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by

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# FUNCTIONAL AND PHENOTYPICAL ASPECTS OF MURINE NATURAL KILLER CELLS IN THE CONTEXT OF EDUCATION

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I hereby confirm that the PhD thesis entitled "**Functional and phenotypical aspects of murine Natural Killer cells in the context of education**" has been written independently and without any other sources than cited. All necessary ethical approvals have been obtained in accordance with the Luxembourgish law (on the use of clinical samples and on the Care and Use of laboratory animals, where applicable).

Luxembourg, 08/06/2022 Neha D. Patil

*Per aspera ad astra*

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**List of Abbreviations**

| | |
|---|---|
| ADCC | Antibody Dependent Cell-mediated Cytotoxicity |
| Ahr | Aryl Hydrocarbon Receptor |
| APC | Allophycocyanin |
| BM | Bone Marrow |
| CAR | Chimeric Antigen Receptor |
| CILCP | Common ILC Progenitor |
| CLP | Common Lymphoid Progenitor |
| Clr | C-type lectin-related proteins |
| CMP | Common Myeloid Progenitor |
| DCP | Dendritic Cell Progenitor |
| DCs | Dendritic cells |
| DNAMs | DNAX accessory Molecule-1 |
| ECP | Eosinophil Cationic Proteins |
| EDN | Eosinophil-Derived Neurotoxin |
| EGF | Epidermal Growth Factor |
| GM-CSF | Granulocyte-Macrophage Colony-Stimulating Factor |
| GvHD | Graft-Versus-Host Disease |
| HCV | Hepatitis C Virus |
| HPCs | Haematopoetic Stem cells |
| HSCs | Haematopoetic Stem Cell |
| IFNγ | Interferon Gamma |
| IL | Interleukin |
| ILCs | Innate Lymphoid Cells |

| | |
|---|---|
| iNK | Immature Natural Killer |
| ITIM | Immunoreceptor tyrosine-based inhibitory motif |
| KIRs | Killer Cell immunoglobins-like receptors |
| KLRG1 | Killer Cell Lectin like Receptor G1 |
| LMP | Lymphoid and Myeloid Primed Progenitor |
| LMPPs | Lymphoid Primed Multipotent Progenitors |
| LNs | Lymph Nodes |
| Mac | Macrophages |
| MAIT | Mucosal-Associated Invariant T |
| MCA | Methylcholanthrene |
| MHC | Major Histocompatibility Complex |
| mNK | Mature Natural Killer |
| MR1 | MHC class I-related gene protein |
| NCAM | Neutral Cell Adhesion Molecule |
| NETs | Neutrophil Extracellular Traps |
| NK | Natural Killer |
| NKP | Natural Killer Cell Progenitor |
| PAMPs | Pathogen Associated Molecular Pattern molecules |
| ROS | Reactive oxygen Species |
| SLAM | Signalling Lymphocytic Activation Molecule |
| SLTs | Secondary Lymphoid Tissues |
| TFs | Transcription Factors |
| TLR | Toll-Like receptors |
| TNF | Tumour Necrosis Factor |
| TOX | Thermocyte selection Associated High Mobility Group Box |

| | |
|---|---|
| TRM | Tissue Resident Macrophages |
| ZEB2 | Zinc Finger E-Box Homeobox 2 |

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**Summary**

The field of Natural Killer cell biology has expanded immensely over the last several decades. These cells form a major class of lymphocytes and are an important part of the host immune system. Their role against viral infections and in cancer has widely been studied. Increasing body of work in the field of bacterial defence has brought forth the contribution of NK cells. Our work in this thesis highlights this.

In the introduction (**Chapter 1)**, we go over the basic biology of NK cells. We aim to understand the different components of the innate immune system, to which NK cells belong. NK cells of humans and mice differ right from their places of origin and development, and in how they differentiate into mature NK cells. We go over the surface markers distinguishing them and how to identify them. NK cell education is a process by which these cells obtain their functionality and how they identify self from non-self. The chapter describes the various models by which the status of education is achieved, and what happens in case of missing self-recognition. After briefly introducing their role in cancer immunosurveillance and in viral infections, we focus on their role in bacterial infections. Experiments demonstrate that NK cells interact directly with the bacteria and indirectly, by cytokines. NK cell functionality differs on depending on their location, and thus when talking about respiratory infections like COPD, we focus on the NK cells in the upper airways and the lungs. NK cells act as a double-edged sword, and it is important to take that into consideration when planning immunotherapies, especially cancer immunotherapies with them. Our review in the same chapter outlines the instances where NK cells have deleterious consequences, for example in autoimmunity or their contribution to a cytokine storm in infections, as well as in transplant biology. Several immune cells interact with NK cells, forming an immune microenvironment. One such being the mucosal associated invariant T cells, described in the last section of the chapter.

Functional NK cells, by virtue of the education achieved by interaction between their major histocompatibility complex class I molecules and self-specific inhibitory receptors, are able to target various infections. In our first paper (**Chapter 2**), we performed various experiments to understand the imprint of this education in four strains of mice: B6 wildtype, TAP1-KO, $B6^{CAST}$ and MR1-KO. We emphasize on the functional aspects of the NK cells and built their phenotypical profile at baseline. We take a look at the inhibitory receptors Ly49C/I and NKG2A, in presence of a combination of cytokines interleukin-2, interleukin-12, interleukin-15 and interleukin-18 and observe the changes in the effector potential. We

reported a loss in the expression levels of Ly49C/I upon prolonged cultures and changes in the corresponding NK cell cytotoxicity towards target cells.

**Chapter 3** documents our efforts to establish a mouse model of chronic lung infection. Mouse models are used in the study of various diseases, in our case specifically, to study the role of NK cells in lung bacterial infections. We wanted to replicate the disease pathology observed in TAP deficient human patients, where recurrent infections of the respiratory tract are found. Commonly found bacteria include *Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae*, *Klebsiella, Escherichia coli, Pseudomonas aeruginosa*. While acute models of infections have been described in literature, the mentions of chronic infection models are far and few. We attempted to establish a *P.* aeruginosa chronic infection and wildtype and KO mice, by combining methods used by different groups.

Microscopy has found its rightful place in biology, right from its first recognised use in the 17$^{th}$ century. Since then, technological advances have enabled us to image and characterise different components of the immune system, their interactions with various infectious agents, and the consequent immune responses. In **chapter 4**, we challenge the traditional imaging techniques by using ImageStreamX, an imaging flow cytometer, to analyse the interactions between NK cells and bacteria. ImageStreamX allows for the usage of fluorescence intensity signals to quantitatively and qualitatively assess the interactions, and performs statistical analysis as well. We found that the interaction is dynamic in nature.

# Chapter 1: General Introduction

**Contribution to the chapter:**

Literature review and writing

## 1.1. Introduction

Every single day, living beings on planet Earth are confronted with the fight for their lives, fending off disease causing organisms. They do so with the help of a set of complex mechanisms, all collectively forming the immune system. This system has had its roots as a simple machinery, morphed by the evolutionary tides of the last several million years. To untangle this clutter, the modern immunologist has to look into the genes, the protein it codes and study them in numerous diverse life forms.

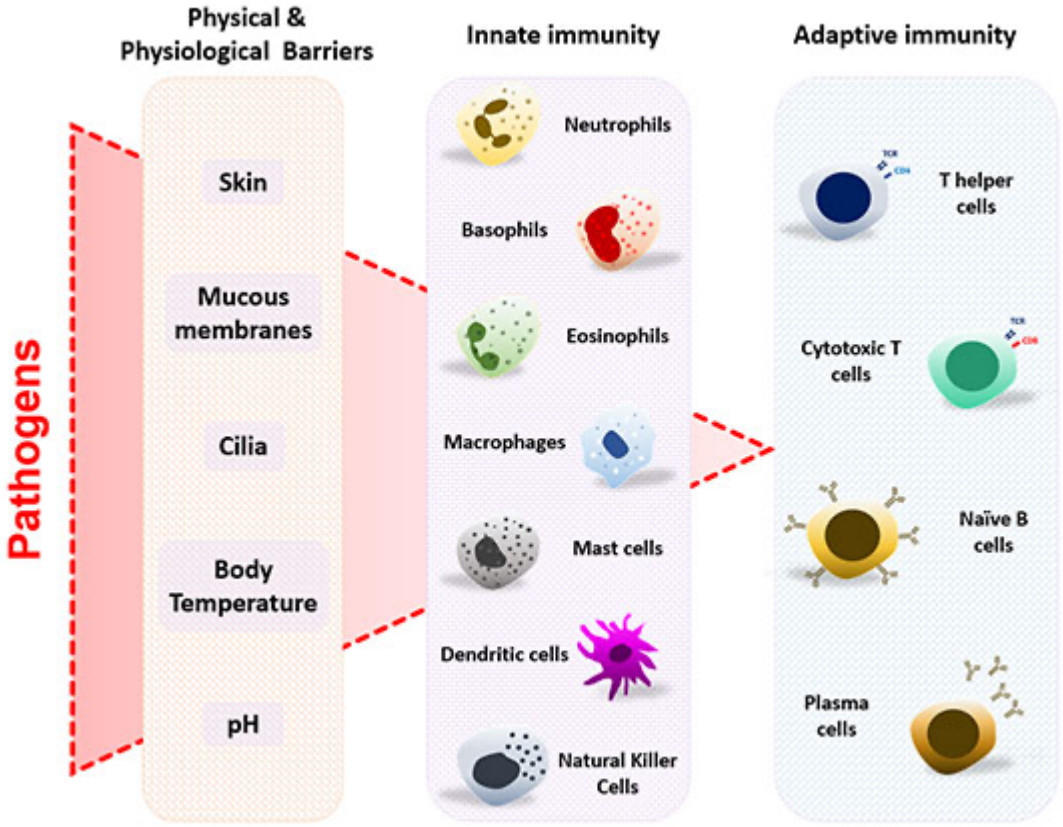
In the first chapter, we will talk about the different cells comprising the innate immune system, explore natural killer cell biology, their role in bacterial infections.

## 1.2. Components of innate immunity

The immune system has classically been divided into innate and adaptive immunity. Innate immunity, also called antigen-nonspecific immunity, is activated within the first few hours of contact with pathogen, and does so by recognizing the pathogen-associated molecular patterns (Amarante-Mendes, Adjemian et al. 2018). Comprising of cells from both hematopoietic and non-hematopoietic background, the innate immune system is armed with phagocytes, antimicrobial peptides, the complement system, soluble moderators like cytokines, chemokines etc., and is an essential first line of defence offering protection until the adaptive system kicks in. Physical barriers, like skin and mucosa, provide safety from the exterior environment. Epithelial cells lining the gastrointestinal, respiratory and genitourinary tracts prevent the entry of pathogens, produce proteins and enzymes such as antimicrobial peptides like defensins (González 2013).

Mast cells, neutrophils, eosinophils, dendritic cells, macrophages and natural killer cells form the hematopoietic repertoire, which are activated during an infection and initiate an inflammatory response (Daha 2011). Present throughout the body, these cells interact and capture the invading pathogen.

Mast cells are granule containing cells that interact with skin and mucosa (Igawa and Di Nardo 2017). These granules or secretory lysosomes contain acid hydrolases, histamine, cytokines, proteases and proteoglycans (Mukai, Tsai et al. 2018). Heavily implicated in IgE-mediated allergic responses, they differentiate in the tissues, and secrete




several cytokines, chemokines and growth factors like TNF, IFNγ, several interleukins (ILs), GM-CSF, and

**Figure 1:** Components of immune system in vertebrates. Source (Esposito 2021)

EGF etc. Their position at the boundary of internal and external milieu makes them a key component in the inflammatory process (Moon, Befus et al. 2014). They can recognize microbes by pattern recognition and toll-like receptors among others, and are recruited by various chemoattractants. Their anti-bacterial response is mediated by secretion of inflammatory mediators by which they can directly kill bacteria while also recruiting other immune cells such as neutrophils (Di Nardo, Yamasaki et al. 2008, Piliponsky and Romani 2018).

Neutrophils, the dominant circulating leukocyte, are mediators of the earliest immune responses towards a wide range of pathogens like protozoa, fungi, and bacteria. With a half-life of mere 8 hours, they have to be constantly generated in the bone marrow, derived from their myeloid precursor, a process controlled by granulocyte colony stimulating factor (Pillay, den Braber et al. 2010). They undergo maturation, transitioning through myeloblast, promyelocyte, myelocyte, metamyelocyte, band cells and into the mature polymorphonuclear neutrophils that are enriched in granules filled with pro-inflammatory

proteins (da Silva, Massart-Leen et al. 1994). Tissue-resident leukocytes upon contact with pathogens release inflammatory cytokines, leukotrienes, etc. that recruit neutrophils to the site of infection (Ley, Laudanna et al. 2007, Phillipson and Kubes 2011). The infiltrating neutrophils are captured by the surrounding endothelial cells with the help of integrins and adhesion molecules (Shattil, Kim et al. 2010). Once at the site of infection, neutrophils can either phagocytose the microbe or target them by degranulating. Once phagocytosed, the microbe is enclosed in a phagosome where it is destroyed by reactive oxygen species (ROS) or by presence of antibacterial peptides and proteases such as lysozyme, defensins, cathepsins and proteinase-3 (Borregaard 2010, Kobayashi, Malachowa et al. 2018). These antibacterial proteins can also be released at the infection site to directly eliminate the microbe. Another highly interesting mechanism is the formation of neutrophil extracellular traps (NETs), a web-like structure, composed of DNA (decondensed chromatin originating from nucleus or mitochondria) and the granule proteins (Yang, Biermann et al. 2016). These NETs trap the microbe, immobilising it and subsequently phagocytosing or directly killing it, a mechanism that has to be tightly regulated in order to not damage the host tissue, and are ultimately dismantled by nuclease DNaseI and ingested by macrophages (Hakkim, Furnrohr et al. 2010, Farrera and Fadeel 2013).

Coming to human eosinophils, the second largest granulocyte population in the blood, make an important defence line against parasitic infections, along with bacteria and viruses. Produced in bone marrow from myeloid precursors (Fulkerson and Rothenberg 2018, Ramirez, Yacoub et al. 2018), their production and activation are highly influenced by IL-5. Once matured, they get their distinct bilobed nucleus, and cationic protein filled granules (Davoine and Lacy 2014). They are terminally differentiated there and do not proliferate further upon leaving the bone marrow. They have secretory granules, known as secondary granules, with characteristic crystalline core, contain eosinophil cationic proteins (ECP), eosinophil-derived neurotoxin (EDN), preformed enzymes, and over 35 cytokines, chemokines and growth factors, making them strong participants in immunomodulation and anti-microbial activities (Davoine and Lacy 2014). These are secreted by (a) classical exocytosis (b) cytolysis with granule release (c) piecemeal degranulation. The cytokines released by them, like IL-4 and IL-13, can help in activation of local epithelial cells, which then recruit other leukocytes to the inflamed tissue. They have been shown to promote B cell activation, survival and proliferation (Wong, Doyle et al. 2014), along with antigen-presentation to T cells (Farhan, Vickers et al. 2016). They also make DNA nets, like neutrophils, forming extracellular traps (Ueki, Melo et al. 2013).

Dendritic cells (DCs) are another bone marrow derived cells, specializing in antigen-presentation, capturing and processing the antigens during their continuous surveillance, acting as envoys between innate and adaptive immune cells. They develop from common dendritic cell progenitor (DCP) and are a heterogeneous cell population with several subsets (Naik, Sathe et al. 2007). The subsets specialize in their interactions with different pathogens, and in their interactions with specific T cell subsets. DCPs circulate, reaching peripheral tissues as immature cells (Merad, Sathe et al. 2013). These immature cells have receptors that interact with various inflammatory signals, TLR-ligands, cytokines, and chemokines. Here, they upregulate surface expression of antigen-presentation equipment that has processed antigen peptide-loaded major histocompatibility complex (MHC) class II to naïve CD4+ T lymphocytes. Antigen uptake is done either via phagocytosis, endocytosis, pinocytosis or micropinocytosis (Land 2018). As they mature, they migrate from periphery to lymph nodes where they activate naïve CD4+ and CD8+ T cells, and even CD4-, CD8- T cells, B and NK cells to become regulatory cells. They also promote peripheral T cell tolerance by presenting self- or innocuous foreign antigens to T cells and can be targeted for therapies for transplantation or autoimmunity (Waisman, Lukas et al. 2017).

Macrophages are large immune cells that phagocytose cancer cells, microbes, cellular debris, foreign substances, etc. Their primary function is to clear pathogens, recognising them with the help of PAMPs and kill the phagocytosed cells by release of reactive oxygen species, nitric oxide (Herb and Schramm 2021). Embryonic development, not the circulating monocytes, leads to most adult tissue macrophages (Epelman, Lavine et al. 2014). They have a role in metabolic functions, for example, the synovial macrophages can induce metabolic reprogramming (Saeki and Imai 2020). Tissue-resident macrophages (TRM) help in tissue repair and remodelling in presence of IL-4 or IL-13 and with apoptotic cells (Bosurgi, Cao et al. 2017). TRMs have also been shown to protect the tissue from neutrophil-mediated inflammatory damage (Uderhardt, Martins et al. 2019). They recognise and present foreign antigens to T cells, they provide the required costimulatory signalling and cytokine secretion for T cell activation. Tumour associated macrophages can suppress naive T cell proliferation and functions (Doedens, Stockmann et al. 2010) and are mediators of tumour immunity (Guerriero 2019).

We will now turn our focus towards the Natural Killer (NK) cells and describe their role in pathogen defence.

## 2. Natural Killer cells

### 2.1. Introduction

The early years of natural killer cell history were shrouded in doubts, with claims of their observances being described as artefacts (Oldham 1983). First mentioned in 1966, the study described them as a cell population that were able to confer a natural immunity to tumours (Smith 1966). By mid-1970s, this 'natural killing' activity was recognised in the mouse as cells cytotoxic towards mouse Moloney leukaemia cells (Kiessling, Klein et al. 1975) as well as in humans, demonstrating cytotoxic activity in the human non-T-lymphocyte fraction (Pross and Jondal 1975). A major development came when human NK cells were finally separated and microscopically viewed by density gradient isolation. They were identified as large, granular lymphocytes, eventually termed as Natural Killer cells (Timonen and Saksela 1980).

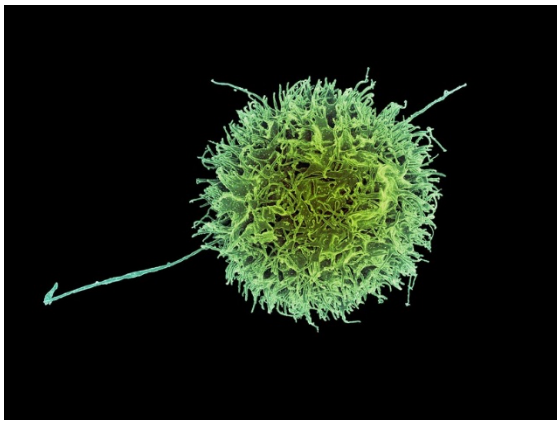


**Figure 2:** Colorized scanning electron micrograph of a human NK cell, National Institute of Allergy and Infectious Diseases

NK cell activity, though initially observed in the mononuclear cell population of human peripheral blood, has been identified in lymphoid and non-lymphoid tissues, including bone marrow, lymph nodes, skin, intestine, spleen, liver, and lung. Part of the group 1 innate lymphoid cells (ILCs) (Spits, Artis et al. 2013), they form about 15% of circulating lymphocyte population in humans (Robertson and Ritz 1990). They are defined by the lack of CD3 expression and presence of CD56 (bright and dim), a neural cell adhesion molecule (NCAM) cell surface marker (Cooper, Fehniger et al. 2001, Freud and Caligiuri 2006, Romagnani, Juelke et al. 2007). The NK population can further be

subdivided based on the relative expression of CD16 (FcγRIII) (Poli, Michel et al. 2009). In mice, about 2-5% of circulating lymphocytes are NK cells (Jiao, Huntington et al. 2016). Similar to human NK cells, mice NK cells are negative for CD3 cell surface expression, while the characteristic NK receptors vary depending on the mouse strain. In C57BL/6mice, $CD3^-$ $NK1.1^+$ cells form the NK cell population. In BALB/c, the population is recognised by the presence of NKp46 and CD49b (Goh and Huntington 2017).

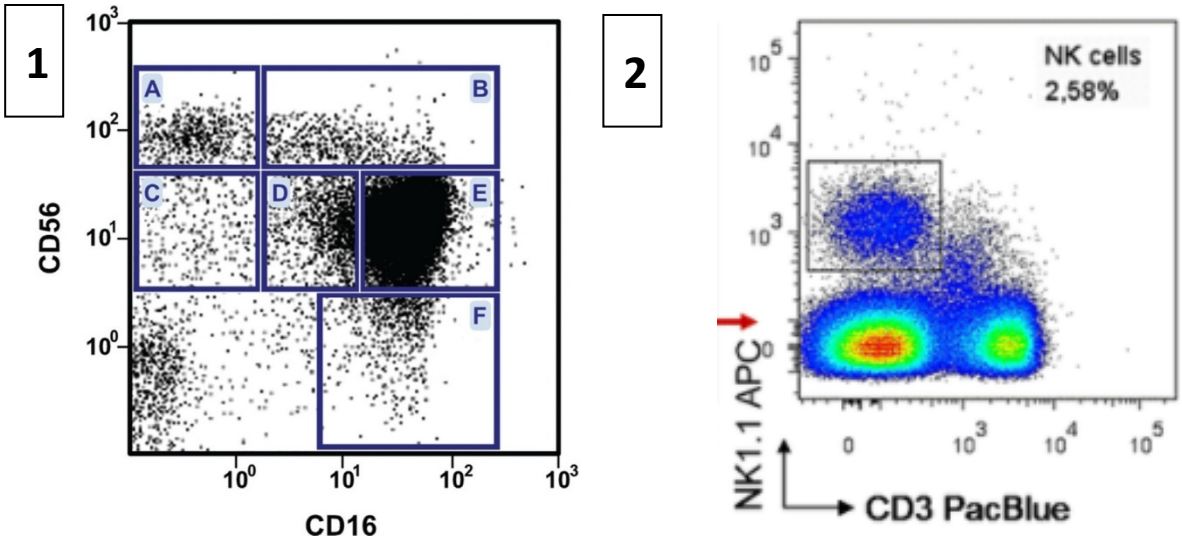



**Figure 3:** (1) Human NK subpopulations – Flow cytometric analysis of $CD56^{bright}$ and $CD56^{dim}$ against CD16 in human peripheral blood derived natural killer (NK) cells. Source: (Zimmer 2020) (2) Mouse NK cells - Flow cytometric analysis of NK1.1 Allophycocyanin (APC) and CD3 Pacific Blue dyes. The NK cell population is defined as $CD3^-NK1.1^+$ and were isolated from mouse spleen. Source: (Hertwig, Hamann et al. 2016)

In the following chapter, we will study NK cells up-close, with focus on their development and differentiation, education and functions. We will also learn their role in anti-bacterial defences and the consequences of them going rogue.

## 2.2. Development and differentiation

All mature blood cells in mammals are produced by haematopoiesis, originating from the self-renewing pluripotent haematopoietic stem cells (HSCs). Residing in the bone marrow, HSCs are responsible for myeloid and lymphoid lineages (Pang, Price et al. 2011). Myeloid precursors differentiate into erythroid/megakaryocytic, and granulocytic/macrophage populations, whereas lymphoid progenitors develop into NK, T,

and B cells (Miranda and Johnson 2007, Ye and Graf 2007). NK, T and B cells differentiate from common lymphoid progenitor (CLP) along with other members of the innate lymphoid cells (ILCs).

The commitment to a single lineage and maturation to specific cell type requires various growth factors, interleukins (IL) and haematopoietic cytokines. The availability of these factors drives the survival and proliferation of a particular cell category (Kaushansky 2006). Understanding the process of differentiation and the factors influencing them has become crucial, as the use of HSCs and their development into target immune cells has a use in cancer and immunotherapies.

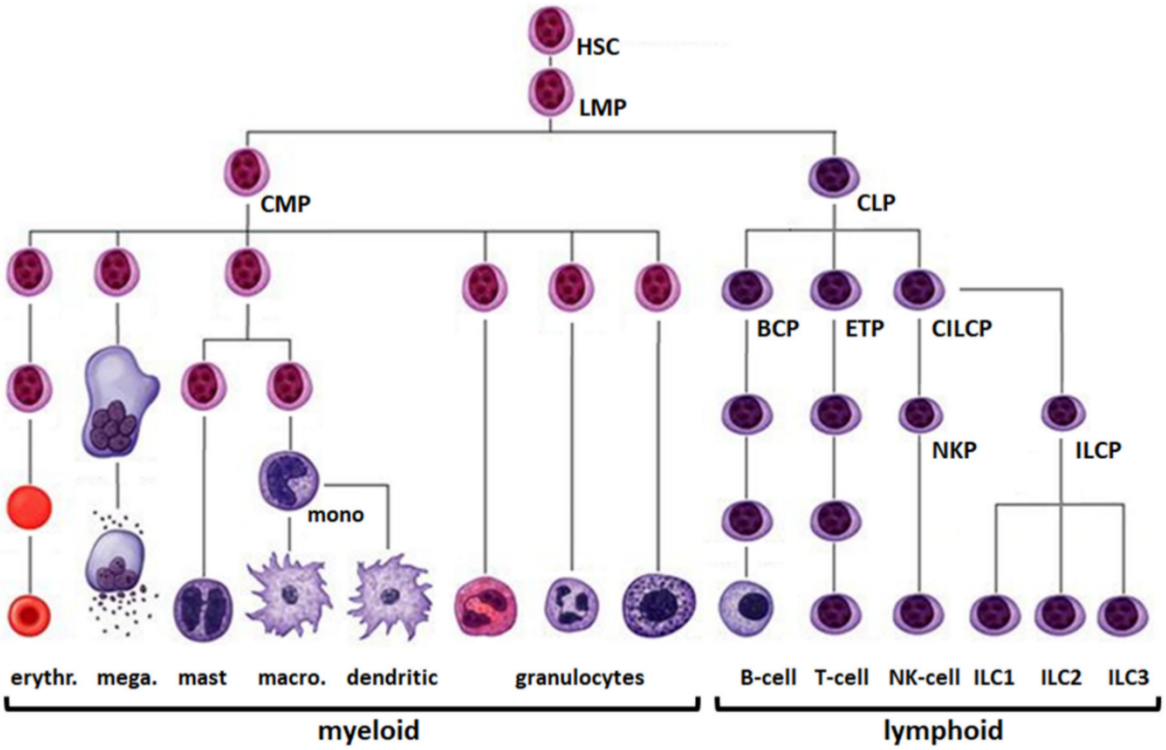



**Figure 4:** Haematopoiesis - Haematopoietic stem cell (HSC) differentiating into a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP). The figure offers a simplified explanation of the further differentiation of these progenitor cells into target cells BCP: B-cell progenitor, CILCP: common ILC progenitor, ETP: early T-cell progenitor, ILC(P): innate lymphoid cell (progenitor), LMP: lymphoid and myeloid primed progenitor, NKP: NK-cell progenitor. Source: (Nagel 2021)

Earlier in their discovery, the assumption was that NK cells belonged with immune group consisting the likes of monocytes, as they are part of innate immune system. Unlike their

adaptive T and B lymphocyte counterparts, NK cells lack the ability to somatically rearrange antigen-specific receptors, rather using their germ line-encoded receptors for target recognition. Later findings have established however, that as an innate immune lymphocyte, they are more closely related to T cells (part of adaptive immune cells), whom they share a common progenitor with (Narni-Mancinelli, Vivier et al. 2011). Both cells partake in similar effector functions, such as lysis of target cells, releasing cytotoxic granules
and their cytokine secretion profiles.

### 2.2.1. Where do NK cells develop?

Bone marrow (BM) was for a long time thought as the exclusive site for NK cell development. BM has high concentration of $CD34^{pos}$ HSCs, including the NK cell progenitors. (Colucci, Caligiuri et al. 2003, Caligiuri 2008). Literature shows that NK cells are naturally supplemented in secondary lymphoid tissues (SLTs), including tonsils, spleen, and lymph nodes (LNs), similar to thymus, liver and uterus (Freud, Becknell et al. 2005, Male, Hughes et al. 2010, Moroso, Famili et al. 2011, Eissens, Spanholtz et al. 2012, Hidalgo, Martinez et al. 2012). In LNs especially, $CD34^{dim}$/$CD45RA^{br}$ haematopoietic precursor cells (HPCs) are present, which differentiate into $CD56^{br}$ NK cells due to activation by T cells residing in LNs (Freud, Becknell et al. 2005). Human liver is a major site of NK cell development, and biopsies have shown evidences of $CD34^{pos}$ HPCs to terminally differentiated NK cells (Moroso, Famili et al. 2011). The progenitors and intermediate populations leading to NK cells are due to the differential expression of lineage-specific surface markers. These markers are sometimes not similar in humans and mice. Nevertheless, the NK cells development process is well studied in BM, and we continue to build upon our knowledge of SLT NK cells.

### 2.2.2. What are the developmental stages for NK cells?

NK cell development is categorized in two stages: lineage commitment and maturation. Murine NK cells develop in specialised BM sites, and differentiate through a series of intermediate progenitors. The HSCs give rise to CLPs (Fig. 5.1), which precede Pro-B, Pre-T, ILCs, and Pre-T/NK cell progenitors (NKPs) lineages among others. Both human and

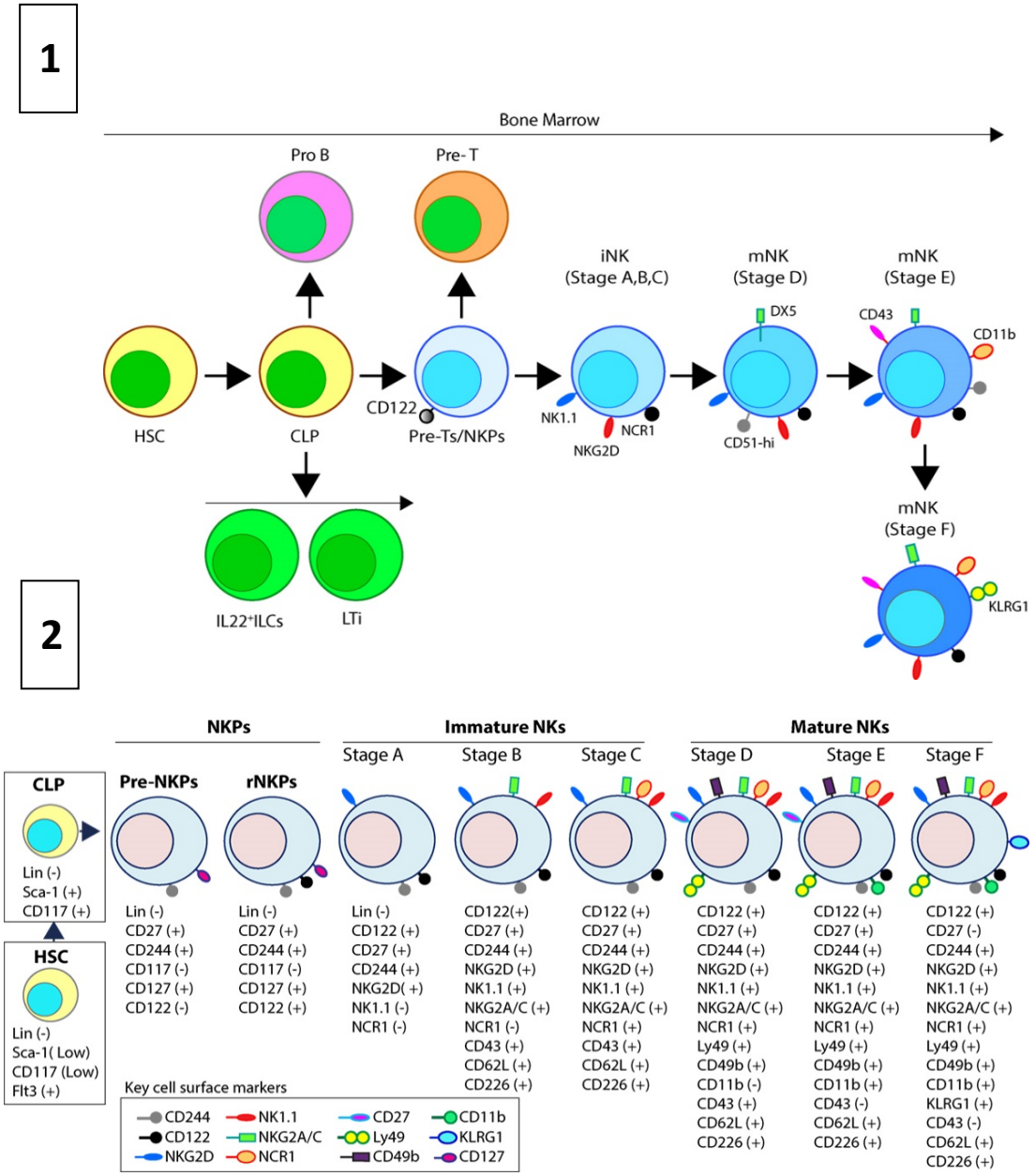



**Figure 5:** (1) Development of mouse NK cells in the bone marrow from the multipotent HSCs. (2) Developmental stages of mouse NK cells is divided in to distinct stages defined by expression and loss of various surface markers. Source (Abel, Yang et al. 2018)

mice NK cells come from CLPs (Kondo, Weissman et al. 1997). CLPs transition into lymphoid lineage, a subset of which forms pre-NKPs, which express IL-2 receptor β chain to then become NKPs (Male, Nisoli et al. 2014). What follows are six stages, through which begins the transition of an immature NK (iNK) cell into a mature NK (mNK) cell. The iNK cells develop in the bone marrow (Kim, Iizuka et al. 2002, Bonanni, Sciume et al. 2019), or

relocate and become mNK cells in the SLTs (Chiossone, Chaix et al. 2009). Different NK cell populations, through the stages of development (Fig. 5.2), express different surface markers. Stage A marks the expression of the NKG2D, an activating receptor and DAP 10, its signalling intermediate and binding partner (Carotta, Pang et al. 2011, Zafirova, Wensveen et al. 2011). Throughout the iNK stages, cells express receptors like NK1.1, NKG2A, the DNAX accessory molecule-1 (DNAM-1), NCR1 (CD335/NKp46) (Goh and Huntington 2017). Various transcription factors (TFs) control this process, by activating/repressing the responsible genes (Fig. 6).

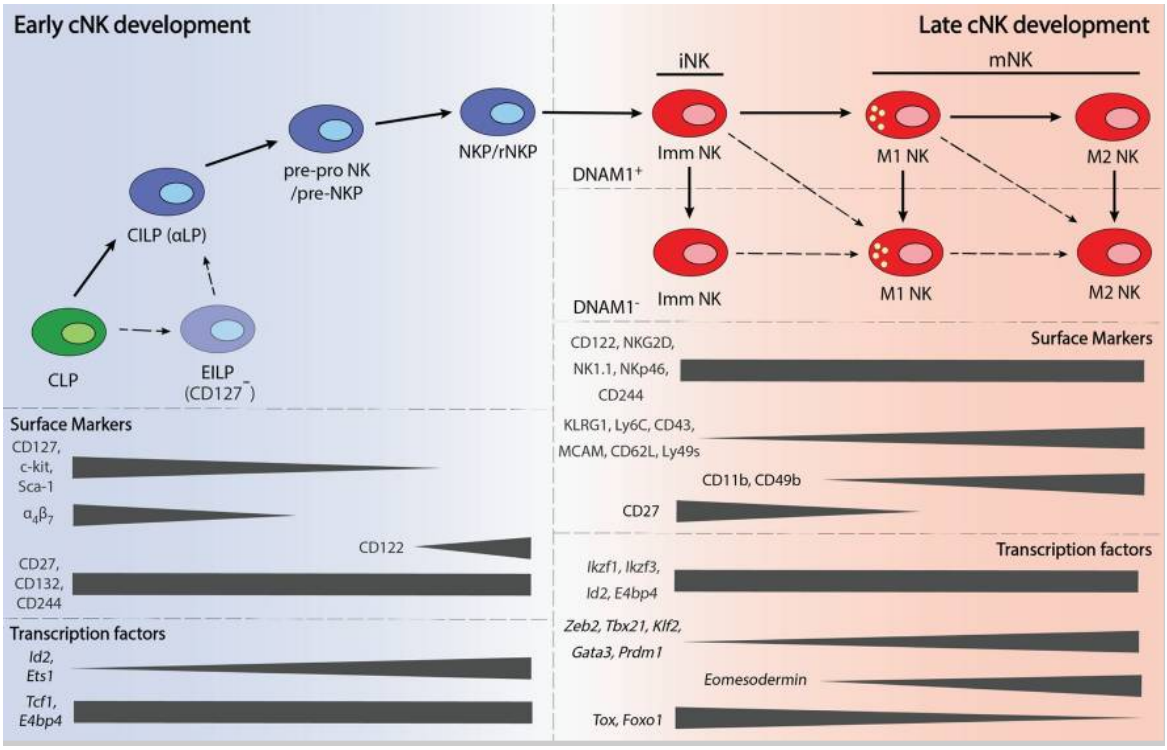



**Figure 6:** Various transcription factors are involved in early and late NK cell development. Source (Goh and Huntington 2017)

The TF ETS proto-oncogene1 (ETS1), a key early NK cell development regulator (Barton, Muthusamy et al. 1998), is responsible for NK cell lineage specification (Ramirez, Chandler et al. 2012). Other TFs include nuclear factor, IL3 regulated (NFIL3) and T cell factor 1 (TCF1) (Male, Nisoli et al. 2014, Seillet, Huntington et al. 2014, Yang, Li et al. 2015). (Fig. 6)

The mNK cells come next, defined by the expression of CD51 (Integrin alpha-V) and CD49b (Integrin alpha 1). Ly49 receptors, both inhibitory (Ly49A, Ly49C/I, Ly49G) and

activating (Ly49D, Ly49H) are expressed on the terminally differentiated mNKs (Stage E). The expression of Killer cell Lectin-like Receptor G1 (KLRG1) signals the NK cells to migrate to secondary lymphoid organs (Stage F) (Kim, Iizuka et al. 2002). A wider array of TFs are responsible for the NK cell maturation. (Fig. 6)

The TF ID2 is a repressor of E2A, and works by controlling the expression of E2A by binding and sequestering E-proteins (Perk, Iavarone et al. 2005). Murine NK cells from the $Id2^{-/-}$ BM did not respond to IL-15 treatment (Yokota, Mansouri et al. 1999), thus proving that titration of E-protein activity by ID2 controls the NK cell sensitivity to IL-15 (Delconte, Shi et al. 2016), and is crucial for their survival. T-$bet^{-/-}$ mice exhibit failed NK cell terminal maturation, and a decreased cell number in peripheral lymphoid organs (Townsend, Weinmann et al. 2004), while $Eomesodermin^{-/-}$ (Eomes) mice shows a block in cNK development (Pikovskaya, Chaix et al. 2016).

Other TFs include the Zinc finger E-box-binding homeobox 2 (ZEB2) – also responsible for IL-15 responsiveness (van Helden, Goossens et al. 2015), Thymocyte selection-associated high mobility group box (TOX) – maintains high expression at iNK stage (Vong, Leung et al. 2014), and any defect can block the development at post-iNK stage (Aliahmad, de la Torre et al. 2010). IKAROS family zinc finger 3 (AIOLOS) can bind to DNA and regulate their gene expression, and is vital for peripheral maturation of NK cells into $CD11b^{high}CD27^{-}$ cells (Holmes, Huntington et al. 2014). NK cell maturation defined on CD11b and CD27 goes through 4 phases: $CD11b^{low}CD27^{low} \rightarrow CD11b^{low}CD27^{high} \rightarrow CD11b^{high}CD27^{high} \rightarrow CD11b^{high}CD27^{low}$ (Chiossone, Chaix et al. 2009). These cells are licensed and fully functional, something we will learn about in detail in the next section.

Human NK cell development has been studied in BM and LNs, and is classically divided into 6 stages as well (Fig 7). They start from $Lin^{-}CD34^{+}CD133^{+}CD244^{+}$ HSCs which become CD45RA+ CD133+ lymphoid primed multipotent progenitors (LMPPs) in stage 1 (Wang and Malarkannan 2020). LMPPs become CLPs, from where they could transition into B, T, NK or other ILCs (Renoux, Zriwil et al. 2015, Scoville, Freud et al. 2019). The NK lineage is secured when CLPs turn into NKPs by expressing CD122, or IL-2 receptor subunit β, which is required for IL-2 and IL-15 signalling pathways, along with the downregulation of CD34 (Freud, Yokohama et al. 2006). Expression of CD56, CD161 and CD94 marks the transition

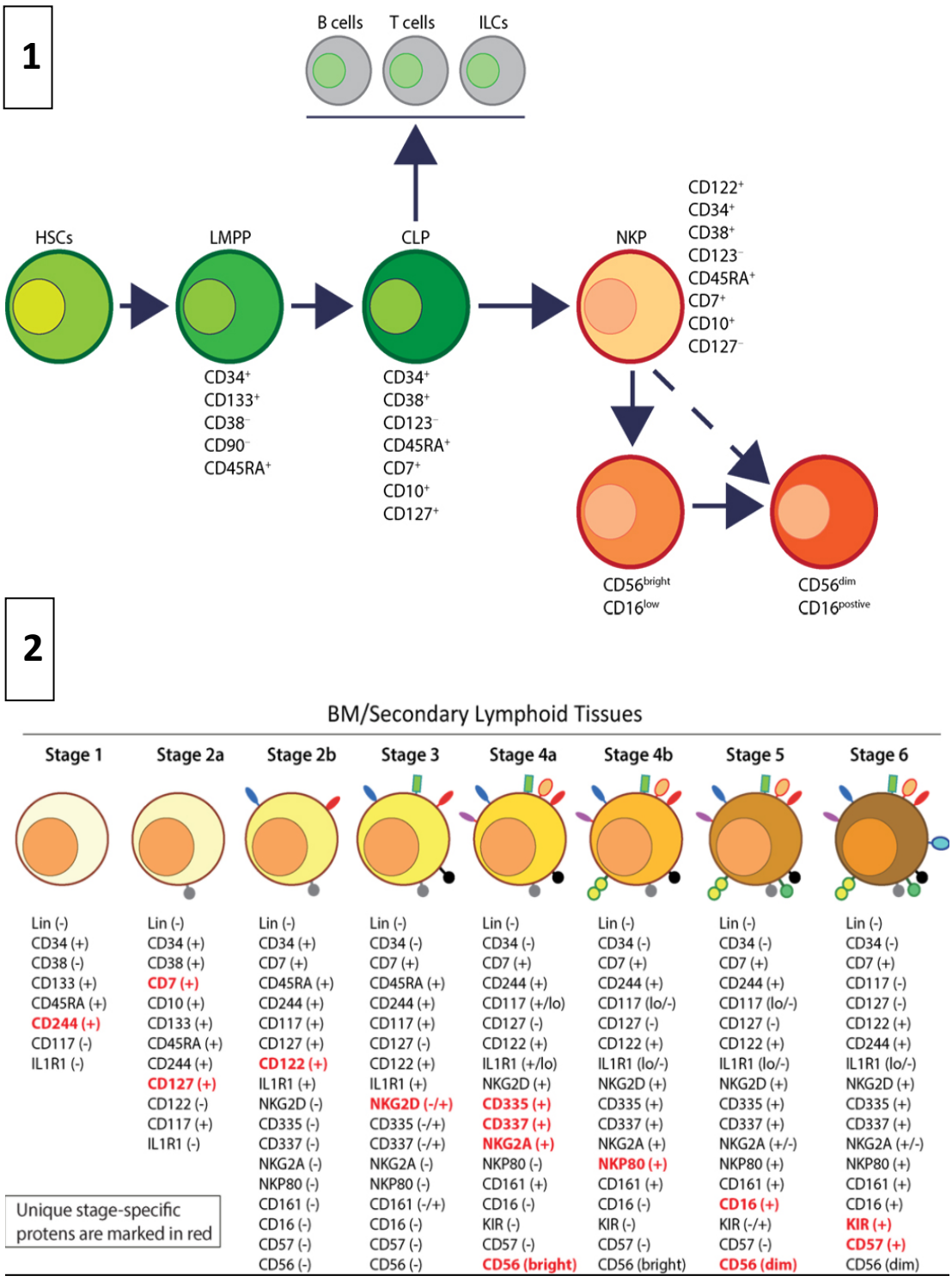



**Figure 7:** (1) Development of human NK cells from Lin−CD34+ hematopoietic stem cells. (2) The development stages of human NK cells are divided in 6 stages as well. Source (Abel, Yang et al. 2018)

from NKPs to mature NK cells (Montaldo, Del Zotto et al. 2013, Chen, Youssef et al. 2018). CD16 and CD56 expression distinguish the mature NK cell developmental stages further. In stage 4a, $CD56^{bright}$ $CD16^{-}$ NK cells represent the cytokine producing immune-regulatory cells, expressing NKG2D, CD335, CD337, NKG2A, and CD161. NKp80 expression, a C-type lectin-like surface-activating receptor, marks the transition from stage 4a to 4b NK cells, which express TFs like T-BET and EOMES (Freud, Keller et al. 2016). NK cells gradually downregulate CD56 and express CD16 and CD94/NKG2C while losing CD94/NKG2A receptor (Vitale, Della Chiesa et al. 2004, Romagnani, Juelke et al. 2007, Pesce, Squillario et al. 2018). These $CD56^{dim}CD16^{+}$ cells have cytolytic functions and are involved in anti-tumour cytotoxicity (Vujanovic, Chuckran et al. 2019). The final stage of NK cell maturation is marked by expression of CD57, a terminally sulfated glycan carbohydrate, and might be suggestive of terminal differentiation (Lopez-Verges, Milush et al. 2010, Collins, Cella et al. 2019).

### 2.3. Educating NK cells

An essential function of NK cells is detection of tumour cells, virally infected cells and their ability to kill these cells, while maintaining tolerance towards healthy cells (Paul and Lal 2017) . NK cells get programmed for this sensitivity by their ability to be inhibited by self-proteins, principally HLA or MHC class I molecules, a process known as education. Their capacity to distinguish between self and non-self was investigated by Klas Kärre and Hans-Gustaf Ljunggren, which led to the ‘missing self’ hypothesis (Ljunggren and Kärre 1990). After the discovery of major NK cells receptors like KIRs and NCRs, the hypothesis could be tested and showed that NK cells targeted tumour or virus-infected cells by recognizing ligands on these cells and by recognising MHC class I expression levels (Long, Burshtyn et al. 1997, Parham 2005, Shifrin, Raulet et al. 2014). Since then, the list of the inhibitory and activating receptors has only increased, (Carrillo-Bustamante, Kesmir et al. 2016, Freud, Mundy-Bosse et al. 2017) and the role of activating receptors as a critical determinant in the response towards target cells has emerged (Champsaur and Lanier 2010, Kruse, Matta et al. 2014, Pazina, Shemesh et al. 2017). It became clear that information other than the absence of MHC class I was required to illicit a proper response from NK cells. The ‘missing-self’ hypothesis, however incomplete, became the basis of further work done in the field of NK cell education.

### 2.3.1. Molecular basis of education

#### 2.3.1.1. Classical MHC class I dependent education

The identification of MHC class I molecules via their corresponding inhibitory receptors is required for creating a fully functional NK cell (Goodridge, Onfelt et al. 2015, Kadri, Thanh et al. 2015). MHC class I inhibitory Ly49 receptors in mice and KIRs (killer cell immunoglobulin-like receptors) in humans have evolved independently, but have similar immunologic roles (de Groot, Blokhuis et al. 2015). Ly49 locus encodes 16 inhibitory and activating lectin-like receptors, while the KIRs are encoded on chromosome 19 in humans. These cells are highly responsive against targets that lack MHC class I, while also maintaining self-tolerance to prevent auto-reactivity by recognising inhibitory signals on MHC class I present cells. These constitute the majority of educated NK cell population (Anfossi, Andre et al. 2006). NK cells that lack the expression of the self-specific inhibitory receptors are surprisingly 'hypo-responsive' instead of being autoreactive (Fernandez, Treiner et al. 2005, Yawata, Yawata et al. 2008). Similarly, in MHC class I deficient mice strains – like β2m KO, TAP-KO and H2-$K^bD^b$ KO– NK cells do not target MHC class I deficient targets (Sun and Lanier 2008). This indicates the need for both NK cell inhibitory receptors and the MHC class I molecules to interact during development to create functional NK cells.

The following paper, "Human natural killer cells in major histocompatibility complex class I deficiency" was written as a comment on the paper "Paths taken towards NK cell-mediated immunotherapy of human cancer-a personal reflection" by Hans-Gustaf Ljunggren. The original paper reviewed the latest state of the art NK cell immunotherapies while reflecting on the general overview of the NK cells, their functional capabilities, NK cell development and differentiation, NK cells in primary immunodeficiencies, viral infections, allergy, autoimmunity and in cancer. In the following part, major histocompatibility complex class I deficiency and the involvement of NK cells has been described. We discuss both the TAP-deficient mice and human TAP1- and TAP2-deficient patients. I contributed to the literature search and writing.

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LETTER TO THE EDITOR

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# Human natural killer cells in major histocompatibility complex class I deficiency

We read with interest the personal reflection of. Hans-Gustaf Ljunggren from the Karolinska Institute (KI) about the path towards natural killer (NK) cell-based cancer immunotherapy.[1] The manuscript includes a paragraph about mouse major histocompatibility complex (MHC) class I deficiency, and as we published several papers about the equivalent entity, Human Leukocyte Antigen (HLA) class I defects, we were inspired to briefly remind the NK cell status in these diseases.

Prof. Ljunggren was among the first to describe that NK cells preferentially kill targets with low or absent MHC class I molecules and postulated the existence of MHC class I-specific inhibitory receptors (IR), which would refrain NK cells from killing normal surrounding cells (missing self-hypothesis).[2] The existence of such receptors was demonstrated by several groups in human, rat and mouse.

The researchers from the KI started, among others, to look at mice genetically deficient in MHC class I molecules, such as beta-2-microglobulin (β2m) knockout (KO) animals. According to the missing self-concept, NK cells were expected to kill autologous cells because they lack expression of self MHC class I molecules, but this is not the case at baseline. The NK cells from such animals are tolerant towards autologous targets and unable to perform missing self-recognition in vitro and in vivo.[3] These observations were confirmed in transporter associated with antigen processing (TAP) KO mice.[4]

An equivalent to TAP-deficient mice was described in 1994 by de la Salle et al[5] in two siblings from a consanguineous marriage, who presented with chronic bacterial infections of the upper and lower respiratory tract. Their serologic HLA class I typing was negative, and the expression of HLA class I molecules assessed by flow cytometry appeared strongly reduced. An autosomal recessive mutation in the TAP-2 gene was identified. Interestingly, ex vivo NK cells from the patients displayed no cytotoxic activity towards K562 (the classical human HLA class I negative NK cell target) nor towards autologous cells.[5,6] Thus, these patients' NK cells were (a) unable to perform missing self-recognition and (b) tolerant to the autologous MHC class I-deficient environment. Upon cytokine-mediated activation, however, they killed several cancer cell lines (including K562), and the autologous B lymphoblastoid cell lines (B-LCL) and skin fibroblasts.[6]

Moins-Teissserenc et al (5) and Furukawa et al. (1) described additional TAP-1 and TAP-2 deficient patients,[7,8] of whom some suffered not only from respiratory infections but had also debilitating granulomatous skin lesions or even destruction of the nasal cartilage.[7] Regarding their NK cells, Furukawa et al confirmed our observations that fresh peripheral blood mononuclear cells were not cytolytic towards K562, Daudi, Molt4 and the HLA class I-negative B-LCL 721.221, in contrast to normal effectors.[8] After activation with interleukin (IL)-2, IL-12, or IL-15, K562 and Molt4 were lysed by the patient's NK cells, but not Daudi nor 721.221, which is opposite to our data.[6] The authors concluded that the tolerant status of TAP-deficient NK cells is maintained even after cytokine stimulation to avoid autoreactivity.[8]

Moins-Teisserenc et al[7] found that four NK cell clones of one patient were not autoreactive, although they killed K562 targets. In contrast, a NK cell line from another case was autoreactive against B-LCL, killed the same cell type from another patient but was inhibited by normal B-LCL, presumably *via* the interaction of HLA class I molecules with specific NK cell IR, the latter being phenotypically and functionally normal in TAP deficiency.[6]

What makes this paper truly interesting and important is the observation that some skin lesions were massively infiltrated with activated NK cells.[7] This suggests a direct involvement of autoreactive NK cells in the pathogenesis of the lung and skin lesions,[7] whereas the self-aggressive peripheral blood NK cells have been stimulated in vitro and were not tested before activation. The initial hypothesis of de la Salle was that the insufficient clearance of viral infections (surprisingly not that severe in TAP-deficient patients) leads to bacterial colonization and superinfection followed by a chronic and deleterious overactivation of NK cells that cannot be inhibited by the insufficient levels of HLA class I molecules in the environment.

In this context, it is interesting to note that tissue NK cells have become a hot topic in recent years, again partly under

the leadership of the KI. It is generally admitted that NK cells in various organs and tissues might not only migrate from peripheral blood, but that different NK cell phenotypes and even lineages might be organ specific.

Although only a bit more than 30 TAP-deficient patients have been described, the clinical presentation and their NK cells appear quite heterogeneous, as illustrated by the discordant findings of our group and those of Furukawa et al[8] and Moins-Teisserenc et al[7] We encountered a patient with TAP deficiency whose NK cells were cytotoxic ex vivo, who had very severe manifestations and died from cerebral vasculitis. In this case, the dogma of the unlicensed NK cells would not apply and might be explained by the clinical status of the patient.

In addition to TAP deficiency, two cases of human β2 microglobulin deficiency were presented by Ardeniz et al.[9] In these patients, not only HLA class I expression is reduced, but also that of the CD1a, CD1b and CD1c molecules, of the FcRn receptor and presumably that of the HLA class I-related molecule MR1, involved in antibacterial defence (as all these structures need to bind β2m for a stable expression). Ex vivo NK cells were not cytotoxic towards K562, in accordance with mouse data (in assays with the appropriate mouse targets).[9]

Overall, NK cells from human HLA class I-deficient patients seem to behave as their mouse counterparts (hypo-responsive ex vivo, auto-aggressive upon cytokine-mediated activation). In both species, NK cells must be educated by the interaction of IR with their cognate MHC class I ligands to become functional, and, in the absence of this interaction, the cells remain hypo-responsive. Nevertheless, when they become stimulated in an infectious and inflammatory context, major auto-aggressive phenomena may occur.

A fundamental difference is that inbred mouse strains are genetically (and maybe even epigenetically) homogeneous, which is not the case when analysing biologic material from different, unrelated human beings. This may explain, at least in part, the discrepancies between our studies and those of Furukawa et al[8] and Moins-Teisserenc et al.[7] In addition, the former stimulated patient cells with cytokines alone for 60 hours,[8] whereas we applied the method based on the co-culture of peripheral blood mononuclear cells with irradiated feeder cells (B-LCL) and IL-2. Moreover, inbred mice usually live in a pathogen-free environment, which might account for the absence of a clinical phenotype.

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## CONFLICT OF INTERESTS

The authors declare that the present letter was written in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

## AUTHOR CONTRIBUTIONS

All authors gave important intellectual input and feedback on the Letter and approved the final submitted version. Jacques Zimmer wrote the Letter.

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#### 2.3.1.2. Non-classical MHC class I dependent education

In addition to the Ly49 receptors/KIRs, other non-classical MHC class I specific inhibitory receptors are involved in regulating NK cell education (Smyth, Sullivan et al. 2013). Non-classical MHC class Ib molecules, Qa-1 in mice and HLA-E for humans interact with CD94/NKG2A and regulate the NK cell education (Sivakumar, Gunturi et al. 1999, Natarajan, Dimasi et al. 2002, Zhang, Feng et al. 2019). NKG2A presenting NK cells target infections and tumour, particularly the ones lacking Qa1/HLA-E ligands (Le Luduec, Boudreau et al. 2019, Shreeve, Depierreux et al. 2021). This process has been observed in case of uterine NK cells and fetal NK cells, where KIRs were found to be lacking in their ability to educate, leading to hyporesponsive NK cells (Ivarsson, Loh et al. 2013, Kieckbusch, Gaynor et al. 2014, Sharkey, Xiong et al. 2015).

Another participant involved is the Ly49A inhibitory receptor that interacts with MHC-I molecule $D^d$, a classical MHC class I molecule and H2-M3, a non-classical one and in part mediate NK cell licensing (Xu, Chun et al. 2006, Chalifour, Scarpellino et al. 2009, Andrews, Sullivan et al. 2012). Ly49A can interact with $D^d$ ligand on other cells in *cis* to gain functional competence and in *trans* to maintain this reactive NK cell (Chalifour, Scarpellino et al. 2009). In MCMV-infection, NK cells are observed to eliminate cells with H2-$D^d$ expression thus controlling viral infections (Parikh, Bern et al. 2020). Ly49A+ NK cells in H2-M3-deficient mice show a lower responsiveness and have substandard tumour response with an increase in risk of Ly49A-dependent tumour invasion (Xu, Chun et al. 2006, Held 2012). CD1d1, a β2-microglobulin-associated non-classical MHC class I molecule, binds to glycolipids on NK cells and inhibits NK cell mediated lysis (Huang, Borszcz et al. 2004). All of these evidences show the role for non-classical MHC class I dependent education of NK cells.

#### 2.3.1.3. MHC class I independent education

Signalling lymphocytic activation molecule (SLAM) family are surface receptors expressed on haematopoietic cells (Wu and Veillette 2016). SLAMF4, SLAMF6 and SLAMF7 are the ones expressed on NK cells. SLAM receptors are homophilic, that is they are their own ligands, with the exception of SLAMF4 (CD244, 2B4), which has CD48 as its ligand (Watzl, Stebbins et al. 2000). In humans, SLAMF4 engagement leads to NK cell enrichment, increased NK cell cytotoxicity and release of cytokines IFNγ and TNFα (Jevremovic, Billadeau et al. 2001, Perez-Quintero, Roncagalli et al. 2014, Gutgemann, Sandusky et al. 2015). In $2B4^{-/-}$ or $CD48^{-/-}$ mice, NK cells could not reject CD48 deficient

tumour cells and the engagement of 2B4 and CD48 proved to be essential for the recognition of CD48-deficient targets (Lee, Forman et al. 2006, Waggoner, Taniguchi et al. 2010). Thus, 2B4 is a non-MHC binding inhibitory receptor for the NK cells (Lee, McNerney et al. 2004). SLAMF4 can also inhibit NK cell mediated lysis of virus-specific T cells, and thus help in clearing of viral infections (Waggoner, Taniguchi et al. 2010). SLAMF6 (CD352) acts as an inhibitory receptor, in the absence of the SLAM-associated proteins (SAM) to enhance NK cell responses via *cis* interactions (Wu, Zhong et al. 2016) and thus is involved in the NK cell education.

Another receptor family looked into is the NKR-P1 protein family, which are C-type lectin-like receptors containing both activating and inhibitory receptors (Giorda, Rudert et al. 1990). Their ligands are also C-type lectin-related proteins (Clr) (Carlyle, Jamieson et al. 2004). NKRP1-B$^{+}$ NK cells cannot clear Clr-b$^{+}$ targets, but can target the Clr-b$^{-}$ cells (Aust, Gays et al. 2009). The absence of NKRP1-B or Clr-b leads to hyporesponsive NK cells with decreased cytotoxic activity (Voigt, Mesci et al. 2007, Rahim, Chen et al. 2015). Therefore, NKRP1-B receptors play a role in NK self-tolerance, recognition of Clr-b$^{-}$ targets.

One more inhibitory receptor involved in the NK cell education is the T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT) which recognizes immunoglobulin superfamily ligand CD155, a non MHC class I ligand. TIGIT$^{+}$ NK cells are highly responsive to various signals, like cells lacking CD155 expression. In CD155$^{-}$ mice, TIGIT$^{+}$ NK cells were hyporesponsive, meaning TIGIT-CD155 interaction was critical in the development of fully functional NK cells, making it yet another MHC class I independent pathway for NK cell education (He, Peng et al. 2017).

### 2.3.2. Models of NK cell education

All of the above-mentioned receptor-ligand interactions have to occur in synchronised manner for the NK cells to become educated. Below, we will discuss some of the proposed mechanisms.

#### 2.3.2.1. Licensing and arming

'Arming model' came up to try to understand how inhibitory receptors aid in NK cell education. It proposes that inhibitory receptors initiate a signalling program or act as a mediator for NK cell educated (Shifrin, Raulet et al. 2014). The 'Licensing model' is similar in its suggestion that inhibitory receptors corresponding to self-MHC ligands endow fully

functional NK cells in a licensing program, which results in self-tolerant NK cells (Kim, Poursine-Laurent et al. 2005). Inhibitory receptors have cytoplasmic tail immunoreceptor tyrosine-based inhibition motif(s) (ITIM) which is(are) crucial for recruitment of phosphatases like SHP-1 [Src homology region 2 (SH2) domain containing phosphatase, encoded by PTPN6], SHIP-1 [SH2 domain-containing inositol 5'- phosphatase, encoded by INPP5D] (Banh, Miah et al. 2012, Viant, Fenis et al. 2014, Chen, Yang et al. 2016). The previously mentioned 2B4 and TIGIT are involved with the SHIP recruitment and $SHIP^-$ phenotype would lead to impaired NK cell education (Wahle, Paraiso et al. 2007).

Similarly, SHP1 deletion in NK cells leads to a change in the Ly49 inhibitory receptor expression and thus changes in the NK cell cytotoxicity (Lowin-Kropf, Kunz et al. 2000). Mutations in the ITIM results in immature NK cells that do not respond to various stimuli (Bern, Beckman et al. 2017), meaning that NK cell education requires ITIM pathway aided by SHP1 or SHIP signalling, providing the proof for the arming model.

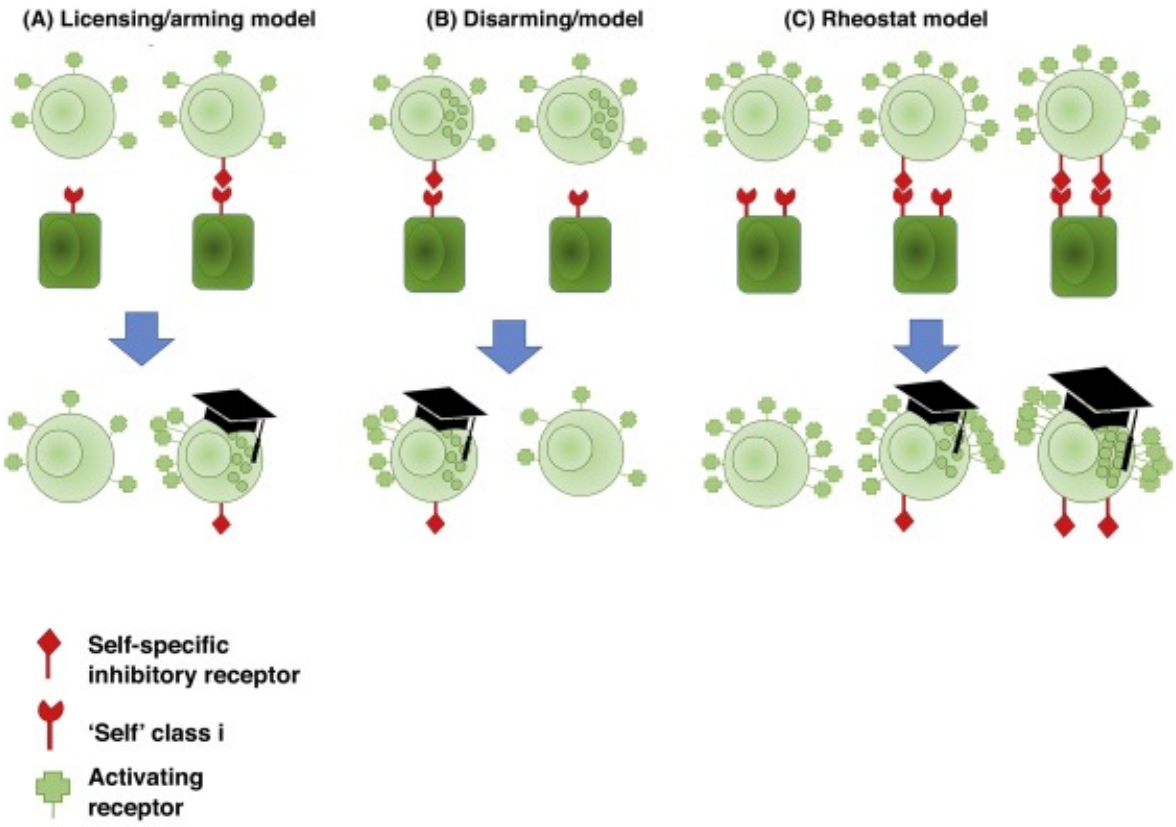


**Figure 8:** Some models of NK cell education. The interaction between various inhibitory receptors, their corresponding ligands and the activating receptors are involved in rendering fully functional NK cells. Source (Boudreau and Hsu 2018)

#### 2.3.2.2. Disarming

Conventionally, inhibitory receptors are associated with the negative regulation of the cells, but evidence shows that their presence is required for making functional and reactive NK cells. The 'Disarming model' aims to clear this inconsistency by speculating that all NK cells start as highly reactive cells that later lose their status due to chronic stimulation induced anergy and become hyporesponsive (Tripathy, Keyel et al. 2008). This was demonstrated in the case of mice with reduced NKG2D receptor expression, and for KIR2DS1 expression in humans (Oppenheim, Roberts et al. 2005, Fauriat, Ivarsson et al. 2010). However, inhibitory signalling mediated by binding with self-MHC class I molecules can counteract the constant activating signals and preserve the reactive status of NK cells (Raulet and Vance 2006).

#### 2.3.2.3. Rheostat model

MHC class I deficient mice studies show that the increase in the number of MHC class I alleles corresponds to the responsiveness of NK cells (Johansson, Johansson et al. 2005, Brodin, Lakshmikanth et al. 2009). Similarly, NK cells that have both NKG2A and Ly49 inhibitory receptors that engage with self-MHC class I molecules, have a better response rate to different stimuli (Joncker, Fernandez et al. 2009), thus establishing that the number of Ly49/KIR and the co-expression of MHC-specific receptors have an influence on the NK cell education (Jamieson, Diefenbach et al. 2002, Frazier, Steiner et al. 2013, Boudreau, Mulrooney et al. 2016). The NK cell responsiveness is also affected by the quality of receptor-ligand interaction, with the stronger interactions favouring stronger NK cell education, its cytokine production capability and degranulation (Brodin, Karre et al. 2009). Rheostat model talks about the quality and quantity of these interactions, in both arming and disarming model to explain that NK cell education process is a continuum.

#### 2.3.2.4. Confining model

NK cell education is affected by the changing levels of MHC molecules present in the surrounding tissue environment, but the above-mentioned mechanisms are not sufficient to completely understand the process. For instance, what dictates the NK cell mediated cytotoxicity? What interactions take place at the cell surface between the various activating and inhibitory receptors to release the cytotoxic granules in a targeted manner? Cellular contacts create synapses, advances in actin remodelling occurs and receptors like

NKG2D and NKp46 aggregate. Here, the activation thresholds are low enhancing the NK cell immune responses (Hadad, Thauland et al. 2015, Lagrue, Carisey et al. 2015). Therefore, adhesion molecules (like LFA-1, a leukocyte integrin) play an important role in forming stable receptor-ligand interactions (Mace, Zhang et al. 2010). Another adhesion molecule important in the context of NK cells is the CD226 or the DNAX Accessory Molecule-1 (DNAM-1). CD226+ NK cells are mature and can recognize various tumours including melanoma, ovarian carcinoma, etc. (Martinet, Ferrari De Andrade et al. 2015). Its expression corresponds to the expression pattern of MHC class I inhibitory receptors, and its co-expression with LFA-1 at the immune synapse facilitates conjugate formation. This contributes to the intensified effector functions of NK cells and thus NK cell education (Enqvist, Ask et al. 2015). This gave rise to the 'Confining model', the idea that the location of the receptors and the involvement of adhesion molecules has an effect on the state of NK cell education and their functionality (He and Tian 2017).

#### 2.3.2.5. Cis- and Trans- interactions

The expression of MHC class I on NK cell surface leads to their education (Elliott, Wahle et al. 2010, Bessoles, Angelov et al. 2013). Ly49 inhibitory receptor family members bind MHC class I molecules in both a -*cis* and -*trans* manner. We already looked at the Ly49A-$D^d$ interactions, where *cis* interaction is necessary for creating functional NK cells, but *trans* interactions are necessary for maintainance. This was studied in mouse models with Ly49-72A variant, which interacts with $D^d$ only in *trans*, and in mouse models with Ly49A Δa1, which recognizes $D^d$ only in *cis* (Chalifour, Scarpellino et al. 2009, Bessoles, Angelov et al. 2013). In both cases, hyporesponsive NK cells were produced, suggesting a failed NK cell education. Inhibitory receptor-MHC interactions in *cis* restrict the inhibitory signalling by the corresponding MHC in *trans*. Thus, this lowers the threshold for activation by an activating ligand, contributing to the regulation of NK cell education. While on the other hand, *trans* interactions are required for NK cell tolerance and to balance the chronic disarming signals (Back, Chalifour et al. 2007).

In humans, *cis* interactions allows the NK cells to maintain their functional properties even when they are transferred to $HLA^-$ hosts. Similarly, acquiring HLA via *trans* interactions was associated with an enhanced KIR+ NK cell responsiveness (Boudreau, Liu et al. 2016). The aforementioned SLAM receptors also were found to be unable to confer education effect with just *trans* interactions (Wu, Zhong et al. 2016, Claus, Urlaub et al. 2019). Further studies are required to understand these interactions in detail.

## 2.4. Functions of NK cells

Circulating NK cells are generally in resting state; however, upon detection of target cells or through activation by a variety of cytokines, NK cells can mediate quick responses and host protection. We will look into their roles in cancers, viral and bacterial infections.

