Trends in Immunology

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Series: Immunometabolism
Review

Reactive Oxygen Species: Involvement in T Cell Signaling and Metabolism

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T cells are a central component of defenses against pathogens and tumors. Their effector functions are sustained by specific metabolic changes that occur upon activation, and these have been the focus of renewed interest. Energy production inevitably generates unwanted products, namely reactive oxygen species (ROS), which have long been known to trigger cell death. However, there is now evidence that ROS also act as intracellular signaling molecules both in steady-state and upon antigen recognition. The levels and localization of ROS contribute to the redox modeling of effector proteins and transcription factors, influencing the outcome of the T cell response. We discuss here how ROS can directly fine-tune metabolism and effector functions of T cells.

Highlights

ROS have long been recognized as markers of stress and inducers of cellular damage.

Low amounts of ROS are emerging as positive contributors to normal signaling pathways implicated in cell growth, controlled cell death, migration, and T cell activation.

Local levels of ROS determine and diversify the outcome of ROS-associated signaling events.

ROS levels are physiologically balanced by antioxidants, of which glutathione is the most abundant. By scavenging ROS, this non-enzymatic system prevents oxidative damage and fine-tunes ROS concentrations in space and time.

Metabolic Reprogramming during T Cell-Mediated Immune Responses

Immunometabolism examines the functions of various immune cell subsets in the context of the metabolic cues that trigger and support their specific responses [1,2]. It is now becoming clear how defined changes in metabolism are integrated with particular immune cell functions, such as the activation of macrophages, or the dynamic transitions occurring throughout the lifespan of a T cell. This review focuses on the contribution of ROS to T cell immunometabolism and signaling.

After emerging from the thymus, naïve T cells (T_n) circulate in the bloodstream and continually migrate through the secondary lymphoid tissues to scan the body for invading pathogens or malignant cells. A T cell is activated when its T cell receptor (TCR) recognizes the antigen presented by antigen-presenting cells (APCs) in the presence of costimulatory molecules (such as CD28). This recognition and the subsequent intracellular signaling lead to a multistep process in which the activated T cells proliferate and differentiate into effector T cells (T_{eff}). $CD4^+$ T_{eff} cells help to activate other immune cells by producing a variety of cytokines, the nature of which depends on the stimulus, antigen dose, and existing cytokine milieu [3]. $CD8^+$ T_{eff} secrete antitumor and antiviral factors [4], and release cytotoxic granules causing caspase-dependent and -independent apoptosis of target cells such as tumor cells or virus-infected cells [5]. Toward the end of a T cell response – when the antigen has been cleared – $CD4^+$ and $CD8^+$ T_{eff} contract and only a small number of long-lived memory T cells (T_m) that can respond to a second appearance of the threat remain [6]. Each of these stages requires a change in the T cell energy requirements that is reflected in modifications to its metabolic state (Figure 1).

Metabolically speaking, T_n emigrating from the thymus rely mainly on ATP derived from the oxidation of glucose-derived pyruvate, a process that involves oxidative phosphorylation (OXPHOS) in the mitochondria [7,8]. T_n may also use fatty-acid oxidation (FAO) to generate ATP. In a resting metabolic state, T_n rely on tonic signaling through the TCR, as well as on extrinsic IL-7 signaling, to sustain survival and homeostasis [9,10]. Tonic TCR transduction induces the production of mRNA encoding **glucose transporter 1** (**GLUT1**, see Glossary) [10], and IL-7 receptor signaling is crucial for GLUT1 trafficking to the membrane [11,12]. To

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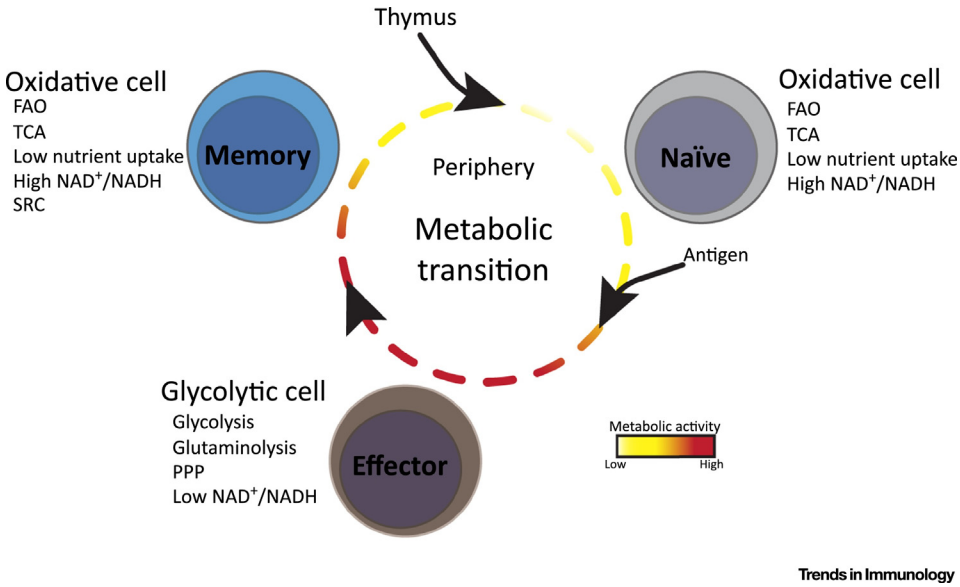

Figure 1. Metabolic Features during the Various Stages of the T Cell Lifespan. Different metabolic pathways are active during different phases of a T cell response. Naive T cells (T_n) are more oxidative and have a lower rate of metabolic activity than effector T cells (T_{eff}). Memory T cells (T_m) switch back to the oxidative metabolism that is necessary for long-term survival, but retain increased energetic capacity (SRC) in case of reactivation by antigen. Abbreviations: FAO, fatty acid oxidation; PPP, pentose phosphate pathway; SRC, spare respiratory capacity; TCA, tricarboxylic acid (cycle).

ensure survival and avoid any oxidative pressure, T_n maintain a balance between oxidizing byproducts (e.g., ROS) and reducing agents (e.g., cellular antioxidants). Indeed, T_n constantly synthesize antioxidant molecules to keep ROS levels in check because excessive ROS are known to precipitate cell death pathways and may contribute to the induction of a persistent pro-oxidative state in cancer cells [13–15].

