The role of reactive oxygen species and mitochondria in T-cell activation

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Declaration

I, Aleksey V. Belikov, hereby declare that the work contained herein has been created independently and has not been submitted elsewhere for any other degree or qualification. The research work was carried out from December 2009 to October 2013 at the Institute of Molecular and Clinical Immunology, Otto-von-Guericke University, Magdeburg.

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Abbreviations

AICD – Activation-induced cell death

AP – Activator protein (transcription factor)

APC – Antigen-presenting cell

ATP – Adenosine triphosphate

BHA – Butylated hydroxyanisole

CCCP – Carbonyl cyanide 3-chlorophenylhydrazone

CD – Cluster of differentiation (surface marker)

CFSE – Carboxyfluorescein succinimidyl ester

ConA – Concanavalin A

CRAC – Calcium release-activated channel

CuZnSOD – Copper-zinc superoxide dismutase, SOD-1

DCFDA – Dichlorofluorescin diacetate

DHE – Dihydroethidium

DMSO – Dimethyl sulfoxide

DPI – Diphenyleneiodonium chloride

DUOX – Dual oxidase

ERK – Extracellular signal-regulated kinase

ETC – Electron transport chain

FACS – Fluorescence-activated cell sorting

FITC – Fluorescein isothiocyanate

GM-CSF – Granulocyte-macrophage colony-stimulating factor

$gp91^{phox}$ – PHOX glycoprotein of 91 kDa

IFN – Interferon

Ig – Immunoglobulin

IL – Interleukin

Iono – Ionomycin

IP_3 – Inositol-1,4,5-trisphosphate

JNK – C-Jun N-terminal kinase

LAT – Linker for activation of T cells (protein)

LCK – Lymphocyte-specific protein tyrosine kinase

mAb – Monoclonal antibody

MEK – MAPK/ERK kinase

MFI – Mean fluorescence intensity

MHC – Major histocompatibility complex

MMP – Mitochondrial membrane potential

MnSOD – Manganese superoxide dismutase, SOD-2

NAC – N-acetyl cysteine

NADH – Nicotinamide adenine dinucleotide reduced

NADPH – Nicotinamide adenine dinucleotide phosphate reduced

NFAT – Nuclear factor of activated T cells

NFkB – Nuclear factor kappa light chain enhancer of activated B cells

NOX – NADPH oxidase

PBMC – Peripheral blood mononuclear cell

PBS – Phosphate buffered saline

PE – Phycoerythrin

PHA – Phytohaemagglutinin

PHOX – Phagocytic NADPH oxidase

PKC – Protein kinase C

PLC – Phosphoinositide phospholipase C

PMA – Phorbol myristate acetate

PRX – Peroxiredoxin

RAC – RAS-related C3 botulinum toxin substrate (protein)

RAS – Rat sarcoma (protein)

ROS – Reactive oxygen species

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOD – Superoxide dismutase

T_c cell – Cytotoxic T cell

TCR – T-cell receptor

T_{eff} cell – Effector T cell

TGF – Transforming growth factor

T_h cell – Helper T cell

T_{mem} cell – Memory T cell

T_{nai} cell – Naive T cell

TNF – Tumor necrosis factor

T_{reg} cell – Regulatory T cell

WT – Wild type

ZAP-70 – Zeta chain-associated protein kinase of 70 kDa

Abstract

Reactive oxygen species (ROS) have been implicated in T-cell hyporesponsiveness, apoptosis, as well as activation and differentiation. However, the majority of the published studies have been performed using the Jurkat leukemic T-cell line or preactivated T cells (T-cell blasts). Moreover, in many cases, non-physiological stimuli or exogenously applied H_2O_2 were employed to induce or to mimic ROS production in T cells, respectively. On the other hand, very few studies have analyzed the role of TCR-triggered ROS under more physiological conditions, for example using human peripheral blood T cells isolated from healthy donors (primary T cells). Therefore, in order to shed light onto the function of ROS in primary T cells, I have analyzed which reactive oxygen species are produced upon T-cell stimulation, where ROS are produced, and their function in T-cell activation.

First, I have shown that, upon TCR ligation, T cells produce superoxide that is released into the extracellular space. Nevertheless, it appears that this reactive species is not required for T-cell functions. Indeed, treatment of cell cultures with antioxidants, such as superoxide dismutase and ascorbate, which remove extracellular superoxide, does not affect activation, proliferation, and cytokine production of CD3xCD28-stimulated primary human T cells. It has been reported that T cells can produce superoxide via the NADPH oxidase 2 (NOX-2). To more specifically analyze the function of superoxide, I have used NOX-2-deficient mice (*gp91$^{phox\ -/-}$*). I have found that T cells from these mice completely lack inducible TCR-mediated superoxide production but display normal upregulation of activation markers and proliferation. Collectively, I have shown that primary T cells produce extracellular superoxide upon TCR triggering via NOX-2, but that this reactive species is not required for T-cell activation, proliferation, and cytokine production.

In the second part of my work, I have investigated whether mitochondria-derived ROS participate in T-cell activation. By using various mitochondrial inhibitors, I have found that mitochondrial membrane potential, but not mitochondria-derived ROS or ATP, is crucial for the activation of primary human T cells.

1 Introduction

1.1 The immune system

One of the inherent properties of any living system is the dynamical maintenance of its structure, despite the harsh impact of the environment and errors in the system itself. In order to achieve this goal, a living system must possess mechanisms to discriminate between “correct” and “incorrect” structures. In multicellular organisms, such a mechanism is called *immunity*, or *immune system*. The natural constituents of the body, which are normally not subject to attack by components of the immune system, are called *self*. On the contrary, the foreign material that enters the body (such as viruses, bacteria or parasites) and the altered structures of the body (such as mutated or malignant cells), which normally stimulate an attack by the immune system, are called *non-self*. Either self or non-self structures that actually do evoke an immune response and that are capable of binding with a product (such as an *antibody*) of the immune response are called *antigens*.

Self and non-self can differ so slightly that the immune system must be extremely sensitive to discriminate between them (1). The lack of sensitivity leads to various *immune deficiencies*, such as recurring infections and cancer. On the other hand, extreme sensitivity necessitates extreme specificity, otherwise many false alarms would be triggered (2, 3). *Autoimmune diseases* are an example of specificity problems, and originate from erroneous recognition of self as non-self.

Achieving high sensitivity and specificity is a difficult, time- and resource-consuming process, and is mediated by the *adaptive* branch of the immune system (also referred to as acquired or specific). Only vertebrates possess this kind of immunity. The cells that mediate adaptive immunity are T and B *lymphocytes*. While B cells produce *antibodies*, T cells destroy infected or malignant cells and help to coordinate the immune response. A more basic component of immunity is the *innate* (or non-specific) immune system, which is present in all multicellular organisms, including plants and fungi (Fig. 1.1). Besides enhanced specificity and delayed response, the main property of the adaptive immune system that differentiates it from the innate one is the *immunological memory*. This allows faster recognition and elimination of reoccurring antigens.

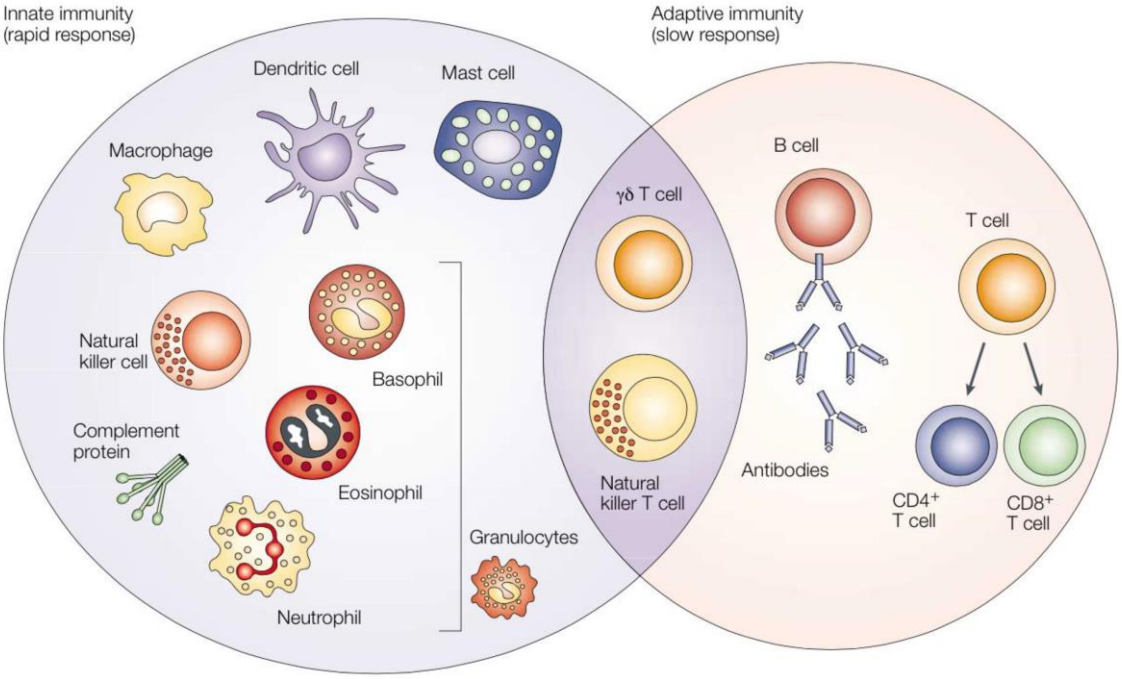

Figure 1.1 Innate and adaptive immunity

The innate immune system functions as the first line of defense against infection. It consists of soluble factors, such as complement proteins, and diverse cellular components, including granulocytes (basophils, eosinophils, and neutrophils), mast cells, macrophages, dendritic cells, and natural killer cells. The adaptive immune response is slower to develop, but it possesses high antigenic specificity and memory. It consists of antibodies, B cells, and $CD4^+$ and $CD8^+$ T lymphocytes. Natural killer T cells and γδ T cells are lymphocytes that bridge the innate and adaptive immunity. Adopted from (4).

1.2 T lymphocytes

1.2.1 T-cell development

"T" in T lymphocytes stands for "thymus-derived", because these cells mature in the *thymus*. Hematopoietic stem cells in bone marrow differentiate into lymphoid progenitor cells that migrate to the thymus, where they become *double-negative* (DN) thymocytes, named after the absence of CD4 and CD8 expression (Fig. 1.2). At the DN stage, thymocytes express a unique β-chain of the *T-cell receptor* (TCR), which is generated upon recombination of V(D)J gene fragments. The expression of a functional TCRβ-chain together with an invariant pre-Tα-chain allows developmental progression (*beta selection*). Once past this step, DN thymocytes rearrange the TCR α-chain through recombination of VJ gene fragments (5). In parallel, DN cells upregulate the expression of CD4 and CD8, thus becoming *double-positive* (DP) thymocytes. If DP thymocytes express a non-functional TCR, they die by apoptosis (*death by neglect*) (Fig. 1.2). Conversely, if DP thymocytes express a functional TCR, which is capable of interacting with self-antigens presented via *major histocompatibility complexes* (MHCs) I or II, which are expressed on the surface of *cortical thymic epithelial cells* (cTECs), they survive (*positive selection*), migrate to the medulla, and develop as either $CD4^+$ or $CD8^+$ (also referred to as *single-positive*, SP) cells (6) (Fig. 1.2).

As I mentioned above, successful immune response requires not only high sensitivity but also high specificity. To minimize the risk of developing autoreactive T cells, thymocytes undergo *negative selection*. This process ensures that thymocytes whose TCRs are capable of strong binding to self-peptides presented on the MHC complexes of cTECs or *medullary TECs* (mTECs) are eliminated via apoptosis (7, 8) (Fig. 1.2). The process by which newly developing lymphocytes are rendered non-reactive to self is called *central tolerance*. It is worth noting that central tolerance is not 100% efficient, and some *autoreactive* T cells escape thymic selection. Additional mechanisms of *peripheral tolerance* exist to silence these cells. They include the suppression of autoreactive cells by *regulatory T* (T_{reg}) cells, the generation of hyporesponsiveness (*anergy*) in lymphocytes that encounter antigen in the absence of co-stimulatory signals, and the existence of *immunologically-privileged* areas (9).

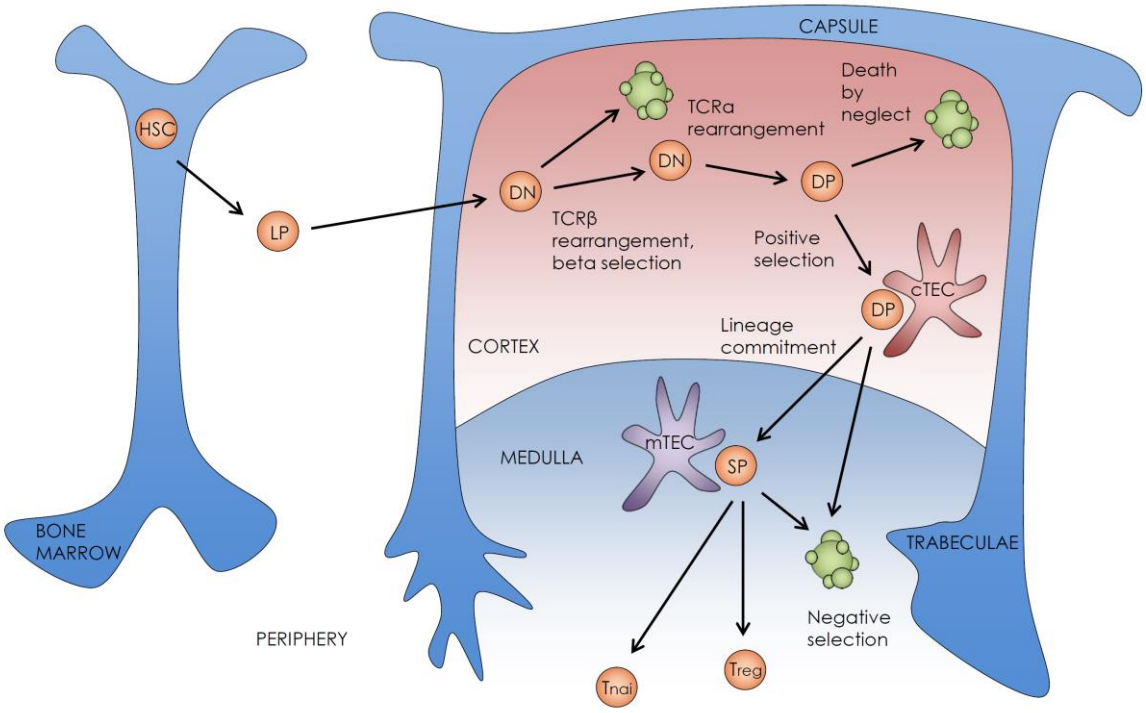

Figure 1.2 T-cell development in the thymus

Hematopoietic stem cells (HSC) in the bone marrow differentiate into lymphoid progenitor cells (LP) that migrate to the thymus, where they become double-negative thymocytes (DN). DNs either successfully rearrange the β-chain of T-cell receptor (TCRβ) or undergo apoptosis. Next, DNs rearrange the TCR α-chain and upregulate CD4 and CD8, thus becoming double-positive thymocytes (DP). If DPs express non-functional TCRs, they die by apoptosis (death by neglect). Conversely, if DPs express TCRs capable of interacting with self-antigens presented by cortical thymic epithelial cells (cTECs), they survive (positive selection), migrate to the medulla, and develop as either $CD4^+$ or $CD8^+$ (single positive thymocyte, SP) (lineage commitment). Thymocytes whose TCRs are capable of strong binding with self-antigens presented by cTECs or medullary TECs (mTECs) are eliminated via apoptosis (negative selection). Positively selected cells exit the thymus as naïve (T_{nai}) or regulatory (T_{reg}) T cells. Green bubbling cells indicate apoptosis.

1.2.2 T-cell subsets

T cells represent a heterogeneous population that consists of several subsets. As I mentioned above, two major T-cell subsets are generated in the thymus - $CD4^+$ T cells and $CD8^+$ T cells.

$CD4^+$ T cells are also called *helper T* (T_h) cells. They are activated by peptide antigens in complex with MHC II, expressed on the surface of *antigen-presenting cells* (APCs). Dendritic cells, macrophages, and B cells can act as APCs. Upon activation, T_h cells proliferate and secrete *cytokines*. Depending on the profile of secreted cytokines, T_h cells are further divided into minor subsets (10) (Fig. 1.3). T_h1 cells secrete IFNγ, IL-2, and Lymphotoxin alpha (LT-α). These cytokines activate macrophages and $CD8^+$ cells. T_h1 cells promote cell-mediated immunity against tumor cells and intracellular pathogens, such as viruses. T_h2 cells secrete IL-4, IL-5, IL-10, and IL-13. These cytokines activate B cells, as well as eosinophils, basophils and mast cells. T_h2 cells promote antibody-mediated immune responses and host defense against extracellular parasites.

In recent years, additional T_h subsets were identified (11). T_h9 cells secrete IL-9 and IL-10 and are involved in host defense against extracellular parasites, especially nematodes. T_h17 cells secrete IL-17a, IL-17f, IL-21, IL-22, IL-26, and Chemokine (C-C motif) Ligand 20 (CCL-20). These cytokines activate neutrophils, as well as B cells. T_h17 cells promote protective immunity against extracellular bacteria and fungi, mainly at mucosal surfaces. Follicular helper T cells (T_{fh}) secrete IL-4 and IL-21. These cytokines activate follicular B cells. T_{fh} cells are involved in promotion of germinal center responses.

Overall, different subsets of T_h cells orchestrate different branches of the immune response. However, it has also become evident that T_h cells can convert from one subset to another and even express mixed cytokine profiles (12, 13) (Fig. 1.3). This plasticity enables quicker and more precise responses to pathogenic threats.

$CD8^+$ T cells are also known as *cytotoxic T* (T_c) cells. They can be activated by peptide antigens in complex with MHC I, expressed on the surface of any nucleated cell. These antigens are usually derived from intracellular pathogens, such as

viruses, or from mutated proteins of tumor cells. Upon activation, T_c cells proliferate, with the help of IL-2 from T_h cells, and release cytotoxic proteins, such as *perforin, granzymes*, and *granulysin*. Perforin makes pores in the plasma membrane, thus allowing serine proteases granzymes to enter the cell and trigger apoptosis (14). On the other hand, granulysin forms pores in the cell wall of intracellular pathogens, which, in turn, allows granzymes to enter and kill them (15). Alternatively, T_c can induce apoptosis via the interaction between Fas ligand on their surface and Fas receptor on the target cell (16).

T_{reg} cells, formerly known as suppressor T cells, are crucial for the maintenance of peripheral tolerance (17). Their major role is to suppress the activation of autoreactive T cells that escaped negative selection in the thymus. Additionally, T_{reg} cells shut down conventional T-cell activity, thus contributing to the termination of the immune response. T_{reg} cells are either directly generated in the thymus from $CD4^+$ thymocytes, referred to as *natural* T_{reg} cells, or originate from activated $CD4^+$ T cells in the periphery, referred to as *induced* T_{reg} cells (18). The various potential suppression mechanisms used by T_{reg} cells can be grouped into four basic modes of action (19): (i) release of inhibitory cytokines (TGFβ, IL-10, and IL-35) or IL-2 deprivation; (ii) granzyme- and perforin-dependent cytolysis; (iii) cAMP- and adenosine-dependent metabolic disruption; (iv) LAG-3- and CTLA-4-dependent suppression of dendritic cell maturation or function.