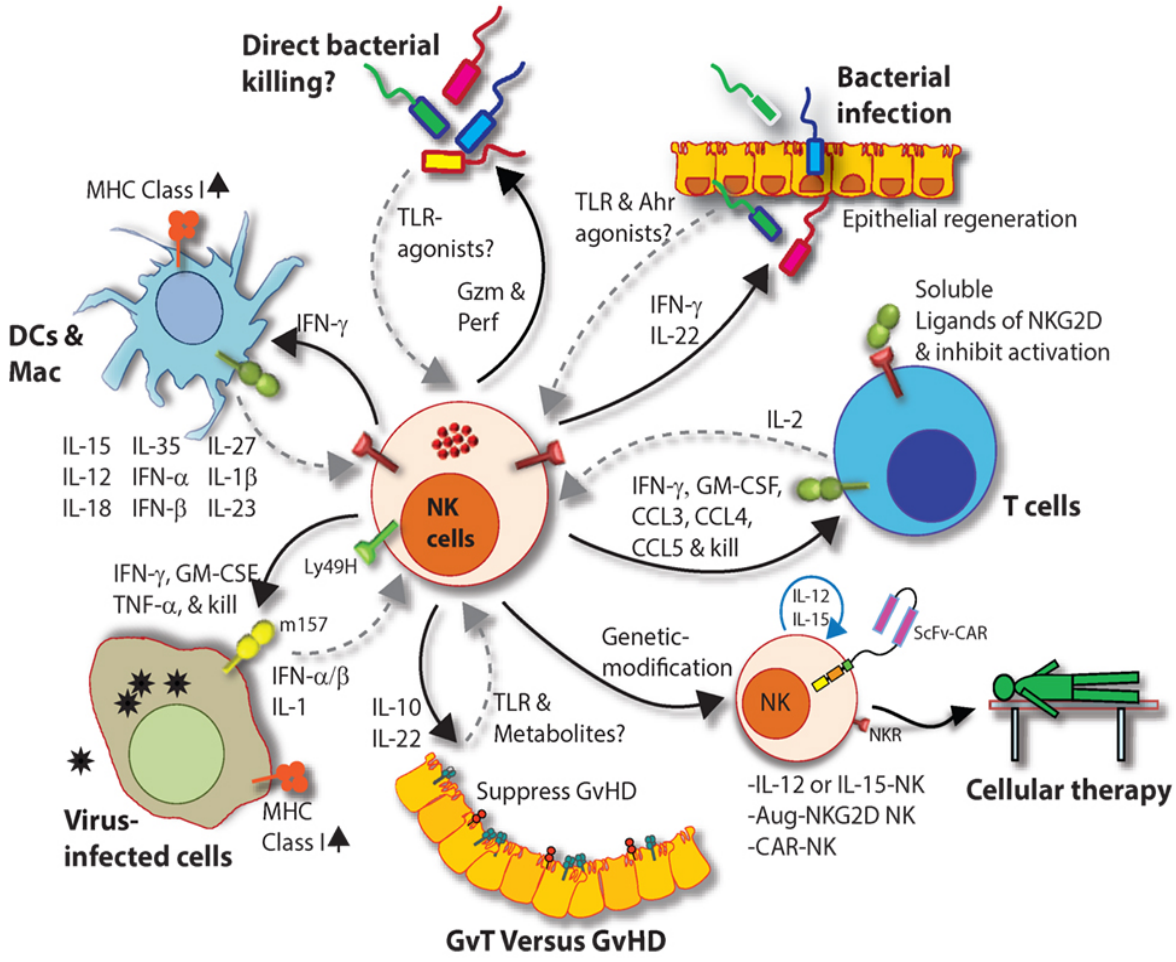


**Figure 9:** The functions of NK cells in various diseases. (1) NK cells interact with dendritic cells (DCs) and macrophages (Mac) leading to priming. (2) Viral infected cells release IFN-α, IFN-β, and IL-1β which are presented to NK cells to activate them. (3) $CD56^{bright}CD16^{-}$ NK cell release IL-10 that leads to a reduction in the graft-versus-host disease (GvHD). (4) Genetically modified NK cells have an increased effector function and are used in cellular therapies including cancer therapies. (5) Interaction of NK cells and T cells is bidirectional and is involved in the regulation of adaptive immunity. (6) NK cells target host cells infected with bacteria via toll-like receptors (TLR)/ aryl hydrocarbon receptor (Ahr) and release cytokines that reduce bacterial load. They also mediate cell lysis by release of granzymes and perforins. Source (Abel, Yang et al. 2018)

### 2.4.1. NK cells in cancer

*In vitro* studies in mice, rats, humans and several other mammalian species has shown that NK cells play a part in cancer immunosurveillance. They can target cancer cells via their cytotoxic granules like perforins and granzymes, inhibit tumour cell proliferation and metastasis (Chiossone, Dumas et al. 2018). In methylcholanthrene (MCA)-induced fibrosarcoma, removing NK cells lead to deteriorating disease progression in mice (Smyth, Crowe et al. 2001). In $RAG2^{-/-}$ (no T/B cells) and $RAG2^{-/-}\gamma c^{-/-}$ (no NK/T/B cells) mice, MCA-induced sarcoma highlighted the role of NK cells in cancer immunoediting. NK cells release IFN-γ, which mediates induction of M1 macrophages (O'Sullivan, Saddawi-Konefka et al. 2012) and other cytokines, influencing the adaptive immune responses (Luetke-Eversloh, Cicek et al. 2014). In B cell lymphomas and mammary carcinomas, NK cells show a perforin-mediated cytotoxicity (Smyth, Thia et al. 2000). However, tumour cells are known for immune-evasion mediated by the secretion of immunosuppressive factors and can escape detection by NK cells (Pietra, Manzini et al. 2012, Baginska, Viry et al. 2013, Paul, Kulkarni et al. 2016). NK cells in tumours have been shown to have reduced cytokine receptors and pro-inflammatory cytokines and thus suppressed cytotoxic activity as seen in melanoma-associated fibroblasts (Balsamo, Scordamaglia et al. 2009). As the role of IL-15 in development and increasing cytotoxic capability of NK cells has emerged, it has been used (in format of *Escherichia coli*-produced rhIL-15 ) in patients with metastatic malignant melanoma or renal cell cancer in clinical trials. The findings support their role in antibody-dependent cell-mediated cytotoxicity (ADCC) and anti-cancer efficacy favouring the use of NK cell based immunotherapies (Conlon, Lugli et al. 2015, Conlon, Potter et al. 2019). Similar results were found with ALT-803, an IL-15 superagonist, in lung cancer (Romee, Cooley et al. 2018, Wrangle, Velcheti et al. 2018). In mice, IL-21 promotes tumour rejection via NKG2D-dependent pathway (Takaki, Hayakawa et al. 2005). IL-12, IL-15 and IL-18 exposed NK cells can retain their effector functions for quite some time and are called cytokine-induced memory-like NK cells (Ni, Miller et al. 2012, Berrien-Elliott, Wagner et al. 2015).

In cetuximab treated head and neck cancer patients, $PD\text{-}1^{+}$ NK cells were associated with better clinical outcomes (Concha-Benavente, Kansy et al. 2018). Similarly, blocking TIGIT receptor prevented NK cell exhaustion and promoted NK cell-dependent tumour specific T cell immune responses in patients with colon cancer (Zhang, Bi et al. 2018). NK cells with lower TIGIT expression also showed higher effector functions, including increased cytokine secretion, degranulation compared to the higher TIGIT expressing NK

cells (Wang, Hou et al. 2015). Chimeric Antigen Receptor (CAR)-transduced cells are efficient killers of tumour cells, both *in vivo* and *in vitro* (Altvater, Landmeier et al. 2009, Chu, Deng et al. 2014). CAR modified NK cells were approved for treatment of recurrent and refractory acute lymphoblastic leukaemia and are currently being followed (Maude, Frey et al. 2014, Mueller, Maude et al. 2017). NK cell therapies, either alone or in combination with other treatments is an exciting opportunity for future clinical trials.

### 2.4.2. NK cells and viruses

NK cells are involved in viral clearance. Virally infected cells are recognised by NK cells, and can be eliminated via CD16-mediated ADCC or direct killing (Ochoa, Minute et al. 2017). This NK cell activation can be due to cytokines secreted by virally infected cells or by activated APCs (Wu, Tian et al. 2017). NK cells respond aggressively in flavivirus infections, irrespective of their education status (Marquardt, Ivarsson et al. 2015, Yao, Strauss-Albee et al. 2017, Zimmer, Cornillet et al. 2019). In case of influenza A virus infections in mice, proliferation of pulmonary NK cells was observed (Carlin, Hemann et al. 2018). While it is known that several cytotoxicity receptors are involved, the exact mechanisms or involvement of NK cells in clearing the infection remains unclear (Luczo, Ronzulli et al. 2021). In chronic viral infections like the HIV-1 infection, NK cells display effector function by eliminating infected host cells, secrete IFNγ, TNF and prevent further viral spread (Scully and Alter 2016, Florez-Alvarez, Hernandez et al. 2018). However, hepatitis C virus (HCV) can establish chronic infection by evading host immune responses. NK cells target $CD4^+$ T cells, leading to $CD8^+$ T cell exhaustion (Khakoo, Thio et al. 2004, Waggoner, Cornberg et al. 2011).

In human SARS-CoV-2 infections, $CD56^{bright}$ and $CD56^{dim}$ NK cells recruitment was observed in lungs (Liao, Liu et al. 2020, Maucourant, Filipovic et al. 2020, Wilk, Rustagi et al. 2020, Xu, Qi et al. 2020). Chemokines like CXCR3 and CCR5 are involved in this recruitment and these NK cells have an increased Ki67, CD69, HLA-DR and CD38 expression and a possible cytokine-driven activation. Further research is needed to understand the antiviral capacity of NK cells against SARS-CoV-2 infection.

### 2.4.3. NK cells and bacterial infections

The following review paper describes the role of NK cells in various bacterial infections. The reviews focus is on the role of airway NK cells in bacterial defence and in health. We talked about the lung-NK cells and how their functions and diminished in the chronic obstructive pulmonary disease. The bacteria we emphasized on were *Pseudomonas aeruginosa*, *Burkholderia cepacia* Complex, *Klebsiella pneumoniae*, along with the other gram-negative and gram-positive bacteria. The idea behind the review was to discuss how NK cell research in anti-bacterial defences has progressed through the years and how they could be part of the therapeutic repertoire for airway infection immunotherapy. I contributed in the literature search.

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REVIEW
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# Airway Natural Killer Cells and Bacteria in Health and Disease

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Natural killer (NK) cells are innate lymphoid cells at the interface between innate and adaptive immunity and mostly studied for their important roles in viral infections and malignant tumors. They can kill diseased cells and produce cytokines and chemokines, thereby shaping the adaptive immune response. Nowadays, NK cells are considered as a strong weapon for cancer immunotherapy and can for example be transduced to express tumor-specific chimeric antigen receptors or harnessed with therapeutic antibodies such as the so-called NK engagers. Whereas a large body of literature exists about the antiviral and antitumoral properties of NK cells, their potential role in bacterial infections is not that well delineated. Furthermore, NK cells are much more heterogeneous than previously thought and have tissue-characteristic features and phenotypes. This review gives an overview of airway NK cells and their position within the immunological army dressed against bacterial infections in the upper and predominantly the lower respiratory tracts. Whereas it appears that in several infections, NK cells play a non-redundant and protective role, they can likewise act as rather detrimental. The use of mouse models and the difficulty of access to human airway tissues for ethical reasons might partly explain the divergent results. However, new methods are appearing that are likely to reduce the heterogeneity between studies and to give a more coherent picture in this field.

**Keywords: natural killer cells, bacteria, infection, lungs, airways, chronic obstructive pulmonary disease, pathogenesis**

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## INTRODUCTION

Historically, human natural killer (NK) cells have mostly been harvested from and studied in peripheral blood (PB), which is an easy way to access them, and where they usually represent 5–20% of all lymphocytes (1–3). Two different subsets have been initially described, called $CD56^{bright}CD16^{-}$ (up to 10% of PB NK cells) and $CD56^{dim}CD16^{bright}$ (at least 90% of PB NK cells). Phenotypic and functional (cytokine production for the former and cytotoxic activity for the latter) characteristics distinguish both populations (1–3). However, things are not that simple, as four additional subpopulations have been identified, which are (i) $CD56^{bright}CD16^{dim}$, (ii) $CD56^{dim}CD16^{-}$, (iii) $CD56^{-}CD16^{bright}$ and finally (iv) $CD56^{dim}CD16^{dim}$ (4), the latter still being almost systematically overlooked in the literature (5). Human NK cell functions are governed by a balance between the messages transmitted through inhibitory receptors (IR), such as KIR, CD94/NKG2A, ILT2, TIGIT, and activating receptors (AR), such as particularly NKG2D and the natural cytotoxicity receptors (NCR) NKp46, NKp30, and NKp44 (6). When stimulated, NK cells exert natural cytotoxic activity against tumor cells and virally infected cells, antibody-dependent

cellular cytotoxicity (ADCC) toward antibody-coated target cells via the Fcγ receptor CD16, and cytokine and growth factor production (2, 6).

Most of the ligands of the IR are represented by Human Leukocyte Antigen (HLA) class I molecules, so that target cells lacking those molecules in part or in total, become killed by the NK cells. The IR nevertheless have another important function, as they are responsible for NK cell education. Indeed, before a developing NK cells becomes functional, its self-specific IR must interact with their ligands expressed by cells in their micro-environment (7, 8). NK cells without such IR, which can represent up to 20% of all PB NK cells, remain uneducated, and hyporesponsive (7, 8). However, they can be activated under certain conditions, such as some viral infections (9).

A hot topic in the NK cell field is of course their potential use as immunotherapeutic anticancer agents. To reach this aim, several approaches are studied, and for example the chimeric antigen receptor (CAR) NK cells, which allow the specific targeting of a tumor antigen (10), or the use of multi-specific antibody constructs directed simultaneously at several NK cell AR and tumor surface molecules (6), appear as particularly promising. It has also been discovered that NK cells, which had been previously considered as exclusively innate immune cells, can develop a memory-like behavior (11). Finally, NK cell metabolism, which appears to be different between educated and uneducated cells, is extensively studied (12, 13).

Another aspect that has changed our view on NK cells in recent years is the observation of a broad heterogeneity of this population. Not only are there many subsets in PB based on the clonal distribution of several IR, immature, partly mature and completely mature fractions based on the relative expression of CD56, CD16 and the IR NKG2A and KIR (14), conventional and adaptive NK cells (14, 15), but there are also heterogeneous aspects between PB and various tissues (15, 16). Very recent data by Dogra et al. (17) suggests a model in which the phenotype, the degree of maturity and the functions of NK cells are closely dependent on the anatomic location, with no influence of age and gender.

## NK CELLS IN THE UPPER AIRWAYS

It is quite difficult to find a substantial amount of references regarding upper airway NK cells. In human, the articles were mostly reporting on the investigation of NK cells in chronic rhinosinusitis, an inflammatory state of the mucosa of the nose and the sinuses (18) with a significant impact on quality of life. Two different forms, one with nasal polyps and one without nasal polyps, are distinguished (19, 20). Bacterial pathogens are considered as one of the etiological factors in this disease (18). However, as the bacteriology of ethmoidal biopsies was the same regardless of the presence or absence of polyps, Niederfuhr et al. questioned the bacterial role in the pathogenesis of the polyps as well as a systematic antibiotic treatment (19). In a study of 18 patients, further subdivided into those responding and those resistant to treatment, and 19 healthy controls, Kim et al. investigated exclusively PB NK cells. The authors demonstrated that the PB NK cells from the patients had decreased effector functions compared to the healthy controls, with the treatment-resistant individuals being most severely affected (18). The recent manuscript by Kaczmarek et al. (20) reported not only on PB NK cells, but also on those from nasal mucosa and from nasal polyps. However, the exploitation of the material was limited to $CD3^{-}CD56^{+}CD16^{+}$ events, which excluded the population of $CD56^{bright}CD16^{-}$ NK cells that might be numerically well represented in these tissues. The phenotypic investigations of this subset in the nose revealed a predominance of relatively immature, $CD27^{+}$ NK cells. Furthermore, the AR NKG2D was expressed at lower frequencies (lower percentages of $NKG2D^{+}$ cells) and lower density of expression in the nasal mucosa and the polyps compared to PB (populations negative for NKG2D were identified in the tissues). Finally, the percentage of NK cells among lymphocytes (mean: 33%) was significantly higher in the polyps than in PB (20).

Okada et al. published a paper about NK cells in the nasal mucosa of the mouse on the C57BL/6 background (21), in which they showed that these NK cells were more immature (according to the relative levels of expression of CD27 and CD11b) and phenotypically more activated (reduced expression of CD62L, higher percentage of $CD69^{+}$ cells) than those from spleen and lung. Around 12% expressed the tissue residency and activation marker CD69 and one third of those also CD103. The pattern of expression of the Ly49 receptor family was different between the three tissues. Functionally [CD107a staining and interferon (IFN)-γ production], nasal NK cells appeared to be hyporesponsive compared to their spleen, but not their lung counterparts (21), which might be related to the possibility that the fraction of $CD69^{+}$ NK cells was not sufficient to significantly activate the global NK cell population in the chosen experimental readouts.

Although this dataset is interesting *per se*, it should not be ignored that Casadei and Salinas (22), in a review about different animal models of nasal infections and immunity, cited several anatomic (functional vomeronasal organ in contrast to human, no Waldeyer's ring) and physiologic (macrosmatic, no coughing-sneezing reflex, lower sensitivity to human viruses) limitations of the mouse in this context, so that such results should always be considered with care before extrapolating to the human situation.

## NK CELLS IN THE LUNGS

Lung NK cells have recently been extensively reviewed in this journal (23, 24), so that a summary of their most important features might be sufficient. Lungs are constantly exposed to microparticles from the environment. Particularly, as the mucosal lung epithelium is at the interface between the outside world and the organism, it can become the entry site for infectious pathogens, be they bacterial, fungal, or viral in nature. Therefore, an extensive and sophisticated local immune response is waiting to be triggered at this anatomic location, and human NK cells, which represent around 5–20% of lung lymphocytes (24), are a part of it. The work of Marquardt et al. has established that most human lung NK cells represented the circulating

subset and had the mature $CD56^{dim}CD16^{bright}$ phenotype (25). They expressed more frequently the differentiation marker CD57 as well as educating KIR than blood NK cells from the same donors but were relatively hyporesponsive upon stimulation with HLA class I-negative target cell lines. In addition, however, a putative tissue-resident subset (around 20% of all lung NK cells), further subdivided into relatively immature $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{-}$ cells (24, 25), expressed the tissue residency markers CD69, CD49a, and CD103. These cells were characterized in detail again by Marquardt et al. (26), who showed that they were functional, especially after stimulation with the cytokine interleukin (IL)-15 and displayed a unique transcriptional profile. Several subpopulations could be distinguished based on the relative expression of CD49a and CD103 (24, 26).

Natural killer cells have likewise been investigated in mouse lungs, particularly by Wang et al. (27) and Michel et al. (28). Both groups found that lung NK cells were more mature than those from the spleen (28) or other organs (27) according to the relative expression of CD27 and CD11b. Whereas the former authors described a higher expression level of the IR CD94/NKG2A and a lower level of the AR NKG2D, the second paper could confirm this data only regarding NKG2D in terms of mean fluorescence intensities. Lung NK cells proliferated less, degranulated less (CD107a assay) and were less cytotoxic than splenic NK cells (28), but these functions were rapidly up-regulated upon bacterial lung infection (27). This suggests that at homeostasis, lung NK cells are inhibited to avoid damage to normal autologous cells, but that they can quickly intervene in case of an infectious insult (27). Michel et al. showed in *in vitro* co-culture systems that both spleen and lung macrophages could significantly up-regulate the cytotoxic activity of lung NK cells through a contact-dependent mechanism (28).

Regarding the homeostatic situation, research in recent years has revealed that in contrast to the older view of the lungs as sterile organs, a lung microbiota is present in the lower airways which exerts significant effects in health and disease, although it is not as abundant as in the gut (29–32). The term "microbiota" refers to all the microorganisms present, namely bacteria, fungi, protozoans, and viruses (29), but here we will only consider the role of bacteria. Six phyla are predominantly represented in the lower airways: Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria (31, 32), Acidobacteria, and Fusobacteria (32). This microbiota is supposed to be transient in healthy donors and to be established from micro-aspiration and inhalation (32) and its composition at any given time point submitted to the parameters of bacterial arrival, bacterial removal, and local immune responses (32, 33). In this way, an equilibrium state is reached that depends also strongly on the gut microbiota through various bacterial metabolites and contributes to the maintenance of homeostasis in the lower airways (gut – lung axis) (32–34). Everything that disturbs this balance, such as some medications and particularly antibiotics, increases in nutrients (high fat diet, low fiber diet), cigarette smoke, infectious agents, chronic inflammation, can disturb the gut as well as the lung microbiota and lead to a state of dysbiosis, characterized by an increased number of airway bacteria and a change in its composition. The dysbiosis is profoundly linked to several severe lung diseases [asthma, chronic obstructive pulmonary disease (COPD), infections, cancer] (29–35).

Natural killer cells have, to our knowledge at least, not been investigated in detail in the context of a normal lung microbiota to date. As most lung NK cells are not activated nor tissue-resident (as illustrated by their negativity for CD69), they might not react very strongly to a normal microbiota. However, as they are expressing several bacteria-specific toll-like receptors (TLRs) that signal in the presence of bacterial pathogens (36), it might be conceivable that they could also mount an immune response toward microbiota components and that this would contribute to homeostasis. The overall immunosuppressed state of lung NK cells at baseline would help to avoid aggression of harmless and useful bacteria and of uninfected autologous cells (31). Yang et al. (31), as well as Fuchs and Colonna (37), discuss data claiming that at steady state, alveolar macrophages secrete immunosuppressive cytokines which keep NK cells in respect. This is in contrast with the results of Michel et al. (28), discussed above. However, the macrophages and dendritic cells (DC) switch to pro-inflammatory cytokine production in case of a bacterial or viral infection and thereby activate the NK cells.

## CHRONIC OBSTRUCTIVE PULMONARY DISEASE

This entity is the third cause of mortality in the United States of America (3) and worldwide (38) and is in most cases the consequence of prolonged cigarette smoking (39). It is characterized by airflow obstruction, emphysema, recurrent infections (24, 39), chronic inflammation, and overproduction of mucus (40). Acute exacerbations significantly limit the quality of life of the patients (38, 39). The exacerbations are in principle caused by viral or bacterial infections, the latter most frequently due to *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* (39). *Pseudomonas aeruginosa* is another bacterium frequently involved and one of the most dangerous ones, based on its highly pathogenic properties (39), and its remarkable level of resistance to antibiotics.

Natural killer cells have been investigated in human COPD as well as in animal models of this disease. Motz et al. demonstrated that exposure of pulmonary leukocytes to viral pathogen-associated molecular patterns (PAMP) induced higher functional properties (degranulation measured with the CD107a assay, and IFN-γ production) *ex vivo* in chronic cigarette smoke exposed than in non-exposed C57BL/6 mice (40). Interestingly, bacterial PAMP appeared to be less efficient in this model, as among five molecules tested, only FSL-1 (bacterial lipopeptide, TLR2/6 agonist) and lipopolysaccharide (LPS, TLR4 ligand) increased the percentage of IFN-$\gamma^{+}$ NK cells above the one of the non-exposed mice. In contrast, other papers reported that NK cell functions are diminished in COPD (41).

It has been further repeatedly demonstrated that in COPD or relevant animal models, NK cell cytotoxic activity is increased relative to non-COPD smokers and healthy individuals (23,

24). Based on the model of lung NK cell hypo-responsiveness at baseline, cigarette smoke and even more the inflammatory state of the lower airways in COPD would activate the alveolar macrophages and induce their production of pro-inflammatory cytokines. These would, in turn, unleash the NK cells and increase their cytotoxic activity, cytokine and chemokine expression, leading to a further aggravation of the inflammation and the clinical status of the patients.

Indeed, in accordance with this concept, Freeman et al. (42) showed that $CD56^+$ cells (in fact a mixture of NK cells and $CD56^+$ T lymphocytes) isolated from lung parenchymal samples of non-COPD smokers and COPD patients with a smoking history, although similar in terms of frequencies between the cohorts, had a different cytotoxic activity toward autologous lung epithelial cells. The $CD56^+$ lymphocytes from the COPD patients were more cytotoxic than the cells from the non-COPD smokers, in an experimental setup without additional stimulation. The target cells were supposed to be mostly epithelial cells based on their positivity for CD326, their size, and their negativity for the hematopoietic cell marker CD45. The cytotoxicity was measured as the percentage of Annexin $V^+$ target cells after the co-culture with the effectors and was around 10% in most samples. This was not a lot, but the NK cells and $CD56^+$ T cells were not otherwise activated. Most of the parenchymal lung NK cells were $CD56^+CD16^+$ and the minor rest $CD56^+CD16^-$ (42).

Another study was provided by the same group in 2018 (43). It showed that isolated, purified lung NK cells induced apoptosis in autologous epithelial cells. This time, the mean level of cytotoxicity was rather high compared to the previous paper, and it was very significantly stronger in COPD patients than in non-COPD smokers. The NK cells, but not the target cells, determined this increased cytotoxic activity, because K562, a HLA class I-negative myeloid leukemia cell line used as the standard NK cell target, was also lysed more efficiently by COPD NK cells than by their non-COPD counterparts. The authors confirmed their data in a mouse model and then showed that the NK cells were primed by DC *via trans*-presentation of IL-15 (43), a phenomenon first described in 2007 by Andreas Diefenbach and his group (44). This would nicely explain the higher level of NK cell cytotoxicity observed in COPD.

Along the same line, Okamoto et al. administered IL-2 and IL-18 to normal mice and observed a lethal effect within 4 days, selectively involving the lungs, with a profound interstitial infiltration of lymphocytes dominated by NK cells (45). High levels of various cytokines and chemokines were found in serum and lungs. Depletion of the NK cells by antibodies completely abrogated the lethal injury, which is a convincing demonstration of the potentially destructive power of NK cell-activating cytokines and NK cells themselves (45). This work was intended as a contribution to the elucidation of the pathogenesis of interstitial pneumonia, but similar mechanisms, in the presence of high levels of pro-inflammatory cytokines in bacterial infections, might contribute to COPD. In human cancer patients, administration of high dose IL-2 induced a vascular leakage syndrome where the so-called lymphokine activated killer cells (equivalent to highly activated NK cells) destroyed endothelial cells, causing a generalized edema, and damaging several organs (46).

Hodge et al. demonstrated a higher number of NK cells in the bronchoalveolar lavage fluid (BALF) of COPD patients (the cohort was composed of current smokers and of ex-smokers) than in healthy smokers (47), a higher content of the cytolytic molecule granzyme B and, most importantly, a significantly increased cytotoxic activity against K562. They also found a reduction in the percentage of BALF NK cells expressing CD94 (which they consider as IR, although it is more a chaperone molecule for the true IR NKG2A). Nevertheless, this indirect measure of a down-regulation of NKG2A could indicate that it contributes to the higher NK cell cytotoxic activity observed in COPD (47).

Recently, Osterburg et al. presented a multiparameter flow cytometry study of PB NK cells from COPD patients compared with smokers and never smokers (38). In contrast to those, COPD patients and smokers highly expressed the maturation marker CD57 as well as the AR NKp46 and NKp44 (normally only present on activated but not on baseline NK cells), but lower levels of CD56. Certain NK cell subpopulations were indicative of prior exacerbations (38).

The AR NKG2C, which is significantly present only on adaptive NK cells from human cytomegalovirus (CMV)-infected individuals, was not differentially expressed in PB of COPD patients with a smoking history and healthy volunteers, but present on a higher percentage of NK cells in the patients with the most frequent exacerbations and the most reduced lean mass (48). A relationship with the bacterial burden cannot be excluded in this context, as there might be a correlation between the viral reactivations and the bacterial colonization, contributing together to the higher number of exacerbations.

Most of the papers discussed above investigated the NK cell cytotoxicity toward autologous cells or conventional NK target cells, but what about a potential direct bacterial killing? NK cells, upon appropriate stimulation, release cytolytic molecules called perforin, granzymes and, in human but not in mice, granulysin, which have an additive or synergistic cytolytic effect toward bacteria (49). They can form pores in the target cell walls and thereby eliminate the microorganisms, but in addition they are able to eliminate some types of eukaryote cells infected by bacteria (41, 49, 50). Furthermore, in addition to the direct effect, NK cells are embedded in the immunological network and react (through an increased cytotoxic activity and cytokine production) to the immune cells and the cytokines/chemokines in their environment (50), which is strongly shaped in case of a bacterial infection ["cellular crosstalk" (50)].

Data about chronic rhinosinusitis, nasal polyposis and COPD are summarized in **Table 1**.

## *Pseudomonas aeruginosa*

As mentioned above, this ubiquitous Gram-negative pathogen is part of those colonizing the lower airways in COPD, but it is also a major problem in cystic fibrosis and in nosocomial infections, with a high morbidity and mortality (51). The role of NK cells in the host defense against this bacterium has been quite extensively studied by the team of Michael T. Borchers (51, 52) in

**TABLE 1 |** Natural Killer (NK) cells in airway diseases.

| Disease | Species | Origin of NK cells | Effect on NK cells | References |
|---|---|---|---|---|
| Chronic rhinosinusitis | Human | Peripheral blood | ↓ Effector functions | (18) |
| Nasal polyps | Human | Nasal mucosa | ↓ NKG2D; ↑ CD27 | (20) |
| COPD model | Mouse | Lung | ↑ IFN-γ | (40) |
| COPD | Human | Lung | ↑ Cytotoxicity | (43) |
| COPD | Human | BALF | ↑ Cytotoxicity | (47) |
| | | | ↑ Number | (47) |
| COPD | Human | Peripheral blood | ↑ CD57, NKp46, NKp44 | (38) |

*Consequences of the indicated diseases on NK cell phenotype and functions. COPD, chronic obstructive pulmonary disease; BALF, bronchoalveolar lavage fluid; and IFN-γ, interferon-gamma.*

mouse models. In the chronologically first work, outbred CD-1 mice were intranasally infected with the *P. aeruginosa* laboratory strain PAO1 (52) and evaluated 24 h later. The findings can be summarized as follows: (i) the infection increased the expression of ligands for the AR NKG2D, present on almost all NK cells but also on a subpopulation of $CD8^+$ T lymphocytes, *in vivo*; (ii) similarly, these ligands increased in an infected human lung epithelial cell line *in vitro*; (iii) the inhibition of the AR NKG2D with a monoclonal antibody significantly reduced the clearance of *P. aeruginosa* from the lungs; (iv) antibody-mediated NKG2D blockade down-regulated the amount of the cytokines IL-1β, IFN-γ and tumor necrosis factor (TNF)-α and in addition of nitric oxide; and finally, (v) the same experiment also revealed a threefold reduction of epithelial cell damage, measured as shedding of these cells into the BALF (52). The latter point brings us again to the recurrent theme of lung cell damage that can be induced by activated NK cells, whereby it would have to be determined if this is beneficial (elimination of infected cells by NK cells) or deleterious (exaggerated damage to the epithelium).

The follow-up paper (51) then presented a conditional mouse model with an inducible expression of NKG2D ligands on lung epithelial cells. Here, the bacterial clearance was significantly higher in those mice that overexpressed the NKG2D ligands. Moreover, the survival up to 96 h post-infection and the level of phagocytosis were significantly increased in the latter group. Similarly, in *in vitro* experiments, where the NK cells were stimulated with LPS, the percentage of NK cells producing IFN-γ was much higher in the mice with the increased expression of NKG2D ligands. As expected, this percentage dropped (but was not completely abolished) in NK cells from infected mice treated with an anti-NKG2D antibody (51).

However, the *P. aeruginosa*-derived exotoxin A, which in combination with IL-1α may induce a dangerous inflammatory state with tissue damage in the host, has also been shown to inhibit NK cell cytotoxic activity against K562, even in the presence of usually stimulating cytokines such as IL-2 (53). The inhibition was almost complete with a high dose of the toxin and still partial with a low dose (53). The effector cells were not purified NK cells but peripheral blood mononuclear cells (PBMC), so that an indirect effect on the NK lymphocytes might play a role in this readout.

Furthermore, Pedersen and Kharamzi described already in 1987 that the *P. aeruginosa*-derived alkaline protease and elastase inhibited NK cell cytotoxic activity against K562, presumably due to a reduction in the effector-target conjugate formation (54). In addition, these molecules strongly reduced the binding of an anti-CD16 (called Leu-11 at that time) antibody (54).

## *Burkholderia cepacia* Complex

This group of pathogenic Gram-negative bacteria is composed of several species, of whom some are dangerous for cystic fibrosis patients, as they are highly resistant to multiple antibiotics (55). Li et al. (55) investigated the interaction between *Burkholderia cenocepacia* and NK cells, and first demonstrated that the NK-like leukemia cell line YT (56), as well as primary purified human NK cells, significantly reduced the number of living bacteria (measured as CFU) after a co-incubation of 2 to 4 h. The results were confirmed with life cell imaging techniques and bacterial uptake of propidium iodide (PI). The authors then wanted to know if the killing activity was contact-dependent or not, and first showed that YT cells bound the fluorochrome-labeled bacteria. Then, they could demonstrate that a direct contact was needed for the killing activity, as nothing happened to the bacteria when they were separated from the NK cells by a porous membrane, allowing passage of soluble molecules but not of cells (55). Most bacteria remained extracellular and were not taken up by the YT cells. Killing was almost completely abrogated after treatment with strontium chloride ($SrCl_2$), which is known to deplete NK cells from their cytotoxic granules (57). Finally, it was established that Src family kinases were activated in YT cells after the contact with *B. cenocepacia* (55). This is a very nice demonstration that NK cells are able to directly kill certain extracellular bacterial species through NK cell – bacteria contact, although the precise mechanism is still unknown. Other possible mechanisms of NK cell-mediated elimination of bacteria are the lysis of intracellular pathogens within the infected cells and the activation of other immune cells, and particularly of macrophages, via NK cell-derived cytokines (such as IFN-γ) (55), and most likely also the killing of bacteria-infected cells expressing ligands for NK cell AR.

## *Klebsiella pneumoniae*

This is another Gram-negative pathogen which poses a major problem due to its frequent causative involvement in nosocomial infections (particularly in pneumonia) and the steady increase of strains multi-resistant to antibiotics (58). Chalifour et al. (59) demonstrated that the outer membrane protein A (KpOmpA) from this microorganism, known to signal via TLR2, induced

IFN-γ and α-defensin (an antimicrobial peptide) synthesis and release in human NK cells. In the mouse, both NK cell-derived IFN-γ (58) and IL-22 (60) have been described to be necessary for bacterial clearance (58, 60). In the paper from Xu et al., it was nicely shown with genetic controls and depletion experiments that the immune defense against this pathogen indeed deeply involved NK cells and that a subset of them produced IL-22. The NK cells had a conventional and mature phenotype (less $CD27^+$, more $KLRG1^+$) distinct from other innate lymphoid cells (ILC) (60). Ivin et al. focused on the fact that IFN-γ production by NK cells, likewise necessary for the elimination of the bacteria through a network with alveolar macrophages, was dependent on the NK cell-intrinsic stimulation by type I IFN, in turn induced by *K. pneumoniae* (58). In contrast to the crucial role of NK cell-derived cytokines, their granzymes (A and B), one of the constituents of the lytic granules, did not seem to play a major role in this model (61). However, this does not rule out that in human, granulysin and perforin together might have a cytotoxic effect on these bacteria.

## OTHER GRAM-NEGATIVE BACTERIA

In the case of *Helicobacter pylori*, responsible for chronic gastric inflammation with the potential to lead to ulcers or cancer, pre-incubation with fixed bacteria increased the cytotoxic activity of NK cell-enriched PBMC toward K562 and other tumor target cells, as well as the release of IFN-γ (62). Furthermore, Rudnicka et al. showed that the bacterial glycine acid extract induced NK cell expansion and IFN-γ production, whereas the LPS from the same bacteria inhibited these parameters, and instead favorized the apparition of IL-10-producing NK cells (63). Although this might just marginally be relevant for NK cells in the lungs, it nevertheless shows to which extent these cells can react to bacteria and how the latter try to manipulate them.

*Legionella pneumophila*, the agent of Legionnaires' disease, is replicating intracellularly in macrophages. Here again, NK cell production of IFN-γ, induced probably through direct TLR messages (64), IL-12 [produced by DC (65)], and IL-18 [produced by neutrophils (66)], was crucial for bacterial clearance from the lungs. In addition, Blanchard et al. had already observed in 1988 in a mouse model that this pathogen stimulated NK cells *in vivo* and *in vitro* to produce IFN-γ and to increase their cytotoxic activity to tumor cell lines, the highest levels having been measured in the lungs (67).

## GRAM-POSITIVE BACTERIA

One of the most frequent culprits in community-acquired pneumonia is *S. pneumoniae*. Regarding the role of NK cells in this infection, their beneficial or detrimental action depended on the pathogen's serotype (68). Thus, the control of serotype 1 depended on NK cells, as demonstrated by Baranek et al. in a mouse model (68). These authors investigated the consequences of a defect in the transcriptional cofactor Four-and-a-half LIM-only protein 2 (FHL-2) on NK cells in general and on pneumococcal infection particularly. It had been previously established that IFN-γ was, once more, the crucial factor in host defense in this context, and that NK cells were one of its major producers (69). In the spleen and the lungs of FHL-2 knockout (KO) mice, the number of NK cells and their expression of the AR NKG2D and NK1.1 (CD161c) were down-regulated and a negative effect of the deficiency on NK cell maturation was observed. Mortality to *S. pneumoniae* lung infection was strongly increased in the KO mice but could be rescued by the adoptive transfer of wildtype NK cells. Finally, the authors showed that IFN-γ production by NK cells was severely reduced and that less neutrophils were recruited to the lungs of the KO animals (68).

The role of the mostly immunosuppressive cytokine IL-10 in dampening the immune response to pneumococcal infection was shown in 1996, when van der Poll et al. administered the pathogen intranasally together with IL-10 and observed early mortality and reduced levels of the pro-inflammatory factors IFN-γ and TNF. Conversely, all this was restored when the mice were pre-treated with an anti-IL-10 antibody (70).

These results were very recently confirmed by Clark et al. (71), who worked with IL-10 reporter and IL-10-KO mice to observe that *S. pneumoniae* induced IL-10 production by NK cells (around 50% of total lung NK cells) with a negative effect on animal survival, and that the bacterial burden was diminished in the lungs of the KO mice compared to wildtype animals. NK cell depletion in the latter induced a strong reduction in the bacterial lung counts and in IL-10. Furthermore, IL-10-deficient mice had significantly more neutrophils and monocytes in the infected lungs. Finally, the virulence protein Spr1875 from *S. pneumoniae* was identified as the IL-10-inducing factor (71).

None of these papers investigated the potential balance between the pro-inflammatory and anti-inflammatory effects of IFN-γ and IL-10, respectively, on the outcome of this infection, which would anyhow have been technically challenging. One might suppose that IL-10 is there to down-regulate an overwhelming immune response that would damage lung tissues, but on the other hand, it might also be counterproductive to dampen it too much and thus to lose control over the pathogens (72). Other groups have described that human as well as mouse NK cells could produce and release IL-10 (73, 74), although, according to Perona-Wright et al., this only occurred in the case of a systemic, but not a localized, pulmonary infection (with the Gram-negative bacterium *Yersinia pestis*) (74). In the case of systemic infections with *Listeria monocytogenes* and *Y. pestis*, approximately 50% of blood NK cells became $IL\text{-}10^+$, and the cytokine was produced by a NK cell subset circulating in blood prior to the infection (74).

Before studying *S. pneumoniae* (71), Clark et al. had already shown that *L. monocytogenes* elicits IL-10 production by NK cells via the virulence factor p60 (with, as a consequence, an inhibition of the recruitment and the activation of myeloid cells) in a mouse model of systemic infection, where the lungs were not further investigated (75).

Another frequently encountered nosocomial and multi-resistant infectious agent is *Staphylococcus aureus*. Small et al. could demonstrate the fundamental role of NK cells in the response to these bacteria in the case of mouse lung infections

(76), as (i) NK cell numbers in the airways increased; (ii) *in vitro* contact with products from the pathogens activated NK cells; (iii) co-culture of NK cells with alveolar macrophages increased the phagocytic activity of the latter, (iv) IL-15-KO mice were much more susceptible to the infection than wildtype mice, whereas they had much more neutrophils and macrophages in the lungs; and (v) NK cell depletion rendered even wildtype mice highly sensitive, despite a conserved IL-15 production (76). These findings demonstrate indeed once again the important role of NK cells in immune defense against extracellular bacteria.

In accordance with this model, Zhao et al. showed that particular matter, associated epidemiologically with enhanced numbers of lung infections, diminished the amount of NK cells migrating to rat lungs in case of infection with *S. aureus*, whereas adoptive NK cell transfer restored a vigorous NK cell response (77). In *ex vivo* experiments, NK cells improved, as in the previous study, the phagocytosis of the pathogens by alveolar macrophages.

It is well known that after influenza, recovering patients are very susceptible to bacterial superinfection, notably by *S. pneumoniae* and *S. aureus* (78). The contribution of NK cells to this phenomenon was demonstrated in a mouse model of H1N1 influenza virus infection followed by intratracheal instillation of *S. aureus*. The sequentially double-infected mice were much more susceptible to the infection (weight loss, survival rate) than those receiving PBS or bacteria only. This went hand in hand with severe changes in the histopathological aspect of the lungs and a marked reduction of local NK cell numbers and TNF-$\alpha^+$ NK cells. Furthermore, the concentrations of TNF-α and of the chemokines IP-10 and MIP-1α were diminished in the BALF. Adoptive transfer of naive NK cells could restore the immune response. The NK cells needed TNF-α to perform their antibacterial effect and this was organized via an interaction with alveolar macrophages and increased phagocytosis (78).

The conclusion that might be drawn from all these papers is that NK cells are very important, at least in mouse models, for the immune response to and the defense against pulmonary infections due to Gram-positive bacteria, with, on the other hand, a detrimental influence of these lymphocytes in case they produce too much immunosuppressive factors [the same old story (72)].

## *Mycobacterium tuberculosis* and Other Mycobacteria

*Mycobacterium tuberculosis* is the agent of tuberculosis (TB), an infectious disease that puts a high burden on the populations in developing but also in developed countries and increasingly shows resistance to conventional antibiotics. It latently infects about 25% of the total population and becomes clinically apparent in ten million patients per year, according to estimations from the World Health Organization (WHO) (79, 80), rendering it a major public health issue. As recently reviewed by Cong and Wei (23), NK cells could interact with this intracellular pathogen through the AR NKp46, NKp44, and NKG2D, as well as TLR2. Although they became activated under these conditions, they seemed to play only a negligible protective role, according to Junqueira-Kipnis et al. (81). These authors showed in a mouse model that lung NK cells augmented in number within the first 3 weeks after exposure to aerosols containing the mycobacteria and up-regulated CD69, IFN-γ and perforin. However, their depletion did not at all change the kinetics of the infection. Human PB NK cells likewise up-regulate IFN-γ after contact with *M. tuberculosis* (23).

Barcelos et al. (82) compared PB NK cells in cohorts of patients with active TB, isolated tuberculin$^+$ skin tests, and tuberculin$^-$ healthy donors. They found a different subset distribution according to the cohorts, with putative TB-exposed but-resistant individuals (defined as those with a positive tuberculin test) having overall less NK cells but an increased percentage of $CD56^-CD16^+$, $CD56^+CD16^-$ and especially $CD56^{bright}CD16^{-/+}$ NK cell subsets compared to the other two donor groups. In contrast, TB patients displayed lower frequencies of $CD56^+CD16^+$ cells. The authors speculated that this different subset distribution might have been related to the resistance or sensitivity to active TB, but of course, as the cells stem from PB, this dataset might have to be interpreted with some caution.

Surprisingly, however, Roy Chowdhury et al. (80), by following a cohort of adolescents from an endemic region in South Africa, could demonstrate with mass cytometry and functional experiments, that latent TB was associated with increased responses of PB NK cells, with a particular role for the AR CD16. Indeed, the percentages of NK cells among total living cells, of total $CD16^+$ cells, of granzyme $B^+$ and of perforin$^+$ cells were significantly higher in patients with latent TB than in healthy, non-infected donors. In addition, ADCC (mediated via CD16) against P815 target cells was also higher in latent TB. By following further cohorts, the authors found that the percentage of PB NK cells was dynamically regulated during latency, progression of the disease and responses to antibiotic medication. This level of NK cells in PB even correlated inversely with inflammation in the lungs of patients with active TB (80). Such observations push NK cells again at the forefront of immune defenses in TB and at a possible role in the maintenance of latency.

With a similar cohort-based approach, Harris et al. (79) evaluated NK cell phenotype and functions in individuals with latent TB compared with healthy controls. Furthermore, participants were separated in infected and non-infected in a TB-endemic region in Kenya and a healthy volunteer cohort from the United States. Among the three groups, the persons from the United States had the significantly lowest percentage of $CD56^-$ cells, which are known to expand in chronic human immunodeficiency virus (HIV) infection and other viral diseases and to be dysfunctional (83). The Kenyan volunteers displayed, among $CD56^{dim}$ NK cells, a higher expression of granzyme B and of the non-MHC class I-specific IR TIGIT, with the highest levels found in the healthy cohort. Furthermore, these individuals had an increased expression of the AR NKp46. Within the $CD56^{bright}$ subpopulation, the Kenyan participants showed increased expression of NKG2D but again decreased levels of NKp46, compared to the cohort from the United States. Functionally, degranulation (CD107a assay), IFN-γ production (intracellular flow cytometry) and CD69 expression

were compared between the three cohorts after co-culture with K562 cells (evaluation of natural cytotoxicity) and P815 mouse cells plus anti-mouse antibody (evaluation of ADCC). Whereas for the latter parameter, no significant differences were observed, frequencies of $CD69^+$, $CD107a^+$, and IFN-$\gamma^+$ NK cells turned out to be significantly higher in the United States study population, such as if an environment endemic for TB would impact the "missing self"-recognition capacities of NK cells (79). The same regional discrepancies were observed after stimulation of total PBMC with three different antigen extracts from *M. tuberculosis*, and the reactivity to these antigens was shown to be at least partially dependent on the presence of IL-12 and IL-18, supposed to be derived from accessory cells.

Conradie et al. (84) described that the level of activation of PB NK cells (frequency of $CD69^+$ and $CD69^+HLA\text{-}DR^+$ events) allowed, among other parameters, to discriminate between *M. tuberculosis*-induced immune reconstitution syndrome, HIV infection and co-infection with both pathogens.

Although these three papers suggest some influence of *M. tuberculosis* on PB NK cells, it is not clear yet to which extent NK cells really intervene in the immune defense against this pathogen that persists in the lungs. An investigation on tuberculous pleurisy (85) revealed a large predominance of $CD56^{bright}CD16^-$ NK cells in the pleural fluid, and an apoptotic effect of soluble factors from this environment predominantly on $CD16^+$ NK cells. *M. tuberculosis* induced IFN-γ production from $CD56^{bright}$ NK cells in the absence of monocytes, T cells and B cells, leaving open the possibility of a direct productive interaction between the bacteria and the NK cells.

Lai et al. (86) presented a work on nontuberculous mycobacterial lung infections, which means due to other mycobacterial species, such as *Mycobacterium abscessus* and *Mycobacterium kansasii*. As the latter become more and more prevalent in developed countries, these authors performed a study in C57BL/6 mice that were infected intratracheally with *M. kansasii*. They found that NK cell depletion increased bacterial burden, mortality, and pathogenetic postinfectious changes (macrophage phagocytosis, DC activation, cytokine production, and development of granuloma). The same observations were made in IFN-γ-KO animals and restored after transfer of wildtype NK cells. These cells were also the most important producer of IFN-γ in this model (86). Lai et al. further cited papers that had demonstrated a similar protective effect of IFN-γ produced by NK cells in the infections with *Bordetella pertussis*, *Francisella tularensis*, and *Chlamydia muridarum* in mouse models of respiratory infection.

Previous publications by the same group had shown that NK cells can directly lyse *M. tuberculosis* and *M. kansasii* via the cytotoxic proteins granulysin and perforin in a contact-dependent manner disrupting mycobacteria cell wall integrity (87), and that in some patients with mycobacterial infections, anti-IFN-γ autoantibodies were detected (88). The killing process involved signaling through NKG2D and NCR as well as MAP kinases, suggesting that similar mechanisms are involved for the killing of bacteria and of eukaryotic target cells (87). This is potentially a very important observation, as it strongly suggests that both conventional cytotoxic mechanisms and cytokine production might be relevant in anti-mycobacterial defense.

Some studies were also performed on *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), an attenuated mycobacterial strain used as an anti-tuberculous vaccine (89). For example, it was demonstrated *in vitro* that $CD56^{bright}$ NK cells reacted to this microorganism by proliferation and IFN-γ production, whereas their $CD56^{dim}$ counterparts better up-regulated the cytolytic proteins perforin and granzyme A (90), all of which was largely expected based on what is known about the functional specialization of these two NK cell subsets (1–3). In a mouse *in vivo* model, where BCG was directly administered (intratracheally) into the lungs, NK cell-mediated production of IFN-γ rapidly increased in the first days after infection, similarly to the number of lung NK cells (89). After NK cell depletion, the reduction of body weight was less pronounced compared to non-depleted mice, whereas the bacterial load remained identical. Importantly, inflammation and injury of the pulmonary structures was much less pronounced in the NK cell-depleted animals, suggesting a pathogenic role for these lymphocytes. Indeed, the level of pro-inflammatory cytokines and chemokines was also reduced in the absence of NK cells, and the percentages of IFN-$\gamma^+CD4^+$ and IFN-$\gamma^+CD8^+$ T cells was significantly increased in these mice. Bacillus Calmette-Guérin-infected macrophages up-regulated NKG2D ligands, which induced their lysis via this receptor-ligand interaction. Finally, the blocking of NKG2D with a monoclonal antibody restored the survival of the macrophages and the T cell-mediated immune response (89).

It is difficult to make a coherent synthesis of all these observations on NK cells and mycobacteria, but it is nevertheless quite appealing that again positive, negative and neutral aspects are described, which may vary according to the models and the experimental setup. This shows that NK cells still hide a lot of secrets regarding their function in anti-mycobacterial infections as well as in bacterial pathogenesis overall. A summary of the relationships between the bacteria discussed and NK cells is presented in **Table 2**.

## CHLAMYDIA

These are obligate intracellular pathogenic bacteria that are responsible for several types of human and mouse diseases. Various studies dedicated to this type of microorganisms illustrated the concept that NK cells usually do not respond as a pure population as may be the case for *in vitro* experiments, but that *in vivo* they are part of a tightly controlled immune network composed of cells, cytokines, chemokines and exosomes.

Thus, in mouse models, NK cells influenced the interaction between DC, T helper (h)1 and Th17 T lymphocytes in *C. muridarum* lung infection (91), modulated the balance between Th1 and Th17 T cells and T regulatory cells (Treg) in the same type of infection (92), and again positively regulated the interactions between DC and T lymphocytes against *Chlamydophila pneumoniae* (93). In all these situations, NK cells exerted a protective and disease-controlling effect via

**TABLE 2 |** Natural Killer (NK) cells and different bacteria.

| Bacteria | Sp. infected | Origin of NK cells | Effects on/of NK cells | References |
|---|---|---|---|---|
| *Pseudomonas aeruginosa* | Mouse | Lung | ↑ Clearance via NKG2D | (51) |
| *P. aeruginosa* exotoxin A | Human | Peripheral blood | ↓ Cytotoxicity (K562) | (53) |
| *Burkholderia cenocepacia* | Human | Peripheral blood | Killing of bacteria | (55) |
| *Klebsiella pneumoniae* | Human | Peripheral blood | ↑ IFN-γ, ↑ α-defensin | (59) |
| *Klebsiella pneumoniae* | Mouse | Lung | ↑ IFN-γ, ↑ IL-22 | (60) |
| *Legionella pneumophila* | Mouse | Lung, spleen | ↑ cytotoxicity, ↑ IFN-γ | (67) |
| *Streptococcus pneumoniae* | Mouse | Lung | ↑ IFN-γ, ↑ IL-10 | (71) |
| *Staphylococcus aureus* | Mouse | Airways | ↑ Number/activation | (76) |
| *Mycobacterium tuberculosis* | Mouse | Lung | ↑ Number, ↑ IFN-γ | (81) |
| *Mycobacterium tuberculosis* | Human | Peripheral blood | ↑ IFN-γ | (23) |
| *Mycobacterium kansasii* | Mouse | Lung | ↑IFN-γ | (86) |
| Mycobacteria | Human | Peripheral blood | Killing of bacteria | (87) |
| *Mycobacterium bovis* BCG | Mouse | Lung | ↑ IFN-γ, ↑ number | (89) |
| *Mycobacterium bovis* BCG | Human | Peripheral blood | ↑ IFN-γ, ↑ PF, ↑ GZM A | (90) |

*Summary of the effects of and/or on NK cells in infections with the listed bacterial pathogens. Sp., species; IFN-γ, interferon-gamma; BCG, bacillus Calmette-Guérin; IL, interleukin; PF, perforin; and GZM A, granzyme A.*

their influence on the bridge between innate and adaptive immune responses.

With the ambitious aim to experimentally investigate the famous "hygiene hypothesis," Han et al. (94) studied mice infected with *C. muridarum* and rendered allergic to ovalbumin (OVA). They observed that prior infection could inhibit at least certain parameters of allergy. However, NK cell depletion partly suppressed the "beneficial" effect of the lung infection. Adoptive transfer of NK cells from infected mice inhibited partially the development of an allergic response in non-infected recipients. NK cell-devoid mice coherently produced more Th2 type cytokines ("pro-allergic" Th2 cytokines, IL-4, and IL-5) than IFN-γ ("anti-allergic" Th1 cytokine).

A detrimental effect of NK cells had been shown for the immune response to the respiratory rodent pathogen *Mycoplasma pulmonis*, related to the human infectious agent *Mycoplasma pneumoniae*. Indeed, in a quite complicated experimental setup, Bodhankar et al. demonstrated that NK cell depletion interfered positively with the development of a protective adaptive immunity after nasal-pulmonary immunization with bacterial antigens (95). This could be explained because NK cells shaped the T cell cytokine response toward more IL-4, IL-13, and IL-17 but away from IFN-γ production.

## NATURAL KILLER CELLS AS CLINICAL INDICATORS IN RESPIRATORY INFECTIOUS DISEASES IN CHILDREN

Wurzel et al. presented large cohort studies of children with protracted bacterial bronchitis (PBB) and mild bronchiectasis, associated or not with human adenovirus co-infection (96, 97). Besides typical socio-economic and clinical factors, an elevated NK cell number relative to the values of healthy children of the same age was observed in the PB of diseased children in general and with adenovirus species C particularly. NK cell phenotype and function were not further investigated.

## A HUMAN KIR RECOGNIZES A CONSERVED BACTERIAL EPITOPE

Recently, Sim et al. (98) made the important discovery that the HLA-C-specific activating KIR2DS4 did recognize a conserved bacterial peptide presented by HLA-C, and more precisely by HLA-C*05:01. The sequence of the peptide required for this recognition was a "rare" self-peptide, but the epitope of interest is conserved in the recombinase A (RecA) of many bacterial species (more than 1000 according to the authors' claims), most of them belonging to serious human pathogens, such as *Helicobacter pylori*, Brucella, *Campylobacter jejuni,* and *Chlamydia trachomatis* (98). Interestingly, activation of resting NK cells via KIR2DS4 alone was sufficient to induce degranulation and cytokine production, whereas all other known AR, except CD16, need at least one co-activating molecule engaged at the same time (99). There was, furthermore, an inverse correlation between the frequency of the KIR2DS4 full length gene and the HLA-C*05:01 allele. Thus, it appears that the KIR family is not only involved in NK cell licensing and in multiple disease associations, but also, most likely, in antibacterial defense. This paper received an accompanying Commentary by Peter Parham, which places the findings in the broader context of KIR and HLA class I molecules (100).

To sum up, NK cells might be directly activated by various bacteria via contact-dependent mechanisms whose modes of functioning are still unknown, via TLR, via KIR2DS4, or more indirectly via the up-regulation of ligands for their AR, such as NKG2D, by infected cells. They might also react to cytokines released into the microenvironment by antigen-presenting cells (macrophages, DC).

## STATE-OF-THE-ART METHODS FOR INVESTIGATION OF THE LUNGS

Dietert et al. published a plea for the histopathological evaluation of the consequences of different infectious lung diseases in

mouse models and described the pathogen-specific features characteristic for each of them (101). Indeed, many variations were observed between the infecting microorganisms, be they bacterial or viral in nature. The authors emphasized that histopathology remains the "most conclusive and practical read out" for the evaluation of the effects of the various infectious models on mouse lungs.

Although this is true, more "modern" and state-of-the-art methods are being developed and are about to enter the laboratories, as a consequence of general scientific and technical progress but also of the "3R" approach regarding experiments with animals.

In 2019, the team of Hans Clevers described the generation of human airway organoids derived from surgical material or from BALF (102). These were long-term proliferating structures that recapitulated a normal airway with different types of cells that are physiologically present *in vivo*. The beauty of the system *per se* was already an accomplishment, but it could be used for the study of various lung diseases, such as cystic fibrosis, cancer, or viral infections (102). Therefore, it is likely that bacterial infections could similarly be investigated in this system, and the data obtained would probably be more relevant to human pathology than the mere mouse models (and save the life of many mice by the way).

The same year, Ross et al. (103) published a review on the "*ex vivo* human lung." They worked with donor lungs not retained for transplantation, extracted primary cells from them and developed an "*ex vivo*-perfused single human lung" that would allow the investigation of different lung diseases. The system seems at first sight less elegant than the lung organoids and is maybe also more limited in the spectrum of possible pathologies that can be investigated. The advantage would be that an entire, complete organ is available and not just an organoid.

Yet another option is the "alveolus-on-a-chip," developed by Deinhardt-Emmer et al. (104). It was a three-dimensional structure with an air phase and a liquid phase, where endothelial cells, epithelial cells and macrophages could be co-cultured. In the presented work, a primary influenza virus infection, followed by a *S. aureus* superinfection, were investigated, and it was shown that the endothelium was seriously damaged under these conditions.

Likewise, single cell transcriptomics is a powerful tool that can reveal huge amounts of details about all kinds of immune cells, and among them NK cells, as exemplified by lung cancer-infiltrating immunocytes in human and mouse (105).

## NATURAL KILLER CELLS AS A THERAPEUTIC OPTION FOR AIRWAY INFECTIONS? CAVE CANEM!

One aspect of NK cells is their putative potential for a dual role as "pro-inflammatory" and "regulatory" effectors, which might be mediated by different subsets (106). Our group has previously touched the problem that NK cells are in fact a double-edged sword, meaning that they might have sometimes beneficial but sometimes rather deleterious effects (72). This has again become clear throughout this review, although the models and studies presented and discussed were all but homogeneous. It might be expected that this will change in the coming years if more and more teams will use the organoid and organ on-a-chip technologies and go into various "omics." Overall, given the current and justified hype for NK cells as efficient agents for cancer immunotherapy, it would be difficult to convince the NK community that their favorite cells might also have a dark side. We emphasize that several methods to improve NK cell antitumoral efficiency, such as particularly CAR-NK cells (10) and NK cell engagers, recently described by the Vivier group (107), should be sufficient to stand up for the use of NK cells in this indication.

However, what about the therapeutic indication of NK cells in infectious diseases in general and in the lungs particularly? Due to the current COVID-19 pandemic, this question has gained increased interest (108, 109), and in addition, most of the papers discussed here that describe an influence of NK cells on the disease course in the airways conclude with the statement that NK cells should be targeted in respiratory infections. But it has clearly been shown that these lymphocytes can have detrimental side effects and cause significant damage to the airways, at least in viral diseases (108, 109). Available literature does not give clear indications regarding bacterial pathogenesis, but the issue was already discussed in 2012, with the question if NK cells are angels or devils in bacterial infectious diseases (50). This problem is, in our opinion, not yet resolved and a lot of research work will be necessary in the field, keeping in mind that the number of multi-resistant bacterial strains is increasing at a terrifying rate and that alternatives to antibiotics must be discovered and developed.

Finally, a general problem in the field and a caveat to many of the presented studies is the difficulty of distinguishing NK cells reliably form ILC1, which also produce IFN-γ as a signature cytokine and have a partially overlapping phenotype. A high plasticity within the ILC family renders even possible the conversion of NK cells, in certain microenvironments, into ILC1-like cells (110–112). However, whereas both NK cells and ILC1 require the transcription factor T-bet for their development and function, NK cells need and express Eomes in addition. ILC1 are preferentially located in tissues and are very rare in peripheral blood, in contrast to NK cells. Thus, one can be confident that the studies discussed here that worked with blood (human) and blood or spleen (mouse) NK cells really investigated NK cells and not ILC1. For tissue-based studies, the differences might be more blunted, although ILC1 are considered as non-cytotoxic cells (110–112). These difficulties are in line with the increasing number of "new" cell types that are currently discovered [for example MR1 T cells (113)], as a consequence of the ever growing diversification and performance of the experimental tools in immunology.

## AUTHOR CONTRIBUTIONS

MT and NP conceived the article, participated to bibliographic research, and critically reviewed the final revised manuscript. JZ conceived the article and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## 3. Revisiting the Functional Impact of NK Cells

The following review paper aims to understand the complexity of the NK cell biology. While everything we described in the preceding sections holds true, it is also important to acknowledge the negative contributions of NK cells in different instances. This particular review compiles the increasing evidence of deleterious effects NK cells cause based on their role in autoimmunity, cytotoxicity in cases such as toxic epidermal necrolysis, in cytokine production leading to the ‘cytokine storm’ due to them being a major source of proinflammatory cytokines such as IFN-g and GM-CSF. Despite these examples, we firmly believe in engineered NK cell-based anti-cancer and anti-infectious immunotherapies. My contribution to the review was literature search and writing.

Trends in Immunology

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Opinion

# Revisiting the Functional Impact of NK Cells

Aurélie Poli,[1,2] Tatiana Michel,[1,2] Neha Patil,[1] and Jacques Zimmer[1,*]

**Immune responses are critical for the maintenance of homeostasis but can also upset the equilibrium, depending on the context and magnitude of the response. Natural killer (NK) cells are well known for their important roles in antiviral and antitumor immune responses, and they are currently used, mostly under optimized forms, as immunotherapeutic agents against cancer. Nevertheless, with accumulating examples of deleterious effects of NK cells, it is paramount to consider their negative contributions. Here, we critically review and comment on the literature surrounding undesirable aspects of NK cell activity, focusing on situations where they play a harmful rather than a protective role.**

## Highlights

NK cells and other innate lymphoid cells may actually be redundant for protective immunity.

NK cells have the potential to induce deleterious effects in multiple settings, including autoimmunity, infections, and cancer.

Depending on the systemic and local microenvironment (cells, cytokines, chemokines, extracellular vesicles), NK cells may hinder a normal immune response or become aggressive towards normal autologous or allogeneic (in the case of transplantation) cells, either via cytotoxic activity or through cytokine production.

There is major controversy about the role of NK cells in the context of reproductive failure, because different influential authors have conflicting views on the topic.

NK cells can be educated through proteins other than MHC class I molecules, for example the inhibitory receptor TIGIT.

## Piecing Together Four Decades of NK Cell Science

NK cells, members of the innate lymphoid cell (ILC) family, are proficient at killing infected and cancerous cells, and at producing **cytokines** (see Glossary). Although they are a part of innate immunity, they participate in adaptive immunity either indirectly by influencing/eliminating adaptive cells or directly by the release of cytokines. In addition, they can adopt a memory phenotype which allows a stronger response to a previously encountered stimulus [1,2]. They share cytotoxicity and cytokine production as well as memory with the cytotoxic $CD8^+$ T lymphocytes, and also their characteristic dependence on **major histocompatibility complex (MHC) class I molecules**; however, they differ in the nature of this dependence because T lymphocytes are activated by MHC association while NK cells are inhibited (Box 1). NK cell functions are tightly regulated by a balance between the signals transmitted through germline-encoded inhibitory and activating receptors. Most of the inhibitory receptors recognize classical or non-classical MHC class I molecules on surrounding cells, and this ligand–receptor interaction protects the healthy host cells. However, during their development, NK cells must express a minimum of one inhibitory receptor (KIR and CD94/NKG2A in human, Ly49 and CD94/NKG2A in the mouse) specific for a self-MHC class I molecule to become optimally functional, a state termed 'licensed' or 'educated' [3,4]. NK cell education is also promoted by the inhibitory receptor TIGIT, whose ligand is a non-MHC related molecule, CD155 (poliovirus receptor) [5,6]. Unlicensed NK cells, which are maintained, represent a non-negligible fraction of the total NK cell pool and can become functional under some conditions [7–10].

In humans, several NK cell subsets are defined based on the relative expression of the adhesion molecule CD56 and the Fcγ receptor CD16. The best-studied are the $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{bright}$ populations, the latter being numerically predominant in peripheral blood and are traditionally considered as the most cytotoxic subtype, whereas the former preferentially produce cytokines [11,12]. While most authors claim that $CD56^{bright}$ NK cells are the immature precursors of the $CD56^{dim}$ subset, this is not definitively established, and the possibility of two different lineages has not been completely ruled out [13]. In a recently proposed classification [14], four subsets are distinguished on the basis of their granule content as well as on the differential expression of several surface molecules and transcription factors: circulating $CD56^{bright}$ NK cells, canonical $CD56^{dim}$ NK cells, adaptive $CD56^{dim}$ NK cells, and

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Box 1. Parallelism and Antiparallelism between NK Cells and Cytotoxic CD8$^+$ T Lymphocytes

NK cells and cytotoxic T lymphocytes have much in common: (i) both are able to rapidly kill target cells, predominantly via the granule exocytosis pathway; (ii) the mechanism of killing is almost identical (apoptosis induction), as is the granule content (perforin, granzymes, granulysin in some species); (iii) both produce cytokines, mostly type 1; (iv) both are derived from common lymphoid progenitor cells in bone marrow; (v) both closely depend on MHC class I molecules for their regulation; (vi) both are important in the defense against viral infections and are capable of lysing infected cells; (vii) both can intervene in the immune response against cancer, and the adoptive transfer of both is proposed as an immunotherapeutic approach against this disease; (viii) both can become memory cells after a first exposure to an antigen, and remain in the organism for a long time, waiting for a new encounter with the same antigen which will immediately induce heightened effector functions; and (ix) importantly, their transcriptional signatures in the mouse are relatively close, as established in a transcriptome-wide analysis by Bezman *et al*. [83].

Needless to say, there are important differences: NK cells are innate lymphocytes without a rearranged antigen receptor, whereas CD8$^+$ cytotoxic T lymphocytes express a rearranged T cell receptor (TCR) that recognizes specific peptides in association with a MHC class I molecule and delivers a strong activation signal to the cell. By contrast, the binding of MHC class I molecules to their inhibitory NK cell receptors blocks NK cell function and maintains tolerance to healthy autologous cells expressing normal MHC class I levels (although NK cells likewise can express activating isoforms of these inhibitory receptors). In addition, CD8$^+$ T cells must undergo positive and negative selection steps in the thymus before being released in a mature, although naïve, state into the periphery, whereas NK cells are thymus-independent. The latter respond relatively rapidly to insults, whereas it takes days to mount efficient T lymphocyte-mediated responses unless memory cells are involved.

finally tissue-resident CD56$^{bright}$ NK cells (Table 1). However, the heterogeneity extends beyond this classification, and it is now increasingly recognized that tissue-resident NK cells can be further separated into 'organ-specific' subsets that can be distinguished phenotypically and functionally from conventional NK cells, probably governed by the specific local microenvironments [15].

Most immune cell types are obviously necessary for immunity but can also be deleterious, sometimes within the same immune response; NK cells, that are traditionally seen as efficient antiviral and antitumoral effectors, are not an exception. In fact, it is now clear that they fall into the same cost–benefit paradigm as other immune cells.

We have chosen here to not describe their widely recognized beneficial effects in detail, but instead focus, mostly in human studies, on their more recently explored negative aspects after briefly addressing the topic of their potential redundancy.

## Redundancy of NK Cells (and Other ILCs)

In a recent key paper by Vély *et al*. [16], **severe combined immunodeficiency** (SCID) patients treated with allogeneic **hematopoietic stem cell** transplantation were followed up for 7–39 years post-transplantation. Individuals without prior myeloablation did not reconstitute ILCs. Nevertheless, none developed severe infections or neoplasms, and two had normal pregnancies. The authors concluded that ILCs seem to be redundant for protective immunity in conditions of modern hygiene and medicine, when T and B cell functions are maintained. A strong argument against an important role of NK cells in protective immunity is the fact that patients with immunodeficiency syndromes predominantly involving NK cells can reach adulthood [17], in contrast to patients with early life-threatening conditions such as SCID where T cells are absent [16,18]. In addition, several papers have suggested a role for ILCs in the pathophysiology of inflammatory diseases (allergy, inflammatory bowel disease, chronic obstructive pulmonary disease, psoriasis), as recently reviewed [19,20]. It is also notable that Vély *et al*. [16] only provided limited investigations on tissue ILCs, whereas in the mouse the majority of these cells are tissue-resident [19]. In an illustration of this principle, Weizman *et al*.

## Glossary

**Antibody-dependent cellular cytotoxicity (ADCC):** killing of an antibody-coated target cell by a cytotoxic effector cell.
**Bronchoalveolar lavage (BAL):** medical procedure using a bronchoscope for the collection of BAL fluid for the diagnosis of lung disease.
**Corticosteroids:** steroid hormones produced by adrenal cortex, also prepared synthetically; commonly used as potent anti-inflammatory drugs.
**Cytokines:** small molecules secreted by immune and other cells; examples include chemokines (attracting immune cells to sites of inflammation), interferons, and interleukins.
**Dendritic cells (DCs):** antigen-presenting cells (APCs) that process antigens and present them to T cells; also control immune responses by secreting cytokines.
**Graft-versus-host disease (GvHD):** a condition in which donor immune cells attack normal tissues of the recipient; GvHD can be mild, severe, or life-threatening.
**Granzyme:** a cytotoxic molecule secreted by immune cells that mediates apoptosis in target cells. The human granzyme family has several members with similar functions (granzymes A, B, H, K, M).
**Hematopoietic stem cells:** multipotent cells residing in the bone marrow that can differentiate into all blood cell lineages.
**Major histocompatibility complex (MHC) class I molecules:** these are expressed on the surface of all nucleated cells; they display peptide fragments from within the cell to cytotoxic T cells and inhibit NK cells through an interaction with inhibitory receptors.
**Neurogenic niche:** a region in which the cytoarchitecture and signaling factors within the microenvironment preserve neural stem cells (NSCs) with self-renewing capacity that give rise to new neurons and glial cells.
**Perforin:** pore-forming protein secreted by NK cells and cytotoxic T cells; acts on the surface of target cells and favors the entry of granzymes.

described that ILC1 cells, but not NK cells, protect mice at the initial sites of viral infection via early and abundant IFN-γ production [21]. One could further argue that the cohort investigated by Vély *et al*. [16] is relatively small, given that SCID is not a frequent disease. These patients also probably benefit from very close medical follow-up, allowing early detection and treatment of infections.

NK cells have been conserved by evolution, and it has been extensively shown that they take part in immune responses against infections and tumors. Should we then consider that these cells, being actively present during immune responses but 'not really needed', could become troublemakers detrimental to healthy immunity? Along the same lines, Weinkove *et al*. [18], in their comment on the paper by Vély *et al*., suggest that ILC depletion in 'inflammatory disorders' might become a therapeutic option given both their detrimental role in these diseases and their assumed redundancy in healthy subjects.

Nevertheless, it is important to note that, as with any intervention on the human immune system, extreme caution should be applied because the consequences of eliminating a big piece of the immunological puzzle are difficult to foresee. In addition, it is not yet clear how to achieve ILC depletion in human.

## NK Cells in Autoimmunity

The role of NK cells in the pathogenesis of autoimmune diseases (Table 2 and Figure 1) has long been suspected, and their defective immunoregulatory functions are gaining increased attention.

For example, in systemic lupus erythematosus (SLE), Ye *et al*. have shown in a cohort of 32 newly diagnosed patients that, although the proportion of NK cells was lower than in healthy controls, there were fewer inhibitory $KIR^+$ and more $NKp46^+$ cells [22], which would theoretically predispose them to be more activated. Interestingly, the percentage of $NKG2C^+$ NK cells was also significantly lower in the patients, whereas it was found to be higher in the cohort described in Hervier *et al*. [23]. NK cells are probably more involved in the early phases of autoimmunity [24]. In most human autoimmune diseases, NK cells in peripheral blood are lower in number than in healthy controls, and/or are functionally altered [25]. They might instead

**Programmed death ligand 1 (PD-L1):** binds to its receptor PD-1 on T cells, B cells, some NK cells, and macrophages (interaction is inhibitory for immune cells).
**Sepsis:** life-threatening organ dysfunction caused by a dysregulated host response to infection.
**Septic shock:** a subset of sepsis in which profound circulatory, cellular, and metabolic abnormalities are associated with a greater risk of mortality than with sepsis alone.
**Severe combined immunodeficiency (SCID):** congenital genetic disorder caused by mutations inhibiting the development of T cells, B cells, and/or NK cells.