The high energy demands of T cell activation require the cell to substantially increase its nutrient uptake and to reprogram its metabolism. Upon antigen stimulation, an activated $CD4^+$ or $CD8^+$ T cell experiences a shift from a catabolic quiescent state to an anabolic state that supports its proliferation and differentiation into T_{eff} by upregulating both glycolysis and mitochondrial metabolism, which is fueled by glutaminolysis. These processes are characterized by a rapid accumulation of biomass and increased generation of macromolecules. This switch to aerobic glycolysis leads to the conversion of glucose to pyruvate and the generation of the metabolic intermediates required for cell growth and proliferation [16] (Figure 2). In addition, aerobic glycolysis helps to maintain redox balance in the cell through the production of NADH. Downstream signaling in activated T cells also leads to induction of the transcription factor Myc, which activates the expression of target genes promoting aerobic glycolysis and glutaminolysis [17,18]. In addition, TCR engagement triggers stimulation of the PI3K/Akt/mTORC1 axis, in which the mTORC1 complex senses the availability of amino acids [19]. mTORC1 signaling results in both sustained aerobic glycolysis through the induction of hypoxia-inducible factor 1α (HIF-1α) [19] and an increase in glutamine metabolism. mTORC1 signaling also favors the pentose phosphate pathway (PPP) [19], which is essential for *de novo* nucleotide synthesis and DNA replication. A key function of the PPP is to produce **NADPH**, which is essential for the regeneration of the major intracellular antioxidant molecule glutathione (GSH) (Figure 2). Finally, T cell activation is accompanied by increases in glucose uptake [20,21] and mitochondrial

Glossary

AMP-activated protein kinase (AMPK): central regulator of fatty acid metabolism.
AP-1: a transcription factor family known to play an important role in cancer progression and development.
CD69: marker antigen that is rapidly expressed on the surfaces of T and B cells during the early stages of activation.
CD95: also known as FAS or Apo-1; belongs to the tumor necrosis factor receptor superfamily (TNFSFR) and has a pleiotropic expression pattern. Upon ligand (CD95L) binding, CD95 induces apoptosis.
Extracellular signal-regulated serine/threonine kinases (ERKs): the ERK pathway plays a crucial role in the regulation of cell growth and differentiation.
Glucose transporter 1 (GLUT1): the major glucose transporter located in the plasma membrane of mammalian cells.
Histone deacetylases (HDACs): this class of enzyme removes acetyl groups from histones.
NADPH: reduced form of NADP. The redox potential of NADPH is required in a variety of reductive synthesic processes.
Spare respiratory capacity (SRC): the difference between the cellular oxygen consumption rate (OCR) at basal and maximal activity.
$\Delta\Psi m$: the transmembrane electrical potential gradient across the mitochondrial inner membrane that is established by pumping of protons into the transmembrane region by the mitochondrial respiratory chain.

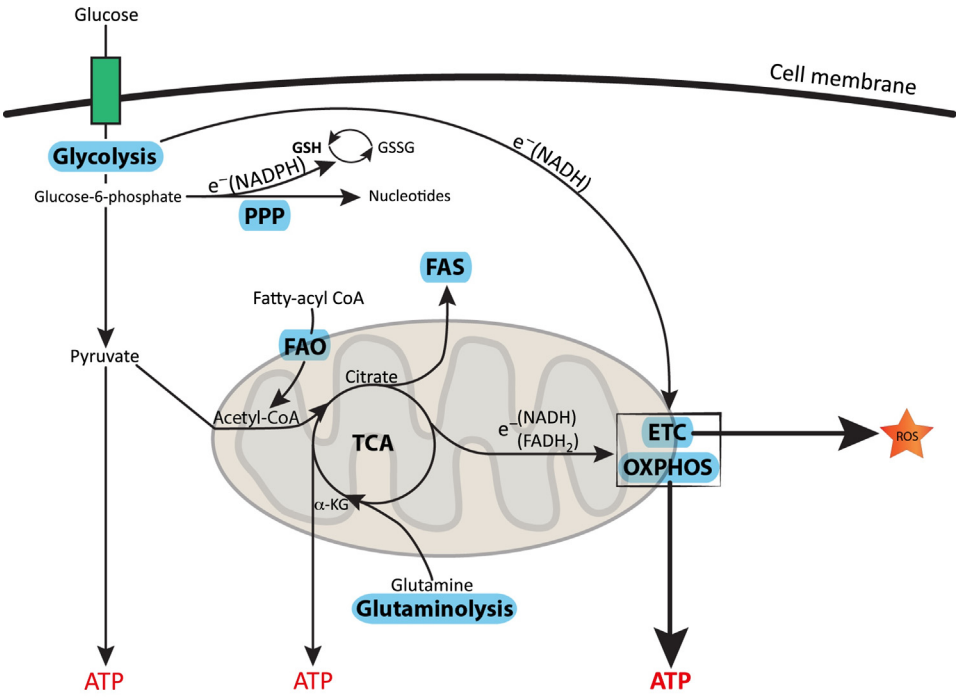

Figure 2. Energy-Generating Pathways in T Cells. Glucose is converted into glucose-6-phosphate in the cytoplasm and is metabolized by glycolysis to produce NADH and pyruvate or via the pentose phosphate pathway (PPP) to synthesize nucleotides and generate NADPH to allow glutathione (GSH) production. Pyruvate is shuttled into the mitochondria where it is converted to acetyl-CoA. Acetyl-CoA can also arise from fatty acid oxidation (FAO). Acetyl-CoA drives the tricarboxylic acid (TCA) cycle in the mitochondria, generating reducing equivalents (NADH and $FADH_2$) that feed oxidative phosphorylation (OXPHOS) executed by the electron transport chain (ETC); this process produces ATP as well as reactive oxygen species (ROS). The TCA cycle also provides biosynthetic precursors for fatty acid synthesis (FAS). Glutamine may fuel the TCA cycle through its provision of α-ketoglutarate (α-KG). The ROS generated by the ETC during OXPHOS act at low levels as secondary intracellular signaling messengers. However, excessive ROS production is harmful to the cell.

activity [8,18,22]. This elevation in glucose uptake is crucial for T_{eff} functions and cytokine secretion, whereas heightened OXPHOS in the mitochondria of activated T cells is needed for their proliferation [22]. Interestingly, Sukumar *et al*. have suggested that elevated mitochondrial membrane potential (**$\Delta\Psi$m**) is associated with high ROS production, and may indicate commitment toward a terminally differentiated state, whereas a low $\Delta\Psi$m is linked to increased levels of oxidized GSH and antioxidant gene expression [23].