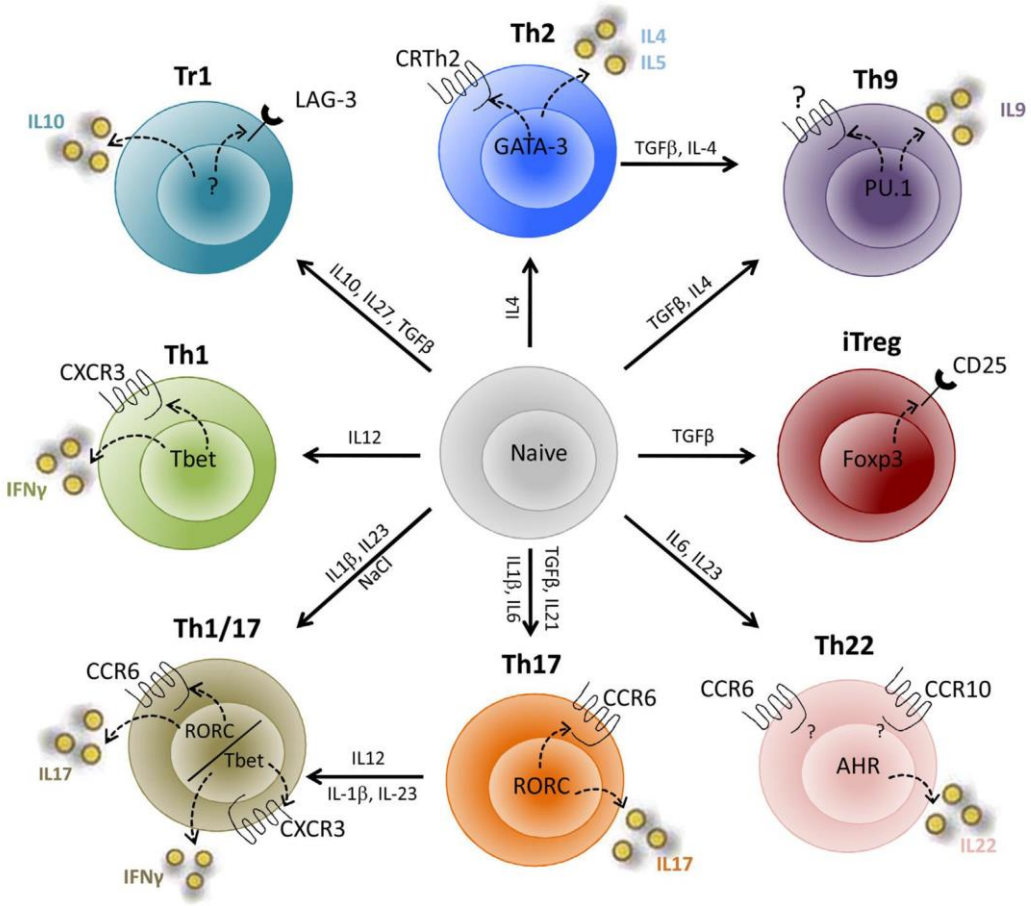

Figure 1.3 CD4⁺ T-cell subsets

Naive T cells can differentiate into various T_h subsets upon priming in response to different cytokines. These helper T-cell subsets express lineage-defining transcription factors that induce specific surface markers in the steady state and release characteristic effector cytokines following TCR stimulation. Adopted from (10).

1.2.3 T-cell activation

T cells that exit the thymus are called *naïve* (T_{nai}) because, unlike *effector* (T_{eff}) or *memory* (T_{mem}) T cells, they have not encountered their specific (*cognate*) antigens yet or, in other words, they have not yet been activated. T_{nai} cells are characterized by the surface expression of CD127 (IL-7Rα), CD62L (L-selectin), CD197 (Chemokine (C-C motif) Receptor (CCR-7)), and RA isoform of CD45 (Protein Tyrosine Phosphatase, Receptor type, C (PTPRC)). T_{nai} cells are quiescent and non-dividing but require periodic contacts with self-peptide-MHC and IL-7 for homeostatic survival (20, 21). L-selectin and CCR-7 lead to the localization (*homing*) of T_{nai} cells to *secondary lymphoid organs*, such as lymph nodes (22, 23). There, they continuously scan APCs for the presence of cognate antigens. When the TCR on the surface of T_{nai} cell recognizes a cognate antigen in complex with MHC-II molecules on the surface of APC, T_{nai} cell becomes activated (see next section). Costimulation via CD28 and/or other surface molecules is necessary for successful activation, otherwise T cells become anergic (24). This is a mechanism to reduce the risk of autoimmunity.

Proper T-cell activation results in the up-regulation of the surface markers CD25 (IL-2Rα), CD44 (Homing Cell Adhesion Molecule (HCAM)), and CD69 (C-Type lectin). Activated T cells secrete IL-2, intensively proliferate, and differentiate into various T_{eff} and T_{mem} subsets (25-28). Activated $CD4^+$ T cells generate T_h and/or T_{reg} progenitors, whereas activated $CD8^+$ T cells are precursors for T_c cells. T_{eff} change the CD45 isoform from RA to RO, lose IL-7 receptor CD127 and lymphoid tissue homing receptors CD62L and CD197, and populate the periphery, to fight infection or cancer *in situ*. After the pathogen or tumor have been cleared, expanded T_{eff} cells die via Fas-mediated apoptosis (*activation-induced cell death, AICD*) or cytokine depletion-induced apoptosis (*death by neglect*), during so-called *contraction phase* of the immune response (29). This limits excessive consumption of nutrients, damage to the body, and development of autoimmunity. T_{mem} cells are preserved in the body much longer than T_{eff} cells. At the second encounter with pathogen, they can initiate a faster and stronger immune response (30). T_{mem} cells are classically distinguished by the expression of the CD45RO isoform, CD44, CD127, and CD215 (IL-15Rα), and require IL-7 and IL-15 for homeostatic survival (20).

1.2.4 T-cell signaling

The TCR consists of one α- and one β-chain, linked by disulfide bond. Both chains include variable, constant, transmembrane, and cytoplasmic regions. The variable parts are generated through genetic recombination during thymic development and are required for the recognition of antigens (31). They are unique for each *T-cell clone*. The other parts are necessary for anchoring of the TCR to the plasma membrane and for the association with accessory molecules, such as CD3 and ζ-chain, to form the TCR/CD3 complex. The *TCR/CD3 complex* consists of a TCRαβ heterodimer, CD3εδ and CD3εγ heterodimers, and a ζζ-chain homodimer (32) (Fig.1.4 A).

The TCR complex does not possess catalytic activity and is dependent on Non-receptor Lymphocyte-specific protein tyrosine Kinase (LCK) to initiate downstream signaling cascade. LCK associates with CD4 and CD8 co-receptors that interact with MHC, and therefore is brought into close proximity of the TCR upon T cell-APC interaction. The main function of LCK is to phosphorylate the *Immunoreceptor Tyrosine-based Activation Motifs* (ITAMs) on CD3 and ζ chains of TCR complex (33) (Fig.1.4 A).

LCK activity is regulated mainly via two tyrosine residues, Tyr505 in the C-terminus and Tyr394 in the catalytic domain (33, 34) (Fig.1.4 B). Phosphorylation of LCK on Tyr505 by C-terminal Src Kinase (CSK) results in the binding of the C-terminus to the SH2 domain of LCK, producing the *closed*, inactive conformation. Tyr505 can be dephosphorylated by Protein Tyrosine Phosphatase, Receptor type, C (PTPRC, CD45), leading to the *primed* form. Primed LCK can autophosphorylate or phosphorylate other primed nearby LCK molecules on Tyr394, resulting in the *open*, active conformation, that is capable of phosphorylating ITAMs and Zeta-chain-Associated Protein kinase of 70 kDa (ZAP-70). Tyr394 can be dephosphorylated by Protein Tyrosine Phosphatase, Non-receptor type, 22 (PTPN-22), SHP-1 (SH2 domain-containing Phosphatase 1), or CD45, thus converting LCK back to the primed state. Moreover, open LCK can be further phosphorylated by CSK on Tyr505 without losing its catalytic activity. ZAP-70 binds to phosphorylated ITAMs via SH2 domains but remains autoinhibited due to the interaction between Tyr319 and the catalytic domain. LCK phosphorylation of ZAP-70 at Tyr319 relieves the autoinhibited

conformation, leading to ZAP-70 activation via trans-autophosphorylation of Tyr493 (33, 34).

Activated ZAP-70 phosphorylates nine tyrosine residues in the Linker for Activation of T cells (LAT). After that, the intracellular domain of LAT becomes able to organize the assembly of a multiprotein complex called the *LAT signalosome*. The LAT signalosome recruits both positive and negative regulators of T-cell signaling and initiates multiple signaling cascades and feedback loops (35) (Fig. 1.5). Ultimately, this culminates in the activation of three major T-cell transcription factors – Nuclear Factor of Activated T cells (NFAT), Nuclear Factor kappa light chain enhancer of activated B cells (NFkB), and Activator Protein 1 (AP-1). These factors initiate the expression of various genes coding for surface receptors, such as CD69 and CD25, and for growth-promoting cytokines, such as IL-2 (36).

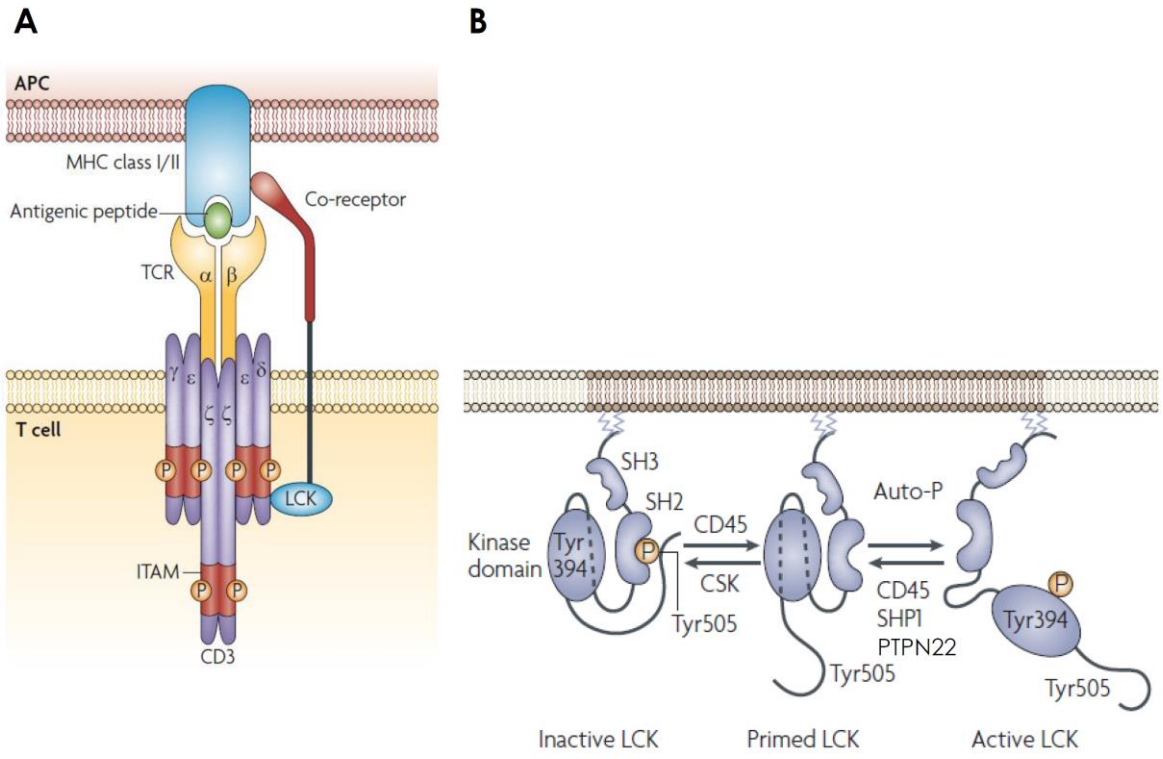

Figure 1.4 TCR structure and LCK regulation

(A) TCR interacts with an antigenic peptide–MHC complex, and the co-receptor (CD4 or CD8) brings LCK in the proximity of the TCR/CD3 complex. LCK can then phosphorylate the CD3-associated ITAMs. Adopted from (37).

(B) Primed LCK can be either phosphorylated on Tyr505 by CSK, resulting in the binding of Tyr505 to the SH2 domain and thus producing the closed, inactive conformation, or can auto- or trans-autophosphorylate on Tyr394, resulting in the open, active conformation. Moreover, open LCK can be further phosphorylated by CSK on Tyr505 without losing its catalytic activity (not shown). Tyr505 can be dephosphorylated by CD45. Tyr394 can be dephosphorylated by CD45, SHP-1, or PTPN-22. Modified from (38).

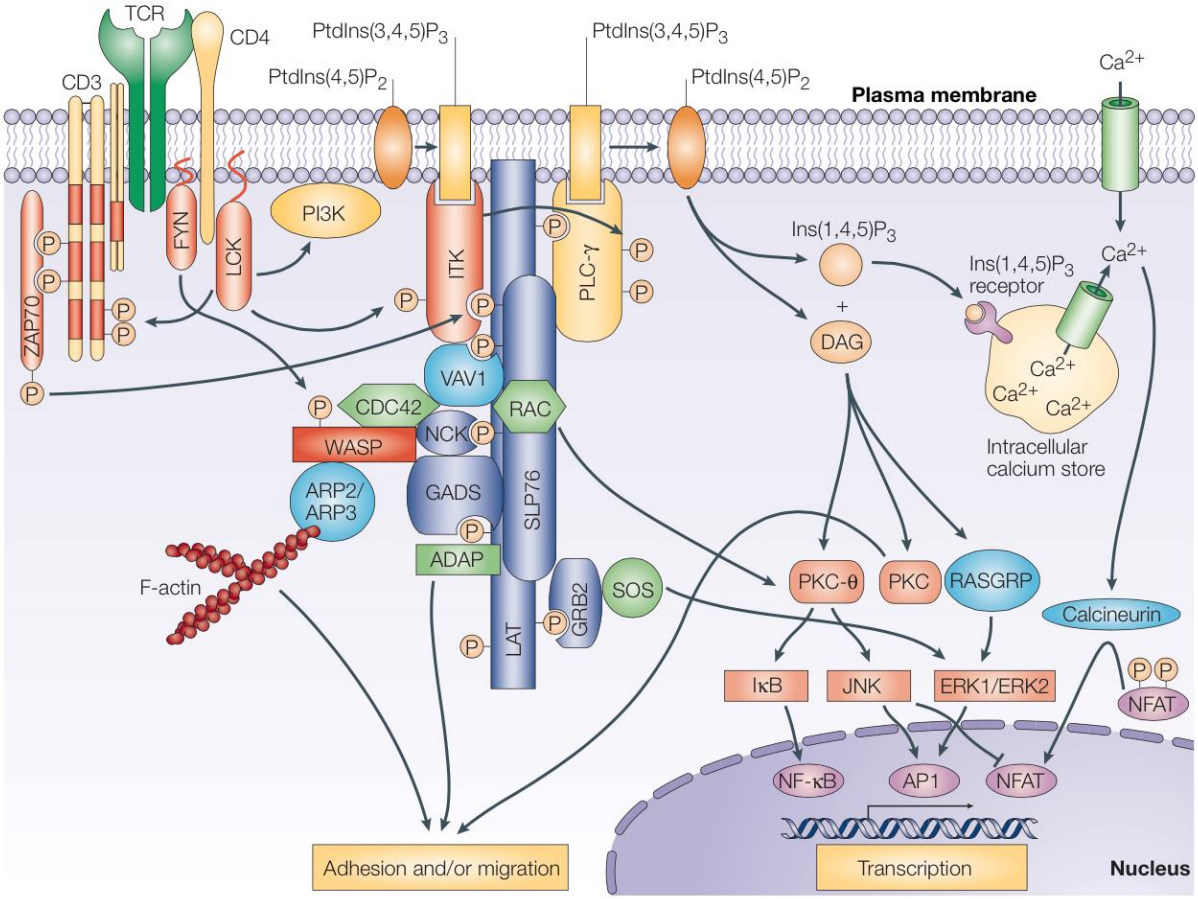

Figure 1.5 TCR signaling pathways

LCK is recruited to the TCR/CD3 complex by CD4 (or CD8) and phosphorylates ITAMs and phospho-ITAM-bound ZAP-70. Activated ZAP-70 phosphorylates LAT, leading to recruitment of SLP-76 (SH2 domain-containing Leukocyte Protein of 76 kDa), ITK (IL-2-inducible T-cell Kinase), and PLCγ-1 (Phospholipase C γ 1). LCK then phosphorylates ITK, which, in turn, phosphorylates PLCγ-1. Activated PLCγ-1 converts PIP_2 (phosphatidylinositol 4,5-bisphosphate) into IP_3 (inositol-1,4,5-trisphosphate) and DAG (diacylglycerol). IP_3 engages specific receptors at the intracellular calcium store, leading to store depletion. This triggers opening of CRAC (Calcium-Release Activated Channels) at the plasma membrane and sustained calcium influx. The resulting rise in intracellular calcium is sensed by calcineurin, which dephosphorylates NFAT, leading to its translocation to the nucleus. DAG activates PKCθ (Protein Kinase C θ) and RASGRP (RAS Guanyl-Releasing Protein). This results in the activation of mitogen-activated protein kinases, such as JNK (C-Jun N-terminal Kinase) and ERK (Extracellular-signal-Regulated Kinase), leading to the activation of AP-1. In addition to DAG, PKCθ and ERK pathways can be activated through RAC (RAS-related C3 botulinum toxin substrate) and SOS (Son of Sevenless), respectively. PKCθ also activates NFkB. Additional proteins and pathways are involved in cell adhesion and migration. Adopted from (39)

1.3 Reactive oxygen species (ROS)

1.3.1 ROS as harmful by-products of metabolism

ROS are small short-lived oxygen-containing molecules that are chemically highly reactive, a property that is mainly due to their unpaired electrons (radicals). Superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), hypochlorous acid (HOCl), lipid peroxides (ROOH), singlet oxygen ($^1O_2$), and ozone (O_3) are some of the most common ROS (40). The first two species are the most important ROS in the regulation of biological processes. *Superoxide* is usually the species from which other ROS originate (Fig. 1.6). Once produced, and if it does not rapidly react with surrounding molecules, superoxide dismutates to H_2O_2, spontaneously or with the help of *superoxide dismutase* (SOD) (41). *Hydrogen peroxide* is more stable, less reactive, can diffuse in the microenvironment, and even cross cell membranes. H_2O_2 can either react with particular amino acids, usually cysteines and methionines, or can be converted to $OH^{\bullet}$ (in the Fenton reaction), to HOCl (by myeloperoxidase), or to H_2O (with the help of catalase, peroxidase, or peroxiredoxin) (42) (Fig. 1.6). Both the $OH^{\bullet}$ and HOCl are highly reactive and usually irreversibly damage nearby molecules.

One of the major sources of ROS in the cell are mitochondria (43, 44) (Fig. 1.6). Mitochondria express the *electron transport chain* (ETC) complexes, which transfer electrons from reduced Nicotinamide Adenine Dinucleotide (NADH) and succinate, along a controlled redox pathway, to oxygen molecule (O_2). Upon receiving four electrons, oxygen is reduced to H_2O. However, the ETC is not perfect, and occasionally O_2 undergoes one- or two-electron reduction to form $O_2^{\bullet-}$ or H_2O_2, respectively. Complexes I and III of the ETC are the main sources of mitochondrial $O_2^{\bullet-}$ (43, 44). Multiple metabolic enzymes, such as Endoplasmic Reticulum Oxidoreductin 1 (ERO-1), cytochromes P-450 and b5, lipoxygenases, cyclooxygenases, α-ketoglutarate- and glycerol phosphate dehydrogenases, as well as hydroxyacid-, urate-, xanthine-, monoamine-, diamine-, polyamine-, and amino acid oxidases, are also producing ROS as necessary intermediates or byproducts of their reactions (45). They can be found in mitochondria, endoplasmic reticulum, peroxisomes, and cytosol. Moreover, a class of membrane-bound *NADPH oxidases*

produces ROS for antimicrobial and signaling purposes (see next section). Finally, there are exogenous sources of ROS, including ultraviolet and gamma radiation, smoke and other air pollutants, several drugs and chemicals, which are usually harmful for the cell.

As ROS can damage proteins, lipids, and nucleic acids, the evolution has created specialized antioxidant systems. They consist of antioxidant enzymes, such as SODs, catalases, *glutathione peroxidases* (GPXs), *peroxiredoxins* (PRXs), *thioredoxins* (TRXs), *glutaredoxins* (GRXs), *sulfiredoxins* (SRXs), thioredoxin reductases, glutathione reductases, and methionine sulfoxide reductases (46), and also small non-enzymatic antioxidant molecules, such as *glutathione*, *ascorbate*, pyruvate, α-ketoglutarate, and oxaloacetate (47). When the rate of ROS production in the cell significantly exceeds the rate of their neutralization by the antioxidant systems, cell undergoes *oxidative stress*. Prolonged or excessive oxidative stress can lead to impairment of cellular functions, cell death, senescence, or malignant transformation (48-50).

