DN) for both markers. Upregulation of PD-1 on CTLs is commonly observed upon activation and during priming by APCs, so the DN CTL population could consists of naive CD8+ T cells, but also central memory TILs (CD44^{hi}CD62L^{hi})[663]. PD-1$^+$Tim-3$^-$ CTLs have been described as good cytokine producer and the highest IFN-γ producing subpopulation, while PD-1$^+$Tim-3$^+$ TILs show severe dysfunction in cytokine production[511,663,664]. In the hierarchy of CTL exhaustion, PD-1$^+$Tim-3$^-$ CTLs have been suggested to represent a mixed population of effector TILs that upon sufficient activation stimulus can exert a strong anti-tumour response but also show early signs of exhaustion. PD-1$^+$Tim-3$^+$ TILs on the other hand represent a terminally exhausted and dysfunctional subpopulation that has been suggested to increase over time and to be unresponsive to mono-ICI therapy. This preliminary data is particularly exciting given that B16F10 is a poorly immunogenic tumour model and suggests that *in vivo* the role of the AR goes beyond tumour cell intrinsic resistance to cytotoxic lymphocyte-mediated lysis with a broader immunosuppressive effect on the tumour (immune) microenvironment.

Ongoing Analysis

Discussion

In this article, we have investigated the actin response as an immune evasion mechanism of cancer and found that the actin response at the immunological synapse (AR-IS) is characterized by a prominent polarization of predominantly inhibitory ligands to the cell-cell contact with NK cells. We further show that this recruitment of MHC-I and PD-L1 to the IS has protective function against NK cell-mediated cytotoxicity, whereas homogenous distribution of these ligands does not impair effector functions. SFPs at the AR-IS are heavily decorated with PD-L1, suggesting fast initiation of inhibitory signalling in PD-1$^+$ effector lymphocytes. Furthermore, we could show that the rate of the AR influences *in vivo* tumour progression and has an impact on CTL and NK cell infiltration and activation. Together, these findings support the hypothesis of the AR as a highly conserved immune evasion mechanism that promotes escape from both, NK cells and CTLs through clustering and stabilization of inhibitory MHC-I and PD-L1 at the IS, preventing effector lymphocyte infiltration and activation both *in vitro* and *in vivo*.

In a recent study, we have reported that patient-derived chronic lymphocytic leukaemia (CLL) cells promote their immune escape potential from NK cells by upregulation of the non-classical HLA-G and formation of an AR-IS[465]. While the inhibitory receptor/ligand interaction can be overcome

with blocking antibodies that help restore IS formation, the formation of an AR-IS was associated with lower levels of transferred granzyme B in target CLL cells and increased survival of AR-competent CLL cells. Here, we report that the AR drives polarization of inhibitory ligands to the IS between resistant cancer cells and NK cells and that this clustering is associated with an inhibition of NK cell effector functions.

By SFPs that are adorned with PD-L1 and that are extended beyond the plasma membrane of prospective target cells a strong inhibitory signal could be induced in PD-1$^+$ effector lymphocytes during the first step of IS formation. Interaction of PD-1/PD-L1 has been reported to not prevent IS formation altogether, but to result in a weak target-effector conjugate through interruption of activating signalling[665,666]. Recruitment of PD-1 to the TCR microclusters and accordingly to the IS has been reported to be dependent on availability of its ligands[667]. Engagement of PD-1 with its ligands results in tyrosine phosphorylation of the immune tyrosine inhibitory motif and immune tyrosine switch motif within its cytoplasmic tail and interference with TCR signalling, actin remodelling and formation and maturation of the lytic IS[665,668]. Furthermore, engagement of PD-1 with its ligands has been linked to inhibition of lytic granule polarization to the IS in PD-1$^+$ NK cells by blockade of outside-in signal of LFA-1[146]. In our model, SFPs could recruit and/or stabilize PD-1 to the IS during the early phase of cell-cell contact, restricting firm adhesion of effector lymphocytes and IS maturation through impairment of actin cytoskeletal dynamics that are initiated by activating receptors and adhesion molecules. Besides PD-L1, we also observed polarization of MHC-I to the AR-IS. While peptide-loaded MHC-I serve as ligands for the TCR, the classical HLA-A/-B/-C are also inhibitory ligands for KIRs on NK cells and involved in “self” recognition. A similar mechanism has been described for DC-NK cell conjugates during NK cell priming[160]. In this study, inhibition of actin cytoskeleton remodelling in DCs induced NK cell effector functions and DC lysis. Through polarization of MHC-I to the IS, cancer cells can suppress execution of NK cell effector functions, as demonstrated by a lack of lytic granule polarization, and increase their immune escape potential. The simultaneous recruitment of both, MHC-I and PD-L1, can inhibit IS formation with NK cells, but also CTLs (under current investigation) and convert the lytic IS into an evasion IS.

Because the AR appears as soon as a contact between a resistant target cell and NK cells is initiated, involvement of adhesion molecules in the formation of an AR-IS is likely. scRNA-Seq as well as proximity-biotinylation assays have suggested the involvement of proteins from the ezrin-radixin-moesin (ERM) family in initiation of the AR and the associated recruitment of PD-L1. ERM proteins serve as linkers between the membrane and the actin cytoskeleton and are involved in initiating actin cytoskeleton remodelling in response to transmembrane protein signalling and intracellular signalling pathways[669]. We observed recruitment of both moesin and ezrin to the AR-IS, that have been suggested to be involved in the translation of ICAM-1 and Ca^{2+} dependent signals into activation of Rho GTPases and actin remodelling. Overexpression of these proteins is commonly observed in cancer and often associated with EMT. An increased expression of ezrin has even been suggested to play a role in the transition of benign lesions into malignant breast cancer[670], a shift that requires effective immune evasion strategies.

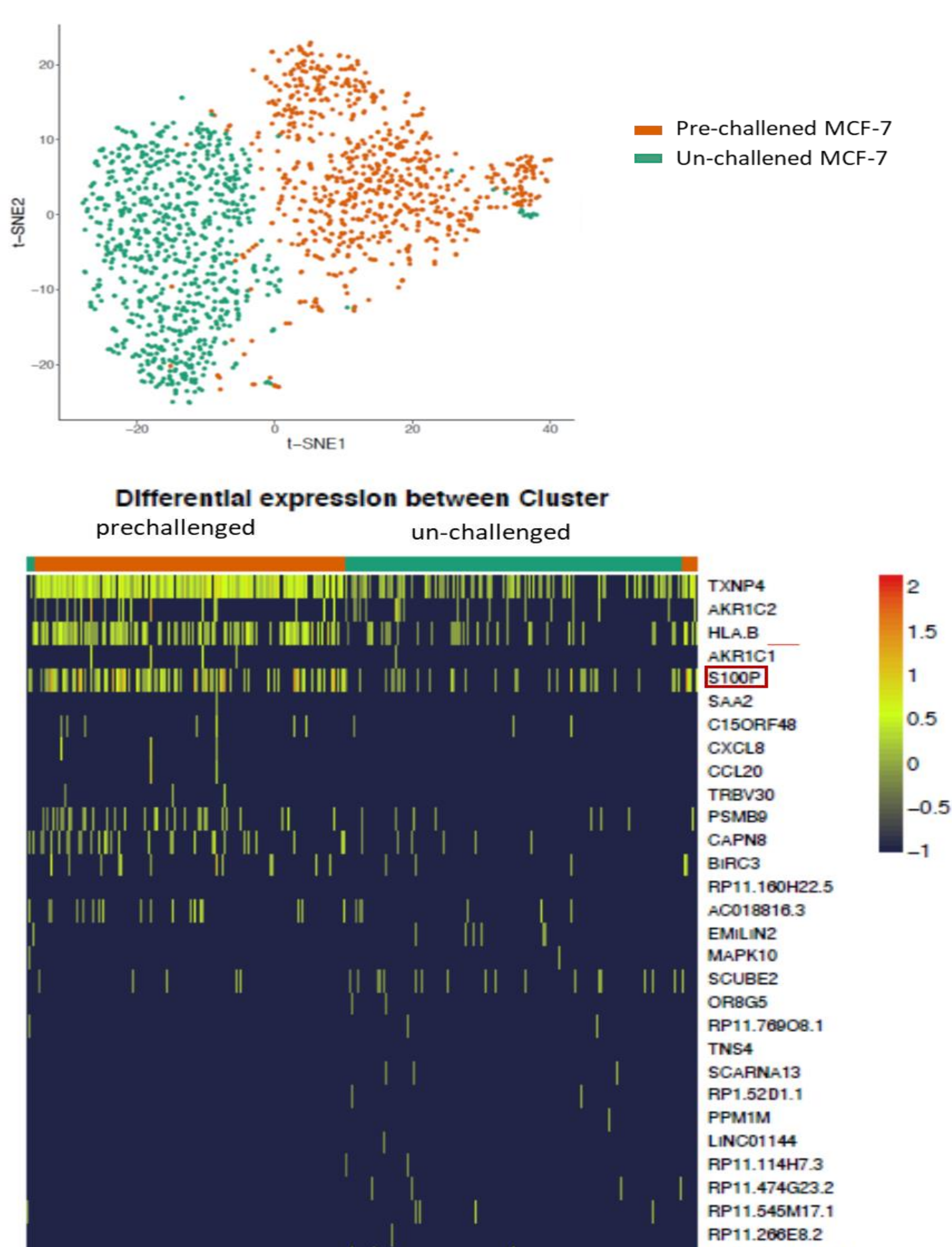
By changing the signal translation capacity of key actin cytoskeleton regulators, we could manipulate the actin response in cancer cell lines. Expression of either constitutively active or dominant negative CDC42 significantly changed the ability of breast cancer or melanoma cells to respond to NK cell attack with an AR. However, we also observed a residual resistant subpopulation, suggesting an alternative signalling pathway utilized by these cancer cell clones to initiate the AR. However, induction or blockade of the CDC42 pathway was associated with an increased or decreased resistance in *ex vivo* cytotoxicity assays, respectively. This suggests, that in the absence of AR-driven polarization of inhibitory ligands, sufficient activation signal enables the formation of

a lytic IS and execution of NK cell effector functions. Indeed, we observed in immune competent mice that are low AR frequency was associated with restoration of an anti-tumour immune response and conversion of a “cold” into a “hot” tumour. The strength of the anti-tumour immune response could be further increased through ICI, as the size of the PD-1$^+$Tim-3$^-$ CTL population was significantly increased in tumours with a small AR subpopulation compared to tumour with a large AR population. It should be noted however that the decrease of the AR rate did not result in complete tumour rejection suggesting that immunoediting could lead to selection of AR-competent tumour cell clones. Similar results from *in vitro* experiments have shown that NK cell attack selects for cancer cells that respond to NK cell binding with the formation of an AR-IS and suggest a similar mechanism *in vivo*.

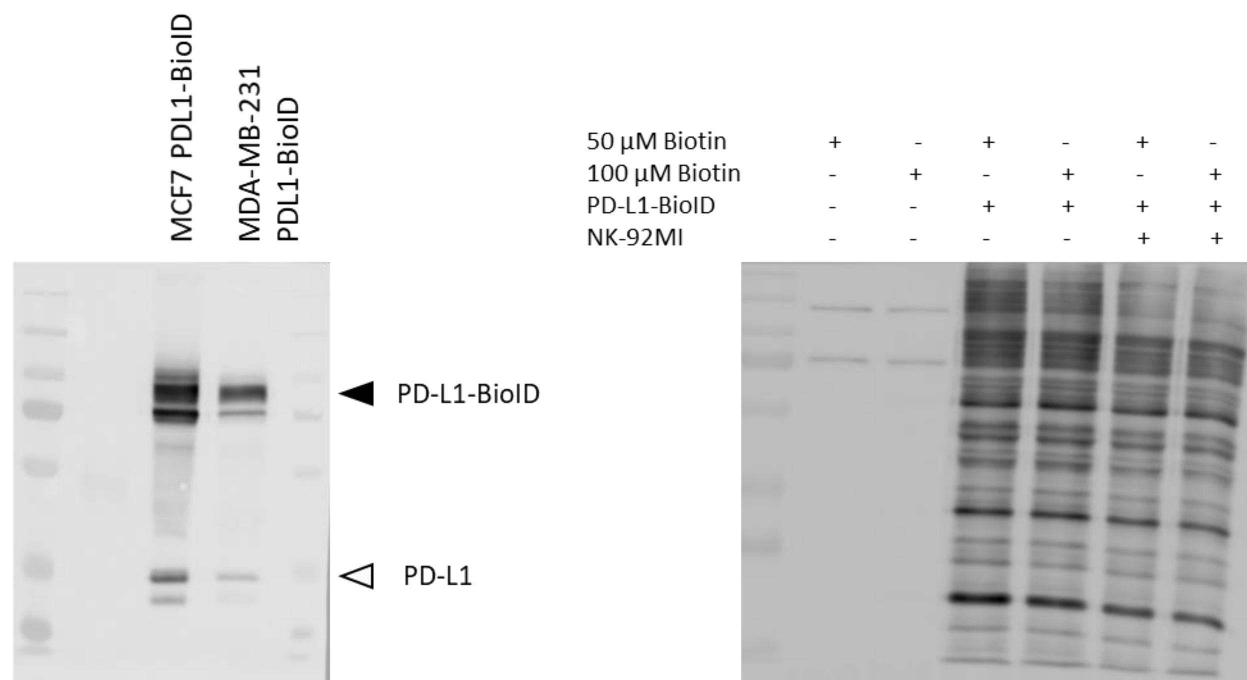
Interestingly, AR-competent cancer cells showed resistance against NK-92MI cells, a cell line lacking inhibitory KIR, suggesting that the AR possesses additional properties that intervene with the delivery of cytotoxic proteins such as granzymes and perforin. Our CLEM analysis suggests that SFPs form a physical fence that alters formation of a tight and fully functional lytic IS. More than simple passive obstacles, SFPs likely exert pushing forces against the NK cell membrane, due to the active actin polymerization at their tip, which further weakens the IS. Such mechanical perturbation could account for the abnormally wide synaptic clefts observed in the presence of an AR, which in turn likely prevents cytotoxic proteins to reach functional concentrations at the post-synaptic target cell membrane. Such a mechanism could have potential implications for NK cell-based immunotherapies, such as CAR-NK cells, where activation of effector functions is linked to recognition of tumour neo-antigens and not the balance between activating and inhibitory receptor signalling. Insufficient transfer of apoptosis-inducing factors could result in immune escape of AR-competent tumour clones from CAR-NK cells. Second, as suggested before, ERM proteins could be involved in the AR, and we have only targeted one of their downstream effectors. This resulted in a significant reduction of the AR in cancer cell lines or increased their immune evasion potential, but some cancer clones resisted restriction of this signalling pathway. Further studies are needed to identify other signalling pathways that are involved in the initiation of the AR and could serve as potential therapeutic targets either alone or in combination with ICI therapy. Third, identification of the AR in a cancer cell currently relies on analysis of individual target-effector conjugates by high-throughput multispectral imaging flow cytometry or high-resolution immunofluorescence microscopy. To establish the AR as a prognostic marker for success of ICI therapy and/or combinational therapy, further investigations are required to identify markers of the AR. We hope that this current study serves as a steppingstone for future investigations of the AR, which may help identify novel anti-cancer therapeutics.

Despite all these considerations, we suggest that the AR is a multi-step immune escape strategy of cancer with the primary goal to prevent IS maturation through polarization of inhibitory ligands to the AR-IS and prevent uptake of granzyme B through steric hindrance as a secondary outcome. By recruiting PD-L1 to SFPs, firm binding of effector lymphocytes to target cells can be inhibited through early blockade of LFA-1 outside-in signalling and actin remodelling at the effector side of the IS. Further, NK cell functions are inhibited through clustering of MHC-I at the IS. We have identified the AR as a critical determinant for *in vivo* tumour immune escape and identification of drug-able targets in the pathways enabling the AR will be an important first step toward restoring anti-tumour immunity and improving immunotherapy.

Supplemental information



Supplemental Figure 3.1 Differential clusters of mock- and pre-challenged MCF-7 cells in scRNA-Seq analysis. Evaluation of expression data indicates IFN-y mediated upregulation of the MHC-I HLA-B on pre-challenged MCF-7, as well as the Ca^{2+} sensor protein S100P.



Supplemental Figure 3.2 Confirmation of PD-L1 BioID expression and ligase function of the fusion protein. MCF-7 and MDA-MB-231 cells were transduced to stably expressed PD-L1 BioID2-myc. Expression of the fusion protein was confirmed in both cell lines. MCF-7 cells do not express endogenous PD-L1, and the band detected at the 33kDa band is probably the result of spontaneous cleavage of the ligase-fusion protein. Both cell lines express a functional ligase-fusion protein that shows extensive biotinylation activity with an excess of biotin in the culture medium.

Conclusion and Perspectives

Immunosurveillance of cancer is responsible for host protection against carcinogenesis and maintenance of cellular homeostasis. Consequently, development of clinically detectable tumours is a consequence of immunoediting, the process by which effector lymphocytes of both the innate and the adaptive immune system continue to destroy cancerous cells they can identify but are blind to tumour cells that have developed strategies to mask themselves against detection. The current concept of immunoediting suggests three phases (elimination, equilibrium, escape) that merge and result in the generation of resistant cancer cell populations, driven by Darwinian selection. Selective pressure is not limited to escape from predators (the immune system), but also includes competition for resources (nutrients, O_2, growth factors) and interaction with the often hostile environment. A concept that can be applied to the dynamic emergence of resistant tumour cell clones is evolutionary game theory that assumes that individual cancer cells (players) drive for fast optimization of their survival and proliferation (payoff) by employing mutations and other accumulated aberrations (strategies). During the equilibrium phase of immunoediting both players, the immune system, and the cancer cells, are engaged in indefinite cyclic “games” (e.g., interaction of effector lymphocytes with target tumour cells), similar to rock-paper-scissor. In this steady state, the bulk of tumour cells are killed. But by detecting and destroying these susceptible cells, the immune system imposes an immense evolutionary selection pressure for resistance while removing all potential competitors. The payoff for the immune system is tumour control, but the payoff for the tumour is the eradication of unfavourable cancer clones that ultimately results in an increased fitness and the acquisition of different strategies to cheat the immune system at its own game. Ultimately, this allows the escape of individual cancer cell clones and their growth into a heterogeneous tumour that no longer participates in the game. This heterogeneity of tumours and their ability to adapt to environmental selection is also what it makes it so difficult to treat cancer, as with each cycle of chemotherapy or radiation a new group of cancer cell clones emerges that was unaffected by the treatment or developed molecular resistance mechanisms. Coming back to the evolutionary game theory, application of systemic drugs in a maximum tolerated dose strategy recalls a “whack-a-mole” tactic, where dominant cancer cell populations are targeted, but hidden resistant populations pop up after a short time and with each round the game accelerates.