Depending on the cytokine milieu, proliferating $CD4^+$ T cells activate particular gene expression programs that drive the generation of specific T cell subsets, such as T helper 1 (Th1), Th2, Th17, and T regulatory (T_{reg}) cells [24]. Each of these subsets has a distinctive metabolic phenotype [25,26]. For instance, T_{reg} show higher levels of OXPHOS and **AMP-activated protein kinase (AMPK)**-dependent FAO, whereas Th17 cells rely mostly on glycolysis [27,28]. So far, less is known concerning the subsets of $CD8^+$ T_{eff} with respect to their metabolic requirements [29].

T_m are able to mount a much faster response to recall challenges with the same pathogen [30]. Resting T_m lose the active glycolytic phenotype characteristic of T_{eff} and acquire metabolic

features resembling those of T_n (Figure 1). This observation suggests that a progressive metabolic conversion occurs during the late phase of a T cell response that supports the generation of T_m, which no longer need to ramp up the energy machinery. Instead, T_m revert to sustaining basic cellular functions while waiting for the next pathogen challenge, and escape cell death through mitochondrial FAO and by maintaining a greater mitochondrial mass. It has been shown that TRAF6-deficient $CD8^+$ T cells displayed reduced FAO owing to an inability to activate AMPK [31], and that blocking FA entry into mitochondria reduced T_m survival [8]. However, He *et al*. found that mTORC1 inhibition by rapamycin permitted AMPK phosphorylation and favored the maintenance of the oxidative signature of T_m [16]. Recent evidence indicates that moderate levels of metabolic activity and ROS (i.e., low $\Delta\Psi$m) promote the longevity and response capacity of T_m [23]. Upon reinfection of a host by a given pathogen, energy demand initially exceeds supply in T_m. Nevertheless, the substantial mitochondrial mass and ATP reserves in T_m enable them to rapidly respond to the invader. The mechanism by which T_m boost their energy output in this way is referred to as **spare respiratory capacity (SRC)** [32]. It is thought that the considerable SRC present in T_m confers a bioenergetic advantage that ensures an effective secondary response [8,33].

In summary, T cells can dynamically adjust their metabolic pathways during their development and differentiation (Figures 1 and 2). The low metabolic needs of the naïve state are supported by OXPHOS and little, but not negligible, glycolysis. Upon stimulation, the cell undergoes metabolic reprogramming that is designed to support the proliferation and production of effector molecules, and this requires a glycolysis-dependent increase in ATP as well as the pentose phosphate pathway (PPP), glutaminolysis, and mitochondrial metabolism for the generation of biosynthetic macromolecules. Following pathogen clearance, however, a small percentage of T cells differentiate to establish the memory T cell pool. T_m rewire their metabolism back to a low energetic state (i.e., OXPHOS) but retain the ability to generate additional energy when needed (i.e., SRC).

Physiological Effects of ROS: A Double-Edged Sword

ROS are chemically reactive free radicals with one unpaired electron in their outer orbit. Physiological generation of ROS results from homeostatic metabolism (Box 1), and cells can benefit from signaling mediated by these low ROS levels. However, given the highly reactive nature of ROS, their presence in excess can result in damage to mitochondrial proteins, organelle membranes, and even DNA, and thus can alter the functional state of the cell. Crucially, the damage to the mitochondria may impair their ability to synthesize the ATP, biosynthetic macromolecules, and importantly ROS that are needed to support activation. Because strict control of ROS is necessary to prevent host pathology, cells possess multiple antioxidant systems that act to limit ROS-induced oxidation [34].

Prolonged oxidative stress due to excessive ROS may induce cells to become senescent, undergo malignant transformation, or die by apoptosis [35]. In particular, the detrimental effects of the $OH^{\bullet}$ radical may outstrip the capacity of cellular DNA repair systems and cause many forms of DNA damage. These DNA alterations may trigger genomic instability, transcription errors, and/or aberrant pathway activation [36]. In addition to DNA damage, the action of ROS on carbon–carbon double bonds in phospholipids may cause lipid peroxidation at the plasma membrane, resulting in a chain reaction that is propagated among all the lipids in this membrane [37]. With respect to proteins, excessive ROS can cause peptide fragmentation or conformational alteration as a result of changes in the electrical charge of amino acids (Cys and Met, in particular). These abnormalities may induce protein dysfunction or may cause partial unfolding of the protein and increase its susceptibility to proteolysis [38].

Box 1. Where Do ROS Come From?

ROS are short-lived chemically reactive radicals, such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen. ROS are generated from oxygen (O_2) by several intracellular mechanisms. Mitochondria mainly generate superoxide by reducing one electron of O_2. Superoxide is converted to the more stable H_2O_2 by superoxide dismutases (SODs).

About $\leq$2% of the total O_2 consumed by a T cell goes into the production of mitochondrial ROS (mROS) [96]. mROS production is mainly associated with the operation of the electron transport chain (ETC), which transfers electrons from NADH and succinate to O_2 in a highly controlled redox pathway that eventually reduces O_2 to H_2O. As the ETC functions, a small fraction of electrons leak out of the chain, resulting in a partial reduction of O_2 that generates free radicals such as $O_2^{\bullet -}$ or H_2O_2. Complexes I and III of the ETC are the main contributors of mROS [96]. There is ongoing debate on the crucial role of the components of the complexes that are involved in $O_2^{\bullet -}$ production, an issue that has been expertly reviewed elsewhere [97].