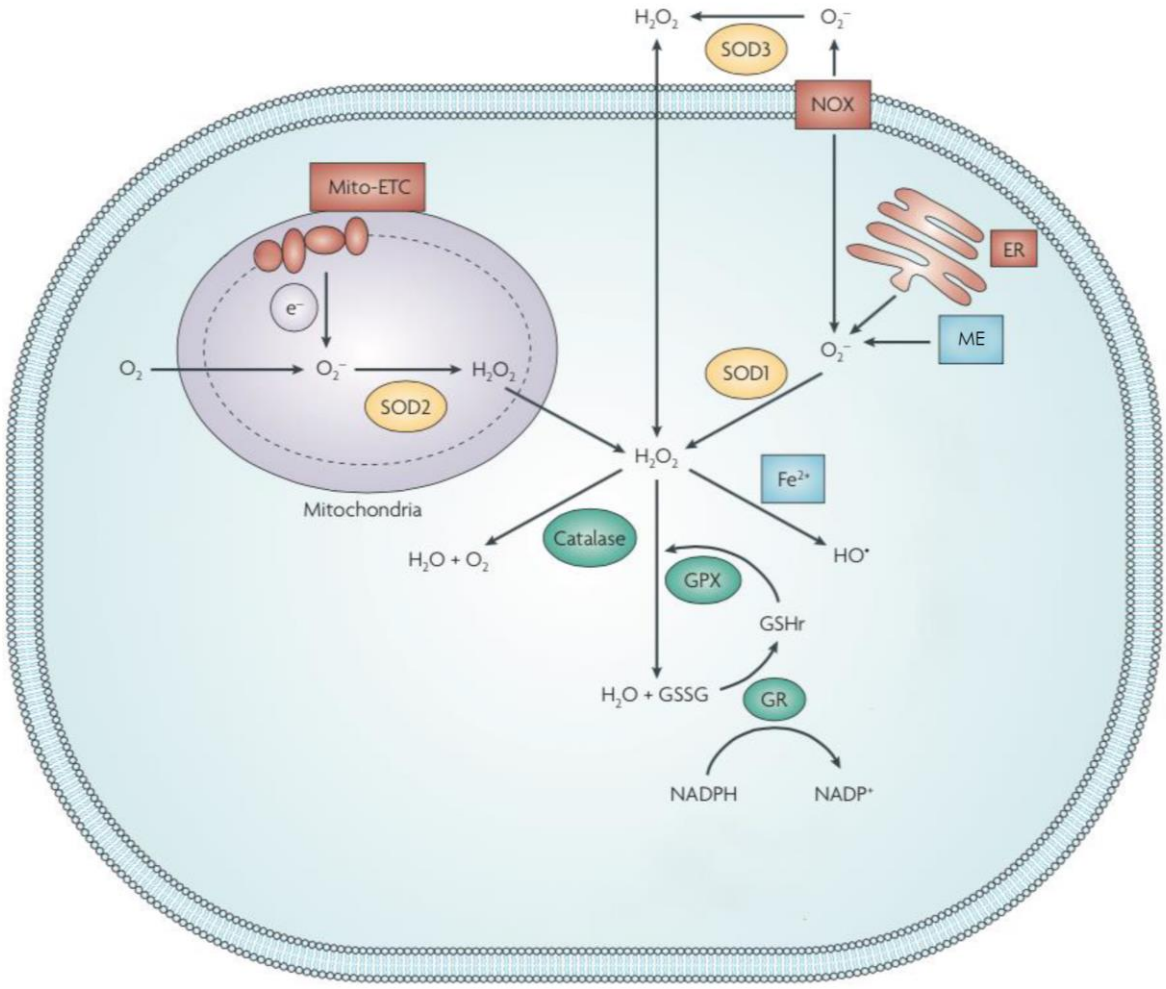

Figure 1.6 Reactive oxygen species, their sources, and scavengers

Superoxide ($O_2^{\bullet -}$) can be produced by the mitochondrial electron transport chain (Mito-ETC) upon one-electron reduction of oxygen (O_2), by NADPH oxidase (NOX) at the plasma membrane, by enzymes at the endoplasmic reticulum (ER), or by cytoplasmic metabolic enzymes (ME). $O_2^{\bullet -}$ then dismutates to hydrogen peroxide (H_2O_2), either spontaneously or with the help of superoxide dismutases (SOD). H_2O_2 can be neutralized by catalase or by glutathione peroxidase (GPX), using oxidation of glutathione (GSHr to GSSG). Glutathione is reduced by glutathione reductase (GR), using oxidation of NADPH. H_2O_2 can participate in the Fenton reaction (Fe^{2+}) and be converted to hydroxyl radical ($HO^{\bullet}$). Modified from (51).

1.3.2 ROS as weapons of phagocytes

Phagocytic cells produce ROS as a strategy to kill engulfed bacteria during the so-called *respiratory burst* (52). In these cells, ROS are produced by the Phagocytic NADPH Oxidase (PHOX), an enzyme consisting of several subunits (53) (Fig. 1.7 B). The catalytic subunit, called PHOX glycoprotein of 91 kDa ($gp91^{phox}$) or NADPH oxidase 2 (NOX-2), is located at either the plasma membrane or the phagosome membrane. PHOX includes also a variety of regulatory subunits: a membrane-anchored $p22^{phox}$ and cytoplasmic $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$, as well as the RAC GTPase. PHOX becomes functional upon assembly of this multisubunit complex, a process that is under tight regulation.

The respiratory burst can be triggered by a large and diverse group of agonists. They include phagocytosable particles (e.g., bacteria, yeast), antibodies, complement, interleukins, chemotactic peptides, and bioactive lipids. The kinetics and overall level of $O_2^{\bullet -}$ production in response to different agonists varies widely (54). Activated PHOX transfers electrons across the membrane from NADPH to O_2, generating $O_2^{\bullet -}$. Superoxide can directly damage bacteria, can react with other antimicrobial molecules, or can dismutate to hydrogen peroxide. H_2O_2, in turn, can react with metal ions, leading to production of highly poisonous $OH^{\bullet}$, or can be converted to even more toxic HOCl by *myeloperoxidase*, an enzyme which is abundant in the azurophil granules of neutrophils (55). PHOX-derived ROS have also been suggested to increase the pH of phagosomes, thus supporting the activity of neutral proteases (56, 57). The importance of PHOX in the immune response has been demonstrated by the fact that patients with mutations in this enzyme suffer from *chronic granulomatous disease*, a clinical condition characterized by recurring bacterial and fungal infections (58).

Interestingly, six homologs of $gp91^{phox}$ (NOX-2) have been identified in different tissues: NOX-1, NOX-3, NOX-4, NOX-5, Dual Oxidase 1 (DUOX-1), and DUOX-2 (59, 60) (Fig. 1.7). As mentioned above, NOXs are usually activated upon triggering of cell receptors by their respective ligands, such as insulin, angiotensin, Platelet-Derived Growth Factor (PDGF), Granulocyte–Macrophage Colony-Stimulating Factor (GM-CSF), Fibroblast Growth Factor (FGF), Nerve Growth Factor (NGF), TNF, chemokines that bind G protein-coupled receptors, complement component 5a

(C5a), sphingosine-1-phosphate (S1P), lysophospholipids (LPLs), and leukotriene B4 (LB4), as well as by cell adhesion and by phagocytosis (47). Because of the widespread yet differential expression of NOX and DUOX isoforms across organelles, cell types, and organisms, $O_2^{\bullet -}$ and H_2O_2 can be potentially considered as ubiquitous chemical signaling messengers.

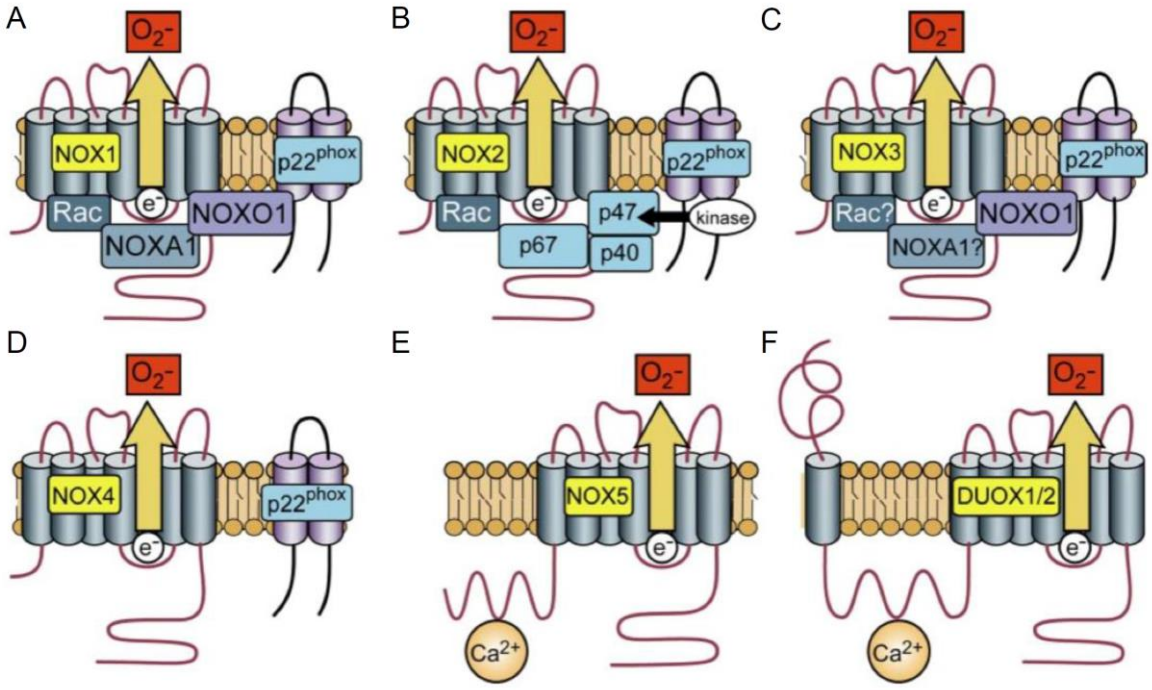

Figure 1.7 Subunit composition, activation, and cellular distribution of NADPH oxidases

(A) NOX-1 activity requires $p22^{phox}$, NOX Organizer 1 (NOXO-1), NOX Activator 1 (NOXA-1), and RAC. It is expressed in colon epithelial cells.

(B) NOX-2 requires $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and RAC. $p47^{phox}$ phosphorylation is required for NOX-2 activation. $p40^{phox}$ also associates with this complex and may contribute to activation. It is expressed in phagocytes and lymphocytes.

(C) NOX-3 requires $p22^{phox}$ and NOXO-1. The requirement for NOXA-1 and RAC in its activation is under debate. It is expressed in cochlear and vestibular sensory epithelial cells.

(D) NOX-4 requires $p22^{phox}$ and appears to be constitutively active. It is expressed in smooth muscle and endothelial cells of kidney and blood vessels.

(E) NOX-5 is activated by Ca^{2+} through EF-hands and does not appear to require subunits. It is expressed in splenic and lymph node lymphocytes and in spermatocytes.

(F) DUOX-1/2 are activated by Ca^{2+} through EF-hands and do not appear to require subunits. They also possess peroxidase-like ectodomains. They are expressed in thyroid epithelial cells.

Adopted from (60).

1.3.3 ROS as ubiquitous signaling messengers

During the last decade, it has become evident that ROS are not just harmful byproducts of metabolism and weapons of phagocytes, but that they also play crucial roles in cellular signaling. ROS-mediated signaling is involved in multiple processes, such as cell growth (59, 60), stem cell renewal (61, 62), tumorigenesis (47, 59, 62), cell death (59, 60), cell senescence (60, 62), cell migration (61), oxygen sensing (60), angiogenesis (60), circadian rhythm maintenance (61), and immune responses (47, 60).

Among ROS, hydrogen peroxide acts as the major signaling messenger and is excellently suited for this function (63). In fact, it is stable enough, is able to cross cell membranes, and is reacting preferentially with cysteine residues (42). It has to be noted that cysteine residues are amongst the most conserved and least abundant protein residues (64), which ensures high selectivity and specificity for oxidation-mediated post-translational modifications. Moreover, only specific *cysteinyl thiols* (R-SH) that can become *thiolate anions* ($R\text{-}S^-$), upon coordination with neighboring amino acid side chains, are able to react with H_2O_2 (65).

When H_2O_2 reacts with a cysteine thiolate anion, a *sulfenic acid* (R-SOH) is formed (66, 67). This process, which is referred to as *sulfenylation,* is reversed by GRXs and TRXs (46, 65) (Fig. 1.8). Thus, sulfenylation is believed to be akin to phosphorylation or other post-translational modifications. Sulfenylation may lead to further post-translational modification, such as glutathionylation and disulfide bond formation (66, 67). Most importantly, it can be involved in the regulation of protein activity. In fact, sulfenylation can induce changes in protein conformation, thus leading to activation or inactivation of the catalytic center or to other functional alterations of the protein. Multiple protein classes have been shown to be regulated by sulfenylation, including phosphatases and kinases, transcription factors and histone deacetylases, antioxidant enzymes and heat-shock proteins, proteases and hydrolases, ion channels and pumps, adaptor molecules and cytoskeleton components (47, 61, 62, 66-68).

In the presence of high levels of ROS, sulfenic acid can be further oxidized to *sulfinic* ($R\text{-}SO_2H$) and then to *sulfonic* ($R\text{-}SO_3H$) acid, the latter being a completely irreversible modification, whereas the former could be reversed by SRXs (66, 67)

(Fig. 1.8). In addition to cysteine, H_2O_2 can also react with methionine, another sulfur-containing amino acid, albeit with a four-fold lower rate. The resulting modification can be reversed by methionine sulfoxide reductase (66). Thus, an additional methionine-based signaling system has been proposed (69). Moreover, various ROS can react with other amino acid residues or protein cofactors (such as [Fe-S] clusters), causing irreversible modifications. Their role in signaling is less defined (47, 61, 68).

Interestingly, in addition to modifying transcription factors, ROS can react directly with DNA. This reaction is believed to activate transcription (47). For example, ROS produced by hypoxic mitochondria oxidize specific bases in the promoter of Vascular Endothelial Growth Factor (VEGF). This event enhances the binding of Hypoxia-Inducible Factor 1 alpha (HIF-1α) transcription factor to the VEGF promoter (70).

It is believed that NOX enzymes are the major source of “signaling” ROS (71). Nevertheless, it is likely that ROS produced by the mitochondrial ETC or metabolic enzymes are also involved in signaling processes (72-75). The levels of ROS that are involved in signal transduction are much lower than the levels occurring during respiratory burst or oxidative stress. Moreover, due to the abundance of antioxidant systems in the cell, ROS cannot travel long distances, and hence they transmit signals only locally, in confined compartments (76, 77). In other words, the source and the corresponding targets of signaling ROS usually have to be in close proximity.

ROS-mediated signaling can be additionally regulated via controlled alterations in the local levels and activity of specific antioxidants (61, 68). For example, intracellular glutathione is a good scavenger for many ROS, such as HOCl, but reacts too slowly with H_2O_2, the major mediator of oxidative signaling. On the contrary, PRXs have remarkably high reaction rates with H_2O_2, and their activity is tightly regulated by phosphorylation and *sulfinilation*. PRXs appear to be the major scavengers of “signaling” ROS, akin to phosphatases in the kinase-phosphatase system, and thus constitute a crucial component of redox signaling (46, 61, 68, 78-81). Moreover, the members of the *aquaglyceroporin* and *aquaporin* families can enhance the permeability of cell membranes to H_2O_2, thus adding an additional level of regulation to ROS-mediated signaling (61) (Fig. 1.8).

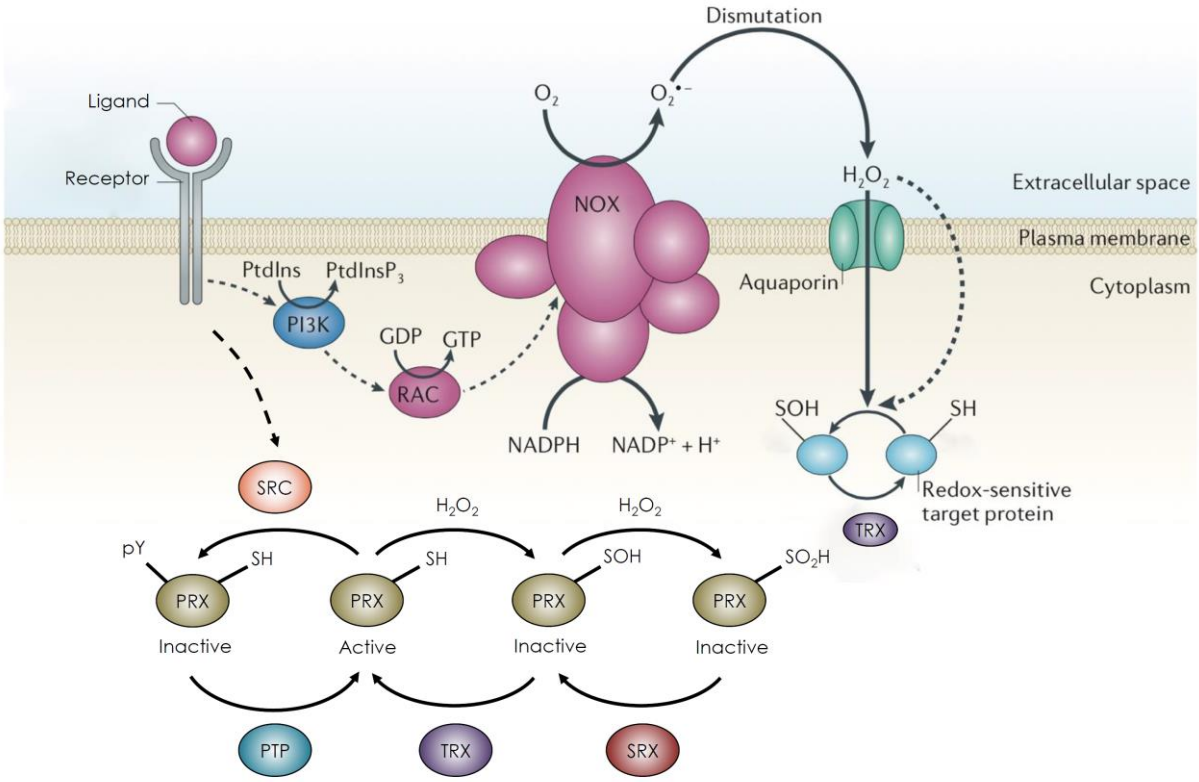

Figure 1.8 Signal transduction by ROS and peroxiredoxins

The binding of the ligand to its cognate receptor leads to the activation of several downstream signaling events. One pathway involves sequential activation of Phosphatidylinositol-3-Kinases (PI3K) and RAC, leading to the assembly and activation of NOX. After activation, NOX produces extracellular $O_2^{\bullet-}$ that spontaneously dismutates to H_2O_2. Small amounts of H_2O_2 diffuse into the cell through lipid bilayer, but much more can enter through aquaporins. Once inside the cell, H_2O_2 alters the activity of redox-sensitive targets by oxidizing specific reactive cysteine residues (-SH) into sulfenic acid. This modification is reversed by TRXs and GRXs (the latter is not depicted in the figure). Another pathway leads to the activation of Sarcoma protein family (SRC) members, which phosphorylate PRXs, thus inactivating them. Inactivated PRXs can be dephosphorylated by tyrosine phosphatases (PTPs). Active PRXs keep H_2O_2 concentrations low by neutralizing it with their cysteinyl thiols. PRXs are reduced back by TRXs and GRXs (not depicted in the figure). However, high concentrations of H_2O_2, produced, for example, upon NOX activation, lead to overoxidation of PRX cysteine residues into sulfinic acid. This modification can be slowly reverted by SRXs. Such temporal inactivation of PRXs by NOX-derived H_2O_2 allows prolonged sulfenylation of target proteins and transduction of the oxidative signal (the “floodgate” model). Modified from (62).