With immunotherapy, scientists and clinicians have tried to change the rules of the game, approaching it more like a game of chess. Cancer immunotherapy can change the dynamics by providing the immune system with new strategies to overcome cancer resistance while simultaneously reducing the fitness of cancer cells. In the context of immune escape, a major playground is the immunological synapse. Recognition of cancerous cells occurs in this cell-cell contact and the balance between engaged activating and inhibitory receptors defines whether a conjugated target cell is destroyed or spared. The initiation of the cancer-immunity cycle depends on the effector function of NK cells and the antigen-presenting and priming capacity of DCs, but antigen-specific identification and lysis of tumour cells relies on adaptive CTLs. But cancer cells are cheaters and can change the playground to their advantage. At the immunological synapse, this includes the downregulation of tumour neo-antigen presenting MHC-I and the upregulation of non-classical MHC-I molecules, as well as other inhibitory immune checkpoint ligands, such as PD-L1, CD80/CD86. These immune checkpoints are targeted in immunotherapy, but primary and acquired resistance are commonly observed and patients can even relapse with more aggressive

tumours (Fig. 4.1). New combinational treatments try to address these resistances by restoring immunosurveillance and identifying trunk mutations as more robust therapeutic targets.

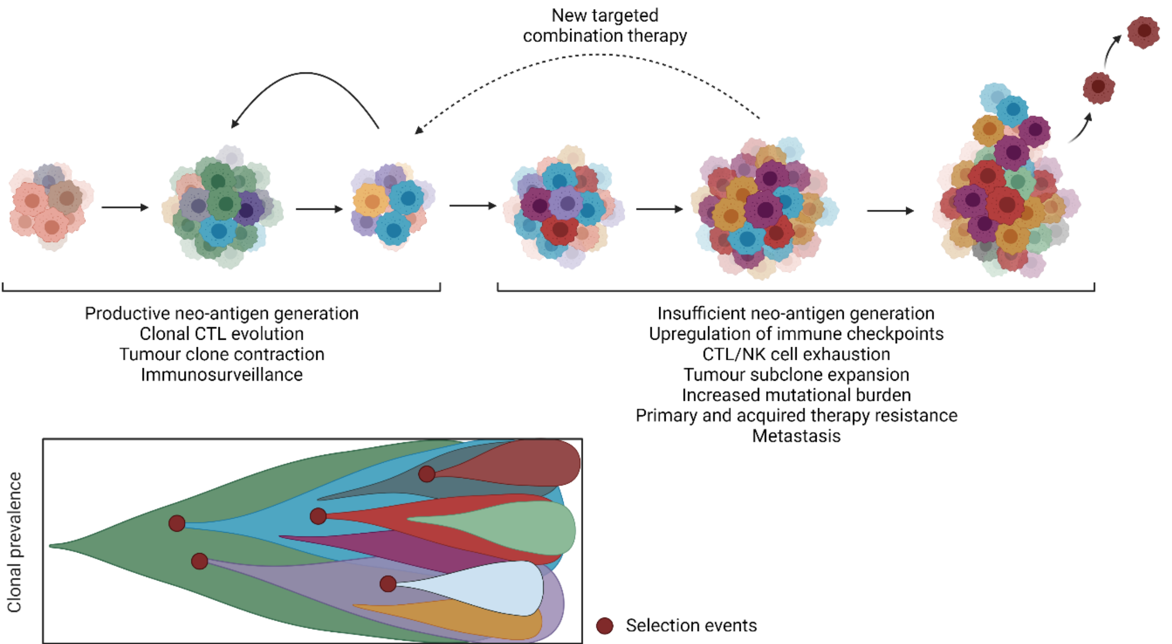


Figure 4.1: Cancer evolution during immunoediting and (immuno) therapy. Along with neutral selection, some tumour clones acquire mutations that are positively selected during immunoediting and later during (immuno) therapy. Over time, repeated cycles of selection result in the generation of poorly immunogenic cancer cell clones, establishment of an immunosuppressive tumour microenvironment, and resistance to standard chemotherapy. New targeted therapies can re-establish immunosurveillance and lead to tumour suppression or even eradication. Created with BioRender.com

I. The role of the immunological synapse in predicting the immune escape potential of cancer cells

The immunological synapse is a locally restricted cell-cell interaction that allows bi-directional exchange of information. During formation of the regulatory IS between DCs and NK cells, the target DC side of the IS shows extensive accumulation of F-actin that is a necessity for clustering and stabilization of inhibitory MHC-I molecules at the IS. In this type of IS, activation of killing by NK cells is suppressed in favour of stimulating survival and proliferation[160]. In contrast, lytic NK-IS formation shows the characteristic symmetrical F-actin accumulation at the NK cell side of the IS that is associated with NK cell polarization and directed degranulation of lytic granules. The third canonical type of NK-IS is the inhibitory IS, a transient contact between healthy cells and NK cells and induction of “self” recognition. In this type of IS inhibitory signals predominate and prevent NK cell attachment to the target cell and induction of effector functions[142].

1.1 Tumour cell evasion from activated NK cells

In **annex I** we have described for the first time another type of NK cell-immunological synapse, the evasion synapse that is formed between resistant cancer cells and NK cells. This interaction is characterized by accumulation of F-actin at the cancer side of the IS, the actin response, that provides protection even against polarized NK cells. In this model, NK cells initiate the formation of a lytic IS, characterized by polarization and firm attachment to target cells, but with low target cell killing efficiency.

A possible explanation for this observation could be increased target cell surface tension. The pore-forming function of perforin has been reported to be enhanced by an increased target cell membrane tension that is generated by the effector lymphocytes by straining the target cell membrane. This is facilitated by the adhesive ring that is formed during IS progression, as well as effector lymphocyte protrusions at the IS that exert mechanical force by pushing into the target cell membrane[202]. An extensive network of F-actin on the other side of the IS could counteract this mechanical stretching, inhibiting the pore-forming activity of perforin and by this limiting perforin-induced lysis, but also diffusion of granzymes into target cells. Moreover, a dense network of actin filaments, as we see it during the actin response, could be a steric hindrance for granzyme diffusion into the target cell, restricting it from the target cell cytoplasm. This described mechanism seems to be the last bastion against activated NK cells and target cell uptake of cytolytic effector molecules, although cell death-inducing granzyme functions can also be hindered by aberrant expression of caspases and pro- and anti-apoptotic proteins. The mechanism of the actin response seems to be largely dependent on signalling of the Rho GTPase CDC42, as targeting of this protein and its downstream effector N-WASp efficiently prevented the actin response in breast cancer cells with a high actin response frequency. However, epithelial breast cancer cells that exhibit a low intrinsic actin response rate showed no changes in their actin response frequency upon confirmed knockdown of CDC42 or N-WASp, suggesting the existence of an alternative actin regulatory pathway that is utilized by a subpopulation of actin response-competent cancer cells.

Another observation we made in **article II** was the increased size of the synaptic cleft at the AR-IS. While this could be indicative of improper IS formation, as discussed later, it could further suggest weak adhesion of NK cells to their prospective tumour target and diffusion of lytic molecules within the synaptic cleft. Studies have demonstrated that the synaptic cleft of the NK-IS is not consistent, but has been suggested to range between 0 and 55 nm[671–673]. This is in parts due to the fact most receptors and ligands can only span an intermembrane distance of 15 to 20 nm, whereas integrins and their ligands interact over a distance of 40 to 50 nm and too close membrane proximity would require membrane bending to allow their interaction[672]. The differences in synaptic cleft distance play also a role in the size-dependent exclusion of particles from the cSMAC. Directed degranulation has been described to occur only within the borders of the adhesive ring[674], resulting in a local increase of perforin and granzymes within a confined space. An increased synaptic cleft size could – in case NK cells even proceed to the last step of lytic IS formation – result in a diffusion of lytic effector molecules and a concentration at the post-synapse that is insufficient to induce target cell death. However, it is more likely that the drastic increase of the synaptic cleft as seen at the AR-IS does not allow for proper NK cell activation and might even prevent stable NK cell attachment to AR-competent cancer cells.

1.2 Triggering the actin response: a first working hypothesis

In **annex I**, we suggested that the size of the actin response-competent subpopulation correlated not only with increased resistance against NK cell-mediated cytotoxicity, but that the actin response was also an escape mechanism that was facilitated by EMT. EMT promotes morphological changes and increases cell motility and the crucial role of actin cytoskeleton dynamics for adoption of the mesenchymal phenotype and associated functions is well documented. The connection between transmembrane proteins, such as the focal adhesion kinase (FAK) and integrins with actin cytoskeleton remodelling to facilitate cell migration correlates with an invasive metastatic cancer

phenotype. During the EMT process, integrin expression is increased, and their engagement plays a crucial role in connecting the environment with cancer cellular responses.

While we yet have to define the initiating event of the actin response, live cell imaging has shown that an actin response occurs as soon as the first contact is initiated by the NK cell. It is worth hypothesizing that transmembrane proteins involved in establishing cell-cell contact could be involved in the initiation of the actin response. The outside-in signalling cascade of the LFA-1 ligand ICAM-1 has been shown to induce co-localization of the intracellular ICAM-1 domains with several actin binding proteins, including proteins of the ERM family, cortactin, and RhoGTPases (Fig. 4.2). While the cytoplasmic tail of ICAM-1 lacks any intrinsic kinase activity or interaction domain, it has a tyrosine residue that allows interaction with SH2-domain carrying proteins upon phosphorylation. Induction of Rho GTPase signalling and actin cytoskeleton remodelling in response to ICAM-1 multimerization has been associated with recruitment of ERM proteins but also coincides with the formation of actin stress fibres and phosphorylation of cortactin[675]. The mechanism of ICAM-1-dependent activation of RhoGTPases is yet not clearly defined, but involvement of Src tyrosine kinases and ERM family proteins has been suggested. Induction of these signalling pathways result

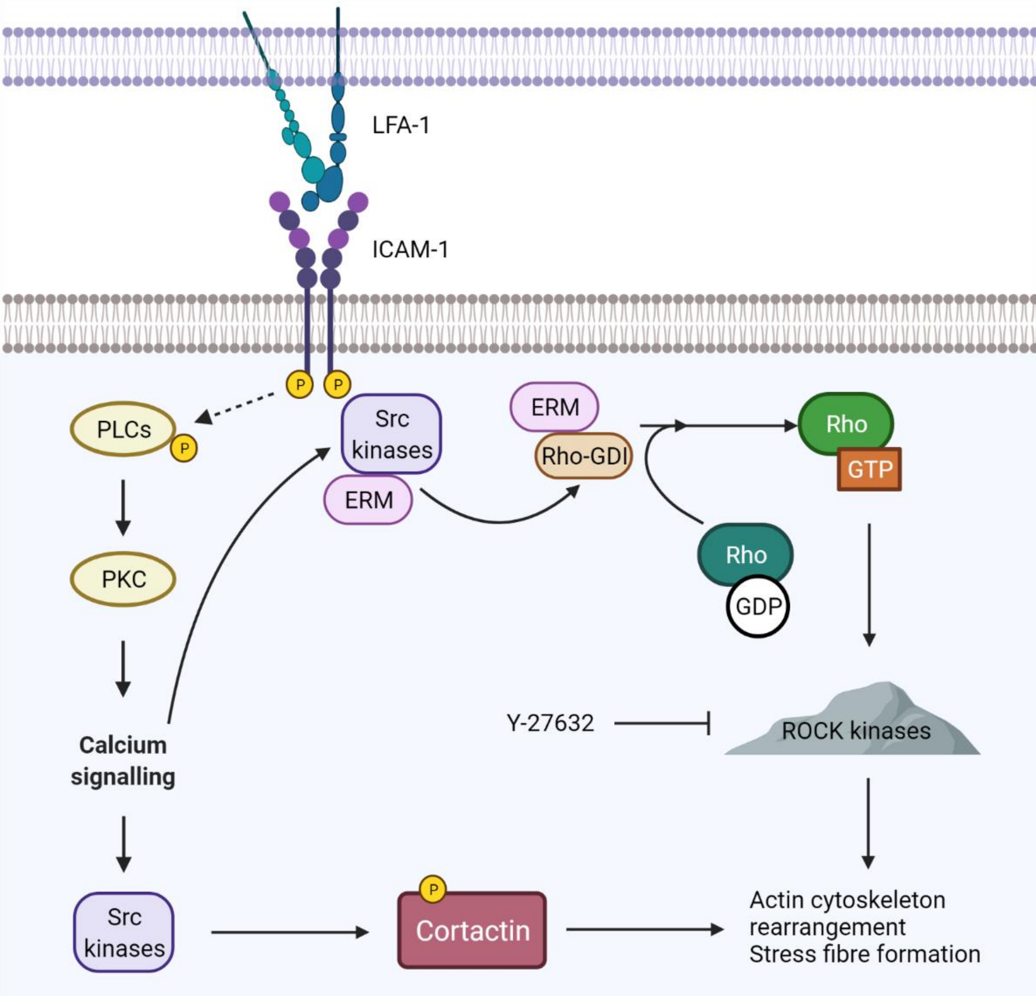


Figure 4.2: Hypothesized "outside-in" signalling pathways of ICAM-1 and its link to actin cytoskeleton remodelling. Ligation of ICAM-1 with its receptor LFA-1 induces the recruitment and activation of Src kinases that in turn activate ERM family proteins and cortactin. Through induction of Rho GTPases, actin cytoskeleton remodelling and stress fibre formation is induced. By activation of phospholipase C (PLC) intracellular calcium signalling is induced, enhancing Src kinase activity and accelerating actin remodelling. Created with BioRender.com

in extensive actin cytoskeleton rearrangement under the regulation of PKC. Although polymorphism of ICAM-1 has not been described to be linked with carcinogenesis, expression of mutated ICAM-1 variants has been associated with stronger adhesion and an increase in Erk-1/-2 phosphorylation in cell expressing polymorphic ICAM-1 variants.

Since adhesion is one of the first steps of IS formation, blockade of LFA-1/ICAM-1 interaction would most likely result in a loss of NK cell-cancer cell conjugates. Instead, targeting of downstream effectors such as the silencing of ERM proteins or ROCK inhibition through pharmacological means could help identify key pathways of the actin response besides CDC42-mediated actin cytoskeleton remodelling. Alternatively, site-directed mutagenesis of the intracellular domain of ICAM-1 to substitute the tyrosine residue with a non-phosphorylatable amino acid such as alanine or phenylalanine using a CRISPR/Cas9-mediated mutagenic (ICM) system could help answer the question whether ICAM-1 signalling is involved in the imitation of the actin response. The phosphorylation site of ICAM-1 could also be mutated to a phosphomimetic, replacing it with either an aspartic acid or glutamic acid, however the side chains and the charge density of these amino acids differ significantly from a phosphotyrosine, making a phosphomimetic of phosphotyrosine more difficult to achieve. Identification of the initiating pathways of the actin response could facilitate the search for a targeted approach to inhibit the actin response *in vitro* and possible also *in vivo*.

1.3 A potential role of p53 transcriptional regulation for enabling the actin response

CLL is a disease that is highly immunosuppressive and patient-derived NK cells are characterized by an extensive hypo-responsiveness, similar to exhaustion observed in CTLs and linked to downregulation of activating receptors[676]. However, upon sufficient activation stimulus, patient-derived NK cells function can be restored, and they can kill target cells through ADCC. This observation was the rational for the development of NK cell-based immunotherapies against CLL, and in first clinical studies CAR-NK cell therapy showed a remarkable success. However, selective pressure, which occurs during chemotherapy or immunotherapy, has been associated with expansion of resistant CLL clone(s) and an aggressive disease shift.

In **article I** we have described the actin response in CLL cell lines and patient-derived CLL cells, for the first-time confirming conservation of this cancer cell intrinsic mechanism in different types of cancer and its existence in patient-derived non-solid tumour samples. Although CLL cell lines are considered to be even more of an artificial model than other cancer cell lines, a high rate of the actin response in these cell lines correlated with a molecular profile associated with poor prognosis. The highest actin response frequency was found in a CLL line that was characterized by a complete loss of functional TP53 expression. TP53 is a transcription factor and as such has no biological function in regulating fast actin cytoskeleton remodelling. However, as a transcription factor p53 governs the expression and activation of integrins and associated molecules in their downstream signalling-pathways, and a loss of p53 function has been associated with increased integrin expression and cancer aggressiveness. Further p53 negatively regulates expression of mechanosensitive signalling proteins, such as FAK and paxillin[677]. FAK-induced actin cytoskeleton remodelling is linked to recruitment of ERM proteins, while the exact mechanism of paxillin-induced Rho GTPase activation in response to external force remains unclear. As mentioned before, interaction of target and NK cells at the IS is characterized by pulling forces. An increased expression of integrins – that have a direct link to the actin cytoskeleton and upon induction of outside-in signalling can induce actin remodelling – and mechanoresponsive adaptor proteins could further

facilitate the process of the actin response. p53 deficiency has further been linked to an increased activity of Rho GTPases, as well as upregulation of their downstream effector proteins. Although and transcription factor, p53 can influence actin cytoskeleton dynamics through directly or indirectly regulating expression of transmembrane receptors and regulators of actin cytoskeleton remodelling, and a loss of functional p53 could accordingly enable CLL cells to respond to NK cell attack with an actin response.