In addition to mitochondria, other ROS-producing entities in a cell include membrane-bound NADPH oxidases (NOX). These enzyme complexes produce large amounts of ROS that are crucial for the oxidative burst employed by many innate immune cells to kill phagocytized pathogens [38]. Several other enzymes can produce ROS, such as cycloxygenases, lipoxygenases, cytochromes P450, and others. These proteins are located in the mitochondria, endoplasmic reticulum, peroxisomes, or cytosol. Exogenous sources of ROS that might affect cell functions include those generated by UV and gamma radiation, air pollutants, and industrial/agricultural chemicals [39].

Because ROS from any source can damage cellular macromolecules and lead to cell death, their presence must be tightly controlled. Cells express several antioxidant enzymes, such as SODs, catalases, peroxiredoxins, thioredoxins, glutaredoxins, and glutathione peroxidases, as well as small antioxidant molecules such as glutathione (GSH), ascorbate, pyruvate, α-ketoglutarate, and oxaloacetate [39,51]. When the levels of ROS produced in a cell exceed the neutralization rate that can be achieved by the totality of the cellular antioxidant systems, the cell experiences oxidative stress that may impede its function, kill it, or transform it.

It has become clear over the past few years that, although excessive ROS are damaging to cells, low to moderate ROS levels are positive contributors to signaling pathways implicated in cell growth, death, and migration, as well as in tumorigenesis, angiogenesis, oxygen-sensing, and immune responses. For example, H_2O_2 causes reversible post-translational modifications of signaling molecules by acting preferentially on their Cys residues. Thiol groups (-SH) on Cys residues are oxidized by H_2O_2 to sulfenic acid (-SOH), which then reacts with GSH and becomes glutathionylated (-SSG). Adjacent thiols can then form disulfide bonds (-SS-) or partner with amides to form sulfenyl amides (-SN-) [38]. These modifications can alter the function of a protein and thus influence the outcome of signaling pathways in which it is involved [39,40]. Sulfenylation has been implicated in the regulation of phosphatases, kinases, transcription factors, **histone deacetylases (HDACs)**, and antioxidant enzymes [41]. For example, tyrosine and serine/threonine phosphatases are regulated by ROS through the transient oxidation of the Cys sulfhydryl that contributes to the active site function [42]. Activation of transcription factors such as Nrf2/Keap1, NF-κB, members of the **AP-1** family, and HIF-1α can be indirectly modulated by ROS-dependent redox signaling [40,43,44] (Figure 3). In addition, p53 and FOXO transcription factors have been shown to respond to increasing ROS concentrations [13]. Together, this can lead to differences in T cell activation, although other intracellular metabolites (NAD^+) can maintain effector functions and improve antitumor activity of T cells [45]. Nrf2 is indirectly regulated by ROS in multiple ways [44]. Most crucially, Cys151 in Keap1 acts as a ROS sensor and mediates the formation of disulfide bonds between two Keap1 molecules. Cystine formation by ROS impedes Keap1 dimerization and initiates Nrf2 translocation to the nucleus where it triggers a cytoprotective transcriptional response [13,46]. ROS also promote the phosphorylation of Ser209 of eIF4E which regulates Nrf2 protein translation. In addition, ROS play a role upstream of the activation of the IκB kinase complex (IKK), which frees NF-κB from its inhibitor (IκB), and activation of IKK by several kinases (Akt, MEKK1) is susceptible to hydrogen peroxide [47].

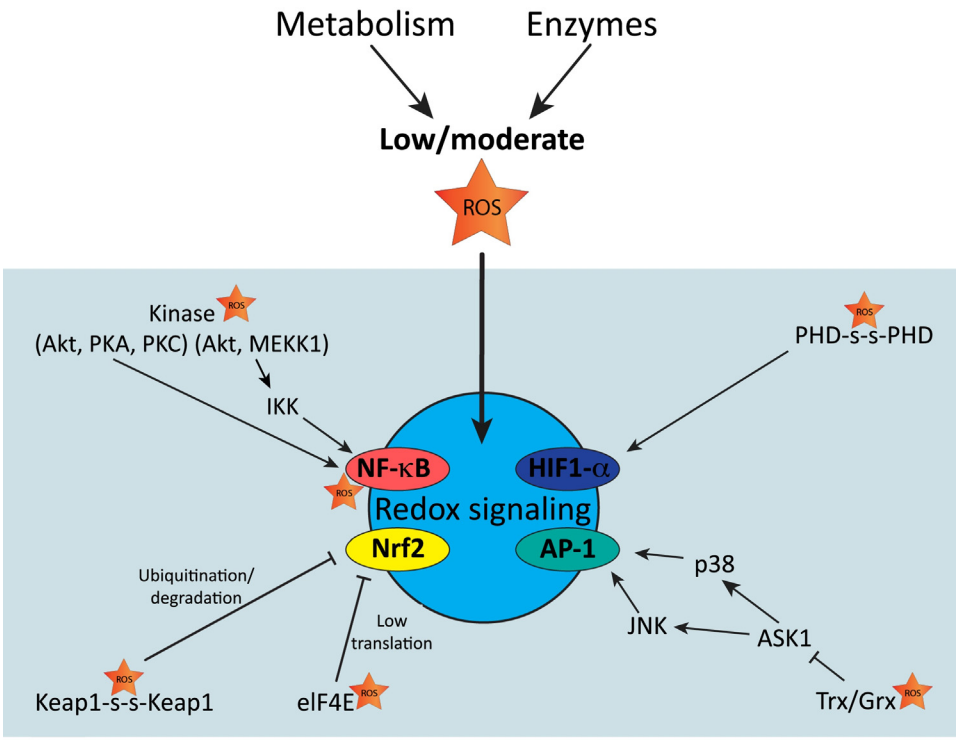

Figure 3. Redox-Sensitive Signaling Pathways for the Regulation of Transcription Factors. Low/moderate levels of reactive oxygen species (ROS) sustain the correct activation/inactivation of transcription factors under homeostatic conditions. This effect can for example be mediated by the formation of disulfide bonds (represented as -s-s-) in prolyl hydroxylase (PHD), a regulator of hypoxia-inducible factor (HIF)-1α, or in the Keap1 (Kelch-like ECH-associated protein 1)–Nrf2 (nuclear factor-like 2) pathway that regulates responses to oxidative stress. ROS can also mediate the sulfenylation of kinases, for example in the nuclear factor (NF)-κB pathway or the AP-1 pathway.