1.4 ROS and T cells

The complex role of ROS in T-cell biology can be simplified by dividing it into two parts: (i) the effects (usually detrimental) of large exogenous quantities of ROS on the overall redox state and function of T cells, known as “oxidative stress”, and (ii) the function of small amount of compartmentalized endogenous ROS in T-cell signaling (hereafter referred to as “signaling ROS”). The latter can be implicated in both activation and apoptosis of T cells (82).

1.4.1 Oxidative stress and T cells

T cells are often present in close proximity to phagocytic cells, which are known to produce large amounts of ROS. Moreover, activated T cells can trigger phagocytic respiratory burst by direct cell-cell contact (83), as well as by secreted cytokines. The produced ROS can, in turn, reach T cells and cause oxidative stress (Fig. 1.9). For example, it has been shown that f-MLP (N-Formyl-Methionyl-Leucyl-Phenylalanine)-activated neutrophils inhibit CD3-mediated DNA synthesis in human T cells and that this inhibition of T-cell proliferation is proportional to superoxide levels (measured by cytochrome c reduction) (84). More importantly, treatment with the glutathione precursor *N-acetyl cysteine* (NAC) or catalase prevents this inhibition (84). Further analysis showed that the inhibition of DNA synthesis is associated with alterations of TCR signaling, such as conformational changes in TCRζ and LCK, reduced PLCγ-1 phosphorylation and calcium flux, and increased ERK phosphorylation (84). Nevertheless, this study shows that activated neutrophils do not induce apoptosis in T cells. An additional study attempted to mimic the effects of phagocyte-derived ROS by treating human T cells with polyamine oxidase-generated H_2O_2 for a prolonged time (85). This treatment suppresses CD3-induced tyrosine phosphorylation and IL-2 production, PHA (Phytohaemagglutinin)-induced calcium flux, and PHAxTPA (Tetradecanoylphorbol acetate)-induced NFAT and NFkB (but not AP-1) activation (85). Thus, it can be concluded from these studies that phagocyte-derived ROS inhibit T-cell signaling, activation, and proliferation, without affecting T-cell survival.

Many investigators have also induced oxidative stress by short-term treatment with high levels (in the millimolar range) of exogenous H_2O_2. Instead of suppression,

which is induced by the prolonged action of intermediate levels of ROS produced by phagocytes (~10 nmol $O_2^{\bullet-}$/min per 10^6 cells (54)), this strong and transient treatment with H_2O_2 has the opposite effect on T cells. Indeed, high amounts (≥5 mM) of H_2O_2 induce tyrosine phosphorylation of multiple molecules and calcium flux in the Jurkat T-cell line even in the absence of other stimulatory agents (86). Among the phosphorylated proteins, important signaling molecules, including SLP-76 (87), LCK (88, 89), ZAP-70 (86), and ERK (87), were found to be activated by high ROS levels. Interestingly, the tyrosine phosphorylation of SLP-76 and the activation of ERK in response to H_2O_2 (ranging from 1 to 10 mM) is absent in the ZAP-70-deficient Jurkat T-cell variant (P116) (87). ERK activation is rescued by reconstituting P116 with ZAP-70, indicating that H_2O_2 acts at the level of ZAP-70 or upstream (87). High H_2O_2 levels (ranging from 1 to 5 mM) also induce strong tyrosine phosphorylation in preactivated human T cells (*T-cell blasts*) (90). Unfortunately, because the high levels of ROS used in these studies are far above the physiological concentrations, it is hard to interpret these data with regard to the function of ROS in T cells.

Phagocytic cells regulate the redox state of the microenvironment not only by releasing ROS, but also by producing antioxidants, especially the glutathione precursor *cysteine* (Fig. 1.9). LPS (Lipopolysaccharide)- or TNF-activated murine macrophages secrete cysteine that can be taken up by T cells, thus resulting in an increased intracellular glutathione level (91). Human dendritic cells also secrete cysteine, as well as TRX, upon co-incubation with alloreactive human T cells (92) (Fig. 1.9). Coculture of antigen-pulsed human dendritic cells (but not B cells) with autologous T cells leads to an increase in the content of both cell-surface and intracellular thiols in antigen-specific (but not in antigen-non-specific) T cells and protects them from H_2O_2-induced apoptosis (93). LPS stimulation or direct contact of DCs with murine T cells leads to release of cysteine by dendritic cells, its subsequent uptake by T cells, and conversion to glutathione (94). This correlates with the acquisition of a reduced state by T-cell surface thiols (94). On the other hand, T_{regs} suppress cysteine release by DCs, thus leading to the oxidation of surface thiols, decrease in intracellular glutathione, and reduction in DNA synthesis, in conventional T cells (94). It has been proposed that this represents a novel mechanisms used by T_{regs} to suppress the activation of peripheral T cells. Additional experiments showed that murine T_{regs} suppress glutathione synthesis and cysteine release by dendritic cells, in a Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4)-dependent manner

(95) (Fig. 1.9). The addition of exogenous cysteine partially reverts T_{reg}-induced inhibition of DNA synthesis in CD3- or peptide-activated T cells (95). In contrast to conventional human T cells, T_{regs} have higher thiol content (but expressed similar levels of catalase, *Manganese SOD* (MnSOD, SOD-2), and *Copper-Zinc SOD* (CuZnSOD, SOD-1)), and are more resistant to cell death induced by granulocyte-secreted H_2O_2 (96). Moreover, human T_{regs} express and secrete more TRX-1 than conventional T cells, and upregulate it stronger upon CD2xCD3xCD28 stimulation (97). Thus, activated phagocytes and dendritic cells secrete cysteine to the extracellular space, which is taken up by T cells and converted to the antioxidant glutathione. Regulatory T cells interfere with this process and, by doing so, inhibit T-cell activation.

Glutathione seems to be indispensable for T-cell proliferation. It is required for protein and DNA synthesis in IL-2-dependent murine T-cell clones (98), (91), for ConA (Concanavalin A)-induced DNA synthesis in murine T cells (99), and for CD2xCD3-, CD3xPMA (Phorbol 12-myristate 13-acetate)-, or diC_8 (1,2-Dioctanoylglycerol)xIono (Ionomycin)-induced DNA synthesis in human T cells (100, 101). Glutathione is also required for proper LAT conformation in human T cells (102), for localization of LAT to the plasma membrane in Jurkat T cells (102), and for CD3-induced calcium flux in Jurkat T cells (103). These findings suggest that sufficient levels of this antioxidant are required to maintain a proper redox balance, which, in turn, appears to be indispensable for T-cell signaling and proliferation. It is also possible that T-cell proliferation depends on some redox-unrelated functions of glutathione.

Overall, under physiological conditions there is likely an equilibrium between ROS and antioxidant systems, thus allowing normal T-cell function (Fig. 1.9). However, perturbation of such equilibrium by ROS overproduction or by depletion of antioxidants can lead to T-cell hyporesponsiveness or hyperactivation, thus resulting in the development of pathological conditions.

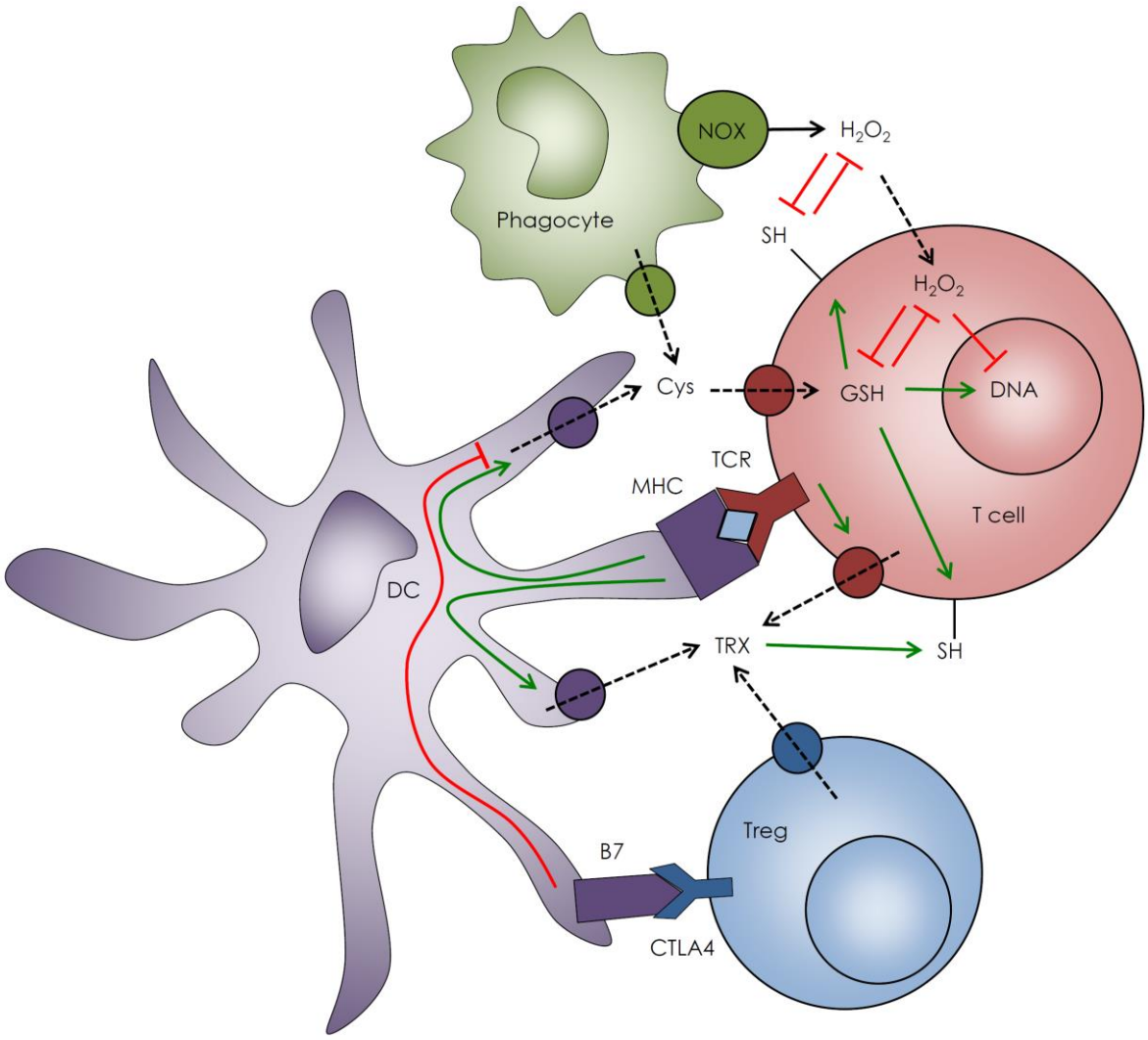

Figure 1.9 Regulation of the T-cell redox state

Activated phagocytes produce H_2O_2 via NOX. H_2O_2 either oxidizes thiols (SH-) on the surface of T cells or enters inside T cells. Intracellularly, H_2O_2 either oxidizes glutathione (GSH) or interferes with DNA synthesis. Activated phagocytes and dendritic cells (DC) secrete cysteine (Cys) to the extracellular space. Cys is taken up by T cells and converted to GSH. GSH keeps surface thiols in a reduced state, neutralizes intracellular H_2O_2, and is required for DNA synthesis. TCR-peptide-MHC interaction leads to the secretion of TRX by T cells, DCs, and T_{reg} cells. TRX helps to keep surface thiols in a reduced state. T_{reg} cells inhibit Cys release by DCs via CTLA-4/B7 interaction. Black solid arrow indicates production, black dashed arrows indicate import/export, green solid arrows indicate activation, red bar-headed lines indicate inhibition.

1.4.2 ROS and T-cell apoptosis

Stimulation of activated T cells – T-cell blasts, as well as Jurkat T cells or other T-cell lines, results in *activation-induced cell death* (AICD) (29). In this section, I summarize the data describing the function of ROS in Jurkat T cells and T-cell blasts (see also Table 1). While the majority of the data on ROS function during AICD have been generated using Jurkat T cells, the results obtained from a transformed cell line should be interpreted with caution. In fact, it is known that Jurkat T cells have mutations in important genes involved in TCR signaling (104).

Jurkat T cells exhibit oxidation of the superoxide-sensitive dye *luminol* upon PMA stimulation (105). In addition to PMA, luminol oxidation is induced by ConA, PHA (105), and ligation of the death receptor Fas (106). PMA or PHA stimulation of Jurkat T cells leads also to the oxidation of the ROS-sensitive dye *2',7'-Dichlorofluorescein diacetate* (DCFDA) (107, 108). Analysis of the molecular mechanisms regulating ROS production showed that PMAxIono and CD3 stimulations induce DCFDA oxidation that is dependent on RAS signaling, in particular on the RAS-like GTPase (RAL), and could be inhibited by RAS-related protein 1 (RAP-1) (109). Studies from another group showed that PMAxIono- and CD3-induced DCFDA oxidation, FasL upregulation, and cell death are dependent on PKCθ and mitochondrial complex I (108, 110) (Fig. 1.10). CD3-induced (but not PMA-induced) DCFDA oxidation is also dependent on ZAP-70, LAT, SLP-76, and PLCγ-1 (110). In other studies, it has been shown that CD3 stimulation induces two waves of DCFDA oxidation that depend on DUOX-1, as well as a wave of oxidation of the superoxide-sensitive dye *Dihydroethidium* (DHE) (111, 112). The first wave of DCFDA oxidation appears to be also dependent on LCK, ZAP-70, SLP-76, PLCγ-1, IP_3 receptor, and intracellular (but not extracellular) calcium (112) (Fig. 1.10). CD3xCD28 stimulation also induces oxidation of the H_2O_2-sensitive mitochondria-targeted dye *Dihydrorhodamine 123* (DHR) (113). Additionally, triggering of Fas increases DHE but, surprisingly, decreases DCFDA oxidation (114). Moreover, FasL induces oxidation of DHE, DHR, and HO-sensitive dye 3”-(p-hydroxyphenyl) fluorescein (HPF) (115). Thus, triggering of the TCR or Fas in Jurkat T cells leads to ROS production from various sources, such as DUOX-1 and mitochondria, via the activation of different signaling cascades (Fig. 1.10).

While the Jurkat T-cell line was used in the studies described above, other studies have employed human and murine T-cell blasts. CD3 stimulation of murine T-cell blasts induces Fas-dependent DCFDA oxidation by NOX-2-derived ROS (116) (Fig. 1.10). Moreover, CD3 stimulation also induces transient DCFDA oxidation that is not dependent on Fas or NOX-2 and Fas-dependent DHE oxidation that is not mediated by NOX-2 (116), indicating that at least two more sources of ROS are involved in AICD. CD3 stimulation of human T-cell blasts triggers MEK-dependent oxidation of DHE and DCFDA (111, 117). Other studies have shown that CD3 stimulation of human T-cell blasts leads to DHE and DCFDA oxidation, FasL expression, and cell death that all depend on mitochondrial complex I (108, 110, 118). Thus, in T-cell blasts, ligation of the TCR or Fas leads to ROS production via NOX-2, mitochondria, and potentially other sources.

Different groups have studied the role of ROS in T-cell signaling and apoptosis using RNA interference and knockout mice. It has been shown that CD3-induced MEK and ERK activation are enhanced, whereas AKT activation is suppressed, in T-cell blasts from $gp91^{phox-/-}$ mice (116) (Fig. 1.10). However, it appears that apoptosis of murine T-cell blasts in response to CD3 stimulation is not dependent on $gp91^{phox}$ (119). Suppression of DUOX-1 with shRNA in Jurkat T cells leads to decreases in calcium flux, in LCK, ZAP-70, and ERK phosphorylation, as well as in AP-1 and NFAT activation, upon CD3 stimulation (112). Similarly, suppression of DUOX1 with siRNA in human $CD4^+$ T-cell blasts leads to decreases in CD3-induced ZAP-70, PLCγ-1, and ERK phosphorylation, PLCγ-1, SHP-2, and GRB-2-Associated Binding protein 2 (GAB-2) association with GRB-2, as well as in SHP-2 oxidation and association with GAB-2 (112) (Fig. 1.10). Thus, both NOX-2 and DUOX-1-derived ROS are involved in TCR-mediated signaling in T-cell blasts and Jurkat T cells.

In order to investigate the role of ROS in T-cell signaling and apoptosis, other groups have used micromolar doses of H_2O_2. The majority of studies have been performed in the Jurkat T-cell line. It has been shown that the addition of 30-50 μM H_2O_2 enhances CD3xCD28-induced p38, JNK, and ERK activation (120, 121). Moreover, 200 μM H_2O_2 induces MEK-dependent ERK activation, as well as SRC- and calcium-dependent Ribosomal S6 Kinase (RSK) activation (122). Similarly, 100-1000 μM H_2O_2 induces MEK and ERK activation, in a PLCγ-1- and calcium-dependent manner (121). In addition, 400 μM H_2O_2 induces p38, JNK, and ERK

activation in a SRC-dependent manner and inhibits the phosphatase activities of CD45, SHP-1, and Hematopoiesis-specific Protein Tyrosine Phosphatase (HePTP) (123). Interestingly, induction of ERK activation by H_2O_2 is dependent on PLCγ-1 and PKC, whereas induction of p38 and JNK activation is not (123). 50-500 μM H_2O_2 induces NFkB and AP-1 (*c-Jun*) (but not NFAT) activation (124-126). Moreover, same concentrations suppress NFAT activation upon PHAxPMA-stimulation (126). Interestingly, 100 μM H_2O_2 enhances PDBU (phorbol dibutyrate)- or TNF-induced activation of NFkB when added simultaneously, but suppresses it when added 20 or 3 hours before stimulation (127). 50-200 μM H_2O_2 induces calcium flux (125). Thus, applications of exogenous H_2O_2 in micromolar range to Jurkat T cells induce calcium flux and the activation of several signaling molecules (including p38, JNK, MEK, ERK, PLCγ-1, PKC, and LCK) and transcription factors (including NFkB and AP-1). Conversely, they suppress CD45, SHP-1, HePTP, and NFAT activation.

It has been additionally shown that exogenous H_2O_2 may induce apoptosis in Jurkat T cells. In fact, 500 μM H_2O_2 induces FasL expression via NFkB (128). However, another report showed that the same concentration of H_2O_2 induces cell death independently of FasL (108). Interestingly, sustained H_2O_2 release from glucose oxidase in combination with ionomycin (but not each of the components alone) induces both FasL expression and FasL-dependent cell death (108). Human T cells seem to be much more sensitive to H_2O_2-induced apoptosis. However, it strongly depends on the T-cell subset that has been used in the study. In fact, 5 μM H_2O_2 induces apoptosis in human $CD45RO^+$ T_{mem}, but not in $CD45RA^+$ T_{nai} cells, via mitochondrial depolarization and caspase activation (129). Similarly, human $CD4^+$ $CD45RA^+$ T_{nai} cells are more resistant to cell death induced by 5-20 μM H_2O_2 than $CD4^+$ $CD45RA^-$ T_{mem} cells (96). In contrast to conventional human T cells, T_{reg} cells have lower intracellular ROS levels, as measured with DCFDA, and are resistant to H_2O_2-induced death (96). Moreover, 10 μM H_2O_2 does not affect T_{reg} cell suppressive capacity (96). Interestingly, 100 μM H_2O_2 completely eliminates human $CD4^+$ T cells, but has no effect on $CD4^+$ T blasts (130).

Various antioxidant compounds and overexpression of antioxidant enzymes are also used in order to understand the function of ROS in T-cell signaling and apoptosis. Jurkat T cells were most commonly used in these studies as well. Overexpression of PRX-2 leads to sustained MEK and ERK (but not JNK) activation

upon CD3 stimulation (111). Overexpression of MnSOD increases general tyrosine phosphorylation induced by CD3xCD28 stimulation, whereas overexpression of CuZnSOD decreases it (131). Moreover, MnSOD, but not CuZnSOD, enhances phosphorylation of JNK and p38 (131). NAC, Vitamin E, *Butylated hydroxyanisole* (BHA) and *Nordihydroguaiaretic acid* (NDGA) block PMA- and TNF-induced NFkB (but not AP-1) activation (124, 132). In another report, NAC, Vitamin E, and overexpression of MnSOD reduce NFkB and AP-1 (but not NFAT) activation upon PMAxIono stimulation (133, 134). Thus, various antioxidants exert diverse effects on signaling molecules, due to their specificity for different reactive oxygen species and distinct localization in the cell.