Restoration of p53 function has been associated with tumour suppression through cell cycle arrest and induction of apoptosis. While speculative, inhibition of transcriptional activity of p53 could also be involved in facilitating the actin response, as a consequence of increased expression of actin cytoskeleton regulators.

1.4 Patient-derived tumour cells use the actin response to escape from NK cell natural cytotoxicity

In this paper we also demonstrated the effectiveness of the CDC42 inhibitor ZCL-278 in preventing the actin response and increasing susceptibility of CLL cells to NK cell lysis. While drug treatment is a more direct mechanism to inhibit the actin response, as it does not rely on transfection efficiency and siRNA sequence specificity, we observed again that a subpopulation of the resistant CLL cells, as well as all CLL cell lines characterized by an intrinsically low actin response resisted this treatment. While this supported our hypothesis of a secondary signalling pathway involved in the formation of an actin response, it also allowed us to target the actin response in cells that are resistant to most transfection methods. This includes patient derived CLL cells.

We initially observed poor IS formation of *ex vivo* CLL cells with NK-92MI cells due to interaction of HLA-G on target cells with its cognate receptor. The physiological role of HLA-G is to induce immune tolerance during pregnancy by excluding NK cells from the placenta through interactions with the inhibitory leukocyte immunoglobulin-like receptor (LIR) subfamily B member 1, ILT-2. On CLL cells, expression of HLA-G is associated with immune escape from host NK cells and induction of immunosuppression. Using blocking antibodies, we could partially restore IS formation and natural cytotoxicity of NK-92MI cells against patient-derived CLL cells. As in cancer cell lines, a high rate of the actin response was linked to lower levels of granzyme B in target cells and accordingly less signs of target cell death in conjugates engaged in an evasion synapse. The targeting of CDC42 activation partially restored susceptibility to NK cell-mediated cytotoxicity, but as observed with CLL cell lines the actin response could not be inhibited completely.

While this study was conducted with a small number of patients, it confirmed the actin response and associated resistance mechanism for the first-time in patient derived tumour samples. It also highlighted a two-step process by which CLL cells avoid destruction by NK cells: inhibition of IS formation through upregulation of inhibitory ligands and the actin response as a mechanism to attempt escape from a lytic NK-IS through changing it into an evasion IS. In the future, studies including larger patient groups and other types of cancer as well as patient-derived NK cells are required to further characterize the actin response in primary tumour cells because in contrast to cancer cell lines the primary tumour is vastly diverse and unique in its subpopulations[678]. Moreover, while inhibition of CDC42 is effective to increase susceptibility of the majority of resistant cancer cell clones, a small subpopulation remains unaffected by this approach. In a clinical setting this would most likely result in a temporary shrinkage of the tumour mass or decline of

leukemic cells, but we would select for cancer cells that use different signalling strategies to manifest an actin response.

II. Stabilization of inhibitory ligands at the IS increases the immune escape potential of cancer cells

In **article II** we confirmed the actin response in a variety of different cancer types. While we previously reported that the actin response is a last obstacle for the transfer of granzyme B and perforin-mediated pore formation that cancer cells utilize to escape from activated NK cells, we here observed that in conjugates with primary NK cells lytic granule polarization in an evasion IS was poor. Polarization of lytic granules is used as an indicator for NK cell activation and commitment to target cell lysis and a lack thereof accordingly associated with insufficient activation signal strength. Evaluation of the size of the synaptic cleft further indicated a wide space between target and NK cells engaged in an AR-IS, indicating an instable IS similar to the inhibitory IS. Because all used cancer cell lines express moderate to high levels of stress-induced ligands, such as CD48, CD155, MIC-A/-B, and ULBPs, our hypothesis for the evasion IS was that inhibitory receptor signalling could counterbalance NK cell activation. To rule out receptor-mediated recruitment of inhibitory ligands to the IS, we used the KIR$^-$/PD-1$^-$ NK-92MI cell line to evaluate positioning of the immune checkpoint ligands MHC-I and PD-L1 on target cells.

We observed an impressive clustering of MHC-I molecules in the absence of its ligands at the AR-IS with NK-92MI cells. Based on this finding, we validated this phenotype with pNK and observed an inhibition of target cell killing in conjugates with MHC-I clustering. In NK cells, regulation of activation is rather defined by the quantity of activating versus inhibitory signal at the IS, with ITIM-domain receptor signalling being faster and stopping IS progression by slowing actin cytoskeleton remodelling. In contrast, CTL activation is controlled by the quality of the signal, meaning the specificity of TCR for the neo-antigen and co-stimulatory signals. We could confirm this inhibitory ligand clustering with PD-L1, though it is worth mentioning that the scientific debate about whether PD-1$^+$ NK cells exists and if they can be found in tumours if they are as strongly inhibited by PD-L1$^+$ tumour cells as CTLs has just started. Expression of PD-L1 on target cells does not interfere with initial conjugate formation, but instead has been shown to result in weak adhesion and inhibition of IS progression in CTLs[666]. Accordingly, clustering of inhibitory ligands at the IS would not inhibit conjugate formation per se but would weaken the activation signal and prevent effector functions. Interestingly, ligand clustering did not affect the overall surface expression, suggesting an actin-driven repositioning of available transmembrane proteins rather than upregulation of inhibitory ligands at the AR-IS. This observation was similar to the previously designated regulatory IS, that was described to be selective for inhibitory MHC-I clustering to the DC-NK cell contact side.

This clustering of inhibitory ligands within the small area of the IS could be more efficient than homogeneous ligand presentation in inducing a strong inhibitory signal in NK cells. While during “normal” ligand distribution around 20% of overall ligands are presented at the IS, providing a balanced mixture of the relative ratio of activating and inhibitory ligands that could potentially engage with their cognate receptors, at an AR-IS up to 60% of overall inhibitory ligands expressed on the surface compete for positioning in a limited area. On the one hand, this could engage more inhibitory NK cell receptors than a homogeneous distribution of ligands, favouring termination of IS formation, and on the other hand this could further exclude activating ligands from the AR-IS through spatial competition. As discussed before, inhibitory receptors transmit their signals faster

than activating receptors, perturbing pre-synapse actin cytoskeleton remodelling and IS maintenance, so the induction of a strong inhibitory signal during initial contact could prevent stable IS formation altogether. This could be a potential function of SFPs that probe the surface of the approaching NK cells with PD-L1 decorated projections, which – upon engagement with their receptor – could set up the stage for an early intervention of LFA-1 induced adhesion and IS formation. Sustained activation of activating receptor signalling pathways in NK cells requires continuous intracellular Ca^{2+} increase and PI3K pathway signalling, and it has been shown in $CD8^+$ T cells that intact actin cytoskeletal rearrangement is required to sustain these signalling pathways[679]. In CTLs, probing of the APC surface and formation of tight adhesion has been shown to be dependent on actin cytoskeleton remodelling that was initiated upon the first pMHC-TCR-mediated Ca^{2+} influx and maintained by serial engagement of TCRs and sustained activation of downstream pathways, includingCa^{2+}-calcineurin, PI3K and PCK[680]. While the lytic IS of CTLs and NK cells differ in their structural architecture, the initiated signalling pathways of activating receptors and actin cytoskeletal dynamics are remarkably similar. Accordingly, a disruption of intracellular Ca^{2+} influx could inhibit firm adhesion of NK cells to their prospective target cells in a quantitatively regulated balance between inhibitory and activating receptor signalling[681]. Moreover, a recent study has reported that while NK cells can spread over large surface areas to integrate sufficient activation signal to initiate IS formation, the degranulation process was dependent on a local signal of sufficient strength[682], as NK cells require a stacked activation signal to execute their lytic functions. This suggests that even in the absence of PD-1 on effector lymphocytes, occupancy of the post-synaptic membrane at the AR-IS by MHC-I or other inhibitory ligands and exclusion of activating receptors could interfere with downstream effector functions such as lytic granule convergence, MTOC polarization, and degranulation.

Furthermore, clustering of inhibitory ligands at the AR-IS is probably not a NK cell-exclusive observation, as it is receptor-independent, and this observation has potential implications for CTLs. While accumulation of pMHC-I at the IS could be considered beneficial for $CD8^+$ T cell activation, combined clustering of PD-L1 and pMHC-I has the potential to terminate TCR signalling, as it has been shown that PD-1 co-localized with TCRs to form negative costimulatory microclusters to inhibit the effector phase of CTL tumour recognition[683,684]. Overall, AR-driven clustering of predominantly inhibitory ligands could therefore allow immune escape from both, NK cells but also CTLs.

Based on these initial findings, we decided to focus on the non-classical HLA-E molecule as a main inhibitory ligands of peripheral and tumour infiltrating NK cells, as it is highly conserved and overexpression is frequently detected in patient tumour tissue[685]. Because this is an ongoing project at the time, I am writing this thesis, I cannot discuss the impact of spatial distribution of HLA-E on NK cell activation here, but our working hypothesis suggests that while HLA-E expression provides protection against NK cell activation, the actin response-driven polarization of HLA-E to the IS could further increase the immunosuppressive potential (Fig. 4.3). Whereas PD-1 expression on NK cells is still debated and accordingly PD-L1-decorated SFPs are probably insufficient to prevent NK cell attack, accumulation of HLA-E on SFPs or at the post-synaptic AR-IS membrane could have a similar effect, as NKG2A acts as an inhibitory IC on NK cells. In this hypothesis, application of blocking antibodies against HLA-E, CD94 and/or NKG2A would not be sufficient to prevent inhibitory receptor/ligand interactions, especially in tumours where antibody distribution

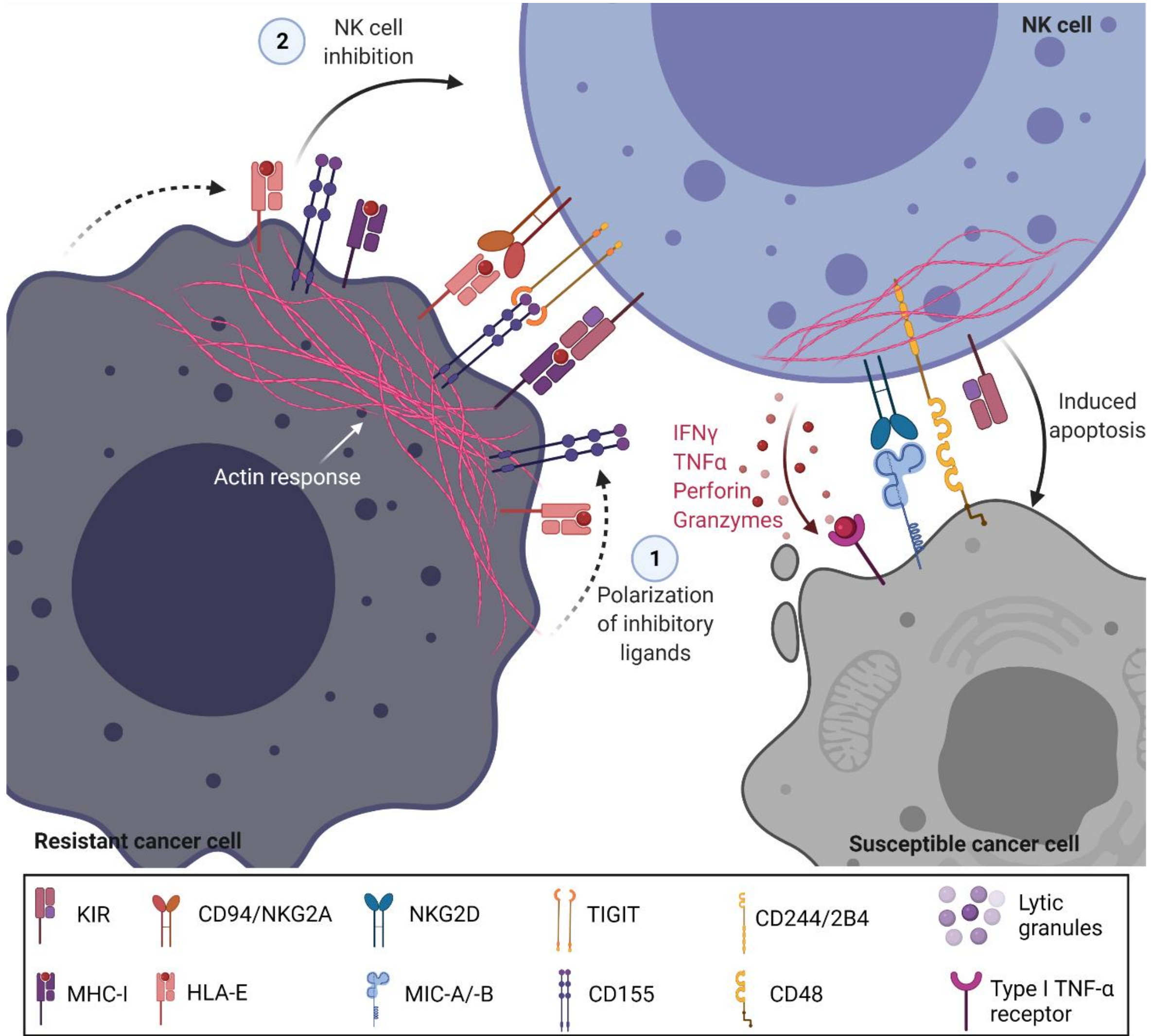


Figure 4.3: The effect of actin response-dependent ligand polarization on NK cell activation and tumour immune escape. The mechanism of the actin response enhances the immune escape capacity of tumour cells by recruiting and stabilizing predominantly inhibitory ligands at the evasion IS between cancer cells and NK cells. This results in inhibition of NK cell effector function and survival of the target cell. Cells unable to form an evasion synapse are engaged in a lytic NK-IS and killed through directed degranulation and/or engagement of death receptors. Created with BioRender.com

is extremely uneven and does not reach concentrations we use in *in vitro* assays[686]. Against cancer cells that cannot produce an actin response, lowering the inhibitory signal strength by blocking some of the interaction of homogeneously distributed inhibitory ligand with their receptors or by providing a secondary activating signal through CDC or ADCC by using tumour-targeting mAbs, immune checkpoint inhibitor therapies are efficient to restore NK cell anti-tumour immunity. But in the presence of an actin response and the associated clustering of inhibitory ligands, the same amount of blocked interactions is not sufficient to lower the inhibitory signal strength to allow immune cell activation. We reason that a combination of blocking antibodies with targeting of the actin response could restore NK cell effector functions against tumour cells by converting the evasion IS into a classical lytic NK-IS.

The observation that the actin response was linked with a decrease in pNK cell activation and consequently increased resistance of cancer cell lines with a large actin response-competent subpopulation led us to develop different mouse cancer models. Through transduction of mutated CDC42 proteins we could modify the actin response rate of these daughter cell lines that we

implanted in immune competent syngeneic mice. In these models, a high actin response frequency was associated with an accelerated tumour grow (4T1-CDC42(Q61L)), while a reduction of the actin response-competent population resulted in small tumours (B16F10-CDC42(T17N)) compared to the parental cell lines. The evaluation of the tumour immune landscape indicated that a high actin response rate was associated with a more immunosuppressive "cold" tumour, supporting our hypothesis that actin response-dependent polarization of inhibitory ligands influences effector lymphocyte activation and can even induce exhaustion. This has also potentially important implications for current (NK cell-based) immunotherapies, as we have also shown that CTLs and even macrophages invoke an actin response and that over time NK cell attack selects for actin response-competent tumour clones *in vitro*. Considering these observations, we could speculate that *in vivo* both CTLs and NK cells apply selection pressure in favour of actin response-competent tumour cell clones. For a comprehensive understanding of the effect of the actin response on the tumour immune landscape, we plan to perform mass cytometry experiments. This approach has been previously used to identify beneficial dual immunotherapy and could be a first step to identify combinational immune checkpoint blockade options for tumours that show the characteristic immune profile of the actin response.

However, we are currently lacking information about the total number of TILs and moreover their localization, underlining the need for a comprehensive mapping of the tumour landscape using whole tumour scanning technologies, such as imaging mass cytometry or multiplex imaging of tumour sections. Using these technologies could help us determine whether actin response-competent tumour cells are mainly found in the invasive margin, correlating with the increase expression of PD-L1 and other IFN-y induced ligands[316], and whether the actin response is also involved in the interaction with other immune cell populations, for example T_{Regs} or MDSCs. This approach would also allow us to investigate, whether actin response-competent tumour cells building a barrier that could prevent infiltration of immune cells, and if by blocking or lowering the actin response, we could increase the number and distribution of TILs in the TME. In this context, ligand polarization not only in murine tumour tissue, but also patient tumour samples would be of great interest. Tumour mapping could indicate the influence of the actin response on TIL distribution, establishment of tertiary lymphoid organs, and most importantly the impact of clinical therapy on the prevalence of actin response-competent tumour cell clones and their apparent ability to shape the TIME during therapy and after relapse. For this analysis, larger cohorts including different types of cancer would be required, to identify recurring clinical features or even establish the actin response as a predictive marker of effective immunotherapy.