Other ROS-sensitive kinases, such as PKA and PKC, can directly activate NF-κB by phosphorylation [47]. Of note, NF-κB itself possesses a redox-sensitive site in the p50 subunit (Cys62) which is associated with its ability to bind to DNA. NF-κB is also dependent on Keap1-dependent degradation of IKK-β [48].

Stress-activated protein kinases, such as c-Jun N-terminal kinase (JNK) and p38, can be sensitive to ROS redox regulation, and their phosphorylated forms activate AP-1-dependent transcription. The main activating sensors, thioredoxin (Trx) and glutaredoxin (Grx), when reduced can bind and sequester the apoptosis-regulating signal kinase 1 (ASK1). ROS-induced oxidation of Cys residues in Trx causes its dissociation from ASK1 [49]. The activation of ASK1 subsequently leads to activation of JNK and p38 that results in induction of cell death [50]. In addition, HIF-1α is a direct target of ROS [50]. Moreover, indirect HIF-1α regulators, for example, prolyl hydroxylases (PHDs) (Figure 3), are susceptible of redox modulation [50,51].

Although ROS were long considered as harmful metabolic byproducts that cause cellular damage, it has been appreciated more recently that ROS are crucial for healthy cell functions in many contexts. In the upcoming sections we focus on the role of ROS in the adaptive immune response, more specifically in T cells.

ROS Influence T Cell Responses

Several lines of evidence have suggested an important role for ROS in T cell activation. Studies of T cells deficient for complex III of the electron transport chain (ETC) have shown that mitochondrial ROS (mROS) are crucial for NFAT activation and IL-2 production by T cells *in vitro* and *in vivo*. This crucial role appears to be due to ROS-mediated alteration of the redox status of the kinases modulating the NFAT and IL-2 pathways so as to support proliferation after antigen stimulation [52]. Consistent with this observation, IL-2 production by complex III-deficient T cells recovers after treatment with exogenous ROS in the form of H_2O_2. By contrast, complex III is dispensable for the homeostatic proliferation of T cells, as shown by adoptive transfer of complex III-deficient T cells into lymphopenic mice [52]. This result indicates that, although complex III is not required for energy production, it is necessary for mROS-mediated effects on signaling in T cells. The observed localization of mitochondria to the immunological synapse after TCR activation also supports the hypothesis that low levels of mROS are vital to the T cell response [53]. In addition, mROS are involved in the activation of mTOR and Myc, which have pivotal functions in regulating metabolism and cell-cycle commitment in lymphocytes. For example, Previte *et al*. demonstrated that ROS scavenging was able to halt $CD4^+$ T cells at the G0/G1 phase of the cell cycle as a result of enhanced AMPK phosphorylation and consequently mTOR/Myc inhibition [54]. These data suggest that mROS are crucial for T cell metabolic reprogramming following activation by antigen.

Local accumulations of ROS modulate the redox status of many proteins containing Tyr or Cys residues that are important in the context of T cell activation [55,56]. Seminal findings have shown that mitogen-activated pathways in rat thymocytes drive their upregulation of glycolysis and glutamine oxidation [57]. Notably, NF-κB activation in antigen-stimulated human T cells can be blocked by antioxidant treatment [58]. Similarly, antioxidant treatment of mice leads to reduced primary T cell responses to viral infections *in vivo* [59]. By contrast, ROS can also inhibit the activation of transcription factors in T cells and lead to a complex interplay of pathways [51]. For example, chronic exposure of human T cells to H_2O_2 *in vitro* selectively suppressed the DNA-binding capacities of NFAT and NF-κB, which led to downregulation of IL-2 transcription. Conversely, AP-1 DNA-binding activity was enhanced by oxidative stress [60].

T cells interact with various immune cells both during activation and at inflamed sites. At these sites, phagocytes and neutrophils can produce large amounts of ROS to efficiently kill invading pathogens, influencing T cells and potentially causing oxidative stress. It has been shown that activated neutrophils can inhibit DNA synthesis in human T cells, and this could be blocked by the addition of ROS-scavenging *N*-acetylcysteine or catalase [61]. Another study showed that viability of $CD4^+$ T cells was decreased upon coculture with granulocytes in a ROS-dependent manner [62]. However, positive interactions exist that may counterbalance the increased environmental ROS levels that are generated by innate immune cells. Activated macrophages and dendritic cells (DCs) secrete antioxidant precursors such as Cys – which can be taken up by T cells and converted to the antioxidant GSH (Box 2) [63,64]. This would allow T cells to be protected from detrimental environmental ROS, for example, during antigen presentation.