Table 1. Subpopulations of Human NK Cells Based on Transcription Factor and Surface Receptor Expression as well as Granule Content [14].

| | Circulating $CD56^{bright}$ NK cells | Canonical $CD56^{dim}$ NK cells | Adaptive $CD56^{dim}$ NK cells | Tissue-resident $CD56^{bright}$ NK cells |
|---|---|---|---|---|
| Transcription factors | | | | |
| EOMES | High | High | High | Low |
| PLZF | Low | + | – | ? |
| Granule content | | | | |
| | Low | High | High | Low |
| Surface molecules | | | | |
| CD62L | + | +/– | – | – |
| CD49a/CD103 | –/– | –/– | –/– | +/+ |

Table 2. Beneficial and Detrimental Effects of NK Cells across Diseases

| Condition | Observations | Detrimental NK cell effects | Beneficial NK cell effects |
|---|---|---|---|
| **Autoimmunity** | | | |
| Systemic lupus erythematosus | ↘ Circulating NK cells [22,23]<br>↗ IFN-γ production [23]<br>↘ $KIR^+$ NK cells [22;23]<br>↗ $NKp46^+$,<br>↘ $NKG2C^+$ [22], or<br>↘ $NKG2C^+$ cells [23] | May induce autoimmune reactions involved in the pathogenesis of these diseases | ? |
| Type I diabetes mellitus | ↗ NK cells in pancreas [27] | | ? |
| Alopecia areata | ↗ NK cells in hair follicles [28] | | ? |
| Juvenile dermatomyositis | ↗ NK cells in muscle [24] | | ? |
| Rheumatoid arthritis | ↗ $CD56^{bright}$ NK cells in synovial fluid<br>↗ IFN-γ secretion [29] | | |
| **Inflammation** | | | |
| Toxic epidermal necrolysis | ↗ NK cells in the skin [31] | Induce keratinocyte apoptosis | ? |
| Multiple sclerosis | ↗ NK cells in the SVZ [34] | Induce death of NSC in CNS | A high percentage of circulating $CD56^{bright}$ NK cells and NK2 cells correlates with remission phase [33] |
| Brain ischemia | ↗ NK cells secreting IFN-γ;<br>↗ NK cells in brain lesions [36] | Increase local inflammation and catalyzed neuronal death | ? |
| **Infectious diseases** | | | |
| Human hantavirus infection | ↗ NK cell activation [37] | NK cells are implicated in the destruction of endothelial cells leading to vascular permeability | Elimination of infected cells [1,2] is a basic feature of NK cells |
| HIV | ↗ $NKp44^+$ NK cells [46] | Killing uninfected $CD4^+$ T cells | |
| Chronic hepatitis B | | Elimination of virus-specific $CD8^+$ T cells via the TRAIL pathway [43] | |
| Sepsis and septic shock | ↗ IFN-γ, TNF-α, and GM-CSF [47] | Involved in the cytokine storm leading to tissue damage and multiple organ failure | |
| **Asthma** | ↗ % NK cell in BALF<br>↗ release of cytolytic molecules<br>↗ NK cells secreting IL-4<br>↘ NK cells secreting IFN-γ [38] | ↘ Lung functions may be implicated in the physiopathology of asthma | Protective effect (may be suppressed in severe asthma) [38] |
| **Cancer** | ↗ IFN-γ secretion [61–63] | May be involved in the selection of less-immunogenic variants of tumor cells and may influence angiogenesis; direct elimination of these T lymphocytes | Elimination of cancerous cells [1,2]; is another basic property of NK cells |

Table 2. (continued)

| Condition | Observations | Detrimental NK cell effects | Beneficial NK cell effects |
|---|---|---|---|
| **Reproductive failure** | Disturbance of the uNK cytokine levels<br>↗ % circulating NK cells<br>↗ cytotoxicity of circulating NK cells [41] | ? Recurrent miscarriage and implantation failure are associated with increased levels and enhanced cytotoxicity of peripheral blood NK cells | ? May favor trophoblast implantation through the release of angiogenic factors [41] |
| **Transplantation** | | | |
| Solid organ transplantation | | NK cells likely participate in graft rejection via ADCC [93] | ? |
| Hematopoietic stem cell transplantation | | Acute GvHD following donor-derived infusions of NK cells stimulated with IL-15 and 4-1BBL [92] | Protective effect against GvHD, likely via (i) killing of APCs, which precludes allogeneic T cell activation; and (ii) direct elimination of these T lymphocytes [91] |

accumulate in the target organs [25] and potentially cause harm there. As reviewed by Spada *et al.*, data from different patient cohorts are often contradictory, whereas mouse models emphasize the need to look at the involved organs, and in particular the kidney, in addition to the spleen [26]. In human studies it is easy to access peripheral blood, but the relevance of the information obtained is not always clear when the pathology is predominantly organ-specific. The NK cells might accumulate in these organs, as shown in the pancreas in patients with type I diabetes mellitus [27], in the hair follicles of alopecia areata patients, and the muscles in juvenile dermatomyositis patients [24,28]. In rheumatoid arthritis, $CD56^{bright}$ NK cells are abundant in the synovial fluid and produce large amounts of IFN-γ [29]. However, for obvious ethical reasons, organ biopsies in patients should be limited to situations of medical need.

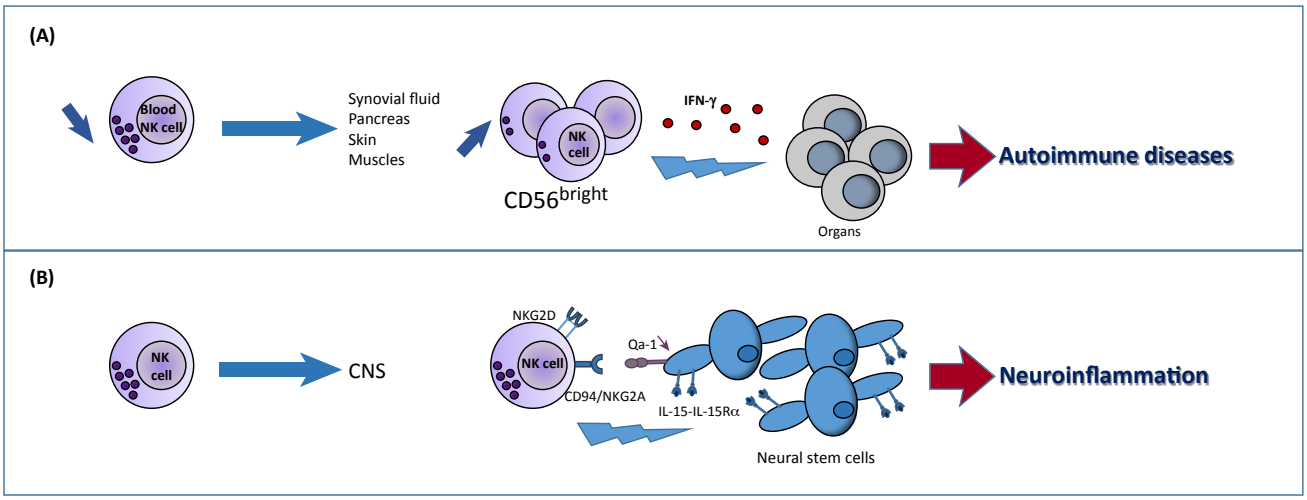


Figure 1. Natural Killer (NK) Cells in Autoimmunity. NK cells exert their influence through the release of cytokines and/or lysis of the target cell. NK cell functions are controlled by ligands expressed on various cells, and interaction with these ligands triggers inhibitory or activating immune-regulatory activities of NK cells leading to an increase in pathologies such as autoimmune diseases. (A) Illustration of human NK cells in rheumatoid arthritis, type I diabetes mellitus, alopecia areata, and juvenile dermatomyositis. (B) Example of the potential effect of mouse NK cells in the EAE model. Abbreviations; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis.

Interestingly, in chronic lymphoproliferative disorders of NK cells, autoimmune cytopenias can occur [30], suggesting that dysfunctional NK cells might indeed become autoaggressive. NK cells can exert their effects through the release of cytotoxic granules containing **perforin**, **granzymes**, and, in humans, granulysin, which kill target cells. In addition and/or in parallel, they secrete diverse cytokines and chemokines that participate in the complex immune responses to infections and tumors. Consequently, the detrimental effects of NK cells can be attributed either to their cytotoxicity and/or to the cytokines they produce, and this is another parallel with $CD8^+$ cytotoxic T lymphocytes (Box 1).

## Detrimental NK Cell Effects Based on Cytotoxicity

An example where both NK cells and $CD8^+$ T cells seem to act in concert with potentially fatal consequences for the patients is toxic epidermal necrolysis, a severe adverse reaction to drugs where keratinocytes are massively destroyed via apoptosis after the release of cytotoxic molecules, in particular granulysin [31]. It is not clear what triggers the NK cells in this pathology, but they might be memory NK cells that react with the disease-inducing drug similarly to the reaction to haptens that leads to the emergence of hapten-specific memory NK cells in the mouse [32].

Not surprisingly, both positive and negative influences of NK cells on human multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), have been described [33]. The group of Shi [34] has recently shown that, in human brain sections from chronic MS patients and in the late phase of mouse EAE, there are abundant NK cells in the subventricular zone (SVZ), and more precisely in the **neurogenic niche**. During the acute phase of EAE, the neural stem cells (NSCs) upregulate IL-15/IL15-Rα complexes on their surface that stimulate the survival of the penetrating NK cells but inhibit their cytotoxic activity through a concomitant increase in the expression of Qa-1, the ligand of the NK cell inhibitory receptor CD94/NKG2A. Finally, in the chronic phase, SVZ NSCs further augment IL-15/IL-15Rα molecules but downmodulate Qa-1, downregulating the inhibitory signal and leading to NK-cell-mediated killing of NSCs (Figure 1). One might conclude that NK cells do what they have learned to do during their education, namely killing target cells when they are sufficiently activated and not inhibited enough, and this can sometimes lead to significant collateral damage. The group of Shi also observed rapid recruitment of NK cells to ischemic brain lesions in correlation with their size [35], killing neurons which downregulate Qa-1 in parallel with an increase of NKG2D expression by the NK cells [36].

In infectious diseases, the cytotoxic activity of NK cells might contribute to pathogenesis. For example, Braun *et al*. [37] demonstrated robust NK cell activation in human hantavirus infection that was attributable to virus-induced expression of IL-15 and IL-15Rα by infected cells. *In vitro*, such overactivated NK cells kill uninfected endothelial cells, and this might in part explain the detrimental increase in vascular permeability observed in these patients. NK cells have also been implicated in a different vascular disorder, atherosclerosis (reviewed in Box 2).

### Box 2. NK Cells in Atherosclerosis

Human NK cells have been described as being both pro- and antiatherogenic [84,85]. Animal investigations based on NK cell depletion, adoptive transfers, and the use of NK-cell-deficient models have likewise given conflicting results. NK cells are relatively abundant in human atherosclerotic plaques [86], and the number of blood NK cells correlates with the severity of this disease [87]. Interestingly, Martinez-Rodriguez *et al*. [88] demonstrated that the expansion of human $NKG2C^+$ NK cells, which occurs upon cytomegalovirus infection, is accompanied by a higher risk of plaque rupture. It is possible that differences in the mouse strains employed could underlie the discrepancies between the different studies. However, the use of sophisticated models now indicates that cholesterol-induced atherosclerosis develops independently from NK cells, except in the additional context of systemic stimulation [e.g., with poly(I:C)] [89]. With regard to human patient cohorts, it is frequently argued that selection biases and/or significant differences in study designs might underlie the discordant results, raising the question of why efforts are not made to adopt a uniform clinical trial design.

Likewise, in a cohort of human patients with severe allergic asthma, Duvall *et al*. [38] showed that (i) **bronchoalveolar lavage** (BAL) NK cell number and lung function were inversely correlated, (ii) peripheral blood NK cells of asthmatic patients released more cytolytic molecules but were less cytotoxic than in healthy donors, and (iii) **corticosteroids**, which are the basis for treatment of most asthma patients, further downregulated NK cell functions. In this situation, NK cells thus appear to be bystanders rather than drivers of asthma, and are affected by both allergic inflammation and treatment.

In another context, the immunogenetic association of maternal KIR expressed by uterine (u)NK cells and fetal HLA-C genotypes expressed by the extravillous trophoblast are associated with reproductive failure. Thus, a KIR AA haplotype will functionally result in strongly inhibitory signals to uNK cells upon presentation of paternal HLA-C2 by the fetus, thereby increasing the risk of pre-eclampsia and recurrent miscarriage. Conversely, the KIR B haplotype, which contains the activating KIR2DS1 that binds C2 ligands, is correlated with protection from pre-eclampsia and increased birth weight [39,40]. To predict and prevent complications of pregnancy, the KIR/HLA system is not the only factor influencing uNK cell functions. Genetic, nutritional, and environmental influences need to be considered in each pregnancy to better understand the biology of uNK–trophoblast interactions [41].

NK cells have the ability to negatively regulate autologous **dendritic cells** (DCs) and T cells [42,43], and consequently can impact on the outcome of an immune response. It has been known for a long time that NK cells can kill immature DCs that express insufficient levels of MHC class I molecules, and even mature DCs with suboptimal surface amounts of these proteins (DC editing) [44], thereby contributing to the control of immune activation processes and potentially limiting T cell responses [43]. NK-cell-mediated lysis of activated T cells occurs through granule release or through death receptor–ligand interactions (FasL and TRAIL): in the former case, these T cells express ligands for NK cell activating receptors [45]. This phenomenon can, depending on the precise challenge to the immune system, be beneficial or detrimental to the host [45], and might in the worst case prevent an efficient defense against pathogens if too many activated T cells are eliminated by NK cells. This occurs for example in HIV infection where uninfected $CD4^+$ T cells upregulate the ligand for the activating receptor NKp44 [46], as well as in chronic hepatitis B infection characterized by the elimination of virus-specific $CD8^+$ T cells via the TRAIL pathway [43] (Figure 2).

## Detrimental NK Cell Effects Based on Cytokine Production

In infectious diseases, NK cells are considered to be one of the major sources of the 'cytokine storm', or cytokine release syndrome, in which massive secretion of proinflammatory cytokines (in the case of NK cells, these are mostly IFN-γ and GM-CSF) in response to an infection can be fatal for the patient – who may not succumb to the pathogen itself but instead to the consequences of the overwhelming immune response it induces, leading to tissue damage and multiple organ failure [47]. This is particularly obvious in bacterial **sepsis** and **septic shock** [47], in several acute airway infections such as influenza [48], and during Ebola virus infection [49]. It is followed by a state of immunosuppression which leaves the patient vulnerable to secondary infectious complications [47]. NK cells could participate to this immunosuppression during *Listeria monocytogenes* infection where human and mouse NK cells, after an initial peak in IFN-γ production, start to express the immunosuppressive cytokine IL-10 that inhibits the activation of myeloid cells involved in the antibacterial response, and this response is therefore blunted [50]. Other authors have likewise reported that NK cells are capable of producing IL-10 [51,52], which can dampen systemic infection caused by *Toxoplasma gondii*, *Yersinia pestis*, and *Listeria monocytogenes* in mice. Human NK cells produce IL-10 during chronic hepatitis C

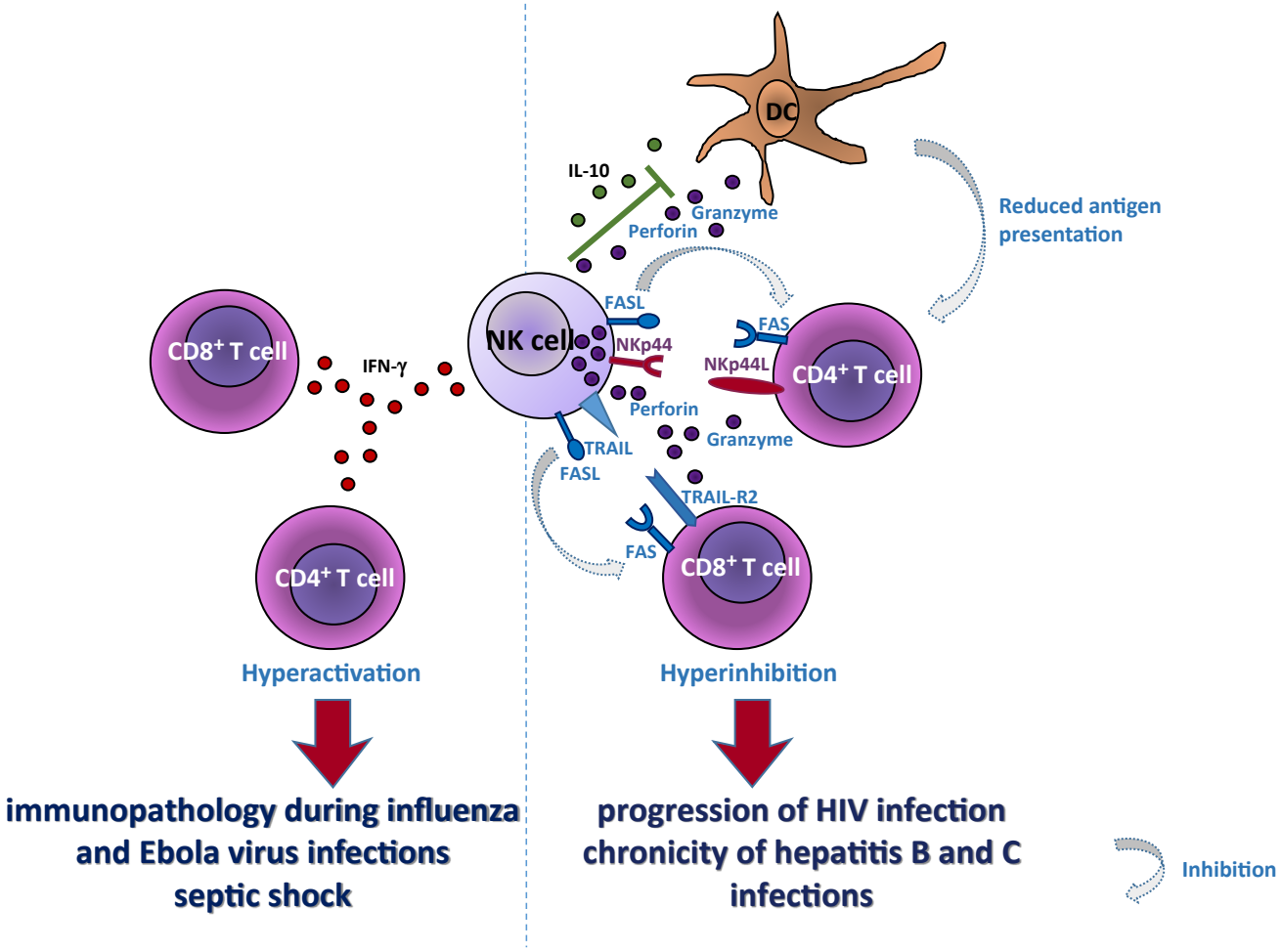


Figure 2. Pathological Consequences of Natural Killer (NK) Cell-Mediated Hyperactivation and Hyperinhibition of Other Immune Cells. NK cells can induce T cell hyperactivation by the release of IFN-γ, and this could lead to exaggerated immunopathology in infectious diseases such as influenza and Ebola virus infections as well as in septic shock. Conversely, NK cells can regulate T cell activities by eliminating them via the cytotoxic activities of granzyme B and perforin, or via the expression of death receptor ligands such as FasL and TRAIL (TNF-related apoptosis-inducing ligand), and, in the case of human NK cells, by the expression of the activating receptor NKp44. Similarly, dendritic cells (DCs) can also be killed or downregulated by the release of IL-10, which leads to a reduced antigen presentation to T lymphocytes. An excess of NK-cell-mediated inhibition could lead, for example, to progression of HIV infection and chronicity of hepatitis B and C infections.

virus infection [53] and such NK cells, termed 'regulatory' NK cells, inhibit antigen-specific T cell proliferation [54].

In human asthma, increased numbers of NK2 cells (i.e., NK cells producing the type 2 cytokine IL-4) have been described in peripheral blood, contrasting with a reduced number of IFN-$\gamma^+$ NK1 cells [38], and suggesting that NK cells may participate in the pathophysiology of asthma. Similarly, in reproduction, disturbance in the local production of uNK cytokines including TNF-α, TGF-β1, and IFN-γ can influence the inhibition of trophoblast invasion [55]. Thus, the upregulation during pre-eclampsia of ULBP1, a ligand of NKG2D, could induce the increase of cytokine secretion by uNK cells. At the peripheral level, several clinical studies have suggested that recurrent miscarriage and implantation failure are associated with increased

levels and cytotoxicity of peripheral blood NK cells [56]. Several therapies including steroids (prednisolone), intravenous immunoglobulins, and TNF-blocking agents are currently proposed to dampen high NK cell activity in reproductive failure. Regardless of the observation that women present increased uNK cells in the endometrium, the real involvement of these cells in causing infertility, recurrent miscarriage, or pre-eclampsia remains controversial, and more data will be required before employing immunotherapies to alter uNK function [57,58]. Discussion is still ongoing to determine whether to treat by suppressing or activating the NK cells, and whether NK cells should even be analyzed in reproductive failure [59,60]. Investigations highlight that uNK cells are likely a heterogeneous population arising from local progenitors and/or from peripheral blood, raising the possibility that specific subsets may mediate detrimental effects.

Immune escape mechanisms are well characterized for the increased expression of inhibitory ligands, such as **programmed death ligand 1** (PD-L1) and MHC class I molecules, by the tumor. This increased expression is associated with the presence of tumor-infiltrating lymphocytes as well as with elevated levels of IFN-γ [61–63]. Thus, as IFN-γ producers, NK cells could also be involved in the enhancement of these inhibitory ligands on tumor cells [62–64]. Indeed, the supernatant of IL-2-activated NK cells increased the expression of PD-L1 on various hematopoietic tumors [63].

In addition, tumor-infiltrating NK cells frequently share phenotypic and functional characteristics of decidual NK cells, displaying reduced cytotoxic function, pro-angiogenic features, and immunoregulatory properties, prompting speculations about their involvement in the pathogenesis of solid tumors [65–67]. Independently of these immune roles, it is also important to note that NK cell leukemias can occur (Box 3).

## Use of NK Cells in Immunotherapy

Major progress in cancer immunotherapy has been made with the introduction into the market of (i) checkpoint inhibitors, that aim to rescue exhausted T cells, and (ii) chimeric-antigen receptor (CAR) T cells, which are transferred to the patient and then directly attack the tumor cells in a specific manner. There is no reason not to apply these principles to NK cells. Should they indeed be redundant, this does not preclude their potential clinical use as immunotherapeutic agents. Thus, autologous or allogeneic NK cells, or the leukemic cell line NK92 [68], can be very efficiently expanded and activated *in vitro* before (re)infusion into the patient. However, clinical success rates are currently not optimal because of tumor escape mechanisms and the lack of NK cell specificity. To improve the latter, CAR NK cells [69] as well as bispecific (BiKE)

### Box 3. Neoplastic NK Cell Diseases

NK cells can become cancerous themselves, and three different types of NK disease have been recognized by the World Health Organisation since 2008: (i) chronic NK cell lymphoproliferative disorder; (ii) extranodal NK/T cell lymphoma, nasal-type or extranasal; and (iii) aggressive NK cell leukemia [90,91]. The first corresponds to a persistent increase in circulating, morphologically normal NK cells (unknown pathogenesis). It can occur in adults of all ages, ethnic groups, and gender. Diagnosis is mostly through an isolated laboratory finding or by disease accompanied by mild anemia and/or neutropenia. Extranodal NK/T cell lymphoma, nasal-type, is characterized by destructive midfacial lesions with nasal symptoms and locoregional invasion. Almost always associated with Epstein–Barr virus (EBV), it predominantly occurs in Asia and Central and South America. Prognosis is good in early stages but poor in late stages. Extranasal NK/T cell lymphoma also has a poor prognosis because it is classically diagnosed in advanced stages. Almost every organ, but only exceptionally the lymph nodes, can be invaded by neoplastic masses or ulcers. Finally, aggressive NK cell leukemia evolves fatally within a few weeks, but is fortunately very rare. Extranodal NK/T cell lymphoma, nasal-type, can represent up to 10% of non-Hodgkin lymphomas in the geographic regions where it usually appears.

### Box 4. NK Cells in Transplantation

NK cells are similarly implicated in hematopoietic stem cell transplantation. Groundbreaking work by the group of Velardi [74,92] showed a protective effect against GvHD likely via (i) NK-mediated killing of APCs, which precludes allogeneic T cell activation, and (ii) direct elimination of these T lymphocytes. A KIR–ligand mismatch in the donor–recipient direction was beneficial in preventing relapses, at least in some hematopoietic malignancies, without increasing the GvHD risk, and further investigations by others confirmed this concept. However, Shah *et al*. described five cases of acute GvHD in a cohort of nine patients receiving an HLA-matched unrelated hematopoietic allograft followed by donor-derived infusions of NK cells stimulated with IL-15 and 4-1BBL [76], bringing the question of the clinical safety of NK cell products to the forefront again. In the case discussed above, it is possible that the additional stimulation rendered the cells more resistant to inhibitory mechanisms and induced the observed toxicity. Heidenreich and Kröger [93] compiled the relevant studies and concluded that unrelated donor grafts can be dangerous in case of a KIR–ligand mismatch, under the influence of multiple factors including the type of disease, the preconditioning regimen, the direction of the mismatch (donor–recipient or recipient–donor), and of course the HLA and KIR genotypes of donor and recipient. To reconcile these studies, Simonetta *et al*. [82] proposed an elegant model in which the cytotoxic activity of NK cells would be beneficial and prevent GvHD, while the production of proinflammatory cytokines such as IFN-γ and TNF-α could favor GvHD.

In solid organ transplantation, NK cells likely participate in graft rejection through ADCC against the graft in the presence of donor-specific antibodies [81]. Parkes *et al*. investigated NK cell CD16-inducible transcripts first *in vitro* and then in biopsies of human kidney grafts. Their data strongly suggest a role for NK cell activation via CD16 in antibody-mediated kidney transplant rejection [80].

### Outstanding Questions

Are NK cells and other innate lymphoid cells really redundant, as strongly suggested by the investigation of SCID patients treated with hematopoietic stem cell transplantation without prior myeloablation?

Are there situations where these cells are useful and needed, for example in tissue reconstruction after an inflammatory insult?

To what extent do NK cells disturb normal immune responses? In other words, could the immune system function better in their absence?

How can we better control the detrimental effects of NK cells in autoimmunity, infectious disease, transplantation, allergy, reproduction, and cancer? Could NK cells be manipulated by small molecules?

How can we influence NK cells in a way that only their positive aspects, in particular their cytotoxic activity against cancer cells and infected cells, are harnessed without deleterious side effects? Do CAR NK cells and TriKE constructs provide the solution?

and trispecific (TriKE) killer engagers aiming to crosslink NK cells with tumor cells [70] have been designed. The latter, although still in the preclinical phase, look very promising because they would circumvent the need for NK cell transfer and would therefore be more cost-effective and easier to handle. These approaches allow simultaneous NK cell activation and specific targeting to tumor cells expressing the antigen(s) recognized by the construct. Such a strategy compensates for the initial disadvantage that NK cells lack target specificity, in contrast to T cells. Memory-like NK cells, antibodies masking inhibitory receptors, and NK cell checkpoint inhibitors are likewise being considered in anticancer immunotherapy [71,72]. In addition, therapeutic anticancer antibodies act, at least in part, through NK-cell-mediated **antibody-dependent cellular cytotoxicity** (ADCC).

It is very important to address safety concerns, particularly in the case of NK cell adoptive transfer. It is usually claimed that NK cells do not induce **graft-versus-host disease** (GvHD) [73], and thus are safe to be infused, at least in the KIR–MHC class I mismatch situation after haploidentical allogeneic hematopoietic stem cell transplantation (Box 4) [15,74]. Moreover, the NK92 cell line was well tolerated in the first clinical applications [75] but, as the work of Shah *et al*. has shown [76], highly activated NK cells might nevertheless induce GvHD. With CAR T cells, important and sometimes fatal side effects have been recorded, represented mostly by (i) B cell aplasia, (ii) a cytokine release syndrome, and (iii) neurological complications [77]. This reflects what was already known from earlier interventions in the human immune system [78], and remind us to exert great caution before unleashing a potent killer army. The most recent TRiKE constructs contain IL-15 to increase NK cell activation and survival, and similar problems might therefore occur [77]. However, and importantly, we should not forget that some of these new immunotherapeutic approaches save a significant number of lives, but improved safety should remain an important concern.

## Concluding Remarks

In immunology there are often two sides to the coin. In the same way as the balance between inhibitory and activating messages governs NK cell functions, there is also a balance between the positive and negative actions of the latter. As new drugs and constructs are increasing our ability to modulate immune responses and populations, it becomes crucial to evaluate these

beneficial and detrimental aspects to balance the outcomes and safety profiles of these therapies (see Outstanding Questions). More generally, the Janus face of NK cells discussed here mirrors similar situations involving other immune cells (T lymphocytes, macrophages, . . . ) as well as their pathways of communication (receptor/ligand interactions, cytokines, chemokines, exosomes). Most frequently, immune responses have a cost – ranging from tissue damage to cytokine storms that might become overwhelming and kill the individual – and these adverse effects might outweigh the potential benefits. However, we obviously need our immune system to resist the continuous microbial pressure from the environment. In the context of this broader discussion, the case of NK cells, and of ILCs in general, is interesting because of their reported redundancy, arguing that impairment of their function could be safe in conditions where immune reactions are dysregulated, such as those discussed in this article.

Of course, this likely represents a superficial take on a complex reality because NK cells cannot be designated as either beneficial or detrimental in any given situation; however, reductionist models can provide an inspiring new look at the topic. We argue that a deeper understanding of the detrimental role of NK cells will not only be crucial for the treatment of autoimmune and inflammatory conditions but will also enable the safe use of therapies that rely on the engineering of NK cell function for improved anticancer and anti-infectious therapies. It is also important to note that contrasting results from animal models might of course be due to subtle variations in the protocols, but also and probably more importantly reflect variations in the local microbiota in the respective facilities. Indeed, a different origin of the mouse lines and even microbiota differences between individuals within the same animal house might affect experimental outcome [79]. It will be crucial to take these factors into account to increase our understanding of NK cell involvement in pathologic mechanisms and to usher in a new era of immunotherapeutics.

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4. Mucosal associated invariant T (MAIT) cells

Mucosal-associated invariant T (MAIT) cells express semi-invariant αβ T cell receptors (TCR) and are an unconventional T cell subset (Porcelli, Yockey et al. 1993, Tilloy, Treiner et al. 1999). Though initially defined in humans on the $CD4^{-}CD8^{-}$ subset, $CD8^{+}$ MAIT cells have been now recognised (Napier, Adams et al. 2015) and have high levels of CD161 (Dusseaux, Martin et al. 2011). Their role in the preservation of homeostasis of the mucosal and non-mucosal barriers is currently the subject of many ongoing studies (Kjer-Nielsen, Patel et al. 2012, Corbett, Eckle et al. 2014, McWilliam, Birkinshaw et al. 2015, Mak, Xu et al. 2017). MAIT cells are present at an elevated frequency in humans, making about 10% of airway T cells (Hinks, Wallington et al. 2016), up to 4% of PBMC T cell population (Gherardin, Souter et al. 2018) and can make up to a remarkable 40% of liver T cells (Billerbeck, Kang et al. 2010) thus playing an important role in immunity.

The presence of TCRs allows them to detect microbial antigens presented by MHC class I-related gene protein (MR1) (Gold, McLaren et al. 2014). They recognize riboflavin (Vit. B12) metabolites presented as MR1-bound antigens such as 5-OP-RU (5-(2-oxopropylideneamino)-6-D-ribitylaminouracil) and 5-OE-RU (5-(2-oxoethylideneamino)-6-D-ribitylaminouracil) (Corbett, Eckle et al. 2014). MAIT cells can identify bacterially infected cells and release cytokines like IFN-γ, IL-4, IL-5, IL-10, and TNF that can lyse these cells (Kawachi, Maldonado et al. 2006, Le Bourhis, Martin et al. 2010, Wong, Ndung'u et al. 2017). They can also release perforin, undergo degranulation and respond to intracellular bacteria like in the case of *Shigella* (Le Bourhis, Dusseaux et al. 2013). In mice, the intracellular Gram-negative coccobacillus, *Francisella tularensis*, induces the expansion of pulmonary MAIT cells. These MAIT cells recruit the inflammatory monocytes to the site of infection by secreting GM-CSF, signifying the important role they play in mucosal immune responses (Meierovics, Yankelevich et al. 2013).

Similarly, MAIT cells have a part in the anti-viral responses. Viruses do not synthesize vit. B2, thus fail to activate MAIT cells via MR1 ligands (Le Bourhis, Martin et al. 2010) but can activate CD161+Vα7.2+ MAIT cells via a cytokine dependent pathway as

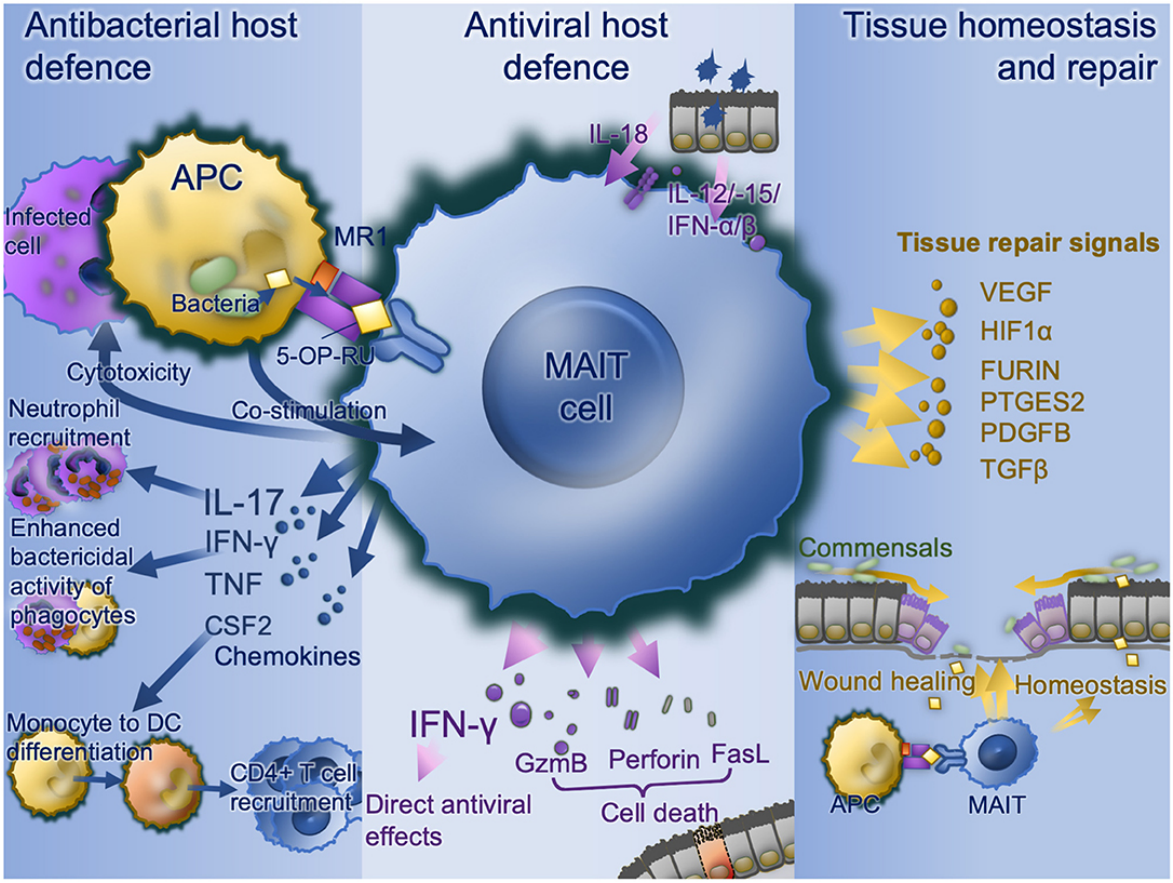


**Figure 10:** Role of MAIT cells in infection and host immune responses. APC, antigen-presenting cell; CSF2, colony stimulating factor 2 (GM-CSF); DC, dendritic cell; FasL, Fas cell surface death receptor ligand; GzmB, granzyme B; IFN, interferon; IL, interleukin; MAIT, mucosal associated invariant T; MR1, major histocompatibility complex-related protein-1; TCR, T cell receptor. Image source (Hinks and Zhang 2020)

-demonstrated in case of influenza, hepatitis C and dengue infections (van Wilgenburg, Scherwitzl et al. 2016). Higher frequency of MAIT calls was reported in patients recovered from H7N9 influenza A virus infections (Loh, Wang et al. 2016) and are associated with immune protection as demonstrated in mice infected with H1N1 influenza A virus (van Wilgenburg, Loh et al. 2018).

Apart from this, a significant role of MAIT cells in tissue repair and homeostasis has emerged, due to their presence on skin, and oral-, intestinal-, respiratory-, urogenital-tracts (Nel, Bertrand et al. 2021). $Mr1^{-/-}$ non-obese diabetic (NOD) mice show increased translocation of gut microbiota along with elevated intestinal permeability, proving the critical role of MAIT cells in preserving GI tract integrity (Rouxel, Da Silva et al. 2017). Similarly,

reduced survival was found in allogeneic bone marrow transplantation in $Mr1^{-/-}$ mice because of increased colonic graft-versus-host disease (GVHD) (Varelias, Bunting et al. 2018). *In vivo* studies using germ-free mice found that the colonization of *Staphylococcus epidermidis* on the skin shows expansion of dermal MAIT cells and could aid in the healing of skin wound, supported by topical application of 5-OP-RU (Constantinides, Link et al. 2019). This finding has an exciting implication in clinical use that could translate into treatment for ulcers, burns, and pressure sores etc.

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## Aims of the study

Natural killer cells, classified as group I innate lymphoid cells (ILCs), form part of early responses against viral infections, cancer cells etc. Their responses include secretion of cytokines, chemokines, degranulation and lysis of infected cells. NK cell education is an important process through which NK cells acquire functional competence while maintaining tolerance towards self. In this thesis, we take a closer look at the education status of NK cells from murine lung and spleen. We used *wildtype* and it's TAP1-KO, along with MAIT CAST variants CAST/EiJ (B6CAST) and MR1-KO in this study. The latter part of the thesis works towards establishing a model of chronic bacterial infection of the lungs in these mice, to assess any alteration in the education status. We also take a visual look into the interaction between NK cells and bacteria.

### Imprint of initial education and loss of Ly49C/I in activated natural killer cells of TAP-KO mice and control strains

We present a study to assess the NK cell functions in relation to their education status. We used the NK cells from lungs and spleens from the previously mentioned mice. The aims of the study were as follows.

(i) Confirming the initial licensing profile of NK cells by antibody-mediated stimulation of the activating receptor NK1.1 (CD161c)
(ii) Assessing the comparative phenotype of B6, TAP1-KO, B6CAST and MR1-KO NK cells
(iii) Studying the differences in activated NK cell IFNγ production according to the initial licensing status
(iv) Observation of the downmodulation of Ly49C/I on activated NK cells at day 6
(v) Testing the Natural killer cell cytotoxic activity, degranulation against target cell lines
(vi) Cytotoxic granule profiling of NK cells
(vii) Phenotype of MAIT cells assessed by MR1 tetramers

**Mouse model of chronic lung infection**

As has widely been reported, NK cells are well known for their role in viral infections and in cancer immunosurveillance. Experiments showing their role in anti-bacterial defence play a part in highlighting their contribution towards other immune response. NK cells can directly interact with bacteria though pattern recognition molecules and engage in production of cytokines, chemokines and other anti-microbial activities. As such, it is important to make animal models that can recreate the immune responses which can then be assessed to create an effective picture of the defence mechanism. The aim of this chapter is exactly that, we intended to establish a mouse model of chronic bacterial lung infection. We chose *Pseudomonas aeruginosa* as the infectious agent to mimic the human chronic lung infections in our mice strains.

**Imaging Natural Killer cells in *Pseudomonas aeruginosa* co-culture**

Both *Escherichia coli* and *Pseudomonas aeruginosa* are both gram negative and extracellular bacteria. As such, we used *E. coli* to establish a novel method to image the dynamic interaction between NK cells and bacteria. This method was then used to assess the interaction between *P. aeruginosa* and NK cells.

**Chapter 2: Imprint of initial education and loss of Ly49C/I in activated natural killer cells of TAP-KO mice and control strains**

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- Design and execution of the experiments, analyses of the data
- Literature search and writing

## Abstract

Natural killer (NK) cells are important effectors of the innate immune system and participate in the first line of defense against infections and tumors. Prior to being functional, these lymphocytes must be educated or licensed through interactions of their major histocompatibility complex class I molecules with self-specific inhibitory receptors that recognize them. In the absence of such contacts, caused by either the lack of expression of the inhibitory receptors or a very low level of major histocompatibility complex class I (MHC class I) proteins, NK cells are hyporesponsive at baseline (ex vivo). After activation however, they can become cytotoxic and produce cytokines. This is particularly the case in transporter associated with antigen processing (TAP)-deficient mice, which we investigated in the present study. Transporter associated with antigen processing transports endogenous peptides from the cytosol to the endoplasmic reticulum, where they are loaded on nascent MHC class I molecules, which then become stable and are expressed at the cell surface. Consequently, TAP-KO mice have very low levels of MHC class I expression. We present a study about phenotypic and functional aspects of NK cells in four mouse strains, B6 wildtype, TAP1-KO, B6CAST and MR1-KO, in spleen and lung. We observed that in the latter as well as in wildtype mice on the same genetic background, the initial pattern of education, conferred to the cells via the inhibitory receptors Ly49C/I and NKG2A, was maintained even after a strong stimulation by the cytokines interleukin-2, interleukin-12, interleukin-15 and interleukin-18. Furthermore, the percentages of activated NK cells expressing Ly49C/I were strongly downmodulated under these conditions. We completed our investigations with phenotypic studies of the NK cells of these mice and compared them with C57BL/6CAST and MR1-knockout animals. The latter are almost devoid of mucosal-associated invariant T (MAIT) cells, but their NK cells have never been investigated in detail before. Despite the fact that MR1 structurally resembles MHC class I molecules, we could not detect major differences between MR1-KO and wildtype NK cells, suggesting a normal NK cell educational process in the context of the MR1-depleted environment.

## Introduction

Natural Killer (NK) cells are the founding members of the innate lymphoid cell (ILC) family comprising in addition several other populations called innate lymphoid cells types 1, 2 and 3, as well as lymphoid tissue inducer cells (1). All the latter are predominantly cytokine producers, whereas NK cells are likewise able to release cytokines and chemokines but are also endowed with cytotoxic properties, further subdivided into natural cytotoxicity (killing of targets without prior sensitization or immunization) and antibody-dependent cellular cytotoxicity (ADCC), which occurs when the Fc portion of antibodies bound to a target cell interacts with the activating NK cell receptor CD16 (Fc$\gamma$RIIIa) (2, 3). Natural Killer cells are extensively studied in human and mouse for their anti-tumoral and anti-infectious properties that are therapeutically exploitable and constitute a true hope for the future of immunotherapy (4,5). Since the first descriptions of NK cells in the mid-seventies, their capacity to kill tumor cells and viral-infected cells has been observed in a huge number of *in vitro* and *in vivo* investigations. Upon recognition of a target cell, the content of the cytolytic granules (perforin, granzymes, and, in human but not in the mouse, granulysin) is released and induces apoptosis of the abnormal cell (6).

Before reaching this step, NK cells integrate signals from their activating and inhibitory receptors, respectively (AR and IR), and the target cell is eliminated when the activating messages predominate (7). The best studied IR are specific for major histocompatibility complex (MHC) class I molecules, and a normal level of the latter is characteristic for healthy cells that are consequently identified as such and spared by the NK cells. Their absence is recognized as “missing self” and induces target cell lysis in the presence of sufficient activating messages (7-9). Besides the concept of the balance between signals transmitted through AR and IR (7), another important factor governing NK cell functions is education or licensing (10-12). These terms designate the interaction of IR with autologous MHC class I molecules during NK cell development, which is necessary for the cell to become functional (although non-MHC class I ligands can educate NK cells via different IR) (10-12). In the absence of this

phenomenon, due either to the lack of expression of one or several self-specific IR on a NK cell or to a general MHC class I deficiency of the cellular environment, such as observed in beta-2 microglobulin (β2m) and/or transporter associated with antigen processing (TAP) defects, NK cells remain uneducated (unlicensed) and hypo-responsive. This observation was made both in human (13-15) and in knockout mouse strains, such as β2m-KO, TAP1-KO, β2m/TAP1 double KO (16,17): *ex vivo* NK cells display low or absent cytotoxicity and low cytokine production, but become functionally very active upon cytokine-mediated stimulation and then kill autologous Con A T cell blasts (16). In human TAP deficiency, activated NK cells kill autologous B lymphoblastoid cells (Epstein-Barr virus-transformed immortalized B lymphocytes) and skin fibroblasts, whereas they surprisingly spare self T-PHA blasts (18-20).

Natural killer cell education is in the focus of interest of several groups and to explain it, different models have been elaborated, such as the arming model, the disarming model, the rheostat model, the *cis-trans* model and the confinement model (11,12). More recently, it has been shown that TRP Calcium channels dynamically regulate NK cell licensing on the level of the content of cytolytic proteins in the secretory lysosomes (that degranulate upon target cell recognition) (21). Moreover, and in accordance with the *cis-trans* interaction model, the role of NK cell-intrinsic MHC class I molecules for tuning has been emphasized (22).

All these different concepts have mostly been established with short-term activated NK cells, for example taken *ex vivo* the day after poly(I:C) or tilorone administration. In the present paper, we wanted to rather explore what happens in mouse NK cells in terms of the production of their major cytokine, namely interferon gamma (IFN-γ), after culture with interleukin 2 (IL-2) for five days and then an overnight re-stimulation with IL-2 alone, (IL-2, IL-12, IL-15) and (IL-2, IL-12, IL-18), respectively. The first condition was the negative control, as in the mouse IL-2 alone does not induce significant IFNγ production. In contrast, the last cytokine combination was intended as the positive control, IL-12 and IL-18 together being known for the stimulation of a maximal amount of IFNγ. Finally, the IL-12 plus IL-15 association was supposed to lead to

an intermediate level of stimulation, for which differences between NK cell subsets, defined by the presence or absence of self-specific IR, could be visible.

We choose to work with the most frequently used mouse model in immunology, the C57BL/6 (B6) strain, and its TAP1-KO littermates. In B6 mice, the self MHC class I-specific IR are Ly49C/I, recognizing the classical class I molecules H-2K$^{b}$ (and H-2D$^{b}$), and CD94/NKG2A (NKG2A), binding to the non-polymorphic molecule Qa-1$^{b}$, the mouse equivalent of HLA-E (23,24). Consequently, NK cells expressing either one or both IR are supposed to be educated, whereas their double negative (Ly49C/I-NKG2A-) counterparts are expected to be hypo-responsive. In contrast, in TAP1-KO mice, the entire NK cell population should in principle be functionally deficient.

Additionally, we were interested in the MHC class I-related molecule MR1, which presents antigens derived from the bacterial and fungal metabolism of vitamin B2 (riboflavin) to mucosal associated invariant T (MAIT) cells in the context of antimicrobial defense (25). Indeed, due to the ubiquitous expression of MR1 and its structural relationship with conventional MHC class I molecules (26), we hypothesized that the presence (wildtype B6-MAIT$^{CAST}$ (B6$^{CAST}$) mice) or absence (MR1-KO mice) of MR1 might have an impact on NK cell phenotype and functions. Indeed, due to the similar structure of MHC class I and MR1 molecules, the latter might interact with NK cell IR and/or AR. With the help of fluorochrome-conjugated MR1 tetramers (27), we also studied the phenotype of spleen and lung MAIT cells from the four mouse strains (wildtype B6, TAP1-KO, B6$^{CAST}$ and MR1-KO).

In summary, our investigations show that the initial pattern of NK cell education leaves its imprint even after six days of intense cytokine-mediated stimulation in B6 animals, and, surprisingly, that there is an unequal functional distribution between NK cell subsets of activated TAP-KO littermates, largely matching the one observed in B6 mice. Regarding MR1, it does not seem to influence NK cells profoundly, at least in the absence of infection.

## Material and Methods

### Ethical statement

The animal studies were approved by the Animal Welfare Structure (AWS) and the experiments were carried out in accordance with the European Union directive 2010/63/EU as incorporated in Luxembourgish law for the care and use of laboratory animals (DII-2017-02).

### Mice

C57BL-6 mice with *wildtype*, TAP1-KO, MAIT CAST variants CAST/EiJ ($B6^{CAST}$) and MR1-KO, aged 8-12 weeks old, were used. Mice were bred and maintained at the Luxembourg Institute of Health's specific pathogen-free animal facility. They were fed a standard maintenance chow and followed a 12-h light dark cycle at 22-23°C and 45-65% relative humidity.

### Cell preparation

Lungs and spleens were extracted from WT, TAP KO, CAST/EiJ and $MR1^{-/-}$ mice. Lungs were incubated in 1.6ml digestion solution (for 15 ml, 20mg collagenase II, 10% FBS, 750U benzonase, 15 µl of 1M $MgCl_2$, PBS) for an hour at 37°C. Spleens and the digested lungs were passed through a 40µm cell strainer (Corning) with the back of a syringe plunger to make a single cell suspension. Red blood cell lysis was done by ACK lysing buffer (Gibco™). Murine T-lymphoma cell lines YAC1 (ATCC), RMA, and $C4.4.25^{-}$ - the $\beta_2$m-deficient variant of EL-4 lymphoma - were chosen as target cell lines. YAC1 and RMA were cultured in suspension with RPMI medium supplemented with 10% FBS, 1% Pen/Strep, 1mM HEPES, while $C4.4.25^{-}$ was cultured in DMEM medium supplemented with 10% FBS, 1% Pen/Strep, 1mM HEPES.

**Flow cytometry**

The cells were washed twice with PBS buffer containing 1% BSA (Miltenyi) (FACS buffer) and were proceeded for surface staining by Fc block anti-mouse CD16/CD32 followed by fluorochrome-conjugated monoclonal antibodies, incubated at 4°C for 30 minutes, in the dark. Cells were then washed twice (100µl, 4°C, 300 x g, 10 minutes, FACS buffer), and fixed with Cytofix/Cytoperm buffer (BD) for 45 minutes. Intracellular staining was performed after fixation, and staining was done in Perm/Wash buffer(BD) at 4°C for 30 minutes, in the dark. The samples were washed again and re-suspended in FACS buffer for further analysis. The acquisition was done on BD LSRFortessa™. The monoclonal antibodies (mAbs) used for phenotypic analysis were: NK1.1, CD3, KLRG1, Qa-1$^{b}$, NKp46, CD27, CD11b, Ly49C/I, Qa2, Ly49D, Ly49G2, Ly49H, NKG2A, H-2K$^{b}$, Ly49A, Ly49F, NKG2D and the fixable viability stain in APC-Cy7.

| Antibody | Clone | Format | Supplier | Identifier |
|---|---|---|---|---|
| NK1.1 | PK136 | BUV-395 | BD Biosciences | 564144 |
| | | BV421 | Biolegend | 108741 |
| CD3 | 145-2C11 | BV510 | BD Biosciences | 563024 |
| | | PE-Cyanine7 | eBioscience™ | 25-0031-82 |
| NKG2A$^{B6}$ | 16a11 | PerCP-eFluor 710 | eBioscience™ | 46-5897-82 |
| Ly-49C and Ly49I | 5E6 | BV786 | BD Biosciences | 744032 |
| CD11b | M1/70 | FITC | Biolegend | 101205 |
| KLRG1 | 2F1 | BV421 | BD Biosciences | 562897 |
| Qa-1$^{b}$ | 6A8.6F10.1A6 | BV421 | BD Biosciences | 744385 |
| CD335 | 29A1.4 | BV421 | BD Biosciences | 562850 |

| | | | | |
|---|---|---|---|---|
| CD27 | LG.3A10 | BV650 | Biolegend | 124233 |
| Qa-2 | 69H1-9-9 | FITC | eBioscience™ | 11-5996-82 |
| Ly-49D | 4 E5 | FITC | BD Biosciences | 555313 |
| Ly-49G2 | 4D11 | FITC | BD Biosciences | 555315 |
| Ly-49H | 3D10 | FITC | BD Biosciences | 562536 |
| H-2K$^{b}$ | AF6-88.5 | PE | Biolegend | 116507 |
| Ly-49F | HBF-719 | PE | BD Biosciences | 550987 |
| Ly-49A | A1 | PE | BD Biosciences | 557424 |
| CD314 (NKG2D) | CX5 | PE | Biolegend | 130207 |
| CD19 | 1D3 | PE-Cy7 | BD Biosciences | 552854 |
| Granzyme A | GzA-3G8.5 | eFluor 450 | eBioscience™ | 48-5831-82 |
| CD226 (DNAM-1) | TX42.1 | BV605 | Biolegend | 133613 |
| IFN-γ | XMG1.2 | BUV737 | BD Biosciences | 564693 |
| Phospho-S6 (Ser235, Ser236) | cupk43k | PE-Cy7 | eBioscience™ | 25-9007-42 |
| Phospho-mTOR (Ser2448) | MRRBY | PE | eBioscience™ | 12-9718-42 |
| TNF alpha Monoclonal | MP6-XT22 | FITC | eBioscience™ | 11-7321-82 |
| CD107a | 1D4B | BV711 | BD Biosciences | 564348 |
| Granzyme B | NGZB | FITC | eBioscience™ | 11-8898-82 |
| | GB11 | Alexa Fluor® 647 | Biolegend | 515405 |

| | | | | |
|---|---|---|---|---|
| Perforin | S16009B | PE | Biolegend | 154405 |
| Fixable Viability Stain 780 | | | BD Biosciences | 565388 |
| Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™) | 2.4G2 | | BD Biosciences | 553142 |

**Degranulation assay**

NK cell degranulation towards target cells was tested by co-culturing them together for four hours at 37°C, in presence of GolgiStop protein transport inhibitor (BD). Target cell lines YAC-1, RMA and C4.4.25$^{-}$ were stained with CellTrace Violet at 0.5 mM concentration. The NK cells used were cultured in IL-2 and stimulated with IL-12/-15 and IL-12/-18 overnight. The Effector:Target (E:T) ratios were 2:1 and 5:1. The cells were then stained with NK1.1, CD3, DNAM-1, Ly49CI, NKG2A, intracellular IFNγ and the fixable viability stain according to the staining protocol. The acquisition was done on BD LSR Fortessa$^{TM}$.

**Cytotoxicity**

NK cell cytotoxicity towards target cells was tested by co-culturing them together for 4 hours at 37°C. The NK cells used were cultured in IL-2 for 6 days and stimulated with IL-12/-15 and IL-12/-18 overnight before co-culture. The tested E:T ratios were 1:1, 5:1, 10:1, 25:1, 50:1. Target cell lines YAC-1, RMA and C4.4.25$^{-}$ were stained with CellTrace Violet at 0.5 mM concentration. At the end of the 4 hours, TO-PRO-3 (1μM, Invitrogen) diluted at 1:1000 was added to each sample, incubated at room temperature for 15 minutes. The samples were acquired on NovoCyte Quanteon.

**Metabolic profiling**

NK cells were cultured for 4 days in IL-2 and then stimulated with IL-12/-15 and IL-12/-18 overnight. The following day, cells were treated with GolgiStop for 1 hour at 37°C and recuperated with EDTA. The cells were then stained with NK1.1, CD3, DNAM-1, Ly49C/I, NKG2A, intracellular markers IFNγ, mTOR(S6), mTOR(S2448) and the fixable viability stain according to the staining protocol. The acquisition was done on BD LSR Fortessa™.

## Results

*1. Antibody-mediated stimulation of the activating receptor NK1.1 (CD161c)*

First, we wanted to confirm prior observations (16,17) about the hypo-responsiveness of MHC class I-KO mouse splenic NK cells after stimulation of the AR NK1.1 by plate-bound anti-NK1.1 antibodies (clone PK136). As expected, the isotype control antibody did not induce IFN-γ production, either in B6 wildtype or in TAP1-KO mice. In contrast, activation of NK1.1 resulted in the production of the readout cytokine in a significant fraction of wildtype NK cells, increasing sometimes to more than 30% of IFNγ+ cells. Interestingly, in all experiments performed (n=4), the wildtype double-licensed Ly49C/I+NKG2A+ subset contained the highest frequency of IFN-producing NK cells, whereas the values were not even half as high in the double negative, unlicensed population (mean of IFNγ+ cells: 10.88% *versus* 27.15% for the double positive cells). Natural killer cells from TAP1-KO mice displayed frequencies inferior to 10% whatever the subset, in accordance with the literature. However, the positive control PMA/ionomycin (strong non-specific stimulation) provoked a very high percentage of IFNγ+ NK cells, similar to the wildtype mice. Somewhat surprisingly, the unlicensed subset (Ly49C/I-NKG2A-) reacted less well to PMA and ionomycin than the three others in both types of mice, whereas this mode of activation is reputed to be non-specific and show the general, receptor-independent capacity of a cell to produce a cytokine for example (28). Thus, TAP1-KO NK cells are indeed hypo-responsive to AR stimulation, but it is possible to activate them non-specifically to the same extent as their wildtype littermates. In turn, this observation suggests

an active education-dependent process in the wildtype mice, whereas traces also seem to remain in the TAP1-KO animals in the PMA/ionomycin condition.

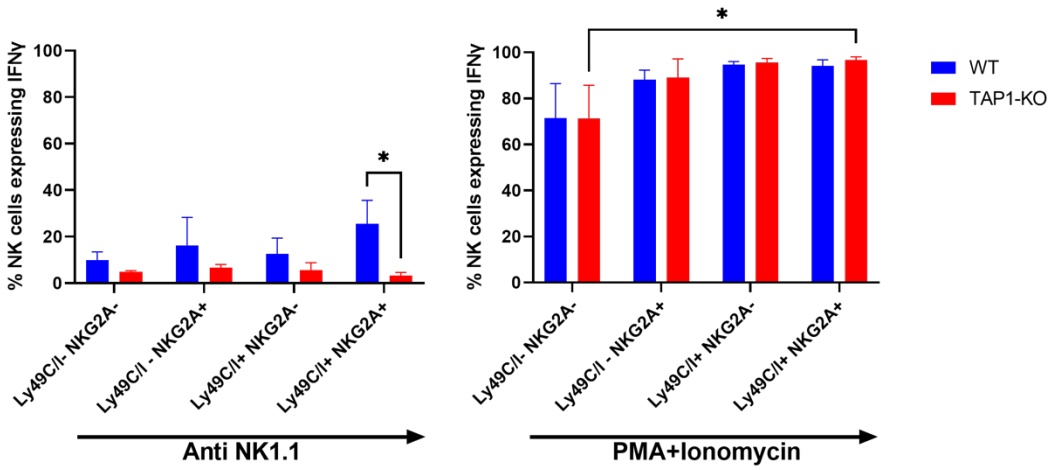



**Figure 1:** NK cells from B6 WT and TAP1-KO mice. The NK cells were stimulated with plate-bound anti-NK1.1 antibodies (clone PK136) and PMA+Ionomycin (positive control). In the stimulated cells, IFNγ was measured in CD3-NK1.1+ NK cells by intracellular staining. Statistical analyses were performed using GraphPad Prism 9.0.0 with an ordinary two-way ANOVA with Sidak multiple comparisons tests (n=4). *, $p<0.05$.

*2. Comparative phenotype of B6, TAP1-KO, B6$^{CAST}$ and MR1-KO NK cells*

We previously described the phenotype of B6 wildtype NK cells from spleen and lung, which displays several significant differences between the two organs. Notably, lung NK cells are more mature than their splenic counterparts (29). These findings, which were confirmed by others (30), have, to the best of our knowledge, not yet been investigated for the TAP-KO, B6$^{CAST}$ and MR1-KO strains. Our rationale for the study of NK cells from the latter two types of

mice was that, although $B6^{CAST}$ mice are supposed to resemble standard B6 animals in terms of NK cell numbers and function, the same might not be true for MR1-KO mice, as outlined above. Splenocytes and lung mononuclear cells were stained with fluorescent antibodies (Table 1) and NK cells comparatively analyzed by flow cytometry.

The proportion of NK cells (percentage of living cells that were CD3-NK1.1+) was almost three- fold higher in lung than in spleen, whereas the absolute numbers were comparable. The only notable exceptions were a significantly higher (i) percentage of NK cells in TAP1-KO lungs compared to $B6^{CAST}$, and (ii) absolute number of NK cells in TAP1-KO mice compared again to $B6^{CAST}$ in the spleen.

Overall, the splenic phenotype of the $B6^{CAST}$ and MR1-KO NK cells was not dramatically different from conventional wildtype B6 mice. This was particularly true for the activating (Ly49D, Ly49H) and inhibitory (Ly49C/I, Ly49G2) members of the Ly49 family, as well as for NKG2A. In general, nevertheless, there was a tendency for an increased frequency and mean fluorescence intensity (MFI) of MR1-KO NK cells expressing these receptors compared to $B6^{CAST}$, but without reaching statistical significance. However, as described for MHC class I-deficient mouse strains (24,31), the percentages (and MFI) of NK cells expressing Ly49C/I and NKG2A were significantly higher in the TAP1-KO background than in the other types of mice.

In the lung, as previously observed (29) in B6 animals, the Ly49 IR were down- and the AR also down (Ly49H)-regulated or unchanged (Ly49D), with NKG2A remaining stable, compared to the spleen. Here again, the same trend towards a slightly higher number of receptor-expressing NK cells from MR1-KO mice in comparison to $B6^{CAST}$ mice was observed. For the TAP1-KO NK cells, the IR, including NKG2A, still displayed an increased frequency of positive cells relative to the other three strains, whereas the AR Ly49D and Ly49H were non significantly down-modulated.

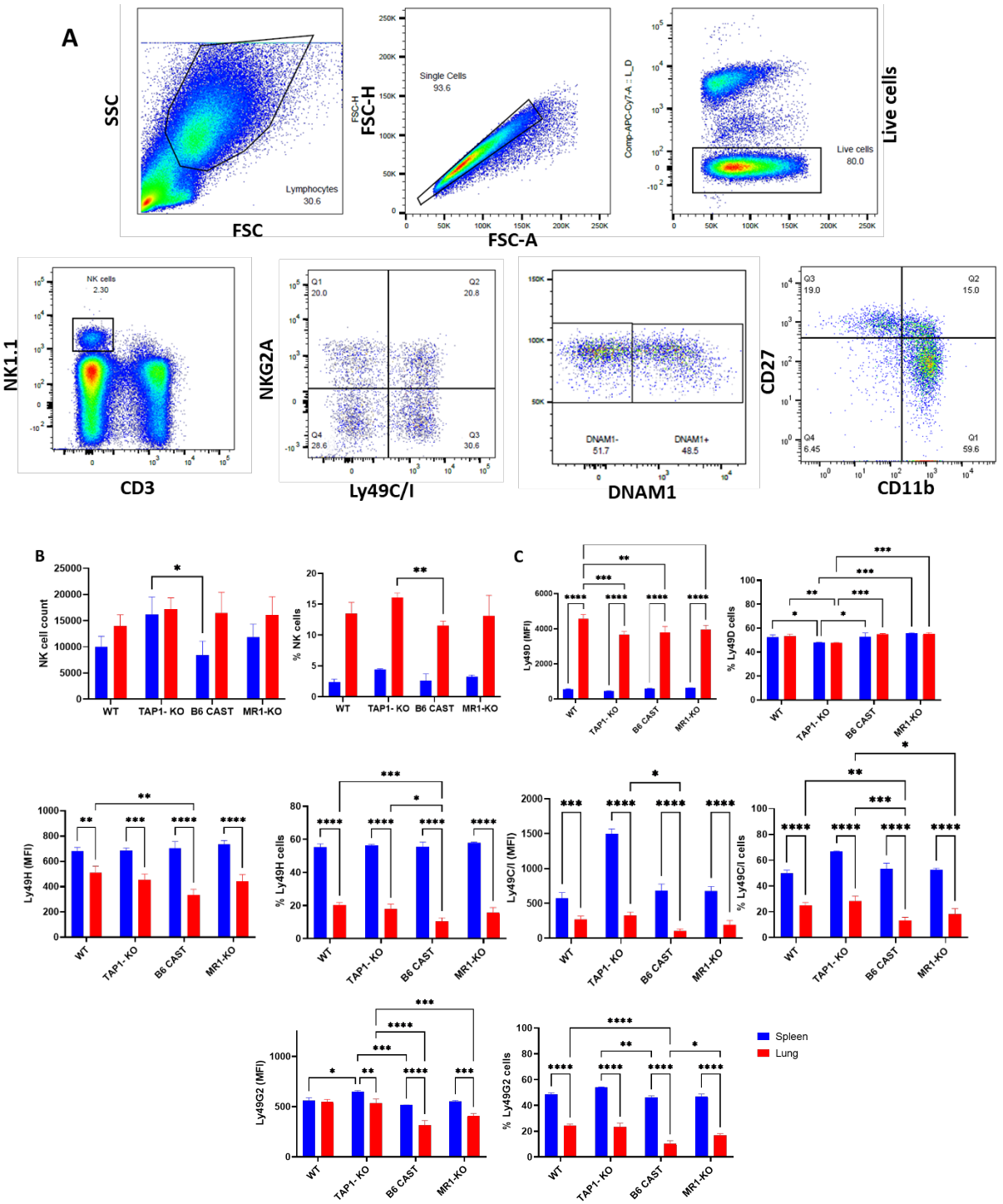



**Figure 2:** (A) Gating strategy used to identify murine NK cells and their subsets. Representative flow cytometry plots of NK cells derived from mouse lung/spleen, gated using the side and forward scatter dot plot display. NK cells were defined as CD3-NK1.1+ and were further differentiated into NK cell subsets based on the expression levels of NKG2A, Ly49C/I, and CD27/CD11b. (B) Comparison of NK cell absolute count and frequency in live cells was compared between various strains derived from lung and spleen. (C) The comparison of

activating (Ly49D, Ly49H) and inhibitory (Ly49C/I, Ly49G2) members of the Ly49 family. Statistical analyses were performed using GraphPad Prism 9.0.0 with an ordinary two-way ANOVA with Sidak multiple comparisons tests (n=4). *, p<0.05, p<0.01, ***, p<0.001 and ****, p<0.0001.

We then turned to the analysis of the comparative expression between strains of the AR NKp46 and NKG2D, which are crucial for the cytotoxic activity of NK cells. The natural cytotoxicity receptor (NCR) NKp46 was present on almost all NK cells in the four types of mice and in spleen as well as in lung, with very similar expression levels per cell (reflected by the MFI), but overall higher MFI in lung than in spleen (statistically not significant). Furthermore, the C-type lectin NKG2D showed a trend for lower frequencies and MFI in spleen and lung (no statistical significance) of TAP1-KO NK cells, and surprisingly also in the lung of $B6^{CAST}$ mice.

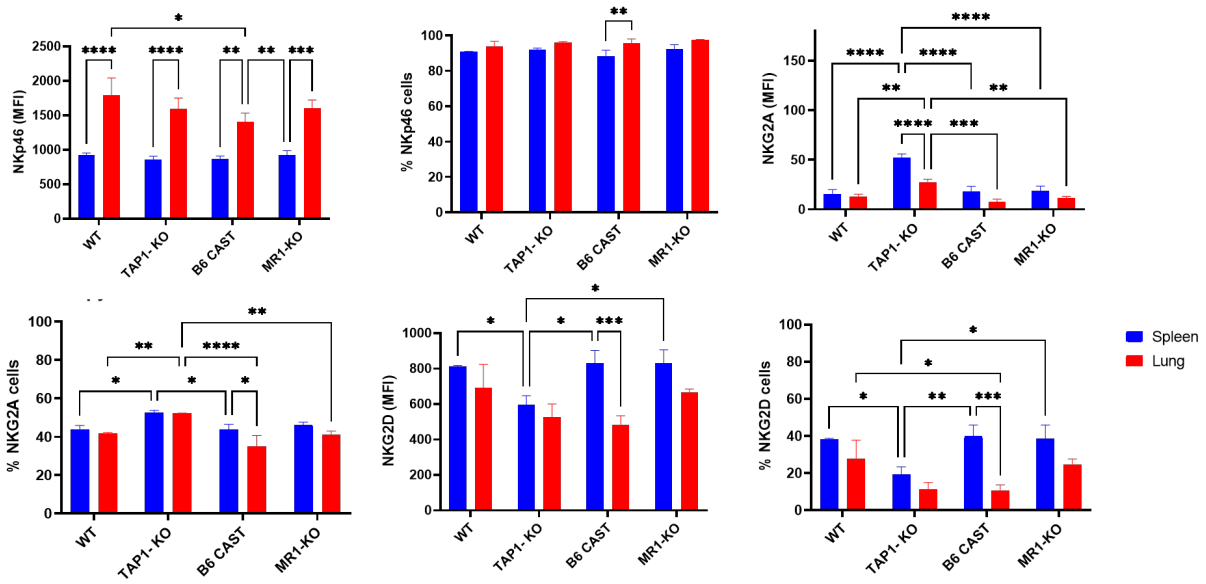



**Figure 3:** The comparison between expression levels of the natural cytotoxicity receptor NKp46, the C-type lectin NKG2D and the IR NKG2A. Statistical analysis was performed using GraphPad Prism 9.0.0 with an ordinary two-way ANOVA with Sidak multiple comparisons tests (n=4). *, p<0.05, p<0.01, ***, p<0.001 and ****, p<0.0001.

Whereas, as previously described (32), the classical MHC class I molecule H-2K$^{b}$ was severely down-modulated but not absent from TAP1-KO cells, the non-polymorphic MHC class I molecule Qa-2 could not at all be revealed, whereas it was present on the totality of the NK cells from the other three strains (with, interestingly, a higher MFI in lung than in spleen). Thus, it can be concluded that this molecule, considered as the mouse equivalent of the human non-classical class I protein HLA-G (33), is entirely TAP-dependent and cannot be loaded with TAP-independent peptides, in contrast to Qa-1$^{b}$ (34).

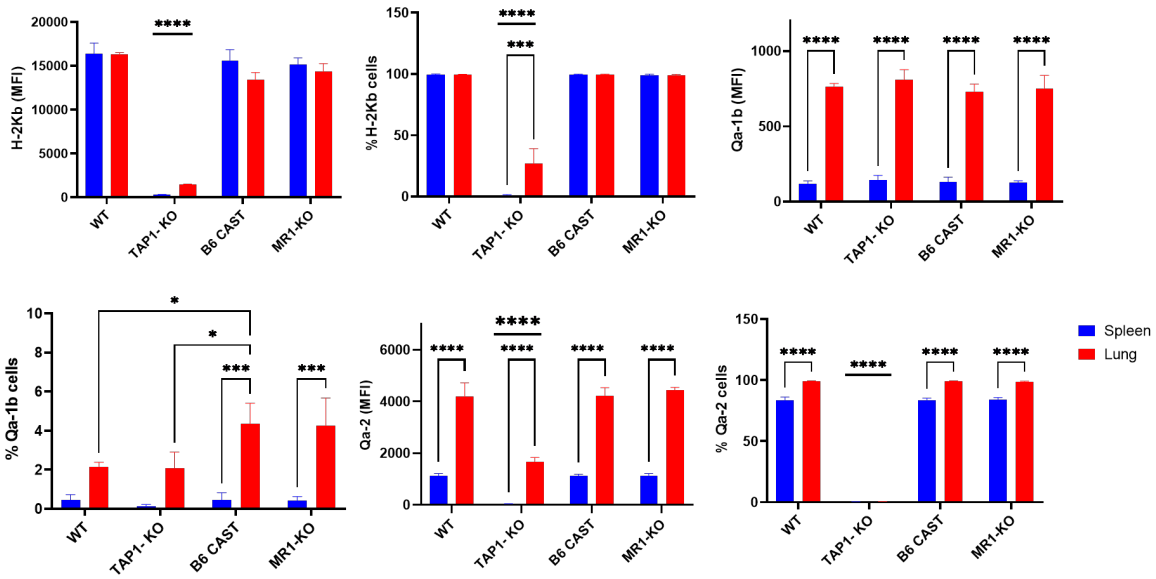



**Figure 4:** Expression and frequency of NK cell subsets based on MHC class I molecules H-2K$^{b}$, Qa-2 and Qa-1$^{b}$ in B6 WT, TAP1-KO, B6$^{CAST}$ and MR1-KO mice. The NK cells were derived from lung and spleen, and stained and acquired by flow cytometry without expansion. Statistical analysis was performed using GraphPad Prism 9.0.0 with an ordinary two-way ANOVA with Sidak multiple comparisons tests (n=4). *, p<0.05, p<0.01, ***, p<0.001 and ****, p<0.0001.