III. DISARMING THE ACTIN RESPONSE BY PREVENTING LIGAND CLUSTERING

Since neither MHC-I nor PD-L1 have been described to directly interact with actin filaments, linker proteins between these transmembrane proteins and the actin cytoskeleton are most likely necessary for actin response-dependent polarization of these transmembrane proteins. For APCs, association of transmembrane MHC-II clusters with lipid rafts has been described, and the TCR and PD-1 microclusters are especially important in transmitting the inhibitory PD-1 signal to intercept TCR signalling. MHC-I presentation pathways are well described, however a link between the actin cytoskeleton and MHC-I molecules has not been described so far. This suggests that the actin response-initiated MHC-I clustering either through linker proteins, or by repositioning of lipid rafts. This is supported by studies that could demonstrate that lipid rafts are associated with increased Src kinase activation and actin cytoskeleton remodelling[687]. However, lipid rafts including both

MHC-I and PD-L1 on target cells have not yet been described. Alternatively, membrane trafficking could explain the repositioning of MHC-I and PD-L1 to the AR-IS. Regulation of PD-L1 surface expression has been associated with recycling endosomes and CKLF-like MARVEL transmembrane domain containing protein 6 (CMTM6), CMTM4, and CMTM7 expression in different types of cancer[688–690]. Whereas we did not observe an increase in overall surface expression for MHC-I and PD-L1 in actin response-competent cancer cells, endosomal trafficking has been shown to be involved in receptor sorting and be highly dependent on actin cytoskeleton dynamics[691–693]. Both, CMTM4 and CMTM6 have been suggested to control the transition of early endosomes containing PD-L1 into recycling endosomes rather than late endosomes[694] and while a direct link between these proteins and the actin cytoskeleton has not yet been described, the actin cytoskeleton plays a critical role in the formation of endosomal tubular domains at sites of transmembrane protein endocytosis and provides the structure for transport of early endosomes[695,696].

In contrast to PD-L1 and HLA-E that are inhibitory ligands for both CTLs and NK cells, peptide-loaded MHC-I molecules are important for the induction of an antigen-specific T cell response against tumour cells. A clustering of pMHC-I to the IS with CTLs could accordingly allow $CD8^+$ T cells to reach their activation threshold faster if cancer cells polarize MHC-I to the IS. Because of these considerations we initially aimed to identify selective ligand-actin cytoskeleton linkers to prevent clustering of PD-L1 or HLA-E to the IS. In proximity-biotinylation assays we could identify moesin, an ERM protein, as one of the top 5 hits of enriched actin linkers after NK cell attack, and expression of ezrin was enriched in epithelial breast cancer cells that survived NK cell attack. As mentioned before, ERM proteins are downstream effectors of Src kinases and can in turn induce Rho GTPase activity. Recent work has further shown that activity of ERM proteins is required for F-actin recruitment during clathrin-mediated endocytosis of membrane proteins and endosomal sorting[697–699]. In immune cells, ligation of MHC-I and outside-in signalling has been shown to induce clustering of ICAM-1 and firm adhesion to endothelial cells through activation of mTOR, and mTOR signalling was associated with an induction of RhoA, ROCK, and ERM activity[700]. If tumour cells have hijacked this mechanism – as they have probably hijacked the actin response from the regulatory DC-NK IS – this could explain the identification of ERM proteins from proximity-biotinylation assays and single cell transcriptome analysis. Whether actin response-associated ligand clustering at the AR-IS is the result of lateral trafficking of transmembrane molecules organized within lipid rafts or intracellular endosomal recycling will require further experiments, but both hypotheses suggest that discriminatory targeting of the polarization of certain inhibitory ligands but not the actin response and pMHC-I clustering itself is unlikely. Moreover, the physical properties of the actin response that were mentioned before in counteracting mechanical pulling forces at the IS and the dense network of F-actin that could prevent transition of granzymes deeper into the target cell would remain. Accordingly, dissecting the clustering of unwanted inhibitory ligands (e.g., PD-L1, HLA-E) to the IS from the actin response and polarization of potential beneficial ligands (e.g., pMHC-I) is most likely an impossible task.

Our preliminary data using primary NK cells has further suggested that an actin response-competent cell has not the intrinsic property to prevent NK cell activation, but instead utilizes the actin response to increase the physical distance between itself and the attacking NK cell and/or prevent NK cell activation through inhibitory ligand clustering. Therefore, prevention of the actin response would be most beneficial, as the high antigen-specificity of CTLs allows for their activation even at low pMHC-I target cell expression levels in the absence of interfering inhibitory signalling,

and NK cell activation could be facilitated by a balanced presentation of the ligand repertoire of target cells.

IV. THE ACTIN RESPONSE AS A MULTI-TIERED RESISTANCE MECHANISM

Usually, NK cells form a lytic IS with a susceptible target cell, as a result of insufficient inhibitory ligand expression and an upregulation of activating ligands on stress cells. The effector functions of NK cells include the directed release of cytotoxic granules containing perforin and granzymes, and the non-directional release of cytokines and chemokines (Fig. 4.4A). An evasion IS however is characterized by the actin response and clustering of inhibitory MHC-I and PD-L1 molecules to the side of the cell-cell contact (Fig. 4.4B). This can result in a stronger inhibitory signal in NK cells due to the engagement of more inhibitory than activating receptors. In the for the cancer cell most beneficial case, this terminates the intention of the NK cell for target cell lysis and can further lead to induction of an exhaustion-like phenotype in NK cells that is characterized by the upregulation of further inhibitory receptors and the downregulation of activating receptors such as NKG2D. In

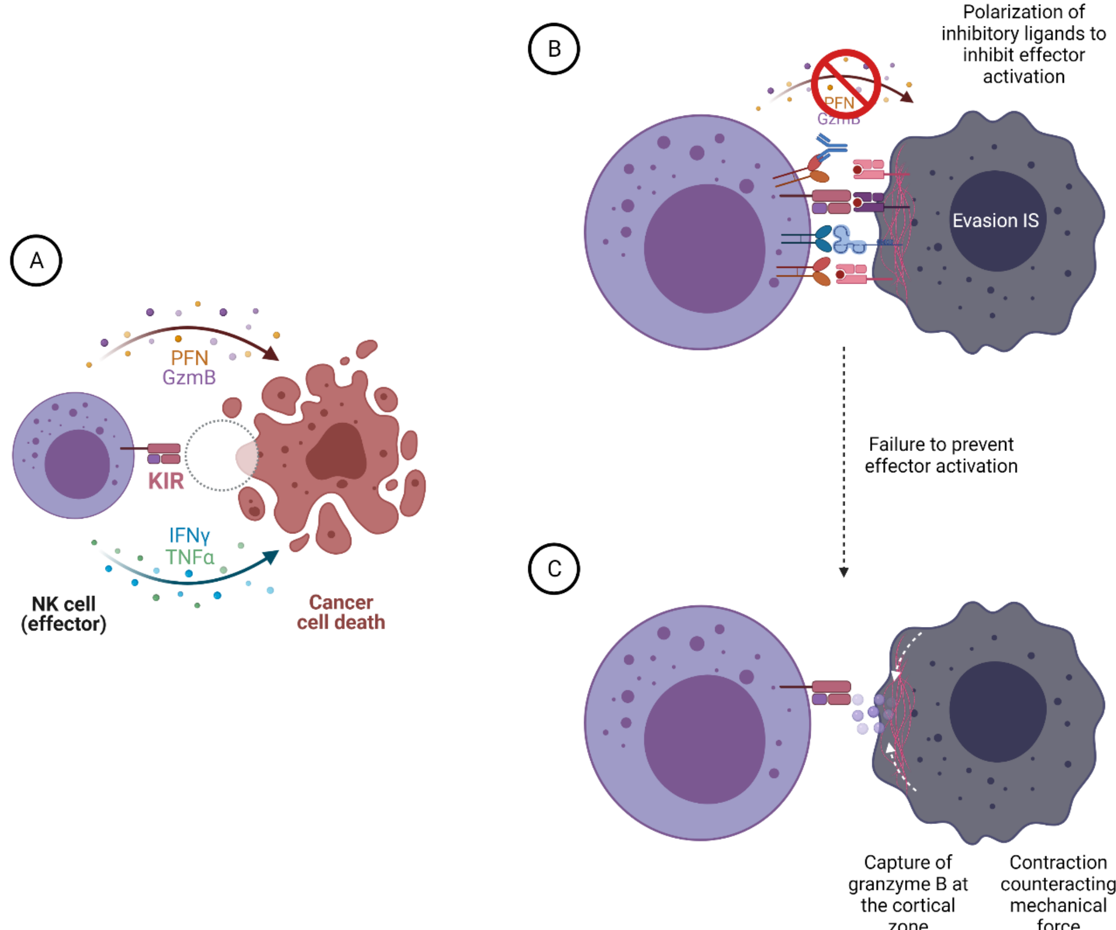


Figure 4.4: The actin response can interfere with effector functions at multiple steps. (A) NK cells engage in lytic IS with susceptible target cells and induce target cell lysis. (B) Formation of an evasion synapse requires sufficient expression of inhibitory ligands in combination with the actin response to prevent NK cell activation. The polarization of inhibitory ligands increases the signal strength of inhibitory receptors. ICI targeting receptors rather than the ligands could be ineffective in lowering the inhibitory signal strength at the AR-IS. If NK cell activation cannot be prevented, either due to strong activation signal or absence of inhibitory receptors (e.g., CAR-NK cells) the actin response can still interfere with the perforin/granzyme cell death pathway through contraction at the IS and exclusion or destruction of granzymes. Created with BioRender.com

the next IS that this NK cells forms the activation threshold is even more difficult to reach and repeated exposure to cancer cells that exhibit ligand polarization, or a high surface expression of inhibitory ligands will result in NK cell hypo-responsiveness and anergy.

But even if an evasion IS cannot be formed and the activation of the NK cell is not prevented by inhibitory ligand polarization, either due to strong activation signal or for example in an IS with a CAR-NK cell, an actin response-competent cancer cells is not left defenceless. The potential contractile physical properties of the actin response can intervene with the pore-forming function of perforin (Fig. 4.4C). This decreases target cell death through lytic pores and can also inhibit the different mechanisms that are responsible for the transfer of granzyme B into target cells. While granzyme can diffuse to perforin pores, the dense network of F-actin bundles could retain it at the peripheral region of the target cell, away from pro-caspases and functionally useless. Another pathway that has been suggested for transfer of granzyme B are so-called gigantosomes, the endocytic uptake of both granzymes and perforin and the subsequent perforation of the endosome membrane by perforin, releasing granzymes in the target cell cytoplasm. Endosomal uptake requires local depolymerisation of the actin cytoskeleton and gigantosomes could therefore be prevented at the AR-IS.

Further, hypoxia induced autophagy has been suggested to play a role in degradation of granzyme B, through fusion of the gigantosomes with the autophagosome, to form an amphisome, and the subsequent fusion with a lysosome[701]. I have not discussed this here, but we have gathered data that indicates a translocation of lysosomes to the AR-IS, that could be involved in the selective degradation of granzyme B, further enhancing the capacity of actin response-competent tumour cells to escape from effector lymphocytes in a lytic IS. Further studies have suggested that lysosomes could be involved in cancer cell resistance and that the post-synaptic IS membrane could function as a secretory domain[702]. Through release of lysosomes the pH of the synaptic cleft could be decreased, which could impair function of perforins but also granzymes. Moreover, fusion of lytic granules with the effector cell membrane has been shown to modify the lipid order of the pre-synaptic membrane in both CTLs and NK cells and play a role in preventing autolysis by hindering perforin binding to the pre-synapse. If the post-synaptic AR-IS domain is indeed a secretory domain, a similar mechanism could prevent execution perforin-mediated pore formation and facilitate tumour immune escape even from an activated effector lymphocyte.

V. FUTURE PERSPECTIVES

Over the past years, researchers have identified different strategies that allow tumour immune escape from NK cells and many of these strategies seem to interfere with the multistep process of lytic IS formation. An intrinsic mechanism such as the actin response that is triggered by the attacking lymphocyte could have the capability to combine these strategies and focus them at the AR-IS and thus allow them to exert their full potential for immune escape from cytotoxic lymphocytes.

The discussed resistance mechanism that we have associated with the actin response could be just a small peak at what is occurring at the AR-IS. Additional to lysosomal recruitment to the side of the cell-cell contact, other granules could also polarize and degranulation at the post-synapse could have a multitude of consequences. Acidification of the synaptic cleft could inhibit cytolytic proteins and the release of soluble death receptors as decoys could intervene induction of target cell death through this pathway as well. A recent study has shown that degranulation at the IS changes the lipid density of the pre-synapse and thereby prevents perforin binding to the NK cell membrane, and has reported that resistant cancer cells also polarize granules to the IS[703]. If the post-synapse of the AR-IS is indeed a secretory domain, increased lipid density as a result of degranulation could

further inhibit the pore-forming activity of perforin. As discussed before, membrane trafficking could help to reorganize cancer cell lipid rafts for signalling and compartmentalization of intracellular transport and signalling machineries, as it is observed at the CTL side of the IS[704], and endosomal recycling of surface proteins could modify the local ligand presentation at the AR-IS. Future experiments will have to show whether lateral transmembrane protein movement or endosomal trafficking are responsible for the clustering of inhibitory ligands at the AR-IS, because the identified ERM proteins could be involved in both mechanisms, as well as in the transmission of the initiation trigger of the actin response.

Some of the most intriguing questions are what initiates the actin response and how can we recognize an actin-response competent tumour cell before it engages in an evasion IS. While I was not able to address these questions in my PhD project, the results presented in this thesis and the established Omics technologies to characterize the expression profiles and the phospho-proteome of cells with an actin response provide a basis for developing *in vitro* and *ex* vivo diagnostic tools to answer these questions. One of these tools is the use of RNA sequencing technologies to identify protein signatures that correlate with the actin response. A remaining challenge for this approach is the fact that we do not have a “pure” actin response-competent cancer cell line, but instead are facing a limited facet of the tumour heterogeneity that is observed in clinical samples. To clearly identify a transcriptomic signature of actin response-competent cells, we would further need a reference cell line. We have tried to address these challenges by using the pre-challenged tumour cell model. However, after 4 hours of co-culture with NK-92MI cells, some susceptible tumour cells remained that again became the dominant subpopulation over time, suggesting that repeated rounds of NK cell-challenges would be required to eliminate these cells. With each cycle the number of resistant cancer cells should increase, although it is not yet clear if this approach will result in a pure actin response-competent cell line, moreover since NK cells that are encountering a high number of actin response-competent tumour cells in a killing assay could be engaged in frustrated lysis and not participate in destruction of susceptible clones. An alternative method could be the establishment of clonal cancer cell lines. This method harbours however the risk that instead of a bulk analysis that addresses the tumour heterogeneity to a certain degree, only a few surviving clones are used to determine the transcriptomic signature of actin response-competent cells. Another limitation is the fact that IFN-γ release triggers upregulation of different proteins, as we have seen in our first scRNA-Seq experiment in which we observed a strong upregulation of HLA-B in pre-challenged MCF-7 cells, even 48 hours post-challenge. Repeated exposure to IFN-γ, but also TNF-α and other inflammatory cytokines during *in vitro* killing assays could further initiate EMT, so analysis of mRNA and protein levels of EMT markers such as E-cadherin, N-cadherin, and vimentin will most likely be crucial to not falsely identify an EMT transcriptome as an actin response transcriptomic signature[705,706]. In a following step, these signatures will have to be confirmed with clinical samples to test if this way to identify actin response-competent clones is translational.

The actin response we observed using live cell imaging was a fast response to an approaching NK cell. With this in mind, signalling pathways that induce phosphorylation or dephosphorylation of proteins are most likely involved in the transmission of the triggering signal and initiation of the actin response. For this reason, quantitative analysis of the phospho-proteome of target cells in conjugation with NK cells is required and we have initiated stable isotope labelling with amino acids in cell culture (SILAC) to detect differences in phosphoprotein levels between non-conjugated and conjugated cancer cells. While these experiments are performed with high actin response-frequency cell lines (MDA-MB-231), this cell line still contains a mixture of susceptible and resistant subpopulations. We are still waiting for the results of the quantitative proteomic analysis, but utilization of the pre-challenged cancer model could also facilitate the read-out of this experimental approach.

Ultimately, there is the question of the translational approach. While the initial *in vivo* experiments included in this thesis suggest that the actin response is conserved and plays a role in shaping the TIME and modifying effector lymphocyte maturation and activation, we have yet to determine if this observation is merely correlative or causative. To establish our cell lines for *in vivo* experiments, we have modified the CDC42 pathway that is involved in a plethora of cellular functions, including migration, endocytosis, but also secretion. A targeted approach that prevents the actin response further upstream without alteration of key actin cytoskeleton regulators would provide a clearer view on the impact of ligand clustering on the TIME. Further, the ligand clustering at the AR-IS within a tumour needs to be confirmed, most likely using multiplex imaging or imaging mass cytometry, as complimentary approaches to confocal microscopy. In a second step, these results will also have to be confirmed in patient tumour samples. While we have preliminary results suggesting PD-L1 polarization not only within the tumour, but also on the subcellular levels towards infiltrating lymphocytes, this analysis requires a larger cohort of patient samples and – in combination with the previously mentioned ongoing experiments – establishment of a marker of the actin response, to facilitate identification of resistant subpopulations during immunohistochemically evaluation of tumour samples.