The T cell response to an antigen is tightly controlled by a contraction phase that succeeds the effector phase, and this is regulated by the integration of a series of complex signals, including transcriptional changes and altered cytokine levels in the local microenvironment [65–67]. T cell numbers decline due to the engagement of various controlled cell death mechanisms in the activated cells [15,68–70]. One of these mechanisms is activation-induced cell death (AICD) that is induced by chronic antigen exposure, and this is mainly mediated by the death receptor **CD95** [71]. There is some evidence that ROS are involved not only in the **extracellular signal-regulated serine/**

Box 2. Glutathione Synthesis

ROS are balanced by antioxidants, which generally include molecules that are sufficiently stable to donate electrons and thus act as ROS scavengers. The most abundant intracellular antioxidant system is glutathione (GSH), a γ-glutamyl–L-cysteinyl–glycine tripeptide. GSH production is regulated in a two-step, ATP-dependent reaction. The GSH precursors cysteine, glycine, and γ-glutamyl amino acid enter a cell via an amino acid (AA) transporter. Within the cytosol, γ-glutamyl amino acid forms 5-oxoproline, which is further converted to glutamate. The enzyme glutamate–cysteine ligase (GCL), which is composed of a regulatory subunit (Gclm) and a catalytic subunit (Gclc), ligates glutamate and cysteine to form a dipeptide in the rate-limiting step of the synthetic pathway. This dipeptide is then covalently linked to glycine by GSH synthase (GS) to generate GSH (Figure I). GSH functions in the GSH peroxidase redox cycle, shuttling electrons between its reduced (GSH) and oxidized (GSSG) forms to buffer ROS generation [77].

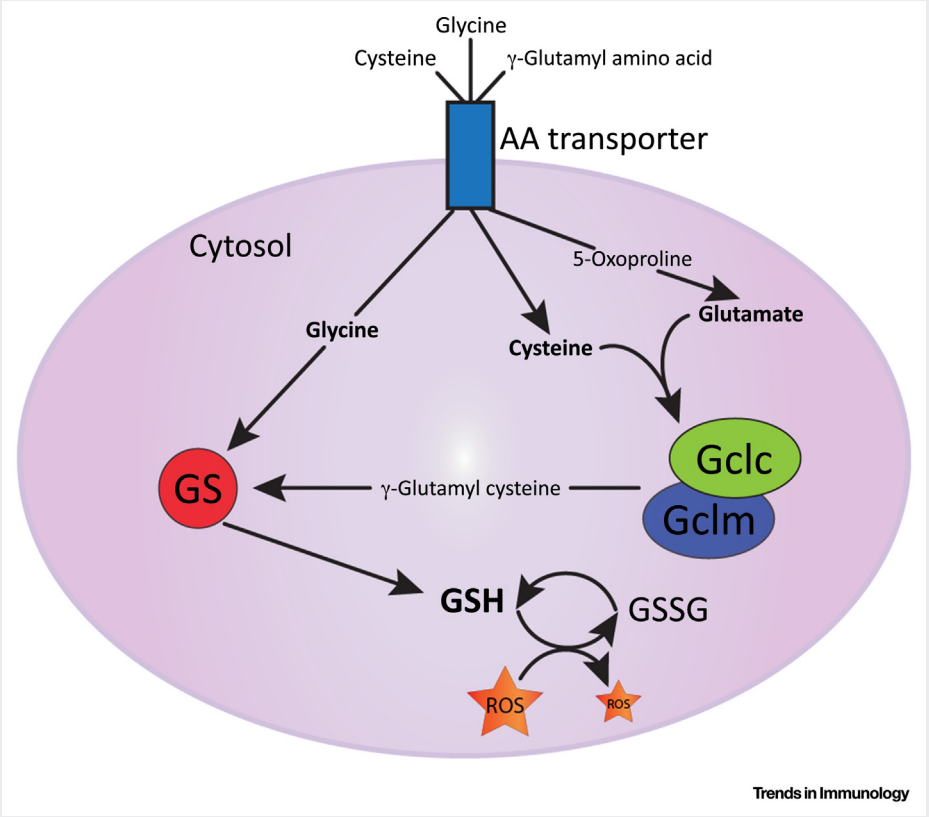

Figure I. Pathway of GSH Synthesis.

threonine kinase (ERK)-mediated proliferative phase of T cell activation but also in AICD [72]. Activated T cells express NADPH oxidase 2 (NOX2) at a low level, and in its absence, or that of other NOX complex components, TCR-stimulated ROS generation is decreased and cytokine production is altered [56] (see Box 1 for ROS sources). NOX2-induced ROS production was originally shown to depend on CD95L and CD95 [56], but more recent findings have shown that NOX2-deficient murine T cells proliferate as expected and show normal expression of the activation markers CD25 and **CD69** [73]. In human T cells, the NOX family member Duox1 has been implicated in the generation of H_2O_2. Accordingly, siRNA-mediated knockdown of Duox1 reduced TCR-mediated H_2O_2 production [74]. It appears that NOX-derived ROS participate in TCR-mediated signaling at multiple levels. Duox1 is involved in proximal TCR signaling, whereas the membrane-bound NOX2 molecule is activated under conditions of chronic TCR stimulation and requires CD95/CD95L expression. Thus, ROS derived from TCR engagement serve to first regulate the ERK proliferative pathway and later the CD95/CD95L proapoptotic pathway, both of which are crucial for a normal T cell response [55].

In addition to the above, TCR plus CD28 stimulation in human T cells induces IL-2 secretion and NF-κB activation that are accompanied by a 5-lipoxygenase-dependent increase in ROS which reduces GSH levels [75]. Similarly, Sena *et al*. showed that CD3/CD28 stimulation of murine $CD4^+$ T cells causes oxidation of mitochondrion-specific redox probes [52], suggesting that both TCR engagement and CD28 costimulation are required for optimal mitochondrial activity. Rotenone-induced inhibition of ETC complex I in T cells leads to decreased IL-2 and IL-4 production as well as greatly reduced production of activation-induced ROS [72,76]. In summary, the triggering of the TCR plus CD28 on a T cell leads to ROS production via various sources, including via NOX activity and the mitochondrial ETC (Box 1).