We made interesting observations by comparing the expression levels of the MHC class I molecules H-2K$^{b}$, Qa-2 and Qa-1$^{b}$ in NK cells of the B6 and TAP-KO strains, after 18 hours of culture of *ex vivo* splenocytes in the presence of either IL-2 alone, (IL-2, IL-12, IL-15), or (IL-2, IL-12, IL-18). The hypothesis here was that maybe these self-molecules would be

differentially expressed by the four subsets defined by the presence or absence of Ly49C/I and NKG2A. As expected, the former two proteins (H-2K$^b$ and Qa-2) were expressed homogeneously and at high levels in B6 mice, without major differences in the MFI between the subpopulations. However, we observed a double peak for H-2K$^b$ in the Ly49C/I-NKG2A+ and Ly49C/I+NKG2A+ NK cells only in the (IL-2, IL-12, IL-18 condition). Our hypothesis held true in the case of Qa-1$^b$ in the B6 strain. Indeed, whereas we found a homogeneous peak almost superposed to the negative control in the NK cells devoid of NKG2A, the two NKG2A+ subsets displayed a MFI overall approximately twice as high and moreover two connected but separate populations (one “bright” and one “dim”) in the three cytokine conditions.

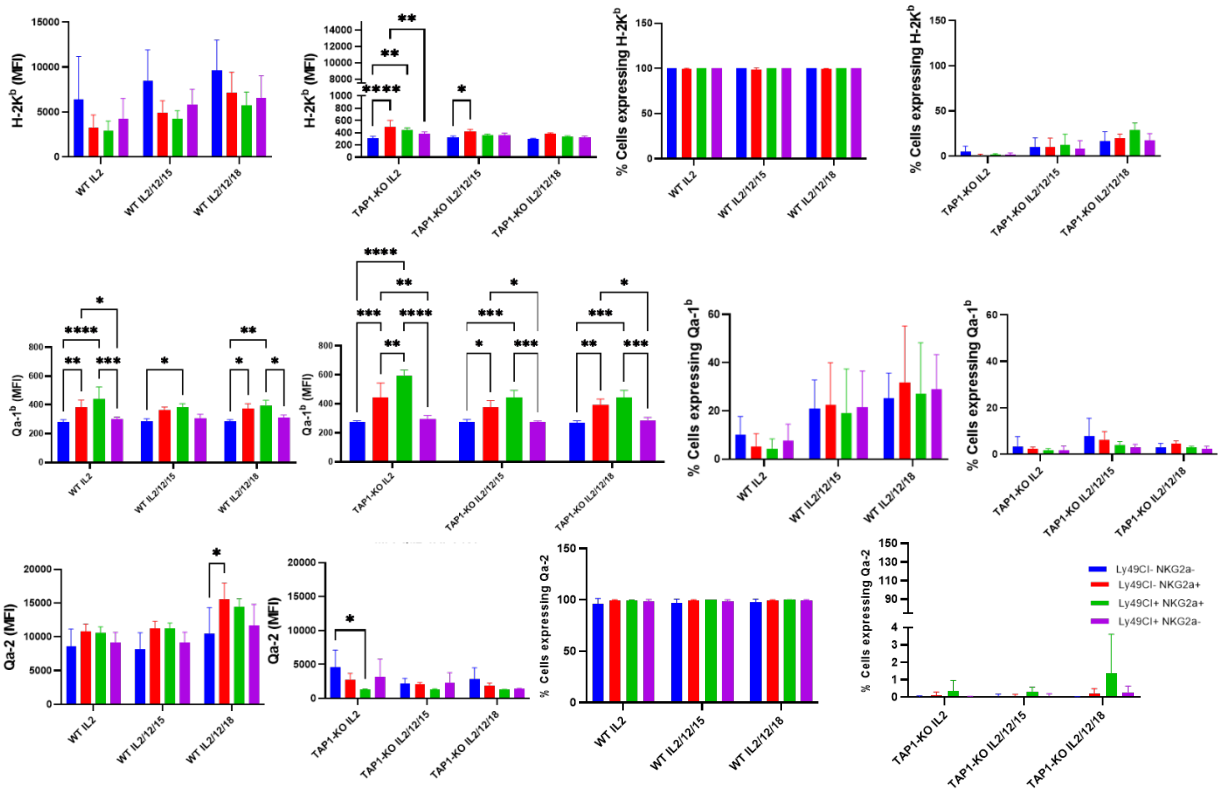



**Figure 5:** Comparison of MHC class I molecules H-2K$^b$, Qa-2 and Qa-1$^b$ in NK cells derived from murine spleens of the B6 and TAP-KO strains. The NK cells were expanded for 18 hours in the presence of IL-2 and stimulated with IL-2, IL-12, IL-15, or IL-2, IL-12, IL-18 with an IL-2 only control group as well. The expression levels were checked in the NK cell subsets defined by the presence or absence of NKG2A and Ly49C/I. Statistical analysis was performed using

GraphPad Prism 9.0.0 with an ordinary two-way ANOVA with Tukey's multiple comparisons tests (n=4). *, p<0.05, p<0.01, ***, p<0.001 and ****, p<0.0001.

Furthermore, while the expression of H-2K$^{b}$ was very low in TAP1-KO NK cells, again as expected, the effect of IL-12 + IL-18 was still observable even in this MHC class I-deficient context. Very interestingly, for Qa-1$^{b}$, the same phenomenon of the double populations appeared selectively in the NKG2A+ NK cells of the three mixtures of cytokines. This suggests both in B6 wildtype and in TAP1-KO NK cells, that the observation is linked to NKG2A rather than to the types of cytokines and/or the MHC class I molecules themselves.

We concluded our phenotypic investigations with the markers CD11b, CD27 and KLRG1. The former two allow the subdivision of NK cells into four subpopulations, the CD27+ cells being considered as the most immature ones (35). The senescence and maturity marker KLRG1 is in contrast better represented on terminally differentiated NK lymphocytes (36). In mouse TAP deficiency, the distribution of the CD27+ and CD11b+ subsets were shown to be different from wildtype mice in that there are almost twice as much CD27$^{high}$ (immature) NK cells in the former (37). However, with our gating strategy, we found very similar values between the four strains in the spleen. In the lungs, the CD27 *versus* CD11b plots showed a mean of 21% of immature NK cells (double negative, CD27$^{high}$CD11b- and double positive) opposed to means of 12%, 7% and 11% in the B6 wildtype, B6$^{CAST}$ and MR1-KO mice, respectively (n=3). In addition, KLRG1 was expressed on a significantly reduced NK cell population of β2m-KO mice of various genetic backgrounds (38), an observation that we could confirm in the spleens of TAP1-KO animals, both in terms of frequency of KLRG1+ cells and of MFI. Interestingly, the percentages of the KLRG1+ NK cells and the MFI of the receptor showed a strong trend to increase in all four strains in the lung compared to the spleen, although TAP1-KO NK cells still lagged behind the others for both of the parameters. As previously observed (29,30), the lung contained more CD11b+ and much less CD27+ NK cells than the spleen in the four types of animals. Overall, the data shows that NK cells from TAP1-

KO mice are relatively immature compared to their wildtype counterparts, and that, in contrast, NK cell maturation does not seem to be affected in MR1-KO animals. Indeed, the subsets defined by the expression of CD27 *versus* CD11b and the percentages of KLRG1+ NK cells were not different in this strain compared to B6 wildtype and $B6^{CAST}$ mice.

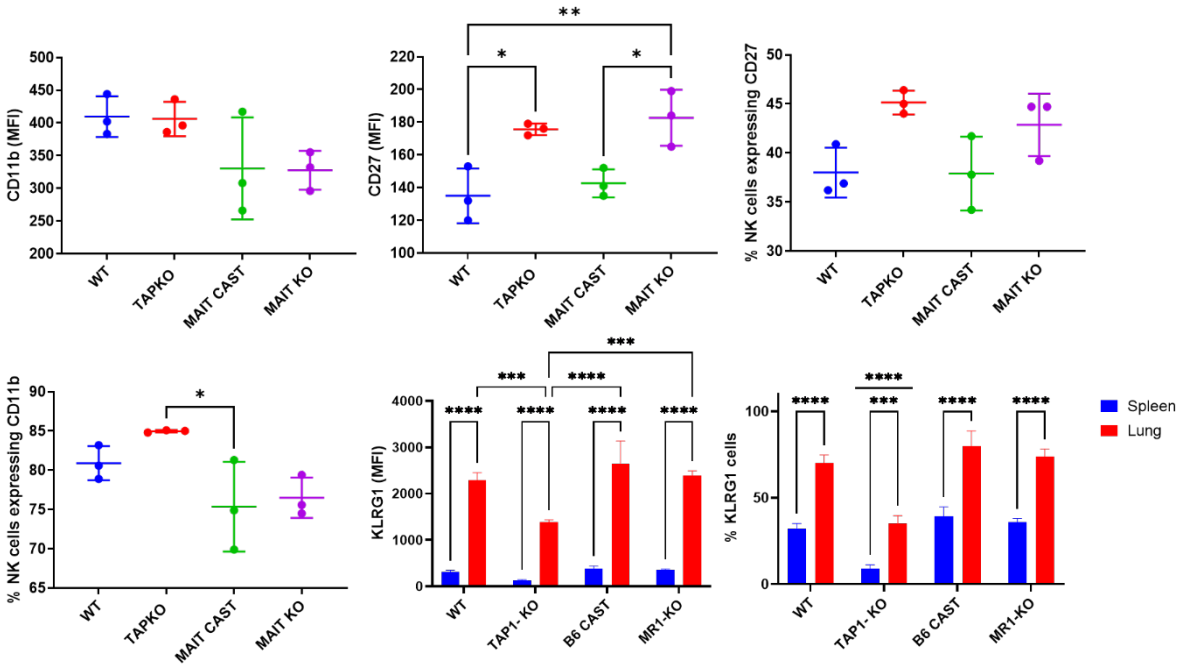



**Figure 6:** (A) Mean fluorescence intensity (MFI) and frequency of NK cells based on CD27 and CD11b expression levels in mouse spleen. Statistical analyses was performed using GraphPad Prism 9.0.0 with a one-way ANOVA (n=3). *, $p<0.05$, **, $p<0.01$. (B) KLRG1 expression levels and frequency measured in murine lung and spleen derived NK cells. Statistical analyses were performed using GraphPad Prism 9.0.0 with an ordinary two-way ANOVA with Tukey's multiple comparisons tests (n=4). *, $p<0.05$, $p<0.01$, ***, $p<0.001$ and ****, $p<0.0001$.

*3) Differences in activated NK cell IFNγ production according to the initial licensing status*

Next, we wanted to compare the production of the signature cytokine of NK cells, IFNγ, in the four subsets defined by the presence or absence of the self-specific and educating IR, Ly49C/I and NKG2A. Theoretically, as outlined above, we expected fewer IFNγ-producing NK cells in the double negative, unlicensed NK cell fraction, as described by Kim et al. (10). This

dataset was nevertheless established in *ex vivo* splenocytes stimulated with plate-bound antibodies. We asked the question if the licensing features would be maintained after a strong stimulation with the cytokines IL-2, (IL-2, IL-12, IL-15) and (IL-2, IL-12, IL-18), and investigated this initially at day 1, *i.e.* after an overnight activation. Here, we found in B6 wildtype NK cells no significant IFNγ production with IL-2 alone, whereas all four subsets defined by Ly49C/I and NKG2A produced the cytokine with values around 30% for the NKG2A- subpopulations *versus* approximately 20% for the NKG2A- ones when stimulated by the (IL-2, IL-12, IL-15) cocktail. In the presence of (IL-2, IL-12, IL-18), only 35 % of the double negative NK cells became IFNγ+, opposed to more than 60 % (Ly49C/I single positive), 70% (NKG2A single positive) and 80% (double positive) of the other subtypes, respectively. This observation fits with current models for NK cell education, notably the licensing model (10) and the rheostat model (10-12,39,40). The latter predicts higher functional capability of NK cells when they express more than one self-specific IR. Natural killer cells from TAP1-KO mice were stimulated under the same conditions. Whereas again no IFNγ production was observed in the presence of IL-2 alone, the two cytokine cocktails surprisingly induced an almost similar distribution of the IFNγ-producing NK cells compared to wildtype mice, with the strongest effect seen in NKG2A+ cells, followed by Ly49C/I single positives and finally the double negatives (barely half the values of the NKG2A+ cells). In principle, as TAP1-KO NK cells cannot be appropriately educated, one should not observe such a difference in IFNγ-producing cells between the NK subsets but, as even MHC class I- NK cells become activated under appropriate cytokine stimulation (18), a more equal distribution.

To get a deeper insight into this topic, we repeated the experiments by first culturing splenocytes with IL-2 alone for five days and then, at day 5, adding the same three cytokine conditions until day 6, when the cells were harvested and stained. We then similarly looked at the percentages of IFNγ+ NK cells in the four subsets defined by the presence or absence of the IR Ly49C/I and NKG2A.

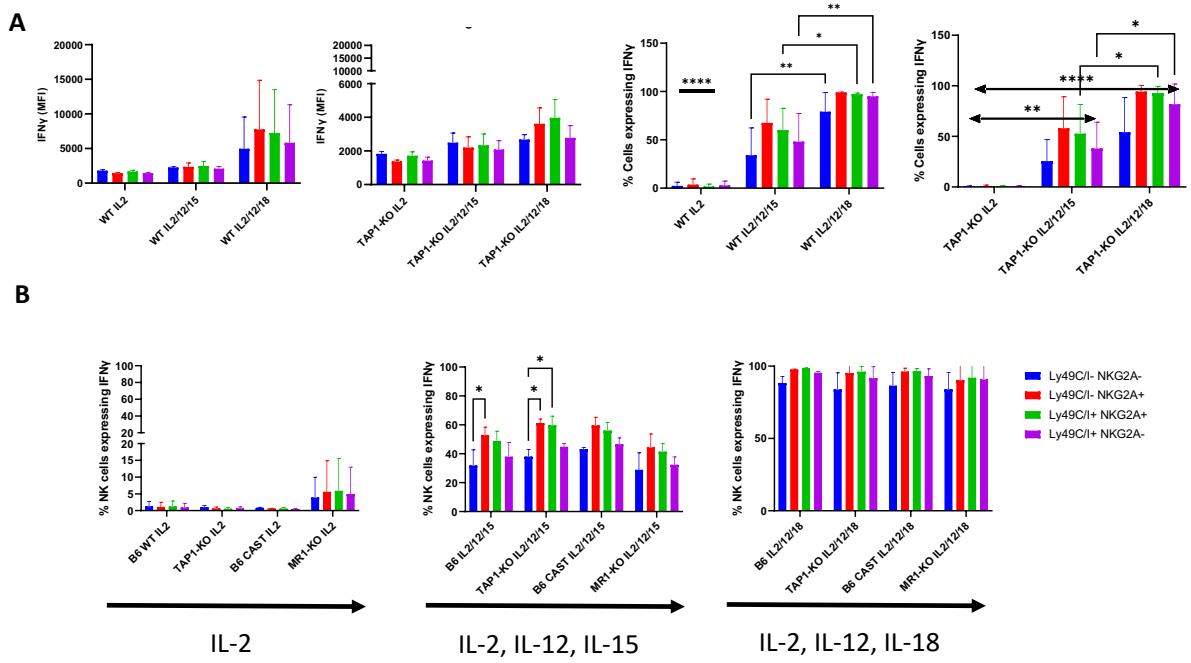



**Figure 7:** (A) IL-2 expanded B6 WT and TAP1-KO NK cells from murine spleen were activated with overnight stimulation of cytokines IL-2, (IL-2, IL-12, IL-15) and (IL-2, IL-12, IL-18) at day 1. IFNγwas measured in CD3-NK1.1+ NK cells by intracellular staining. Statistical analysis was performed using GraphPad Prism 9.0.0 with an ordinary two-way ANOVA with Sidak multiple comparisons tests (n=4). *, p<0.05, p<0.01, ***, p<0.001 and ****, p<0.0001. (B)B6 WT and TAP1-KO NK cells from murine spleen were expanded in presence of IL-2 for 5 days. On day 5, NK cells were activated with overnight stimulation of cytokines IL-2, (IL-2, IL-12, IL-15) and (IL-2, IL-12, IL-18). IFNγ was measured in CD3-NK1.1+ NK cells by intracellular staining. Statistical analysis was performed using GraphPad Prism 9.0.0 with an ordinary two-way ANOVA with Sidak multiple comparisons tests (n=4). *, p<0.05, p<0.01, ***, p<0.001 and ****, p<0.0001.

Overall, we observed the same results than at day 1, although the global cytokine production levels were higher in each subset. The double negative population was still the less proficient in intracellular IFNγ accumulation in the (IL-2, IL-12, IL-15) condition, whereas the NKG2A+ subsets remained the most productive ones. With (IL-2, IL-12, IL-18), almost 100% of the four subpopulations became IFNγ+. However, concordant differences were still observed

in the MFI, reflecting in these cases the quantity of IFNγ per cell and not, as the percentages parameter, the fraction of NK cells producing the cytokine. Similar to day 1, we could not observe significant differences between B6 wildtype and TAP1-KO mice, meaning that (i) the wildtype NK cells maintained to some degree the *ex vivo* educational profile that let an imprint even after a very strong cytokine stimulation at days 1 and 5, and (ii) this imprint was also present in the TAP1-KO context, although NK cells are not supposed to be educated through classical and non-classical MHC class I molecules in this genetic background (as the expression of these proteins is severely reduced). Moreover, our data validate the licensing (10) and the rheostat models (10-12,39,40) even in a situation of major NK cell activation. Globally similar results were gathered on day 6 in the $B6^{CAST}$ and MR1-KO mice.

*4) Downmodulation of Ly49C/I on activated NK cells at day 6*

When we undertook the phenotyping of the spleens of the four strains of mice at day 6 for the purpose of measuring intracellular IFNγ production by NK cells, we observed that the percentages of Ly49C/I+ events were dramatically downmodulated compared with days 0 (*ex vivo*) and 1. This phenomenon, not noticed in the case of NKG2A, was significant for each mouse type, either between day 0 and day 6 (NKG2A+Ly49C/I+) or between day 1 and day 6 (double positive), whereas rather minor and not systematically significant differences could be seen between day 0 and day 1. In a mirror image, the percentages of NKG2A+Ly49C/I- as well as double negative NK cells increased. A reduction of Ly49C/I+ NK cells in the context of activated tumor-infiltrating lymphocytes has been previously described by Shi et al. (41).

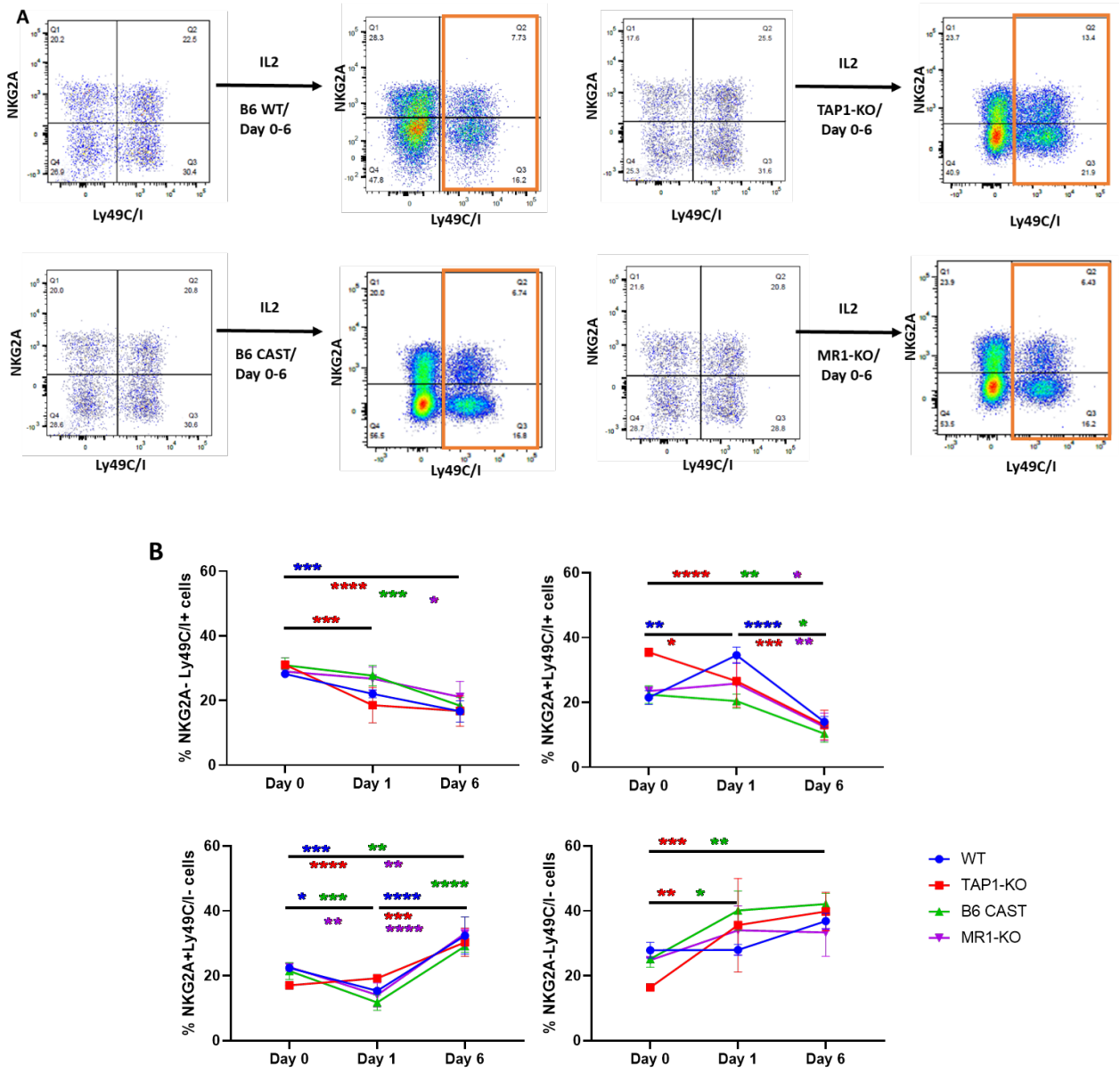



**Figure 8:** (A) Representative flow cytometric dot plots of splenic NK cells distinguised according to NKG2A and Ly49C/I expression. The NK cells were expanded in presence of IL-2 for 6 days and the expression levels of NKG2A and Ly49C/I were compared with that of Day 0. (B) Analyses of the frequency of NK cell subsets based on NKG2A and Ly49C/I expression levels. The frequency between Day 0, Day 1 and Day 6 for IL-2 expanded NK cells was compared. Statistical analysis was performed using GraphPad Prism 9.0.0 with an ordinary two-way ANOVA with Sidak multiple comparisons tests (n=4). *, p<0.05, p<0.01, ***, p<0.001 and ****, p0.0001.The colors of the * correspond to the strains of mice used.

*4) Natural killer cell cytotoxic activity*

Besides cytokine production, natural cytotoxicity against tumor cells and viral-infected cells is the second major property of NK cells. In the mouse, the lymphoma cell line YAC-1 is the standard target, due to its exquisite NK cell sensitivity (42). Another T cell lymphoma, RMA, is rather resistant to these cells, whereas C4.4-25$^-$, a β2m-deficient variant of EL4, the parental cell line of RMA (43), is again susceptible because of the almost absent expression of MHC class I molecules (44). As the latter two possess the B6 genetic background, they can serve for the determination of the missing self-recognition by NK cells from B6 origin.

We performed a four-hour cytotoxicity assay in duplicates, with splenocytes from the four types of mice cultured overnight with IL-2 alone and with each of the two cytokine cocktails and evaluated the lysis of the targets YAC-1, RMA and C4.4-25$^-$. It is known that a relatively short incubation time with IL-2 is enough to significantly increase natural cytotoxicity (45), and this was the case in the four strains, as YAC-1 was killed at levels between 50% and nearly 70% at all E/T ratios. Interestingly, the addition of the other cytokines did not further level up the cytotoxicity, but even induced a minimal trend towards a decrease.

RMA cells resisted quite well as described (44) and the residual level of cytotoxic activity, that became significant at the highest E/T ratios, was again observed to a comparable degree between the four types of splenocytes. Furthermore, we demonstrated that B6 wildtype, TAP1-KO, B6$^{CAST}$ and MR1-KO NK cells were able to perform missing self-recognition, as they abundantly killed the MHC class I-deficient cell line C4.4-25$^-$, which lacks the B6 class I molecules H-2D$^b$ and H-2K$^b$, highly expressed by RMA (data not shown). Thus, after cytokine stimulation, NK cells from TAP1-KO and MR1-KO mice become functional to the same extent as their wildtype counterparts.

Then, we repeated the cytotoxicity experiments with spleen and lung NK cells cultured during five days in the presence of IL-2 and then re-stimulated overnight with IL-2 alone. Here again, YAC-1 and C4.4-25$^-$ were very efficiently lysed by NK cells from the four strains and of

both organs, with the latter being even more exquisitely sensitive. Lung NK cells showed a tendency to a stronger killing activity than their splenic counterparts. RMA cells were significantly susceptible to spleen and lung NK cells (especially at higher E/T ratios), which might be related to the longer stimulation time of these effectors compared with only one day. Importantly, we could not detect statistically significant differences in the lysis intensity between B6, TAP1-KO, B6$^{CAST}$ and MR1-KO NK cells, confirming the results obtained at day 1 with the spleen and extending them at day 6 to the lung.

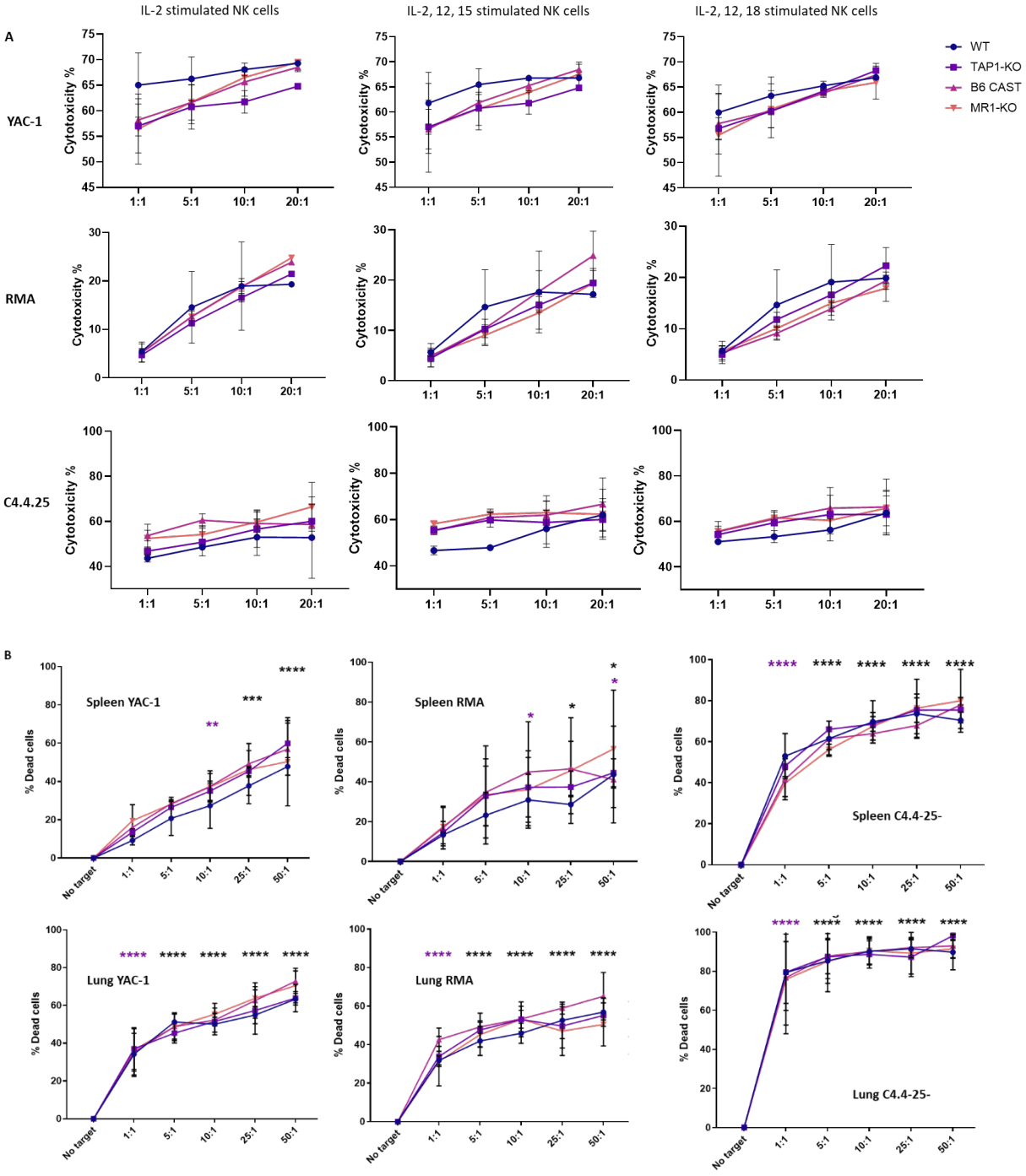


**Figure 9:** (A) NK cells derived from murine spleen were expanded overnight in IL-2 and stimulated with IL-2 alone, or IL-2, -12, -15 or IL-2, -12, -18 cytokines overnight. These cells were then co-cultured with the targets YAC-1, RMA and C4.4-25$^{-}$ for four hours. The cytotoxicity was measured with TO-PRO-3 staining. (B) NK cells derived from murine spleen and lung were expanded in the presence of IL-2 for 5 days and then restimulated with IL-2 alone overnight. These cells were then co-cultured with the targets YAC-1, RMA and C4.4-25$^{-}$ for four hours. The cytotoxicity was measured with TO-PRO-3 staining.

*5) Natural killer cell degranulation*

Degranulation assays consist in the measurement, by flow cytometry, of the percentage of NK cells expressing the marker CD107a at the surface after, for example, incubation with target cells (46). This molecule is part of the membrane of the NK cytotoxic granules and accompanies their secretion. It is considered as a surrogate for the cytolytic activity (46). We analyzed degranulation of *ex vivo* spleen and lung NK cells from the four types of mice (three mice per strain) after overnight stimulation with the same cell lines and cytokine cocktails than used for the cytotoxicity evaluation in the various quadrants defined by the presence or absence of Ly49C/I and NKG2A. Quite surprisingly, there was a significant level of degranulation (CD107a+ NK cells) at baseline, *i.e.* in the absence of targets. This background was higher with IL-2 alone than with the two mixtures of interleukins, but predominantly among the NKG2A single positive and the double positive NK cells. The values did not change significantly after addition of the three target cells but continued to be highest with IL-2 alone. Furthermore, we observed the same distribution of the percentages of de-granulating cells than in the experiments about IFN$\gamma$ production, namely that the most important fractions of CD107a+ NK cells were reached in the two subsets expressing NKG2A, compared to the Ly49C/I single positive and the double negative populations. Regarding the different strains, the tendency was the same, although overall more B6 wildtype and TAP1-KO NK cells became CD107a+ than NK cells on the B6$^{CAST}$ background. Whereas IL-2 was most efficient in stimulating spontaneous and target cell-induced CD107a cell surface mobilization, it came a

bit unexpected that the cytokine cocktails were less active here. In any case, the degranulation experiments confirmed once more published data about NK cell licensing and education.

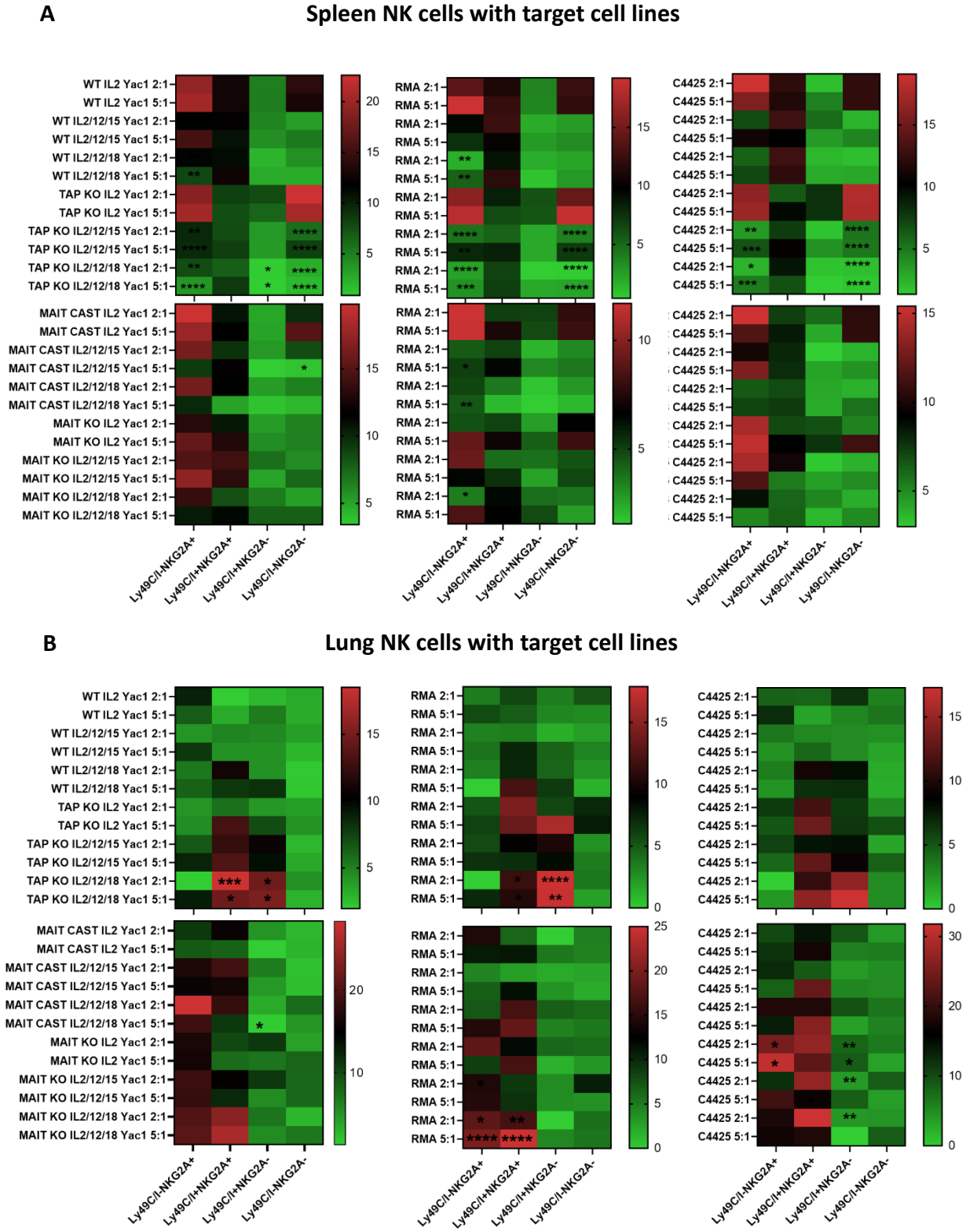


**Figure 10:** (A) NK cells derived from murine spleen were expanded in the presence of IL-2 for 5 days and then restimulated with IL-2 alone, or IL-2, -12, -15 or IL-2, -12, -18 cytokines overnight. These cells were then co-cultured with the targets YAC-1, RMA and C4.4-25$^{-}$ for four hours. The stimulated cells were investigated for CD107a expression in CD3-NK1.1+ NK cells by intracellular staining. The expression levels were divided into four NK cell subsets based on presence of NKG2A and Ly49C/I. Statistical analysis was performed using GraphPad Prism 9.0.0 with an ordinary two-way ANOVA with Tukey's multiple comparisons tests (n=4). *, p<0.05, **, p<0.01, ***, p<0.001 and ****, p<0.0001. (B) Same with NK cells derived from murine lungs.

In addition, we stained the NK cells with an anti-DNAM-1 antibody, as it has been described that this AR defines two NK cell subsets, DNAM-1+ and DNAM-1-, with different phenotypic and functional properties (47). In all strains and culture conditions, the fraction of de-granulating NK cells was higher among the DNAM-1+ subpopulation.

*6) Cytotoxic granule profiling of NK cells*

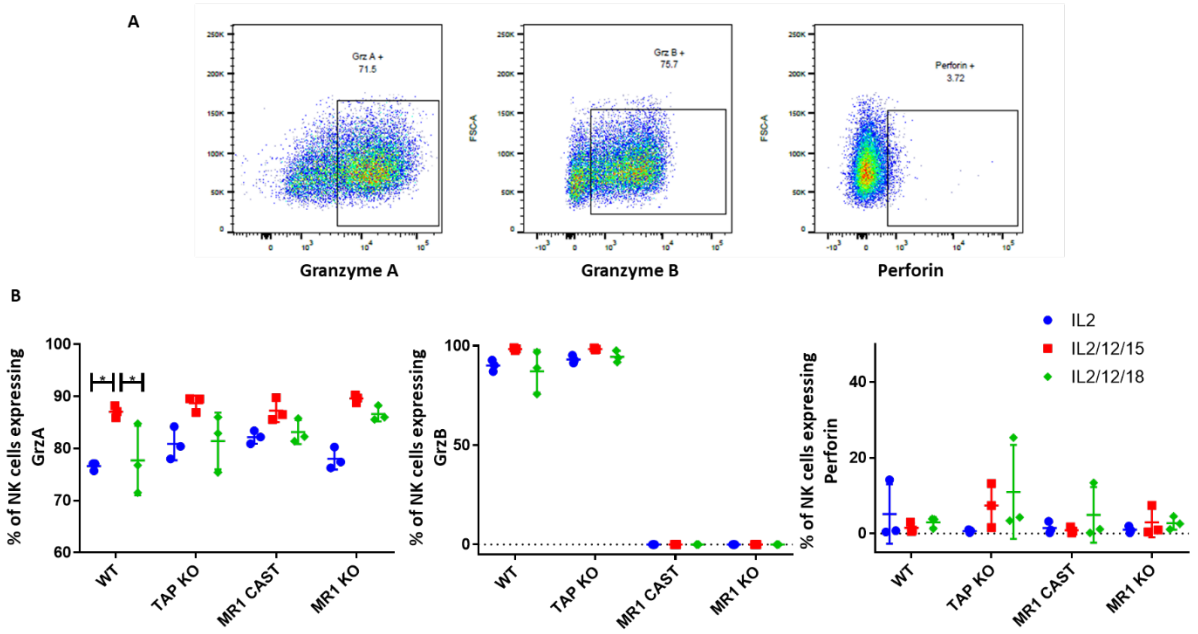


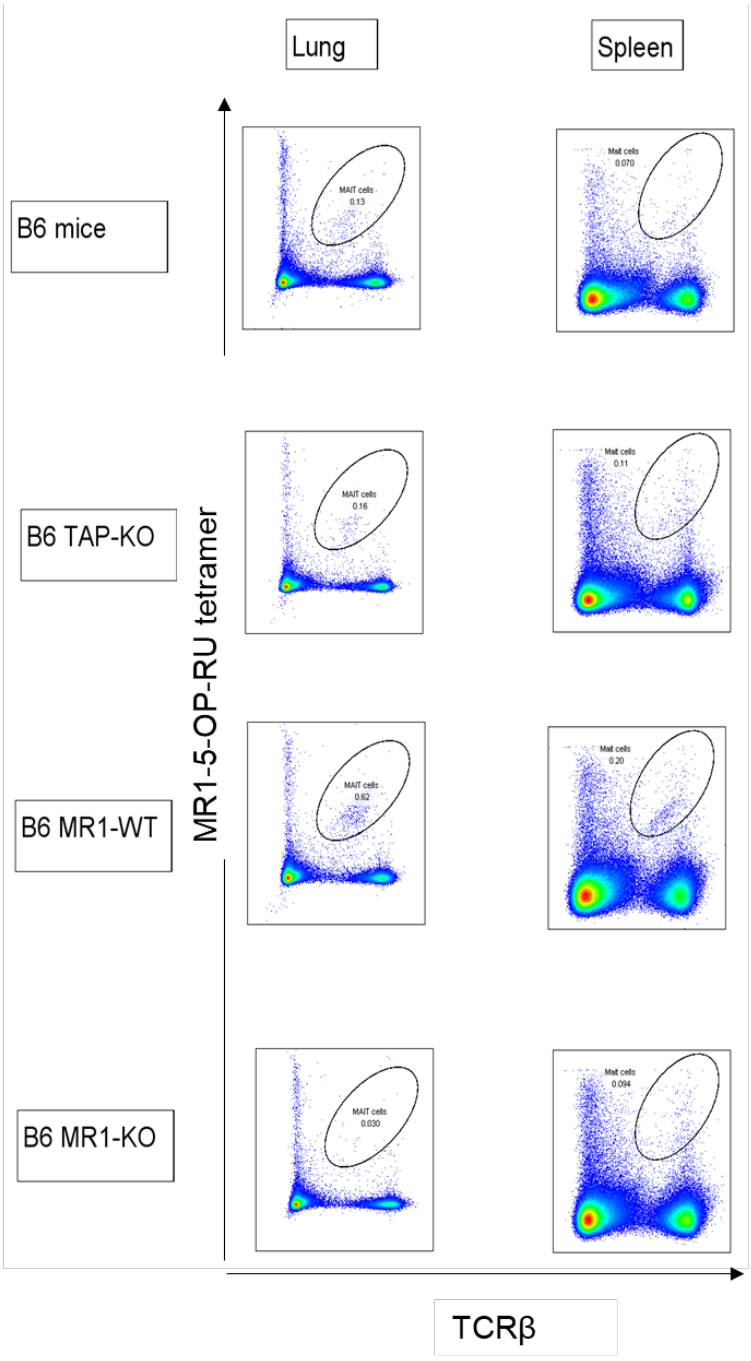
**Figure 11:** (A) Representative plots showing granzyme A, granzyme B and perforin against forward scatter in splenic NK cells. (B) Analyses of the frequency of granzyme A, B and perforin in the NK cell population. Statistical analyses was performed using GraphPad Prism 9.0.0 with an ordinary two-way ANOVA with Tukey's multiple comparisons tests (n=4). *, $p<0.05$.

*7) Phenotype of MAIT cells assessed by MR1 tetramers*

Finally, we took advantage of the availability of MR1 tetramers (48) to stain MAIT cells in the four types of mice in spleen and in lung. These tetramers are specific for MR1-restricted T cell receptors (TCR) and therefore identify the vast majority of MAIT cells (48). In all the strains and organs, a discrete MR1 tetramer+ population (TCRβ+MR1 tetramer+) could be identified. However, as expected, this fraction was extremely low in the MR1-KO mice and appeared only when we acquired a very high number of events by flow cytometry. We then looked at several surface molecules described for MAIT cells, namely the activation markers CD44 and CD69, the homing marker CD103, the adhesion molecule CD62L, the NK cell AR NK1.1 (CD161c), which is also expressed by NKT-like lymphocytes, the cytokine receptors CD127 (IL-7 receptor) and CD218 (IL-18 receptor α chain), and finally the chemokine receptors CCR9 and CXCR6, in the gated (TCRβ+MR1 tetramer+) population. In the spleens, CD44 was expressed on approximately 90% of the cells. We found a low percentage of positivity for CD69 and CD103 except in the two KO strains where CD69 was present at a much higher level. Interestingly, 75% – 80% of the gated TAP1-KO and MR1-KO T cells were CD62L+, versus only 9% and 23%, respectively, for the two wildtype mouse spleens. The receptors NK1.1 and CCR9 were almost absent in all strains. In contrast, CD127 was present on the majority of cells, whereas CD218 expression appeared as surprisingly low (16% - 50% of all tetramer+ events). Finally, CXCR6 appeared on around 30% of the cells. Coming to the lungs, CD44 was expressed by almost all cells, CD69 at a very low percentage except in the MR1-KO strain (18%), CD103 by half of the cells, CD62L again at low percentages with the MR1-KO cells being outliers (18% positive), NK1.1 and CCR9 almost absent, CXCR6, CD127 and CD218 by

the majority of cells. However, in the MR1-KO strain, the percentage of CD218+ cells was lower (70%), suggesting together with other markers that the tetramer MR1+ cells in these mice are indeed MR1-restricted but are not MAIT cells.

**A**



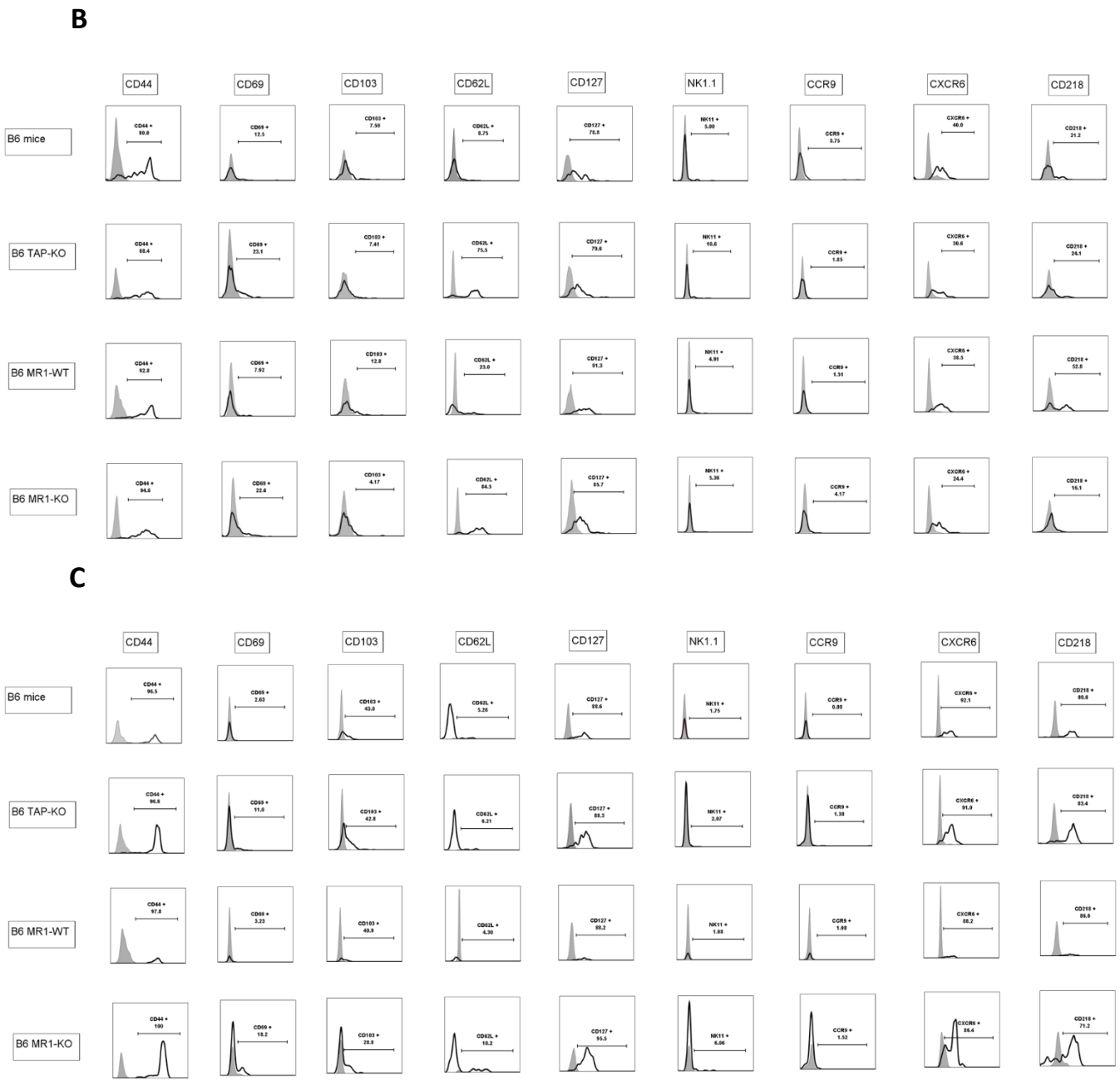



**Figure 12:** (A) Representative flow cytometry plots for the murine MAIT cells from lung and spleen stained with MR1-restricted T cell receptors (TCR) specific tetramers (B) Surface molecule staining for splenic MAIT cells including CD44, CD69, CD103, CD62L, CD127, NK1.1, CCR9, CXCR6 and CD218 for various strains. (C) Surface molecule staining for lung MAIT cells including CD44, CD69, CD103, CD62L, CD127, NK1.1, CCR9, CXCR6 and CD218 for various strains.

## Discussion

In this paper, we confirm the initial licensing profile of NK cells in the B6 wildtype mouse genetic background, corresponding to the fact that the expression of self-specific IR for autologous (self) MHC class I molecules educates these cells and renders them functionally active, although the licensing does not automatically lead to the activation of the entire population of self-specific IR+ NK cells but only of a fraction of them, in accordance with the literature (10,11). Furthermore, we show that the phenotype and functional behavior of NK cells from $B6^{CAST}$ and MR1-KO animals are similar to the B6 wildtype. This is not surprising in the former, because these mice, initially deriving from captured wild animals, have been abundantly backcrossed to B6 for many generations (49).

For the MR1-KO NK cells however, we expected to see some differences because of the structural similarity of the MR1 molecule with classical MHC class I molecules. It could be conceivable that NK cells express an AR or an IR specific for MR1, which would implicate these lymphocytes in antibacterial defense (the principal role of MAIT cells) and that they recognize MR1 together with a vitamin B2-derived bacterial metabolite when this complex appears on the surface of cells infected by bacteria. Antibacterial effects of NK cells have been previously described (50); however, they were, to the best of our knowledge, not yet investigated in the context of MR1. Our experiments do not rule out this possibility, but we observed no major effect of MR1 deficiency on NK cell phenotype and function, compared to the other three mouse strains. On the other hand, the phenotype and functions of TAP1-KO NK cells are not that different from their wildtype counterparts, and in many knockout models of NK cell receptors or members of signaling cascades, the surface phenotype remains unaffected. An interesting experiment would be to stain NK cells with the MR1 tetramers and to check if some binding occurs, because this would suggest the existence of a receptor for MR1 on NK cells.

Our experiments furthermore confirmed the phenotypic differences between spleen and lung NK cells that we (29), and others (30), have previously shown. This is fully in line with

the current paradigm of NK cell heterogeneity dependent on the organ that is looked at (51), and so the mere investigation of peripheral blood NK cells in human and splenic NK cells in the mouse, although they are easily accessible, is to some extent a limitation.

Interestingly, even if the phenotype of TAP1-KO NK cells was only minimally different from wildtype, as previously described (52), we found that after a strong cytokine-mediated activation, these cells resembled wildtype NK cells in terms of licensing. Indeed, the level of autologous MHC class I molecules being very low in their cellular environment, they are hyporesponsive *ex vivo* and can in principle not be educated. Nevertheless, after stimulation with (IL-2, IL-12, IL-15) or (IL-2, IL-12, IL-18), they become significantly active in terms of cytokine production and cytotoxicity. What was really surprising was the observation that, although the four NK cell subsets defined by the presence or absence of the self-specific IR were significantly active regarding IFN$\gamma$ production, which was largely expected in the wildtype mice, the initial education profile was to some extent conserved at day 6, and moreover, also present and clearly identifiable in the TAP1-KO NK cells. Thus, there must be molecular processes that can license these cells independently of MHC class I molecules and on the other hand are likely related to them, as the differences are defined by the presence or absence of Ly49C/I and NKG2A.

The phenotypic analysis of the NK cells from the four strains revealed some interesting differences which might be worth investigating in more detail and with more fluorochromes for flow cytometry and/or an investigation by mass cytometry. We were particularly impressed by the total absence of staining of TAP1-KO cells by the anti-Qa2 antibody, revealing the complete dependence of this non classical HLA class I molecule on a functional TAP.

The downmodulation of Ly49C/I, whereas NKG2A expression was spared, has previously been described by Shi et al. (41). These authors envisaged several possibilities to explain the observation, namely receptor downmodulation (internalization?), contraction of the subset or

relative expansion of the Ly49C/I- NK cells, to finally suggest after an experimental approach that the most likely explanation might be receptor shedding from the cell surface**.** In any case, the phenomenon could be reproduced after cytokine-mediated stimulation of NK cells as an incidental finding in our study. If the reason behind it was receptor internalization after prolonged contact with cognate MHC class I ligands in the cell culture system, it would most likely not have been observed in TAP1-KO mice. Comparative evaluation of the proliferation of Ly49C/I- compared with Ly49C/I+ NK cells, and the amount of soluble Ly49C/I in the supernatant of strongly activated *versus* less activated splenocyte cultures could be interesting perspectives for future experiments. Likewise, Korten et al. reported a reduction in the percentages of Ly49C+, Ly49G2+ and Ly49A+ NK cells in helminth infection, in the context of a global NK cell expansion (53). However, these observations stem from the Balb/c mouse strain, which carries another genetic background and other polymorphic forms of the Ly49 family. Tay et al showed that in murine cytomegalovirus infection, the percentage of Ly49C+ NK cells declines in the spleen but not in the peritoneal exudate, a phenomenon that is not present in lymphocytic choriomeningitis virus (LCMV) infection (54). Other aspects of Ly49C/I receptors are their presence on memory NK cells and their peptide sensitivity (recognition of the peptide presented by the cognate MHC class I molecules), as reviewed by Wight et al. (55). One might hypothesize that the loss of Ly49C/I by activated NK cells renders these effectors more efficient against targets expressing classical MHC class I ligands. After cytokine-mediated stimulation however, the most efficient activation resides in the NKG2A-expressing subsets, which seem to educate NK cells “better” than Ly49C/I. Finally, Ly49C also interacts with its ligand in cis, i.e. in the plane of the same cell membrane (56,57), which corresponds in fact to the physiological situation. This phenomenon, initially described for Ly49A, has important consequences for the education and function of NK cells (58).

Despite their biological immune suppression (low number of CD8+ T cells, hypo-responsive NK cells), TAP1-KO mice display no clinical phenotype, if they live in a specific pathogen free or a “dirty” animal facility. Moreover, their short life span and the normal humoral

immune response, as well as the possibility of activating their NK cells in an infectious context, might contribute to the explanation. This is in sharp contrast to human TAP deficiency, a very are autosomal recessive disease (around 35 cases described in the literature) characterized by repeated bacterial infections of the respiratory tract, bronchiectasis, deep skin ulcers and a granulomatous destruction of the nasal cartilage ending up in a clinical picture resembling NK/T cell lymphoma, nasal type (lethal midline granuloma) (59). These patients usually reach early adulthood but have a low quality of life. Their NK cells are hypo-responsive ex vivo as in the mouse, but they become strongly activated after cytokine stimulation (IL-2). They present aroused NK cells within the skin lesions, so that we cannot exclude that the former contribute to the pathophysiology of the disease (13,14,18). A mouse model mimicking skin ulcers and/or bacterial respiratory infections would be necessary to address these questions in-depth.

Our limited investigations of the phenotype of MAIT cells do not yet allow drawing firm conclusions. There were some differences between spleen and lung, such as the somewhat surprising result of a relatively low percentage of cells expressing the IL18 receptor $\alpha$ (CD218) in the spleens of the four mouse strains. This data needs to be confirmed and extended.

Overall, we present a study about phenotypic and functional aspects of NK cells in four mouse strains, B6 wildtype, TAP1-KO, B6CAST and MR1-KO, in spleen and lung. We confirm the licensing and the rheostat models that surprisingly also seem to apply to the TAP1-KO NK cells, whereas this is theoretically not possible. The only option would be that Qa-$1^b$ might educate the cells, as its expression is possible with TAP-independent peptides (60). An argument for this possibility could be that the NKG2A+ NK cell subsets react stronger than the NKG2A- ones, at least in the case of the parameters we investigated. We present some limited insight into MAIT cells from the phenotypic point of view. An interesting observation is the loss of Ly49C/I (for whatever reason), that could indicate, if similar observations would be made for human KIR and/or NKG2A, the possibility of selecting subsets with lower percentages of IR+ NK cells.

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# Chapter 3: Mouse model of chronic lung infection

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**Contribution to the chapter:**

- Literature review and writing
- Culturing bacteria, creating bacteria embedded agar beads
- Standardising the infection protocol on various strains of mice, performing histology, follow-up on the mice throughout the infection period
- Analysing the experiments

**Abstract**

Even with the growing evidence of the involvement of natural killer cells in anti-bacterial defences, the mechanisms remain unclear. In the previous chapter, we had a look at the cell surface markers on the murine NK at baseline in absence of pathogen. In the following study, we aim to establish a mouse model of chronic bacterial lung infection. With *Pseudomonas aeruginosa* as the infectious agent, we emphasized on lungs as they are a primary entry site for various pathogens. Models of chronic lung infections have previously been used to study diseases such as cystic fibrosis and Chronic obstructive pulmonary disease (COPD). We combined the existing models to create a non-invasive model to introduce *P. aeruginosa* into mouse lungs, as an attempt to mimic human lung infections. Animal models have previously helped uncover specific immune mechanisms; time taken for responses, aided in drug discovery and vaccine trials among others. The established model will aid in elucidating the mechanisms of NK cell action in anti-bacterial defences.

**Introduction**

Lungs are constantly exposed to an influx of microbes from air inhalation passing through the upper respiratory tract. Lungs play host to a diverse community of microbiota - such as viruses, bacteria, fungi- in sickness and health and understanding the dynamic between these is essential to understand disease progression (Hilty, Burke et al. 2010, Morris, Beck et al. 2013, Dickson, Erb-Downward et al. 2015, Dickson, Erb-Downward et al. 2016). In absence of infections, the immune cells in the lung have basal response and maintain homeostasis and tolerance towards the resident commensal microbiome (Segal, Clemente et al. 2016). This balance is disturbed when a pathogen, for example, *S. pneumoniae*, *P. aeruginosa*, etc invade. The consequences can lead to significant lung diseases

Cell culture models are essential to understand and analyse the functional and physical interaction between the host cells and microbes. However, they aren't a true reflection of the microenvironment and do not mimic the cellular processes in the system. The cell lines used lack the necessary biological properties needed to replicate the host-pathogen interactions, for example human epithelial cell lines lack BEAS-2B are often used to study airway epithelial structures, however they do not form tight junctions (Rothen-Rutishauser, Blank et al. 2008). To form a comprehensive picture, we would have to create a realistic model in our culture dishes, which is currently immensely difficult due to our technical limitations.

The anatomical and physical similarities between animals, especially mammals, and humans has made animal models an essential tool in biomedical research. They are used to answer questions pertaining from basic science to understanding disease biology and address the mechanisms of novel drugs and therapies. For example, the mouse model for *Acinetobacter baumannii* helped us understand the role of innate immune response in its early infection stage (Knapp, Wieland et al. 2006, Kim, Kim et al. 2014, Zeng, Gu et al.

2019). Animal models are crucial for developing antibacterial drugs and vaccines (Byrne, Waack et al. 2020).

To understand the role of natural killer (NK) cells in long-term bacteria mediated lung infections, we decided to establish a model of bacterial lung infection in different strains of C57BL/6 mice, with *Pseudomonas aeruginosa* (*P. aeruginosa,* PA) as the infection mediator. The mouse model of chronic broncho-lung infection using PA-laden agar beads is used to study diseases like cystic fibrosis (CF) and chronic obstructive pulmonary disorder (COPD) (Bayes, Ritchie et al. 2016). We decided to repurpose this model to answer our needs. Embedding bacteria in arresting agent like agar beads helps in formation of micro-colonies of the pathogen and developing characteristics similar to human clinical pathology such as airway inflammation. However, establishing this model is very difficult, as there are very few papers that showed the persistence of the infection in long term, and perhaps the bacteria is actively cleared from the host as the days progress.

As previously reported by Bragonzi *et al*, usage of *P. aeruginosa* clinical strain RP73 over PA01 laboratory strain resulted in lower mortality, severe lesions, and also showed chronic infection up to three months (Facchini, De Fino et al. 2014). *P. aeruginosa* RP73 is a clinical strain isolated from a CF patient, 16.9 years after the onset of airways colonization. PA RP73 has been reported to be resistant to most antibiotic classes, and we handled experiments with the strain at biosafety level 3 facility.

## Materials and Methods

### Ethical statement

The animal studies were approved by the Animal Welfare Structure (AWS) of the Luxembourg Institute of Health (LIH) and the ministry of health of Luxembourg and the experiments were carried out in accordance with the European Union directive 2010/63/EU as incorporated in Luxembourgish law for the care and use of laboratory animals.

Protocol no. DII-2017-02

### Animals

C57BL/6 mice with *wildtype*, TAP1 KO, MAIT CAST variants, aged 8-12 weeks old, were used (bred and maintained at the Luxembourg Institute of Health's SPF animal facility).

### PA-laden agar beads

Two days prior to the mouse challenge, we inoculated *P. aeruginosa* RP73 (PA RP73) strain from the frozen glycerol stocks on a Trypticase Soy Agar (TSA, Sigma) plate and incubated at 37ºC overnight. A single colony is then further inoculated into 5 ml Trypticase Soy Broth (TSB) and incubated at 37ºC overnight. A small aliquot of this overnight culture was diluted 1:50 in phosphate buffered saline (PBS) and optical density (OD) was measured at 600 nm using a spectrophotometer. Up to 2 OD of this overnight culture was added to 20 ml of fresh TSB in a new tube and incubated at 37ºC for 3-4 hours, till the log phase of 10-15 OD was reached. Freshly prepared sterile and autoclaved TSA (TSB mixed with 1.5% of agar) was kept in dry bath at a temperature of 50ºC along with 150 ml of heavy mineral oil (Sigma). Once PA RP73 reached its log phase, the bacterial cells were centrifuged at 2700g for 15

min at 4ºC and the supernatant was discarded. The obtained bacterial pellet was re-suspended in 1 ml PBS and was thoroughly mixed and brought up to 10 ml with the pre-warmed liquid TSA. This mix was then added to the heavy mineral oil (kept at 50ºC) and stirred at a high speed for 6 min at room temperature with the help of a magnetic stirrer. The mixture was cooled rapidly to 4ºC, and stirred at a minimum speed for 35 min. This agar-oil mix was rested for an additional 20 min at 4ºC, then transferred to a 50 ml falcon and centrifuged at 2700g for 15 min at 4ºC. The oil was cautiously removed and washed with PBS at 2700g for 15 min and repeated for 6 times or till the oil clears. We found that the final washes worked better with gravity, instead of centrifugation. The agar beads were re-suspended in 20-25 ml of sterile PBS and were stored at 4ºC overnight.

An aliquot of about 1 ml of the beads was homogenized by passing serially through syringes with decreasing gauge (BD 26G, 23 G and 18G) and observed under microscope to examine the bead diameter between 100-200 µm. 100 µl of this homogenized mix was serially diluted by 1:10 6 times and plated on TSA plates, incubated at 37ºC overnight. The colony forming units (CFU) were counted at the end of the incubation period, to determine

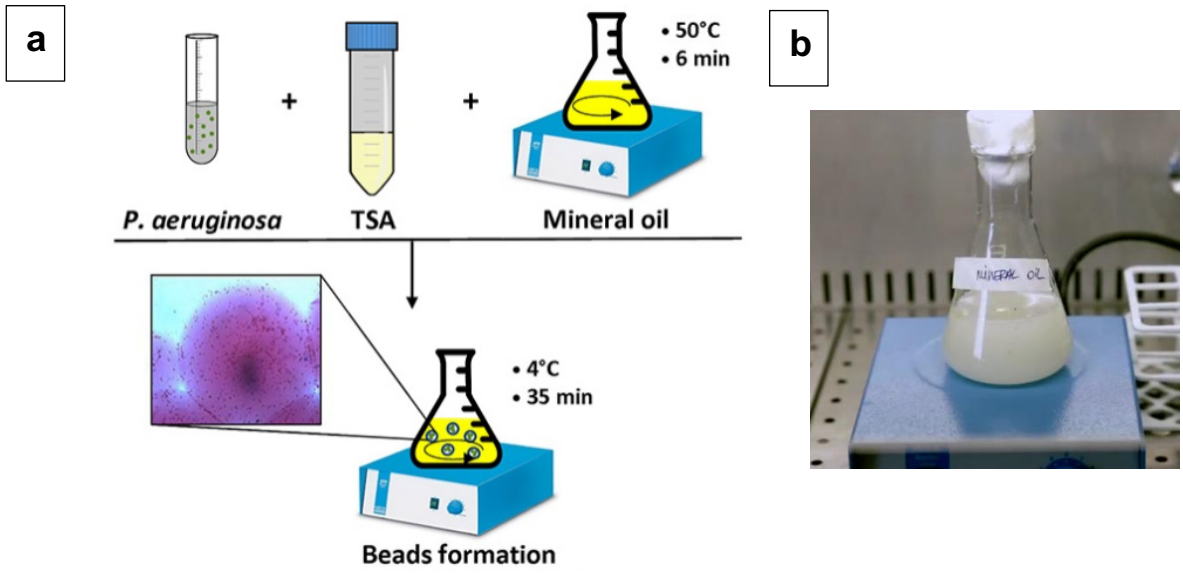


**Figure 1:** (a) Graphical representation of the bead formation protocol. (b) agarose beads in mineral oil

the number of the bacterial CFU/ml in the homogenized agar-bead suspension with the following formula:

$$\text{CFU/ml} = \frac{no.of\ colonies\ x\ dilution\ factor}{volume\ of\ culture\ plate}$$

Once determined, agar-beads solution was diluted with sterile PBS to 2-4 x $10^7$ CFU/ml i.e. 1-2 x $10^6$ in 50 µl. This 50 µl suspension with the required concentration was taken up by a 1 ml syringe for mouse challenge.

**Orotracheal intubation of mice**

Mice were anesthetized intraperitoneally with a mix of fentanyl (0.05mg/kg), midazolam (5mg/kg) and medetomidine (0.5mg/kg). Using a simple support stand, the mice were placed with their ventral side facing the administrator. The mice were suspended vertically, by their upper incisors. The tongue was pulled out softly, and lidocaine was applied (Fig 2).

With otoscope as a light source, epiglottis and the vocal cords were identified. A 20 gauge IV catheter (BD) was very carefully inserted in between the vocal cords. This catheter was connected to a syringe containing a pre-determined concentration of PA-laden agar beads

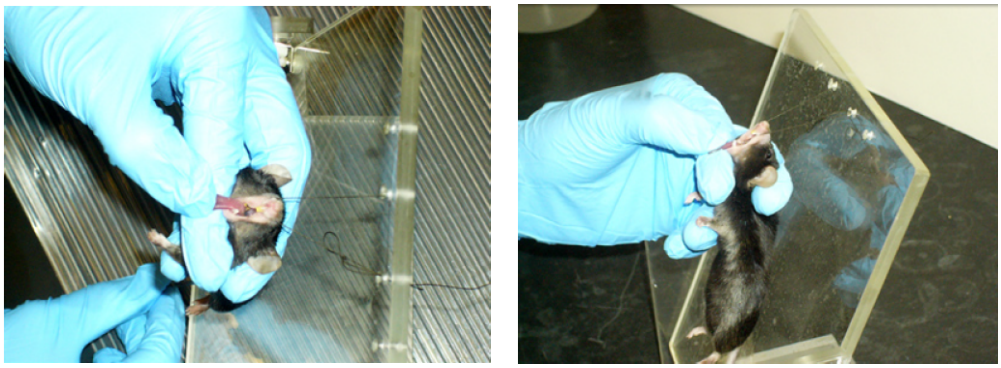

**Figure 2:** Anesthetized mice on the support stand being prepared for orotracheal intubation of agarose beads.

Or sterile PBS agar beads in case of control group, which were gently pushed into the mouse lung via tracheal route. Once intubated, the mouse was carefully removed from the support stand, and was injected intraperitoneally with the atipamezole (brand name antisedan) (1mg/kg). The mice were then placed on a heating pad, set at 38ºC, until a full recovery was observed. The mice were returned to their respective cages based on the beads intubated.

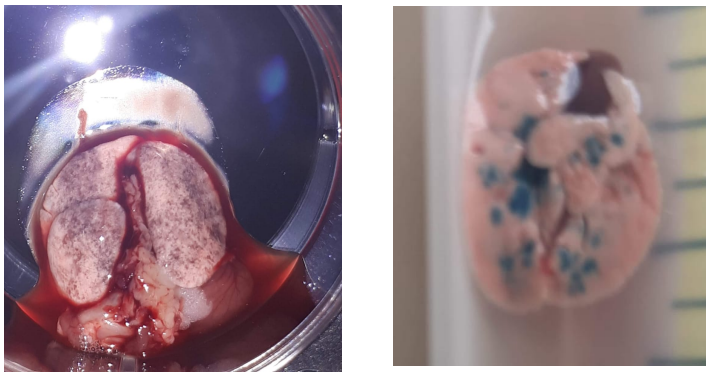

**Figure 3:** Lungs of mice intubated with Evans blue dye, to confirm the successful intubation.

**Mice evaluation**

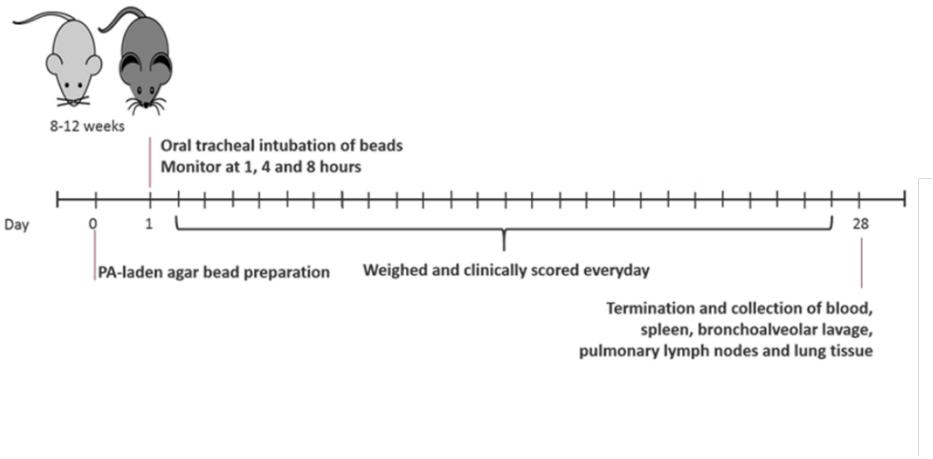


**Figure 4: Layout of the chronic lung infection model.**

The mice were observed for specific points listed below and scored according to the table defined in the authorized protocol. For the first three days following the challenge, the mice were observed and scored twice daily, and then once daily till the termination of experiment. The weight was monitored daily, and mice that lost ≥20% of their starting body weight were euthanized. The mice were observed for 28 days, and samples were acquired at specific time intervals for the purposes of optimization.

| **Observation/Points** | **0** | **1** | **2** | **3** |
|---|---|---|---|---|
| *Coat quality* | Normal | Decrease | Secretion from eyes and nose | Piloerection, kyphosis |
| *Weight* | Normal | <5% | Between 5% and 15% | >15% |
| *Natural behaviour/Posture* | Normal | Slight change | Slight reduction of movement, reduced alertness | Immobility, isolation |
| *Clinical symptoms* | Normal breathing | Slight change | Increase of respiratory frequency with mild abdominal breathing | Prominent abdominal breathing |

Table 1: Score sheet for mice evaluation

Evaluation:

- 0-4 : Daily supervision
- 5-6 : Close supervision (2x/Day)
- >7 : Euthanasia

**Measurement of bacterial load in BAL fluid and lung**

The selected mouse, from whom the sample collection is required, was euthanized by cervical dislocation. The mouse was placed on the dissection surface with the ventral side facing up. The coat was disinfected with 70% ethanol. With dissection tools, the abdominal cavity was opened, taking care that no damage was done to any visceral organs.