Concluding Remarks

This PhD thesis provides new insights into how tumour cells can exploit their actin cytoskeleton to enhance their immune evasive potential against NK cells. When we first described this mechanism in breast cancer cells, we termed it “actin response” and defined it as a characteristic of a resistant cancer cell subpopulation (**annex I**). Listed below is a summary of the main findings from scientific publications and the manuscript in progress:

- The actin response is conserved in chronic lymphocytic leukaemia (CLL) cell lines and patient derived CLL cells and a high frequency correlates with a molecular phenotype that is associated with a poor prognosis (**article I**)
- HLA-G expression on patient-derived CLL cells hinders stable IS formation with ILT-2^{+}/CD16^{-} NK-92MI cells that can be overcome with HLA-G blocking antibodies without modulation of the actin response frequency due to target cell opsonisation (**article I**)
- Pharmacological inhibition of CDC42 activation in CLL cell lines and *ex vivo* CLL cells partially reduces the actin response and increases susceptibility though improved transfer of granzyme B, but target cells resisting this treatment support the existence of a secondary signalling pathway involved in actin response initiation (**article I**)
- An actin response-competent subpopulation is found in a variety of solid and hematologic malignancies and its size correlates with resistance against NK cell-mediated target cell lysis in *in vitro* cytotoxicity assays (**article II**)
- An immunological synapse with an actin response is characterized by a wider synaptic cleft and insufficient activation of primary health donor NK cells as demonstrated by a lack of lytic granule polarization (**article II**)
- Polarization of the actin cytoskeleton in tumour cells is associated with the selective recruitment of inhibitory immune checkpoint ligands and this polarization to the immunological synapse correlates with inhibition of NK cell effector function and increased target cell survival (**article II**)
- The actin response-dependent ligand clustering is conserved in murine models of cancer and modulation of the actin response frequency in this cell lines leads to changes in susceptibility in *ex vivo* cytotoxicity assays (**article II**)
- A large actin response-competent cancer cell subpopulation correlates with accelerated tumour grow *in vivo* and reduced numbers of activated tumour infiltrating lymphocytes suggesting enhanced immune evasion (**article II**)
- The actin response is associated with a cold tumour and efficient immunosuppression of CTLs and NK cells (**article II**)

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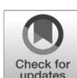
Annex I

Published OnlineFirst August 13, 2018; DOI: 10.1158/0008-5472.CAN-18-0441

Cancer Research

Tumor Biology and Immunology

Actin Cytoskeleton Remodeling Drives Breast Cancer Cell Escape from Natural Killer–Mediated Cytotoxicity



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Abstract

Elucidation of the underlying molecular mechanisms of immune evasion in cancer is critical for the development of immunotherapies aimed to restore and stimulate effective antitumor immunity. Here, we evaluate the role of the actin cytoskeleton in breast cancer cell resistance to cytotoxic natural killer (NK) cells. A significant fraction of breast cancer cells responded to NK-cell attack via a surprisingly rapid and massive accumulation of F-actin near the immunologic synapse, a process we termed "actin response." Live-cell imaging provided direct evidence that the actin response is associated with tumor cell resistance to NK-cell–mediated cell death. High-throughput imaging flow cytometry analyses showed that breast cancer cell lines highly resistant to NK cells were significantly enriched in actin response-competent cells as compared with susceptible cell lines. The actin response was not associated with a defect in NK-cell activation but correlated with reduced intracellular levels of the cytotoxic protease granzyme B and a lower rate of apoptosis in target cells. Inhibition of the actin response by knocking down CDC42 or N-WASP led to a significant increase in granzyme B levels in target cells and was sufficient to convert resistant breast cancer cell lines into a highly susceptible phenotype. The actin response and its protective effects were fully recapitulated using donor-derived primary NK cells as effector cells. Together, these findings establish the pivotal role of actin remodeling in breast cancer cell resistance to NK-cell–mediated killing.

Significance: These findings establish the pivotal role of the actin cytoskeleton in driving breast cancer cell resistance to natural killer cells, a subset of cytotoxic lymphocytes with important roles in innate antitumor immunity.

Cancer Res; 78(19); 5631–43. ©2018 AACR.

Introduction

Natural killer (NK) cells are lymphocytes of the innate immune system with cytotoxic activity that contributes to ridding the organism of pathogen-induced infections and cancer cells. Unlike other cytotoxic cells of the adaptive immune system, such as CD8$^+$ cytotoxic T lymphocytes (CTL), NK cells kill their targets on a short time scale without requiring preactivation via prior antigen exposure. Accordingly, they are frequently referred to as the first line of defense against cancer (1). NK cells recognize malignant cells by sensing a loss of inhibitory MHC class I molecules (missing-self) and/or an overexpression of activating stress-induced ligands (altered-self), such as the NKG2D receptor ligands ULBP1-6 and MICA/B (2, 3). The balance between inhibitory and activating signals critically determines the activation of NK-cell–mediated cytotoxicity toward their targets, although some complementary mechanisms have also been described (4). In addition to Ca2$^+$-dependent exocytosis of cytotoxic enzymes (perforin and granzymes) leading to caspase-dependent and -independent apoptosis, NK cells can also promote slow, caspase-dependent, cancer cell death via the engagement of death receptors (e.g., Fas/CD95, DR4, and DR5) on target cells by their cognate ligands FasL and TRAIL (5). Besides their direct cytotoxicity and the production of proinflammatory cytokines, NK cells modulate the activity of other immune cells (6, 7) and can prime dendritic cells to activate antitumor-specific CTL responses (8, 9).

Although NK cells hold promising cytotoxic activity against transformed cells, their numbers in solid tumors are usually low. To overcome the poor infiltration, an autologous or allogeneic NK cell transfer followed by adjuvant high dose IL2 has been clinically evaluated (10–12). Both treatment regimens proved to be partially effective but failed to induce durable remissions. Major

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-18-0441

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Published OnlineFirst August 13, 2018; DOI: 10.1158/0008-5472.CAN-18-0441

bottlenecks to clinical efficacy remain, including lack of consistent *in vivo* NK-cell expansion and tumor-induced immunosuppressive mechanisms. With the advent of efficient and FDA-approved autologous T-cell immunotherapy, interest in exploring the applicability of NK cells for use in cancer immunotherapies has been reawakened (13). Genetic modification of NK cells, as demonstrated for CAR T cells, could help to circumvent challenges posed by the tolerance to self-cells, helping establish NK cells as potent effectors against malignancy (14, 15). However, evasion mechanisms mounted by cancer cells and immunosuppressive tumor microenvironments remain major hurdles (13, 16, 17).

Upon recognition of tumor cells, NK cells undergo a series of specific steps leading to directed secretion of preformed lytic granules containing cytotoxic mediators, such as perforin and granzyme B (GzB). An early and key event in NK-mediated cell death is the formation of an immunologic synapse (IS) between the immune cell and its target, the ultimate function of which is to focus lytic granule secretion toward the target cell. Both the formation and activity of the IS critically rely on actin cytoskeleton rearrangements in NK cells (18–22). The initial steps of IS formation are associated with a prominent accumulation of actin filaments (AF), which facilitates the formation and stabilization of the so-called peripheral supramolecular activation cluster SMAC (23). Following actin reorganization, lytic granules converge on the microtubule-organizing center (MTOC) along microtubules. These lytic granules, together with the MTOC, subsequently polarize within the IS (24). High-resolution imaging studies have demonstrated that a dynamic meshwork of fine AFs is located at the center of the IS and this meshwork controls the delivery of myosin IIA-associated granules to specific membrane areas (25–28). The two major classes of actin nucleators (the ARP2/3 complex and formins) were found to play critical but distinct roles during NK-cell–mediated cytotoxicity (19). Recently, coronin 1A was reported to promote deconstruction of the synaptic cortical actin network (29), a process resulting in permissive "clearances" where granule secretion preferentially occurs (25).

In striking contrast to the well-characterized roles of the cell actin cytoskeleton of NK cells during target cell recognition and killing, little is known about actin configurations and functions in target cells during these processes. In the present study, we address this gap and provide for the first time direct and compelling evidence of the pivotal role of the tumor cell actin cytoskeleton in resistance to NK-cell–mediated cell lysis.

Materials and Methods

Cell lines and cell culture conditions

The breast adenocarcinoma cell lines used as target cells in this study were purchased from ATCC and include MCF-7, MDA-MB-231, T47D, and Hs578T cells. Each of these cell lines was authenticated and checked for not being cross-contaminated through STR profiling analysis (Microsynth). All cell lines were maintained in DMEM high glucose with L-glutamine medium (Lonza). The NK-92MI cell line was purchased from ATCC and cultured in RPMI-1640 (Lonza) supplemented with 10% (v/v) fetal bovine serum (FBS, Life Technologies) and 10% (v/v) horse serum (ATCC). All other media were supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Sigma-Aldrich). Cell lines were cultured in a humidified atmosphere at 5% CO_2 and 37°C and routinely checked for *Mycoplasma* contamination using the MycoAlert *Mycoplasma* detection kit (Lonza).

Isolation of human primary NK cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats provided by the Luxembourg Red Cross using Lymphoprep density gradient medium (StemCell). Briefly, samples were diluted 1:5 with PBS supplemented with 10% FBS. Thirty milliliters of diluted buffy coat was poured onto 15 mL of Lymphoprep medium in LeucoSep centrifuge tubes (Greiner Bio-One) and centrifuged for 30 minutes at $800 \times g$ in a swinging-bucket rotor with slow acceleration and no brake. The enriched PBMC fraction was harvested and washed twice with PBS containing 10% FBS. Remaining erythrocytes were lysed with 1× ACK lysis buffer for 5 minutes before NK-cell isolation using the MACS NK cell negative isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Human NK cells were cultured in RPMI medium (Lonza) supplemented with 10% (v/v) FBS, 10% (v/v) horse serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, and 100 U/mL recombinant human IL2 (PeproTech).

Cell transfection and pharmacologic treatments

MCF-7 cells were transfected with wild-type SNAIL and SNAIL-6SA (a constitutively active mutant of SNAIL) expression vectors. These vectors were obtained from Addgene (gift from Mien-Chie Hung; Addgene plasmids #16128 and #16221). Parental cells were transfected 48 hours prior to subsequent analyses using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific). The expression of recombinant SNAIL/SNAIL-6SA was confirmed by Western blotting. N-WASP and CDC42 knockdown was achieved by transfecting MCF-7 and MDA-MB-231 cell lines with *N-WASP* and *CDC42* siRNAs (siWASP#1 5′-GGUUUGUCGUAAUCCUCUATT-3′, siWASP#2 5′-CCUUAAUGUAAUUUACUUA-3′, siCdc42#1 5′-CAGCAAUG-CAGACAUUAATT-3′, and siCdc42#2 5′-CGAUGGUGCUGUUG-GUAAATT-3′). The nontargeting siCtrl was purchased from Qiagen. All cell lines were transfected 48 hours prior to each assay using DharmaFECT transfection reagent (GE Dharmacon), and knockdowns were confirmed by a Western blotting. The LifeAct-mEGFP-7 plasmid was obtained from Addgene (gift from Michael Davidson, Addgene plasmid #54610). The LifeAct-mEGFP fragment was subcloned into the lentiviral plasmid pCDH-EF1α-MCS-IRES-Puro (CD532A-2; System Biosciences) using the XbaI and BamHI restriction enzyme sites and NEBuilder HiFi DNA Assembly (NEB) to generate the pCDH-LifeAct-mEGFP expression plasmid used for lentiviral transduction. Transduced cells were selected with puromycin (0.5 μg/mL, Sigma-Aldrich).

In addition to SNAIL-induced epithelial-to-mesenchymal transition (EMT), MCF-7 cells were treated by either 5 ng/mL TGFβ (PeproTech) for 6 days or 10 ng/mL TNFα (BioLegend) for 3 days. Prior to TGFβ treatment, MCF-7 cells were starved for 24 hours (1% FBS).

For actin drug-based assays, target cells were treated with 0.5 μmol/L cytochalasin D or DMSO (Sigma; control) for 30 minutes and washed twice prior to presentation to NK cells.

Imaging flow cytometry

For conjugate formation, NK cells were counted, stained with anti-human CD56-PE-Cy7 (BioLegend, clone: HCD56), and washed before the direct contact with LifeAct-mEGFP-target cells at an effector-to-target (E:T) ratio of 5:1. Cells were coincubated for 30 minutes prior to fixation with 2% paraformaldehyde

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(Thermo Fisher Scientific) and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich). Afterward, cells were washed twice with PBS and then stained with anti-human GzB monoclonal antibody-APC (Thermo Fisher Scientific, clone: GB12), anti-γ-tubulin antibody-PE (Santa Cruz, clone: D-10), and DAPI (0.3 μg/mL, Sigma-Aldrich) for 20 minutes. The Amnis brand ImageStreamX Mark II (EMD Millipore) imaging flow cytometer with five built-in lasers (405, 488, 561, 640, and 785 nm) was used for acquisition. Using INSPIRE (EMD Millipore), 2×10^4 events were collected per tube at 60× magnification on a low speed and high-sensitivity settings. Unstained, single-stained, and Fluorescence Minus One-(FMO-) stained samples were collected for each experiment as controls. For better visualization of lytic granules, the extended depth of field was activated during the acquisition of GzB-containing samples. Ideas software (IDEAS 6.2.64.0, EMD Millipore) was used for data analysis. The gating strategy, masks, and features to analyze conjugates were created and applied. Features are used to calculate, analyze, and measure specific intensities in the cell while masks are designed to define a specific area of the cell where features could be applied. Masks can be established on the basis of the bright field or florescence image. The mask overlaying the synaptic region was created by Boolean logics of mEGFP-LifeAct and CD56-PeCy7, Dilate (mEGFP-LifeAct, 1) and Dilate (CD56-PE-Cy7,2). For actin response measurements, the mean fluorescence intensity (MFI) of mEGFP-LifeAct was calculated at the IS mask. To better calculate the intensity of the MTOC and improve the detection of γ-tubulin, the feature of Bright Detail Intensity R7 (BDI) was used. The software's BDI feature can compute the intensity of the bright spot that have radii smaller than 7 pixels. For GzB quantifications in target cells, the mask was defined on the mEGFP-LifeAct after subtracting the region of the immune synapse in order to exclude the intensity of GzB derived from NK cells. In order to assess apoptosis in target cells upon the contact with NK cells, the intensity of the signal of Annexin V–APC signal and DAPI was measured at the mask covering the mEGFP-LifeAct. The intensity of the Annexin V–PE was plotted versus the intensity of DAPI to distinguish between viable (Annexin V^-, $DAPI^-$), early apoptotic (Annexin V^+, $DAPI^-$), late apoptotic (Annexin V^+, $DAPI^+$), and necrotic cells (Annexin V^-, $DAPI^+$).

Target cell surface antigens were stained for 30 minutes prior incubation with NK cells, using the following antibodies: anti-human HLA-A, -B, -C-Brilliant Violet 605 (BioLegend; clone: W6/32), anti-human MICA/MICB-APC (BioLegend; clone: 6D4) and anti-human PD-L1-Brilliant Violet 605 (BioLegend; B7-H1, CD274) antibodies. The data were acquired on our imaging flow cytometer, and MFI values for each ligand were determined at the synaptic region using the above described IS mask.

Overall ligand expression at the cell membrane of control and N-WASP- and CDC42-depleted target cells was analyzed using a BD FACSAria II flow cytometer (BD Biosciences).

Statistical analysis

The unpaired Student *t* test in Microsoft Excel 2016 and GraphPad Prism was used to determine the statistical significance of the results obtained. For Annexin V experiment, a *Z*-score test for two population proportions was used to determine the statistical significance between samples. *, $P \leq 0.05$; **, $P \leq 0.01$ and ***, $P \leq 0.001$.

Results

Breast cancer cell resistance to NK-cell–mediated cell lysis is associated with a prominent "actin response"

To examine AF configurations in tumor cells during NK-cell attack, two breast adenocarcinoma cell lines were transduced with lentivirus to achieve stable expression of the actin reporter LifeAct-mEGFP (Fig. 1A; Supplementary Fig. S1A; ref. 30). The epithelial-like MCF-7 cell line (Supplementary Fig. S1B) was chosen for its high susceptibility to NK-cell–mediated lysis, whereas the mesenchymal-like MDA-MB-231 cell line was chosen for its highly resistant phenotype. As shown in Fig. 1B, MDA-MB-231 cells were almost three times less susceptible to NK-mediated lysis when compared with MCF7 cells. Only 13% of MDA-MB-231 cells were killed by NK-92MI cells at an E:T ratio of 5:1 after 4 hours, while 42% of MCF-7 cells were killed under the same conditions. A confocal microscopy analysis conducted 30 minutes after tumor cells were exposed to NK cells revealed that most NK-cell–conjugated MDA-MB-231 cells exhibited a massive accumulation of actin near the IS, hereinafter referred to as the "actin response" (Fig. 1C; Supplementary Movie S1). In contrast, this actin response was rarely observed in NK-cell–conjugated MCF-7 cells. Instead, most conjugated MCF-7 cells exhibited a rather homogeneous distribution of actin at their cortex (Fig. 1D; Supplementary Movie S2).

The actin response was induced remarkably fast, as evidenced by the many MDA-MB-231 cells showing synaptic actin accumulation, in as little as 2 minutes after presentation to NK cells. Target–effector cell conjugates were tracked over long periods of time using live-cell imaging, and time-lapse movies were assembled. In MDA-MB-231 cells, the actin response persisted throughout the whole duration of the interaction between the cancer and immune cells (Fig. 1E; Supplementary Movie S3).

After about 30 minutes, the NK cell detached without achieving lysis of its target, and the actin response in the escaped cancer cell rapidly ceased. As exemplified in Fig. 1E and Supplementary Movie S3, the escaped cells were still alive at the end of the recording (120 minutes; Fig. 1E; Supplementary Movie S3). In most MCF-7 cells, no actin response could be observed at any time during the interaction (Fig. 1F; Supplementary Movie S4). Multiple membrane blebs were detected at the target cell surface after 1 hour, indicating that the target cell entered apoptosis. A few minutes later, the target cell lysed, and the green fluorescence of the actin reporter disappeared.