Antioxidants can also block AICD in Jurkat T cells. Indeed, dithiothreitol, NAC, and overexpression of MnSOD prevent PMAxIono-induced FasL expression (108, 118, 128). Accordingly, MnSOD overexpression reduces PMAxIono-triggered apoptosis (118). FasL upregulation upon CD3 stimulation is prevented by glutathione, NAC, and dithiothreitol (108, 128) (Fig. 1.10). In agreement with these observations, NAC diminishes CD3-induced cell death (108). Interestingly, only the thiol antioxidants glutathione, NAC, and 2-mercaptopropionylglycine (but not MnSOD overexpression, BHA, NDGA, Vitamin E analog Trolox, SOD mimetic MnTE-2-PyP, xanthine oxidase inhibitor allopurinol, or cyclooxygenase inhibitor indomethacin) reduce FasL-triggered apoptosis (115, 118, 135) (Fig. 1.10). Surprisingly, high GGT expression increases susceptibility to Fas-induced apoptosis (136). Interestingly, T cells targeted for apoptosis try to counteract the anti-apoptotic action of antioxidants. Indeed, FasL triggers glutathione efflux via specific anion-exchange transporter, and this is required for the activation of caspases and execution of the apoptotic program (137). Moreover, Fas ligation induces preferential calcium-dependent caspase-mediated degradation of MnSOD in mitochondria (114) (Fig. 1.10).

There is a limited amount of data on the effects of antioxidants on human T-cell blasts. NAC and overexpression of PRX-2 lead to sustained MEK and ERK phosphorylation but delayed AKT phosphorylation, upon CD3 stimulation (111). Additionally, NAC diminishes CD3-induced general tyrosine phosphorylation (90). Overexpression of CuZnSOD and MnSOD (but not catalase or TRX peroxidase) abrogates CD3-induced FasL expression (117) (Fig. 1.10). FasL upregulation and cell death upon CD3 stimulation are prevented by NAC (108, 118). Thus, FasL

expression (most likely via NFkB) in Jurkat and T-cell blasts is triggered by superoxide and can be prevented by overexpressing superoxide dismutase or by thiol antioxidants, whereas apoptotic cascades downstream of Fas can be blocked only by thiol antioxidants. Notably, Fas ligation triggers MnSOD degradation and glutathione efflux from the cell.

In summary, it seems that several different sources of ROS are involved in AICD of T cells (Fig. 1.10). First, H_2O_2 produced by DUOX-1 in response to depletion of intracellular Ca^{2+} stores serves to amplify the initial T-cell receptor signaling, creating a positive feedback loop that involves DUOX-1, LCK, and PLCγ-1. Next, $O_2^{\bullet-}$ released from mitochondrial Complex I, downstream of DAG-dependent pathways, participates in the activation of NFkB and in the expression of FasL. Finally, Fas ligation activates NOX-2, which probably assists the execution of the apoptotic program via H_2O_2-mediated activation of p38, JNK, and AKT. Moreover, intracellular antioxidant glutathione interferes with FasL expression and caspase activation, thus counteracting AICD (Fig.1.10).

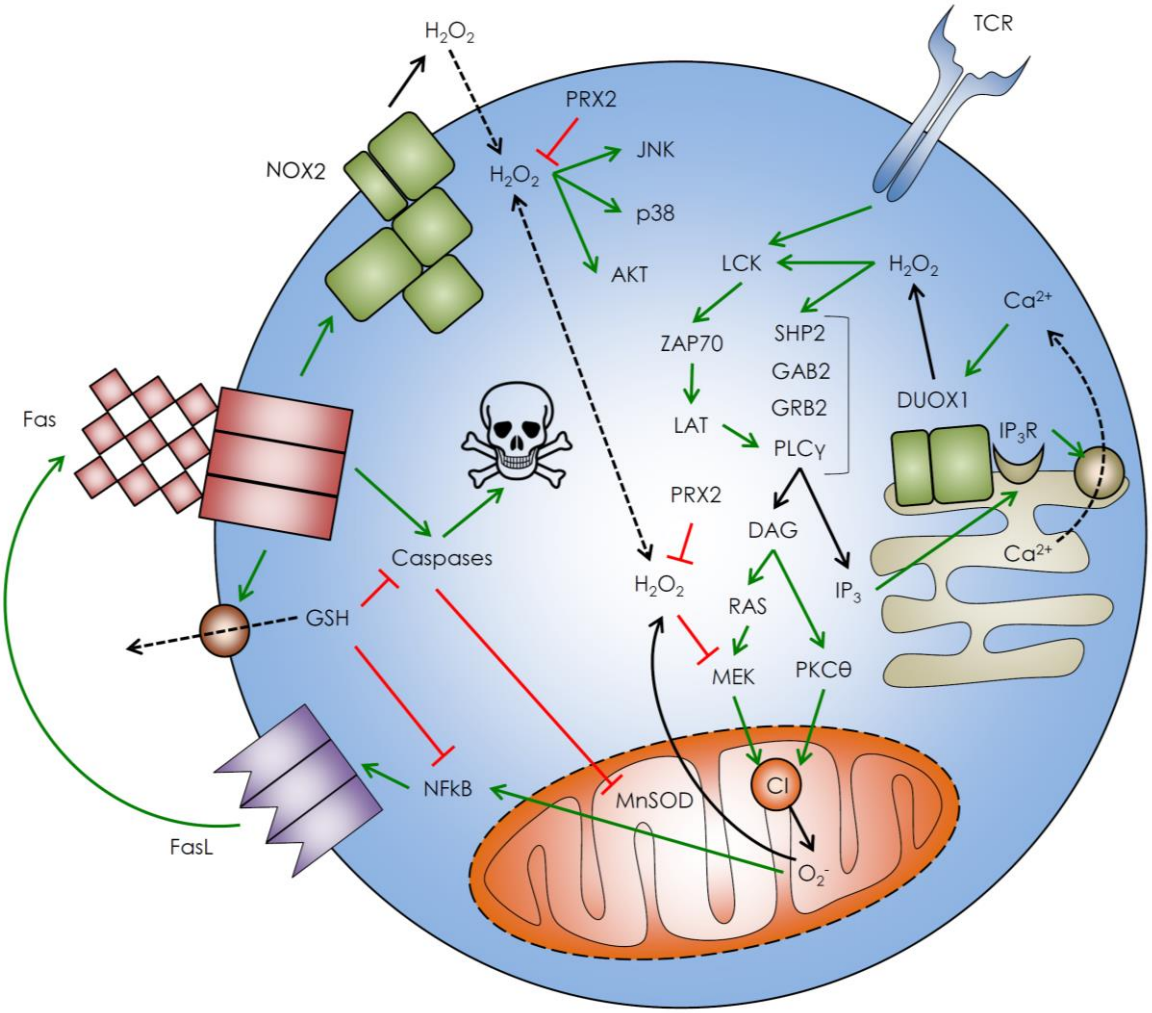

Figure 1.10 The role of ROS in T-cell apoptosis

TCR triggering leads to the activation of LCK, ZAP-70, LAT, and PLCγ-1. PLCγ-1 converts PIP_2 into IP_3 and DAG. IP_3 triggers its receptor (IP_3R), thus leading to the efflux of calcium from endoplasmic reticulum. Released calcium activates DUOX-1, which is associated with IP_3R. DUOX-1 produces H_2O_2 that oxidizes SHP-2 and leads to the formation of a SHP-2-GAB-2-GRB-2-PLCγ-1 complex, as well as to the activation of LCK. Thus, positive feedback loops involving DUOX-1, LCK, and PLCγ-1 are formed. DAG activates $O_2^{\bullet-}$ production by mitochondrial complex I (CI), through RAS-MEK and PKCθ. $O_2^{\bullet-}$ production leads to the activation of NFkB and expression of FasL. FasL triggers Fas that leads to the activation of caspases and cell death. Caspases selectively cleave MnSOD that converts part of $O_2^{\bullet-}$ into H_2O_2. Glutathione (GSH) interferes with the activation of NFkB and caspases, but Fas triggers GSH efflux from the cell. Additionally, Fas activates NOX-2. NOX-2 produces H_2O_2 that enters the cell and activates JNK, p38, and AKT but inhibits MEK. H_2O_2 is neutralized by PRX-2. Black solid arrows indicate production, black dashed arrows indicate export/import, green solid arrows indicate activation, red bar-headed lines indicate inhibition. Skull and bones indicate apoptosis.

Table 1. ROS and T-cell apoptosis

Cell type	Stimulatory agent	ROS dye/ antioxidant	ROS source	Observed ROS effects	Ref.
Jurkat T cells	PMA, PHA, ConA, FasL	Luminol		ROS production	(105, 106)
	PMA, PHA, PMAxIono, CD3	DCFDA		ROS production	(107-109)
	PMAxIono, CD3	DCFDA	Mitochondria	ROS production	(108, 110)
	CD3	DCFDA	DUOX-1	Calcium flux; activation of LCK, ZAP-70, ERK, AP-1, NFAT	(111, 112)
	CD3, FasL	DHE		ROS production	(111, 112, 114, 115)
	CD3xCD28, FasL	DHR	Mitochondria	ROS production	(113, 115)
	FasL	HPF		ROS production	(115)
	CD3	PRX-2		Inhibition of MEK, ERK	(111)
	CD3xCD28	MnSOD	Mitochondria	Inhibition of tyrosine phosphorylation, JNK, p38	(131)
	CD3xCD28	CuZnSOD	Cytosol	Tyrosine phosphorylation	(131)
	PMA, TNF	NAC, Vitamin E, BHA, NDGA		Activation of NFkB	(124, 132)
	PMAxIono	DTT, NAC, Vitamin E, MnSOD		Activation of NFkB, AP-1; expression of FasL; apoptosis	(108, 118, 128, 133, 134)
	CD3	GSH, NAC, DTT		Expression of FasL	(108, 128)
	CD3, FasL	GSH, NAC, 2-MPG		Apoptosis	(108, 115, 118, 135)
Human T-cell blasts	CD3	DCFDA, NAC, PRX-2		ROS production; activation of AKT; inhibition of MEK, ERK	(111)
		DHE, CuZnSOD, MnSOD		ROS production; expression of FasL	(117)
		DCFDA, DHE	Mitochondria	ROS production	(108, 110, 118)
		NAC		Tyrosine phosphorylation; expression of FasL; apoptosis	(90, 108, 118)
Human $CD4^+$ T-cell blasts	CD3	DCFDA	DUOX-1	ROS production; activation of ZAP-70, PLCγ-1, ERK; association of PLCγ-1, SHP-2, GAB-2 and GRB-2	(112)
Murine T-cell blasts	CD3, FasL	DCFDA	NOX-2	ROS production; activation of MEK, ERK; inhibition of AKT	(116)
	CD3	DCFDA	Other than NOX-2	ROS production	(116)
	FasL	DHE	Other than NOX-2	ROS production	(116)

1.4.3 ROS and T-cell activation

The role of ROS in T-cell activation, correlating with productive T-cell responses, such as proliferation and differentiation, can be studied only in primary T cells. T-cell blasts, as well as Jurkat T cells or other T-cell lines, are, on the contrary, already activated cells, and will undergo AICD upon TCR stimulation (29). There is evidence that primary human and murine T cells produce ROS upon stimulation with different agents. However, it cannot be excluded that the detected ROS originate from contaminating phagocytic cells, which are almost inevitably present in any preparation of primary T cells. Phagocytes can be activated by various agents used to stimulate T cells, for example by PMA or by anti-TCR antibodies via Fc receptors (138). Ideally, the purity of T-cell preparation should be very high (>99%), and an isotype-matched control antibody should be used to discriminate between the T cell-derived ROS and the ROS of phagocytic origin. Unfortunately, these conditions have been rarely met, if ever. Moreover, activated T cells can induce respiratory burst in phagocytes by direct contacts (83). Thus, the detection of specific T cell-derived ROS is a challenging task. In the following paragraph, I will describe the studies that attempted to measure ROS production in human or murine primary T cells (see also Table 2).

PMA or PHA stimulation of human T cells induces DCFDA oxidation (107, 139). CD3 and especially CD28 stimulation induces DCFDA oxidation and decreases the intracellular glutathione levels in human T cells (140). This ROS production is mediated by 5-lipoxygenase (140) (Fig. 1.11). Human $CD45RA^+$ T_{nai} cells induce more DCFDA oxidation than $CD45RO^+$ T_{mem} cells, upon CD3, CD28, and CD3xCD28 stimulation (141). Notably, $CD45RO^+$ T_{mem} cells do not induce DCFDA oxidation upon CD28 stimulation alone (141). CD3, CD28, PMA, and ionomycin induce DCFDA oxidation in murine $CD8^+$ T cells (139). CD3 stimulation of murine $CD4^+$ T cells induces $p47^{phox}$-dependent DCFDA oxidation (142) (Fig. 1.11). Surprisingly, CD3xCD28-induced oxidation of the chloromethyl DCFDA derivative (CM-DCFDA) is enhanced in T cells from $gp91^{phox-/-}$ mice (119). CD3 or CD3xCD28 (but not CD28 alone) stimulation of murine $CD4^+$ T cells induces oxidation of mitochondrial reduction-oxidation sensitive Green Fluorescent Protein (mito-roGFP) that is dependent on mitochondrial calcium uptake (143). Moreover, CD3xCD28 or PMAxIono stimulation of murine $CD4^+$ T cells induces oxidation of the mitochondria-

targeted superoxide-sensitive dye MitoSOX that is dependent on mitochondrial Rieske Iron-sulfur Protein (RISP) (143) (Fig. 1.11). Chemokine (C-X-C Motif) Ligand 12 (CXCL-12) stimulation of murine $CD4^+$ T cells induces CM-DCFDA oxidation that is abrogated by catalase and is markedly reduced in aquaporin 3-deficient cells, indicating the involvement of extracellular H_2O_2 (121). Collectively, the data reported above suggest that triggering of the TCR, CD28, or CXCR-4 in primary T cells leads to ROS production by 5-lipoxygenase, NOX-2, and mitochondria.

T cells from mice lacking components of NOX-2 could serve as a good model to study the function of NOX-2-derived ROS in T-cell activation. Nevertheless, the interpretation of the results obtained using this model should be taken with caution, as phagocytes from these mice also lack NOX-2, and this may indirectly affect T-cell functions. T cells from $p47^{phox-/-}$ mice have diminished expression of T-bet, Signal Transducer and Activator of Transcription 1 (STAT-1), and STAT-4, reduced DNA synthesis, and decreased production of IL-2, IL-4, IFNγ, TNFα, and GM-CSF, upon CD3xCD28 stimulation (142). Additionally, those T cells have increased phosphorylation of STAT-3 and production of IL-10, IL-17, and TGFβ (142). $CD4^+$ T cells from $gp91^{phox-/-}$ mice produce less IL-4 and IL-5 but more IL-17 and IFNγ than WT counterparts, upon CD3xCD28 stimulation (144) (Fig. 1.11). Moreover, they have reduced GATA-3 expression and STAT-5 and STAT-6 phosphorylation, but increased T-bet expression (144). Interestingly, they have no defect in CD25 expression or IL-2 production (144). Thus, NOX-2 is required for proper differentiation and migration but not for the activation of primary murine T cells.

Another approach to elucidate the role of ROS in the activation of primary T cells is the exogenous addition of H_2O_2 and the evaluation of its effects on T-cell functions. 10 μM H_2O_2 reduce thapsigargin-triggered calcium flux in $CD4^+$ human T cells (130). However, 10-100 μM H_2O_2 induce CRAC channel-independent calcium flux (130). 10-1000 μM H_2O_2 induce NFkB activation in human $CD45RA^+$ T_{nai} cells stronger than in $CD45RO^+$ T_{mem} cells (145). Nevertheless, another study showed that 25-100 μM H_2O_2 suppress CD3-induced NFkB activation in human T cells (146). High concentrations (≥30 μM) of H_2O_2 inhibit ConA-stimulated DNA synthesis in murine T cells, whereas low concentrations (3-10 μM) augment it (147). Furthermore, 100 μM H_2O_2 reduce IL-2 production by CD3xCD28-stimulated $CD4^+$ human T cells (130). 100 μM H_2O_2 induce ITK and Cell Division Cycle protein 42 (CDC-42) activation in

murine $CD4^+$ T cells (148). Moreover, CXCL-12-induced activation of CDC-42 and ITK, polymerization of actin, and migration of murine $CD4^+$ T cells are reduced in aquaporin 3-deficient cells but are reconstituted upon the addition of 100 μM H_2O_2 (148). In summary, the effects of exogenous H_2O_2 on primary T cells strongly depend on H_2O_2 concentration and are rather contradictory, which might be due to non-physiological nature of such stimuli and to the experimental conditions used in these studies.

Another alternative approach that has been used to understand the functional importance of ROS in primary T-cell activation is based on the addition of antioxidants. NDGA inhibits IL-2 production by PHA-stimulated human T cells (149). On the other side, BHA blocks DNA synthesis and CD25 expression (but not IL-2 production) in murine T cells stimulated with PMAxIono (150). IL-2R expression by murine T cells upon stimulation with ConA or PMAxA23187 (calcium ionophore) is suppressed by NDGA and BHA (151). Vitamin E suppresses IL-4 production in PMAxIono- or CD3xCD28-stimulated human T cells (133). Mitochondria-targeted vitamin E abrogates CD3xCD28- or PMAxIono-induced IL-2 production in murine $CD4^+$ T cells (143). The NAC and NOX-2 inhibitor *apocynin* reduces STAT-5 phosphorylation in murine $CD4^+$ T cells upon CD3xCD28-stimulation to the levels observed in $gp91^{phox-/-}$ mice (144). Catalase suppresses CXCL-12-induced activation of CDC-42 and ITK, polymerization of actin, and migration of murine $CD4^+$ T cells (148). It seems that antioxidants that inhibit lipid peroxidation and/or *lipoxygenase* activity (such as NDGA, BHA, Vitamin E, and Mitovitamin E) can interfere with CD25 expression, cytokine production, and DNA synthesis in primary T cells (Fig. 1.11). Therefore, it is likely that the reduction in the synthesis of leukotrienes (proinflammatory molecules downstream of lipoxygenase), rather than the reduction in ROS levels, could be the reason for the impairment of T-cell activation by those antioxidants.

Signaling ROS exert their function by interacting with particular cysteine residues, thus modulating protein functions. Stimulation of murine $CD8^+$ T cells with CD3xCD28 or PMAxIono induces protein sulfenylation (as assessed by the *dimedone* method (152)) that could be reduced by NAC (139). It has been shown that SHP-1 and SHP-2 are transiently sulfenylated (139). Moreover, it has been shown that dimedone binding to sulfenic acids interferes with CD3xCD28- or

PMAxIono-induced proliferation of murine $CD4^+$ and $CD8^+$ T cells, but only if dimedone is continuously present in the medium (139). Dimedone also interferes with PMAxIono-induced calcium flux, ERK phosphorylation, protein and DNA synthesis, and cell growth, but not p38 or JNK phosphorylation or cell viability, in murine $CD8^+$ T cells (139). Thus, sulfenylation is likely involved in a wide range of processes within T cells.

Collectively, it seems that ROS produced by NOX-2 are involved in differentiation and migration of T cells, but not in the initial phases of T-cell activation. The functional importance of ROS from other sources, such as mitochondria, is not yet clear. Moreover, a possibility remains that even NOX-2-derived ROS are produced by surrounding phagocytic cells and not by T cells themselves.