The opening of the trachea was located at the base of the mouse head, and exposed by removing muscles around the trachea. The middle of the trachea was then punctured with BD insyte-W 20G needle, and inserted about 0.5 cm in. The needle was carefully removed, leaving the catheter in. About 700 µl of sterile PBS was injected via the catheter slowly into the lung, and then the solution was aspirated after a few seconds. Care was taken that the catheter doesn't move in too much, as it might damage the lung structure. Normally, 300-700 µl of bronchoalveolar lavage (BAL) fluid was recovered and collected in a 15 ml falcon tube, at 4ºC. A portion (80% of the total volume) was centrifuged at 400g for 7 min, and the resultant cell pellet was stored at -80ºC for analysing the cellular influx in the lungs. If the cell pellet is red, a treatment of ACK (Ammonium-Chloride-Potassium) lysing buffer was applied to lyse the red blood cells, washed with medium, before storing.

Upon BAL fluid extraction, the lung was immediately cut out, rinsed with sterile PBS and the upper left lobe (superior lobe) was separated from the rest of the lung. This lobe was placed upon a 40 µm cell strainer (BD Falcon) positioned in a 50 ml falcon tube. About 1-2 ml culture medium was passed through the strainer, wetting its surface, before the lung was

crushed with the back of a syringe plunger. Another 2-3 ml of culture medium was poured to wash the strainer, collecting the cells in the falcon tube below.

The subsequent aliquoted BAL fluid and the cells obtained from superior lobe of the lung were used for quantification of the bacteria present. The sample can be serially diluted (1:10) as required and plated on a TSA plate, and then incubated overnight at 37ºC. The total bacterial load is the sum of CFUs found in BAL fluid and the lung.

**Histological examination**

We performed histological analysis on lungs from which BAL wasn't aspirated, as well as lungs from which BAL was aspirated.

The excised lungs (along with other visceral organs like spleen, stomach, liver as control) were immediately transferred to 10% formalin (4% formaldehyde), at 4ºC. The lungs were embedded in paraffin following standardized procedures and sliced at 5 µm thickness using a microtome. The slides were then stained with haematoxylin and eosin, and observed with an inverted brightfield microscope.

The samples were processed at Laboratoire National de Santé (LNS), by Prof. Dr Mittelbornn's group.

**Results**

The experiment was performed for a sum of 4 times, with varying concentration of PA RP73 bacteria, from 2-4 x $10^7$ CFU/ml. The beads measured between 100-200 µm. The first experiment was performed on TAP1-KO mice. PBS-agar beads were administered to control mice, while the PA RP73 laden agar beads were administered at a concentration of 4x$10^7$ CFU/ml to the test group (Fig. 5). At 3 days post-infection, 1 of the mice was found deceased and in rigor mortis, 2 were sacrificed as the weight lost was ≥20% of the starting weight (Fig.5). The remaining 2 mice scored low on the observation chart and survived till

the end of the experiment. The control mice showed no signs of infection and continued weight gain as normal throughout the duration of the experiment. At 28 days post-infection, the experiment was terminated, and organs were collected. Crushed lung and stomach extracts were serially diluted and plated on TSA plates, to evaluate bacterial growth. No bacterial colony was recorded on lung-TSA plates, indicating absence of bacteria. Stomach-TSA plates showed CFUs as expected. Another read-out was lung histopathology (Fig.6).

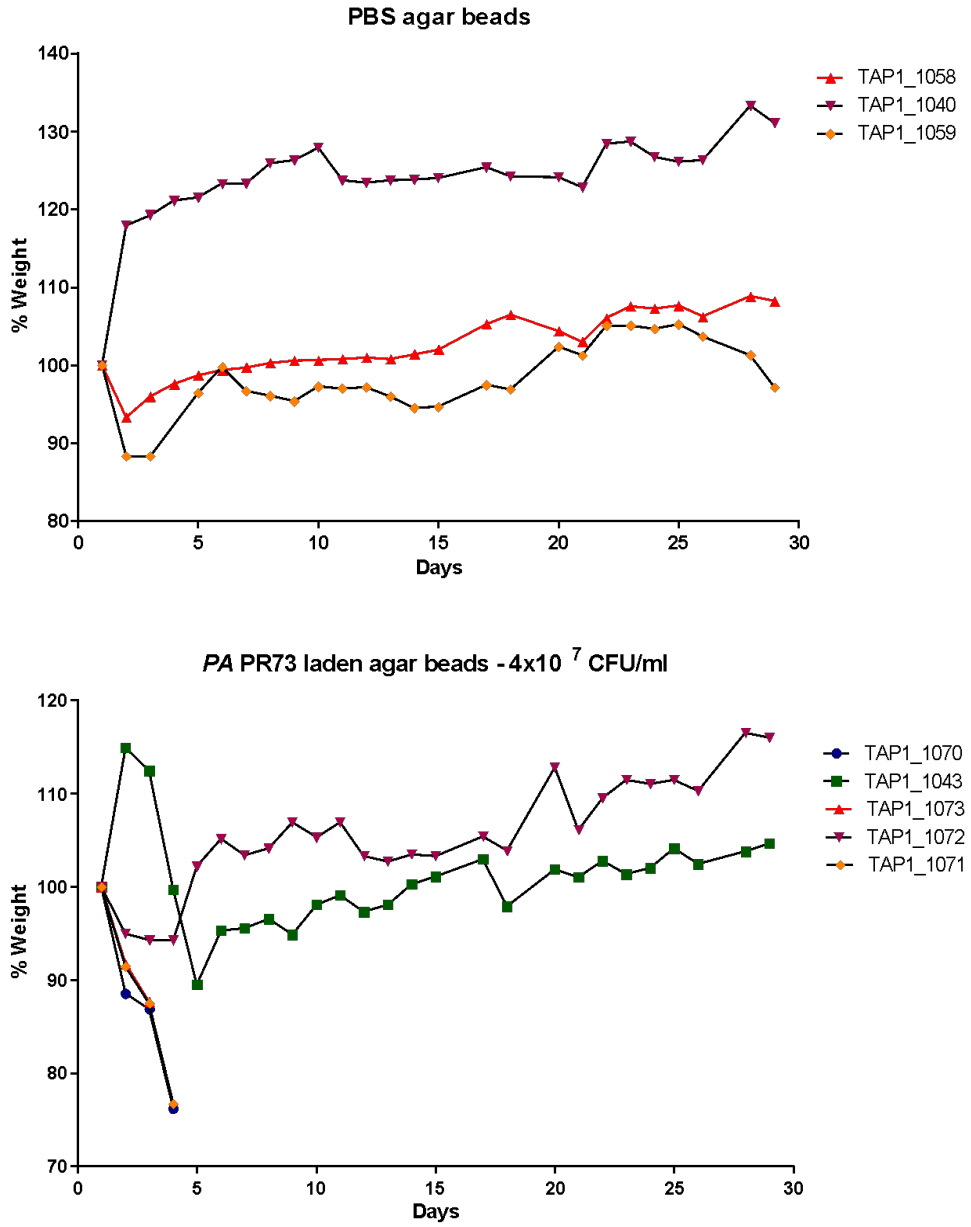

**Figure 5:** Graphs representing the daily weight measurement for B6 mice intubated with PBS-agar beads and *PA*-laden agar beads.

Lung tissue samples were stained with haematoxylin and eosin. Lung tissue from the control mice group showed typical histology with intact alveolar structures (Fig.6a). Lung tissue image from mice sacrificed day 3 post-infection (20% weight loss upon PA RP73 infection) showed collapsed alveolar structure, corresponding to difficulty in breathing in these mice (Fig.6b) while those from mice 28 days post-infection showing recovery in the alveolar structure indicating clearance of the infection over the course of the study.

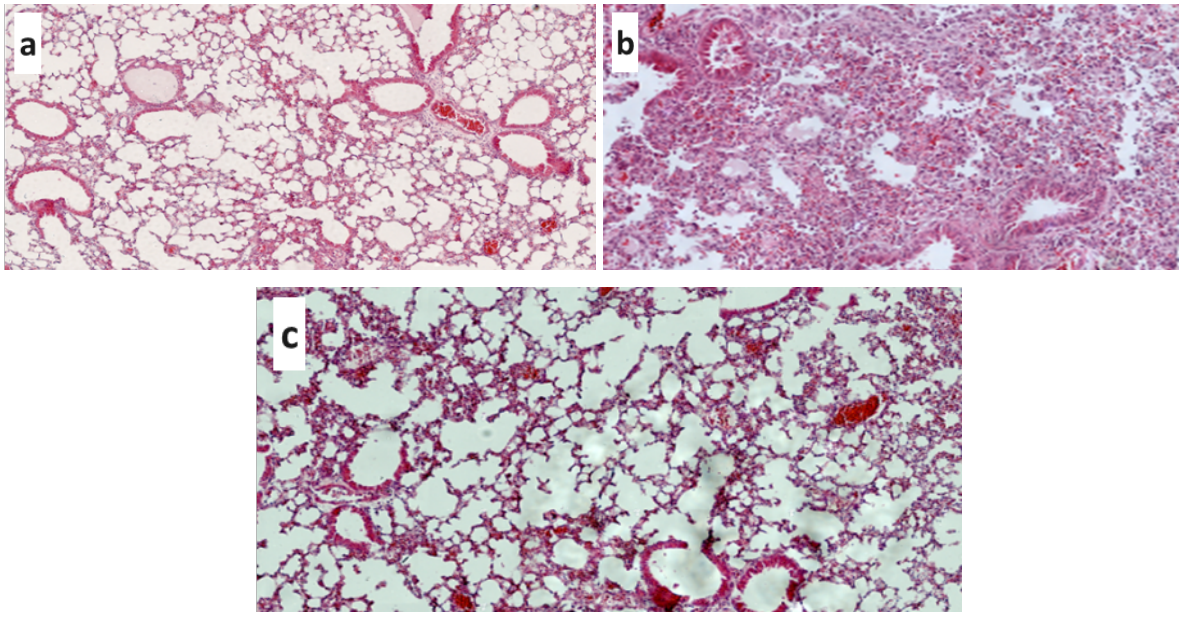


**Figure 6:** Histological analyses of lungs from (a) control mice (b) mice sacrificed day 3 post-infection and (c) mice sacrificed 28 days post-infection.

The PA RP73 concentration of $4x10^7$ CFU/ml caused a mortality of 60% in infected mice. This concentration was reduced to $2x10^7$ CFU/ml ($1x10^6$ in 50 µl) and performed on mice with CAST/EiJ background, enriched in mucosal-associated invariant T (MAIT) cells. 15 mice were intubated with PA RP73 laden-agar beads, 2 of which died during the intubation procedure. All the mice showed normal weight gain (Fig. 7).

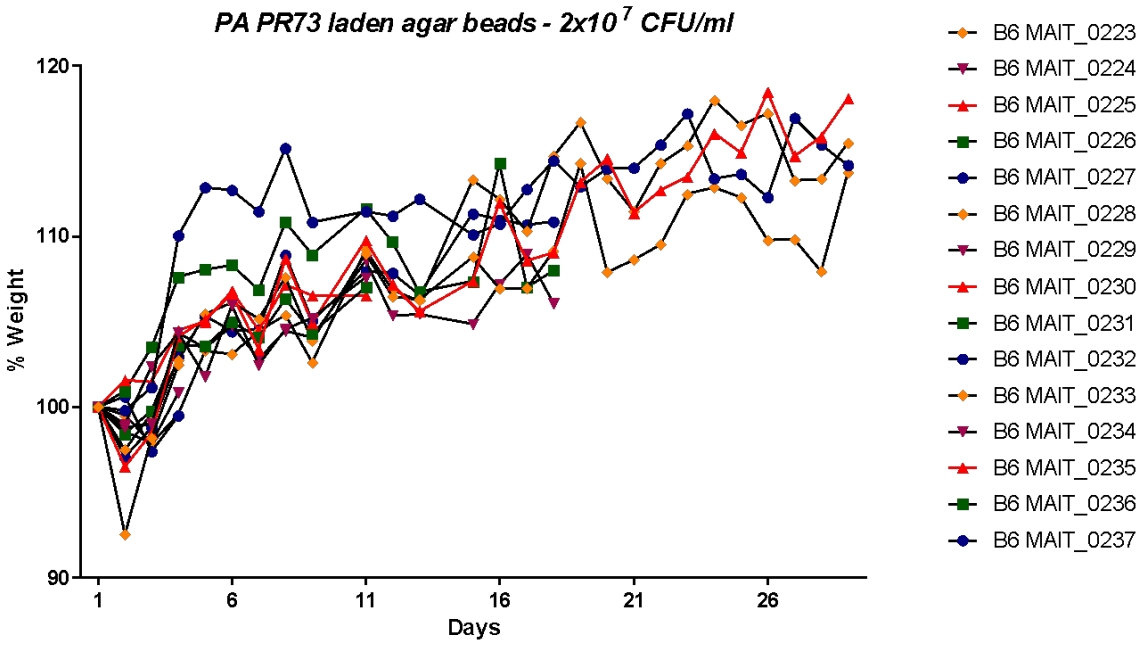


**Figure 7:** Graph for daily weight measurement for B6 MAIT mice intubated with *PA RP73*-laden agar beads over the course of 28 days.

To understand the infection progression, 3 mice were sacrificed each at day 3, 10, 18 post-infection, lung and BALF was collected and plated on TSA plates. The lung and BALF-TSA plates from day 3 post-infection mice all presented bacterial growth, with BALF plate showing significant CFUs, over lung plates. This number dropped in day 10 post-infection cultures to an absence of colonies in day 18 and day 29 post-infection culture plates, indicating the clearance of infection from the mice. (Table 2) Chronic infection could not be established.

Table 2:

| ID | Day Post-infection | CFU/lung | CFU/lung |
|---|---|---|---|
| B6.MAIT_0223 | Day 3 | $9.4x10^2$ | $7\ x10^2$ |
| B6.MAIT_0232 | | $6.8\ x10^2$ | $1\ x10^2$ |
| B6.MAIT_0234 | | $6.1\ x10^2$ | $5\ x10^2$ |
| B6.MAIT_0224 | Day 10 | $0.6\ x10^2$ | 0 |
| B6.MAIT_0226 | | 0 | 0 |
| B6.MAIT_0230 | | $0.5\ x10^2$ | $26\ x10^2$ |
| B6.MAIT_0229 | Day 18 | 0 | 0 |
| B6.MAIT_0231 | | 0 | 0 |
| B6.MAIT_0237 | | 0 | Uncountable |
| B6.MAIT_0225 | Day 29 | 0 | 0 |
| B6.MAIT_0227 | | 0 | 0 |
| B6.MAIT_0228 | | 0 | 0 |
| B6.MAIT_0233 | | 0 | 0 |

After observing the clearance of infection at day 10 post-infection, we decided to re-intubate the mice with PA RP73-laden agar beads on day 7 post-infection, to boost the initial bacterial load.

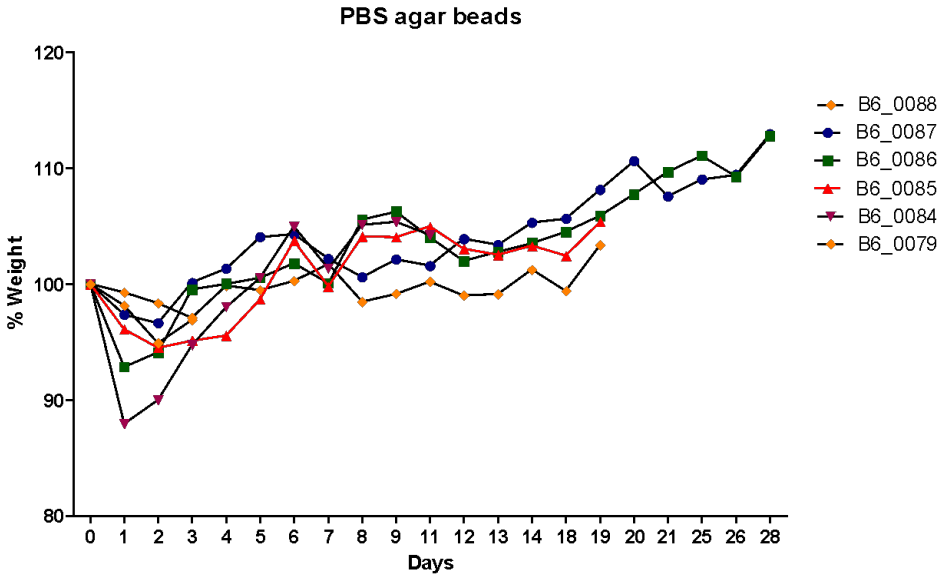


**Figure 8:** Daily weight measurement of B6 mice intubated with PBS agar beads.

20 WT C57BL/6 mice were selected, 6 of whom received sterile PBS agar bead intubation, and 14 received PA RP73-laden agar beads. The mice in this case too however, continued to gain weight in the duration of the experiment, with no significant symptom of distress or infection (Fig. 8, 9).

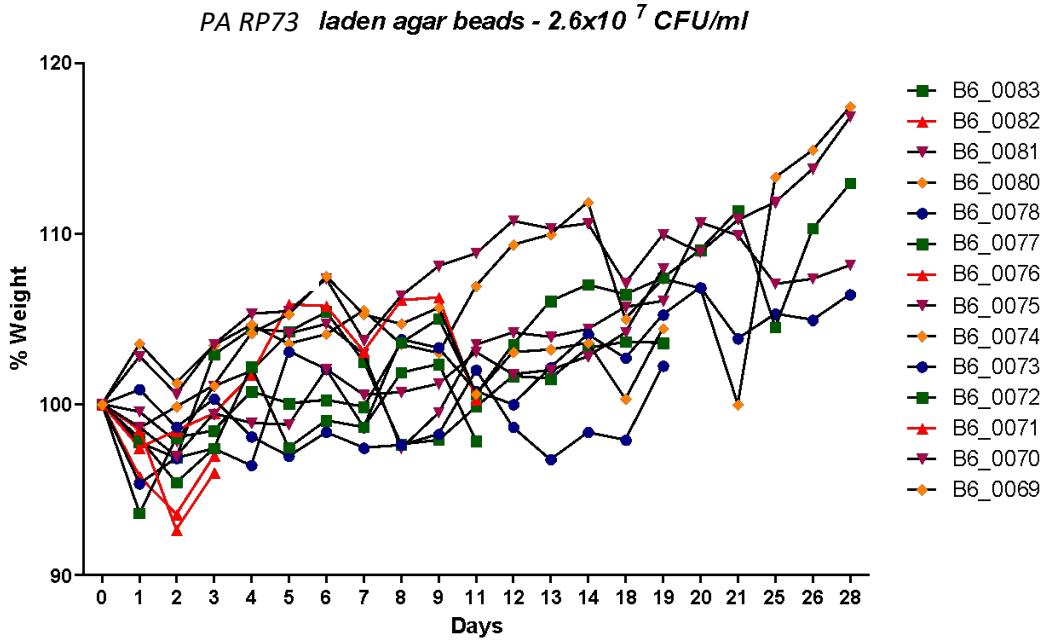


**Figure 9:** Daily weight measurements for mice intubated with *PA RP73*-laden agar beads.

Lung and BALF extracts were plated on TSA plates per usual. Re-intubation didn't ensure the establishment of chronic infection (Table 3a).

Table 3a:

| Mice ID | Treatment | Intubation date | | Colonies | |
|---|---|---|---|---|---|
| | | First | Second | BALF | Lung |
| B6_0069 | PA RP73 intubation | 12/06/2020 | | | |
| B6_0070 | PA RP73 intubation | 12/06/2020 | | No | No |
| B6_0071 | PA RP73 intubation | 12/06/2020 | | No | Yes |
| B6_0072 | PA RP73 intubation | 12/06/2020 | | Yes | No |
| B6_0073 | PA RP73 intubation | 12/06/2020 | | No | Yes |
| B6_0074 | PA RP73 intubation | 12/06/2020 | 19/06/2020 | Yes | Yes |
| B6_0075 | PA RP73 intubation | 12/06/2020 | | Yes | Yes |
| B6_0076 | PA RP73 intubation | 12/06/2020 | | Yes | No |
| B6_0077 | PA RP73 intubation | 12/06/2020 | | No | Yes |
| B6_0078 | PA RP73 intubation | 12/06/2020 | | Yes | Yes |

| | | | | | |
|---|---|---|---|---|---|
| B6_0080 | PA RP73 intubation | 12/06/2020 | 19/06/2020 | No | Yes |
| B6_0081 | PA RP73 intubation | 12/06/2020 | 19/06/2020 | No | No |
| B6_0082 | PA RP73 intubation | 12/06/2020 | | No | Yes |
| B6_0083 | PA RP73 intubation | 12/06/2020 | 19/06/2020 | No | Yes |

On the other hand, mice intubated twice with sterile PBS beads showed bacterial growth on BALF-TSA plates (Table 3b), indicating secondary infection from repeated oral intubation.

Table 3b:

| **Mice ID** | **Treatment** | **Intubation date** | | **Colonies** | |
|---|---|---|---|---|---|
| | | **First** | **Second** | **BALF** | **Lung** |
| B6_0079 | PBS beads intubation | 12/06/2020 | | No | No |
| B6_0084 | PBS beads intubation | 12/06/2020 | | No | No |
| B6_0085 | PBS beads intubation | 12/06/2020 | | No | No |
| B6_0086 | PBS beads intubation | 12/06/2020 | | - | No |
| B6_0087 | PBS beads intubation | 12/06/2020 | 19/06/2020 | No | Yes |
| B6_0088 | PBS beads intubation | 12/06/2020 | 19/06/2020 | No | Yes |

To better understand the lung histology, it was decided to intubate WT and TAP KO mice with PBS and PA RP73-laden agar beads, and directly proceed for tissue processing without collecting BALF. This ensures the preservation of lung aspect and properties.

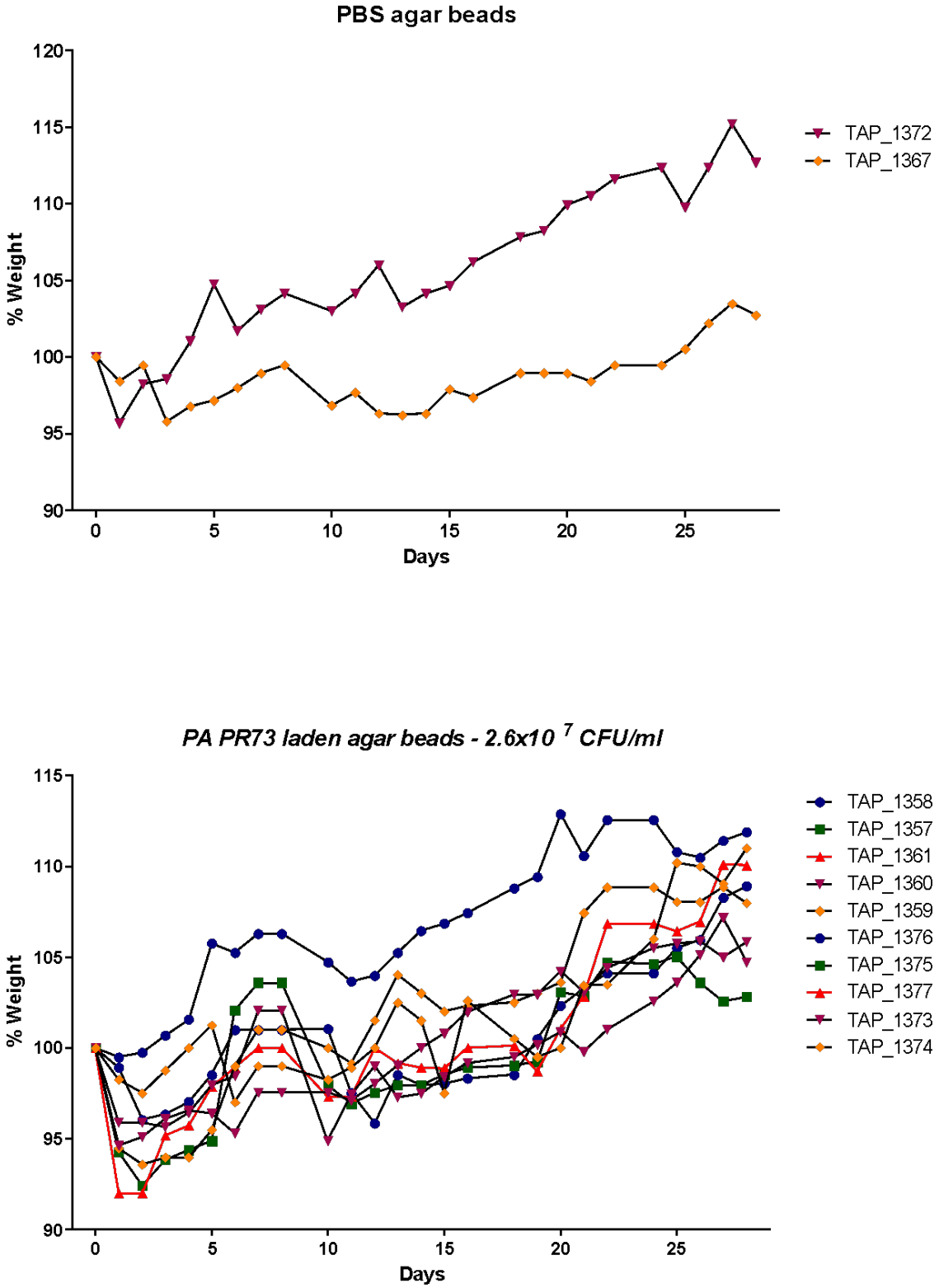


**Figure 10:** Daily weight measurement for mice intubated with PBS agar beads and *PA RP73*-laden agar beads.

Two mice were intubated with PBS agar beads, and 10 were intubated with PA RP73-laden agar beads, at a concentration of $2.6x10^7$ CFU/ml. Two of the infected mice didn't recover after the intubation procedure and had to be sacrificed. No re-intubation was made. The mice gained weight as normal, and there was no notable difference between WT and TAP KO.

The samples were stored in 10% formalin and transported to LNS for processing. In all the cases but one, there was some degree of alveolar oedema with reactivity of the pneumocytes and an increased number of alveolar macrophages. There was no sign of broncho-pneumonia or abscess formation. LNS findings noted acute lung injury but saw no signs of infection or presence of bacteria. There was no establishment of chronic lung infection.

**Discussion and future direction**

As part of their biology, lungs are faced with a constant barrage of various particles, toxic and non-toxic substances, infectious and non-infectious microbes, through the process of respiration (Speert 2006). However, these rarely cause infection. Lung has physical defences such as the 'mucociliary escalator', resident immune cells such as macrophages, phagocytic cells, cells of innate and adaptive immunity (Alonso 2008). However, in immunocompromised patients, such as those suffering from cystic fibrosis or COPD, bacterial infections are commonplace (Kukavica-Ibrulj and Levesque 2008). These recurrent infections cause lung inflammation and injury, leading to a lower quality of life.

To improve our understanding and to solve the mechanisms of these chronic infections, it is pertinent to come up with models that can effectively demonstrate the course

of infection and the subsequent response. The PA bead model is widely known for pulmonary studies, especially at earlier phases of infection and inflammation. Successful attempts to focus on using the model to mimic long term infections were made by the Bragonzi group and the Evans group (Facchini, De Fino et al. 2014, Bayes, Ritchie et al. 2016). Both groups noted the host's ability in clearing the bacterial cells in the weeks following the infection and opted for intratracheal instillation of the PA-laden agar beads. Both groups worked with PA strains obtained from clinical samples, *P. aeruginosa* RP73 (clinical strain from CF isolates) and *P. aeruginosa* strain NH57388A (derived from sputum of a CF patient), respectively and both groups reported a low mortality and persistent infection.

Whereas we opted for the non-invasive orotracheal intubation in our protocol. Our earlier attempts at learning the protocol involved intubation with the Evans blue dye to appreciate the visual confirmation of successful procedure, as shown in figure 3. However, it is possible that the orotracheal intubation of the agar beads lead to its lodging in the upper airway, or the swallowing of the beads by mice. This would in turn lead to clearance of bacterial load over the time.

Despite our numerous attempts at standardizing the protocol, we were not able to mount a chronic bacterial lung infection. The main challenge we faced was maintaining the bacterial burden over the duration of the protocol, especially after 7 days post-infection. One possible alteration could be re-intubation between 7-10 days post-infection. Future attempts must be made to either change the method of intubation, for e.g., intratracheal surgery, or to revise the concentration at which the beads are intubated. Sensitive methods such as PA-specific IgM and IgG ELISA detection could be used to quantify the presence of bacteria, along with the Luria-Bertini (LB) agar cultures.

Alternatively, an attempt must be made to establish an *in vitro* model of chronic bacterial infection, to understand the role NK cells play in resolving them. For example, the Wilmes group at the university of Luxembourg built a 'Human-Microbial X(cross)-talk' or

HuMiX model for studying gut microbiome (Shah, Fritz et al. 2016). This 'organ-on-a-chip' model studies the interaction between the microbes and the human host, to better understand how the change in gut microbiome could cause diseases. Similar model should be established for mimicking the lung microenvironment, opening the avenue to then infect the said model with bacteria. We could also test the effect of antibiotics/cytokines in this model and further our understanding of lung infections.

**Conflict of Interest:**

The authors all declare that they have no conflict of interest.

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**Chapter 4: Imaging Natural Killer cells and *Pseudomonas aeruginosa* interactions**

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**My contributions to the chapter:**

- Design and execution of the experiments, analyses of the data
- Literature search and writing

**Abstract**

Natural killer cells have been established to take part in anti-microbial defences of the immune system. NK cells can directly detect the presence of bacteria in their environment with the help of pathogen-associated molecular patterns (PAMPs). Upon recognition, NK cells are stimulated to produce molecules such as the cytokine IFNγ, well known for its role against bacteria. Traditionally, NK cell interactions with their targets have been studied using functional assays or via flow cytometry. In this study, we devised a way to image the interaction via an imaging flow cytometer. We co-cultured human NK cells with *E. coli* or with *P. aeruginosa* and then stained them for surface markers. These interactions were validated with the Quanteon flow cytometer. We want to highlight the use of an imaging flow cytometer as a unique way to image the dynamic interaction between an immune cell and bacteria, and to assess the functional capacities of NK cells.

## Introduction

Mounting an effective immune response is key to prevent severe infections. Human body plays host to commensal microbes, and differentiating between these microbes and the pathogens is crucial in order to avoid persistent immune reaction and inflammation (Sommer and Backhed 2013). Toll-like receptors (TLRs) and Nod-like receptors, C-type lectin receptors (CLRs), and retinoic acid-inducible gene-I-like receptors (RLRs) are all part of the host's pattern-recognition receptor (PRR) machinery. They recognise the danger-/microbe-associated molecular patterns (DAMP/MAMP) such as lipopolysaccharide (LPS), virulence factors, nucleic acids, peptidoglycan etc presented by the pathogens as well as the commensal microbes (Rakoff-Nahoum, Paglino et al. 2004), which in turn activate several downstream signalling pathways.

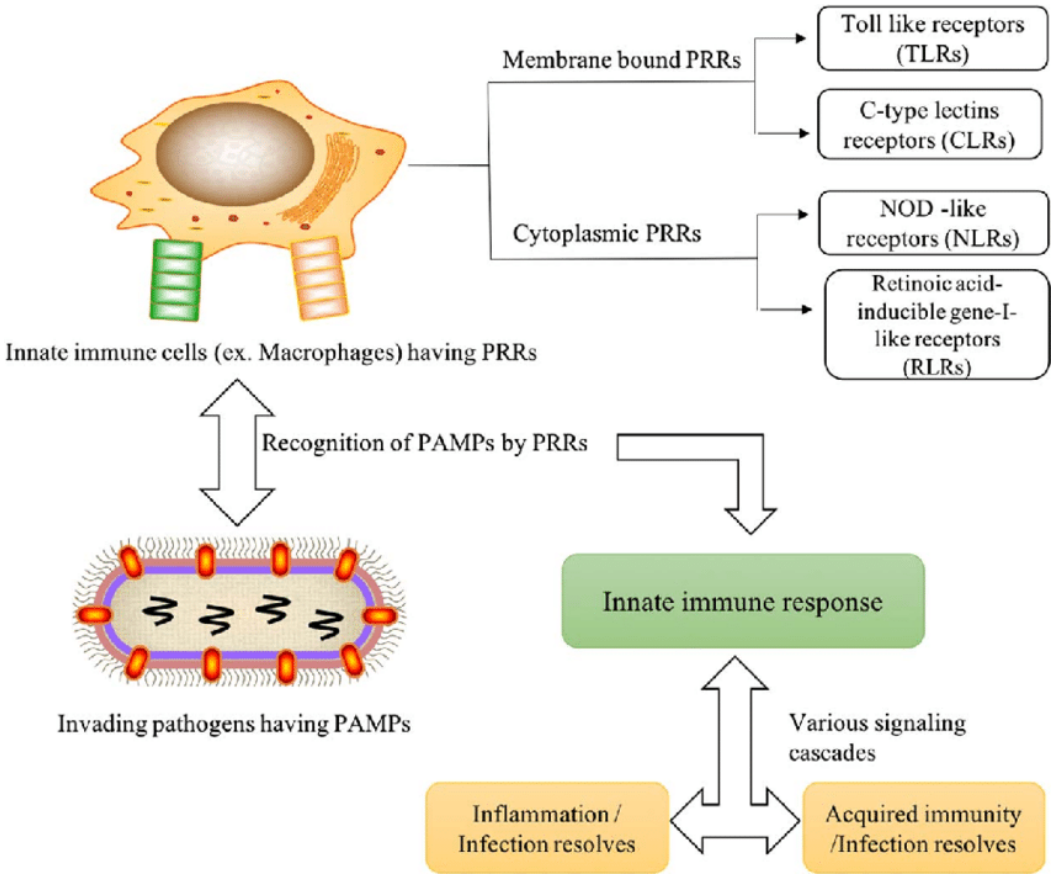


Figure 1: Interaction between the pattern-recognition receptors on the immune cells with the pathogen-associated molecular patterns on the invading pathogens (Kaur, Patiyal et al. 2019)

TLRs were the earliest recognised PRRs and are part of the host inflammatory response (Fitzgerald and Kagan 2020). Emerging evidence points towards the involvement of the NLRs in the recognition of the presence of the extracellular bacteria, contrary to the earlier belief that only TLRs participated. CLRs, in presence of the $Ca^{+}$ recognise the carbohydrates present on the surface of the microbes and belong the phagocytic class of PRRs (Ebner, Sharon et al. 2003). TLRs, NLRs etc engaging with their respective PAMPs results in activation of various immune processes including the recruitment of antimicrobial mediators (Martinon and Tschopp 2005, Kapetanovic and Cavaillon 2007).

The discovery of the of TLRs on the natural killer (NK) cells and their interaction with PAMPs points out the role of innate immune cells in anti-bacterial defences (Noh, Yoon et al. 2020). They are known to activate NK cell functions such as cytotoxicity and cytokine production which in turn slows down bacterial growth and fights infection (Lauzon, Mian et al. 2006). It has taken the scientific community some convincing to recognise the role of NK cells in bacterial infections, starting from the evidence that NK cells could lyse HeLa cells infected with *Shigella flexneri* (Klimpel, Niesel et al. 1986), which was then strengthened by the *in vivo* activity of NK cells in *M.avium* infection in mice (Harshan and Gangadharam 1991). The mechanisms are still poorly understood, and we have long ways to go before we can firmly establish their exact role.

In this chapter, our focus is mainly in establishing the physical interaction between NK cells and *Pseudomonas* aeruginosa. First described and isolated in 1882 by the French pharmacist Carle Gessard (1984), *Pseudomonas aeruginosa* are highly prevalent gram-negative, rod-shaped bacterium. Ranging from 1–5 μm long and 0.5–1.0 μm wide, they are

facultative aerobes and grow well at 37 °C but can persist at temperatures between 4–42 °C (Diggle and Whiteley 2020). *P. aeruginosa* rarely infect healthy individuals but are a major nuisance to immunocompromised individuals thus earning the name 'opportunistic' pathogen. They manifest as chronic airway infections in patients suffering from chronic obstructive pulmonary disease (COPD) or cystic fibrosis (CF) (Murphy 2009, Johnson 2019).

*P. aeruginosa* shows a high resistance to various antimicrobial agents, obtained through means such as production of antibiotic-inactivating enzymes, decreased outer membrane permeability, multidrug resistance pump expression. They also acquire antibiotic resistance by mutational changes, or by the gaining of resistance genes from same or different bacterial species via horizontal gene transfer (Cox and Wright 2013, Hall, Hinz et al. 2018, Pang, Raudonis et al. 2019) . In addition to this, they also form biofilms, aiding the attachment of *P. aeruginosa* on the surface, altering the microenvironment (Rasamiravaka, Labtani et al. 2015). This has led to major concerns for global public health and the need to develop a new class of antibiotics to treat the infections.

In this chapter, we look at a non-traditional way to image and quantify the interactions between *PA* and the human NK cell line.

## Material and Methods

### Bacteria

*Pseudomonas aeruginosa* O11 (ATCC 33358, glycerol stocks stored at -80°C) was grown on Trypticase Soy Agar (TSA), overnight at 26°C. Individual colonies were selected using sterile pipette tip and dropped in 10 ml Trypticase Soy Broth (TSB) and incubated at 26°C overnight in a shaking incubator. *Pseudomonas aeruginosa* RP73 and *Escherichia coli* (E. coli), was used for imaging experiments and was grown similarly at 37°C.

### Imaging flow cytometer and Quanteon

The human NK-92 MI (IL-2 independent) NK Cell line was used along with PA RP73 to image the interaction between NK cells and bacteria. The overnight culture of PA RP73 was pelleted down at 2000rpm, 10 minutes and washed twice in PBS. The pellet was resuspended in 100ul PBS and was then stained with 1uL PKH26/PKH67 and 40ul Diluent C. This mix was gently pipetted for 30s while PKH26/PKH67 incorporates into the lipid bilayers of the bacteria, for 3 min at room temperature. This mix was quenched by adding 2mL 10% BSA in PBS and the volume was brought up to 8.5mL in serum-free media. Simultaneously, NK-92 MI was stained with intracellular granzyme B or surface marker CD56 using the protocol mentioned below. The stained NK-92 MI cells and the stained bacteria were co-cultured for 15 minutes or 30 minutes in a 96 well plate. After the incubation, 2µl DAPI stain (diluted at 1:1000) was added to each sample, incubated for 5 minutes and proceeded for fixation using the BD Cytofix solution for 30 min at 4°C. The samples were washed and suspended in 50µl FACS buffer for further analysis. The acquisition was done on Imagestream$^X$ Mk II imaging flow cytometer and the Novocyte Quanteon.

**Results:**

1) Imaging the interaction between bacteria and NK cells

Traditional flow cytometer analyses of bacteria are complicated due to the size of the bacteria, the background noise, and the inability to discriminate between individual bacteria. We used the imaging cytometer, Amnis ImageStream$^X$ Mk II (IS$^X$) to study the interaction between the bacteria PA RP 73/E.coli and NK-92 MI. The data was analysed using the software IDEAS™, which allows for single cell analyses. For E. coli co-cultured with the granzyme B-stained NK-92 MI cells, we observe the co-localization (marked in figure 1 by yellow arrows) of the bacteria alongside the NK cell line. In some cases, the polarization of granzyme B at the contact point of E. coli was also noted.

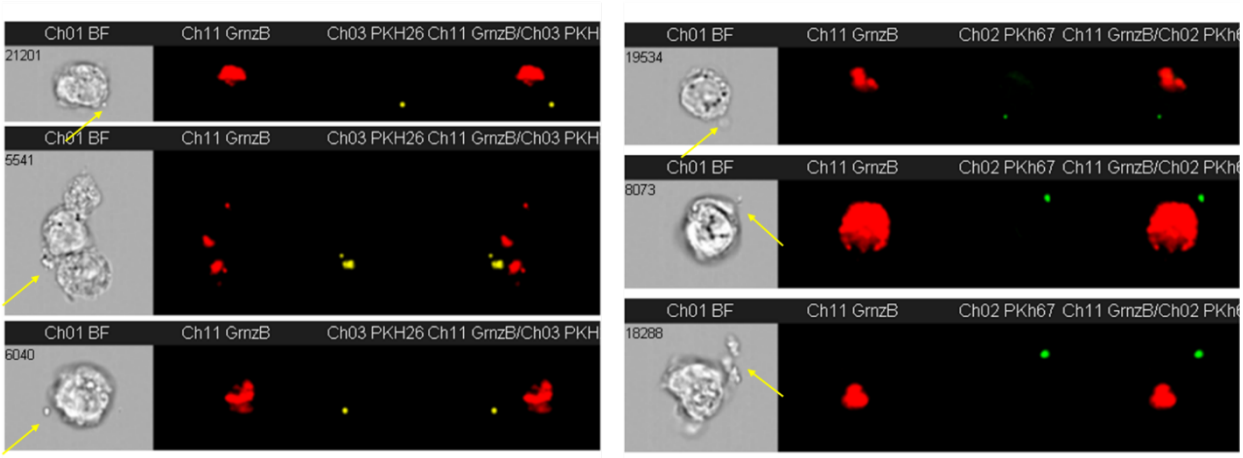
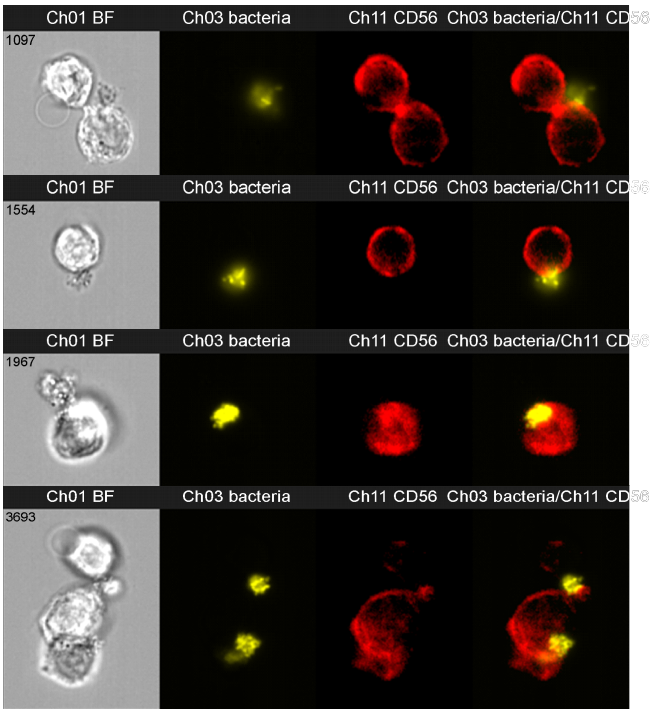



**Figure 1:** *E.coli* stained with PKH26/PKH67 (illustrated in channel 3) in the yellow or green colour respectively. The yellow arrow points at the location of the bacteria with respect to the NK-92 MI cell, and the granzyme B staining is visible in channel 11 as red.

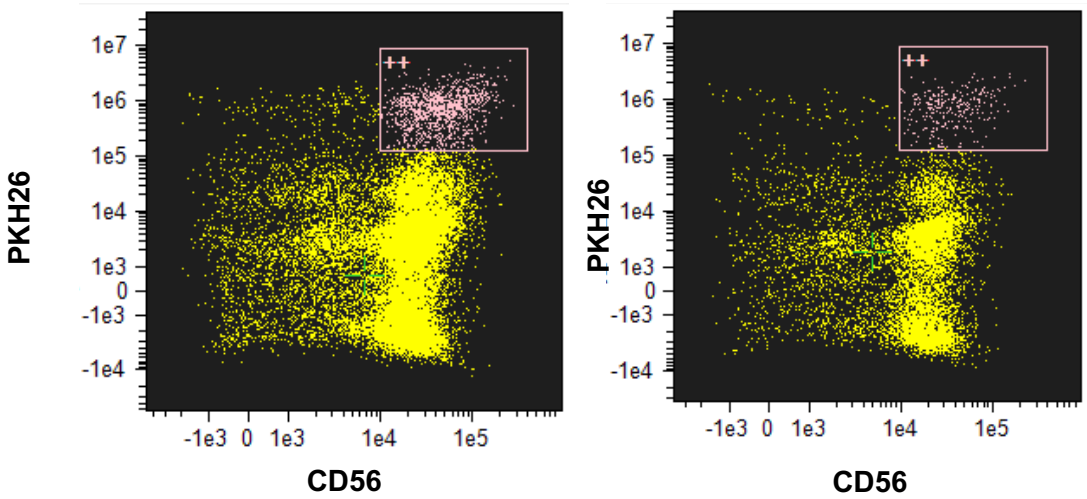
Similarly, *PA* RP73 was stained with the PKH26 dye and incubated with NK-92 MI cells. To better visualize the NK cells, they were stained with the surface marker CD56, seen in IDEAS in channel 11. Surprisingly, in the 15 minutes incubation period, more

associations, up to 4.87% were noted than that in the 20 minutes of co-culture, at 3.16%. This fall could be explained by the dynamic interaction between the two cells, however further experiments are necessary.

**A**




**B** **15 mins of incubation vs 30 mins of incubation**




| Population | Count | %Total | %Gated |
|---|---|---|---|
| cells & focus | 23552 | 93,1 | 100 |
| ++ & cells & focus | 1147 | 4,54 | 4,87 |

| Population | Count | %Total | %Gated |
|---|---|---|---|
| cells & focus | 8431 | 92,8 | 100 |
| ++ & cells & focus | 266 | 2,93 | 3,16 |

**Figure 2:** (A) *PA* RP73 stained with PKH26 dye as seen in channel 3 in the yellow colour. NK-92 MI cells are stained with CD56 and are visible in channel 11 as red. (B) Representative dot plots of the distribution of the cells at 15 minutes and 30 minutes of culture. The CD56+PKH26+ cells represent the population where the bacteria are co-localised with the NK cells.

2) Quantification of the interaction between bacteria and NK cells

To validate our findings from IS$^X$, we looked at the Novocyte quanteon, a flow cytometer that allowed us to differentiate between the bacterial events and provide statistical output. The initial setup was performed on *E. coli* stained with PKH26 and PKH67, incubated with NK-92 MI for 30 minutes. The PKH26 stained *E. coli* showed a higher percentage of association with the NK cells compared to the PKH67 stained *E. coli*. For this reason, further experiments were conducted with PKH26 stained bacteria.

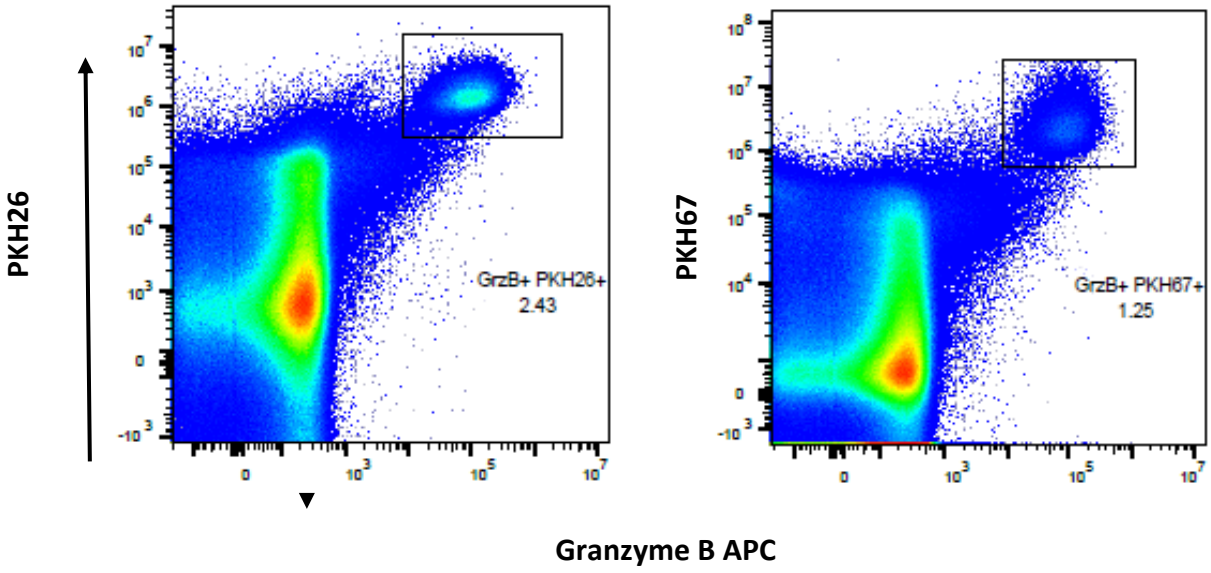



**Figure 3:** *E.coli* stained with either PKH26 or PKH67 co-cultured with granzyme B stained NK cells

The PKH26 stained *PA* RP73 bacteria was co-cultured with the NK-92 MI cell line for 15 minutes and 30 minutes. The findings were consistent with what was observed in case of $IS^X$ experiment. We observed almost twice the PKH26+CD56+ population in case of 15 minutes of incubation (6.29%) than in the case of 30 minutes of co-culture (3.99%).

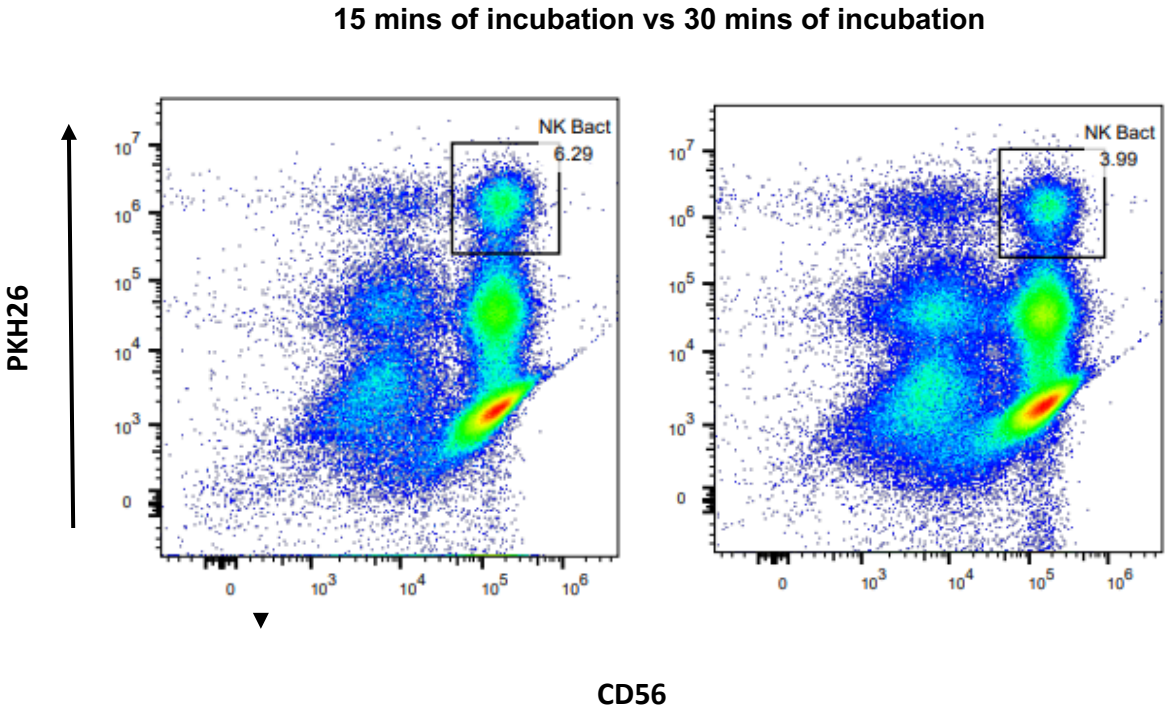



**Figure 4:** *PA RP73* stained with either PKH26 Co-cultured with CD56 stained NK cells at either 15 minutes or 30 minutes.

Surprisingly, we observed three distinct stages of cell population depending on the PKH26 expression levels. While further experiments are necessary to validate this, we assume that this might correspond to the various levels at which the *PA* RP73 bacteria bound to the NK92 MI cells.

**Discussion and future direction:**

*Pseudomonas aeruginosa* is an opportunistic pathogen that often makes home in individuals with compromised immune system, especially in the soft tissues, such as the eyes and the ears along with the respiratory tract and the lungs. (Gellatly and Hancock 2013, Faure, Kwong et al. 2018). *P. aeruginosa* is known cause for chronic infections in cystic fibrosis, with bacterial persistence ranging up to several decades (Stoltz, Meyerholz et al. 2015). Several factors influence this such as the bacterial flagellin and its motility, protease secretions, extracellular polysaccharides, lipopolysaccharides (Faure, Kwong et al. 2018). In addition, *P. aeruginosa* also form multicellular biofilms that can suppress the immune system, and cause tissue damage over time (Jensen, Bjarnsholt et al. 2007, Boisvert, Cheng et al. 2016).

Natural killer cells form a crucial part of the innate immune system, the host's first line of defence. NK cells can be activated by the pathogen associated molecular patterns, however it has to be complemented by the participation of other cytokine producing immune cells (Beutler, Eidenschenk et al. 2007). Various TLRs have been reported on both human (TLR2, TLR3, TLR5, TLR6) (Hornung, Rothenfusser et al. 2002) and murine (TLR2, TL4, TLR8, TLR9) (Martinez, Huang et al. 2010) NK cells. Given that NK cells are heterogenous, population from different NK cells would express various TLRs differently. Similarly, NLRs and RIG-I-like receptors are other pattern recognition receptors expressed by the NK cells and are involved in the activation. The earlier mentioned flagellin interacts with the TLR5 and the NLR-neuronal apoptosis-inhibitory protein (NAIP) (Hayashi, Smith et al. 2001, Zhao, Yang et al. 2011).

The aim of this chapter was to elucidate the mechanisms of the NK cell-bacterial interactions. Previous studies with NK cells were done with traditional methods such as flow cytometer, functional assays with bioluminescent assay kits, or with confocal microscopy. All these methods work well. However confocal microscopy is usually a time-consuming method, with several constraints such as the kind of experiments, co-culture studies that

could be done. Similarly, traditional flow cytometry gives exceptional information on the functional status of NK cell, however the dimension lacking is the inability to find specific location of the markers present on the cell surface. With an imaging flow cytometer, we were provided with an incredible flexibility with the experiment setup, along with being able to locate the exact location of the interaction between human NK cells and *E. coli* and *P. aeruginosa*. To this end, we stained the human NK cells and the bacteria, which were then identified based on bright field morphology, the bacteria were gated based on their smaller size and low side scatter. Recently, this method has demonstrated the interactions between *P. aeruginosa* and the *Acanthamoeba polyphaga* (Dey, Rieger et al. 2019).

Our earlier attempt of staining NK cells with Granzyme B was due to our belief that NK cells could kill bacteria by releasing the cytotoxic granule which was also recently demonstrated by Mody et al (Feehan, Jamil et al. 2022). This secretion of cytotoxic granules, i.e., granzymes and perforins happens in a polarized fashion at the site of contact between NK cells and the pathogen (de Saint Basile, Menasche et al. 2010). In our experiments, we observed the localization of granzyme B where *E. coli* contact with NK cells was established. However, to clearly define the NK cells-bacteria boundary, we opted staining the former with the surface marker CD56. We were able to successfully identify this interaction as well. We observed lower interactions at higher co-culture incubation timings and our preliminary hypothesis is that the synapses formed at the site of contact are highly dynamic in nature and need to be tightly regulated in both spatial and temporal manner.

Further optimisations in experiments could be done to add more markers, such as adding markers perforin, other granzymes like granzyme A, granzyme H to understand the polarization of these molecules. Longer incubation times need to be done to visualize the lysis of the bacteria or alternatively, the phagocytosis-induced apoptosis of NK cells by *P. aeruginosa* as reported by Choi et al (Chung, Piao et al. 2009). This method of imaging the interaction provides a way for rapid analysis and could be adapted for various biological investigations. Additionally, this experiment can be extended to transmission electron

microscopy (TEM), as we originally intended, to identify other key receptors involved in direct recognition of bacteria by NK cells, such as the cytotoxicity receptor NKp44 in case of *Mycobacterium bovis* bacillus Calmette-Guerin (Esin, Batoni et al. 2008).

**Conflict of Interest:**

The authors all declare that they have no conflict of interest.

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# Chapter 6: General Discussion and Outlook

**Contribution to the chapter:**

Literature review and writing

## General Discussion and Outlook

Over the past few years, remarkable research in the field of natural killer cell biology is happening all over the world. Their involvement in host immune response towards pathogens, cancer immunosurveillance, transplantation, pregnancy has solidified their position in the immune system. This thesis is an attempt to further our understanding in how NK cells regulate these functions, how they maintain tolerance to self and their responses in anti-bacterial defence.

In chapter 1, we talked about the various components that are part of the innate immune system and their function. We go through the vast literature available on NK cells in an attempt to understand their origin, development, and education. NK cells belong to the innate lymphoid cell (ILC) family and form between 5-20% of human circulating lymphocytes (Perera Molligoda Arachchige 2021). Human NK cells are defined by the lack of CD3 surface expression and by the presence of CD56. CD3−CD56bright CD16− cells and CD3−CD56dim CD16+ cells are two major subsets, and the latte is functionally more mature. In mice, NK cells are defined as CD3-NK1.1+ or CD3-NKp46+.

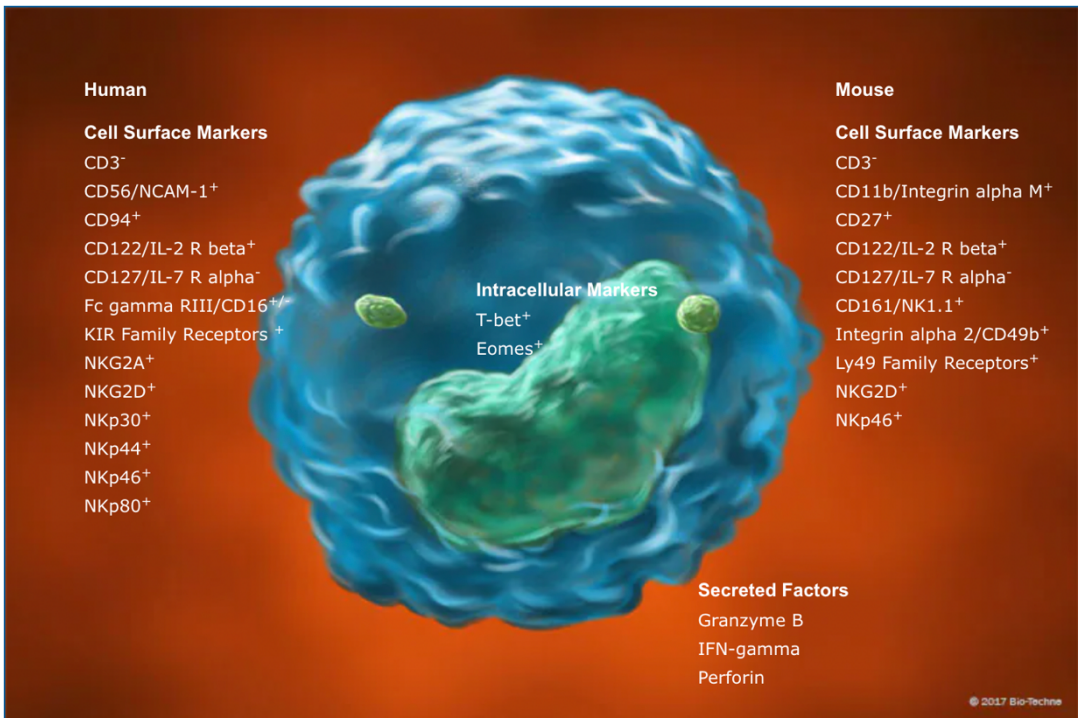


**Figure 1:** Representation of human and mouse NK cell markers (Source: biotechne)

NK cells are produced by haematopoiesis, from the self-renewing pluripotent haematopoietic stem cells. They are formed in the bone marrow, as well as secondary lymphoid tissues such as spleen, lymph nodes, tonsils, uterus, etc. The development and maturation of NK cells is tightly regulated by various transcription factors, signalling pathways, and cytokines (Bi and Wang 2020). Mature NK cells are functionally competent and take part in immune surveillance against cancer and pathogens (Orange 2013, van Helden, Goossens et al. 2015).

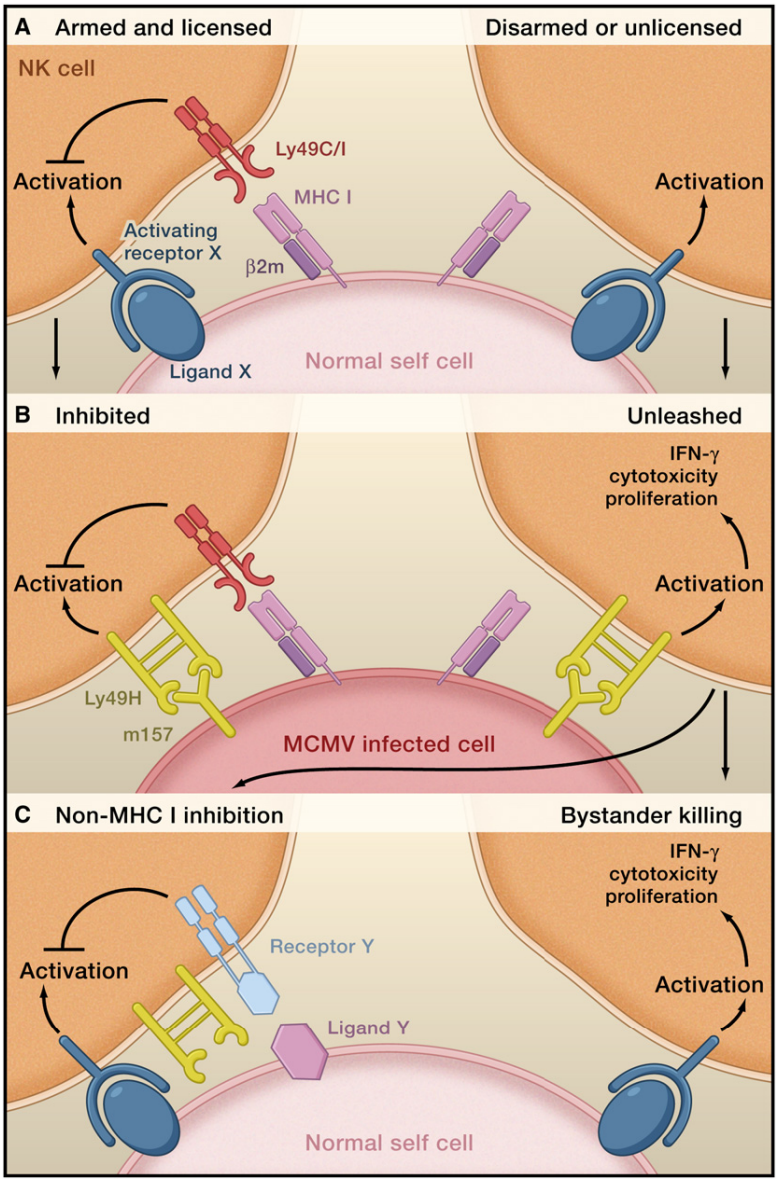


**Figure 2:** Representation of NK cell models of tolerance. Source: (Orr and Lanier 2010)

The process of education, maintained by the complex interactions between various receptor-ligand pairs, mediates the functional response of the NK cells, which in turn varies between individuals and according to the challenge presented (Boudreau and Hsu 2018). Under equilibrium conditions, activation receptors on NK cells interact with their endogenous ligands and are anergized/disarmed. When inhibitory receptors engage with MHC I, the earlier activation is opposed, and NK cells are licensed. Under infectious conditions, the disarmed NK cells become activated and control the infection. If NK cells lack MHC I inhibitory receptors, they may kill healthy cells that express activating ligands (Fig.2). The knowledge of NK cell education can lead to the development of widely applicable immunotherapies. With this in mind, we explored the state of NK cell education in various strains of mice upon stimulation by cytokine cocktails in chapter 2.

Before we dive into chapter 2, we should discuss 'Bare lymphocyte syndrome'. BLS is an inherited disorder of the immune system, where the major histocompatibility complex (MHC) or human leukocyte antigen (HLA) expression is either partially or completely absent (Nekrep, Fontes et al. 2003). As such, lymphocytes are devoid of the surface expression of MHC molecules. As discussed in the introduction, the presence of MHC molecules helps in training for the recognition of self from non-self, along with regulation of other immune activities, both innate and adaptive. Their absence renders individuals immunocompromised, and thus BLS is classified as a form of severe combined immunodeficiency (DeSandro, Nagarajan et al. 2000). Depending on the surface MHC expression, BLS can be further classified into type I, II and III.

1) Type I BLS: Type I is defined by the low surface expression of MHC class I. MHC class I is involved in the regulation of NK cell activities, presenting endogenous peptides to cytotoxic T lymphocytes, etc. As described in the figure 1, the presence of TAP1/2, tapasin is critical in the formation of a stable MHC I assembly. In type I BLS, mutations of the TAP are very commonly found, along with cases of tapasin

mutation and absence of $\beta_2$-microglobulin ($\beta_2$m) too (Zimmer, Andres et al. 2005, Parcej and Tampe 2010).

BLS I is a very rare disorder with around 40 reported cases worldwide. Patients with 10% of normal MHC class I expression are typically asymptomatic with no increased risk of infections. Patients showing 1-3% of MHC class I expression are develop recurrent bacterial sino-pulmonary infections around 4-7 years of age leading to bronchiectasis. Patients that have significantly reduced MHC class I expression present the severest phenotype and present with a combined immunodeficiency in the first year of life. They are inflicted with fungal, parasitic, and bacterial infections. Pathogens reported in patients include *Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae, Klebsiella pneumoniae, Escherichia coli*, and *Pseudomonas*. Bacterial infections of upper respiratory tract is a recurring theme in these patients often accompanying nasal septum perforation and nasal polyps, sinusitis and otitis media (necrotizing granulomatous inflammation with a close resemblance to Wegener's granulomatosis (WG)), while viral infections have not been observed (Gadola, Moins-Teisserenc et al. 2000).

Investigation of patient blood sample showed an expansion of $CD56^{bright}$ NK and low number of CD8+ αβ T cells. Functional tests on NK cells from samples showed no cytotoxic activity towards Class I deficient targets, however it has been reported that upon cytokine-mediated activation, the NK cells regain the cytotoxic activity towards tumour cells as well as normal autologous cells (Zimmer, Donato et al. 1998). We suspected that these non-functional NK cells in TAP deficient patients might be responsible for the recurrent bacterial infection.

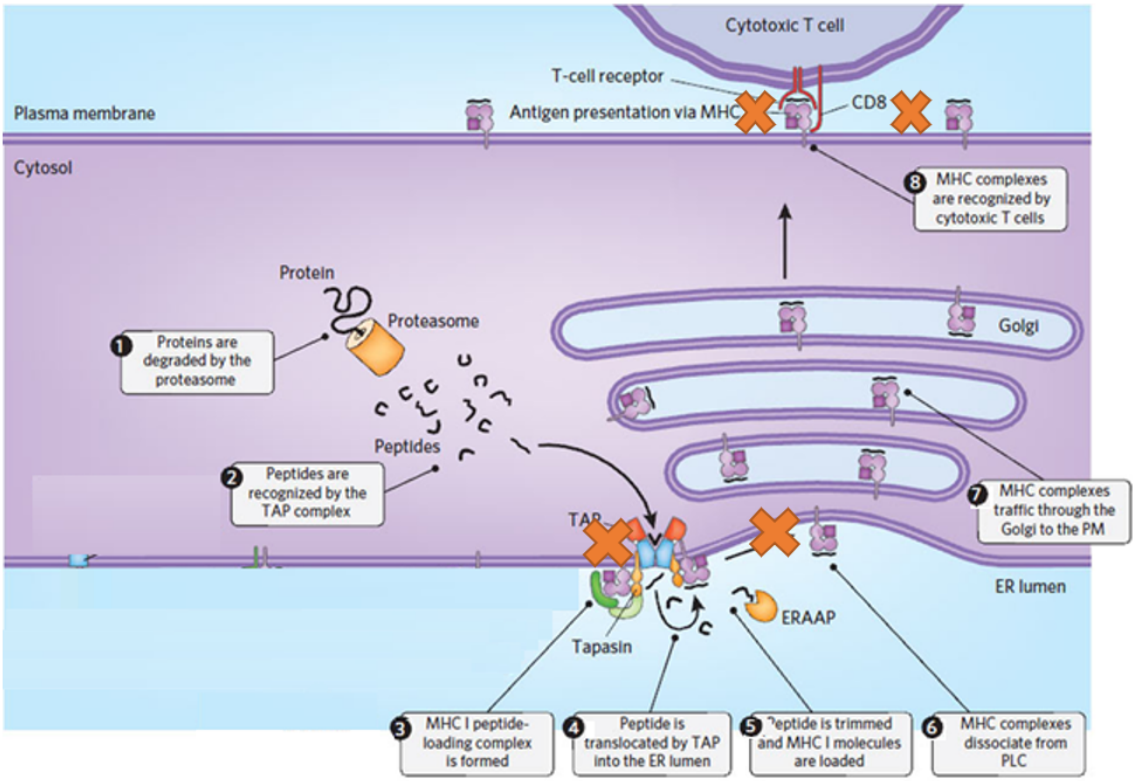


**Figure 3:** Overview of the MHC I antigen-processing pathway where peptides degraded from the proteins are recognised by transporter associated with antigen processing (TAP)1–TAP2 or tapasin and translocated into the ER lumen for loading onto MHC I. This translocation helps in formation of stable MHC I complex that are trafficked by golgi complex onto the cell surface. These MHC molecules are then recognised by cytotoxic T lymphocytes or by NK cells. (Image adapted and modified from: (Parcej and Tampe 2010))

2) Type II BLS: Loss of MHC class II molecules defines type II BLS. By far the highest number of BLS cases belong in this category. MHC II present exogenous protein molecules to helper T cells and is thus an important part of adaptive immune system (Gelin, Sloma et al. 2009). Patients suffer from infections of bacteria, viruses, or protozoa and immunoglobulin deficiency has also been reported.

3) Type III BLS: Type III BLS is defined by a loss of both MHC I and MHC II molecules and is very aggressive. Like in the previous two types, patients suffering from type III also present recurrent bacterial infections and have very low life expectancy.

For the purposes of this thesis, we will be focusing on BLS type I, specifically on the TAP deficiency.

As beneficial as human or mouse cell lines are, mouse models play an important role in elucidating several immune mechanisms. To our advantage, we had the B6 WT and TAP1-KO mice, the latter of which have defective assembly and transport of class I molecules. These mice show severely low levels of surface class I molecules. We also received MAIT CAST variants CAST/EiJ ($B6^{CAST}$) and MR1-KO from the group of Olivier Lantz (Salou, Legoux et al. 2021). Mucosal-associated invariant T (MAIT) cells are a subtype of αβ T cells in mammals, whose development exclusively depends on the expression of MHC class Ib molecule MR1 and are activated during bacterial infections. As such, we were interested to understand the NK cell and MAIT cell profile from these mice.