Quantification of GFP fluorescence showed that, in MDA-MB-231 cells, the cortical region that included the IS contained on average about two times more F-actin as compared with the opposite side of the cell, whereas no statistically significant asymmetrical distribution of actin was found in MCF-7 cells ($n = 25$; Fig. 2A). Nevertheless, we noticed that a few MDA-MB-231 cells in conjugate with NK cells did not show the typical actin response; conversely, a number of MCF-7 cells accumulated F-actin at the IS. We accordingly assumed that each cell line comprised two cell subpopulations differing in their ability to remodel their actin cytoskeleton in response to NK-cell attack. To further characterize these subpopulations, we used high-throughput imaging flow cytometry and analyzed a large number of cell conjugates. Breast cancer cells were cocultured with NK cells for 30 minutes prior to fixation and permeabilization. Typically, 2×10^4 events were acquired on the imaging flow cytometer, and a gating strategy was applied to select in-focus, live, and interacting

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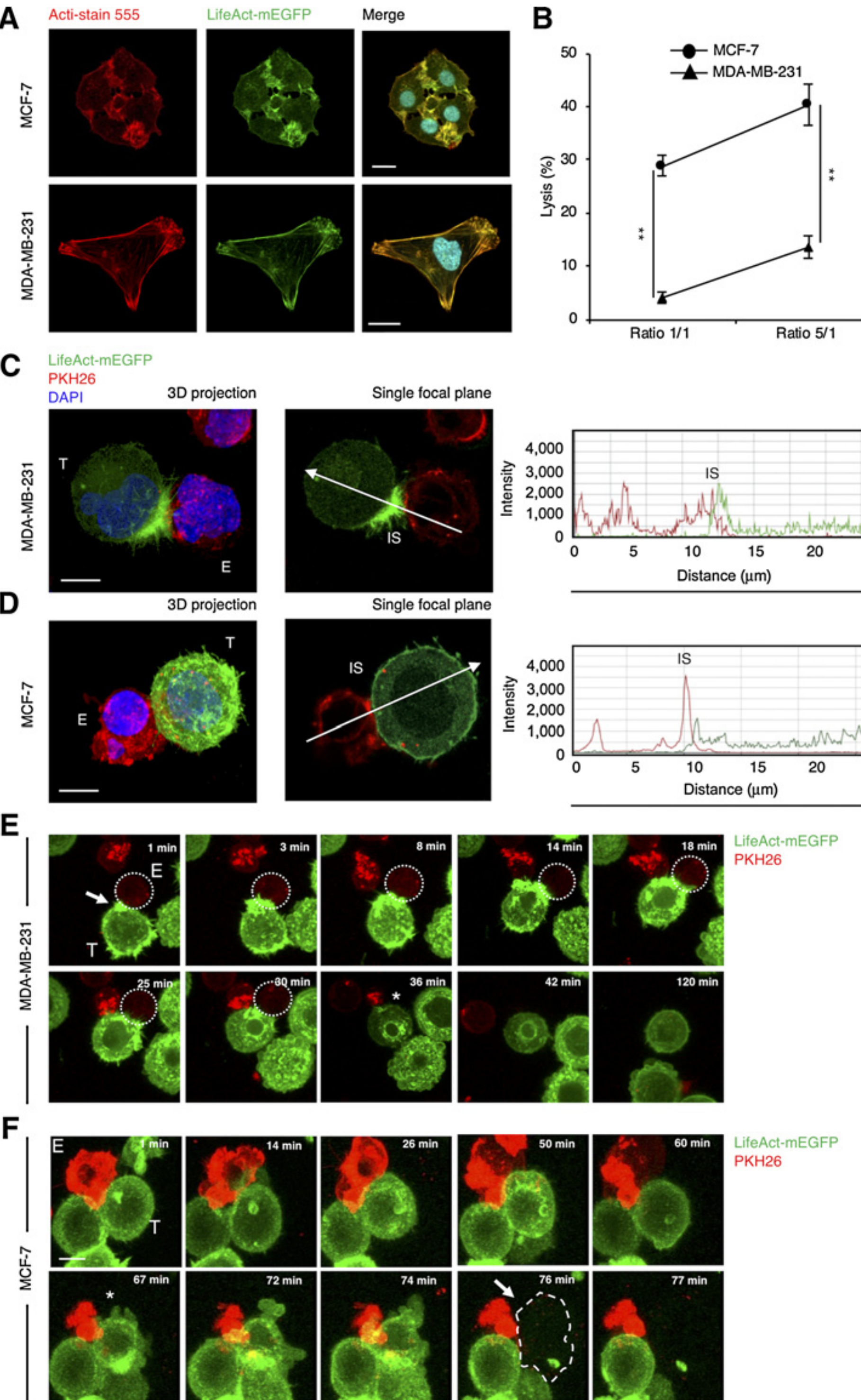

Figure 1.
Breast cancer cell line resistance to NK-cell–mediated cell death is associated with prominent F-actin accumulation near the immunologic synapse. **A,** Both MCF-7 and MDA-MB-231 cells were transduced to express the actin cytoskeleton reporter LifeAct-mEGFP (green). Stable cell lines were stained with Acti-stain 555 phalloidin (red) and DAPI (cyan). The yellow-orange signal in the merged images shows an extensive colocalization between the two actin probes. **B,** Cytotoxicity assays performed in three independent experiments with NK92MI cells (effectors) and MCF-7 or MDA-MB-231 cells (targets) at 1:1 and 5:1 effector:target ratios. The unpaired Student *t* test was applied to determine the statistical significance; **, $P < 0.01$. **C** and **D,** Confocal microscopy images showing the typical actin cytoskeleton organization in most MDA-MB-231 (**C**) and MCF-7 (**D**) target cells (green; T) in conjugation with PKH26-stained effector NK92MI cells (red; E). The charts on the right show the relative fluorescence intensities of LifeAct-mEGFP and PKH26 along the trajectories indicated on the corresponding confocal pictures. The region of the immunologic synapse is indicated by "IS." Note the spike in the fluorescent actin signal at the IS in MDA-MB-231 cells. Scale bars, 20 μm (**A**); 10 μm (**C** and **D**). Images are examples of at least 25 cells and three independent experiments. **E** and **F,** Time lapse imaging showing typical actin configurations over time in MDA-MB-231 (**E**) and MCF-7 (**F**) cells upon NK-cell attack. The arrow and the asterisk in **E** indicate the initiation of the actin response, and the detachment of the NK cell from its target and the subsequent cessation of the actin response in the escaped cancer cell, respectively. The asterisk and the arrow in **F** indicate the appearance of membrane blebs in the dying target cell and subsequent target cell lysis. Supplementary Movies S3 and S4 show the entire time-lapse movies (120 minutes) corresponding to **E** and **F**, respectively. Scale bars, 10 μm.

effector–target cell pairs (Fig. 2B). For analysis, a mask was manually generated using the IDEAS software to define the region of interest corresponding to the IS and its surrounding intracellular areas, hereinafter referred to as the "synaptic region" (Supplementary Fig. S1C). Such a mask was automatically applied to the data sets generated by imaging flow cytometry and confirmed to efficiently capture the actin response as exemplified in Fig. 2C.

For each cell line, the subpopulations of cells with or without an actin response were discriminated by comparing the relative intensity of LifeAct-mEGFP in the synaptic region and in the entire cell (Supplementary Fig. S1D). From three independent experiments, including a total of at least 500 conjugates, we calculated that 62% of MDA-MB-231 cells conjugated with NK cells exhibited an actin response, whereas the remaining 38% did

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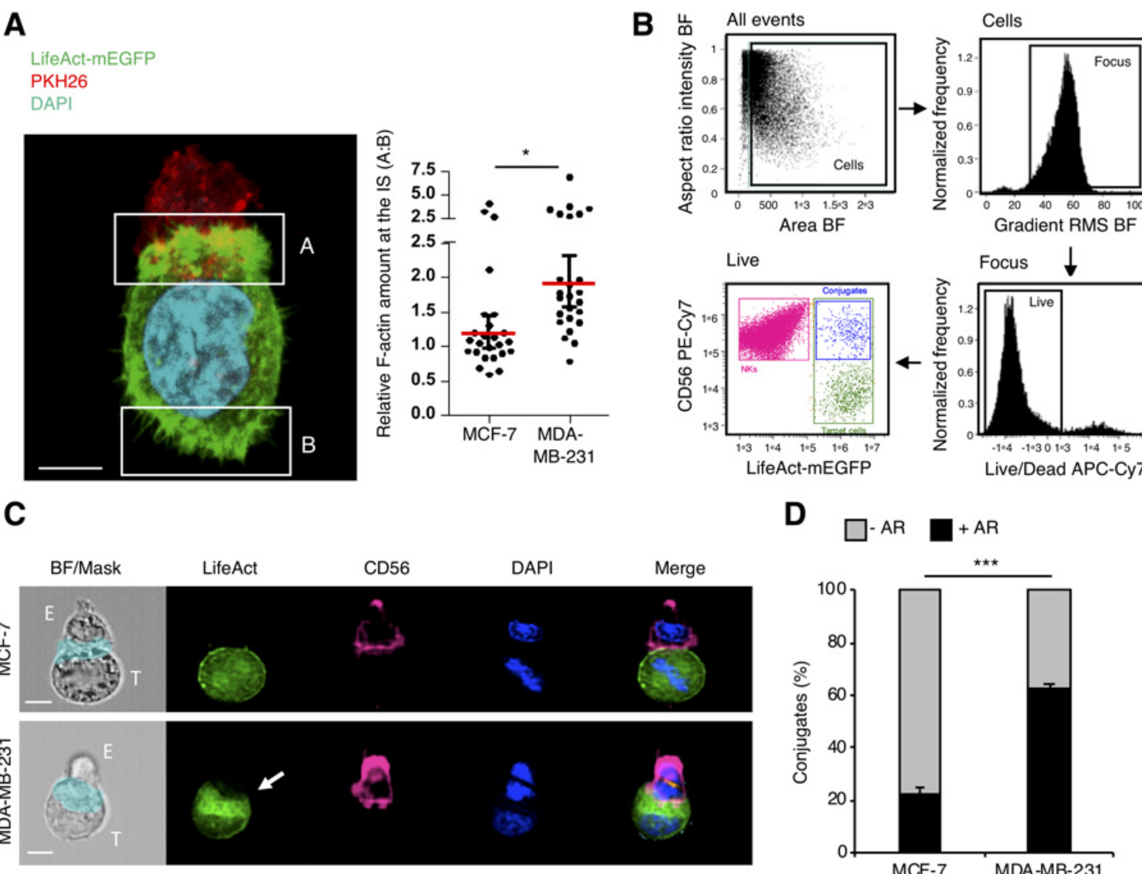

Figure 2.

Quantitative analysis of the actin response in susceptible and resistant breast cancer lines. **A,** Relative amounts of F-actin in tumor cells near the immunologic synapse. The left confocal image shows a typical conjugate between NK (red) and MDA-MB-231 (green) cells. The two white rectangles labeled A and B depict the regions of interest used for LifeAct-mEGFP fluorescence quantification. The right chart shows F-actin enrichment in the synaptic region of 25 MCF-7 and MDA-MB-231 cells conjugated with NK cells, as calculated by the ratio A:B. **B,** Gating strategy was used to identify NK-cell–target cell doublets using imaging flow cytometry. Cells were selected based on size, in order to exclude beads, by setting threshold values in a plot of area versus aspect ratio of events measured in the bright-field channel (top left). Focused events were then selected from a histogram plot of gradient root-mean-square of the bright-field channel (top right). Live cells were then selected for by setting threshold values for APC-Cy7 (Live/Dead) intensity (bottom right). Finally, the double-positive population (LifeAct-mEGFP for target cells and CD56-PeCy7 for NK cells) was selected as conjugates (bottom left). **C,** Typical examples of imaging flow cytometry panels obtained for conjugates between NK cells and MCF-7 cells (top row) or MDA-MB-231 cells (bottom row). The cyan area in the BF image depicts the mask applied to capture LifeAct signal near the immunologic synapse. Note the prominent actin response (arrow) in the MDA-MB-231 cell. **D,** Percentage of NK-cell–conjugated target cells with (black; +AR) and without (light gray; −AR) actin response in each cell line. Data are graphed as mean ± SE from three independent experiments including a total of 500 cell conjugates. The two-tailed unpaired Student *t* test was applied to determine the statistical significance. ***, $P < 0.001$. Scale bars, 10 μm (**A**); 7 μm (**C**).

not (Fig. 2D). The opposite results were obtained for the MCF-7 cell line, in which only 22% of conjugated cells showed an actin response.

To extend the above data and further evaluate the relationship between tumor cell resistance to NK-mediated cell death and the actin response, we analyzed two additional breast cancer cell lines, the epithelial-like T47D cell line, and the mesenchymal-like Hs578T cell line (Supplementary Fig. S1B). As previously described, these cell lines were transduced to stably express LifeAct-mEGFP in order to image actin in target cells only, and to exclude any potential spillover effects from the actin cytoskeleton of NK cells (Supplementary Figs. S1A and S2A). Cytotoxic assays revealed that, similar to MDA-MB-231 cells, Hs578T cells were highly resistant to NK-cell lysis, displaying an NK-cell–mediated cytotoxicity of 13% at an E:T ratio of 5:1 (Fig. 3A). In addition, Hs578T cells were highly competent for the actin response, and more than 60% of conjugated tumor cells exhibited F-actin accumulation in the synaptic region (Fig. 3B; Supplementary Fig. S2B). In comparison, T47D cells were significantly more susceptible to NK-cell–induced lysis, and 36% displayed immune cell–mediated lysis at an E:T ratio of 5:1 (Fig. 3A). These cells also exhibited a reduced ability to respond to NK-cell attack by an actin response (<30% of conjugated cells; Fig. 3B; Supplementary Fig. S2B). Interestingly, the amplitude of the actin response was relatively similar across cell lines with 30% to 45% of total cell F-actin accumulated at the synapse (Fig. 3C).

Taken together, our data reveal that a subpopulation of tumor cells responds to NK-cell attack by fast remodeling of their actin cytoskeleton, leading to a massive F-actin accumulation in the synaptic region. This is a process we termed the actin response.

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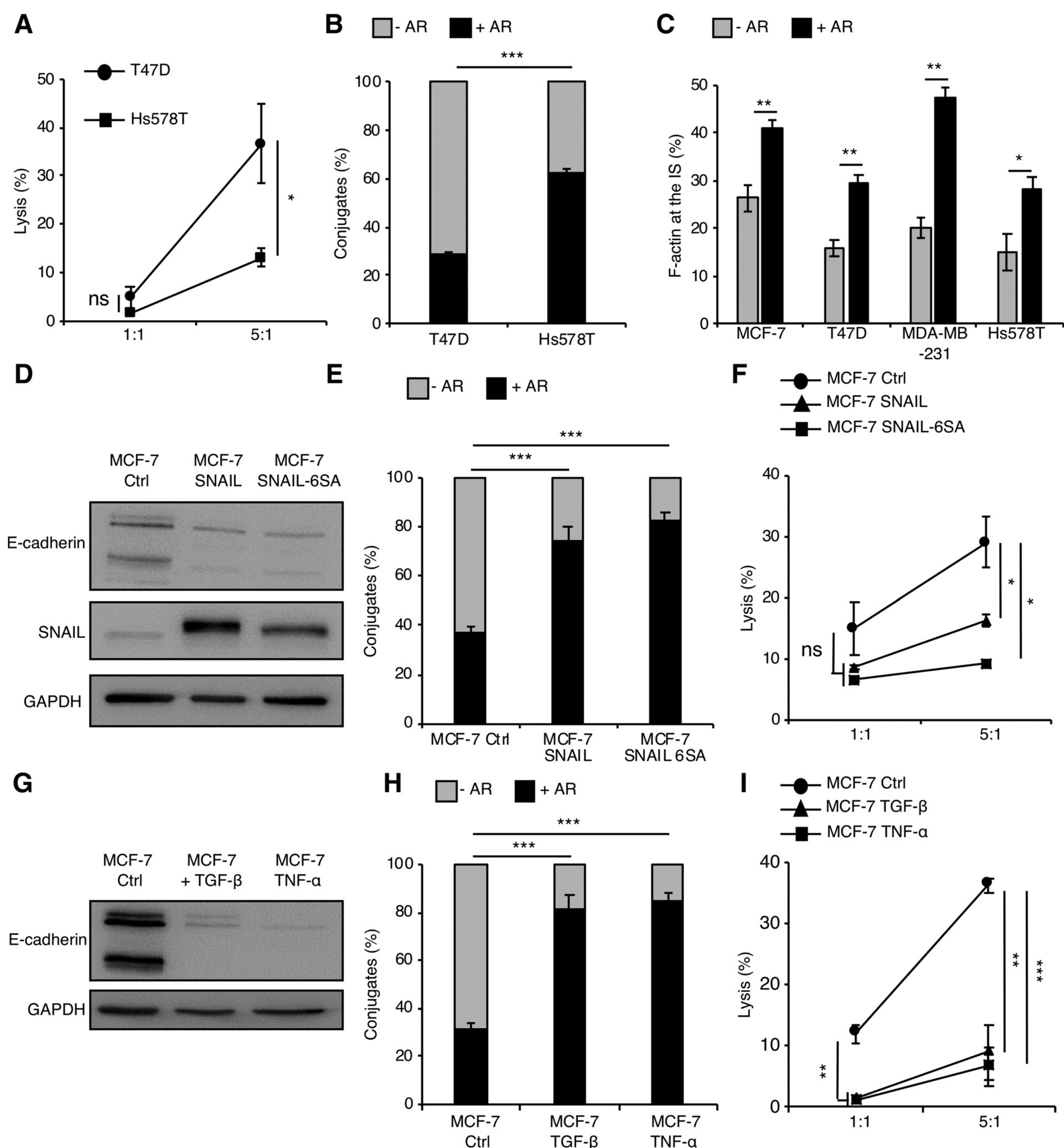

Figure 3.