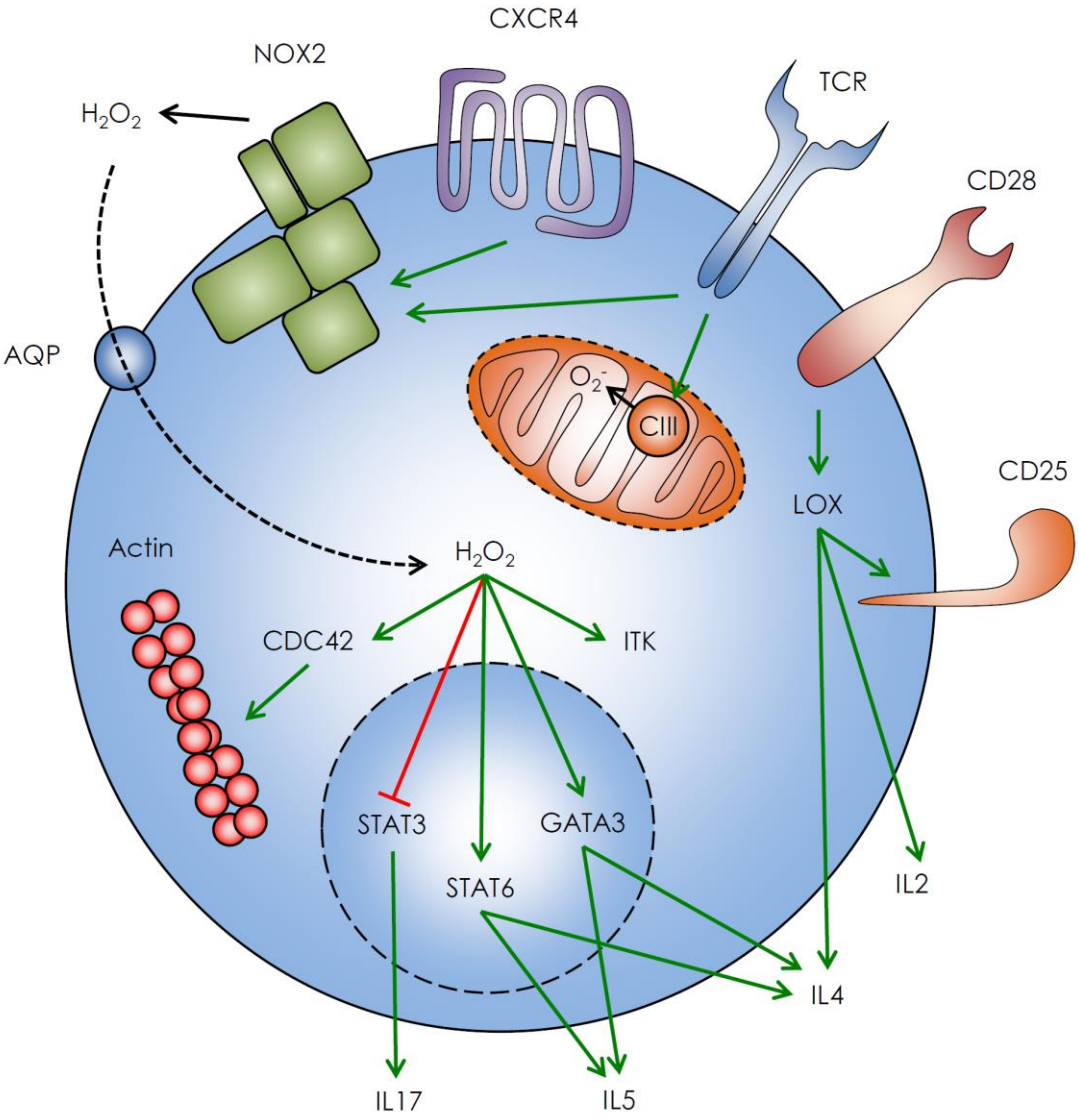

Figure 1.11 The role of ROS in T-cell activation

The ligation of CD28 leads to the activation of the lipoxygenase pathway (LOX). This enhances the expression of CD25, IL-2, and IL-4. The triggering of TCR leads to $O_2^{\bullet-}$ production by mitochondrial complex III (CIII). Whether this superoxide has any function is not clear. Additionally, the ligation of TCR or CXCR-4 leads to the activation of NOX-2. NOX-2 produces H_2O_2 that enters the cell predominantly via aquaporin channels (AQP). Inside the cell, H_2O_2 activates CDC-42, ITK, GATA-3, and STAT-6, but inhibits STAT-3. Activated CDC-42 initiates actin polymerization and cell migration. ITK is involved in multiple processes. GATA-3 and STAT-6 direct T-cell differentiation towards T_h2 phenotype and production of IL-4 and IL-5. STAT-3 leads to T_h17 differentiation and IL-17 production. Black dashed arrow indicates import, green solid arrows indicate activation, red bar-headed line indicates inhibition.

Table 2. ROS and T-cell activation

Cell type	Stimulatory agent	ROS dye/ antioxidant	ROS source	Observed ROS effects	Ref.
Human T cells	PMA, PHA, CD3, CD28, CD3xCD28	DCFDA		ROS production	(107, 139, 141)
	CD28, CD3	DCFDA	Lipoxygenase	ROS production	(140)
	PHA	NDGA	Lipoxygenase?	IL-2 production	(149)
	PMAxIono, CD3xCD28	Vitamin E	Lipoxygenase?	IL-4 production	(133)
Murine T cells	ConA, PMAxIono, PMAxA23187	NDGA, BHA	Lipoxygenase?	DNA synthesis; IL-2R (CD25) expression	(150, 151)
	CD3xCD28	CM-DCFDA	Other than NOX-2	ROS production	(119)
Murine $CD4^+$ T cells	CD3, CD3xCD28	DCFDA	NOX-2	ROS production; activation of T-bet, STAT-1, STAT-4; DNA synthesis; production of IL-2, IL-4, IFNγ, TNFα, GM-CSF; inhibition of STAT-3, IL-10, Il-17, TGFβ	(142)
	CD3xCD28	Apocynin, NAC	NOX-2	Activation of GATA-3, STAT-5, STAT-6; production of IL-4, IL-5; inhibition of T-bet, IL-17, IFNγ	(144)
	CD3, CD3xCD28, PMAxIono	Mito-roGFP, MitoSOX	Mitochondria	ROS production	(143)
	CD3xCD28, PMAxIono	Mitovitamin E	Mitochondria or lipoxygenase?	IL-2 production	(143)
	CXCL-12	CM-DCFDA, catalase	Extracellular	ROS production; activation of CDC-42, ITK; polymerization of actin; migration	(148)
Murine $CD8^+$ T cells	CD3, CD28, PMA, ionomycin	DCFDA		ROS production	(139)

1.5 Mitochondria and T-cell activation

Despite the widespread idea that, upon activation, T cells switch from mitochondrial respiration and fatty acid oxidation to aerobic glycolysis (153, 154), it has been recently shown that $CD4^+$ T cells can use both metabolic pathways for proliferation (143, 155), although it appears that glycolysis is required mainly for T_{eff} cell function (155). While T_h cells depend on glycolysis, T_{reg} cells require mitochondrial lipid oxidation (156). Interestingly, $CD8^+$ T_{mem} cells have more mitochondria than T_{eff} cells and depend on fatty acid oxidation (157, 158). Thus, mitochondrial metabolism is essential for T_{mem} and T_{reg} cell function.

Several mitochondrial processes participate in the activation of T cells. First of all, mitochondrial ATP production is required for CD25 and CD44 expression and initial proliferation of murine $CD4^+$ T cells (155) (Fig.1.12). In addition, calcium buffering by mitochondria is necessary for sustained CRAC-mediated calcium flux and NFAT activation in Jurkat T cells (113, 159, 160). To mediate efficient calcium uptake, mitochondria translocate to the immunological synapse (161-164). Thus, mitochondrial ATP production and calcium buffering are required for T-cell activation.

Both ATP synthesis and calcium uptake are powered by mitochondrial membrane potential (MMP) (165, 166) (Fig.1.12). Murine $CD8^+$ T cells, activated *in vivo* by lymphocytic choriomeningitis virus (LCMV), have elevated MMP, which then decreases during the contraction phase of the immune response (167). Mitochondrial biogenesis and MMP increase after stimulation of murine T cells (168). Interestingly, in human $CD4^+$ T cells, MMP drops immediately after stimulation, but is required for IL-2 expression (113). Thus, MMP seems to be proportional to the activation state of T cells, at least in mice.

MMP is maintained by the activity of the ETC, which consists of complexes I-IV. Mitochondrial complex I is required for CD95L expression and AICD in Jurkat T cells and human T-cell blasts (110) and also for ERK phosphorylation, CD69 expression, proliferation, and TNFα production in murine $CD8^+$ T cells (169), as well as for IL-2 and IL-4 production in human T cells (113, 134). The mitochondrial complex III subunit RISP is required for NFAT (but, surprisingly, not for ERK or for NFkB) activation, CD25 and CD69 expression, and IL-2 production in murine $CD4^+$ T cells, as well as *in vivo* antigen-specific expansion of $CD4^+$ and $CD8^+$ T cells (143). Specific

involvement of complex III in the activation of NFAT suggests that MMP-dependent calcium buffering is the primary function of mitochondria in T-cell activation. Thus, ETC complexes I and III have been shown to be required for T-cell activation, proliferation, and cytokine production, as well as for AICD.

In conclusion, MMP seems to correlate with the state of T-cell activation. Indeed, MMP is required for mitochondrial ATP synthesis and calcium buffering – processes that were shown to be essential for the activation of T cells. Moreover, disruption of MMP by inhibition or by knockout of ETC complexes I or III leads to severe defects in T-cell activation, proliferation, cytokine production, and AICD.

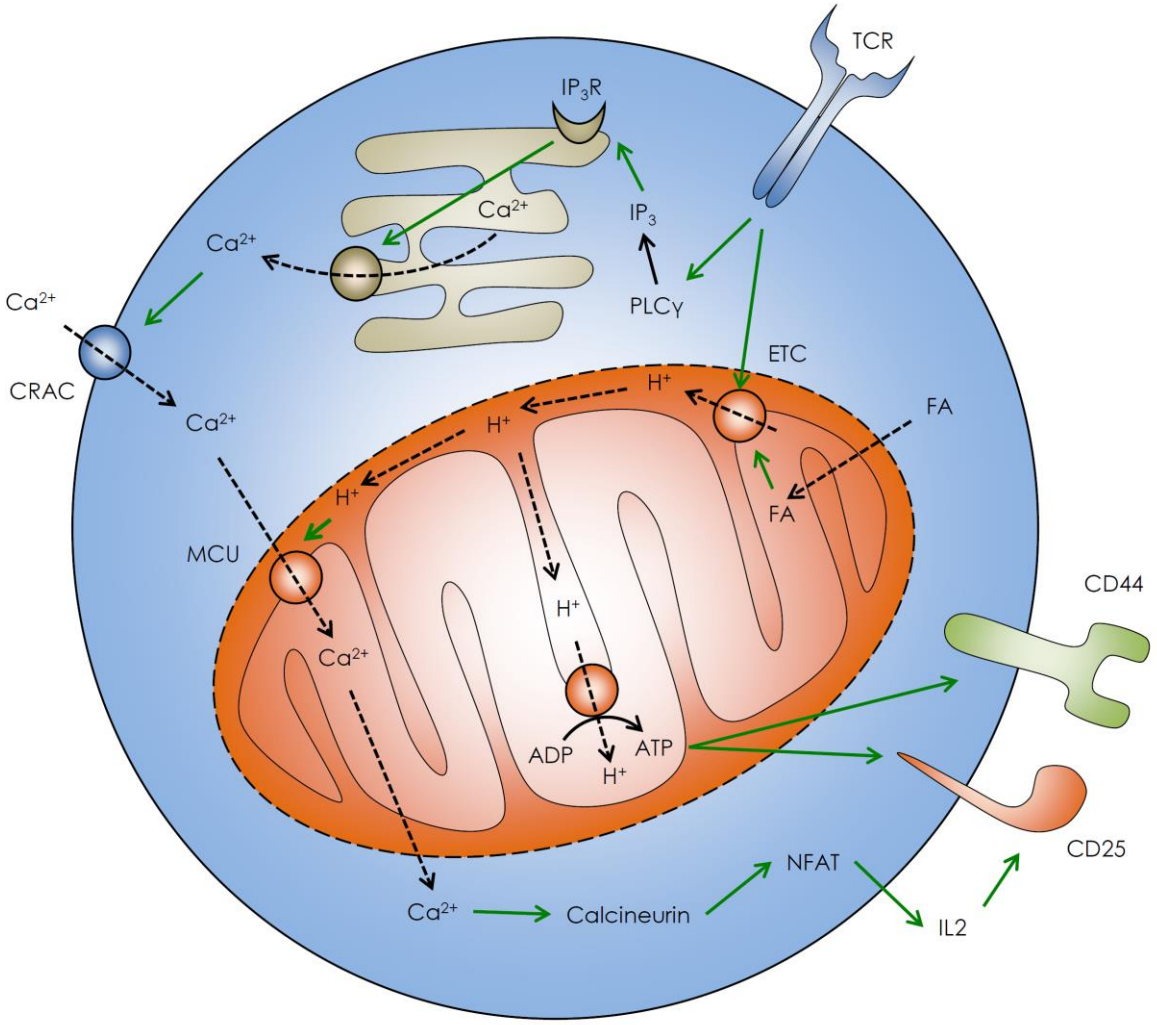

Figure 1.12 The role of mitochondria in T-cell activation

The triggering of the TCR leads to an increase in the activity of mitochondrial ETC, which pumps protons (H^+) across the inner mitochondrial membrane, creating MMP. ETC is fueled mainly by the catabolism of fatty acids (FA). TCR ligation also activates PLCγ-1, which generates IP_3 from PIP_2. IP_3 triggers its receptor on endoplasmic reticulum, leading to the release of Ca^{2+}. This event activates CRAC, resulting in the influx of extracellular Ca^{2+}. Mitochondria scavenge Ca^{2+} with mitochondrial calcium uniporter (MCU), which is powered by MMP. Ca^{2+} is then slowly released, allowing the sustained activation of NFAT via calcineurin. NFAT activation leads to the expression of IL-2. MMP also fuels mitochondrial ATP synthase. Mitochondria-derived ATP is required for the expression of CD25 and CD44. Black solid arrows indicate production, black dashed arrows indicate import/export, green solid arrows indicate activation.

1.6 The aim of this work

Investigations on the role of ROS in T cells have an almost 30-year-long history (98, 100, 107, 147, 150). It has been shown that phagocytic cells can induce oxidative stress in T cells, leading to unresponsiveness, but also that dendritic cells can secrete antioxidant precursors to help T-cell proliferation (see section 1.4.1). It has been additionally shown that the re-stimulation of previously activated T cells, such as T-cell blasts and Jurkat T cells, triggers ROS production that leads to death receptor upregulation and activation-induced cell death (see section 1.4.2). However, studies investigating the function of ROS in the activation of primary T cells are limited (see section 1.4.3). There is especially a profound lack of knowledge about the role of ROS in the activation of *human* primary T cells, the most relevant cell target for clinical applications.

Another important issue to take into account is the agent used for stimulation. Many studies employed relatively non-specific and/or artificial substances, such as Concanavalin A, PHA, PMA, or ionomycin, which complicate the interpretation of the results. Even in cases where stimulation with CD3 and CD28 antibodies was used, they were often added in solution, not immobilized on plates or microbeads. It has been recently shown that soluble stimulation leads to T-cell unresponsiveness, whereas stimulation with immobilized antibodies results in T-cell activation (170, 171). Thus, it is important to study the function of ROS under stimulation conditions correlating with productive T-cell responses, such as proliferation. Finally, it also has to be considered that phagocytic cells are almost inevitably present in T-cell preparations from human peripheral blood. These cells can be activated by anti-TCR antibodies through Fc receptors (138). Therefore, to differentiate between ROS produced by T cells and those released by phagocytes, isotype control antibodies have to be used in each experiment. This has almost never been done.

When antioxidants are used to assess the role of ROS in T-cell activation, it is important to choose substances with minimal off-target effects. For example, most antioxidants that were shown to affect the activation of T cells are also inhibitors of the lipoxygenase pathway – a pathway crucial for the synthesis of proinflammatory leukotrienes (see section 1.4.3) – and hence may modulate T-cell function in an oxidation-independent manner. Therefore, natural and more specific antioxidants,

such as superoxide dismutase, catalase, and ascorbate, are a better choice to elucidate the importance of ROS in T cells. Mice with the specific knockouts of ROS-producing enzymes, such as gp91$^{phox-/-}$ mice, are also excellent tools. However, it is not possible to perform a specific knockdown in human T cells without inducing cellular stress, which will inevitably lead to stress-mediated ROS generation.

Based upon these concerns, the aim of this work was to elucidate the involvement of ROS in the activation, proliferation, and differentiation of T cells under conditions that mimic physiological settings as closely as currently possible. To avoid the problems described above, I have measured ROS production in primary human and mouse T lymphocytes upon stimulation with CD3 and CD28 monoclonal antibodies immobilized on microbeads and used isotype-coated beads as control. Next, I have investigated the effects of superoxide dismutase, catalase, and ascorbate on the activation, proliferation, and cytokine profile of primary human T cells. Finally, I have characterized primary T cells from gp91$^{phox-/-}$ mice to assess whether NOX-2-derived ROS play any role in the activation of T cells. I have found that primary T cells produce extracellular superoxide via NOX-2 upon TCR stimulation, but this superoxide is not required for T-cell activation, proliferation, and differentiation.

Mitochondria have been implicated in T-cell activation, proliferation, cytokine production, and AICD (see section 1.5). Fatty acid catabolism, ATP synthesis, calcium uptake, and ROS production have been proposed to participate in these processes. However, it is not clear which mitochondrial function plays the major role. Again, very little is known about the involvement of mitochondria in the activation of human primary T cells. Therefore, in the second part of this work, I have studied the effects of various mitochondrial inhibitors on signaling, expression of activation markers and ROS production in primary human T cells. I have found that mitochondrial membrane potential, but not mitochondria-derived ROS or ATP, is crucial for the activation of primary human T cells.

2 Results

2.1 Measurements of TCR-triggered ROS

There are few studies investigating ROS production in primary T cells, especially in human primary T cells. Moreover, the majority of those studies have employed non-physiological types of stimulation, such as PMA and ionomycin or CD3 and CD28 antibodies added in solution, which lead to T-cell hyperactivation or unresponsiveness (170), respectively. In addition, many studies have not discriminated between the T cell-derived ROS and the ROS generated by phagocytic cells. In fact, phagocytes are usually present in preparations of primary T cells and can be easily activated by the same stimulatory agents.

Based upon these concerns, the goal of the first part of my work was to measure ROS production specifically in primary T cells using more physiological stimulation. To this aim, I have measured ROS production in primary human and mouse T lymphocytes upon stimulation with CD3 and CD28 monoclonal antibodies immobilized on microbeads, and I have used isotype-coated beads as control.

2.1.1 Phagocytes interfere with ROS measurements in primary T-cell cultures

Firstly, I have decided to measure ROS production in primary human T cells with the most commonly used ROS-sensitive fluorescent indicator – DCFDA. Peripheral blood T cells were isolated from healthy volunteers by negative magnetic sorting to avoid the preactivation of the cells. Subsequently, the cells were loaded with DCFDA and stimulated with anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) immobilized on microbeads (CD3xCD28) to mimic a physiological stimulation (170). At 15 and 30 minutes after stimulation, the cells were diluted in ice-cold PBS, to terminate the stimulation, and were analyzed by flow cytometry, gating on lymphocytes according to their physical parameters (on the FSC-SSC dot plot). I have detected a moderate increase in the fluorescence of the dye upon CD3xCD28 stimulation (Fig. 2.1 A). However, the oxidation of the dye induced by microbeads coated with the isotype control antibodies was identical to that observed upon

CD3xCD28 stimulation. Therefore, I concluded that the ROS production was triggered in a TCR-independent fashion.

Microbeads coated with immunoglobulins mimic opsonized pathogens and thus may activate phagocytic cells via Fc receptors (138, 172). I have indeed identified a small percentage of $CD14^+$ and $CD16^+$ (the markers for monocytes and neutrophils) cells in my T-cell preparations (Fig. 2.1 B). If ROS detectable with DCFDA are produced by phagocytic cells present in T-cell preparations, then I should have observed more dye oxidation in cell cultures enriched in phagocytes, such as peripheral blood mononuclear cells (PBMCs). Indeed, as shown in Fig. 2.1 C, lymphocytes gated within isolated PBMCs exhibit very strong DCFDA oxidation upon stimulation with microbeads coated with either anti-CD3 and anti-CD28 mAbs or the isotype controls. Conversely, no detectable ROS production is observed upon stimulation of *in vitro* expanded T-cell blasts or Jurkat T cells, which do not contain contaminating phagocytic cells (Fig. 2.1 C). Interestingly, when PBMCs are stimulated with beads not coupled to antibodies, the increase in DCFDA oxidation within lymphocytes is much weaker (Fig. 2.1 D). This implies that ROS production is indeed triggered mostly by Fc receptor-mediated phagocyte activation.