Our study uncovered several interesting findings. We confirmed the licensing profile of the B6 WT and TAP1-KO mice and showed the same for $B6^{CAST}$ and MR1-KO mice. Despite the expression levels of MHC class I molecules on TAP1-KO mice being low, strong cytokine stimulation can activate the cytotoxic capabilities of NK cells derived from them. We compared the phenotype of B6, TAP1-KO, B6CAST and MR1-KO NK cells from lung and spleens. The proportion of NK cells in lungs is significantly higher than those in spleen, however, the absolute numbers were comparable. We could not detect the non-polymorphic MHC class I molecule Qa-2 on TAP1-KO cells, and the classical MHC class I molecule $H\text{-}2K^b$ was severely down-modulated as well. We concluded that Qa-2 is TAP-dependent and cannot be loaded with TAP-independent peptides. We also looked at the markers CD11b and CD27, which subdivided NK cells into four distinct subsets and levels of maturity. CD27+ subsets are the most immature ones. The lung contained higher levels of CD11b+ NK cells and less CD27+

NK cells compared to the spleen in the four types of animals. We also saw that NK cells from TAP1-KO mice are relatively immature compared to B6 WT mice.

We could not identify the expression of granzyme B in mice with $B6^{CAST}$ and MR1-KO background, even after using different clones of the granzyme B antibody. These mice were originally derived from wild mice trapped in Thailand. It is known that the granzyme B gene is highly polymorphic in wild mice (Thia and Trapani 2007). Subsequent studies should focus on the sequencing of this gene from the $B6^{CAST}$ and MR1-KO mice and compared to the B6 WT granzyme B genes to offer an explanation.

A surprising discovery was the downmodulation of Ly49C/I levels across all strains of mice upon 6 days of culture, even in presence of cytokine-mediated stimulation of NK cells. We did not observe the same for NKG2A. Ly49 receptors play a key role in NK cell memory with Ly49C/I in particular being implicated in the peptide presentation on the MHC class I molecule, and this peptide sensitivity might be responsible for the Ag specific memory NK cell responses. This might have effects on how NK cells respond, as demonstrated in the MCMV and helminth infections (Korten, Volkmann et al. 2002, Pyzik, Gendron-Pontbriand et al. 2011). The mechanism of this loss is currently not elucidated. We speculate that the downmodulation could be due to the receptor internalization, contraction of the subset or the relative expansion of Ly49C/I- cells. The most likely explanation could be receptor shedding. One might hypothesize that the loss of Ly49C/I by activated NK cells renders these effectors more efficient against targets expressing classical MHC class I ligands. Future experiments should focus on the time progression of this loss, and perhaps the transcriptomic analyses of the receptor under various stimulation conditions. A similar phenomenon has not yet been reported in the case of humans.

We did not observe any clinical phenotype in our TAP deficient mice. This could be explained by their short life span and normal humoral immune responses, as well as living in a specific pathogen free animal facility. This contrasts with TAP-deficient patients. Thus, in addition to studying TAP1-KO mice at baseline, we need to develop mouse models that can

mimic TAP-deficient patient clinical phenotype, such as skin ulcers and/or bacterial respiratory infections. This will facilitate our understanding of the disease.

The part played by NK cells in cancer immunology, against viral infections is quite well known, while we still lack the clarity when it comes to their response against bacteria. It is now known that NK cells detect the presence of bacteria with the help of pattern recognition receptors such as nucleotide oligomerization domain (NOD)-like receptors (NLRs) and Toll-like receptors (TLRs)(Souza-Fonseca-Guimaraes, Adib-Conquy et al. 2012). They recognise PAMPs such as lipopolysaccharide (LPS), flagellin, bacterial membrane proteins among others. Different subsets of NK cells might respond differently to bacterial infections. In the case of bacillus Calmette-Guérin (BCG), human $CD56^{dim}$ subset presented with higher levels of perforin and granzyme A compared to $CD56^{bright}$ NK cells where IFNγ production was observed (Batoni, Esin et al. 2005). Similarly, the nature of NK cells derived from different species differs in functions. Accessory cells, interacting with the bacteria, release cytokines such as IL-12, IL-18 that could also stimulate NK cells, thus activating them.

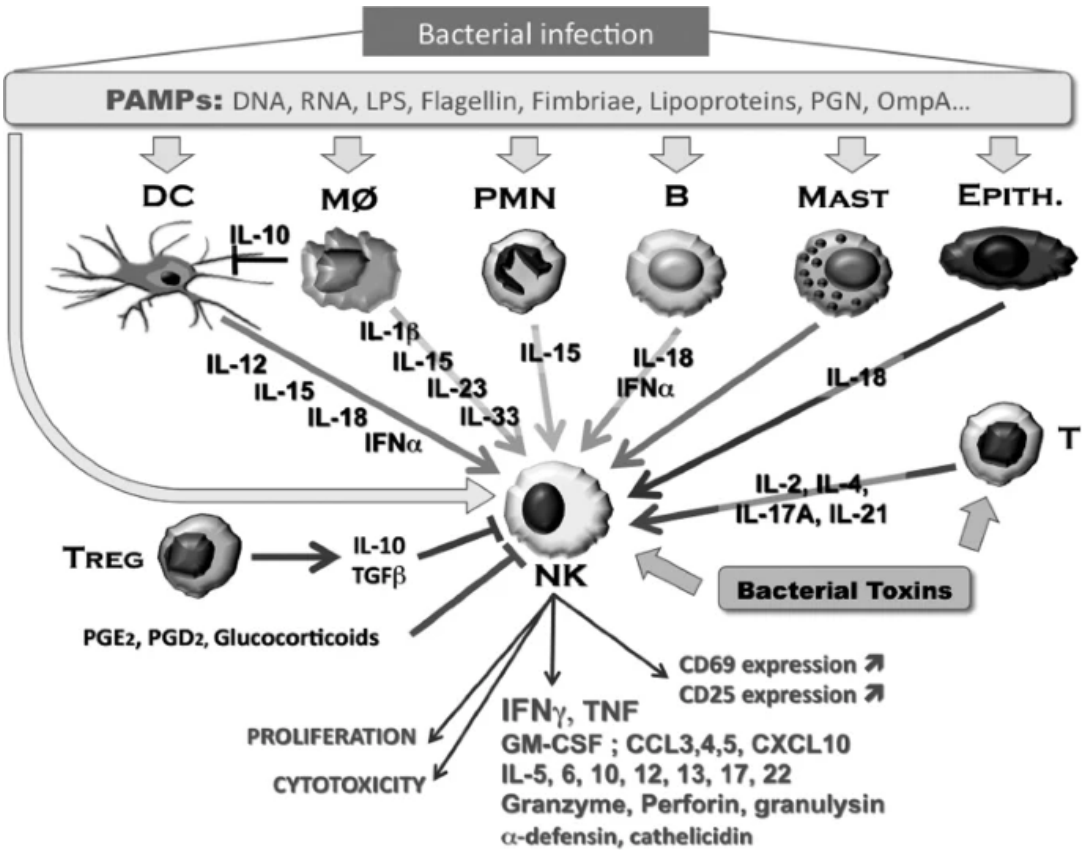

**Figure 4:** Activation of NK cells by bacterial pathogen-associated molecular patterns and accessory cells that detect bacterial PAMPs. Detection of bacteria by accessory cells leads to the cytokine production that in turn activate NK cells, whose reactivity is further strengthened by NK cell PAMP detection. PGN, peptidoglycan; OmpA, outer-membrane protein A, MØ, macrophages; PMN, polymorphonuclear leukocytes (neutrophils); B, B lympocytes; MAST, mast cell; EPITH., epithelial cell; PGE2, prostaglandin E2; PGD2, prostaglandin D2. (Image source: (Souza-Fonseca-Guimaraes, Adib-Conquy et al. 2012))

In chapter 3, we attempted to create the mouse model for chronic lung infection, to better understand NK cells in infection. The aim was to establish a mouse model with *Pseudomonas aeruginosa* lung infection lasting for 28 days, and use the blood, lung, spleen, etc samples from these mice to study the response of NK cells to the infection. The infection however did not last beyond 1 week, despite our best efforts. Previous successful reports with PA laden agar bead model were performed either with a different strain of bacteria or with trans-tracheal instillation (Bayes, Ritchie et al. 2016). It is possible that the orotracheal intubation of the agar beads lead to its lodging in the upper airway, or the swallowing of the beads by mice. This would in turn lead to clearance of bacterial load over the time. Future attempts could focus on replicating this method, exploring other strains of bacteria or starting with shorter infection period. A different method to introduce the agar beads, such as intratracheal surgery could be opted along with revising the concentration at which the beads are intubated.

Another way to study the role of NK cells in bacterial infection could be by exploiting specialised plates such as transwells (Bucior, Tran et al. 2014, Kusek, Pazos et al. 2014). *In vitro* models could be the answer to overcoming the complex setup process of mouse chronic lung infection models. Transwells inserts have permeable membranes with pore sizes ranging from 0.4 µm to 8.0 µm and can be inserted into six well plates. The basolateral surface could have epithelial cell lines, like LA-4, a mouse lung epithelial line. The transwell insert could have

live or fixed bacteria, either alone or in co-culture with immune cells such as purified NK cells. The purified NK cells can also be replaced by whole crushed murine lungs to mimic lung microenvironment.

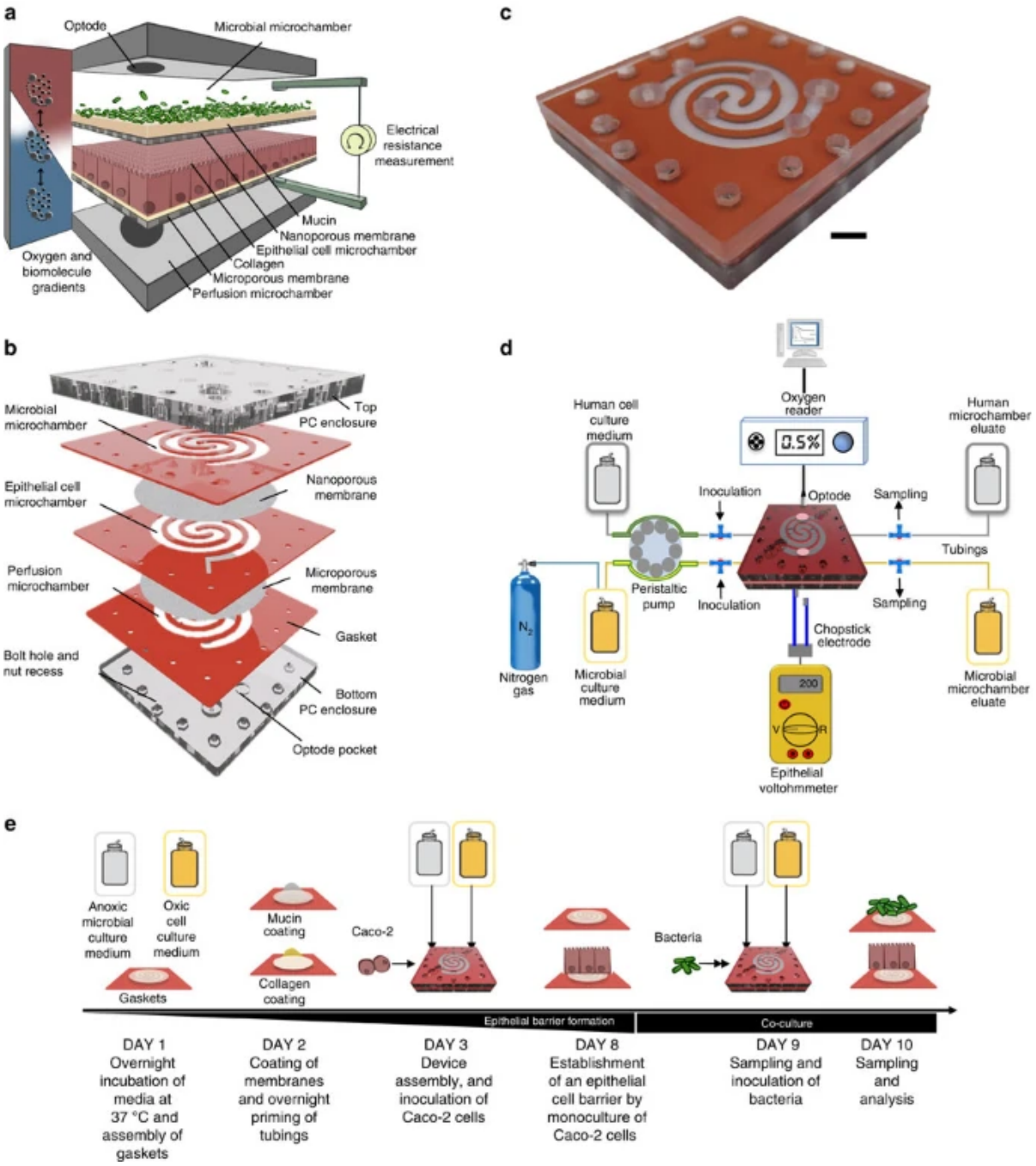


**Figure 5:** Pictorial representation of the HuMiX working model with co-culture of human epithelial cell line and gut microbiota and the protocol

The group of Paul Wilmes, in case of gastrointestinal human-microbe interaction, described a microfluidics-based in vitro model (Shah, Fritz et al. 2016). The human-microbial crosstalk or HuMiX model was developed to allow for systematic manipulation of various conditions affecting the gut microbiota and to study the effects on health. The model consists of a medium perfusion microchamber, a human epithelial cell culture microchamber and a microbial culture microchamber, each of which has an inlet and outlet for controlling various factors. A similar model could be attempted for understanding the lung microbiota and the interaction with host immune system. This model could allow for methodical studies with various strains of bacteria, a host of human or murine epithelial cell lines and NK cells. Specific aspects, such as alteration in the phenotypic aspects of NK cells or changes in the effector functions can be measured and help in coming up with the pathway of anti-bacterial NK cell action.

In the chapter 4, we looked at a way to image NK cell interaction with bacteria, instead of traditional imaging techniques. Confocal microscopy is usually a time-consuming method, with several constraints such as the kind of experiments, co-culture studies that could be done. Similarly, traditional flow cytometry gives exceptional information on the functional status of NK cell, however the dimension lacking is the inability to find specific location of the markers present on the cell surface. The ImagestreamX allows for visualising individual bacteria and focus of the specific contact points with NK cells. Experiments to look at the polarization of cytotoxic granules at the contact points, or the remodelling of the cytoskeletal proteins could be designed to further explore the topic. We could add markers such as perforin, other granzymes like granzyme A, granzyme H to understand the polarization of these molecules. Longer incubation times need to be done to visualize the lysis of the bacteria.

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**Further publication and collaborations**

Along with my PhD project, I had the opportunity to work with collaborators working on various topics. Through these collaborations, the following papers were published.

**RESEARCH HIGHLIGHT**

# Trained through generations

**Neha D. Patil**[1], Jonathan D. Turner[1], Mahesh S. Desai[1,2] and Jacques Zimmer[1#]

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The paper is a comment on the paper, “Transmission of trained immunity and heterologous resistance to infections across generations” (Katzmarski, Dominguez-Andres et al. 2021). The original article highlights the intergenerational immune traits in mice and how their inheritance is linked to the epigenetic modifications. I performed literature review and made the figure.

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RESEARCH HIGHLIGHT

# Trained through generations

Neha D. Patil[1], Jonathan D. Turner[1], Mahesh S. Desai[1,2] and Jacques Zimmer[1]✉



*Cellular & Molecular Immunology* (2022) 19:653–654; https://doi.org/10.1038/s41423-022-00846-5

The first scientists to coin the term "trained immunity" were Mihai G. Netea, Jessica Quintin, and Jos W.M. van der Meer in a 'Perspective' article in 2011 [2]. With the publication of vast amounts of experimental data and follow-up reviews, the concept that the innate immune system can develop a memory function in parallel to the well-recognized adaptive memory, mediated by B and T lymphocytes, is largely accepted. The cells that are responsible for trained immunity are mainly myeloid cells (monocytes, macrophages) and innate lymphoid cells (ILCs), such as natural killer (NK) cells and helper ILCs [3]. Studies of trained immunity frequently focus on infections, revealing increased protection against the same or a different pathogen after the first encounter with a microbe.

Among the infectious agents investigated in this context, Bacillus Calmette Guerin (BCG), an attenuated *Mycobacterium bovis* strain used in many countries as a protection against tuberculosis caused by *Mycobacterium tuberculosis*, is predominant. This vaccination can indeed induce some cross-protection against other bacteria (nontuberculous mycobacteria, various agents of lower respiratory tract infections), fungi (*Candida albicans*), and viruses (among them the influenza A virus and possibly to some extent the agent of COVID-19, SARS-CoV-2) [4].

Regarding the mechanisms underlying trained immunity, epigenetic and transcriptional changes as well as metabolic reprogramming are considered the main factors and have been abundantly documented [4, 5].

Not only infections but also psychological stress, such as early life adversity (ELA), may impact innate immune cells. Thus, when a human cohort of young adults who were institutionalized after birth and subsequently adopted were compared with controls raised by their natural parents in Luxembourg, significant phenotypic (accelerated maturation) and functional (reduced degranulation capacity) alterations in NK cells from the former were observed [6]. Likewise, when rat pups were separated from their mother during the early postnatal phase for 180 min daily and exposed to restraint stress in adulthood, their NK cells became phenotypically more mature than their control, non-ELA-exposed counterparts, and they were found to be functionally severely deficient in cytotoxic activity against the YAC-1 tumor cell line [6].

All the examples of trained immunity mentioned above take place within a single organism and can better protect this organism against pathogens or other forms of stress encountered at a later stage.

In contrast, Katzmarski et al. [1] investigated the trained immunity phenomenon across generations in vertebrates, namely, in C57BL/6 mice. The authors first sublethally infected male mice with *C. albicans* and bred them one month later with healthy females. The control group consisted of noninfected (PBS-treated) males and similarly naïve females. As systemic nonlethal candidiasis is known to lead to resistance to subsequent bacterial infection (through epigenetic mechanisms) [7], it was tempting to check whether this would hold true in the offspring of these mice, called the F1 exposed generation. Exposed and control F1 animals were then systemically infected with the Gram-negative bacterium *Escherichia coli*. Very interestingly, the exposed group quickly showed a lower bacterial burden in different target organs, accompanied by stronger immune cell infiltration and increased cytokine production (intergenerational protection; Fig. 1).

To go a step further and analyze whether the transmission of the resistance would be transgenerational, that is, affect the F2 offspring of F1 mice themselves not exposed to fungal or bacterial infections, the authors mated F1 exposed and F1 control males with control females and repeated the same for the F2 exposed and F2 control generations (giving rise to F3 offspring). Whereas the reduced burden of *E. coli* was still observed in F2 exposed mice, the effect had disappeared in F3 offspring (Fig. 1). For Katzmarski et al., these data underline the concept of "a nongenetic transgenerational transmission of trained immunity from F1 to F2 mice" [1].

Furthermore, they confirmed their results in a second experimental model generated in a different animal facility, where F0 mice were intraperitoneally injected with zymosan from *Saccharomyces cerevisiae*, and then the F1 exposed or F1 control offspring were challenged with the Gram-positive pathogen *Listeria monocytogenes*. Again, the F1 exposed mice resisted the infection much better (better survival, lower pathogen burden, reduced weight loss) [1].

More mechanistically, as trained immunity has previously been related to changes in the bone marrow myeloid compartment [7], it was established that in the transgenerational model of resistance to infection, this niche was modified toward less 'patrolling' monocytes and a higher activation level of common monocyte progenitors [1]. Genomic regions located close to those involved in myeloid cell development and activation were more accessible in the F1 exposed group than in the F1 control group. Gene set enrichment analysis revealed a bias toward the differentiation and activation of the monocyte lineage [1].

Interestingly, another study, also published in *Nature Immunology* [8] and with a very similar overall experimental approach as in Katzmarski et al., concluded that there is no intergenerational transmission of immunity to *M. tuberculosis*, *C. albicans* and influenza virus after the priming of the immune system with BCG, *Candida albicans* and the pathogen-associated molecular pattern β-glucan.

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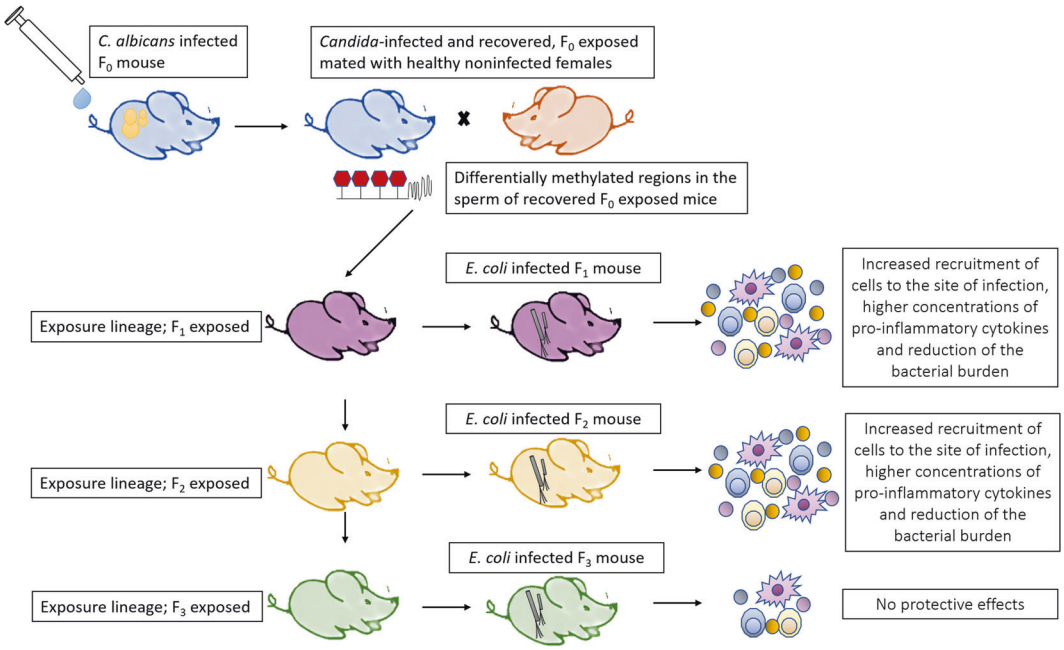


**Fig. 1** Schematic representation of the inter- and transgenerational transmission of trained immunity.

In their reply, Katzmarski et al. highlight several papers strongly suggesting the existence of intergenerational transmission of trained immunity [9]. They speculate that, among other factors, the discrepant conclusions of the two independent studies could be caused by the respective housing, diet, and microbiome of the experimental animals.

Indeed, the composition of the mouse microbiome is significantly influenced by the characteristics of a given animal facility [10], which most likely can then seriously impact the results. This nicely completes the circle, as in our human ELA cohort, we were also able to demonstrate a triangular relationship among ELA, trained immunity, and long-term alterations in the microbiome [11]. Therefore, to further clarify the fascinating topic of transgenerational trained immunity to infectious diseases, which would clearly make sense from an evolutionary point of view and regarding mammalian adaptation to an environment rich in pathogens, better controlled experimental designs to account for microbiome variation need to be developed.

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## COMPETING INTERESTS

MSD works as a consultant and an advisory board member at Theralution GmbH, Germany. The other authors declare no conflicts of interest.

## ADDITIONAL INFORMATION

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## Unbiased Screening Identifies Functional Differences in NK Cells After Early Life Psychosocial Stress

Sara B. Fernandes [1,2], **Neha D. Patil** [1,2], Sophie Meriaux [1], Maud Theresine[1], Claude. P. Muller[1], Fleur A. D. Leenen[1], Martha M. C. Elwenspoek [1], Jacques Zimmer [1,2] and Jonathan D. Turner [1*]

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This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology. The paper focused on the early life adversity and how they are closely associated with the risk of developing autoimmune diseases, type-2 diabetes, and cardiovascular diseases. For this study, rat maternal deprivation model was used to study the immune system. I was involved in designing the natural killer cell cytotoxicity and degranulation assays and performing them with the lead author. We found that NK cells in maternally separated rats are less cytotoxic towards their target cells (YAC-1) have a lesser degranulation capacity and show an increase in the maturation markers.

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# Unbiased Screening Identifies Functional Differences in NK Cells After Early Life Psychosocial Stress

*Sara B. Fernandes[1,2], Neha D. Patil[1,2], Sophie Meriaux[1], Maud Theresine[1], Claude. P. Muller[1], Fleur A. D. Leenen[1†], Martha M. C. Elwenspoek[1†], Jacques Zimmer[1,2] and Jonathan D. Turner[1*]*

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Early Life Adversity (ELA) is closely associated with the risk for developing diseases later in life, such as autoimmune diseases, type-2 diabetes and cardiovascular diseases. In humans, early parental separation, physical and sexual abuse or low social-economic status during childhood are known to have great impact on brain development, in the hormonal system and immune responses. Maternal deprivation (MD) is the closest animal model available to the human situation. This paradigm induces long lasting behavioral effects, causes changes in the HPA axis and affects the immune system. However, the mechanisms underlying changes in the immune response after ELA are still not fully understood. In this study we investigated how ELA changes the immune system, through an unbiased analysis, viSNE, and addressed specially the NK immune cell population and its functionality. We have demonstrated that maternal separation, in both humans and rats, significantly affects the sensitivity of the immune system in adulthood. Particularly, NK cells' profile and response to target cell lines are significantly changed after ELA. These immune cells in rats are not only less cytotoxic towards YAC-1 cells, but also show a clear increase in the expression of maturation markers after 3h of maternal separation. Similarly, individuals who suffered from ELA display significant changes in the cytotoxic profile of NK cells together with decreased degranulation capacity. These results suggest that one of the key mechanisms by which the immune system becomes impaired after ELA might be due to a shift on the senescent state of the cells, specifically NK cells. Elucidation of such a mechanism highlights the importance of ELA prevention and how NK targeted immunotherapy might help attenuating ELA consequences.

**Keywords: early life stress, NK cell, maternal deprivation, immune system, natural killer cells**

## INTRODUCTION

Early life adversity (ELA), which means stressful events occurring in the first 1000 days of life (1–3), plays a major role in adult-onset illness (4–6). These stressful events include a series of negative situations such as poor socio-economic status, parental mental disease, abandonment and/or institutionalization. Exposure to ELA has long-lasting effects on both mental and physical health as well as having negative behavioral consequences. It is associated with an increased risk of developing cardiovascular diseases (7), asthma (8), cancer (9) and mental disorders such as depression and anxiety later in life in both humans and in rodent models (10–14). There is now growing clinical and pre-clinical evidence that ELA and the associated negative health risk behaviors act through an altered immune system to induce the later-life disease risk (15–19).

Development of the immune system starts in early gestation. Innate immune cells such as monocytes, neutrophils and NK cells appear in the first trimester of pregnancy. Adaptive immune cells (T and B-lymphocytes), emerge around the start of the second trimester (20–22). During the pre- and perinatal period, this development is highly affected by several maternal factors such as obesity (23), malnutrition (24, 25), anxiety (19, 26–28) and smoking (29, 30), but also environmental factors such as parturition (31, 32), breastfeeding (33, 34) and antibiotic treatment (35). Alterations in specific cellular subsets in the immune system resulting from ELA have been widely documented in clinical studies. Individuals subjected to parental separation and subsequent adoption displayed a higher activation state of the immune system, with decreased levels of circulating central memory T cells and CD8+ T regulatory cells (18). Teenagers who suffered from childhood maltreatment, such as sexual and physical abuse, physical and emotional neglect showed increased circulating levels of NK and NKT cells after ELA (36). In addition, blood levels of the inflammatory marker C - reactive protein (CRP) in teenagers who had a low social-economic status as children was found to be increased when compared to individuals not exposed to early stress (37). Individuals with poor maternal care and harsh discipline during childhood also presented increased levels of CRP in the blood (38). Studies with rhesus monkeys, early isolated from their mothers at early age, also show a significant decrease in the CD4+/CD8+ ratio and increase in the circulating levels of NK cells (39). Even though some animals studies do not show any difference in the cell number after maternal separation (40), others document a decrease of the CD8+ T cells with subsequent increase of the CD4+/CD8+ ratio (13), opposite to what was documented in monkeys. Furthermore, prenatal exposure to alcohol lead to an increase in the CRP serum levels, indicating an inflammatory state of the immune system (41), similarly to what was previously observed in clinical studies (37, 38). Despite these numerous investigations, the literature is still lacking an unbiased overview of the complete cellular immune system.

Although the mechanisms through which these events occur are still not fully understood, increasing evidence shows that the mechanism by which ELA influences the function of CD8+ T cells and, consequently, viral responses, may be through the HPA axis (42). The neuro-endocrine axis plays an important role in the adaptive response to stress. When facing insults, corticotropin-releasing factor (CRF) is released from the hypothalamus, which in turn stimulates the production and release of adrenocorticotropin (ACTH). This hormone's main target is the adrenal cortex where the production and release of glucocorticoids (GCs) happens. Release of GCs into the bloodstream will trigger the adaptive mechanisms, in a negative feedback manner (43, 44). Stress events in an early period of life are known to have an impact in the HPA axis, programming its effects and responses in adulthood. This leads to decreasing levels of blood corticosterone and cortisol, which consequently affects the response of the peripheral immune system, leading to compromised viral responses (13, 45, 46). Such dysregulation of the HPA axis is thought to occur through the glucocorticoid receptor (GR), by regulation of gene transcription and negative feedback on the HPA axis, which in turn decreases the expression of certain cytokines (47). Clinical studies show an association between increased GR1F promoter methylation and ELA (48, 49). However GR/GC signaling remains undisturbed after ELA despite a slight increase in GR1F promoter methylation (50), raising doubts as to the importance of single-digit changes in promoter methylation levels (51).

ELA also accelerates immunosenescence, the natural aging process by which the immune cells begin to deteriorate and lead to weakened immune responses (52). Immunosenescence is accelerated not only after exposure to ELA (53), but also with depression after physical injury (54). T cells are strongly affected. Naïve T cell numbers decrease while memory T cell and terminally differentiated effector T cell (TEMRA) numbers increase, with concurrent telomere shortening (55–58). Furthermore, these cells have decreased expression of the co-stimulatory CD28 molecule and increased expression of the glycopeptide CD57, which leads to increased cytotoxicity and decreased proliferative capacity (53, 55, 59, 60). This increase in T cell senescence after ELA has been reported to be influenced by the exposure to and subsequent reactivation of cytomegalovirus (CMV), as levels of CD57+ cells are increased in patients seropositive for CMV (53, 61–63). Moreover, CMV in ELA individuals was recently reported to be linked to the presence of certain gut bacteria and CD8+CD57+ cells (64), suggesting an impact of ELA through the immune-brain-gut axis.

Although it is not as well documented as for T cells, immunosenescence also occurs in other cell types, such as B (65, 66) and NK cells (67, 68). The expression of CD57 in natural killer cells does not necessarily mean they are senescent but rather that they reached a higher maturation state, which is accompanied by functional changes similar to those observed in senescent T cells: less proliferation and higher cytotoxic capacity

**Abbreviations:** MD, Maternal deprivation; NK cell, Natural killer cell; NKT, Natural killer-like T cell; PND, Post-natal day; ACTH, Adrenocorticotropin hormone; CRH, Corticotropin-releasing hormone; GCs, Glucocorticoids; HPA axis, Hypothalamic–pituitary–adrenal axis; CRP, C-reactive protein; PBMCs, Peripheral blood mononuclear cells; CMV, cytomegalovirus.

(28, 67, 69). Moreover, NK cells are clearly involved in the response to CMV infections (70, 71) and links between NK cells, CMV and immunosenescence are starting to emerge (69, 70, 72).

Using an unbiased screening tool for flow cytometry data visualization, viSNE (73), this study provides a detailed description of the overall immune changes induced in the rat maternal deprivation (MD) model of ELA, identifying unexpected, but clear changes in NK cell properties. Furthermore, we describe the functional profile of NK cells, showing a shift in the maturity and cytotoxic capacities. We validated the NK cell phenotype in samples from our EpiPath ELA cohort (53, 74). This cohort consists of young adults (average age 24) institutionalized or otherwise separated from their biological parents at birth and adopted in early childhood (mean age of adoption 4.5 months) together with control participants in their natal families, all brought up in Luxembourg under similar societal and socioeconomic conditions.

# MATERIAL AND METHODS

## Human Samples

Peripheral blood mononuclear cells (PBMCs) from individuals that had experienced ELA in the form of institutionalization and subsequent adoption were obtained from our previously published EpiPath cohort (18, 50, 74). Briefly, participants aged between 18 and 35 years old with a prior history of ELA (institutionalization followed by adoption) or raised by their natural parents were recruited in Luxembourg between 2014 and 2016. Baseline EDTA anti-coagulated blood samples were drawn at a fixed time (11 am). Peripheral blood mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation as previously reported (53). and stored in liquid nitrogen until analyzed. All participants provided written informed consent, and the study was performed in accordance with the Declaration of Helsinki. The study was approved by the Luxembourg National Research Ethics Committee (CNER, No 201303/10 v1.4) and the Ethics Review Panel (ERP, University of Luxembourg, No 13-002).

## Animals

Ten to twelve week old 2-day timed-pregnant Wistar rats were obtained from Janvier Labs (Le Genest-Saint-Isle, France). Pregnant dams were housed in groups of 3 in 48 × 37.5 × 21 cm clear plastic isolator cages (Tecniplast, Varese, Italy) under a conventional 12-h light-dark cycle at 21°C and 49-54% relative humidity with food and water provided ad libitum. During pregnancy only routine husbandry was performed. Nesting material was provided for all females from gestational day (GD) 16 onwards and the cage was not changed between GD17 and post-natal day (PND) 2. Litters were naturally delivered between days 21-23 of gestation and size was adjusted to 12 pups/dam. Dams were randomly assigned to give birth to pups for one of the following groups (one condition per litter; two litters per group): 3 hours Maternal Deprivation from PND2 to PND14 ($MD_{180}$), 15 minutes Maternal Deprivation from PND2 to PND14 ($MD_{15}$) and no separation (CTR). Study outcomes are thus from two independent experiments. The experiments were carried out in accordance with the European Union directive 2010/63/EU as incorporated in Luxembourgish law for the care and use of laboratory animals. The study protocol was approved by the local Animal Welfare Structure (DII-2017-18).

## Rat Maternal Deprivation

Pups from both MD groups underwent a separation from the dam at a fixed time every day ($MD_{180}$: 9 am - 12 am, $MD_{15}$: 9 am - 9:15 am) from PND 2 to PND 14. Separated pups were placed in a clean bedding-free cage and maintained at 33°C in a heated vented animal cabinet (Noroit, France). At the end of the daily separation period, pups were returned to their mothers in the original home cage. Control litters were only handled for regular husbandry (e.g. cage cleaning) and otherwise left undisturbed until weaning. All animals were weaned on PND21, and subsequently housed (2 to 3 per cage) by sex and experimental group, and only received regular husbandry until further experiments.

## Rat Restraint Stress

All animals underwent a 1-hour restraint stress on PND49 +/- 1 day. Restraint stress was performed between 9 and 12 am during the inactive (light) phase. Animals were immobilized in a 50mm diameter dark grey PVC tube, closed at the front and with an adjustable lock in the back. Breathing of the animals was controlled during the whole procedure.

## Rat Corticosterone and Glucose Levels

Blood samples were drawn from the tail vein using a SAFETY Blood Collection/Infusion Set (Greiner Bio-One, Germany), immediately on being placed in the restrainer and in the minutes preceding their release. At the same time, a single blood drop was used to measure glucose levels, using an electronic glucometer (Accu-Chek, Roche). All blood samples were centrifuged at 2000 x g for 5 minutes and the plasma collected and stored at -80°C, until further analysis. Plasma corticosterone levels were measured by ELISA (IBL International, Hamburg, Germany), according to the manufacturer's instructions. A 4-parameter curve was fitted to the calibrator sample OD values; sample concentrations were calculated and, for glucose, presented as delta values (values after stress – values before stress).

## Rat Immunophenotyping

At PND56 animals were euthanized by CO2 inhalation and cardiac puncture was performed post- mortem to collect blood. Post-mortem blood (100µL per animal) was used for immunophenotyping by flow cytometry (LSR Fortessa, BD Biosciences, NJ, USA). Cell surface specific antibodies (see **Supplementary Table 1**) were diluted in flow cytometry staining (FACS) buffer (1X PBS, 1% BSA, 2mM EDTA), added to each individual sample and incubated for 30 minutes, at 4°C in the dark. Subsequently, samples were washed three times (100µl, 4°C, 300 x g, 10 minutes, FACS buffer) and erythrocytes lysed with Lysis buffer (BD Biosciences) for 10 minutes at room temperature in the dark. Cells were fixed with fixation buffer

(Invitrogen, CA, USA) for 1h, washed (100µl, 4°C, 300 x g, 10 minutes, FACS buffer) and permeabilized for 1 hour with permeabilization buffer (Invitrogen, CA, USA). Intracellular markers (**Supplementary Table 1**) were diluted in FACS buffer and added to the samples. After 30 minutes incubation (4°C, protected from light), the samples were washed three times (100µl, 4°C, 300 x g, 10 minutes) and re-suspended in FACS buffer for further analysis.

## Natural Killer Cell Phenotyping

NK cell phenotyping was performed on both rat splenocytes and human PBMCs. Single-cell splenocyte suspensions were prepared on the day of the sacrifice and stored in liquid nitrogen in FBS (Sigma Aldrich, MO, USA) containing 10% DMSO (Sigma Aldrich) until analyzed. On the day of the assay, vials were thawed at 37°C and washed with RPMI-1640 (Lonza, Basel, Switzerland) complemented with 10% FBS, 1% Penicillin/Streptomycin (Lonza), 1% Glutamine (Lonza) and 50µM of β-mercaptoethanol (Invitrogen). Cells were diluted to 106 cells/ml and 200µL aliquots distributed in 96 well plates, prior to incubation for 1 hour at 37°C, 95% humidity and 5% CO2. NK cell maturation state was assessed by flow cytometry (antibodies in **Supplementary Table 1**) as described above. Cell viability was measured using the Zombie NIR™ Fixable Viability Kit (Biolegend, San Diego, CA, USA).

## Natural Killer Cell Cytotoxicity Assays

The cytotoxic response of rat NK cells was determined against YAC-1, a murine lymphoma cell line. Target cells were thawed and cultured in suspension in flasks with complete RPMI medium (RPMI-1640, 10% FBS, 1% Pen/Strep, 1% Glutamine, 1 mM HEPES, 50µM β-mercaptoethanol). Only cells in the exponential growth phase were used in the assays. Single-cell splenocyte suspensions were cultured for 72 hours in complete RPMI-1640, with 200U/mL of recombinant rat IL-2 (Sigma Aldrich), at 37°C, 95% humidity and 5% CO2. Before the challenge, YAC-1 cells were stained with 1µM Cell Trace Violet (CTV) in 1XPBS for 20 minutes and washed twice with 1X PBS. Similarly, human PBMCs from the EpiPath cohort (18) were cultured in complete medium with 200U/mL of recombinant human IL-2 (R&D Systems Inc., MN, USA) and left undisturbed overnight. For human NK cells, the cytotoxic response was determined against K562, a human myeloid leukemia cell line. Cells were cultured in suspension in flasks with complete DMEM (DMEM, 10% FBS, 1% Pen/Strep, 1% Glutamine, 1 mM HEPES) and only taken for the assays at the exponential growth phase. Before the assay, K562 were pre-incubated with 1µM CTV for 20 minutes and washed twice with complete RPMI-1640 (RPMI-1640, 10% FBS, 1% Pen/Strep, 1% Glutamine). Effector NK cells (E) and YAC-1 or K562 target cells (T) were plated at E:T ratios ranging from 1:1 to 100:1 for rat splenocytes and 1:1 to 25:1 for PBMCs, for four hours. Fifteen minutes before acquisition, 15µM of TO-PRO3 (Invitrogen, Karlsruhe, Germany) was added, to discriminate viable cells from dead cells (TO-PRO3+).

## Natural Killer Cell Degranulation Assay

Human PBMCs were cultured overnight in complete medium with 200U/ml of IL-2 and stimulated with CTV labelled K562 target cells at ratios of (E:T): 1:1, 5:1, 10:1 and 25:1 as described above. At the same time, anti-CD107a antibody was added to each well. After 1h incubation, 0.1µL of GolgiStop (BD Biosciences) was added per well and the plate was incubated at 37°C, 5% CO2 for a further three hours. Cells were washed with FACS buffer (10 minutes, 300 x g) and stained for NK cell surface markers (**Supplementary Table 1**) followed by intracellular staining for IFN-γ as described above.

## Flow Cytometry

A minimum of 50,000 events were recorded for all the experiments. Immunophenotyping, NK cell maturity and degranulation assays were performed on BD LSR Fortessa (BD BioSciences using FACSDiva software (BD BioSciences, version 8.0). The NK cytotoxicity assays were analyzed on a NovoCyte Quanteon Flow Cytometer (Agilent).

## Data Analysis

Flow cytometry data was analyzed with FlowJo (Tree Star, Ashland, OR, USA), visNE software (Cytobank, Inc., CA, USA) and Tableau (Seattle, WA, USA). After processing the raw data, 36 flow cytometry.fsc files (12 per experimental group) from the 12-colour initial panel were uploaded onto Cytobank and used to generate viSNE plots according to the following parameters: Events = 50.000; Channels = all 12 antibodies; Compensation = uncompensated; Iterations = 5000; Perplexity = 30. For the illustration menu, the gating of all channels was set for minimum of -2000 and the argument at 200. For further and more detailed analysis, FlowSOM was used with the default settings and all channels and files were selected. Event sampling was set at 50.000; Number of metaclusters at 10; Iterations at 10; and Number of clusters at 49. Results of this analysis were plotted into t-SNEs maps and cell populations were separated according to the presence of each cell marker across the different cell populations.

## Statistical Analyses and Data Presentation

Statistical analyses were performed in GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA, USA) and FlowSOM (Cytobank, Inc., CA, USA). Tests used to assess statistical differences were One-way ANOVA (Tukey's multiple comparisons test) or Two-way ANOVA (Dunnett's or Sidak's multiple comparisons tests), depending on the number of animals and parameters in the assay. Figures were subsequently generated using GraphPad Prism and Adobe Illustrator CS6 (version 16.00).

All biosecurity and institutional safety procedures were adhered to.

# RESULTS

## Maternal Deprived Animals Subjected to Stress in Adulthood Have an Increased Physiological Response to Acute Stress

At PND49, all animals were subjected to a restraint stress in order to evaluate HPA axis function. Corticosterone and glucose were measured from plasma and whole blood before and upon

completion of the acute stressor, respectively. The restraint stress did not induce any significant changes in corticosterone and glucose levels in the two MD groups (**Figures 1A, B**; Tukey's multiple comparisons test, p=0.81 for $MD_{15}$ and p=0.6 for $MD_{180}$ for corticosterone; p= 0.43 for $MD_{15}$ and p=0.14 for $MD_{180}$, for glucose). However, exposure to MD had a clear effect on the baseline glucose level, with exposure to either 15 or 180 minutes MD significantly lowering the baseline glucose level, compared to the control group [**Figure 1C**; $MD_{15}$ 115.9 ± 1.4 *vs* 136.4 ± 7.2, p=0.0008; $MD_{180}$ 116 ± 2.8 *vs* 136.4 ± 7.2, p=0.005, Dunnett's multiple comparisons test)]. Although there was not a significant two-way interaction between the change in glucose and the groups, the stressor significantly increased the absolute glucose levels in the $MD_{180}$ group (115.9mg/dL ± 1.4 *vs* 134.4mg/dL ±3.7, Sidak's multiple comparisons test p=0.014) (**Figure 1C**). This shows an activation of gluconeogenesis in the liver (75) and release into the blood stream, indicative of the fight-or-flight response of a system in need of energy supply.

## Unbiased Immunophenotyping

Flow cytometry was performed for all animals at PND56. After basic data quality checks, t-SNE maps were generated from viSNE and flowSOM analysis. The t-SNE maps show clear differences in the clustering of the data through the abundance and spatial distribution of certain regions (**Figure 2A**). The map regions represent the different immune cell subsets from the animals, that were color defined based on the cell markers used (**Supplementary Figure 1**). In total, 49 different clusters were identified, twenty of which were found to be statistically different between both maternally separated groups and the control, according to the antibodies that define each cluster (**Supplementary Table 2**; **Figure 2B**). As seen in previous reports from other experimental paradigms (76–78), T ($CD3^+$), T helper ($CD4^+$) and T cytotoxic ($CD8^+$) cells were the most clearly delineated populations within the viSNE plot and where we found the most significant changes after MD (**Supplementary Figure 1**). B cells ($CD45RA^+$) (79, 80) together with clusters containing macrophages, dendritic ($CD11b^+$) and T regulatory ($CD25^+$, $FoxP3^+$) cell types were also readily identified (**Supplementary Figure 2**). Surprisingly, some of the most significantly different clusters were associated with the CD161a cell marker, which is one of the primary cell surface markers for NK cells. (**Figure 2C**).

## Maternal Deprivation Induces Long-Term Changes in the Immune System

The clusters identified in our viSNE analysis were examined in detail with FlowJo. The percentage of $CD3^+$ T cells was significantly decreased in the animals subjected to 15 minutes of MD (30.5 ± 0.95, Dunnett's multiple comparisons test, p=0.001), although no significant changes were found in the group separated for 3 hours, when compared to the control group (37.9 ± 1.98 *vs* 38.8 ± 1.62) (**Figure 3A**). B cells, on the other side, were found to be significantly increased in both groups ($MD_{15}$: 34.2 ± 1.4, p<0.0008; $MD_{180}$: 29.7 ± 1.04,

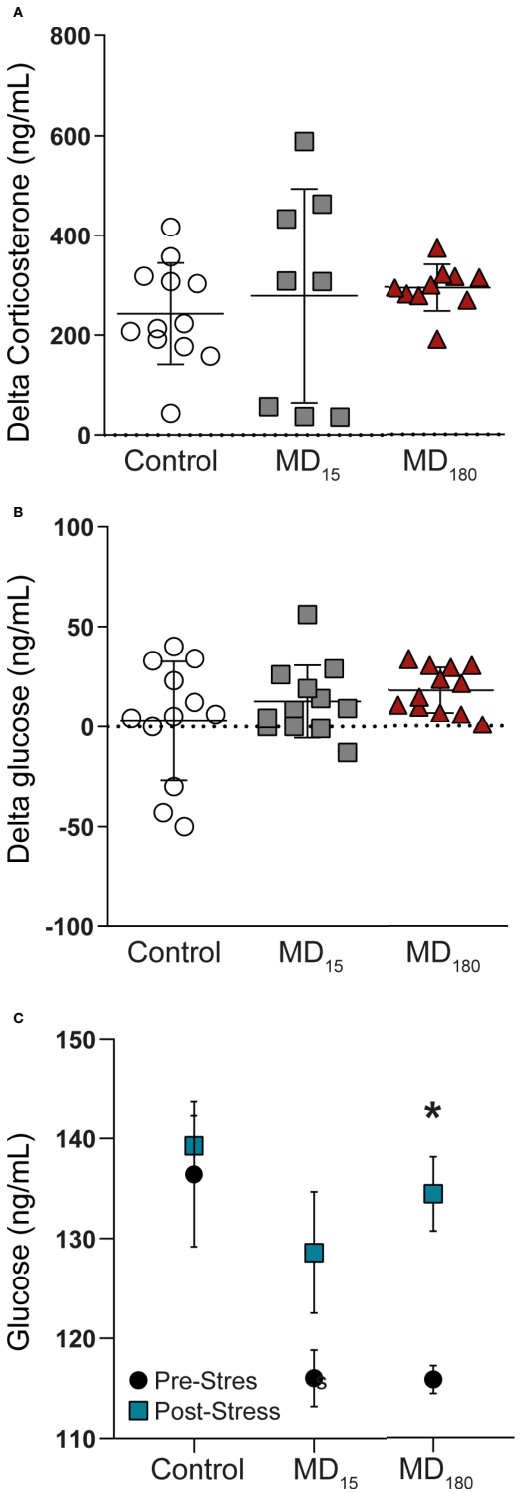


**FIGURE 1** | Acute stress in adulthood has no significant impact on the HPA axis of maternal deprived animals, but changes the glucose absolute levels. **(A)** Delta corticosterone levels; **(B)** Delta glucose levels; **(C)** Absolute glucose levels before and after an acute stress. Data is presented as mean ± SEM of 9 to 12 animals per group. Statistics: One-way and Two-way ANOVA. *p = 0.014.

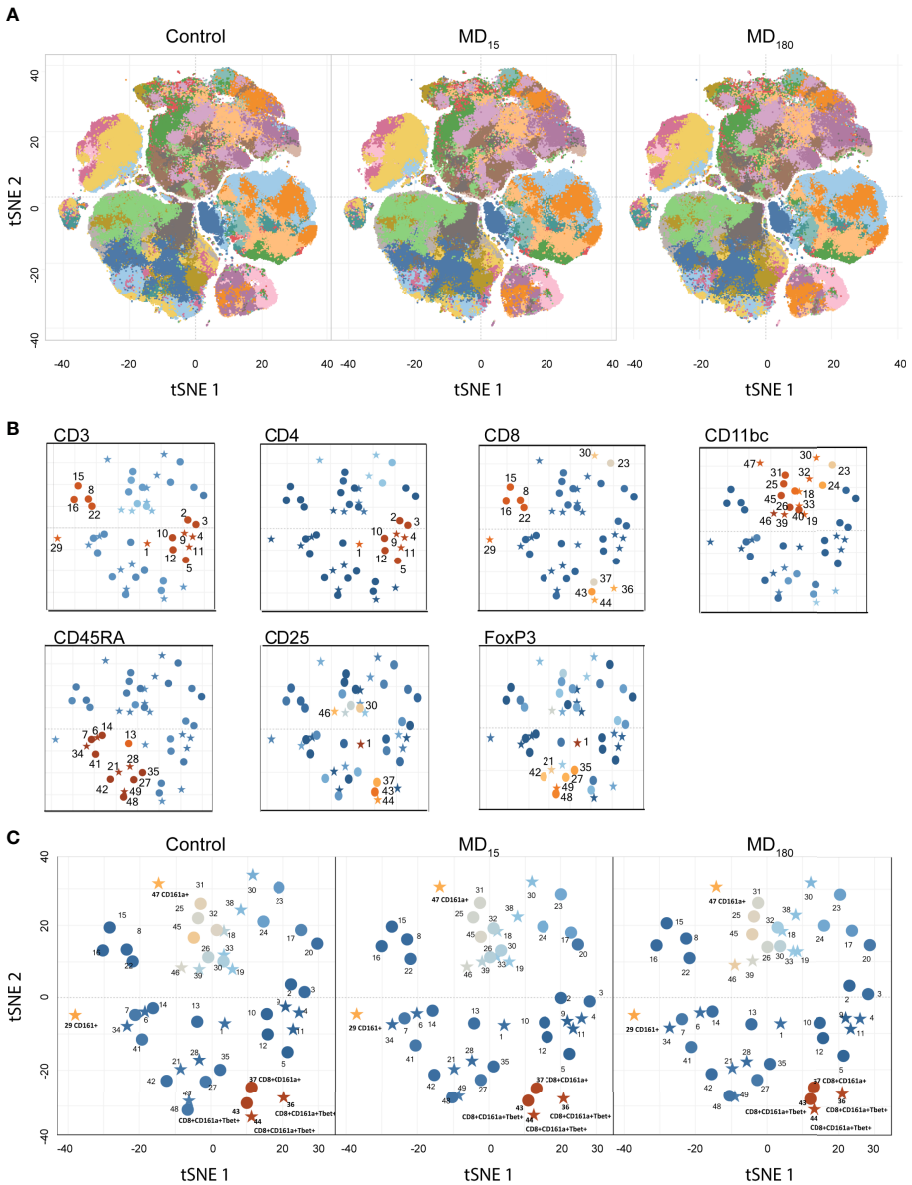


**FIGURE 2** | Unbiased immunophenotyping with viSNE. **(A)** visNE map obtained through cytobank with the markers a single 13-color antibody combination. Each panel represents the mean of all 12 animals in the control (left), $MD_{15}$ (middle) and $MD_{30}$ (right) groups; **(B)** Identification of different clusters by the principal rat cell surface markers – CD3 CD4, CD8, CD11b/c, CD45RA, FoxP3. Stars represent significantly different clusters, between the different treatment groups at an FDR corrected $p < 0.05$; **(C)** Clusters colored by CD161a (NK cell marker) intensity. Blue to orange: low to high expression of the marker.

$p<0.0085$), when compared to controls (25.3 ± 0.81) (**Figure 3A**). To further investigate how the immune system was impacted, we also looked at the different types of T cells. $CD4^+$ helper T cells were found to be significantly decreased in both $MD_{15}$ (57.7 ± 0.71, $p<0.0001$) and $MD_{180}$ (59.8 ± 0.74, $p=0.001$), compared to the control group (65.5 ± 0.51). The cytotoxic $CD8^+$ T cells were significantly increased in the $MD_{15}$ group compared to controls (42.3 ± 0.86 *vs* 39.0 ± 0.48, $p=0.022$) but not in the $MD_{180}$ (**Figure 3A**). Activated B cells and subsets of $CD4^+$ and $CD8^+$ T cells, which are involved in Th1, Th2 and Th17 types of responses, characterized by the transcription factors T-bet, GATA3 and RORγT, respectively, were also analyzed and quantified but did not produce significant changes upon early stress (data not shown), although assessed with anti-mouse antibodies.

## Levels of NK and NKT-Like Cells Changed After ELA

After our unbiased analysis with viSNE, we quantified the levels of both NK and NKT-like cells using a classical gating strategy

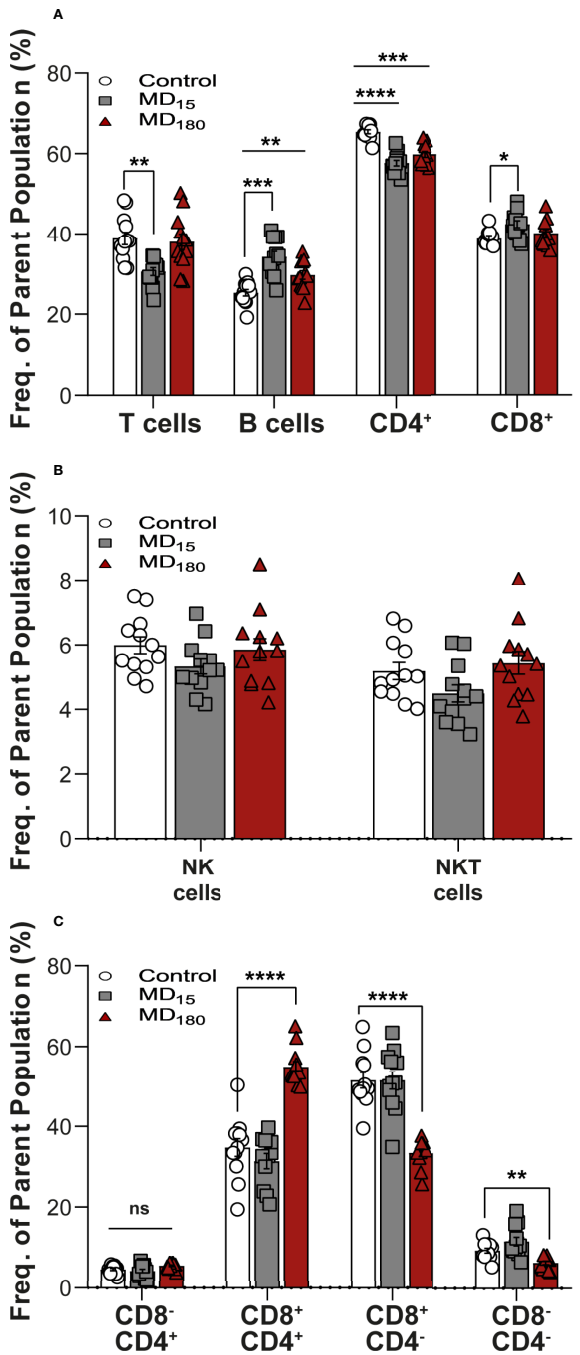


**FIGURE 3** | Maternal deprivation induces long-term changes in the immune system. **(A)** Adaptive immune cell population analysis. T cells gated as CD3+ from total lymphocytes. B cells gated as CD45RA+ gated from total lymphocytes; CD4+ and CD8+ gated from within the T cell population **(B)** expression levels of NK and NKT-like cells. NK and NKT cells were gated from total lymphocytes as the brighter population expressing CD161a+ and being CD3- or CD3+, respectively.; **(C)** Sub-gating of the NKT-cell population from panel **(B)** by $CD4^+$ and $CD8^+$ markers. Data is presented as mean ± SEM of 12 animals per group. Statistics: Two-way ANOVA; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, NS, Not Significant. Representative Figures for flow data are included in the **Supplementary Material**.

(**Supplementary Figure 3**) in FlowJO. For the two populations, both MD groups did not show significant differences when compared to control (**Figure 3B**), but when separating the NKT cells into their functional subgroups defined by CD4 and CD8 expression (Seino and Taniguchi 2005), statistically significant differences appeared (**Figure 3C**). Double positive ($CD4^+CD8^+$) NKT-like cells were significantly increased in the $MD_{180}$ (54.9 ± 1.31, Dunnett's multiple comparisons test, p<0.0001), whereas 15 minutes of MD had no effect, when compared to the control group (34.77 ± 2.21). On the other hand, double negative ($CD4^-CD8^-$) NKT-like cells were significantly decreased in the $MD_{180}$ group (6.19 ± 0.43, p<0.0036) but suffered no changes in the $MD_{15}$ group, compared to controls (9.24 ± 0.62). Furthermore, $CD8^+$ NKT-like cells were also found to be significantly decreased in the group separated for 3 hours in relation to the control group (33.4 ± 0.97 *vs* 51.5 ± 1.92, p<0.0001).

## Maternal Deprivation Changes the Maturation State of NK Cells

To evaluate the effect of MD on NK cell functionality, we quantified the maturation state of these cells using the markers CD27 and CD11b (81–83). The process begins with no expression of either receptors (immature NK cells, iNK), followed by gain of CD27 and CD11b receptors, and ends with loss of CD27, representing the most mature NK cells (mNK) (gating strategy: **Supplementary Figure 4**). The double negative cell population ($CD11b^-$, $CD27^-$; Q4 from **Supplementary Figure 4**) does not appear to be influenced by our MD paradigm (**Figure 4A**). Following that, the NK cell population that gained CD27 but not CD11b (Q1 from **Supplementary Figure 4**) was shown to be significantly decreased in the $MD_{180}$ group compared to the control group (67.0 ± 2.79 *vs* 73.9 ± 3.70, Dunnett's multiple comparisons test, p=0.0119). The double positive (DP) population (Q2 from **Supplementary Figure 4A**) was significantly increased in both maternal deprived groups ($MD_{15}$ 18.28 ± 1.61, p =0.019; $MD_{180}$ 16.48 ± 1.29, p=0.0042) compared to the control group (9.76 ± 0.87). Finally, $CD27^-$ $CD11b^+$ population (Q3 from **Supplementary Figure 4**), representing the most mature NK cells, was significantly increased in both maternal separated groups ($MD_{15}$ 5.07 ± 0.66, p=0.0153; $MD_{180}$ 5.03 ± 0.79, p=0.0023), compared to the control group (3.10 ± 0.56) (**Figure 4A**).

## Long Maternal Deprivation Changes the Cytotoxicity of NK Cells

The cytotoxic capacity of rat NK cells after MD was measured against the mouse target cell line YAC-1, as previously described in the literature (84). Cells from animals that underwent 3 hours of MD exhibited a significantly decreased cytotoxicity from E:T ratio 12.5:1 (5.94 ± 0.38, Dunnett's multiple comparisons test, p=0.0032) to the highest E:T ratio, 100:1 (16.57 ± 1.77, p=0.0057), when compared to the cells of the group that did not suffer any type of early stress (12.99 ± 1.03; 38.16 ± 3.48) (**Figure 4B**). Animals that were maternal separated for 15 minutes displayed a similar response to the control group.

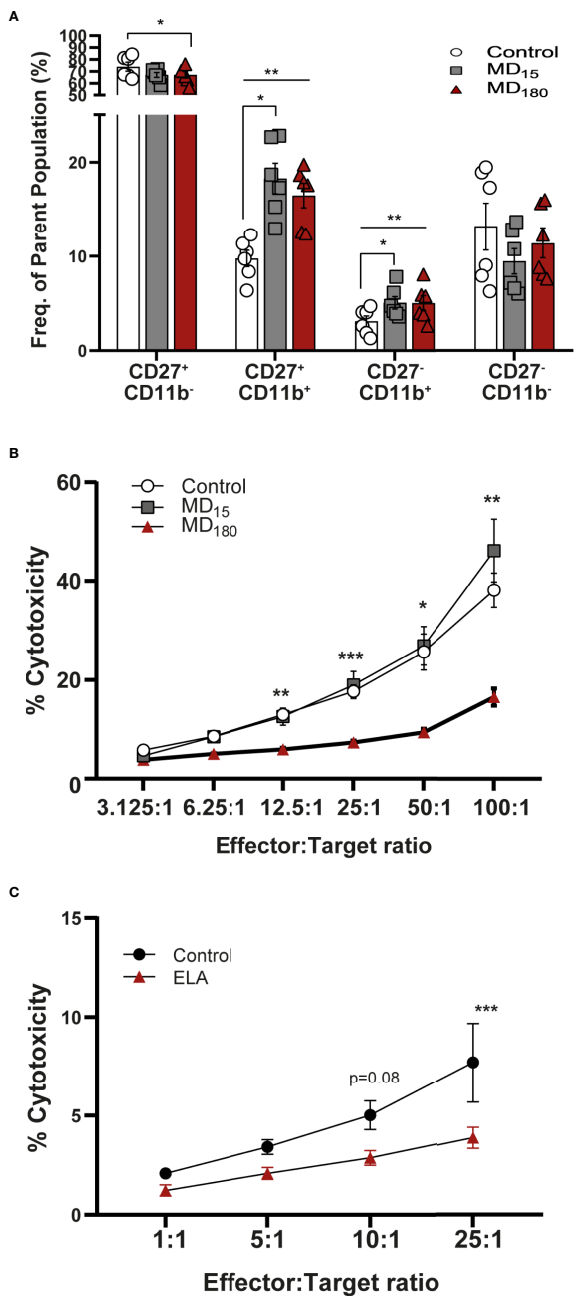


**FIGURE 4** | Early life separation induces functional changes in rat and human NK cells. **(A)** Maturation state of rat NK cells defined by CD27 and CD11b markers. Cells were gated from CD161a+/Nkp46+ NK cells; **(B)** Cytotoxic response of rat NK cells to YAC-1 cells; **(C)** Cytotoxic response of NK cells from institutionalized individuals to K562 cells. Data is presented as mean ± SEM of 6 animals per group or 14 donors per group. Statistics: Two-way ANOVA; *p < 0.05; **p < 0.01; ***p < 0.001. Representative Figures for flow data are included in the **Supplementary Material**.

## NK Cell Changes Are Reproduced in the EpiPath ELA Cohort

The cytotoxic response of human NK cells from the EpiPath cohort was measured against K562 cells. Similarly to the rat, NK cells from the individuals that were exposed to ELA had a lower response than the cells from the control group, reaching statistical significance at the highest ratio (E:T, 25:1) (3.93 ± 1.78 *vs* 7.71 ± 6.52, Sidak's multiple comparison test, p=0.0004) (**Figure 4C**). As previously seen in our study (53), increased titers of CMV could be associated with such a decrease in the cytotoxicity of NK cells. However, there is no statistical correlation in any of the ratios, between CMV titers and NK cytotoxicity (**Supplementary Figure 5**).

## Early Life Stress Reduces Degranulation of NK Cells in the EpiPath Cohort

Similar to what was previously described (85, 86), six populations were obtained in the flow cytometry CD16 *vs* CD56 dot plot: $CD56^{bright}CD16^{-}$, $CD56^{bright}CD16d^{im}$, $CD56dimCD16^{bright}$, $CD56^{-}CD16^{bright}$, $CD56^{dim}CD16^{-}$ and $CD56^{dim}CD16^{dim}$ (**Figure 5A**). In all these populations, the expression of CD107a and IFN-γ were measured. Although the majority of NK populations displayed less degranulation capacity in donors that suffered ELA (**Supplementary Figure 6**), only $CD56^{dim}$, or CD56 negative NK cells ($CD56^{dim}CD16^{dim}$; $CD56^{-}CD16^{bright}$, $CD56^{dim}CD16^{bright}$), the most mature populations, reached statistical significance (**Figure 5**). $CD56^{dim}CD16^{bright}$ (population 5) NK cells from the control group displayed significantly higher levels of CD107a expression for all ratios, but did not show any differences when target cells were not presented (25:1 – 7.2 ± 8.4 *vs* 21.9 ± 8.4, p<0.0001; 10:1 – 9.9 ± 12.4 *vs* 28.2 ± 10.1, p<0.0001; 5:1 – 12.9 ± 15.2 *vs* 33.2 ± 11.4, p<0.0001; 1:1 – 11.04 ± 12.6 *vs* 29.8 ± 14.6, p<0.0001) (**Figure 5B**). The same was observed for the expression of IFN-γ, reaching statistical difference for all ratios except E:T 1:1 (No target – 0.87 ± 0.91 *vs* 2.5 ± 3.5, p=0.0171; 25:1 – 1.12 ± 1.08 *vs* 2.9 ± 4.3, p=0.0061; 10:1 – 0.88 ± 0.9 *vs* 2.7 ± 3.7, p=0.0072; 5:1 – 0.6 ± 0.63 *vs* 2.1 ± 3.2, p=0.0262) (**Figure 5D**). Double dim NK cells from ELA donors (population 4: $CD56^{dim}CD16^{dim}$) showed significantly decreased secretion of IFN-γ for all E:T ratios except 5:1 and 1:1, when compared to control donors [Sidak's multiple comparisons test: No target - 5.48± 3.2 *vs* 9.71 ± 9.02, p=0.0009; 25:1 – 7.9 ± 4.01 *vs* 10.9 ± 7.9, p=0.033; 10:1 –6.3± 3.1*vs* 10.7 ± 7.2, p=0.0006] (**Figure 5E**). Expression of CD107a was not different between the groups (**Supplementary Figure 6**). Finally, the $CD56^{-}CD16^{bright}$ (population 6) NK cell population displayed significant differences at the E:T ratios 10:1 and 5:1 with lower expression in the ELA group for both CD107a (10:1 - 49.6 ± 18.6 *vs* 63.9 ± 24.4, p=0.0084; 5:1 – 47.9 ± 13.9 *vs* 63.6 ± 24.1, p=0.003)) and IFN-γ (10:1 -9.7 ± 7.9 *vs* 26.7 ± 25.4, p=0.0011; 5:1 – 9.2 ± 5.9 *vs* 28.5 ± 27.2, p= 0.0002) (**Figures 5C, F**). CD107a expression was also significantly decreased in the ELA group at the E:T ratio 1:1 (39.5 ± 17.7 *vs* 51.7 ± 28.5, p=0.0298) (**Figure 5C**).

# DISCUSSION

In this study, we demonstrated how ELA, in the form of maternal separation, has a more widespread influence on the immune system than previously thought. To our knowledge, this is the first unbiased viSNE analysis linking ELA with clear changes in the overall immune profile and, more specifically, the first to

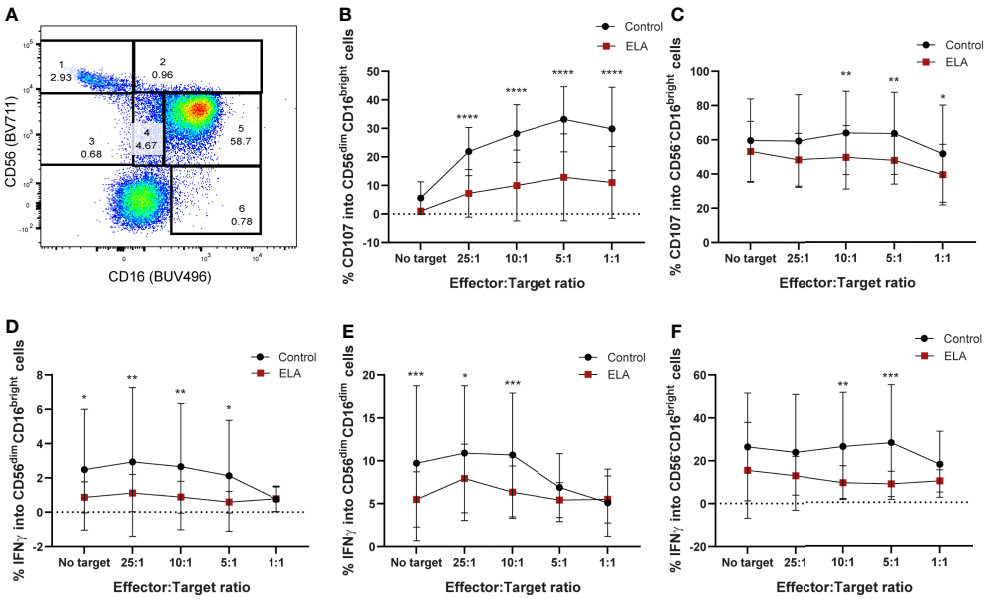


**FIGURE 5** | Maternal deprivation changes the degranulation capacity of the NK cells from ELA individuals. Cells were gated as lymphocytes from FSC-H, SSC-H; singlets from SSC-H, SSC-A; then from CD3-CD19- cells. **(A)** representative image of the NK cell population gating strategy: 1– $CD56^{bright}CD16^{-}$; 2 – $CD56^{bright}CD16^{+}$; 3- $CD56^{dim}CD16^{+}$; 4– $CD56^{dim}CD16^{dim}$; 5- $CD56^{dim}CD16^{bright}$; 6 – $CD56^{-}CD16^{bright}$; **(B, C)** expression of CD107a by populations 5 and 6; **(D–F)** expression of IFN-γ by populations 4, 5 and 6. Data is presented as mean ± SEM of 14 donors per group. Statistics: Two-way ANOVA; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

provide specific mechanisms of maturation, senescence, and changes in cytotoxicity and degranulation profiles of NK cells after ELA. We initially examined the effect of ELA using the rat MD model. As previously reported by ourselves and others (87–89), the separation and deprivation of maternal care during this period has been associated with increased cognitive impairment, HPA-axis dysregulation and anxious-like behavior (13, 90–93).