The actin response is conserved among breast cancer cell lines and is enhanced by epithelial–mesenchymal transition. **A,** Cytotoxicity assays performed in three independent experiments with NK92MI cells (effectors) and T47D and Hs578T cells (targets) at 1:1 and 5:1 effector:target ratios. **B,** Percentage of NK-cell–conjugated target cells with (black; +AR) and without (light gray; −AR) actin response in T47D or Hs578T cell lines. Data are graphed as mean ± SE from three independent experiments including a total of 500 cell conjugates. **C,** Percentage of total cell F-actin in the synaptic region of target cells with (black; +AR) or without (light gray; −AR) actin response for different breast cancer cell lines. Data are shown as mean ± SE calculated from three independent experiments including a total of at least 300 cell conjugates. **D,** Western blot showing the expression of SNAIL and E-cadherin in transfected-only control MCF-7 cells (MCF-7 Ctrl), SNAIL-transfected MCF-7 cells (MCF-7 SNAIL), and SNAIL-6SA transfected MCF-7 cells (MCF-7 SNAIL-6SA). GAPDH was used as a loading control. **E,** Percentage of NK-cell–conjugated target cells with (black; +AR) and without (light gray; − AR) actin response in MCF-7 Ctrl, MCF-7 SNAIL, and MCF-7 SNAIL-6SA. **F,** Cytotoxicity assays performed in three independent experiments with NK92MI cells and either MCF-7 Ctrl, MCF-7 SNAIL, and MCF-7 SNAIL-6SA cells at 1:1 and 5:1 effector:target ratios. **G,** Western blot showing the expression of E-cadherin in mock-treated MCF-7 cells (MCF-7 Ctrl), TGFβ-treated (6 days, 5 ng/mL) MCF-7 cells (MCF-7 TGFβ) and TNFα-treated (3 days, 10 ng/mL) MCF-7 cells (MCF-7 TNFα). GAPDH was used as a loading control. **H,** Percentage of NK-cell–conjugated target cells with (black; +AR) and without (light gray; −AR) actin response in MCF-7 Ctrl, MCF-7 TGFβ, and MCF-7 TNFα. **I,** Cytotoxicity assays performed in three independent experiments with NK92MI cells and either MCF-7 Ctrl, MCF-7 TGFβ, or MCF-7 TNFα at 1:1 and 5:1 effector:target ratios. The two-tailed unpaired Student *t* test was applied to determine the statistical significance. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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Remarkably, this subpopulation was significantly larger in NK cell-resistant breast cancer cell lines than in more susceptible cell lines, suggesting that the actin response contributes to the resistant phenotype. In addition, our data point to a potential link between the EMT status of tumor cells and the actin response. Indeed, mesenchymal-like breast cancer cell lines, including MDA-MB-231 and Hs578T, proved to be significantly more competent for the actin response when compared with the epithelial-like ones, including MCF-7 and T47D (Figs. 2D and 3B; Supplementary Fig. S1B).

EMT enhances the actin response frequency and breast cancer cell line resistance to NK-cell–mediated lysis

To further evaluate the relationship between EMT, actin response, and resistance to NK-cell lysis, EMT was experimentally induced in MCF-7 cells through transient expression of the wild-type EMT inducer SNAIL, or its degradation-resistant variant SNAIL-6SA variant. The mesenchymal transition of SNAIL- and SNAIL-6SA–expressing cell lines was evident by the adaption of a spindle-like morphology and reduced cell–cell contacts, as well as by the loss of E-cadherin (Fig. 3D). Both SNAIL- and SNAIL-6SA–overexpressing cells exhibited a robust increase (>2-fold) in the subpopulation of cells responding to NK-cell attack via synaptic F-actin accumulation as compared with control, transfected only, MCF-7 cells (Fig. 3E; Supplementary Fig. S2B). Consistent with its enhanced stability, SNAIL-6SA had a stronger effect than wild-type SNAIL, resulting in 82% and 74% of conjugated cells exhibiting an actin response, respectively (Fig. 3E). Forced expression of SNAIL or SNAIL-6SA also dramatically decreased MCF-7 cell susceptibility to NK-cell–mediated lysis. At an E:T ratio of 5:1, SNAIL- and SNAIL-6SA–expressing MCF-7 cells were 2- and 3-fold more resistant to NK-cell lysis when compared with the control cells, respectively (Fig. 3F).

In addition to SNAIL-induced EMT, we treated MCF-7 cells with either TGFβ or TNFα, and subsequently analyzed their competency for the actin response and their susceptibility to NK-cell–mediated cell lysis. Treatment efficacy was validated by E-cadherin downregulation and changes in cell morphology (Fig. 3G). Consistent with the above data, both treatments strongly increased the rate of actin response in MCF-7 cells as well as MCF-7 cell resistance to NK cells (Fig. 3H and I).

Together, our data indicate that the EMT status is a critical determinant of the capacity of breast cancer cells to remodel their actin cytoskeleton upon NK-cell attack, and to escape from NK-cell–mediated lysis.

The actin response drives breast cancer cell resistance to NK-mediated cell lysis

We next aimed to evaluate the causal link between the actin response and breast cancer cell resistance to NK-cell–mediated lysis. First, we examined whether inhibition of synaptic F-actin accumulation affected breast cancer cell survival in cytotoxicity assays. Actin polymerization was impaired in target cells by knocking down either N-WASP (a key regulator of the ARP2/3 complex) or the Rho-subfamily small GTPase CDC42 (its upstream regulator; ref. 31). Resistant MDA-MB-231 and susceptible MCF-7 cell lines were transfected with small interfering RNA constructs (siRNAs) targeting *N-WASP* or *CDC42* transcripts (siN-WASP and siCDC42, respectively). The two independent siRNAs used to target each transcript significantly decreased the corresponding protein expression levels in the two cell lines (Fig. 4A). Imaging flow cytometry analysis showed that N-WASP- and CDC42 depletion reduced the subpopulation of actin response-competent cells in MDA-MB-231 cells by 2- to 3-fold (Fig. 4B). This inhibition of the actin response was associated with a significant increase in target cell susceptibility to NK-cell–mediated lysis, ranging from 50% to 150%. Conversely, N-WASP or CDC42 knockdown did not significantly affect the already reduced size of this subpopulation (around 20%) of MCF-7 cells that produce an actin response upon NK-cell attack (Fig. 4C), nor did it increase the susceptibility of MCF-7 cells to NK-cell lysis. The depletion of N-WASP or CDC42 was verified not to modify the overall expression of important ligands of NK-cell receptors at the target cell surface, including HLA-A, -B, -C, MICA/B, and program cell death-ligand 1 (PD-L1; Supplementary Fig. S3A–S3C), supporting our hypothesis that the increase of MDA-MB-231 cell susceptibility following N-WASP or CDC42 depletion is indeed the result of actin response inhibition.

We next quantified the extent of target cell apoptosis associated with the actin response. Briefly, target cells were incubated for 30 minutes with NK cells, labeled with Annexin V–APC and DAPI, and subsequently analyzed by imaging flow cytometry. For the analysis, a new mask was designed to capture Annexin V–APC and DAPI signals only in the LifeAct-mEGFP–expressing target cell from conjugates (Fig. 4D). For both MDA-MB-231 and MCF-7 cell lines, the number of apoptotic cells was considerably higher in the subpopulation of cells without an actin response when compared with the subpopulation of cells with an actin response. Consistent with the reduced subpopulation of actin response-competent cells in the MCF-7 cell line, this cell line contained a much higher total number of apoptotic cells when compared with the MDA-MB-231 cell line. Altogether, the above data provide further evidence that the actin response mediates breast cancer cell resistance to NK-cell–mediated lysis. The data also show that the inhibition of this process is sufficient to restore susceptibility in otherwise resistant breast cancer cells.

The actin response is associated with changes in NK-cell receptor ligand density in the synaptic region of target cells

The possibility that the actin response was associated with local changes in the density of HLA-A, -B, -C, MICA/B, and PD-L1 on target cell surface was evaluated using imaging flow cytometry. Very interestingly, both HLA-A, -B, -C ligands and PD-L1 levels were increased (by 100% and 50%, respectively), while MICA/B ligands were modestly reduced (30%), at the synaptic region of MDA-MB-231 cells with an actin response as compared with MDA-MB-231 cells without an actin response (Supplementary Fig. S3D–S3F). Likewise, HLA-A, -B, -C ligands were significantly enriched at the synapse of MCF-7 cells with an actin response as compared with MCF-7 cells without an actin response (Supplementary Fig. S3D). However, synaptic MICA/B levels did not significantly differ in the two MCF-7 cells subpopulations (Supplementary Fig. S3E). PD-L1 was not amenable to quantification in the synaptic region of MCF-7 cells by imaging flow cytometry in our experimental setup. Together, these data suggest that the actin response increases inhibitory ligands, and possibly decreases activating ligands, at the synaptic region of target cells, respectively, which in turn may alter NK-cell function.

We then evaluated if the actin response was associated with a defect in NK-cell activation. To do so, we quantified MTOC polarization in NK cells conjugated with MCF-7 or MDA-MB-

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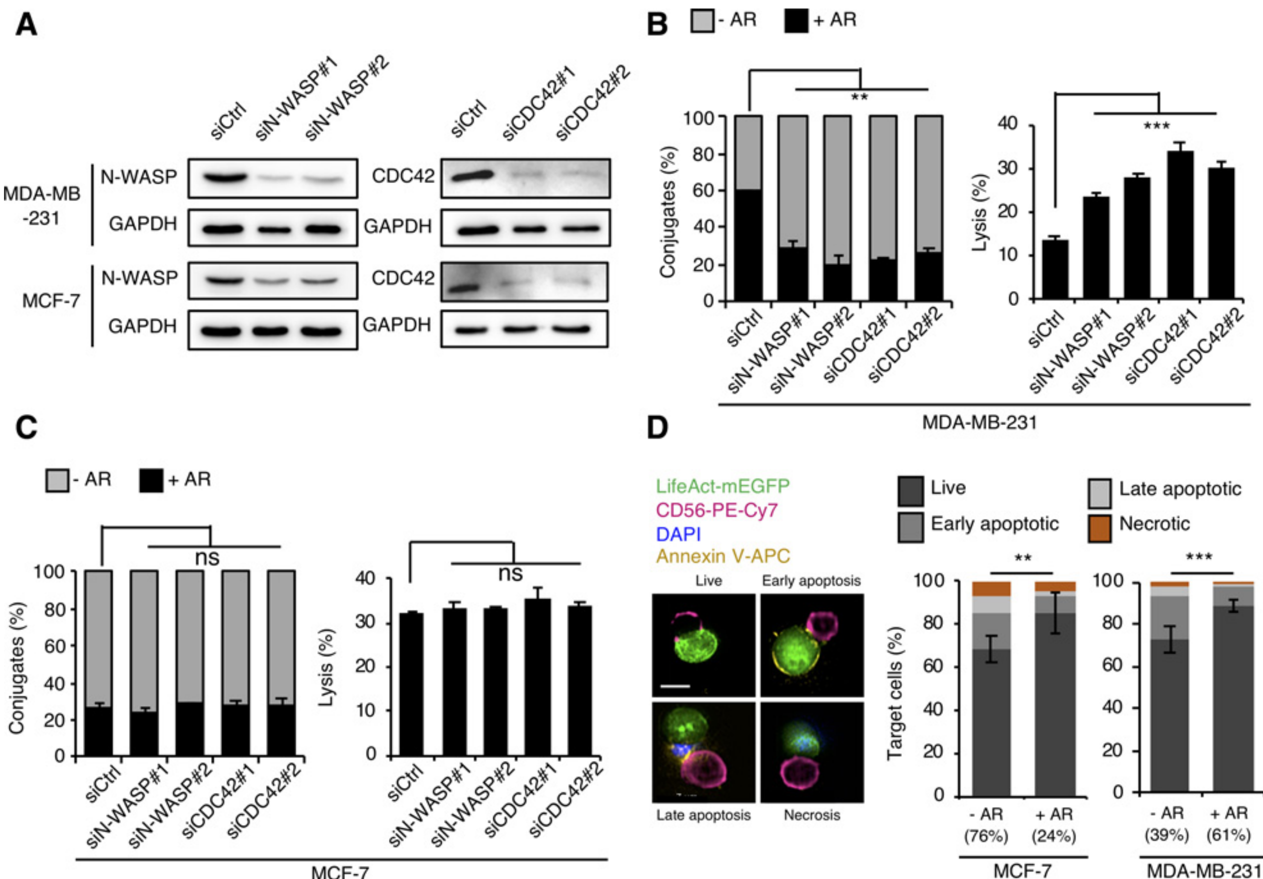

Figure 4.

Targeting the actin response increases tumor cell susceptibility to NK-cell–mediated cell lysis. **A,** MDA-MB-231 and MCF-7 cells were transfected with a control siRNA or different siRNAs targeting N-WASP or CDC42. Forty-eight hours after transfection, cells were lysed and levels of the specified proteins were analyzed by Western blotting. GAPDH was used as a loading control. The blots shown are representative of three independent experiments. **B** and **C,** Actin response frequency and cell susceptibility to NK-cell–induced lysis in N-WASP– and CDC42-deplete MDA-MB-231 and MCF-7 cells. Left, percentage of NK-cell–conjugated target cells with (black; +AR) and without (light gray; −AR) actin response in the indicated siRNA-transfected cell lines. Data are graphed as mean ± SE from three independent experiments including a total of 350 cell conjugates. Right, cytotoxicity assays performed in three independent experiments with NK92MI cells (effectors) and the indicated siRNA-transfected cell lines at 1:1 and 5:1 effector:target ratios. The two-tailed unpaired Student *t* test was used to determine statistical differences between the groups. **D,** MCF-7 and MDA-MB-231 cells were incubated with NK cells for 30 minutes and the percentage of unaffected ($DAPI^-$; Annexin V^-), early apoptotic ($DAPI^-$; Annexin V^+), late apoptotic ($DAPI^+$; Annexin V^+) and necrotic ($DAPI^+$; Annexin V^-) target cells conjugated with an NK cell was determined using imaging flow cytometry. Data are graphed as mean ± SE from three independent experiments including a total of 300 cell conjugates and in respect to the presence (+ AR) or absence (−AR) of an actin response in target cells. The percentage of cells with or without an actin response is indicated below each bar of the chart. A *Z*-score test for two population proportions was used to determine the statistical significance between samples. Representative images of each possible type of cell conjugate are shown above the chart. Scale bar, 7 μm. Statistically significant differences are indicated by asterisks (**, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant).

231 cells. The two cell lines induced a similar overall polarization of the MTOC in NK cells (Fig. 5A). Moreover, MTOC polarization was not statistically different in NK cells conjugated with target cells with or without an actin response (Fig. 5B). In addition, MCF-7 and MDA-MB-231 cell lines induced similar NK-cell degranulation as evaluated by the degranulation marker lysosome-associated membrane protein-1 (LAMP-1)/CD107a (Fig. 5C; refs. 32, 33). However, these results do not totally rule out a role for the actin response in blocking NK-cell activation through modifying the ligand density at the synaptic region. Noticeably, NK92MI cells are a highly cytotoxic NK cell line lacking most of the killer inhibitory receptors (KIR) and, accordingly, are insensitive to an increase of HLA-A, -B, -C ligands (Supplementary Fig. S3D; refs. 34–36).

The actin response reduces GzB levels in NK-cell–conjugated target cells

Following IS formation, NK cells directionally exocytose specialized secretory lysosomes containing cytotoxic proteins. Among these, the serine protease GzB is one of the central mediators of target cell death. We assessed the possibility that the actin response may alter GzB levels in target cells. First, we quantified GzB levels in NK-cell–conjugated target cells with or without an actin response using imaging flow cytometry. To exclude the bulk of GzB contained in effector cells and exclusively focus on the GzB fraction that is transferred to target cells, an appropriate mask was designed for data analysis (Fig. 5D). The poorly susceptible MDA-MB-231 cells were used as targets. We found that GzB levels were markedly reduced (by about 50%) in

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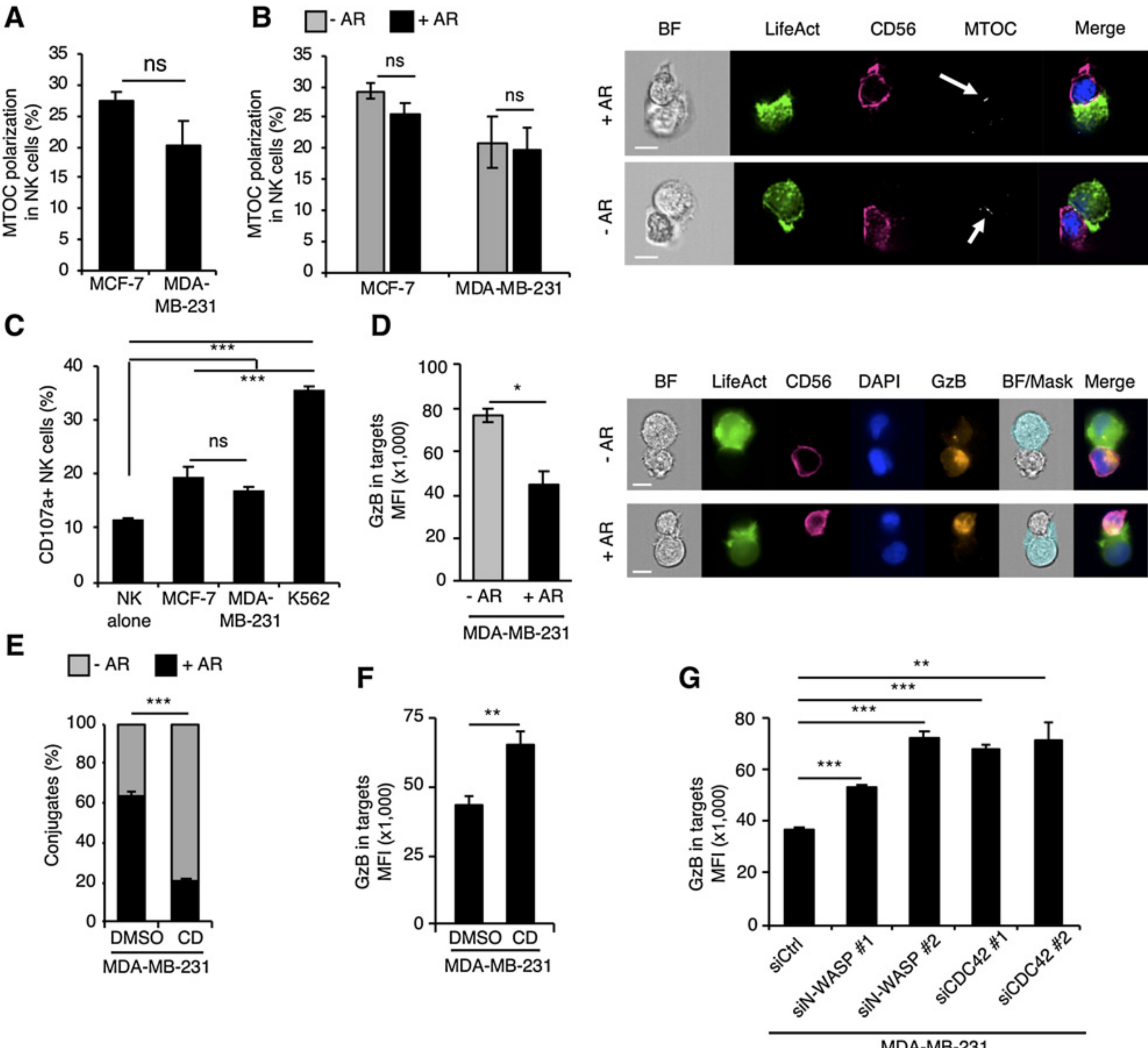

Figure 5.