From these experiments, I have concluded that the employed method is not suitable to detect ROS upon TCR triggering in primary cultures of human T cells. Even if T cells produce very low amounts of ROS, these cannot be detected, as they are masked by ROS from phagocytic cells. Therefore, a more sensitive method to detect ROS is required to address the question of whether T cells produce ROS upon TCR triggering. Moreover, it is known that DCFDA has low sensitivity for superoxide ($O_2^{\bullet-}$) (173). Therefore, even if $O_2^{\bullet-}$ is the main reactive species produced by T cells in response to TCR stimulation, it is not detectable by DCFDA.

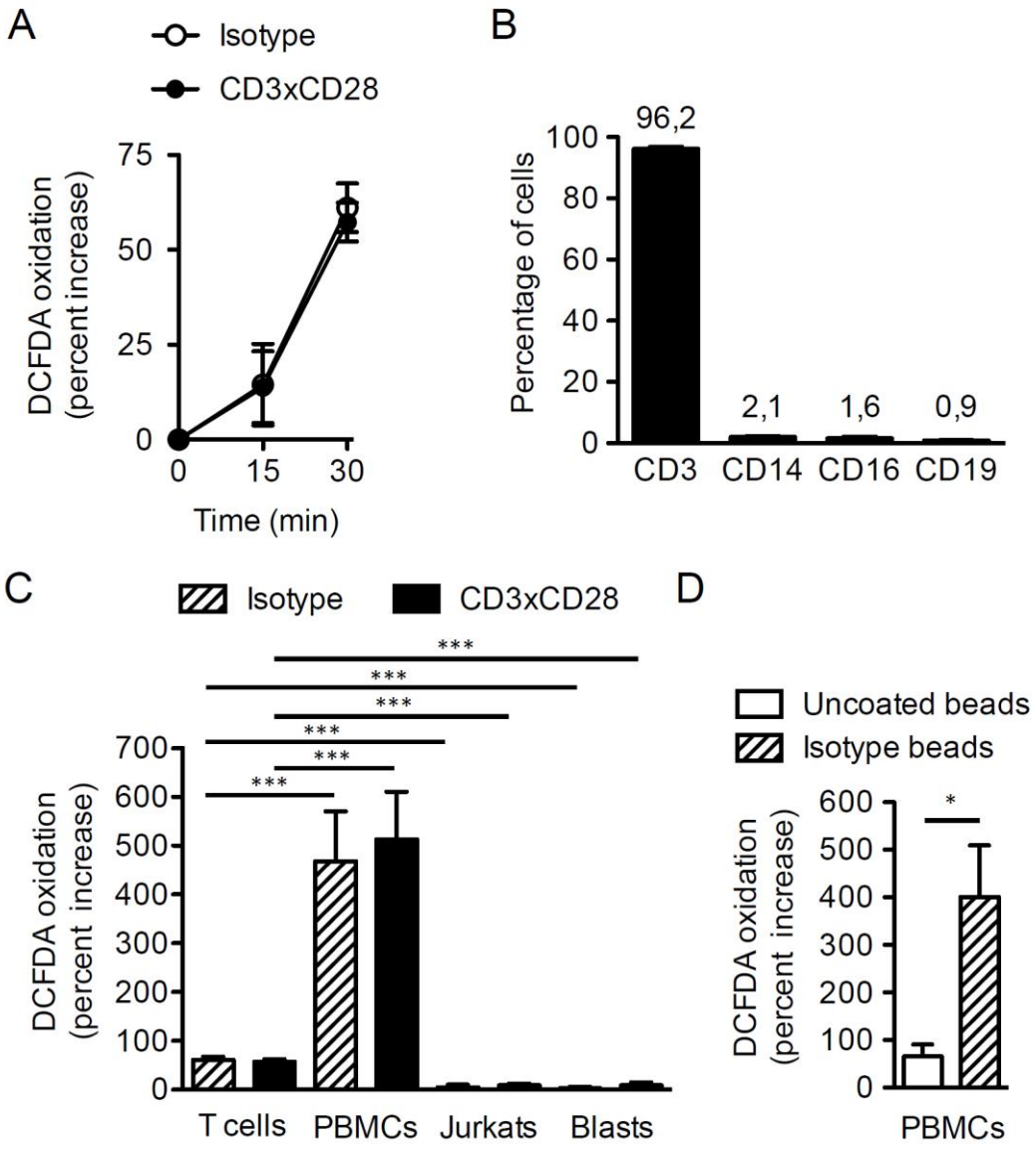

Figure 2.1 Phagocytes interfere with ROS measurements in primary T-cell cultures

In (A), (C) and (D), the cells were loaded with DCFDA and stimulated with the indicated antibodies. DCFDA fluorescence was measured by FACS upon setting a gate on lymphocytes according to their physical parameters (on the FSC-SSC dot plot). The values indicate the increase in the mean fluorescence intensity in the stimulated vs. unstimulated samples. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(A) Primary human T cells were stimulated with CD3xCD28- or isotype-coated microbeads for 15 min (n=3) or 30 min (n=15).

(B) A preparation of primary human T cells was stained with the indicated fluorescently labeled antibodies and analyzed by FACS (n=3).

(C) Primary human T cells (n=15), PBMCs (n=4), Jurkat T cells (n=7), or human T-cell blasts (n=5) were stimulated with CD3xCD28- or isotype-coated microbeads for 30 min.

(D) PBMCs were stimulated with uncoated or isotype-coated microbeads for 30 min (n=3).

2.1.2 TCR stimulation induces the extracellular release of superoxide in primary human T cells

In order to shed more light onto ROS production in T cells, I have decided to use the luminol-based assay Diogenes, which predominantly detects $O_2^{\bullet-}$. In line with the DCFDA measurements, I have detected some oxidation of luminol upon the addition of isotype-coated microbeads to T-cell preparations, likely indicating $O_2^{\bullet-}$ production by contaminating phagocytic cells (Fig. 2.2 A). However, CD3xCD28-coated microbeads induce the significantly stronger oxidation of luminol (Fig. 2.2 A). When the data are presented as the ratio of the oxidation induced by CD3xCD28 vs. the oxidation induced by isotype controls, the specific increase in $O_2^{\bullet-}$ production by CD3xCD28-stimulated T cells becomes even more clear (Fig. 2.2 B). The peak (about 35% increase over the isotype controls) in the TCR-specific $O_2^{\bullet-}$ production occurs 10 min after TCR-triggering, whereas ROS levels decrease gradually afterwards.

The superoxide-neutralizing enzyme superoxide dismutase (SOD), but not the hydrogen peroxide-neutralizing enzyme catalase, prevents the luminol oxidation, indicating that the detected reactive species is indeed $O_2^{\bullet-}$ (Fig. 2.2 C). These results also suggest that $O_2^{\bullet-}$ is released into the extracellular space, as SOD is membrane-impermeable. Interestingly, there is no detectable extracellular $O_2^{\bullet-}$ production upon CD3xCD28 stimulation of human T-cell blasts and Jurkat T cells (data not shown). On the contrary, PBMCs, which contain phagocytic cells, induce very strong $O_2^{\bullet-}$ production (Fig. 2.2 D). In summary, my data show that primary human T cells produce $O_2^{\bullet-}$ upon TCR stimulation and release it into the extracellular space.

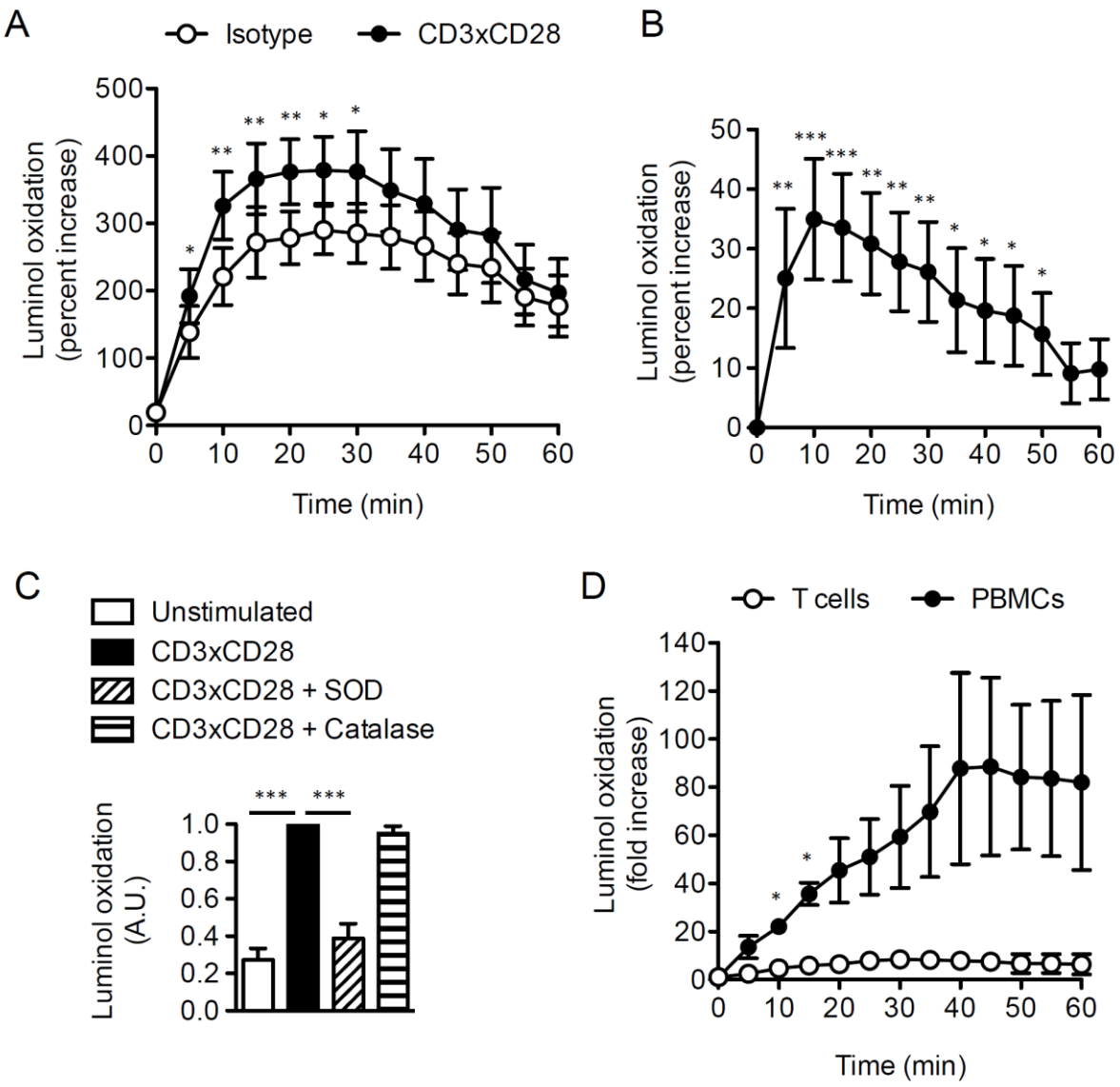

Figure 2.2 TCR stimulation induces the extracellular release of superoxide in primary human T cells

$O_2^{\bullet-}$ levels were measured using the Diogenes assay. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(A) Primary human T cells were stimulated with CD3xCD28- or isotype-coated microbeads (n=16). The values indicate the increase in luminescence in the bead-stimulated samples relative to the unstimulated samples.

(B) Primary human T cells were stimulated with CD3xCD28- or isotype-coated microbeads (n=16). The values indicate the increase in luminescence in the CD3xCD28-stimulated samples relative to the isotype-stimulated samples.

(C) Primary human T cells were stimulated for 10 min with CD3xCD28-coated microbeads alone or in the presence of SOD or catalase (n=4). The values indicate the luminescence signal normalized to the stimulated control.

(D) Primary human T cells or PBMCs were stimulated with CD3xCD28-coated microbeads (n=2). The values indicate the increase in luminescence in the CD3xCD28-stimulated samples relative to the unstimulated samples.

2.2 The function of extracellular superoxide in T-cell activation

Most of the antioxidants used in many studies are also inhibitors of leukotriene production and hence may modulate T-cell activation in a ROS-independent manner. Based upon this concern, the goal of this part of the work was to elucidate the function of ROS in primary T cells using natural antioxidants and specific knockouts. To this aim, I have investigated the effects of SOD, catalase, and ascorbate on the activation, proliferation, and cytokine profile of primary human T cells upon stimulation with CD3xCD28-coated microbeads. I have also analyzed primary T cells from gp91$^{phox-/-}$ mice to assess whether NOX-2-derived ROS play any role in the activation of T cells.

2.2.1 Natural antioxidants efficiently neutralize extracellular ROS

In the previous part, I have shown that the main detectable reactive species produced by T cells upon TCR triggering is $O_2^{\bullet-}$. Moreover, I have shown that extracellular $O_2^{\bullet-}$ can be neutralized by the addition of SOD (Fig. 2.2 C). This allowed me to investigate its functional role in T cells, using SOD as a tool. Two additional natural antioxidants were also tested – ascorbate and glutathione. Ascorbate is as efficient as SOD, whereas glutathione is two-fold less efficient (scavenging only half of inducible $O_2^{\bullet-}$) (Fig. 2.3 A). Therefore, glutathione was not further used in my studies. As $O_2^{\bullet-}$ can naturally dismutate to hydrogen peroxide (H_2O_2), which potentially can also affect T cells, I have included samples treated with catalase in the functional assays. The efficiency of catalase in removing H_2O_2 is confirmed using DCFDA (Fig. 2.3 B), as the Diogenes detects almost exclusively $O_2^{\bullet-}$. SOD, ascorbate, and catalase are essential parts of the cell-intrinsic antioxidant defense system and therefore can be used for functional assays without inducing off-target effects.

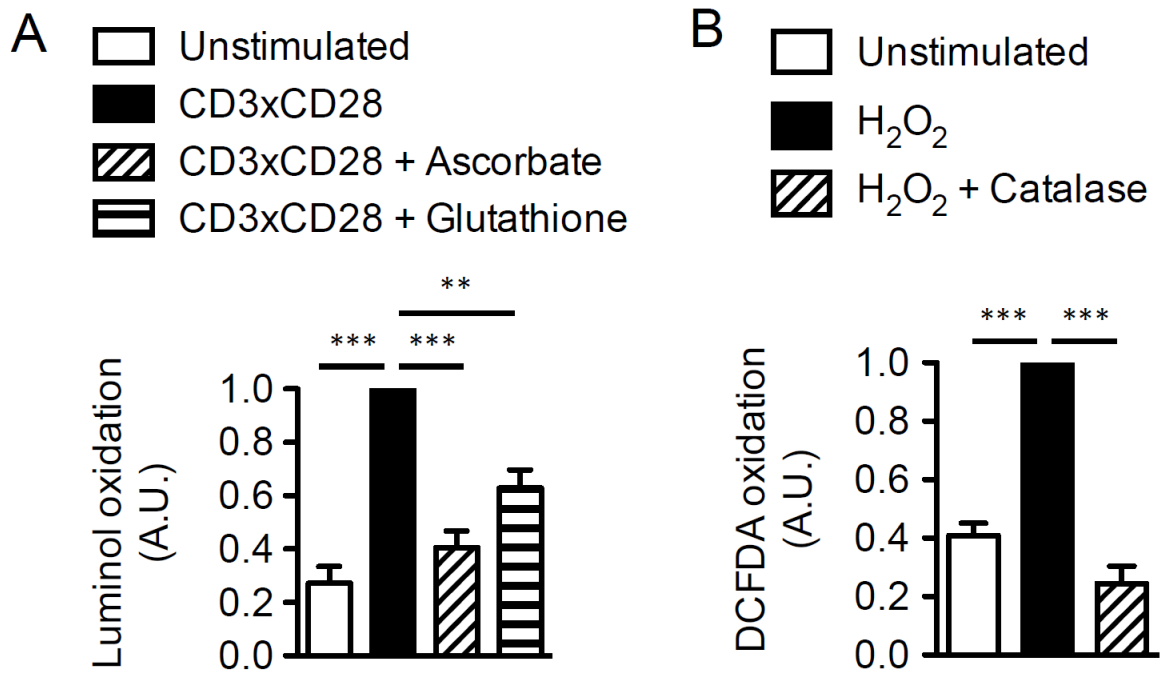

Figure 2.3 Natural antioxidants efficiently neutralize extracellular ROS

(A) Primary human T cells were stimulated for 10 min with CD3xCD28-coated microbeads alone or in the presence of ascorbate or glutathione (n=4). $O_2^{\bullet-}$ was measured using the Diogenes assay. The values indicate the luminescence intensities normalized to the stimulated control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(B) Primary human T cells were loaded with DCFDA and stimulated for 30 min with 100 µM H_2O_2 alone or in the presence of catalase (n=3). DCFDA fluorescence was measured by FACS. The values indicate the mean fluorescence intensities normalized to the stimulated control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

2.2.2 Extracellular superoxide is not required for T-cell activation, proliferation, and cytokine production

In the previous section, I have shown that SOD, ascorbate, and catalase can be used to remove extracellular $O_2^{\bullet-}$ and hence to assess its importance for T-cell activation. To investigate the role of extracellular $O_2^{\bullet-}$ in T cells, I have first stimulated primary human T cells with CD3xCD28-coated microbeads for 16 hours in the presence of the antioxidants and assessed T-cell activation by FACS, using fluorescently labeled antibodies against surface activation markers (Fig. 2.4 A). To my surprise, the addition of SOD, ascorbate, or catalase had no major effect either on the expression of CD25 and CD69 (Fig. 2.4 B) or on the percentage of $CD25^+CD69^+$ cells (Fig. 2.4 C).

Next, I have investigated proliferation of CD3xCD28-stimulated human T cells in the presence of SOD, ascorbate, and catalase using a CFSE dilution assay (Fig. 2.5 A). Consistent with the results presented above, I have observed normal percentages of the proliferating cells 3 days after the stimulation in the presence of the antioxidants (Fig. 2.5 B).

I have then decided to investigate if extracellular $O_2^{\bullet-}$ is involved in the regulation of $CD4^+$ T-cell differentiation. To this aim, I have stimulated human $CD4^+$ T_{nai} cells with CD3xCD28-coated microbeads in the presence of the antioxidants and measured the concentrations of various cytokines in the supernatants after 48 hours, using the Bio-Plex system. No significant differences were observed between the samples (Fig. 2.6).

Overall, these data demonstrate that in human T cells extracellular $O_2^{\bullet-}$ is dispensable for activation, proliferation, and cytokine production.

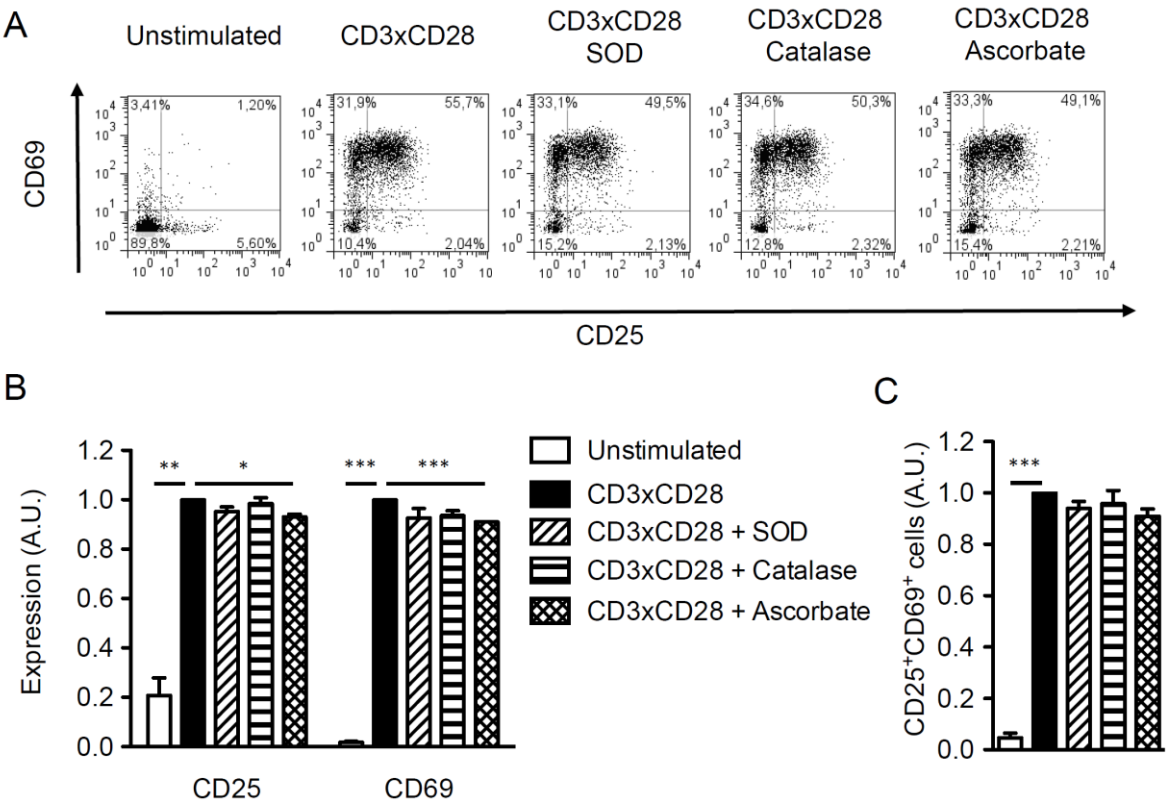

Figure 2.4 Extracellular superoxide is not required for the activation of human T cells

(A) Primary human T cells were stimulated with CD3xCD28-coated microbeads alone or in the presence of either SOD, catalase, or ascorbate (n=3). After 16 hours, the cells were stained with CD25-FITC and CD69-PE mAbs and analyzed by FACS.