Regulation of the Redox State during T Cell Activation and Metabolic Reprogramming

Although ROS are clearly important for T cell activation and metabolic reprogramming, it is the local level of these mediators that appear to determine and diversify the outcome of ROS-associated signaling events. Intracellular ROS accumulation is limited by intracellular anti-oxidants, of which the ubiquitously expressed GSH is the most abundant [77]. Buffering of ROS by GSH prevents their intracellular accumulation to a dangerous concentration and modulates their effects. In addition, GSH can reverse ROS-mediated translational modifications such as sulfenylation. GSH scavenges ROS by forming oxidized glutathione disulfide (GSSG), which can be regenerated in the cytoplasm to reduced GSH by glutathione reductase [78]. GSH is synthesized through a pathway in which glutamate–cysteine ligase (GCL) is the rate-limiting enzyme. GCL contains two components: the catalytic Gclc subunit and the regulatory Gclm subunit (Box 2). Upon TCR triggering, Gclc is transcriptionally upregulated and GSH production increases [79] (Figure 4). Although genetic ablation of Gclc specifically in T cells has only a modest effect on ROS accumulation, and does not impair TCR proximal signaling or the early steps of T cell activation, this enzyme is crucial for the ensuing proliferation of these cells and T_{eff} differentiation *in vivo* [79]. As noted above, these processes involve mTOR and Myc, whose activities initiate and support the rewiring of the T cell metabolic program [18]. GSH promotes mTOR activation, most likely by increasing cellular glutamine uptake and glutaminolysis, and is crucial for NFAT activation [79]. In T cells, NFAT is activated through dephosphorylation by the phosphatase calcineurin. Calcineurin activity is sensitive to increased ROS because these radicals oxidize the iron and zinc atoms in the enzyme active center [80,81]. Upon activation, the phosphatase function of calcineurin is induced by a rise in intracellular Ca^{2+} concentrations, which depend on the endoplasmic reticulum (ER) membrane proteins stromal interaction molecule (STIM)1 and STIM2. Intriguingly, the T cell responses of T cell-specific Gclc-deficient mice (which have no GSH and elevated intracellular ROS), and T cell-specific STIM1 and STIM2 double-knockout mice (which have impaired store-operated Ca^{2+} entry), are strikingly similar, implying that rising intracellular ROS may indeed inactivate calcineurin [79,82] (Figure 4). In line with this observation, NFAT and the calcineurin-dependent signaling pathway have been linked to the expression of glucose transporters and genes implicated in glycolysis [82,83]. Inhibition of either calcineurin or mTOR reduced Myc protein levels in activated murine T cells, but mTOR inhibition was less effective than calcineurin blockade [79]. These results suggest that, in addition to the positive effect of calcineurin on mTOR, alternative pathways must exist to drive Myc expression. In this vein, a weak NFAT-binding site has been identified in the *Myc* promoter region, and expression of an active, calcineurin-insensitive NFATc1 mutant protein induced *Myc* expression in calcineurin-inhibited murine T cells by more than 50-fold [82]. In addition, NFAT activation has been linked to *MYC* expression in a variety of cancer cell lines [84–87].

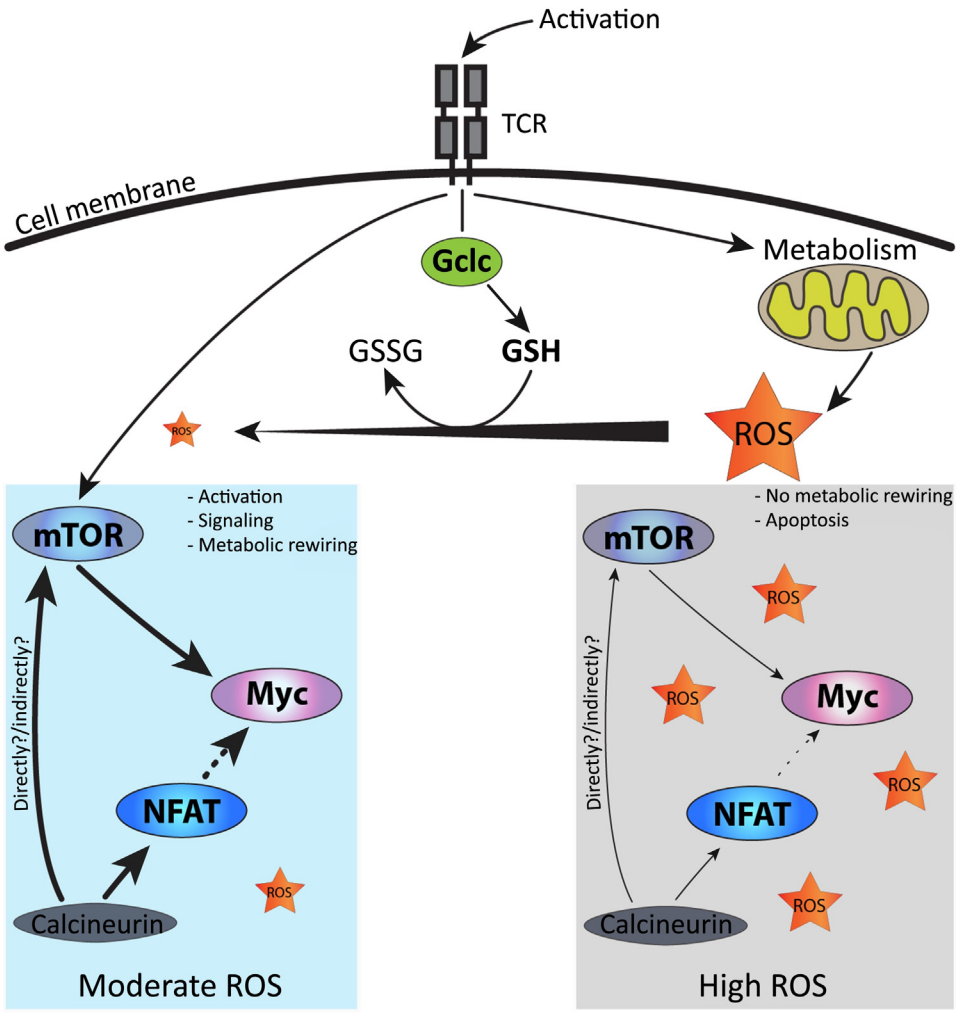

Figure 4. ROS/GSH Balance Allows Correct Control of Transcriptional Regulators and T Cell Activation. TCR engagement leads to the activation of mTOR and a concomitant increase in ROS content. Moreover, T cell activation also induces the expression of Gclc which stimulates GSH production. Control of ROS by GSH buffering activity (GSH → GSSG) ensures the integrity of the T cell energy setup and metabolic rewiring upon antigen-triggered activation, permitting effective activation of mTOR and Myc (left side of the figure), which otherwise would result in inability of the T cell to mount a functional response (right side of the figure). Abbreviations: GSH, glutathione; GSSG, glutathione disulfide; ROS, reactive oxygen species; TCR, T cell receptor.