The actin response is associated with significant decrease in GzB levels in target cells. **A,** Overall percentage of NK cells conjugated with MCF-7 or MDA-MB-231 cells exhibiting MTOC polarization toward the IS after 30 minutes of coincubation. Data are given as mean ± SE from three independent experiments including a total of 300 cell conjugates. **B,** Percentage of NK cells exhibiting MTOC polarization toward the IS in the presence (black; +AR) or absence (light gray; −AR) of actin response in the conjugated target cells. The panels on the right show examples of imaging flow cytometry images with polarized MTOC (arrows) in NK cells conjugated with MDA-MB-231 cells with (top; +AR) and without actin response (bottom; −AR). **C,** Percentage of CD107a-positive NK cells after 4 hours of coincubation with MCF-7 and MDA-MB-231 cell lines. NK cells without target cell were used as a negative control, while coincubation with K562 cells was used as a positive control. Data are given as mean ± SE from three independent experiments. **D,** GzB levels in NK-cell–conjugated MDA-MB-231 cells with (black; +AR) and without (light gray; − AR) actin response. Right, representative imaging flow cytometry images used for quantification. The light blue mask on the bright-field channel depicts the region of interest in which the GzB fluorescent signal was quantified. Note that NK-cell–contained GzB was totally excluded. Scale bars, 7 μm. **E,** Percentage of NK-cell–conjugated MDA-MB-231 cells with (black; +AR) and without (light gray; −AR) actin response after control (DMSO) and CD treatment. Data are given as mean ± SE from three independent experiments including a total of 300 cell conjugates. **F,** GzB levels in control (DMSO) and CD-pretreated MDA-MB-231 cells conjugated with NK cells. In all, about 200 conjugated target cells were scored. Data originate from three independent experiments and are expressed as MFI. **G,** MDA-MB-231 cells were transfected with a control siRNA or different siRNAs targeting N-WASP or CDC42. GzB levels in NK-cell–conjugated target cells were analyzed imaging flow cytometry. In all, about 350 conjugated target cells were scored. Data originate from three independent experiments and are expressed as MFI. The two-tailed unpaired Student *t* test was used to measure the statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant).

the cell subpopulation with an actin response compared with the cell subpopulation lacking an actin response. Next, we analyzed the effects of pharmacologic disruption of the actin response on GzB levels in target cells. The concentration of the actin-depolymerizing drug cytochalasin D (CD) and the treatment duration were optimized to disrupt most filamentous actin in

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MDA-MB-231 cells, without compromising cell viability (Supplementary Fig. S4A and S4B). In addition, CD was carefully washed out prior to target cell incubation with NK cells in order not to impair their function, leaving a reduced, but sufficient, time window for analysis before substantial actin cytoskeleton recovery (Supplementary Fig. S4C). As shown in Fig. 5E, CD treatment strongly reduced the subpopulation of actin response–competent target cells by 3-fold. Along with this effect, CD treatment significantly increased the overall average level of GzB in conjugated target cells, indicating that the actin response was causally linked to GzB level reduction (Fig. 5F). To rule out any potential effect of residual amounts of CD on NK-cell activity, we also quantified GzB levels in target cells in which the actin response was genetically impaired via N-WASP or CDC42 knockdown (see Fig. 4). Consistent with the above data, N-WASP and CDC42 depletion significantly increased the overall average level of GzB in target cells by about 2-fold (Fig. 5G). Stratifying the data according to the presence or absence of an actin response revealed that the fraction of cells that resisted F-actin disruption and responded to NK attack by synaptic actin accumulation maintained reduced levels of GzB (Supplementary Fig. S5). Collectively, our data indicate that the actin response protects target cells from lysis by limiting GzB accumulation.

The actin response is similarly induced by donor-derived NK-cell–mediated lysis

We asked whether the protection of breast cancer cells by the actin response was restricted to cytotoxicity induced by the NK-92MI cell line used in this study. To address this question, primary NK cells were isolated from buffy coats of five human donors by negative selection (>99% purity; Supplementary Fig. S6), and they were used in the subsequent analyses as effector cells. First, we evaluated primary NK-cell cytotoxicity against the MCF-7 and MDA-MB-231 cell lines (Fig. 6A). As expected, donor-derived NK cells exhibited reduced cytotoxic activity compared with the highly cytotoxic NK-92MI cell line, and only modest target cell killing (1%–10%) was achieved at an E:T ratio of 1:1. However, significant target cell death (5%–33%) was induced at an E:T ratio of 5:1, confirming that isolated primary NK cells were functional. Despite interindividual variability in the cytotoxic potential, NK cells from all five donors more effectively killed MCF-7 cells when compared with MDA-MB-231 cells. Consistent with our previous data, confocal microscopy revealed that a majority of MDA-MB-231 cells conjugated with primary NK cells exhibited an actin response (Fig. 6B), whereas most MCF-7 cells did not. Quantitative analysis using imaging flow cytometry analyses established that the actin response was at least twice as frequent in MDA-MB-231 cells (60%–76%) as compared with MCF-7 cells (26%–37%; Fig. 6C). In addition, for each of the combinations of the five sources of primary NK cells and the two target cell lines, we found significantly lower GzB levels in target cells exhibiting an actin response when compared with those without an actin response (Fig. 6D and E). In conclusion, the actin response and its protective effects were fully recapitulated using donor-derived primary NK cells as effector cells.

Discussion

Tumor escape from cytotoxic immune cells is a major hurdle for achieving efficacious immunotherapies. Here, we demonstrate a critical role for the actin cytoskeleton in driving breast cancer cell resistance to NK-cell–mediated lysis. Using high-throughput imaging flow cytometry, we found that common tumor cell lines contain two subpopulations of cells differing in their capacity to respond to NK-cell attack via fast and prominent accumulation of AFs near the IS. Remarkably, the rate of the "actin response" in a given cell line is inversely correlated with the overall susceptibility of this cell line to NK-cell–mediated lysis. Live-cell imaging provided direct evidence that tumor cells exhibiting an actin response survive NK-cell attack and remain alive after immune cell detachment, while tumor cells without an actin response are efficiently lysed. Accordingly, apoptosis in NK-cell–conjugated target cells is markedly reduced in the actin response–competent cell subpopulation. Moreover, inhibition of the actin response is sufficient to convert the initially resistant MDA-MB-231 cell line into a highly susceptible phenotype. Altogether, these findings demonstrate a causal relationship between the actin response and resistance to NK cells.

The actin response is a remarkably fast and localized process that takes place almost immediately after physical contact between the target and effector cells. Tracking individual cell conjugates over time revealed that the actin response lasts throughout the entire period of interaction and rapidly stops after effector cell detachment. This suggests a model in which a signal from the IS is transmitted to the proximal tumor cell cortex where it induces sustained actin polymerization. Although the upstream components of the signaling pathway remain to be identified, the robust inhibition of the actin response induced by N-WASP or CDC42 knockdown suggests that the ARP2/3 complex is a key downstream effector responsible for the burst of actin polymerization following NK-cell attack. The role of N-WASP and CDC42 in driving ARP2/3 complex-dependent actin polymerization at the membrane has been extensively documented (37, 38). Notably, CDC42 is a key regulator of membrane protrusions (such as filopodia) and polarity (39, 40). Consistent with this, the actin response is associated with spike-like projections (Fig. 1C and E). Similar to filopodia or invadopodia, actin nucleators of the formin family (41) and AF crosslinking proteins, such as fascin (42) or CSRP2 (43), are likely to be required for the extension of actin response-associated protrusions.

From a functional standpoint, the actin response was associated with a significant reduction in GzB levels in target cells. Moreover, inhibiting the actin response through pharmacologic impairment of actin dynamics or genetic ablation of CDC42 or N-WASP restored high GzB levels in target cells. Interestingly, our data show that the actin response is associated with modifications in the density of NK-cell receptor ligands in the region of the synapse. Most noticeably, both MCF-7 cells and MDA-MB-231 cells with an actin response exhibited a significant increase (+100%) of HLA-A, -B, -C inhibitory ligands at the synapse as compared with the respective cells without an actin response. Such modifications provide a mechanistic insight into how the actin response mediates target cell resistance to NK-cell–mediated lysis. However, we found no association between the actin response and abnormal MTOC polarization or degranulation activity in the effector cells. These results can be explained by the lack of expression of most KIR receptors in the NK cell line (NK92MI) used in our study, which renders this cell line insensitive to HLA-A, -B, -C inhibitory ligands (34–36). Consequently, the decrease in GzB levels observed in target cells with an actin response must originate from an additional mechanism, e.g., actin response-mediated obstruction of GzB

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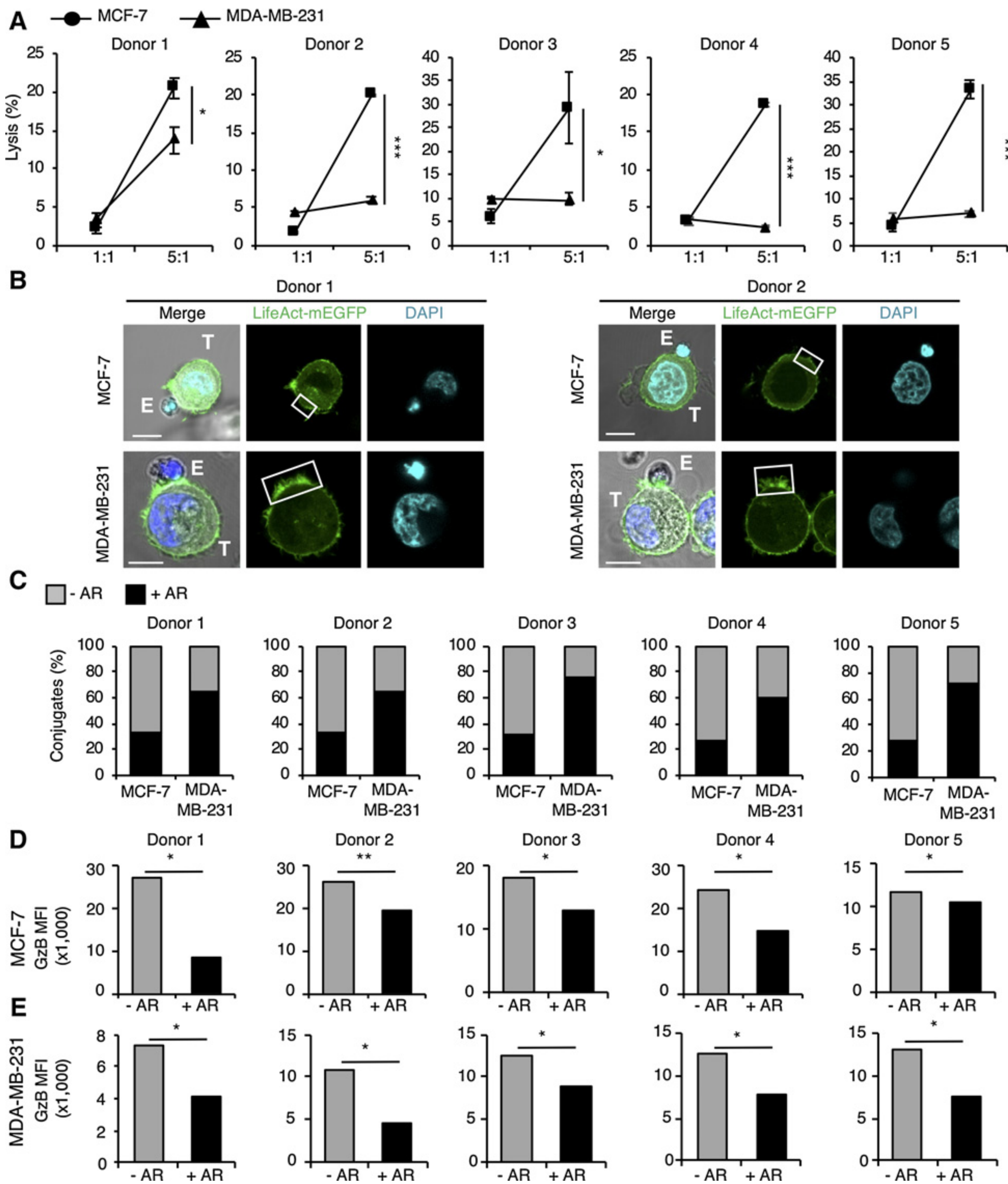

Figure 6.

The actin response is induced by human donor NK cells. **A,** Cytotoxicity assays performed in triplicates using NK cells isolated from five healthy donors on MCF-7 and MDA-MB-231 cells (targets) at 1:1 and 5:1 effector:target ratios. **B,** Representative confocal microscopy images showing the typical actin cytoskeleton organization in most MCF-7 (top) and MDA-MB-231 (bottom) target cells (green; T) in conjugation with primary NK cells (E) isolated from two donors. The white rectangles indicate the position of the synaptic region. Scale bar, 10 μm. **C,** Percentage of NK-cell–conjugated target cells with (black; +AR) and without (light gray; −AR) actin response in each cell line and for each donor. In all, about 300 conjugated target cells were scored. **D** and **E,** GzB levels in NK-cell–conjugated MCF-7 (**D**) and MDA-MB-231 (**E**) target cells with (black; +AR) and without (light gray; −AR) actin response. At least 200 conjugated target cells were scored for each cell line and each donor. Data are expressed as MFI. The two-tailed unpaired Student *t* test was used to determine statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

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uptake into target cells or actin response-mediated GzB degradation inside target cells.

In this regard, we recently reported that autophagy promotes NK-cell–derived GzB degradation in hypoxic MCF-7 cells, thereby reducing target cell susceptibility to NK-cell–mediated lysis (44, 45). In keeping with this, inhibition of autophagy in target cells improves tumor elimination by NK cells in *in vivo* mouse models of breast cancer and melanoma (45). Considering the multiple and critical roles of the actin cytoskeleton during various steps of autophagy (46), follow-up studies should elucidate the link between the actin response and autophagy-mediated GzB degradation in tumor cells.

There is mounting evidence that EMT promotes tumor cells' escape from cytotoxic immune cells, such as CTL- and NK cells (47–50). In support of this, our data show that the mesenchymal-like breast cancer cell lines have a much higher capacity to generate an actin response as compared with epithelial-like breast cancer cell lines. Furthermore, both genetic (SNAIL or SNAIL-6A overexpression) and pharmacologic (TGFβ or TNFα treatment) induction of EMT increases tumor cell competency for the actin response and resistance to NK-cell–mediated cell death. During EMT, extensive actin cytoskeleton remodeling is required to drive morphologic and functional adaptations, such as the acquisition of migratory and invasive properties (51). Thus, EMT-associated cytoskeletal changes likely confer tumor cells with an increased capacity to undergo rapid actin remodeling in response to immune attack.

An intriguing finding of our study is that breast cancer cell lines contain two main subpopulations of cells differing in their capacity to mobilize the actin cytoskeleton in response to NK cell attack and to survive this attack. A direct and important implication of this previously unknown facet of intra-cancer cell line heterogeneity is that the apparently high susceptibility of a given cell line, such as MCF-7, may actually mask the existence of a minor subpopulation of immune-resistant (and actin response–competent) cells. This knowledge should be taken into consideration in cytotoxicity assay-based studies, and efforts should be made to target the actin response–competent cell subpopulation. The characterization of the signaling pathway(s) controlling the actin response and the identification of druggable molecular targets to impair this process are promising future directions. These efforts might help sensitize intrinsically resistant cancer cells to immune cell–mediated cytotoxicity and improve the efficacy of immunotherapies.

Disclosure of Potential Conflicts of Interest

J.P. Thiery is CSO at Biocheetah Singapore, has ownership interest (including stock, patents, etc.) in CNRS Paris, is a consultant/advisory board member for AIM Biotech Singapore, Biosyngen Singapore, and ACT Genomics Taiwan. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Al Absi, C. Guerin, C. Hoffmann, J.-P. Thiery, G. Berchem, C. Thomas
Writing, review, and/or revision of the manuscript: A. Al Absi, H. Wurzer, J. Brown-Clay, J.-P. Thiery, S. Chouaib, G. Berchem, B. Janji, C. Thomas
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Dieterle
Study supervision: A. Al Absi, B. Janji, C. Thomas

Acknowledgments

The authors are grateful to Tony Kaoma (bioinformatics and modeling, Department of Oncology) for support in statistical analyses and to Vanessa Marani (LECR) for technical assistance.

A. Al Absi and H. Wurzer are recipients of PhD fellowships from the National Research Fund (FNR), Luxembourg (AFR7892325 and PRIDE15/10675146/CANBIO, respectively). J. Brown-Clay is recipient of a Postdoctoral fellowship from "Fonds De La Recherche Scientifique", FNRS "Télévie" (7.4512.16). Work in C. Thomas's team is supported by National Research Fund, Luxembourg (C16/BM/11297905) and the Cancer Foundation (FC/2016/02).

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Received February 9, 2018; revised June 14, 2018; accepted July 31, 2018; published first August 13, 2018.

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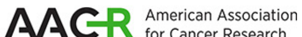

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Actin Cytoskeleton Remodeling Drives Breast Cancer Cell Escape from Natural Killer–Mediated Cytotoxicity

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Cancer Res 2018;78:5631-5643. Published OnlineFirst August 13, 2018.

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