Stress, in all types of forms, either in early life, adolescence or adulthood activates the HPA axis, leading to the release of cortisol that will bind to GRs and ultimately initiate a cascade of molecular and cellular events (94–96). GRs activation is reported to impact gene transcription (97) and to inhibit immune responses (47). However, we previously reported that this process is not always so clear-cut (50). We saw clear clinical consequences later in life, specifically in the immune system and in the development of chronic and psychological disorders (18, 53), however, these changes were not accompanied by alterations in the expression or response of GRs, although the HPA axis was hypo-responsive (50, 74). Although we did not directly assess the functioning of the GRs, our data confirm our previous report that HPA axis hormones and receptors might not be as intimately involved in the long-term consequences of early life stress as thought. Importantly, we were able to reproduce the immune phenotype previously seen in our EpiPath cohort (18), where CD8+ T cells were found to be more activated (18) and more senescent (53) than the cells from the individuals in the control group. In our paradigm, T cells (CD3+) and their subsets (CD4+ and CD8+) were significantly changed, with CD8+ T cells following the same trend as in our ELA (18), confirming the relevance of the MD model for the biological consequences of ELA. Furthermore, we expanded changes in the immune system to B and NK cells. Our unbiased viSNE analysis did not clearly distinguish NK and NKT-like cells, the latter being T cells that share and express NK cell receptors bridging the innate and adaptive immune responses that are implicated in tumor rejection, cardiovascular and neurological diseases (98–101). Little is known about the long-term effects on NK cells after early-life psychosocial stressors, although NK cell numbers have been reported to be impacted (102). However, our previous report (18), together with the data reported here, suggest that ELA has a minor impact on circulating NK cell numbers, but is accompanied by a higher activation state and a trend towards increased senescence. Our data suggest that the NK cells have a similar phenotype to the CD8 T cells, previously reported. We see a different secretion of CD107a and IFN-γ from the CD56dim NK cell subsets (CD56dimCD16dim and CD56dimCD16+), as well as from the CD56-CD16+. As discussed by Emily Mace (103) and others (104, 105), these subsets are thought to be the most differentiated ones, as loss of CD56 expression and acquisition of CD16 was proposed to be part of the maturation process. These results follow the increased expression of maturation markers observed in the rats. Altogether, this seems to indicate that, although immature cells in both adoptees and stressed animals are still functional, as they become more mature, they lose their functionality, both in terms of cytotoxicity and degranulation. In a similar way to the increased activation (CD25) and senescence (CD57) of CD8+ T cells, the NK cells appear to lose functionality as they mature, although unlike CD8 T

cells, this was independent of CMV exposure and titers (18). It would appear that this mechanism is not applicable in NK cells, as there was no correlation between either CMV titers or seropositivity and NK cell activity. Furthermore, as there was no clear HPA axis phenotype, although there was a trend towards an increase in stress-induced gluconeogenesis, we conclude that the HPA axis is unaffected in our MD paradigm and, as such, cannot be responsible for the NK cell phenotype either. We saw clear differences in baseline glucose levels in both our MD groups. We initially hypothesized that this was a function of the altered HPA axis (106). However, it would appear that changes in the external environment did not induce changes in the HPA axis stress response nor in the overall glucocorticoid levels. As such, our recent hypothesis that the disturbed HPA axis fundamentally alters hepatic metabolism such as gluconeogenesis to mechanistically crystalize the early-life adversity associated risk of metabolic diseases may, in fact be erroneous, and that this may be independent of the HPA axis.

NK cells are known to be affected by current acute and chronic stress. The early work by Schedlowski et al. showed that acute stress in adulthood, in this case novice parachute jumpers, had significant changes in the circulating lymphocyte subsets as well as functional differences in NK cells immediately post-stress. We expanded on this to demonstrate the kinetics of NK cell redistribution throughout the day, coupled to the circadian HPA axis rhythm, though in the work of Schedlowski it appeared to be associated with noradrenaline levels (107, 108). Both studies suggested that this rapid mobilization of NK cells was a natural physiological reaction to an external stressor, in agreement with both their natural role as an immediate initiator of the immune response before adaptation starts, and as an evolutionary mechanism, preparing the body to fight injury or infection after encountering an acute stressor. Sympathetic nervous system control of NK cell action *via* noradrenaline has been suggested to be an advantage because of the speed with which the immune system can be primed to act after a stressful encounter, as well as the speed in which the priming can be terminated and homeostasis re-established (109) through the inflammatory reflex (110). There is a similar dearth of literature on the effects of chronic stress on NK cell functioning. In a similar manner to our observation of decreased NK cell functionality, chronic low-dose glucocorticoid administration reduced histone acetylation levels around promoters for two essential NK cell produced effectors: perforin and granzyme B. This was associated with lower mRNA transcript levels, lower protein levels, and NK cells were functionally impaired in a manner similar to what we report in both our rat model and in the EpiPath cohort. The lower perforin and granzyme B levels decreased their cytolytic activity. Inversely, the same administration regime increased histone acetylation of the IFN-γ and IL-6 promoters, up-regulating transcription and functional protein levels (111). The situation is, however, far from clear-cut. Children with current chronic stress from maternal mental health had higher levels of psychiatric symptoms as well as an increase in the number of illness episodes that was associated with increased NK cell cytotoxicity (102). However, none of the data available so far addresses the long-term effect of early life psychosocial stress and adversity, and the differences in NK cell functionality when the stressor is no longer present. In our previous report from the EpiPath cohort, multiple correction testing during our survey of the complete immune system meant that NK cells only narrowly missed significance (18), and the only other comparable study did not investigate NK cells (64, 112). Both of these studies reported that ELA induced a long-term immunosenescence and reduced T-cell functionality that was most probably due to continued re-activation of viruses such as CMV. It would seem logical that the exposure to a period of chronic stress in both models presented here has had a similar effect on NK cells. The two experimental systems show that once the period of ELA has resolved, NK cells are programmed with a long-term hypo-reactivity. As for acute stress preparing the NK cells to deal with an immediate infectious threat or potential wound, we suggest that this long-term hypo-reactivity is a similar evolution. The sensitive early life period would appear to have prepared the NK cells for an environment in which they can expect to be more regularly activated, and as such, to avoid any negative effects associated with NK cell secreted effector molecules or cytokines.

Although NK cells are often associated with a positive regulation of the immune response, they are also associated with the development of immunopathologies. In chronic hepatitis B virus infection, NK cells contribute to both liver inflammation and injury (113, 114), and aggravate and increase the lethality of bacterial infections in murine models (115, 116). NK cell activity was found to be impaired in patients that suffered from multiple sclerosis (117, 118), type-1 diabetes (119) and cardiovascular diseases (120–122), and found to sustain joint inflammation in rheumatoid arthritis patients (123); the risk of the latter three are all increased by exposure to ELA (16, 124–126). This raises the possibility of the long-term alteration of the NK cell phenotype underlying the pathophysiological effects of ELA. It may seem counterintuitive that we see an accumulation of NK cells of a more mature phenotype, but of lower functionality. This does, however, very clearly mirror our prior data on CD8+ T cells. Although it is not as well documented as for T cells, immunosenescence also occurs in other cell types, such as B (65, 66) and NK cells (67, 68). Our data suggests that, as suggested by Judge et al. (67), this corresponds to the accumulation of senescent NK-cells. Although NK cells have received far less attention, anergy, senescence and exhaustion are distinct dysfunctional entities that parallel the same processes in T cells and are all characterized by a significant reduction in both proliferation and NK-effector functions. There is the caveat that the expression of CD57 in natural killer cells does not necessarily mean they are senescent but rather that they reached a higher maturation state, which is accompanied by functional changes similar to those observed in senescent T cells: less proliferation and higher cytotoxic capacity (28, 67, 69). Furthermore, an alternative interpretation may be that at the single cell level ELA reduces NK cell effector functions, while at the population level the NK cells were more mature. This is somewhat confirmed by our previous report of ELA reducing activation CD69 levels on both total NK cells and CD56dimCD16+ NK

cells. Unfortunately, in our previous report, this result was highly significant prior to, but not after, post-hoc multiple testing correction (18)

The limitations of our pre-clinical study include potential litter effects and the absence of a clear HPA axis phenotype. Similarly, the number of EpiPath participants analyzed was limited, however, based on the rat MD data, our power calculation suggested that to see the same phenotype in the cohort only 14 participants were required to have 80% power at alpha 0.05, which we largely exceeded. However, these are clearly outweighed by the reproduction of the functional NK cell phenotype in the EpiPath cohort. Furthermore, the identical phenotype in the MD model and the cohort allowed us to exclude the two most prominent mechanistic hypotheses from the literature – HPA axis control, and continual CMV reactivation. Nevertheless, ELA has a direct impact in the maturation state and later exhaustion of NK cells that may impair their activity and lead to uncontrolled reactions in adulthood.

It is clear that all cells of the immune system are not equally affected by ELA. Here, we have expanded our prior observation of T cell immunosenescence to a novel, unbiased examination of the immune system, identifying NK cells as functionally affected by ELA in both the rat MD model and in our human institutionalization – adoption cohort and NKT-like cells to be differently expressed in the rats. The immature NK cells appear to retain their functionality, however, as they mature towards CD56dim NK cell subsets and CD56- phenotype, their cytotoxic and degranulation potential are reduced. It is now evident that alterations in the HPA axis, either as stress-induced cortisol/corticosterone production or gluconeogenesis, are not responsible for the immune phenotype. The challenge is now to understand how ELA is inducing such changes and the role of both T cell and NK/NKT-like cells functional and expression loss in the long-term ELA-induced disease risk.

## 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 Luxembourg National Research Ethics Committee (CNER, No 201303/10 v1.4) and the Ethics Review Panel (ERP, University of Luxembourg, No 13-002). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by local Animal Welfare Structure (DII-2017-18).

## AUTHOR CONTRIBUTIONS

Conceptualization: SF and JT. Literature review: SF and JT. Data collection: SF, NP, SM, ME, FL, and MT. Data analysis: SF, NP, JZ, and JT. Manuscript writing and editing: SF, NP, JZ, and JT. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.674532/full#supplementary-material

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# FAM13A regulates KLRG1 expression and interferon gamma production of natural killer cells

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This paper concerns with the polymorphic gene family with sequence similarity 13, member A (*FAM13A*). It has been linked to an increased risk associated with lung cancer and chronic obstructive pulmonary diseased (COPD). The authors used a *Fam13a* knock out (KO) mouse model to show that the upregulation in the expression of terminal differentiation and inhibitory marker, KLRG1 (killer cell lectin-like receptor G1) in natural killer (NK) cells is caused by the depletion of the *Fam13a.* Similarly, we saw an impaired NK cell IFN-γ production from *Fam13a*-deficient mice. I contributed in literature search.

# FAM13A regulates KLRG1 expression and interferon gamma production of natural killer cells

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## Abstract

**The polymorphism of the gene *FAM13A* (family with sequence similarity 13, member A) is strongly linked to the risk of lung cancer and chronic obstructive pulmonary disease, which are among the leading causes of mortality and morbidity in lung-related diseases worldwide. However, the underlying molecular and cellular mechanisms through which *FAM13A* contributes to the pathogenesis of these diseases largely remain unclear. Here, using a *Fam13a* knock out (KO) mouse model, we showed that *Fam13a* depletion upregulated the expression of the terminal differentiation and inhibitory marker, KLRG1 (killer cell lectin-like receptor G1) in natural killer (NK) cells. NK cells from *Fam13a*-deficient mice showed impaired IFN-γ production either against target tumor cells or following various cytokine cocktail stimulations. Furthermore, the number of lung metastases induced by B16F10 melanoma cells was increased in *Fam13a*-KO mice. Collectively, our data suggest a key role of *FAM13A* in regulating NK cell functions, indicating that the key lung-disease risk gene *FAM13A* might contribute to the pathogenesis of several lung diseases via regulating NK cells.**

## Keywords

NK cells; KLRG1; Interferon Gamma (IFNG); Lung metastasis

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## Abbreviations

APC, Antigen-presenting cells

AR, Activating receptor

AWS, Animal welfare structure

CFSE, Carboxy fluorescein succinimidyl ester

CM, Central memory

COPD, Chronic obstructive lung disease

EM, Effector memory

eQTL, Expression quantitative trait loci

FACS, Flow cytometry

Fam13a, Family with sequence similarity 13, member A

FBS, Fetal bovine serum

GWAS, Genome-wide association study

HET, Heterozygous

IFN-γ, Interferon gamma

IL-2/-12/-15/-18, Interleukin 2/12/15/18

IR, Inhibitory receptor

KIR, Killer immunoglobulin-like receptors

KLRG1, Killer cell lectin-like receptor G1

KO, Knockout

MFI, Mean fluorescent intensity

MHC, Major histocompatibility complex

NK, Nature Killer

PBS, Phosphate-buffered saline

PCR, Polymerase chain reaction

pLN, peripheral lymph nodes

Tconv, Conventional CD4 T cells

Treg, CD4 regulatory T cells

WT, Wildtype

## Introduction

Several independent genome-wide association studies (GWAS) among various populations have already reproducibly shown the strong link between the polymorphism of *Family with sequence similarity 13, member A (FAM13A)* and chronic obstructive pulmonary disease (COPD) and lung cancer, the leading entities causing mortality in lung-related diseases worldwide [1-12]. Functional studies have revealed that *Fam13a* depletion reduces the susceptibility to COPD via inhibiting the WNT/β-catenin pathway in a mouse model [13]. In human lung tumor cell lines, knocking-down *FAM13A* reduced tumor cell proliferation but induced cell migration *in vitro* [14]. However, the exact *in vivo* physiological role of FAM13A in the complicated process of tumor onset and metastasis still remains mysterious. Recently, a few studies indicated that FAM13A might be involved in the regulation of immune responses. Eisenhut et al. observed the upregulation of FAM13A in $CD4^+CD25^-$ effector T cells but reduced expression in T regulatory cells (Tregs) of human blood [14]. In contrast, in our previous study [15], the expression of FAM13A was increased in human Tregs vs. $CD4^+$ effector T cells following TCR stimulation. Another work using the expression quantitative trait loci (eQTL) method has briefly investigated the functional effect of *FAM13A* knockdown on human naïve $CD4^+$ T cells *in vitro* [16]. Meanwhile, microRNA-328 in M2 macrophage-derived exosomes has been demonstrated to regulate the progression of pulmonary fibrosis via *Fam13a* in an animal model [17]. Those different reports all suggest potential roles of *Fam13a*, although sometimes even controversial, in immune cells, which, however, highlights a need of more investigation to clarify further the complicated *invivo* functions of *Fam13a* in the immune system.

Here we utilized a *Fam13a* whole-body knockout (KO) mouse model to study the potential *in vivo* impact of *Fam13a* on cellular phenotypes and functions of major immune cells, including NK cells, T and B lymphocytes. We found that *Fam13a* did not affect the homeostatic composition and basic functional markers of total B cells, $CD4^+$ T cells, $CD4^+$ Tregs and $CD8^+$ T cells. Interestingly, *Fam13a* depletion upregulated the expression of the critical maturation and inhibitory marker, KLRG1 [18, 19] while impairing the IFN-γ production of NK cells. Notably, *Fam13a* depletion exacerbated lung metastasis induced by B16F10 melanoma cells in the C57BL/6 (B6) mouse model. Altogether, our results provide strong evidence that *Fam13a* is an important component regulating the effector functions of NK cells, mostly through modulating KLRG1 expression and IFN-γ production.

## Results

### *Fam13a* depletion has no spontaneous effects on homeostatic cellularity and functions of major immune subsets

To identify the potential effect of *Fam13a* on the homeostatic phenotypes of the immune system, we first analysed the composition of different major immune cell types in various lymphoid organs and

tissues of *Fam13a* KO mice [Fam13a$^{tm2a\ (KOMP)Wtsi}$] with B6 background obtained from the Knockout Mouse Project. The same mice have been characterized elsewhere [13], but not in the context of immunology. As a starting point, we compared the mRNA expression of *Fam13a* in the lung tissue between *Fam13a* knockout (KO) and wild type (WT) mice and that in NK cells isolated from spleen. As expected, *Fam13a* KO mice relative to WT littermates exhibited a clear reduction in the transcript expression of *Fam13a* in both the lung tissue (**S1a Fig**) and NK cells (**S1b Fig**). We checked the frequency of CD19$^-$CD3$^-$NK1.1$^+$ NK cells in several relevant tissues including spleen (**S1c and 1d Fig**), peripheral lymph nodes (pLNs) (**S1e Fig**), bone marrow (BM) (**S1f Fig**) and lung (**S1g Fig**) and no significant difference between homeostatic *Fam13a* KO and WT mice was observed. We also could not see any significant difference in the frequencies of several other major types of lymphocytes, such as CD3$^+$ total T cells, CD19$^+$ B cells, CD8$^+$ T cells, CD4$^+$ T cells as well as CD4$^+$ Treg cells (**S1 Table**). We further characterized the naïve and memory compartment of CD4$^+$ and CD8$^+$ T cells. Again, no significant difference was found in the percentages of naïve (CD44$^{low}$CD62L$^{high}$) and effector memory (CD44$^{high}$CD62L$^{low}$, EM) CD4$^+$ T cells and naïve, EM and central memory (CD44$^{high}$CD62L$^{high}$, CM) CD8$^+$ T cells at the tested age (8-12 wks) (**S1 Table**). In conclusion, *Fam13a* depletion does not lead to spontaneous abnormalities in the development of various major immune cells under homeostasis.

To further evaluate whether *Fam13a* influences the basic homeostatic functions of those immune cells, we analysed the expression of an activation marker (CD69), an exhaustion/activation marker (PD-1) and a proliferation marker (Ki-67) of CD4$^+$ and CD8$^+$ T cells. No significant difference in the frequency of the cells expressing any of those markers among CD4$^+$ T cells and CD8$^+$ T cells in spleen exhibited between *Fam13a* KO and WT mice (**S1 Table**). We also analyzed a key subset of T cells, Tregs, which play a key role in suppressing responses of effector immune cells. Therefore, we evaluated their suppressive function by co-culturing the CFSE-labelled CD4$^+$ conventional T cells (Tconv), antigen-presenting cells (APCs) and Tregs. Loss of *Fam13a* did not compromise Treg suppressor function against Tconv proliferation (**S2a Fig**). In short, *Fam13a* depletion causes deficiency neither in the development, nor in the activation or proliferation of major lymphoid subsets such as CD4$^+$ and CD8$^+$ T cells, at least in the analyzed mice lines aged 8-12 weeks under homeostatic conditions.

### *Fam13a* depletion upregulates the expression of KLRG1 in NK cells

As demonstrated above, *Fam13a* depletion did not generate differences in the frequency of total NK cells under homeostatic conditions, which, however, cannot exclude the potential effect of *Fam13a* on certain NK functions. To explore whether *Fam13a* affects major functional markers of NK cells, we first investigated the maturation profile of NK cells by checking the co-expression of CD27 and

CD11b. Interestingly, we noticed that *Fam13a* depletion led to a significant but only modest change in the proportions of immature $CD11b^{-}CD27^{+}$and mature $CD11b^{+}CD27^{-}$ NK cells (**S3a-c Fig**). Since $CD11b^{+}CD27^{-}$ NK cells are mature NK cells, representing the major KLRG1-expressing NK subset [19-21], we also analyzed the expression of the inhibitory receptor KLRG1 among NK cells. In line with the data related to CD27 and CD11b subsets, we observed a much higher percentage of KLRG1-expressing cells among total NK cells in both spleen (**Fig 1a, b**) and pLNs (**Fig 1c, d**) of the *Fam13a*-KO mice.

To get a more comprehensive picture, we further analyzed different inhibitory receptors (IR) and activation receptors (AR) of NK cells, whose engagement is critical to regulate and balance NK cell activities. For the AR, the frequency of $NKp46^{+}$, $Ly49H^{+}$ and $Ly49D^{+}$ NK cells (**S3d-f Fig**), as well as the expression of 2B4 and NKG2D (**S3g-h Fig**) were not significantly altered in *Fam13a* KO NK cells. For the IR, we did not observe any significant difference in the expression of Ly49A, Ly49C/I and NKG2A (**S3i-k Fig**) on NK cells between *Fam13a* KO and WT mice. In summary, loss of *Fam13a* critically upregulated KLRG1 expression, indicating that *Fam13a* has an inhibiting or regulatory effect on NK cell maturation or preventing to some extent premature entry into the senescent state.

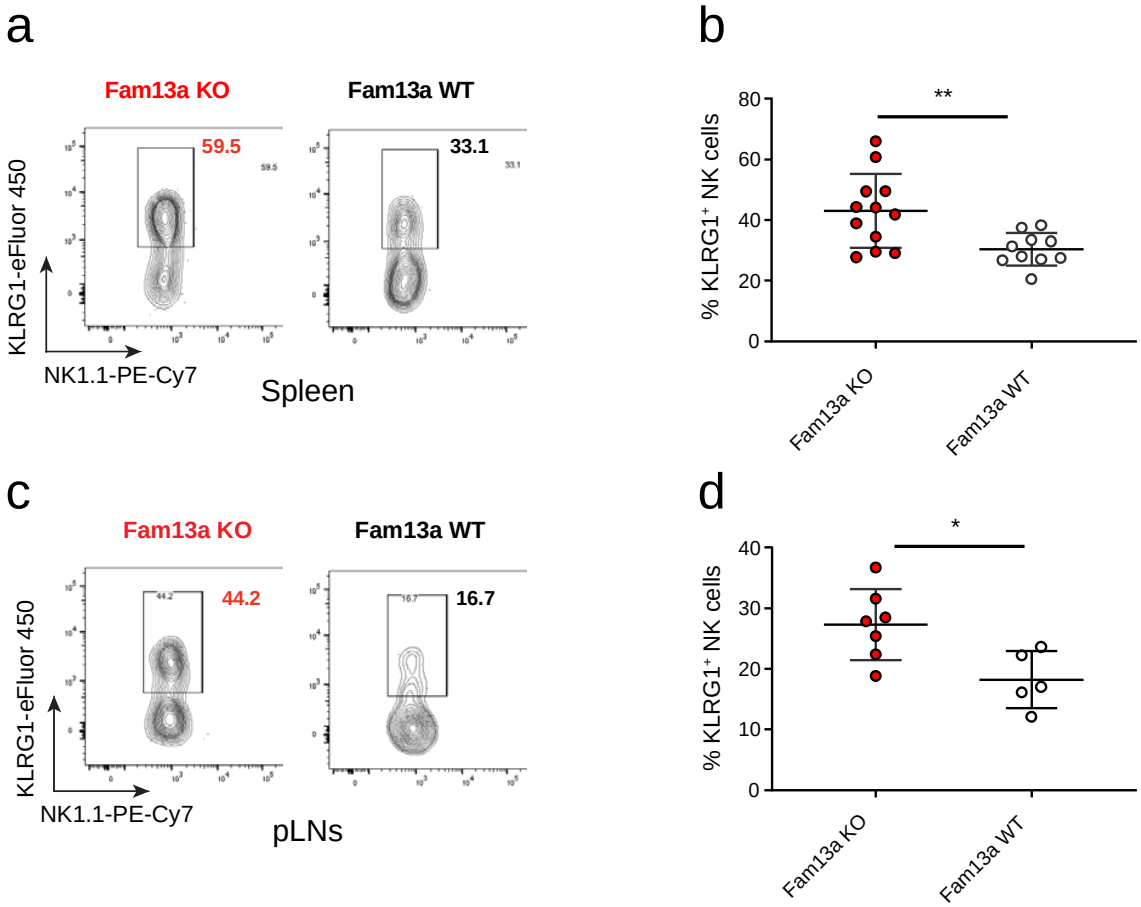


**Fig 1. *Fam13a* depletion upregulates the expression of KLRG1 in NK cells**. **(a)** Representative FACS plots of KLRG1 and NK1.1 expression on $CD19^-CD3^-$ $NK1.1^+$cells in spleen. **(b)** Percentages of $KLRG1^+CD19^-CD3^-$ $NK1.1^+$ among NK cells in spleen of *Fam13a* KO and WT littermates (KO, n=12; WT, n=10). **(c)** Representative FACS plots of KLRG1 and NK1.1 expression on $CD19^-CD3^-$ $NK1.1^+$cells in pLNs of *Fam13a* KO and WT littermates. **(d)** Percentage of $KLRG1^+$ subpopulation among NK cell in pLNs of *Fam13a* KO and WT littermates (KO, n=7; WT, n=5). Results represent four independent experiments. Data are mean ± s.d. The p-values were determined by a two-tailed Student's t-test. n.s. or unlabeled, not significant, *p<=0.05, **p<=0.01 and ***p<=0.001.

## *Fam13a* depletion impairs NK-cell IFN-γ production

A few studies have already demonstrated that KLRG1 inhibited NK-cell IFN-γ production as well as cytotoxicity [18, 22, 23]. Since we observed a significant upregulation in the frequency of the $KLRG1^+$ NK cells, we further assessed IFN-γ production and degranulation of NK cells. We expanded NK cells by culturing total splenocytes isolated from *Fam13a* KO or WT littermates in the presence of a high concentration of IL-2 for 5 days**.** Then the cells were restimulated via different cytokine cocktails to check IFN-γ production **(Fig 2a)**. We found that *Fam13a* deficiency did not affect the survival and expansion of NK cells *in vitro* (**Fig 2b-c**). However, when stimulated with the cytokine cocktail containing IL-2, IL-12 and IL-15, *Fam13a*-deficient NK cells produced a significantly lower amount of IFN-γ compared with their WT counterparts (**Fig 2d-e**). Furthermore, even when stimulated with the strong activation cytokine cocktail composed of IL-2, IL-12 and IL-18, the deficiency in IFN-γ production capacity *of Fam13a*-KO NK cells was not compensated (**Fig 2f-g**). Taken together, our data demonstrate that *Fam13a* depletion impairs IFN-γ production in NK cells under various cytokine-cocktail stimulations *in vitro*.

To further check the killing capacity of NK cells towards tumor cells, we pre-activated NK cells *in vivo* by injecting the TLR3 agonist poly(I:C) into *Fam13a* KO and WT littermates (**Fig 2h**). We then evaluated the expression of IFN-γ and the degranulation marker CD107a of NK cells following the incubation with the target tumor cell line YAC-1. In line with the *in vitro* observations, lower amounts of IFN-γ were produced by *Fam13a* KO NK cells, as shown by different readouts, such as the lower frequency of IFN-γ-expressing NK cells (**Fig 2i**), the smaller absolute number of IFN-γ-expressing NK cells (**Fig 2j**) and the decreased MFI of IFN-γ per NK cell in IFN-$\gamma^+$ NK cells (**Fig 2k**). CD107a reflects the cytotoxic activity of NK cells and cytotoxic $CD8^+$ T lymphocytes [24, 25]. No significant difference was observed in the percentages of CD107a-expressing cells among total NK cells (**Fig 2l**) and in the absolute number of $CD107a^+$ NK cells (**Fig 2m**) between *Fam13a* KO and WT mice following poly(I:C) injection. Interestingly, loss of *Fam13a* caused a modest but significant decrease in the degranulation capacity per individual NK cell against YAC-1, as indicated by the decreased MFI of CD107a in *Fam13a* deficient NK cells (**Fig 2n**). In conclusion, *Fam13a* depletion essentially impairs NK-cell IFN-γ production against YAC-1 tumor cells.

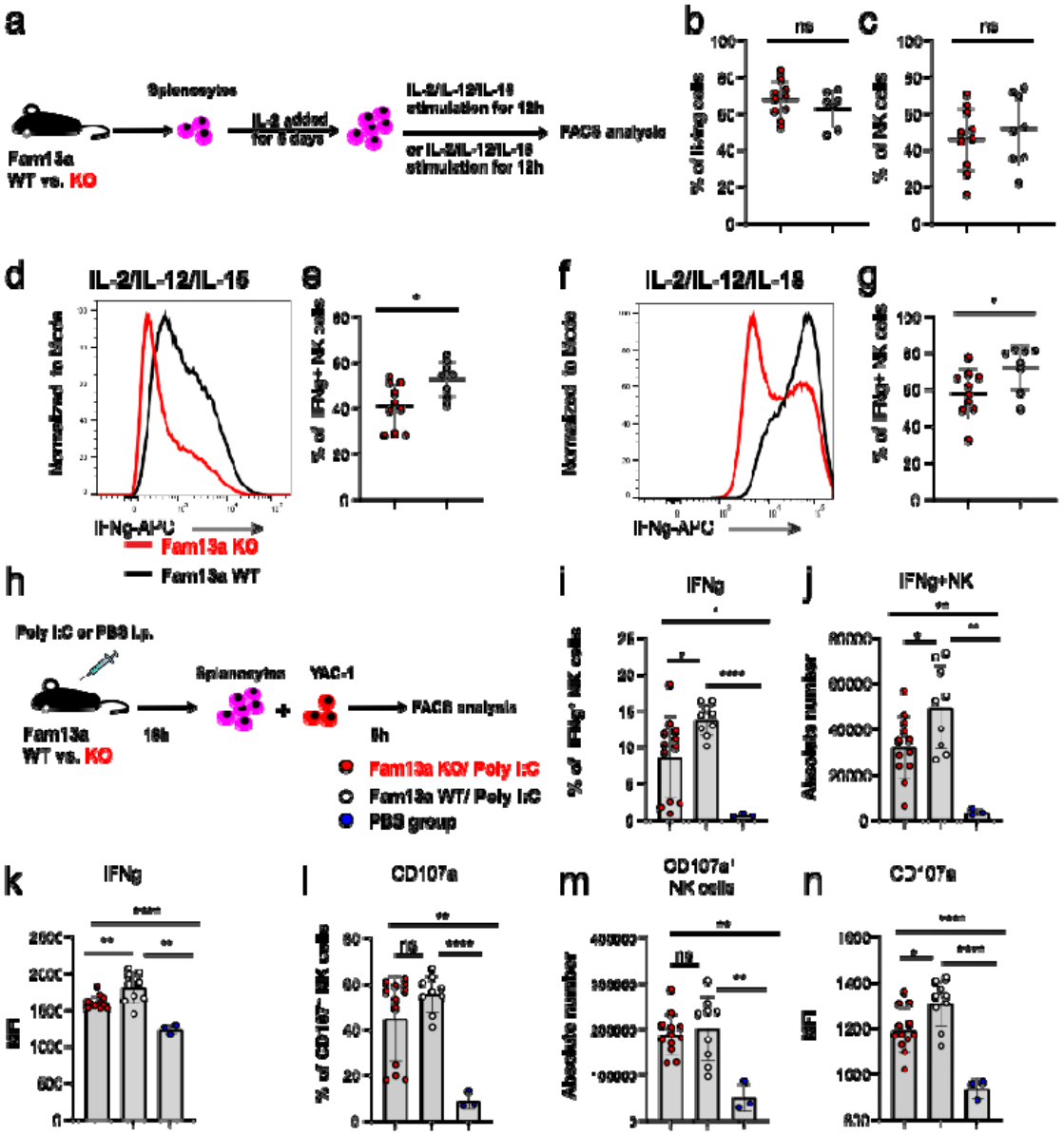



**Fig 2. *Fam13a* deficiency impairs NK-cell IFN-γ production following *in vitro* or *ex vivo* stimulation. (a)** Schematic of the experimental setup to stimulate NK cells with different cytokine cocktails. **(b)** Percentage of living cells after 5-day expansion in the presence of IL-2 (KO, n=10; WT, n=7). **(c)** Percentage of NK1.1$^{+}$ NK cells after 5-day expansion in the presence of IL-2 (KO, n=10; WT, n=8). **(d)** Representative FACS histogram overlay of IFN-γ production of *Fam13a* KO (red line) and WT (black line) NK cells following 5-day IL-2 expansion and IL-2, IL-12 and IL-15 re-stimulation. **(e)** Percentage of IFN-γ producing cells among total NK cells after IL-2, IL-12 and IL-15 re-stimulation (KO, n=12; WT, n=9). **(f)** Representative FACS histogram overlay of IFN-γ production of *Fam13a* KO (red line) and WT (black line) NK cells after 5-day IL-2 expansion and IL-2, IL-12 and IL-18 re-stimulation. **(g)** Percentage of IFN-γ producing cells among total NK cells after IL-2, IL-12 and IL-18 re-stimulation. **(h)** Schematic of the experimental setup to analyze the ex-vivo degranulation capacity of NK cells. *Fam13a* KO or WT littermates were injected intraperitoneally (i.p.) with 150 µg poly(I:C) or PBS and then the next day splenocytes were incubated with YAC-1 cells to evaluate NK cell IFN-γ production and degranulation. **(i)** Percentage of IFN-γ producing NK cells of *Fam13a* KO and WT NK cells against YAC-1 tumor cells. **(j)** Absolute number of IFN-γ producing NK cells of *Fam13a* KO and WT NK cells against YAC-1 tumor cells. **(k)** Geometric mean of IFN-γ expression intensity in total IFN-γ$^{+}$ NK cells of *Fam13a* KO and WT NK cells. **(l)** Percentage of CD107a producing NK cells of *Fam13a* KO and WT NK cells against YAC-1 tumor cells. **(m)** Absolute number of CD107a producing NK cells of *Fam13a* KO and WT NK

cells against YAC-1 tumor cells. **(n)** Geometric mean (MFI) of CD107a expression intensity in total CD107a$^+$ NK cells of *Fam13a* KO and WT NK cells (KO, n=12, WT, n=9). Results are representative of three (**b, c, e, g**) and two (**i-n**) independent experiments. Data are mean ± s.d. The *p*-values were calculated by a two-tailed Student's t-test. n.s. or unlabeled, not significant, **p*<=0.05, ***p* <=0.01 and ****p* <=0.001.

## *Fam13a* deficiency worsens lung metastasis induced by melanoma cells *in vivo*

Having shown that *Fam13a* depletion impaired certain critical effector functions of NK cells *in vitro* and *ex vivo*, we sought to further investigate the *in vivo* effects. Since NK cells are critical in the control of tumor metastasis [26], we induced lung metastasis by intravenous injection of B16F10 melanoma cells into *Fam13a* whole-body KO and WT littermates. After 16 days post inoculation, we evaluated the lung metastases (**Fig 3a**). Compared with WT littermates, *Fam13a* KO mice had developed much more tumor metastases in the lung (**Fig 3b-c**). For cellular composition phenotypes, we first analyzed NK cells in the spleen and lung tissues. The frequency and absolute number of NKp46$^+$NK1.1$^+$ NK cells among CD3$^-$CD19$^-$ splenocytes was much lower in B16F10-inoculated *Fam13a* KO mice compared with that in WT littermates (**Fig 3d-f**). Importantly, *Fam13a* KO mice also had a lower percentage of infiltrated NKp46$^+$NK1.1$^+$ NK cells among CD3$^-$CD19$^-$ cells in the tumor tissue, i.e., lung in comparison to WT littermates (**Fig 3g**). Furthermore, similar to the homeostatic phenotype of *Fam13a* KO NK cells (**Fig 1a-b**), a much higher frequency of KLRG1$^+$ cells were also observed among total NK cells in both spleen (**Fig 3h-i**) and lung (**Fig 3j**) of *Fam13a* KO mice in comparison with that in WT littermates. In the chronic infection model, such as chronic hepatitis C virus infection, KLRG1$^+$ NK cells represent an exhausted phenotype and lower capacity for IFN-γ production [27-29]. This indicates that both the decreased number and the high level of KLRG1 of NK cells in *Fam13a* KO mice together aggravate tumor metastasis.

Although NK cells are indispensable in suppressing B16F10-induced lung metastasis, we hitherto cannot exclude the potential role of other immune cells [30]. We therefore assayed lung-infiltrating T lymphocytes, CD4$^+$ T cells and cytotoxic CD8$^+$ T cells. We observed a higher percentage of CD4$^+$ T cells and CD25$^{high}$CD4$^+$ Tregs **(S3a-b Fig)**, but not CD8 T cells (**S3c Fig**) in *Fam13a* KO compared to that in WT littermates. Furthermore, there was a higher frequency of effector memory (EM) but a lower percentage of naïve CD4$^+$ T cells in *Fam13a* KO mice, indicating more CD4$^+$ T cells were participating in the fight against melanoma, which could be either a driving factor or a secondary response of deteriorated lung metastasis (**S3d-e Fig**). This effect on naïve or memory compartment was not so obvious yet in infiltrated CD8$^+$ T cells (**S3f Fig**). We also found there were much higher percentages of PD-1$^+$ CD4$^+$ T cells in the lung (**S3g-h Fig**), indicating more exhausted or activated CD4$^+$ T cells in *Fam13a* KO mice. Again, the effect on PD-1 expression was not so significant in infiltrated CD8$^+$ T cells of *Fam13a* KO mice (**S3i Fig**). These results indicate that, although a major role of *Fam13a*-deficient NK cells in the rejection of melanoma cells is likely, we cannot definitively exclude a potential contribution from other *Fam13a*-deficient immune cells, e.g., CD4$^+$ T cells.

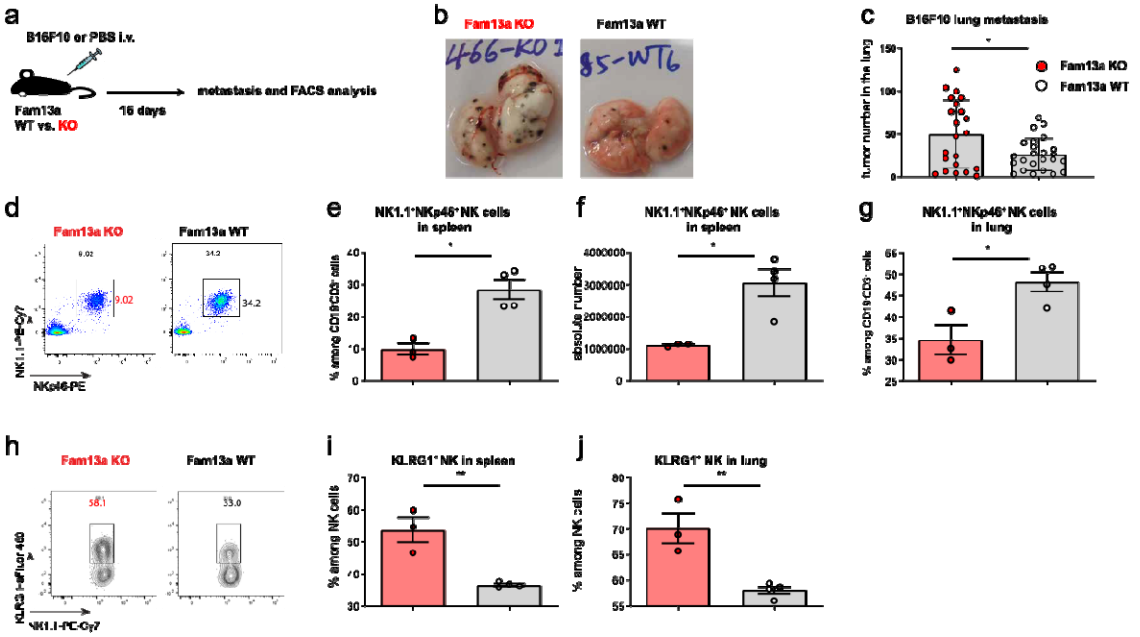



**Fig 3. *Fam13a* deficiency aggravates lung metastasis induced by melanoma cells. (a)** Schematic of the experimental setup for lung metastasis model induced by B16F10 melanoma cells. **(b)** Representative photographs of freshly isolated lungs after B16F10 cell inoculation. **(c)** Quantified metastatic foci of freshly isolated lungs after B16F10 cell inoculation (pooled KO, n=21; pooled WT, n=23). **(d)** Representative FACS plot of NKp46 and NK1.1 expression on *Fam13a* KO and WT $CD3^-CD19^-$ living singlet lymphocytes. **(e, g)** Percentage of $NKp46^+NK1.1^+$ NK cells out of $CD3^-CD19^-$ cells in spleen (**e**) or lung (**g**) of *Fam13a* KO and WT littermates. **(f)** Absolute number of $NKp46^+NK1.1^+$ NK cells out of $CD3^-CD19^-$ cells in spleen of *Fam13a* KO and WT littermates. **(h)** Representative FACS plot of KLRG1 and NK1.1 expression on *Fam13a* KO and WT NK cells in the spleen. **(i, j)** Percentage of $KLRG1^+$ NK cells among total NK cells in spleen (**i**) or lung (**j**) (KO, n=3, WT, n=4). Data are mean ± s.d. The *p*-values were calculated by a two-tailed Student's t-test. n.s. or unlabeled, not significant, **p*<=0.05, ***p* <=0.01 and ****p* <=0.001.

## Discussion

In this work, we showed that (i) *Fam13a* depletion upregulates the expression of the inhibitory and maturation marker, KLRG1 of NK cells, (ii) *Fam13a* is required for an optimal NK cell IFN-γ production, (iii) *in vivo*, the KO mice develop significantly more lung metastases after intravenous injection of a melanoma cell line, and (iv) the absence of *Fam13a* does not generate a phenotypic impact on B and T cell numbers and subset distribution, nor on Treg function (at least under homeostasis).

For *ex vivo* phenotyping analysis of *Fam13a*-KO mice, the most striking observation we found is the enhanced expression of KLRG1 in *Fam13a*-KO NK cells vs. WT NK cells. KLRG1 is an IR of the C-type lectin superfamily able to inhibit NK cell functions (cytotoxicity and cytokine production) upon recognition of its non-MHC class I ligands, which are E-, N- and R-cadherins [18, 22, 23]. The latter are adhesion molecules downregulated on cancer cells. In mice, KLRG1 is expressed on roughly a third of NK cells, but it increases upon infections [22, 23]. In *Fam13a*-KO mice, particularly in spleen,

this value was almost doubled in most of the animals. Furthermore, KLRG1 is regarded as a maturation and terminal differentiation marker of NK cells. In line with this notion, we also observed modestly increased percentages of mature $CD11b^{+}CD27^{-}$ NK cells, which suggests that *Fam13a* has an inhibiting or regulatory effect on NK cell maturation or preventing to some extent premature entry into the senescent state.

Our *in vitro* NK cell functional studies showed that *Fam13a*-deficient NK cells displayed an impaired IFN-γ production following IL-2/IL-12/IL-15 or IL-18 cytokine cocktails stimulation. Furthermore, after an *in vivo* pre-activation of NK cells via the TLR3 agonist poly(I:C), *Fam13a*-KO NK cells produced less IFN-γ compared to WT NK cells. We also observed a mild effect of *Fam13a* depletion on NK degranulation, i.e., no significant difference in the percentages of CD107a expressing NK cells, except for a modest decrease in the MFI of $CD107a^{+}$ NK cells. Since KLRG1 inhibits IFN-γ expression in NK cells at least in chronic viral infections [27-29], it is not surprising to observe impaired IFN-γ production in NK cells following various types of activation.

In our *in vivo* B16F10 melanoma models, we observed a strong reduction in the number of NK cells in spleen and lung of the KO mice compared to their WT counterparts, but a significantly higher frequency of $KLRG1^{+}$ NK cells. This might suggest at first sight that more NK cells were inhibited following the induction of lung metastasis. Moreover, *Fam13a*-KO mice displayed a significantly higher number of lung metastases compared to the WT animals, which is in accordance with our *in vitro* findings**,** showing a hypofunctional state of KO NK cells. Since IFN-γ production by NK cells is critical to control B16F10-induced lung metastasis [31], the observed reduced capability of IFN-γ production in *Fam13a* KO NK cells, might at least partially contribute to more severe metastasis *in vivo*. In line with our observation about a higher expression of KLRG1 and the *in vitro* hypofunctional state of NK cells in *Fam13a* KO mice, KLRG1 neutralization antibody plus anti-PD-1 combinatory treatment vs. the anti-PD-1 therapy alone has achieved better survival rate and response to tumor volume in B16F10 melanoma models [32]. Furthermore, $KLRG1^{+}$ NK cells possibly produced, as previously described, less IFN-γ than $KLRG1^{-}$ NK cells in mice [19]. These data together can already well explain more severe metastasis observed in *Fam13a*-KO mice.

We also observed certain effects of *Fam13a* on filtrated T cells, especially on $CD4^{+}$ T cells, in the induced lung metastasis experiments, so that further depletion experiments and cell-type specific knockout animal models might be required to figure out whether our observation is driven by NK cells alone or together with other cell types. In short, here we demonstrated a previously unrecognized critical role of *FAM13A* in regulating KLRG1 expression and IFN-γ production of NK cells using both *in vitro* and *in vivo* models. As *FAM13A* is strongly associated with the risk of several common lung diseases, our discovery paves the way to develop a novel potential target to mediate the functions

of immune cells, especially NK cells in the fight against complex lung diseases, one of the major health threats globally. Encouragingly, the IFN-γ production in NK cells from patients with another *FAM13A*-strongly-linked lung disease, i.e., COPD, was reported to be impaired [33], indicating that our discovery might have translational potential.

## Materials and Methods

### Animals

*Fam13a* knock-out (KO) mice [Fam13a$^{tm2a\ (KOMP)Wtsi}$] from the project *CSD70561* with C57BL/6N background were obtained from the Knockout Mouse Project (KOMP) Repository at UC Davis. The *Fam13a*$^{-/-}$ (KO), *Fam13a*$^{+/-}$ (HET) and *Fam13a*$^{+/+}$ (WT) mice used in the experiments were age- and gender-matched littermates generated from *Fam13a*$^{+/-}$ heterozygous breeding pairs. All mice were bred and maintained in our specific pathogen-free animal facilities. Both genders were used in our experiments depending on the availability. But we only employed one gender for each individual experiment.

### Ethics statement

All animal experimental protocols were performed following the approval of the Animal Welfare Structure (AWS) of the University of Luxembourg and the Luxembourg Institute of Health, and authorization from Luxembourg Ministry of Agriculture.

### Flow cytometry analysis of immunophenotype

Cell suspensions were obtained by the mechanical disruption of mouse spleen, peripheral lymph nodes, bone marrow and lung. Red blood cells were then lysed in 1x lysing buffer (555899, BD Biosciences). One million cells per sample were pre-incubated with purified anti-mouse CD16/CD32 antibody (Fc Block™, 553141, BD Biosciences). Live/dead cells were discriminated by staining cells with the LIVE/DEAD® Fixable Near-IR Dead Cell Stain kit (L10119, Thermo Fisher Scientific) (dilution 1:500). Detailed antibody information was provide in Supplementary **Table S2**. Cell surface markers were stained by the combination of the following antibodies: anti-mouse CD3-BUV496 (612955, BD Biosciences) (dilution 1:100), anti-mouse CD3-BV421(562600, BD Biosciences) (dilution 1:100), anti-mouse CD19-BV510 (562956, BD Biosciences) (dilution 1:100), anti-mouse CD19-FITC (11-0193-82, eBioscience) (dilution 1:100), anti-mouse NK1.1-PE-Cy7 (108713, Biolegend) (dilution 1:50), anti-mouse NK1.1-BV421 (562921, BD Biosciences) (dilution 1:100), anti-mouse CD27-FITC (11-0271-82, eBioscience), anti-mouse CD11b-APC (553312, BD Biosciences) (dilution 1:100), anti-mouse CD11b-BUV395 (565976, BD Biosciences) (dilution 1:100), anti-mouse KLRG1-eFluor 450 (48-5893-82, eBioscience) (dilution 1:100), anti-mouse KLRG1-PerCP-Cy5.5 (138417, Biolegend) (dilution 1:100), anti-mouse CD69-PE (553237, BD Biosciences) (dilution 1:200), anti-mouse Ly49C/I-FITC (553276, BD Biosciences) (dilution 1:100), anti-mouse NKG2A-PE (142803, Biolegend) (dilution 1:100), anti-mouse CD69-BV605 (104529,

Biolegend) (dilution 1:200), anti-mouse Ly49A-BUV395 (742370, BD Biosciences) (dilution 1:100), anti-mouse Ly49D-BV711 (742559, BD Biosciences) (dilution 1:100), anti-mouse 2B4-FITC (553305, BD Biosciences) (dilution 1:100), anti-mouse Ly49H-BUV395 (744266, BD Biosciences) (dilution 1:100), anti-mouse NKp46-PE (137603, Biolegend) (dilution 1:100), anti-mouse NKG2D-APC (130211, Biolegend) (dilution 1:100), anti-mouse CD4-FITC (11-0042-82, eBioscience) (dilution 1:200), anti-mouse CD8-BUV805 (564920, BD Biosciences) (dilution 1:200), anti-mouse CD25-APC (557192, BD Bioscience) (dilution 1:100), anti-mouse CD44-PE-Cy7 (560569, BD Biosciences) (dilution 1:200), anti-mouse CD62L-PerCP-Cy5.5 (560513, BD Biosciences) (dilution 1:200), anti-mouse PD-1-BV711 (744547, BD Biosciences) (dilution 1:200). Intracellular staining for Foxp3-APC (126403, Biolegend) (dilution 1:200), Ki-67-BV605 (652413, Biolegend) (dilution 1:200) or Ki-67-eFluor 450 (48-5698-82, eBioscience) (dilution 1:200) was performed by using the Foxp3 Staining Kit (00-5523-00, eBioscience). The choice of the combination of the antibodies was decided not only by the specific experimental purposes, spectrum compatibility, but also by our Fortessa laser settings. Samples were measured on a BD LSR Fortessa$^{TM}$ and data were analysed with FlowJo (v10, Tree Star).

### Primary murine NK cell *in vitro* expansion and cytokine activation

Five million of splenocytes per well in 12-well plates were cultured in 2.5 ml of complete DMEM medium (for details refer below) with 1000 U/ml of recombinant human (rh) IL-2 (202-IL-010, R&D Systems) at 37°C, in 5% $CO_2$ incubators until day 5, when the cells were stimulated with 1000 U/ml of rhIL-2 and the combination of 10 ng/ml of recombinant murine (rm) IL-12 (210-12, PeproTech Inc) and 40 ng/ml of rm IL-15 (210-15, PeproTech Inc) or 100 ng/ml of rm IL-18 (B004-5, MBL) for one night. Before FACS staining, the cells were incubated with Golgistop (554724, BD Biosciences) (dilution 1:1500) and Golgiplug (555029, BD Biosciences) (dilution 1:1000) for 5 hrs. Cell surface markers were stained with anti-mouse CD3-BUV496 (612955, BD Biosciences) (dilution 1:100) and anti-mouse NK1.1-PE-CY7 (108713, Biolegend) (dilution 1:50). Dead cells were stained by using the LIVE/DEAD® Fixable Near-IR Dead Cell Stain kit (dilution 1:500). For intracellular cytokine staining, cells were first fixed/permeabilized with Cytofix/Cytoperm buffer (554714, BD Biosciences) and then stained with anti-mouse IFN-γ-APC antibody (554413, BD Biosciences) (dilution 1:100) diluted in Perm/Wash buffer (554714, BD Biosciences). NK cell culture medium is the complete DMEM medium (41965039, Thermo Fisher Scientific) with 10% of Fetal Bovine Serum (FBS, 10500-064, Thermo Fisher Scientific), 100 U/ml Penicillin-Streptomycin (15070-063, Thermo Fisher Scientific), 10 mM HEPES (15630080, Thermo Fisher Scientific) and 55 µM β-mercaptoethanol (M7522, Sigma-Aldrich).

### NK cell degranulation assay

*Fam13a* KO and WT littermates were injected with 150 mg of toxin-free poly(I:C) (tlrl-pic, InvivoGen) in 100 µl PBS via intraperitoneal injection. Eighteen hours later, the mice were sacrificed

and the spleens were harvested. Splenocytes (1E6) were cultured with 5E5 of YAC-1 target cells (2:1 ratio) in the presence of anti-mouse CD107a-BV421 antibody (564347, BD Biosciences) (1:100) for 1 hr. Golgiplug and Golgistop were then added for an additional 4-hr incubation. Cells were stained with anti-mouse CD3-BUV496 (612955, BD Biosciences) (dilution 1:100), anti-mouse NK1.1-PE-Cy7 (108713, Biolegend) (dilution 1:50) plus Live/Dead staining kit (dilution 1:500). Intracellular cytokine IFN-γ staining was performed as described above.

### Melanoma model for pulmonary metastasis

*Fam13a* KO and matched *Fam13a* WT littermate mice aged 8 to 10 weeks were used for lung metastasis study following intravenous injection of 2E5 of B16F10 melanoma cells in 100 µl of PBS. 16 days later, mice were sacrificed. The lung was perfused to remove the excessive blood by slowly injecting cold PBS through the right ventricle. Lung tumor nodules were counted. In addition, the spleens, pLNs and lungs were collected for FACS analysis. For cell isolation from the lung, the organs were cut into small pieces and the tissue digested in the lysis buffer (PBS containing 1.3 mg/ml collagenase Type II (234155, MERCK), 10% FBS, 50 U/ml benzonase endonuclease (101654, MERCK), 1mM $MgCl_2$) in a 37 °C incubator for 1 h. Single cell suspensions for FACS staining were made by filtering of the cells through 40 µM cell strainers (734-2760, VWR).

### Real time PCR

Spleen and/or lung were collected from *Fam13a* KO, *Fam13a* Het and *Fam13a* WT mice. NK cells in the spleen were isolated and purified by using mouse NK cell isolation kit II (130-096-892, Miltenyi Biotec). The purity of NK cells was analyzed by FACS. RNA from lung and NK cells of spleen was isolated via using the RNeasy Mini Spin Kit (74104, Qiagen). Genomic DNA was then removed by on-column DNase digestion (79254, Qiagen). The SuperScript III Reverse Transcriptase Kit (18080-044, Invitrogen) was employed for cDNA synthesis. qPCR was performed with the LightCycler 480 SYBR Green I Master Mix (04707516001, Roche Applied Science) using the LightCycler 480 system as previously described [15, 34]. *Fam13a* mRNA RT-PCR primers are referred according to another *Fam13a* related work [35] (*fwd*: CCG CTG CGA AGC TCA CAG GAA GAT G; *rev*: TTG GTC TCC AGC GTT GCT GAC ATC A). The housekeeping gene for normalizing *Fam13a* mRNA expression was *Rps13* in lung and *18s* in splenic NK cells, respectively.

### Treg suppressive assay

Treg suppressive assay was performed in a way similar to our previous work [34]. To ease the comprehension, were here described the major steps again. Total T cells from spleen of *Fam13a* KO mice and WT littermates were isolated with CD90.2 microbeads (130-121-278, Miltenyi Biotec). The cells were then stained with the antibody mix containing LIVE/DEAD® Fixable Near-IR (dilution 1:500), anti-mouse CD25-PE-Cy7 (552880, BD Biosciences) (dilution 1:200) and CD4-FITC (11-0042-82, eBioscience) (dilution 1:200) antibodies at 4 °C for 30 min. After staining, Tregs ($CD4^+CD25^{high}$) and conventional $CD4^+CD25^-$ T cells (Tconv) were sorted by BD FACSAria™ III

sorter. Tconv cells from WT mice were labelled with a final concentration of 1 µM CellTrace™ CFSE (C34554, Life Technology). Splenocytes depleted of T cells were used as antigen-presenting cells (APCs)/feeder cells and were irradiated in RS2000 (Rad Source Technologies) with a total dose of 30 Gy within a period of 10 mins. $1x10^5$ Tconv cells were co-cultured with Treg cells at different ratios in the presence of $2x10^5$ irradiated APCs and 1 µg/ml soluble anti-CD3 antibody ( 554829, BD Biosciences). The cells were cultured in T-cell complete media which consists of RPMI 1640 medium supplemented with 50 U/ml penicillin, 10 mM HEPES, 10% heat-inactivated fetal bovine serum (FBS), 50 µg streptomycin, 2 mM GlutaMAX (35050061, Thermo Fisher Scientific), 1 mM sodium pyruvate (11360070, Thermo Fisher Scientific), 0.1 mM non-essential amino acids (M7145, Sigma Aldrich) and 50 µM beta-mercaptoethanol at 37°C, 5% $CO_2$ incubator. The proliferation of Tconv cells labeled by CFSE was measured after three-day co-culturing using a BD LSR Fortessa™.

### Statistical analysis

The P-values were calculated by Graphpad Prism software with non-paired two-tailed Student t test as presented in the corresponding figure legends. The P-values under 0.05 were considered as statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n.s, not significant). Data were presented as mean ± standard deviation (s.d.).

## Supporting Information

**Supplementary Table 1. Frequency summary of unaffected immune subsets in homeostatic *Fam13a* KO vs WT mice.** Data are provided as mean ± s.d.. The number of analyzed mice per group was also provided.

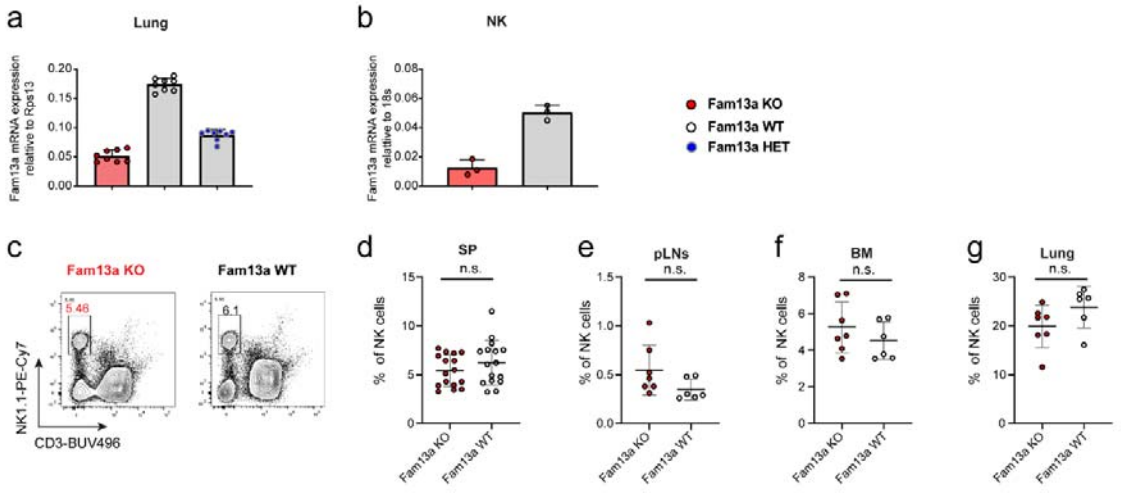
| | Fam13a KO | Fam13a WT |
|---|---|---|
| **cell type (in Spleen)** | **% among the parent gate (%, mean± standard deviation)** | |
| $CD3^+$ T cells (KO=12,WT=11) | 32.28±3.38 | 29.29±8.73 |
| $CD19^+$ B cell (KO=12,WT=11) | 48.6±11.61 | 55.34±11.67 |
| $CD4^+$ T cells (KO,WT N=5) | 12.99±2.29 | 12.23±2.11 |
| $CD44^{low}CD62L^{high}$ naïve CD4 (KO,WT N=5) | 46.35±15.28 | 53.75±3.31 |
| $CD44^{high}CD62L^{low}$ EM CD4 (KO,WT N=5) | 34.45±14.3 | 28.80±3.00 |
| $CD69^+CD4^+$ T cells (KO,WT N=5) | 9.73±1.35 | 11.91±0.96 |
| $KI\text{-}67^+CD4^+$ T cells (KO,WT N=5) | 13.85±4.47 | 12.36±1.20 |
| $PD\text{-}1^+CD4^+$ T cells (KO,WT N=5) | 19.47±6.47 | 18.16±3.49 |
| $FOXP3^+CD4^+$ Tregs (KO,WT N=5) | 19.03±5.40 | 15.66±1.87 |
| $CD8^+$ T cells (KO,WT N=5) | 9.89±1.75 | 8.70±1.77 |
| naïve $CD8^+$ T cells (KO,WT N=5) | 53.08±16.35 | 64.14±1.62 |
| EM $CD8^+$ T cells (KO,WT N=5) | 3.85±1.95 | 2.78±0.79 |
| $CD44^{high}CD62L^{high}$ CM $CD8^+$ T cells (KO,WT N=5) | 37.87±16.42 | 27.53±0.95 |

| | | |
|---|---|---|
| $CD69^+CD8^+$ T cells (KO,WT N=5) | 1.81±0.40 | 2.07±0.35 |
| $Ki\text{-}67^+CD8^+$ T cells (KO,WT N=5) | 7.23±1.60 | 6.94±0.71 |
| $PD\text{-}1^+CD8^+$ T cells (KO,WT N=5) | 2.75±0.76 | 2.29±0.20 |

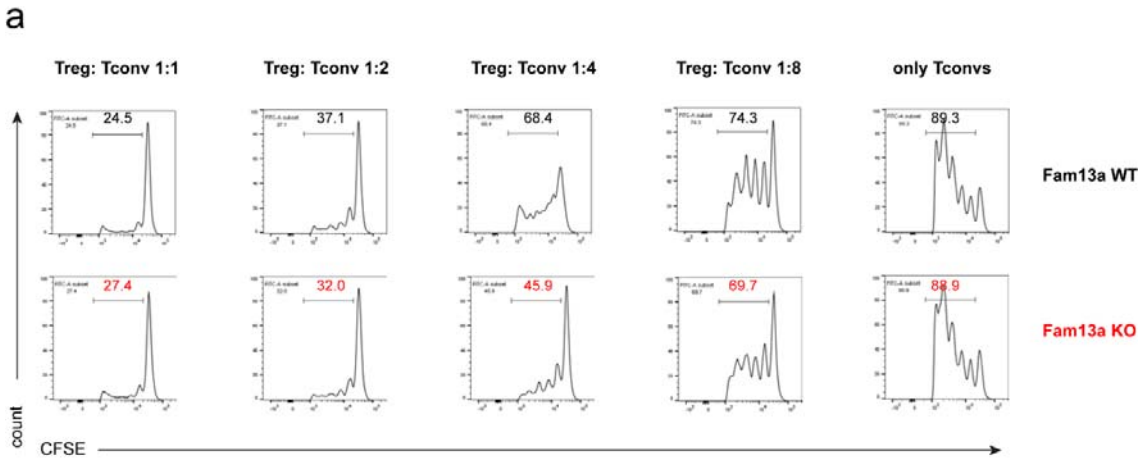
**Supplementary Table 2. List of antibodies with the information required for flow cytometry analysis.**

| Antibody | Clone | Company | Catalogue number | Dilution factor |
|---|---|---|---|---|
| Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™) | 2.4G2 | BD Biosciences | 553141 | 1:50 |
| CD3-BUV496 | 145-2C11 | BD Biosciences | 612955 | 1:100 |
| CD3-BV421 | 145-2C11 | BD Biosciences | 562600 | 1:100 |
| CD19-BV510 | 1D3 | BD Biosciences | 562956 | 1:100 |
| CD19-FITC | 1D3 | eBioscience | 11-0193-82 | 1:100 |
| NK1.1-PE-Cy7 | PK136 | Biolegend | 108713 | 1:50 |
| NK1.1-BV421 | PK136 | BD Biosciences | 562921 | 1:100 |
| CD27-FITC | LG.7F9 | eBioscience | 11-0271-82 | 1:50 |
| CD11b-APC | M1/70 | BD Biosciences | 553312 | 1:100 |
| CD11b-BUV395 | M1/70 | BD Biosciences | 565976 | 1:100 |
| KLRG1-PerCP-Cy5.5 | 2F1/KLRG1 | Biolegend | 138417 | 1:100 |
| CD69-PE | H1.2F3 | BD Biosciences | 553237 | 1:200 |
| CD69-BV605 | H1.2F3 | Biolegend | 104529 | 1:200 |
| NKG2A-PE | 16A11 | Biolegend | 142803 | 1:100 |
| Ly49C/I-FITC | 5E6 | BD Biosciences | 553276 | 1:100 |
| Ly49A-BUV395 | A1 | BD Biosciences | 742370 | 1:100 |
| Ly49D-BV711 | 4E5 | BD Biosciences | 742559 | 1:100 |
| Ly49H-BUV395 | 3D10 | BD Biosciences | 744266 | 1:100 |
| 2B4-FITC | 2B4 | BD Biosciences | 553305 | 1:100 |
| NKp46-PE | 29A1.4 | Biolegend | 137603 | 1:100 |
| NKG2D-APC | CX5 | Biolegend | 130211 | 1:100 |
| CD4-FITC | RM4-5 | eBioscience | 11-0042-82 | 1:200 |
| CD8-BUV805 | 53-6.7 | BD Biosciences | 564920 | 1:200 |
| CD25-APC | PC61 | BD Biosciences | 557192 | 1:100 |
| CD44-PE-Cy7 | IM7 | BD Biosciences | 560569 | 1:200 |
| CD62L-PerCP-Cy5.5 | MEL-14 | BD Biosciences | 560513 | 1:200 |
| PD-1-BV711 | J43 | BD | 744547 | 1:200 |

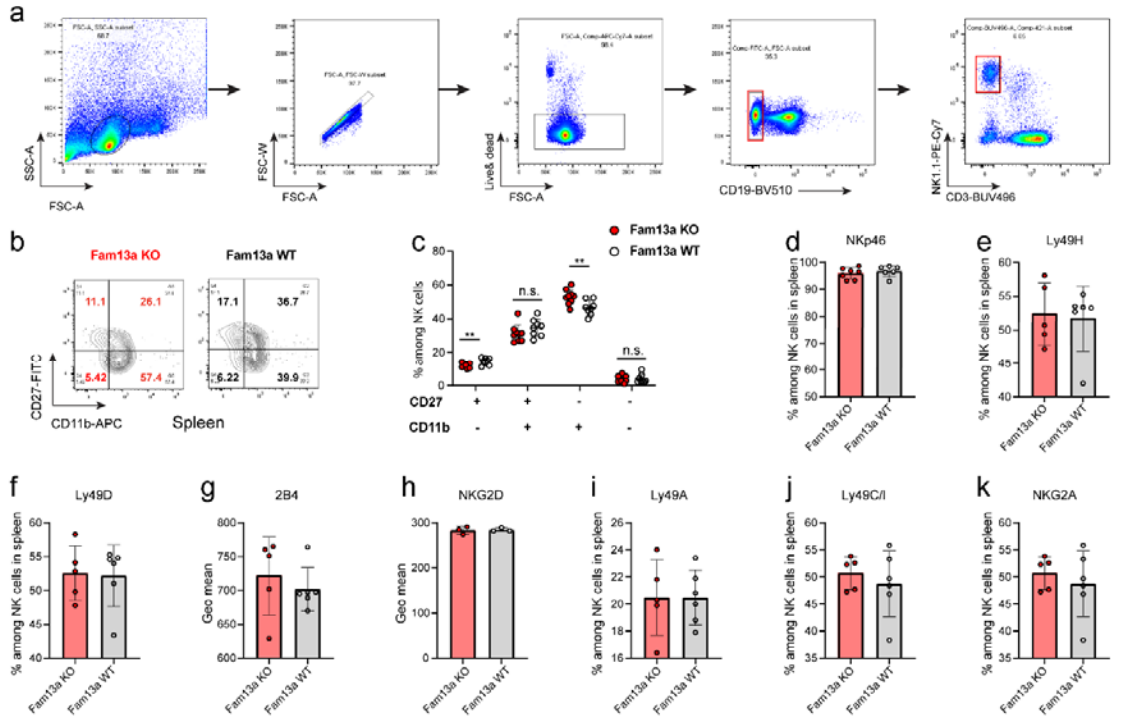
| | | | | |
|---|---|---|---|---|
| | | Biosciences | | |
| Ki-67-BV605 (used in T cell phenotyping) | 16A8 | Biolegend | 652413 | 1:200 |
| Ki-67-eFluor 450 (used in melanoma experiment) | SolA15 | eBioscience | 48-5698-82 | 1:200 |
| FOXP3 Antibody | FJK-16s | eBioscience | 17-5773-82 | 1:200 |
| IFN-γ-APC antibody | XMG1.2 | BD Biosciences | 554413 | 1:100 |
| CD107a-BV421 antibody | 1D4B | BD Biosciences | 564347 | 1:100 |




**S1 Fig. Frequency of NK cells in the different organs and tissues of *Fam13a* KO vs. WT mice.** **(a-b)** Relative *Fam13a* mRNA expression in lung tissue (**a**) or fresh NK cells (**b**) isolated from *Fam13a* KO or WT littermates or Fam13a heterozygous (HET) mice as quantified by real-time PCR. **(c)** Representative flow-cytometry (FACS) plots of $CD3^-$ $NK1.1^+$ NK cells among $CD19^-$ cells in spleen of *Fam13a* KO and WT littermates. **(d-g)** Percentages of NK cells in spleen (**d**, KO, n=16; WT, n=16), peripheral lymph nodes (pLNs) (**e**, KO, n=7; WT, n=6), bone marrow (BM) (**f**, KO, n=7; WT, n=6) and lung (**g**, KO, n=7; WT, n=6) of *Fam13a* KO and WT littermates. Results are representative of three (**c-g**) and two (**a-b**) independent experiments. Data are mean ± s.d. The *p*-values were calculated by a two-tailed Student's t-test. n.s. or unlabeled, not significant, **p*<=0.05, ***p* <=0.01 and ****p* <=0.001.

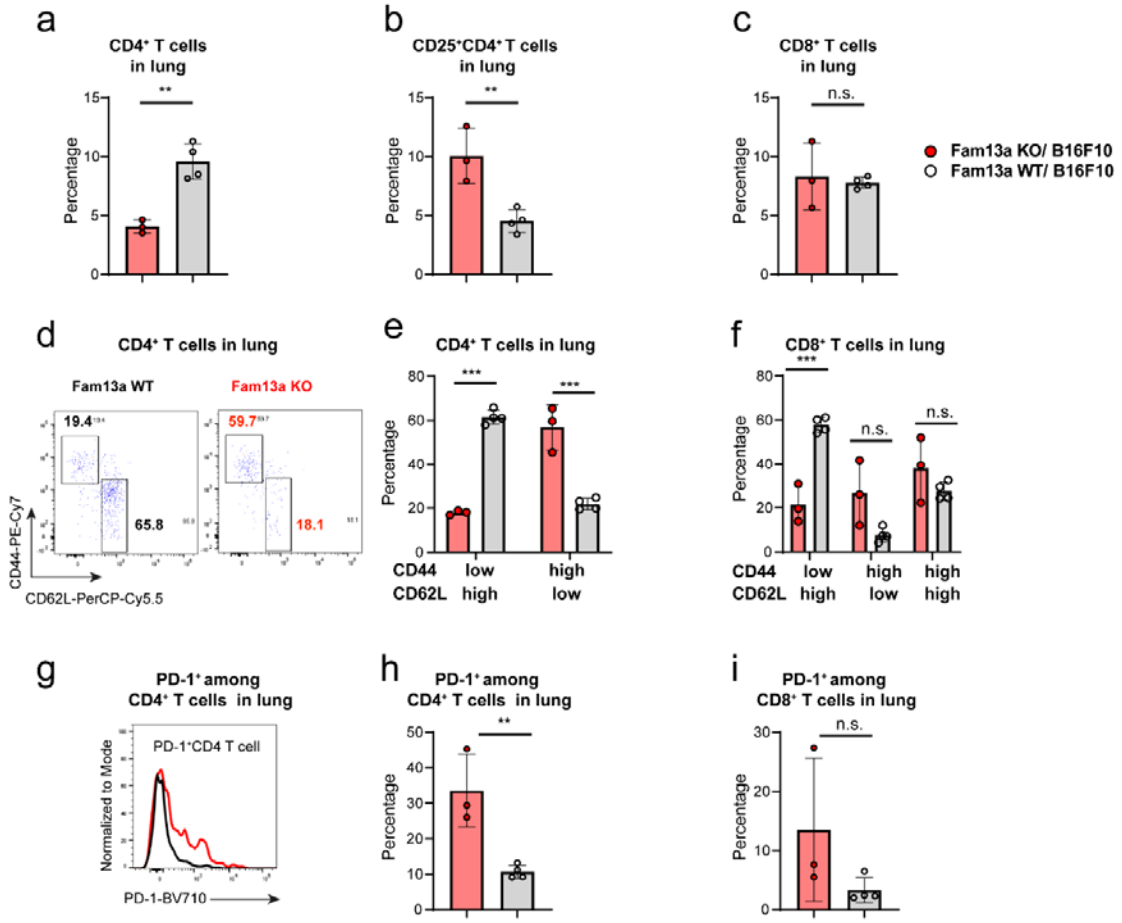



**S2 Fig. Treg suppressive assay. (a)** *In-vitro* suppression assay of *Fam13a* KO or WT Tregs in co-culture with CFSE-labelled Tconv cells at different ratios and irradiated feeder cells in the presence of anti-CD3 antibody for 3 days. Enlarged number in each histogram represents percentage of dividing cells from the total living Tconv population. Results represent four independent experiments.