However, the genetic deletion of two of the three calcineurin-regulated NFAT family members in murine T cells (NFATc1, NFATc2) did not significantly reduce *Myc* mRNA expression [82]. Most importantly, scavenging of ROS by endogenous GSH or exogenously supplemented antioxidants is crucial to prevent redox imbalance and permit the normal induction of *Myc* expression in activated T cells [79] (Figure 4). Myc then triggers the switch of activated T cells from oxidative metabolism to activation-induced aerobic glycolysis [17]. Thus, control of ROS by intracellular GSH ensures the integrity of T cell energy metabolism and metabolic rewiring upon antigen-triggered activation [79]. Evidence exists indicating that either excess or insufficient levels of ROS impair T cell functionality and productive intracellular signaling, and a previous study linked

ROS levels to stimulation of NFAT activation and IL-2 expression [40]. It appears that GSH-mediated scavenging in T cells defines a window that allows ROS-dependent activation of NFAT and mTOR, and induction of *Myc* expression, and thus T cell metabolic reprogramming.

Collectively, the data discussed above imply that the ability of a given T cell to temper its ROS levels directly controls its metabolic reprogramming. The regulation of these ROS must extend beyond classical allosteric and post-translational modifications (i.e., sulfenylation and phosphorylation) of effector molecules because ROS are also known to directly interfere with glycolysis by inhibiting the enzymatic activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [88]. Where ROS are uncontrolled, T cells are non-functional and do not develop T_{eff} or T_m responses. However, ROS at a yet-to-be-defined concentration and/or within a particular cellular compartment(s) are clearly needed for normal intracellular signaling. Therefore, ROS levels must be buffered to a 'sweet spot' to permit the metabolic reprogramming, clonal expansion, and differentiation of an activated T cell.

Concluding Remarks

T cell activation relies on aerobic glycolysis, despite its low efficiency in producing ATP, as well as on mitochondrial metabolism for the delivery of energy and biosynthetic precursors [89]. However, how T cells control their metabolic configuration is partially unknown, and alterations of these mechanisms can lead to an unbalanced use of cellular metabolic pathways, triggering oxidative stress and cell death. This dependence on aerobic glycolysis was identified more than two decades ago [7] and remained a puzzle until it was discovered that low levels of endogenous ROS play an important role in the T cell activation cascade downstream of TCR/CD3 and CD28 engagement. Similarly, little was known about the role of ROS in the context of the B cell response until a recent study found that B cells with augmented mitochondrial mass, respiration, and mROS more readily underwent the immunoglobulin class-switch recombination that is crucial for the humoral response [90]. In fact, by attenuating heme synthesis, mROS indirectly regulate heme-binding factors, including Bach2 and Blimp-1, and therefore play a key role in cell fate determination of activated B cells [90,91]. These results support the growing interest of the field in understanding the molecular mechanisms underpinning the effects of endogenous ROS on lymphocyte metabolism and effector functions (see Outstanding Questions).

Owing to their chemically reactive nature, unscavenged ROS can lead to chronic oxidative stress. Depending on the magnitude of the ROS accumulation, this stress can promote malignant progression [14]. Moreover, tumor microenvironments have been shown to exhibit increased ROS levels [92] which can interfere with antitumor therapies. Chimeric antigen receptor (CAR) T cells represent a promising option for the treatment of several types of cancer, and clinical trials are currently ongoing [93]. Unfortunately, CAR T cells are particularly susceptible to hostile inflammatory conditions, and modulation of their ROS buffering capacity might be beneficial to maintain both their effector functions and their viability. Ando *et al*. [94] studied the functionality of T cells exogenously transduced with the antioxidant enzyme catalase. *In vitro* results showed that engineered T cells were resistant to oxidative stress and cell death [94], implying that targeting T cell redox status in cancer patients through a combination of antioxidant T cell gene therapy and adoptive T cell transfer might be a promising therapeutic strategy. Further studies from the same group showed that CAR T cells that expressed catalase exerted bystander protection of other tumor-infiltrating cells, such as natural killer (NK) cells [95], in favor of better antitumorigenic activity.

Recent work has shed light on the importance of metabolic fitness and functional mitochondria in promoting T cell survival and effector functions to increase therapeutic efficiency [23]. These

Outstanding Questions

What are the molecular targets of ROS in T cells that control immune cell fate?

Why do different T cell subsets (Th1, Th17, T_{reg}) rely on distinct metabolic pathways?

How does the microenvironment influence T cell production of ROS *in vivo*, and how do various types of immune cells (T cells, DCs, T_{reg}) influence each other through ROS production and signaling?

Do tricarboxylic acid (TCA) cycle intermediates, such as acetyl-coA, α-ketoglutarate, and citrate, play a role in altering T cell fate, and how?

How can ROS dynamics, compartmentalization, and production be manipulated in a therapeutically beneficial way?

possibilities have prompted the dissection of ROS signaling and its role in the regulation of immunometabolism in unprecedented detail. The data discussed above hold promise for the use of ROS modulators for therapies directed against cancer or inflammatory diseases. It is important to point out that combinatorial approaches might represent the right compromise to overcome therapy resistance and achieve better efficacy [26] because pre-emptive metabolic adaptation might contribute to the overall efficacy of the immunotherapy.

Acknowledgments

We thank the Luxembourg National Research Fund (FNR) for support. D.B. is funded through the FNR-ATTRACT program (A14/BM/7632103) and the FNR-CORE (C15/BM/10355103) and the FNR-PRIDE (PRIDE/11012546/NEXTIMMUNE) funding schemes. D.B. and D.G.F. are supported through the FNR-RIKEN (TregBar/11228353) grant.

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