**S3 Fig. Extended NK cell immunophenotyping analysis of *Fam13a* KO vs. WT mice. (a)** Representative FACS gating strategy of NK cells from splenocyte singlets stained with live and dead staining dye, anti-CD19, anti-CD3 and anti-NK1.1antibodies. **(b)** Representative FACS plots of CD27 and CD11b expression gated on $CD19^{-}CD3^{-}$ $NK1.1^{+}$cells in spleen. **(c)** Percentages of four developmental stages of NK cells, $CD27^{-}CD11b^{-}$, $CD27^{+}CD11b^{-}$, $CD27^{+}CD11b^{+}$, $CD27^{-}CD11b^{+}$ NK cells in spleen of *Fam13a* KO and WT littermates (KO, n=9; WT, n=8). **(d-f)** Percentages of NK cells expressing activating receptors NKp46 (**d**), Ly49H (**e**) and Ly49D (**f**) among *Fam13a* KO and WT

littermates (KO, n=5; WT, n=6). **(g-h)** Geometric mean of 2B4 (**g**) and NKG2D (**h**) expression on *Fam13a* KO and WT NK cells. **(i-k)** Percentages of NK cells expression inhibitory receptors Ly49A (**i**), Ly49C/I (**j**) and NKG2A (**k**) among *Fam13a* KO and WT littermates (KO, n=5; WT, n=6). Results represent four (**c**) and three (**d-k**) independent experiments. Data are mean± s.d. The p-values were determined by a two-tailed Student's t-test. n.s. or unlabeled, not significant, *p<=0.05, **p<=0.01 and ***p<=0.001.




**S4 Fig. Extended analysis of immune cells in the B16F10-melanoma lung metastasis model. (a-c)** Frequency of $CD4^+$ T cells **(a)**, $CD25^{hi}CD4^+$ Tregs (**b**) and $CD8^+$ T cells (**c**) in the lung of *Fam13a* KO and WT littermates. **(d)** Representative FACS plot of naïve ($CD62L^{hi}$ $CD44^{low}$) and effector memory (EM) ($CD62L^{low}$ $CD44^{hi}$ $CD4^+$ T cells in the lung of *Fam13a* KO and WT littermates. **(e)** Percentage of naïve and EM among total $CD4^+$ T cells in the lung of *Fam13a* KO and WT littermates. **(f)** Percentage of naïve, EM and CM among total $CD8^+$ T cells in the lung of *Fam13a* KO and WT littermates. **(g)** Representative histogram overlays of PD-1 expression in $CD4^+$ T cells in the lung of *Fam13a* KO and WT littermates. **(h-i)** Percentage of PD-$1^+$ $CD4^+$ T cells (**h**) and PD-$1^+CD8^+$ T cells (**i**) in the lung of *Fam13a* KO and WT littermates. Results represent two independent experiments.

Data are mean ± s.d. The *p*-values were calculated by a two-tailed Student's t-test. n.s. or unlabeled, not significant, *$p$<=0.05, **$p$ <=0.01 and ***$p$ <=0.001.

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# Imprint of initial education and loss of Ly49C/I in activated natural killer cells of TAP-KO mice and control strains and C57BL/6 Wildtype Mice

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# Imprint of Initial Education and Loss of Ly49C/I in Activated Natural Killer Cells of TAP1-KO and C57BL/6 Wildtype Mice

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Natural killer (NK) cells are important effectors of the innate immune system and participate in the first line of defense against infections and tumors. Prior to being functional, these lymphocytes must be educated or licensed through interactions of their major histocompatibility complex class I molecules with self-specific inhibitory receptors that recognize them. In the absence of such contacts, caused by either the lack of expression of the inhibitory receptors or a very low level of major histocompatibility complex class I (MHC class I) proteins, NK cells are hypo-reactive at baseline (*ex vivo*). After stimulation (assessed through plate-bound antibodies against activating receptors or culture in the presence of cytokines such as interleukin (IL)-2 or IL-15) however, they can become cytotoxic and produce cytokines. This is particularly the case in transporter associated with antigen processing (TAP)-deficient mice, which we investigated in the present study. Transporter associated with antigen processing transports endogenous peptides from the cytosol to the endoplasmic reticulum, where they are loaded on nascent MHC class I molecules, which then become stable and expressed at the cell surface. Consequently, TAP-KO mice have very low levels of MHC class I expression. We present a study about phenotypic and functional aspects of NK cells in two mouse strains, C57BL/6 wildtype and TAP1-KOin spleen and lung. We observed that in both types of mice, on the same genetic background, the initial pattern of education, conferred to the cells *via* the inhibitory receptors Ly49C/I and NKG2A, was maintained even after a strong stimulation by the cytokines interleukin-2, interleukin-12, interleukin-15 and interleukin-18. Furthermore, the percentages of activated NK cells expressing Ly49C/I and Ly49I were strongly down-modulated under these conditions. We completed our investigations with phenotypic studies of NK cells from these mice.

**Keywords: natural killer cells, NK cells, education, major histocompatibility complex class I, inhibitory receptors, TAP-KO, MR1-KO**

## INTRODUCTION

Natural Killer (NK) cells are the founding members of the innate lymphoid cell (ILC) family comprising in addition several other populations called innate lymphoid cells types 1, 2 and 3, as well as lymphoid tissue inducer cells (1). All the latter are predominantly cytokine producers, whereas NK cells are likewise able to release cytokines and chemokines but are also endowed with cytotoxic properties, further subdivided into natural cytotoxicity (killing of targets without prior sensitization or immunization) and antibody-dependent cellular cytotoxicity (ADCC), which occurs when the Fc portion of antibodies bound to a target cell interacts with the activating NK cell receptor CD16 (FcγRIIIa) (2, 3). Natural Killer cells are extensively studied in human and mouse for their anti-tumoral and anti-infectious properties that are therapeutically exploitable and constitute a true hope for the future of immunotherapy (4, 5). Since the first descriptions of NK cells in the mid-seventies, their capacity to kill tumor cells and viral-infected cells has been observed in many *in vitro* and *in vivo* investigations. Upon recognition of a target cell, the granule content of the cytolytic vesicles (perforin, granzymes, and, in human but not in the mouse, granulysin) is released and induces apoptosis of the abnormal cell (6).

Before reaching this step, NK cells integrate signals from their activating and inhibitory receptors, respectively (AR and IR), and the target cell is eliminated when the activating messages predominate (7). The best studied IR are specific for major histocompatibility complex (MHC) class I molecules, and a normal level of the latter is characteristic for healthy cells that are consequently identified as such and spared by the NK cells. Their absence is recognized as "missing self" and induces target cell lysis in the presence of sufficient activating messages (7–9). Besides the concept of the balance between signals transmitted through AR and IR (7), another important factor governing NK cell functions is education or licensing (10–12). These terms designate the interaction of IR with autologous MHC class I molecules during NK cell development, which is necessary for the cell to become functional *via* (10–12), although non-MHC class I ligands can educate NK cells *via* different IR (11). Therefore, NK cell education relies on the paradox that they only become active after having received a signal through an IR that later can inhibit NK cell functions. In the absence of this phenomenon, due either to the lack of expression of one or several self-specific IR on a NK cell or to a general MHC class I deficiency of the cellular environment, such as observed in beta-2 microglobulin (β2m) and/or transporter associated with antigen processing (TAP) defects, NK cells remain uneducated (unlicensed) and hypo-reactive. This observation was made both in human (13–15) and in knockout mouse strains, such as β2m-KO, TAP1-KO, β2m/TAP1 double KO (16, 17): *ex vivo* NK cells display low or absent cytotoxicity and low cytokine production, but become functionally very active upon cytokine-mediated stimulation and then kill autologous Con A T cell blasts (16). In human TAP deficiency, activated NK cells kill autologous B lymphoblastoid cells (Epstein-Barr virus-transformed immortalized B lymphocytes) and skin fibroblasts, whereas they surprisingly spare self T-PHA blasts (18–20).

Natural killer cell education is in the focus of interest of several groups. Different models have been elaborated, such as the arming model (Raulet, 2006), the disarming model (Raulet, 2006; Joncker and Raulet, 2008), the rheostat model (21bis; 22), the *cis-trans* model (Zimmer et al., 2001; 23) and the confinement model (11, 12). More recently, it has been shown that TRP Calcium channels dynamically regulate NK cell licensing on the level of the content of cytolytic proteins in the secretory lysosomes (that degranulate upon target cell recognition) (24). Moreover, and in accordance with the *cis-trans* interaction model, the role of NK cell-intrinsic MHC class I molecules for tuning has been emphasized (25).

All these different concepts have mostly been established with short-term activated NK cells, for example taken *ex vivo* the day after poly(I:C) or tilorone administration. In the present work, we wanted to rather explore what happens in mouse NK cells in terms of the production of their major cytokine, namely interferon gamma (IFN-γ), after culture with interleukin 2 (IL-2) for five days and then an overnight re-stimulation with IL-2 alone, (IL-2, IL-12, IL-15) and (IL-2, IL-12, IL-18), respectively. The first condition was the negative control, as in the mouse IL-2 alone does not induce significant IFNγ production. In contrast, the last cytokine combination represented the positive control, as IL-12 and IL-18 together activate the production of a maximal amount of IFNγ. Finally, the IL-12 plus IL-15 association was supposed to lead to an intermediate level of stimulation, for which differences between NK cell subsets, defined by the presence or absence of self-specific IR, could be visible.

We choose to work with the most frequently used mouse model in immunology, the C57BL/6 (B6) strain, and its TAP1-KO littermates. In B6 mice, the self MHC class I-specific IR are Ly49C/I, recognizing the classical MHC class I molecules H-2K$^b$ (and H-2D$^b$), and CD94/NKG2A (NKG2A), binding to the non-polymorphic molecule Qa-1$^b$, the mouse equivalent of HLA-E (26, 27). Consequently, NK cells expressing either one or both IR are supposed to be educated, whereas their double negative (Ly49C/I-NKG2A-) counterparts are expected to be hypo-reactive. In contrast, in TAP1-KO mice, the entire NK cell population should in principle be functionally deficient. The reason for the choice of TAP1-KO animals was to undertake an *in vitro* study of their NK cells before switching to a mouse *in vivo* model of a chronic bacterial infection of the airways in order to detect what goes wrong in this context. Transporter associated with antigen processing-deficient human patients suffer severely from such pathologies, which usually evolve to bronchiectasis (13, 14). Understanding the pathophysiology of this phenomenon and the potential link between NK cells and bacterial infections might be useful even for patients without a MHC class I deficiency.

An additional layer of complexity in NK cell education stems from the observation that Ly49C interacts with its ligand H-2K$^b$ in *cis*, on the same NK cell membrane, and simultaneously in *trans*, with the same ligand on surrounding target cells, which leads to an optimal calibration of NK cell reactivity even in the

presence of only a minor down-regulation of the MHC class I molecule in the cellular environment. This phenomenon had initially been demonstrated by the Held group for the receptor-ligand pair Ly49A – H-D$^d$ (23). As the 5E6 monoclonal antibody binds to both the IR Ly49C and Ly49I, the former might actually not been well recognized by the antibody due to its *cis* interaction, which could render the epitope inaccessible. We unsuccessfully tried to obtain aliquots of the non-commercially available Ly49C-specific antibody clone 4LO3311 (Brennan et al., 1996) from several groups, and attempted acid stripping to disrupt the cis interaction. By staining Ly49I with the specific antibody YLI-90, the problem was resolved at least indirectly, by showing the "educational" effect of Ly49C compared with no such effect for Ly49I.

In summary, our investigations show that the initial pattern of NK cell education leaves its imprint even after six days of intense cytokine-mediated stimulation in B6 animals, and, surprisingly, that there is an unequal functional distribution between NK cell subsets of activated TAP-KO littermates, largely matching the one observed in B6 mice. Overall, we confirm that a defective TAP results in somewhat reduced NK cell functions *ex vivo*, but that the cells display the same educational profile than their wildtype counterparts after a strong cytokine-mediated stimulation, which should in principle not be the case. The IR Ly49I is well expressed on NK cells from both strains, *ex vivo* and after activation, but does not really seem to play a major role in NK cell education. Furthermore, we were able to detect Ly49C from a functional point of view (acting as an educating IR), but not with absolute certainty as a reliable phenotypic NK cell marker.

# MATERIAL AND METHODS

## Ethical Statement

The animal studies were approved by the Animal Welfare Structure (AWS) and the experiments were carried out in accordance with the European Union directive 2010/63/EU as incorporated in Luxembourgish law for the care and use of laboratory animals The official authorization for our protocol was given by the Luxembourgish Ministry of Health under the number DII-2017-02.

## Mice

Wildtype (TAP1+/TAP1+) and TAP1-KO (TAP1-/TAP1-) littermates on the C57BL/6 (H-2b) genetic background, obtained through intercross of heterozygous F1 (TAP1 +/TAP1-) mice aged 8-12 weeks, were used. The genetic characteristics (homozygous wildtype, heterozygous, and homozygous TAP1-) were established through genotyping. Mice were bred and maintained at the Luxembourg Institute of Health's specific pathogen-free animal facility. They were fed a standard maintenance chow and followed a 12-h light dark cycle at 22-23°C and 45-65% relative humidity.

## Cell Preparation

Lungs and spleens were extracted from WT and TAP1-KOmice. Lungs were incubated in 1.6 ml digestion solution (for 15 ml, 20 mg collagenase II, 10% FBS, 750 U benzonase, 15 µl of 1M $MgCl_2$, PBS) for one hour at 37°C. Spleens and the digested lungs were passed through a 40 µm cell strainer (Corning) with the back of a syringe plunger to make a single cell suspension. Red blood cell lysis was performed by ACK lysing buffer (Gibco™). Murine T-lymphoma cell lines YAC-1 (ATCC), RMA, and C4.4.25$^-$,the $\beta_2$m-deficient variant of the EL-4 lymphoma, were chosen as target cell lines. YAC-1 and RMA were cultured in suspension with RPMI1640 medium supplemented with 10% FBS, 1% Pen/Strep, 1 mM HEPES, while C4.4.25$^-$ was cultured in DMEM medium supplemented with 10% FBS, 1% Pen/Strep, 1 mM HEPES.

## Flow Cytometry

The cells were washed twice with PBS buffer containing 1% BSA (Miltenyi) (FACS buffer) and were proceeded for surface staining by Fc block anti-mouse CD16/CD32 followed by fluorochrome-conjugated monoclonal antibodies, incubated at 4°C for 30 minutes, in the dark. Cells were then washed twice (100 µl, 4° C, 300 x g, 10 minutes, FACS buffer), and fixed with Cytofix/ Cytoperm buffer (BD) for 45 minutes. Intracellular staining was performed after fixation/permeabilization, and staining was done in Perm/Wash buffer (BD) at 4° C for 30 minutes, in the dark. The samples were washed again and re-suspended in FACS buffer for further analysis. The acquisition was done on BD LSR Fortessa™. The monoclonal antibodies (mAbs) used for phenotypic and functional analyses are listed in **Table 1**.

## Degranulation Assay

NK cell degranulation towards target cells was tested by co-culturing them together for four hours at 37°C, in the presence of GolgiStop protein transport inhibitor (BD). Target cell lines YAC-1, RMA and C4.4.25$^-$ were stained with CellTrace Violet at 0.5 mM concentration. The NK cells used were cultured in IL-2 and stimulated with IL-12/IL-15 and IL-12/IL-18 overnight. The Effector : Target (E:T) ratios were 2:1 and 5:1. The cells were then stained with mAbs to NK1.1, CD3, Ly49C/I, Ly49I, NKG2A, intracellular IFNγ and the fixable viability dye according to the staining protocol. The acquisition was done on BD LSR Fortessa™.

## Cytotoxicity

NK cell cytotoxicity towards target cells was tested by co-culturing them together for four hours at 37°C. The NK cells used were cultured in IL-2 for six days and stimulated with IL-12/IL-15 and IL-12/IL-18 overnight before co-culture. The tested E:T ratios were 1:1, 5:1, 10:1, 25:1, 50:1. Target cell lines YAC-1, RMA and C4.4.25$^-$ were stained with CellTrace Violet at 0.5 mM concentration. At the end of the four hours, TO-PRO-3 (1 µM, Invitrogen) diluted at 1:1000 was added to each sample. The aliquots were incubated at room temperature for 15 minutes and acquired on a NovoCyte Quanteon instrument.

**TABLE 1** | List of antibodies used.

| Antibody to | Clone | Format | Supplier | Identifier |
|---|---|---|---|---|
| NK1.1 | PK136 | BUV-395 | BD Biosciences | 564144 |
| | | BV421 | Biolegend | 108741 |
| CD3 | 145-2C11 | BV510 | BD Biosciences | 563024 |
| | | PE-Cyanine7 | eBioscience™ | 25-0031-82 |
| NKG2A$^{B6}$ | 16a11 | PerCP-eFluor 710 | eBioscience™ | 46-5897-82 |
| Ly49C and Ly49I | 5E6 | BV786 | BD Biosciences | 744032 |
| Ly49I | YLI-90 | unconjugated | | |
| | | FITC | | |
| | | BV | | |
| KLRG1 | 2F1 | BV421 | BD Biosciences | 562897 |
| Qa-1$^b$ | 6A8.6F10.1A6 | BV421 | BD Biosciences | 744385 |
| CD335 (NKp46) | 29A1.4 | BV421 | BD Biosciences | 562850 |
| Qa-2 | 69H1-9-9 | FITC | eBioscience™ | 11-5996-82 |
| Ly49D | 4 E5 | FITC | BD Biosciences | 555313 |
| Ly49G2 | 4D11 | FITC | BD Biosciences | 555315 |
| Ly49H | 3D10 | FITC | BD Biosciences | 562536 |
| H-2K$^b$ | AF6-88.5 | PE | Biolegend | 116507 |
| Ly49F | HBF-719 | PE | BD Biosciences | 550987 |
| CD314 (NKG2D) | CX5 | PE | Biolegend | 130207 |
| CD19 | 1D3 | PE/Cyanine7 | BD Biosciences | 552854 |
| IFNγ | XMG1.2 | BUV737 | BD Biosciences | 564693 |
| TNFα | MP6-XT22 | FITC | eBioscience™ | 11-7321-82 |
| CD107a | 1D4B | BV711 | BD Biosciences | 564348 |
| Fixable Viability Stain 780 | | | BD Biosciences | 565388 |
| CD16/CD32 (Mouse BD Fc Block™) | 2.4G2 | unconjugated | BD Biosciences | 553142 |

## Statistics

Statistical analyses were performed in GraphPad Prism Version 9.3.1 for Windows (GraphPad Software, San Diego, CA, USA). Data points were plotted in grouped tables and the tests used to determine the statistical differences were One-way ANOVA (Tukey's multiple comparisons test) or Two-way ANOVA (Dunnett's or Sidak's multiple comparisons tests), depending on the number of animals and parameters in the assay. We used $p < 0.05$ as the limit for statistical significance.

# RESULTS

## Comparative Phenotype of B6 and TAP1-KO NK Cells

We previously described the phenotype of B6 wildtype NK cells from spleen and lung, which displays several significant differences between the two organs. Notably, lung NK cells are more mature than their splenic counterparts (28). These findings, which were confirmed by others (29), have, to the best of our knowledge, not yet been investigated for the TAP-KO strain splenocytes and lung mononuclear cells were stained with fluorescent antibodies (**Table 1**) and NK cells comparatively analyzed by flow cytometry.

The proportion of NK cells (percentage of living cells that were CD3-NK1.1+) was almost threefold higher in lung than in spleen, whereas the absolute numbers were comparable (**Figure 1B**).

As described earlier for MHC class I-deficient mouse strains (27, 30), the splenic percentages (and MFI) of NK cells expressing Ly49C/I and NKG2A were significantly higher in the TAP1-KO background than in the wildtype littermates.

In the lung, as previously observed (28) in B6 animals, the Ly49 IR were down- and the AR also down (Ly49H)-regulated or unchanged (Ly49D), with NKG2A remaining stable, compared to the spleen. For the TAP1-KO NK cells, the IR, including NKG2A, still displayed an increased frequency of positive cells relative to theB6 wildtype mice, whereas the AR Ly49D and Ly49H were non significantly down-modulated.

We then turned to the analysis of the comparative expression between mice of the AR NKp46 and NKG2D (**Figure 2**), which are crucial for the cytotoxic activity of NK cells. The natural cytotoxicity receptor (NCR) NKp46 was present on almost all NK cells in the two types of mice and in spleen as well as in lung, with very similar expression levels per cell (reflected by the MFI), but overall higher MFI in lung than in spleen. Furthermore, the C-type lectin-type AR NKG2D showed a trend for lower frequencies and MFI in spleen and lung (no statistical significance) of TAP1-KO NK cells However, the overall percentage of NKG2D+ NK cells was rather low compared to previous studies (Zafirova et al., 2009), which might be related to the antibody clone used.

Although, as previously described (31), the classical MHC class I molecule H-2K$^b$ was severely down-modulated but not absent from TAP1-KO cells (**Figure 3**), the non-polymorphic MHC class I molecule Qa-2 could not at all be revealed, whereas it was present on the totality of the NK cells from the B6 littermates (with, interestingly, a higher MFI in lung than in spleen). Thus, it can be concluded that this molecule, considered as the mouse equivalent of the human non-classical class I protein HLA-G (32), is entirely TAP-dependent and cannot be loaded with TAP-independent peptides, in contrast to Qa-1$^b$ (33).

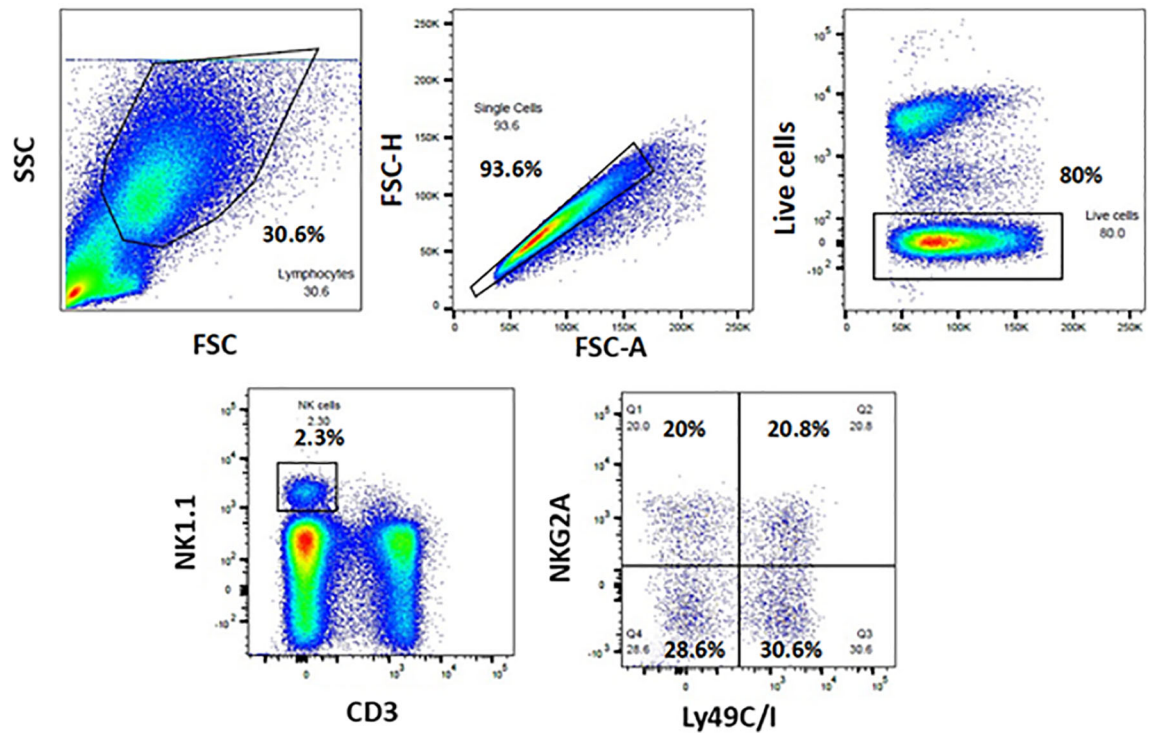



FIGURE 1 |

We made interesting observations by comparing the expression levels of the MHC class I molecules H-2K$^{b}$, Qa-2 and Qa-1$^{b}$ in NK cells of the B6 and TAP-KO strains, after 18 hours of culture of *ex vivo* splenocytes in the presence of either IL-2 alone, (IL-2, IL-12, IL-15), or (IL-2, IL-12, IL-18) (**Figure 4**). The hypothesis here was that maybe these self-molecules would be differentially expressed by the four subsets defined by the presence or absence of Ly49C/I and NKG2A. As expected, the

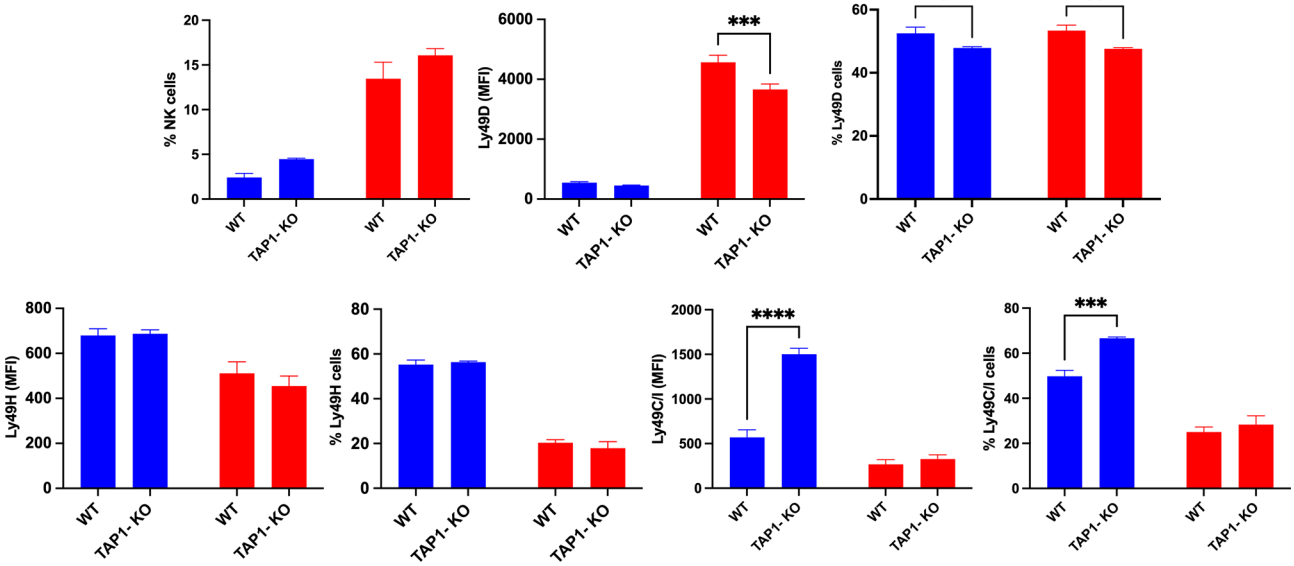



FIGURE 2 |

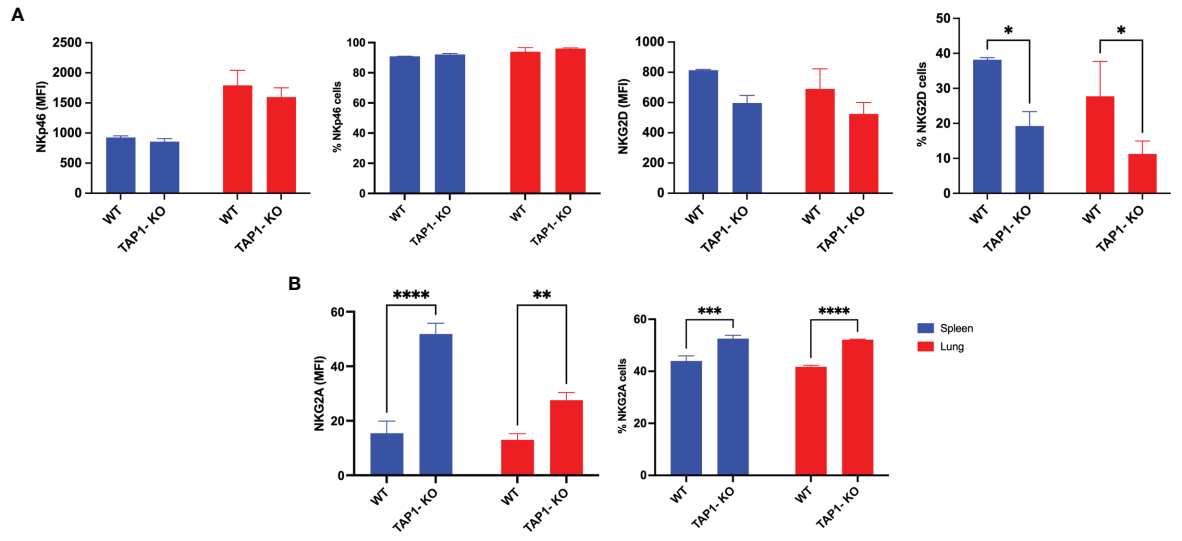



**FIGURE 3** |

former two proteins (H-2K$^b$ and Qa-2) were expressed homogeneously and at high levels in B6 mice, without major differences in the MFI between the subpopulations. However, we observed a double peak for H-2K$^b$ in the Ly49C/I-NKG2A+ and Ly49C/I+NKG2A+ NK cells only in the (IL-2, IL-12, IL-18 condition). Our hypothesis held true in the case of Qa-1$^b$ in the B6 strain. Indeed, whereas we found a homogeneous peak almost superposed to the negative control in the NK cells devoid of NKG2A, the two NKG2A+ subsets displayed a MFI overall approximately twice as high and moreover two connected but separate populations (one "bright" and one "dim") in the three cytokine conditions. The significance of this data will have to be analyzed in further experiments.

Furthermore, while the expression of H-2K$^b$ was very low in TAP1-KO NK cells, again as expected, the effect of IL-12 + IL-18 was still observable even in this MHC class I-deficient context

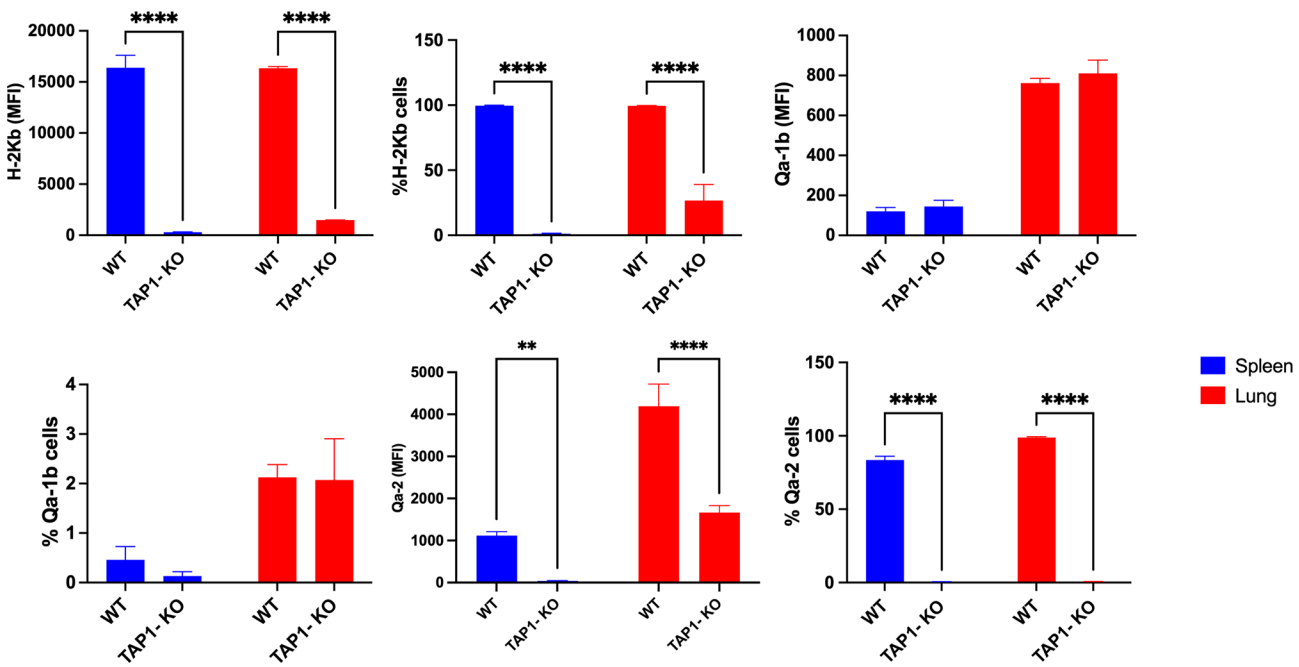



**FIGURE 4** |

(**Figure 4**). Very interestingly, for Qa-1$^b$, the same phenomenon of the double populations appeared selectively in the NKG2A+ NK cells of the three mixtures of cytokines. This suggests both in B6 wildtype and in TAP1-KO NK cells, that the observation is linked to NKG2A rather than to the types of cytokines and/or the MHC class I molecules themselves.

We concluded our phenotypic investigations with the senescence and activation marker KLRG1

(**Figure 5**), better represented on terminally differentiated NK lymphocytes (34). KLRG1 was expressed on a significantly reduced NK cell population of β2m-KO mice of various genetic backgrounds (35), an observation that we could confirm in the spleens of TAP1-KO animals, both in terms of frequency of KLRG1+ cells and of MFI. Interestingly, the percentages of the KLRG1+ NK cells and the MFI of the receptor showed a strong trend to increase in both types of mice in the lung compared to the spleen, although TAP1-KO NK cells still lagged behind wildtype for both of the parameters. Overall, the data shows that NK cells from TAP1-KO mice are relatively less senescent and activated compared to their wildtype counterparts.

## Differences in Activated NK cell IFN-γ Production According to the Initial Licensing Status

Next, we wanted to compare the production of the signature cytokine of NK cells, IFNγ, in the four subsets defined by the presence or absence of the self-specific and educating IR, Ly49C/I and NKG2A. Theoretically, as outlined above, we expected fewer IFNγ-producing NK cells in the double negative, unlicensed NK cell fraction, as described by Kim et al. (10). This dataset was nevertheless established in *ex vivo* splenocytes stimulated with plate-bound antibodies. We asked the question if the licensing features would be maintained after a strong stimulation with the cytokines IL-2, (IL-2, IL-12, IL-15) and (IL-2, IL-12, IL-18), and investigated this initially at day 1, *i.e.* after an overnight activation (**Figure 6A**). Here, we found in B6 wildtype NK cells no significant IFNγ production with IL-2 alone, whereas all four subsets defined by Ly49C/I and NKG2A produced the cytokine with significant values around or above 50% for the two NKG2A+ subpopulations *versus* approximately 30% to 40% for the NKG2A- ones when stimulated by the (IL-2, IL-12, IL-15) cocktail. Ly49C/I+ NK cells contained more IFNγ-producers than the double negative subset. In the presence of (IL-2, IL-12, IL-18), between 80% and 100% of NK cells produced the cytokine. This observation, at least regarding (IL-2, IL-12, IL-15), fits with current models for NK cell education, notably the licensing model (10) and the rheostat model (10–12, 21, 22). The latter predicts higher functional capability of NK cells when they express more than one self-specific IR. Natural killer cells from TAP1-KO mice were stimulated under the same conditions. Whereas again no IFNγ production was observed in the presence of IL-2 alone, the two cytokine cocktails surprisingly induced an almost similar distribution of the IFNγ-producing NK cells compared to wildtype mice, with the strongest effect seen in NKG2A+ cells, followed by Ly49C/I single positives and finally the double negatives (barely half the values of the NKG2A+ cells). In principle, as TAP1-KO NK cells cannot be appropriately educated, one should not observe such a difference in IFNγ-producing cells between the NK subsets but, as even MHC class I- NK cells become activated under appropriate cytokine stimulation (18), a more equal distribution.

To get a deeper insight into this topic, we repeated the experiments by first culturing splenocytes with IL-2 alone for five days and then, at day 5, adding the same three cytokine

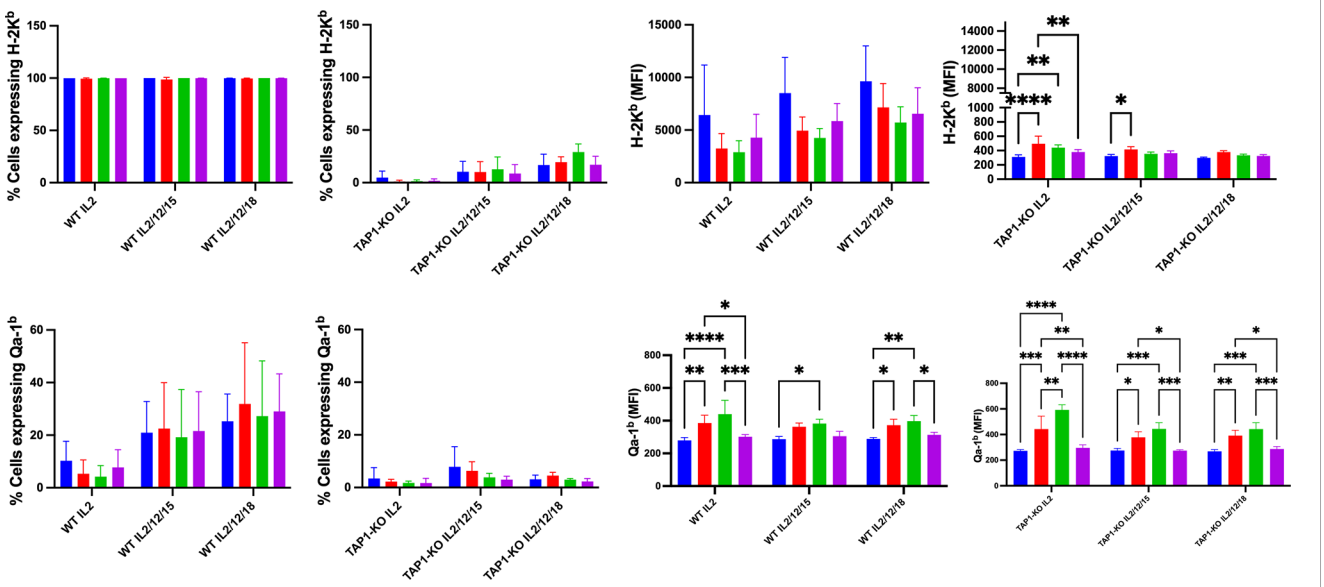



**FIGURE 5** |

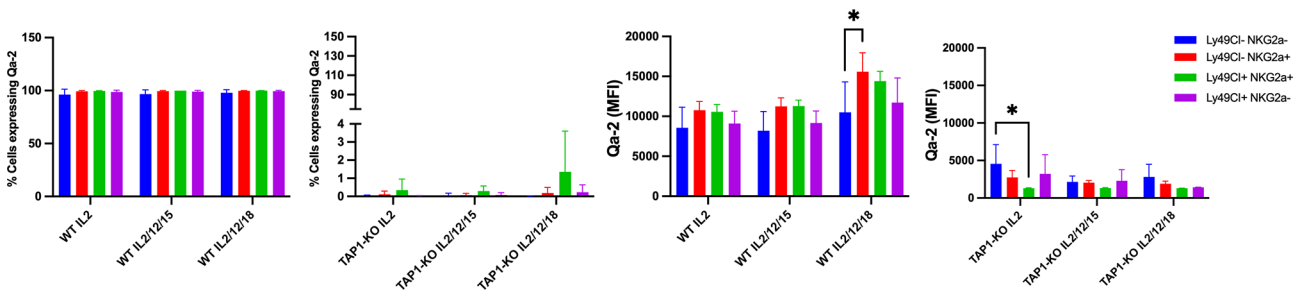



**FIGURE 6** |

combinations until day 6, when the cells were harvested and stained (**Figure 6B**). We then similarly looked at the percentages of IFNγ+ NK cells in the four subsets defined by the presence or absence of the IR Ly49C/I and NKG2A.

Overall, we observed the same results as at day 1, although the global cytokine production levels were higher in each subset. The double negative population was still the less proficient in intracellular IFNγ accumulation in the (IL-2, IL-12, IL-15) condition, whereas the NKG2A+ subsets remained the most productive ones. With (IL-2, IL-12, IL-18), almost 100% of the four subpopulations became IFNγ+. However, concordant differences were still observed in the MFI, reflecting in these cases the quantity of IFNγ per cell and not, as the percentages parameter, the fraction of NK cells producing the cytokine. Similar to day 1, we could not observe significant differences between B6 wildtype and TAP1-KO mice, meaning that (i) the wildtype NK cells maintained to some degree the *ex vivo* educational profile that left an imprint even after a very strong cytokine stimulation at days 1 and 5, and (ii) this imprint was also present in the TAP1-KO context, although NK cells are not supposed to be educated through classical and non-classical MHC class I molecules in this genetic background (as the expression of these proteins is severely reduced). Moreover, our data validate the licensing (10) and the rheostat models (10–12, 21, 22) even in a situation of major NK cell activation.

Given the previously mentioned fact that the 5E6 antibody recognizes both Ly49C and Ly49I, but that in the B6 wildtype animals, Ly49C might be masked due to its cis interaction with H-2Kb, we also stained the cells with the Ly49I-specific antibody YLI-90. Here, we found at day 0 (ex vivo) nicely separated subpopulations of Ly49I+/- and/or NKG2A+/- NK cells (**Figure 7**) in both wildtype and TAP1-KO mice, the latter displaying a highly increased MFI (155% relative to B6 wildtype) for Ly49I. In contrast, the Ly49C/I versus Ly49I staining revealed a double positive diagonal population, as observed in flow cytometry by staining the same molecules or closely related epitopes. Finally, the percentages of Ly49C/I+ cells *versus* NKG2A+ events in the wildtype mice were abnormally low, which could reflect steric hindrance between 5E6 and YLI-90.

We then repeated the IFNγ-producing experiments at day 6 and measured the level of the cytokine in the Ly49C/I, Ly49I and NKG2A NK cell subsets compared with the receptor-negative counterparts. Overall, for Ly49C/I and NKG2A, the data shown in **Figure 6B** were confirmed (**Figure 8**). Interestingly, Ly49I did not seem to contribute to NK cell licensing, as the Ly49I+ subset did not produce more IFNγ than the Ly49I- populations. In contrast, the Ly49C/I+ NK cells harbored a distinctly higher percentage of cytokine-producing NK cells than their counterparts negative for IR. Thus, as this increase cannot

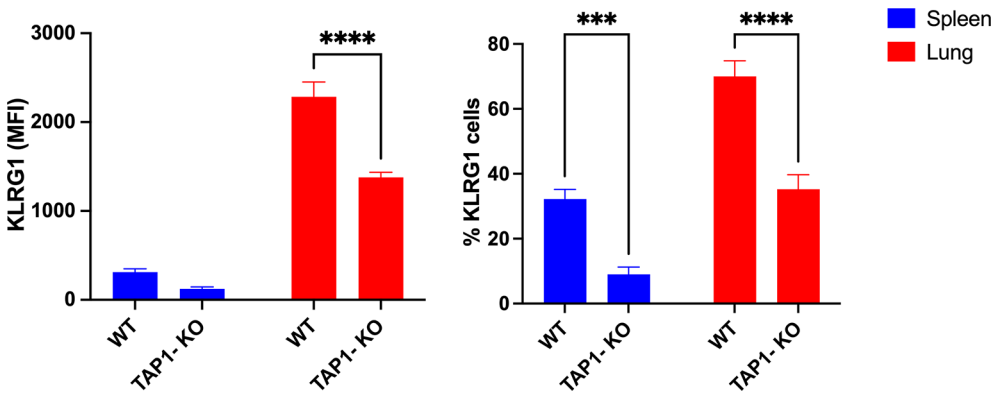



**FIGURE 7** |

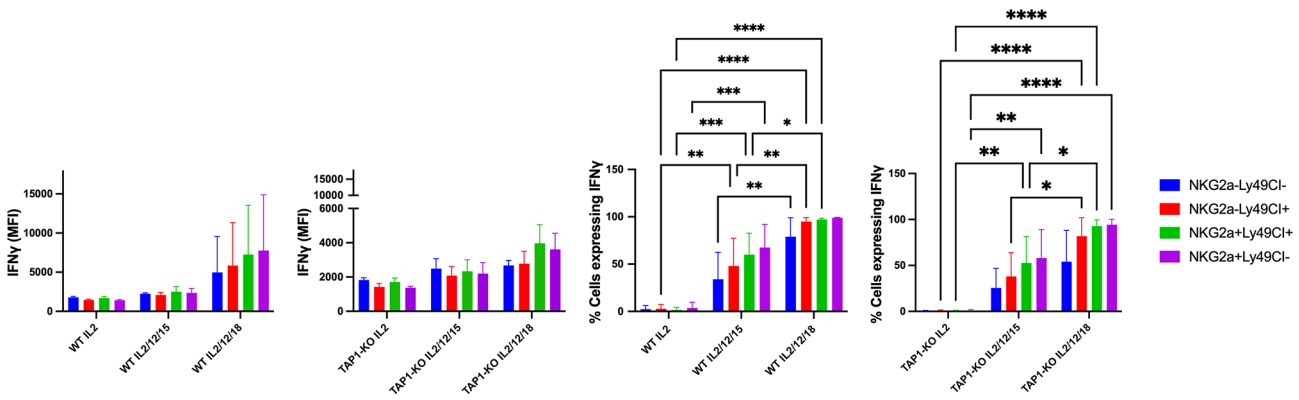



**FIGURE 8** | **(A)** Representative flow cytometric dot plots of splenic NK cells distinguished according to NKG2A and Ly49C/I expression as frequency. The NK cells were expanded in the presence of IL-2 for six days and the expression levels of NKG2A and Ly49C/I were compared with that of Day 0. **(B)** Analyses of the frequency of NK cell subsets based on NKG2A and Ly49C/I expression levels. The frequency between Day 0, Day 1 and Day 6 for IL-2 expanded NK cells was compared. Statistical analysis was performed using GraphPad Prism 9.0.0 with an ordinary two-way ANOVA with Sidak multiple comparisons tests (n=3). *p < 0.05, p < 0.01, ***p < 0.001 and ****p < 0.0001.The colors of the * correspond to the strains of mice used.

stem from Ly49I, we consider our results as evidence for the educational imprint of Ly49C.

## Downmodulation of Ly49C/I and Ly49I on Activated NK Cells at Day 6

When we undertook the phenotyping of the spleens at day 6 for the purpose of measuring intracellular IFNγ production by NK cells, we observed that the percentages of Ly49C/I+ events were dramatically down-modulated compared with days 0 (*ex vivo*) and 1. This phenomenon also noticed in the case of Ly49I but not of NKG2A) was significant for each mouse type, either between day 0 and day 6 (NKG2A-Ly49C/I+) or between day 1 and day 6 (double positive), whereas rather minor and not systematically significant differences could be seen between day 0 and day 1 (**Figure 9**). In a mirror image, the percentages of NKG2A +Ly49C/I- as well as double negative NK cells increased. A reduction of Ly49C/I+ NK cells in the context of activated tumor-infiltrating lymphocytes has been previously described by Shi et al. (36).

## Natural Killer Cell Cytotoxic Activity

Besides cytokine production, natural cytotoxicity against tumor cells and viral-infected cells is the second major property of NK cells. In the mouse, the lymphoma cell line YAC-1 is the standard target, due to its exquisite NK cell sensitivity (37). Another T cell lymphoma, RMA, is rather resistant to these cells, whereas C4.4-25$^-$, a β2m-deficient variant of EL4, the parental cell line of RMA (38), is again susceptible because of the almost absent expression of MHC class I molecules (39). As the latter two possess the B6 genetic background, they can serve for the determination of the missing self-recognition by NK cells from B6 origin.

We performed a four-hour cytotoxicity assay in duplicates, with splenocytes from the two types of mice cultured overnight with IL-2 alone and with each of the two cytokine cocktails and evaluated the lysis of the targets YAC-1, RMA and C4.4-25$^-$ (**Figure 10A**). It is known that a relatively short incubation time with IL-2 is enough to significantly increase natural cytotoxicity (40), and this was the case in both strains, as YAC-1 was killed at levels between 50% and nearly 70% at all E/T ratios.

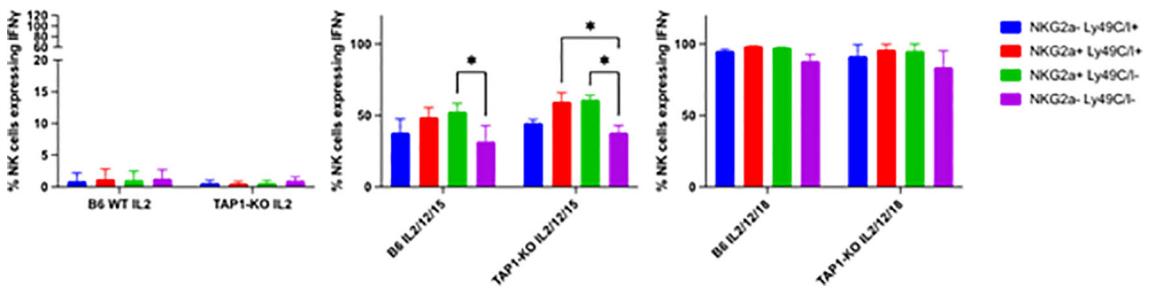
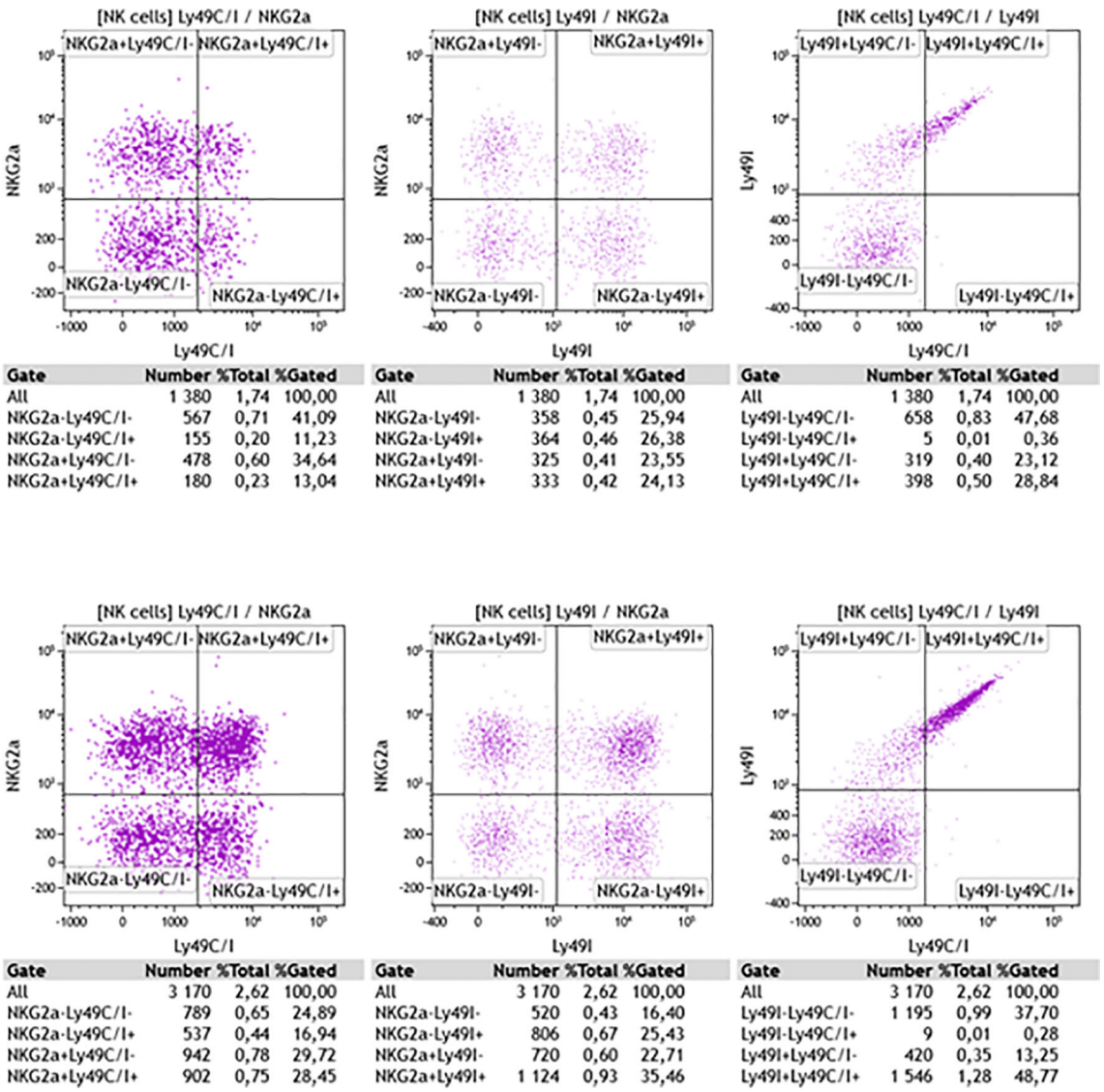



**FIGURE 9** | **(A)** NK cells derived from murine spleen were expanded overnight in IL-2 and stimulated with IL-2 alone, or IL-2, -12, -15 or IL-2, -12, -18 cytokines overnight. These cells were then co-cultured with the targets YAC-1, RMA and C4.4-25$^-$ for four hours. The cytotoxicity was measured with TO-PRO-3 staining. **(B)** NK cells derived from murine spleen and lung were expanded in the presence of IL-2 for 5 days and then restimulated with IL-2 alone overnight. These cells were then co-cultured with the targets YAC-1, RMA and C4.4-25$^-$.




| Gate | Number | %Total | %Gated |
|---|---|---|---|
| All | 1 380 | 1,74 | 100,00 |
| NKG2a-Ly49C/I- | 567 | 0,71 | 41,09 |
| NKG2a-Ly49C/I+ | 155 | 0,20 | 11,23 |
| NKG2a+Ly49C/I- | 478 | 0,60 | 34,64 |
| NKG2a+Ly49C/I+ | 180 | 0,23 | 13,04 |

| Gate | Number | %Total | %Gated |
|---|---|---|---|
| All | 1 380 | 1,74 | 100,00 |
| NKG2a-Ly49I- | 358 | 0,45 | 25,94 |
| NKG2a-Ly49I+ | 364 | 0,46 | 26,38 |
| NKG2a+Ly49I- | 325 | 0,41 | 23,55 |
| NKG2a+Ly49I+ | 333 | 0,42 | 24,13 |

| Gate | Number | %Total | %Gated |
|---|---|---|---|
| All | 1 380 | 1,74 | 100,00 |
| Ly49I-Ly49C/I- | 658 | 0,83 | 47,68 |
| Ly49I-Ly49C/I+ | 5 | 0,01 | 0,36 |
| Ly49I+Ly49C/I- | 319 | 0,40 | 23,12 |
| Ly49I+Ly49C/I+ | 398 | 0,50 | 28,84 |

| Gate | Number | %Total | %Gated |
|---|---|---|---|
| All | 3 170 | 2,62 | 100,00 |
| NKG2a-Ly49C/I- | 789 | 0,65 | 24,89 |
| NKG2a-Ly49C/I+ | 537 | 0,44 | 16,94 |
| NKG2a+Ly49C/I- | 942 | 0,78 | 29,72 |
| NKG2a+Ly49C/I+ | 902 | 0,75 | 28,45 |

| Gate | Number | %Total | %Gated |
|---|---|---|---|
| All | 3 170 | 2,62 | 100,00 |
| NKG2a-Ly49I- | 520 | 0,43 | 16,40 |
| NKG2a-Ly49I+ | 806 | 0,67 | 25,43 |
| NKG2a+Ly49I- | 720 | 0,60 | 22,71 |
| NKG2a+Ly49I+ | 1 124 | 0,93 | 35,46 |

| Gate | Number | %Total | %Gated |
|---|---|---|---|
| All | 3 170 | 2,62 | 100,00 |
| Ly49I-Ly49C/I- | 1 195 | 0,99 | 37,70 |
| Ly49I-Ly49C/I+ | 9 | 0,01 | 0,28 |
| Ly49I+Ly49C/I- | 420 | 0,35 | 13,25 |
| Ly49I+Ly49C/I+ | 1 546 | 1,28 | 48,77 |

**FIGURE 10** |

Interestingly, the addition of the other cytokines did not further level up the cytotoxicity, but even induced a minimal trend towards a decrease.

RMA cells resisted quite well as described (39) and the residual level of cytotoxic activity, that became significant at the highest E/T ratios, was again observed to a comparable degree between the two types of splenocytes. Furthermore, we demonstrated that B6 wildtype and TAP1-KO, NK cells were able to perform missing self-recognition, as they abundantly killed the MHC class I-deficient cell line C4.4-25$^{-}$, which lacks the B6 class I molecules H-2D$^{b}$ and H-2K$^{b}$, highly expressed by RMA (data not shown). Thus, after cytokine stimulation, NK cells from TAP1-KO become functional to the same extent as their wildtype counterparts.

Then, we repeated the cytotoxicity experiments with spleen and lung NK cells cultured during five days in the presence of IL-2 and re-stimulated overnight with IL-2 alone (**Figure 10B**). Here again, YAC-1 and C4.4-25$^{-}$ were very efficiently lysed by NK cells from the two strains and of both organs. Lung NK cells showed a tendency to a stronger killing activity than their splenic counterparts. RMA cells were significantly susceptible to spleen and lung NK cells (especially at higher E/T ratios), which might be related to the longer stimulation time of these effectors compared with only one day. Importantly, we could not detect statistically significant differences in the lysis intensity between B6 and TAP1-KO, NK cells, confirming the results obtained at day 1 with the spleen and extending them at day 6 to the lung.

## Natural Killer Cell Degranulation

Degranulation assays consist in the measurement, by flow cytometry, of the percentage of NK cells expressing the marker CD107a at the surface after, for example, incubation with target cells (41). This molecule is part of the membrane of the NK cytotoxic granules and accompanies their secretion. It is considered as a surrogate for the cytolytic activity (41). We analyzed degranulation of *ex vivo* spleen and lung NK cells from the two types of mice (three mice per strain) after overnight stimulation with the same cell lines and cytokine cocktails used for the cytotoxicity evaluation in the various quadrants defined by the presence or absence of Ly49C/I and NKG2A (**Figures 11A, B**). Quite surprisingly, there was a significant level of degranulation (CD107a+ NK cells) at baseline, *i.e.* in the absence of targets. This background was higher with IL-2 alone than with the two mixtures of interleukins, but predominantly among the NKG2A single positive and the double positive NK cells. The values did not change significantly after addition of the three target cells but continued to be highest with IL-2 alone. Furthermore, we observed the same distribution of the percentages of de-granulating cells than in the experiments about IFNγ production, namely that the most important fractions of CD107a+ NK cells were reached in the two subsets expressing NKG2A, compared to the Ly49C/I single positive and the double negative populations. Whereas IL-2 was most efficient in stimulating spontaneous and target cell-induced CD107a cell surface mobilization, it came a bit unexpected that the cytokine cocktails were less active here. In any case, the degranulation experiments confirmed once more published data about NK cell licensing and education.

## DISCUSSION

In this study, we confirm the initial licensing profile of NK cells in the B6 wildtype mouse genetic background, corresponding to the fact that the expression of self-specific IR for autologous (self) MHC class I molecules educates these cells and renders them functionally active, although the licensing does not automatically lead to the activation of the entire population of self-specific IR+ NK cells but only of a fraction of them, in accordance with the literature (10, 11). The choice of TAP1-KO mice stems from the fact that their NK cells are usually considered as non-educated and hypo-reactive (31), in contrast to standard B6 mice, and are therefore an optimal model for a study about licensing.

However, the phenotype and functions of TAP1-KO NK cells are not that different from their wildtype counterparts, and in many knockout models of NK cell receptors or members of signaling cascades, the surface phenotype remains unaffected.

Our experiments furthermore confirmed the phenotypic differences between spleen and lung NK cells that we (28), and others (29), have previously shown. The latter appear as more mature at least in the mouse (28, 29). This is fully in line with the current paradigm of NK cell heterogeneity dependent on the

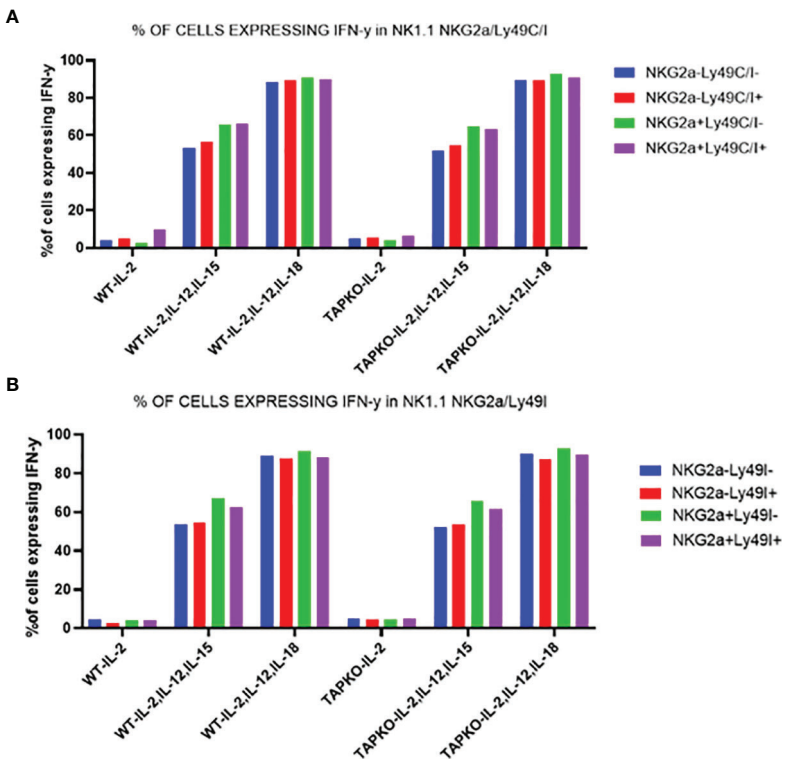
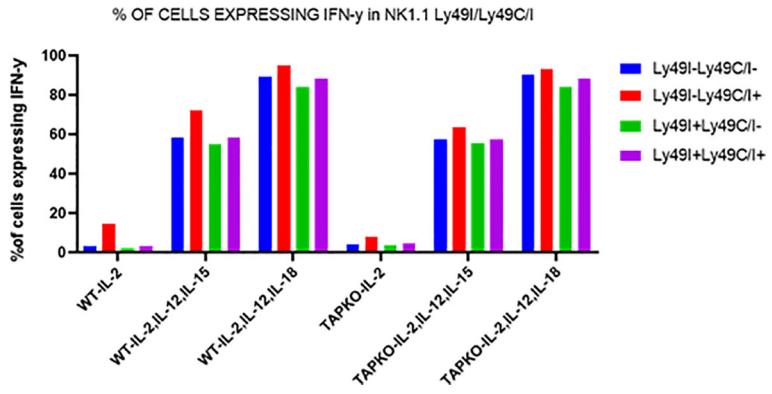



**FIGURE 11** |




Q27 **FIGURE 12** |

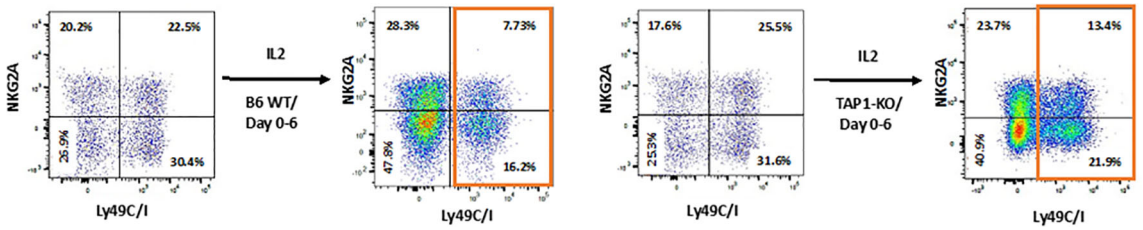



**FIGURE 13** |

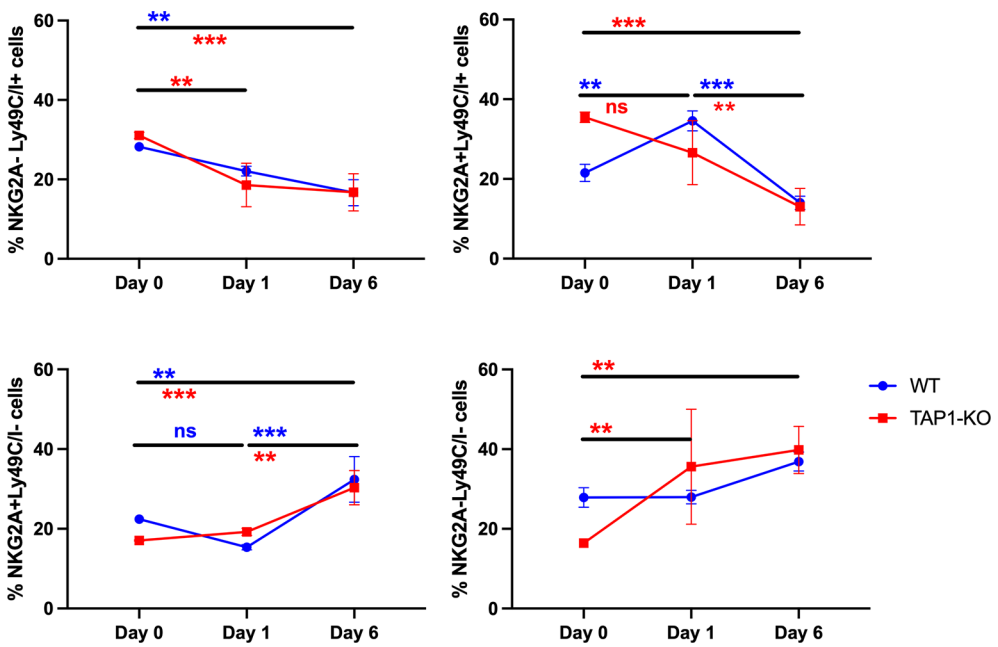



**FIGURE 14** |

organ that is looked at (42), and so the mere investigation of peripheral blood NK cells in human and/or splenic NK cells in the mouse, although they are easily accessible, is to some extent a limitation. This might have clinical consequences in human patients, if for example an immunotherapeutic modality targeting NK cells would have organ-specific effects that could be either favorable or detrimental depending on the specific population.

Interestingly, the phenotype of TAP1-KO NK cells was only minimally different from wildtype, as previously described (31). However we found that after a strong cytokine-mediated activation, these cells resembled wildtype NK cells in terms of licensing. Indeed, the level of autologous MHC class I molecules being very low in their cellular environment, they are hyporeactive *ex vivo* and can in principle not be educated. Nevertheless, after stimulation with (IL-2, IL-12, IL-15) or (IL-2, IL-12, IL-18), they become significantly active in terms of cytokine production and cytotoxicity. What was really surprising was the observation that, although the four NK cell subsets defined by the presence or absence of the self-specific IR were significantly active regarding IFNγ production, which was largely expected in the wildtype mice, the initial education profile was to some extent conserved at day 6, and moreover, also present and clearly identifiable in the TAP1-KO NK cells. Thus, there must be molecular processes that can license these cells independently of MHC class I molecules and on the other hand are likely related to them, as the differences are defined by the presence or absence of Ly49C/I and NKG2A.

The phenotypic analysis of the NK cells from the two strains revealed some interesting differences, which might be worth investigating in more detail and with more fluorochromes for flow cytometry and/or an investigation by mass cytometry. We were particularly impressed by the total absence of staining of TAP1-KO cells by the anti-Qa2 antibody, revealing the complete dependence of this non-classical HLA class I molecule on a functional TAP.

The downmodulation of Ly49C/I, whereas NKG2A expression was spared, has previously been described by Shi et al. (36). These authors envisaged several possibilities to explain the observation, namely receptor down-modulation (internalization)?, contraction of the subset or relative expansion of the Ly49C/I- NK cells, to finally suggest after an experimental approach that the most likely explanation might be receptor shedding from the cell surface. In any case, the phenomenon could be reproduced after cytokine-mediated stimulation of NK cells as an incidental finding in our study. If the reason behind it was receptor internalization after prolonged contact with cognate MHC class I ligands in the cell culture system, it would most likely not have been observed in TAP1-KO mice. Comparative evaluation of the proliferation of Ly49C/I- compared with Ly49C/I+ NK cells, and the amount of soluble Ly49C/I in the supernatant of strongly activated *versus* less activated splenocyte cultures could be interesting perspectives for future experiments. Likewise, Korten et al. reported a reduction in the percentages of Ly49C+, Ly49G2+ and Ly49A+ NK cells in helminth infection, in the context of a global NK cell expansion (43). However, these observations stem from the Balb/c mouse strain, which carries another genetic background and other polymorphic forms of the Ly49 family. Tay et al. showed that in murine cytomegalovirus infection, the percentage of Ly49C+ NK cells declines in the spleen but not in the peritoneal exudate, a phenomenon that is not present in lymphocytic choriomeningitis virus (LCMV) infection (44). Other aspects of Ly49C/I receptors are their presence on memory NK cells and their peptide sensitivity (recognition of the peptide presented by the cognate MHC class I molecules), as reviewed by Wight et al. (45). One might hypothesize that the loss of Ly49C/I by activated NK cells renders these effectors more efficient against targets expressing classical MHC class I ligands. After cytokine-mediated stimulation however, the most efficient activation resides in the NKG2A-expressing subsets, which seem to educate NK cells "better" than Ly49C/I. Finally, Ly49C also interacts with its ligand in *cis*, *i.e.* in the plane of the same cell membrane (46, 47), which corresponds in fact to the physiological situation. This phenomenon, initially described for Ly49A, has important consequences for the education and function of NK cells (23).

Despite their biological immune suppression (low number of CD8+ T cells, hypo-reactive NK cells), TAP1-KO mice display no clinical phenotype, if they live in a specific pathogen free or a "dirty" animal facility. Moreover, their short life span and the normal humoral immune response, as well as the possibility of activating their NK cells in an infectious context, might contribute to the explanation. In addition and importantly, Barbet et al. recently described that a "non-canonical" way of cross-presentation by TAP-deficient dendritic cells allows the stimulation of CD8+ T cells (48). This is in sharp contrast to human TAP deficiency, a very rare autosomal recessive disease (around 40 cases described in the literature) characterized by repeated bacterial infections of the respiratory tract, bronchiectasis, deep skin ulcers and a granulomatous destruction of the nasal cartilage ending up in a clinical picture resembling NK/T cell lymphoma, nasal type (lethal midline granuloma) (49). These patients usually reach early adulthood but have a low quality of life. Their NK cells are hypo-reactive *ex vivo* as in the mouse, but they become strongly activated after cytokine stimulation (IL-2). They present aroused NK cells within the skin lesions, so that we cannot exclude that the former contributes to the pathophysiology of the disease (13, 14, 18). A mouse model mimicking skin ulcers and/or bacterial respiratory infections would be necessary to address these questions in-depth.

Overall, we confirm the licensing (an IR on the NK cell must interact with an autologous MHC class I molecule) and the rheostat (the more IR there are on a NK cell, the better it is educated – quantitative aspect) models that surprisingly also seem to apply to the TAP1-KO NK cells, whereas this is theoretically not possible. The only explanation would be that Qa-1$^b$ might educate the cells, as its expression is possible with TAP-independent peptides (50). An argument for this possibility could be that the NKG2A+ NK cell subsets react stronger than the NKG2A- ones, at least in the case of the parameters we

investigated. An interesting observation is the loss of Ly49C/I, that could indicate, if similar observations would be made for human KIR and/or NKG2A, the possibility of selecting subsets with lower percentages of IR+ NK cells. This would in turn optimize adoptive NK cell therapies, because the majority of the cells would be free from inhibition by the host's MHC class I molecules and therefore, at least in theory, be more effective than a bulk population in eradicating tumors or infections.

## 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 animal study was reviewed and approved by Ministry of Health, Luxembourg Animal Welfare Structure, Luxembourg Institute of Health.

## AUTHOR CONTRIBUTIONS

NP, CM, MT, OS, and MT performed experiments and analyzed data. CS-D and HS gave critical input to the study. MO and JZ conceived and supervised the work. NP and JZ wrote the paper. All authors edited the paper and agreed with the final version.

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