(B) Quantification of (A). The values indicate the mean fluorescence intensities of CD25 and CD69 staining normalized to the stimulated control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(C) Quantification of (A). The values indicate the percentages of $CD25^{+}CD69^{+}$ cells normalized to the stimulated control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

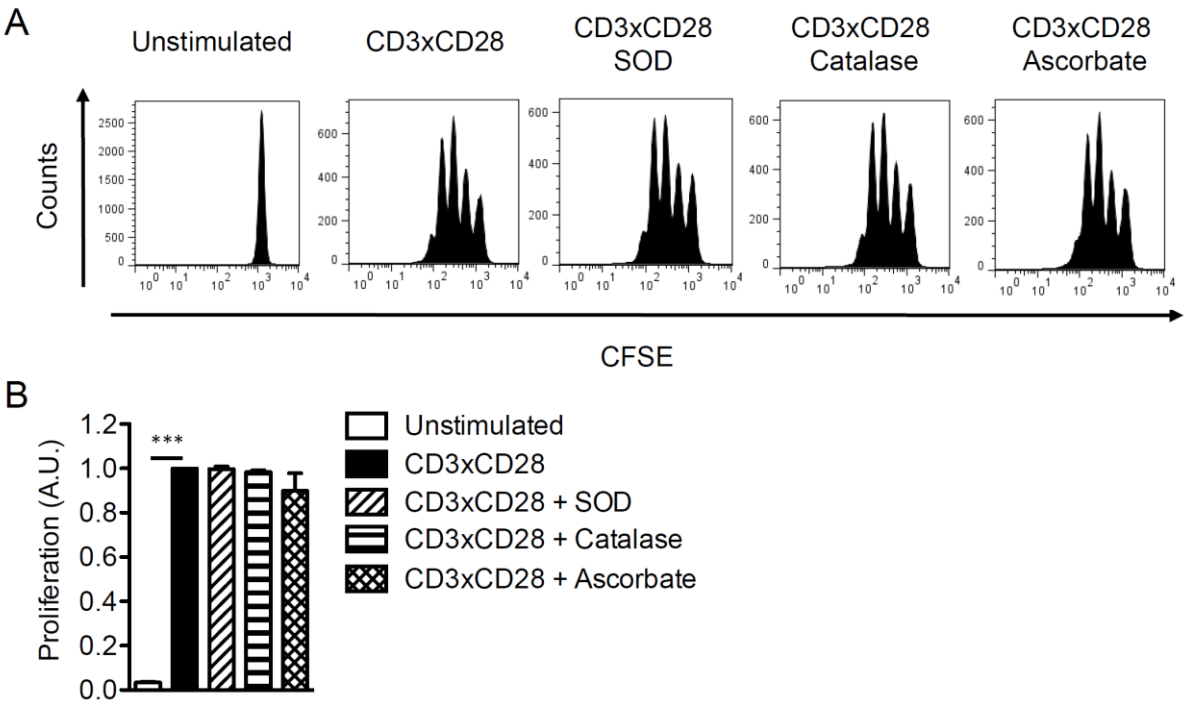

Figure 2.5 Extracellular superoxide is not required for the proliferation of human T cells

(A) Primary human T cells were loaded with CFSE and stimulated with CD3xCD28-coated microbeads alone or in the presence of either SOD, catalase, or ascorbate (n=3). After 72 hours, the CFSE dilution was analyzed by FACS.

(B) Quantification of (A). The values indicate the percentages of the proliferating cells normalized to the stimulated control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

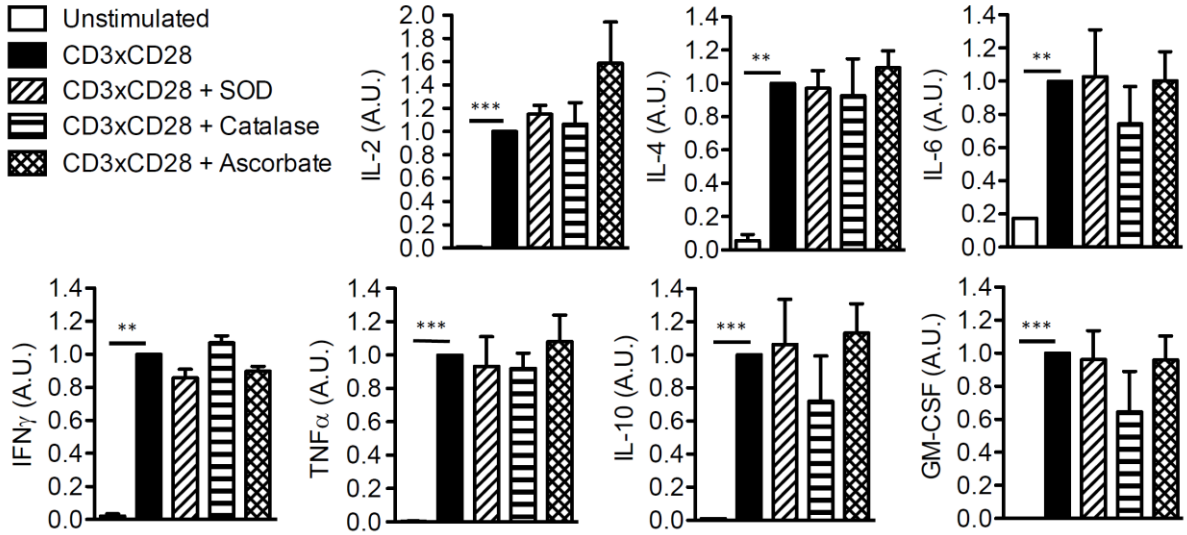

Figure 2.6 Extracellular superoxide is not required for cytokine production by human CD4⁺ T cells

Human $CD4^+$ T_{nai} cells were stimulated with CD3xCD28-coated microbeads alone or in the presence of either SOD, catalase, or ascorbate (n=3). After 48 hours, supernatants were collected. The cytokine concentrations were measured using the Bio-Plex Pro assay. The values indicate the cytokine concentrations normalized to the stimulated controls. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

2.3 The role of NOX-2 in T cells

2.3.1 TCR-triggered superoxide production is mediated by NOX-2 in primary T cells

I have shown that primary T cells produce extracellular $O_2^{\bullet-}$ upon TCR stimulation. One of the most-well described sources of inducible $O_2^{\bullet-}$ production is the phagocytic NADPH oxidase 2 (NOX-2). Remarkably, NOX-2 has been shown to be expressed in human and murine T-cell blasts (116, 119). I have decided to use T cells from NOX-2-deficient mice (*gp91$^{phox-/-}$*) to investigate whether NOX-2 is the source of the TCR-triggered $O_2^{\bullet-}$ production. Splenic T cells from wild type (WT) mice produce $O_2^{\bullet-}$ upon the CD3xCD28 microbead stimulation, similarly to the human T cells albeit with faster kinetics (Fig. 2.7 A). On the contrary, T cells from *gp91$^{phox-/-}$* mice show no inducible $O_2^{\bullet-}$ production upon TCR stimulation (Fig. 2.7 A). Moreover, isotype-coated microbeads induce $O_2^{\bullet-}$ production in WT but not in *gp91$^{phox-/-}$* cells, thus confirming my hypothesis that phagocytic cells produce ROS upon antibody stimulation (Fig. 2.7 B). Therefore, these data demonstrate that NOX-2 is indeed activated upon TCR triggering in primary T cells and that NOX-2 is responsible for the rapid generation of $O_2^{\bullet-}$.

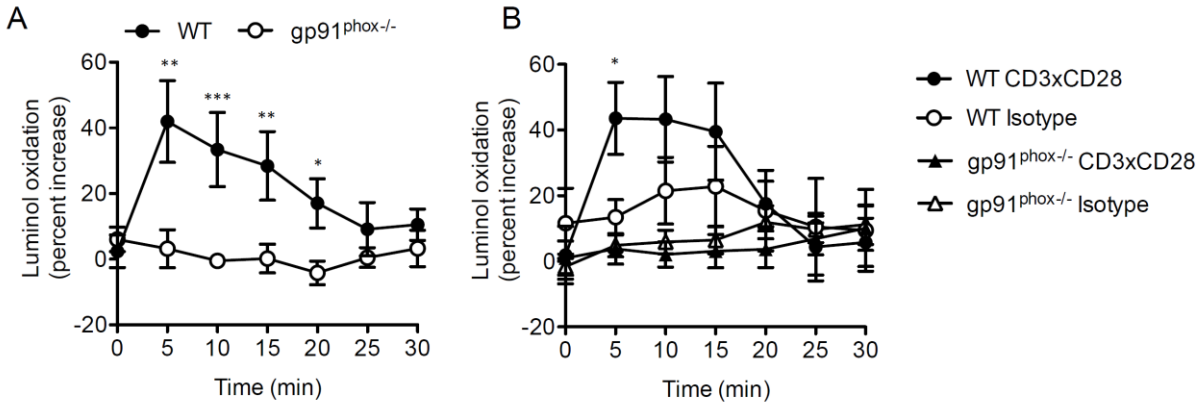

Figure 2.7 TCR-triggered superoxide production is mediated by NOX-2 in primary T cells

(A and B) Splenic T cells from either WT (n=6) or *gp91$^{phox-/-}$* (n=12) mice were stimulated with CD3xCD28- or isotype-coated microbeads. $O_2^{\bullet-}$ was measured using the Diogenes assay at 5 min intervals. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(A) The values indicate the increase in luminescence intensity in the CD3xCD28- relative to the isotype-stimulated samples.

(B) The values indicate the increase in luminescence intensity in the bead-stimulated samples relative to the unstimulated samples.

2.3.2 NOX-2 deficiency does not affect T-cell activation and proliferation

To assess the importance of NOX-2 in T-cell functions, I have compared the activation and proliferation of *gp91$^{phox-/-}$* and WT T cells upon CD3xCD28 microbead stimulation. In line with my observations of human T cells treated with antioxidants (Fig. 2.4, Fig. 2.5), the upregulation of the activation markers CD69 and CD25 (Fig. 2.8 A, B), the percentage of $CD69^{+}CD25^{+}$ cells (Fig. 2.8 A, C), and proliferation (Fig. 2.9) are not affected in the T cells from *gp91$^{phox-/-}$* mice. Altogether, my data suggest that TCR-triggered extracellular $O_2^{\bullet-}$ production via NOX-2 is dispensable for the activation of primary T cells.

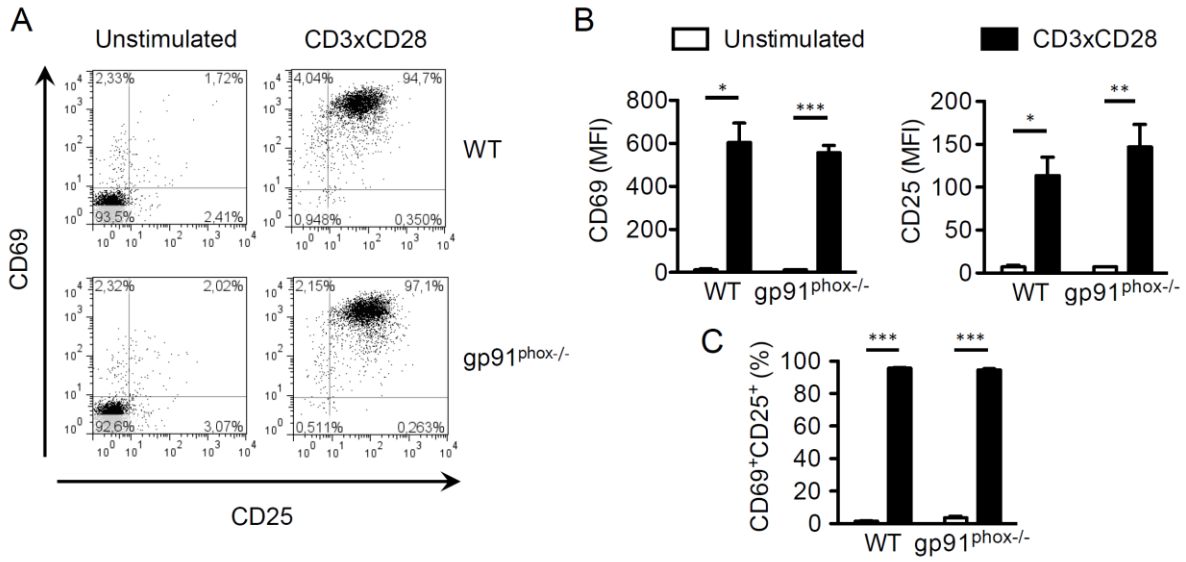

Figure 2.8 NOX-2 is not required T-cell activation

(A) Splenic T cells from WT (n=2) or *gp91$^{phox-/-}$* (n=4) mice were stimulated with CD3xCD28-coated microbeads. After 16 hours, cells were stained with CD25-FITC and CD69-PE mAbs and analyzed by FACS.

(B) Quantification of (A). The values indicate the mean fluorescence intensities. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(C) Quantification of (A). The values indicate the percentages of $CD69^+CD25^+$ cells. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

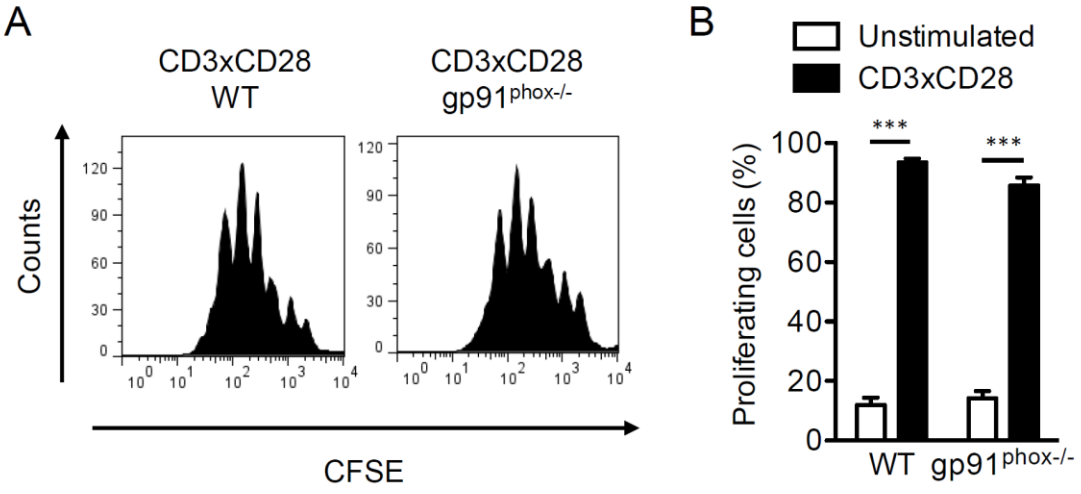

Figure 2.9 NOX-2 is not required for T-cell proliferation

(A) Splenic T cells from WT (n=4) or *gp91$^{phox-/-}$* (n=8) mice were loaded with CFSE and stimulated with CD3xCD28-coated microbeads. After 72 hours, the CFSE dilution was analyzed by FACS.

(B) Quantification of (A). The values indicate the percentages of the proliferating cells. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

2.4 The role of mitochondria in T-cell activation

Mitochondria have been implicated in T-cell activation, proliferation, cytokine production, and AICD, but little is known about the role of mitochondria in human primary T cells. Fatty acid catabolism, ATP synthesis, calcium uptake, and ROS production are among the mitochondrial processes that have been proposed to participate in T-cell functions. Accordingly, the goal of this part of the work was to identify the mitochondrial process that plays the key role in T-cell activation. To this aim, I have studied the effects of various mitochondrial inhibitors on signaling, expression of activation markers, and ROS production in primary human T cells.

2.4.1 Flavin-containing proteins are crucial for T-cell activation

Since the discovery that *diphenyleneiodonium* (DPI), a flavin-domain inhibitor, is able to inhibit phagocytic NADPH oxidase (174), it has been often used to study the involvement of NADPH oxidases in various cell processes (175-177). In particular, by using DPI, it has been suggested that T cells require NADPH oxidase-generated ROS for proper signaling (117). However, in the previous section, I have shown that NOX-2 is dispensable for T-cell activation and proliferation. Therefore, I was interested to investigate whether DPI can still affect activation of NOX-2-deficient mice by other mechanisms. To this aim, I have compared T-cell activation in WT and *gp91$^{phox-/-}$* mice in the presence of DPI. I have found that DPI strongly affects the expression of both CD69 and CD25 activation markers in primary T cells from both WT and NOX-2-knockout mice (Fig. 2.10). These data indicate that the effect of DPI on T-cell activation is NOX-2-independent.

DPI is a general inhibitor of flavin-containing proteins, which include but are not limited to cytochrome b5 (methemoglobin), methylenetetrahydrofolate, and thioredoxin reductases; D-amino acid, monoamine, xantine, and NADPH oxidases; Acyl CoA, butyryl CoA, dihydrolipoamide, NADH, and succinate dehydrogenases.

NADH dehydrogenase (ubiquinone) and succinate dehydrogenase are also called mitochondrial electron transport chain (ETC) Complexes I and II, respectively. However, it has been shown that DPI selectively inhibits Complex I but not II (178-181). By blocking Complex I, DPI decreases electron flow through the ETC and

hence affects the generation and maintenance of mitochondrial membrane potential (MMP) (182). There are two major processes dependent on MMP: mitochondrial ATP production (oxidative phosphorylation) and mitochondrial calcium uptake (165, 166). The latter is especially important in case of T cells, as it has been shown to be necessary for sustained CRAC-mediated calcium influx and NFAT activation in Jurkat T cells (159, 160). Therefore, I have hypothesized that DPI may affect T-cell activation by disrupting these mitochondrial processes, rather than by blocking NOX-2-dependent ROS production.

Indeed, I have found that DPI can dramatically affect primary human T cells. It decreases the phosphorylation of Zap70, PLCγ, LAT, and ERK (Fig. 2.11 A) and CD69 and CD25 expression (Fig. 2.11 B, C), upon stimulation with CD3xCD28-coated microbeads. Interestingly, it also decreases the phosphorylation of LCK in unstimulated T cells (Fig. 2.12 A), the phosphorylation of MEK and ERK after PMA stimulation (Fig. 2.12 B), and general tyrosine phosphorylation after pervanadate treatment (Fig. 2.12 C).

Thus, I have shown that DPI strongly affects activation of primary T cells. Those effects could be due to the inhibition of mitochondrial Complex I. However, as DPI can potentially target all flavin-containing proteins, which include many metabolic enzymes, it is still possible that its effects on T-cell activation are mediated by alterations of mitochondria-unrelated processes (183, 184). Therefore, I have decided to investigate the effects of the specific mitochondrial Complex I inhibitor *rotenone* on T-cell functions.

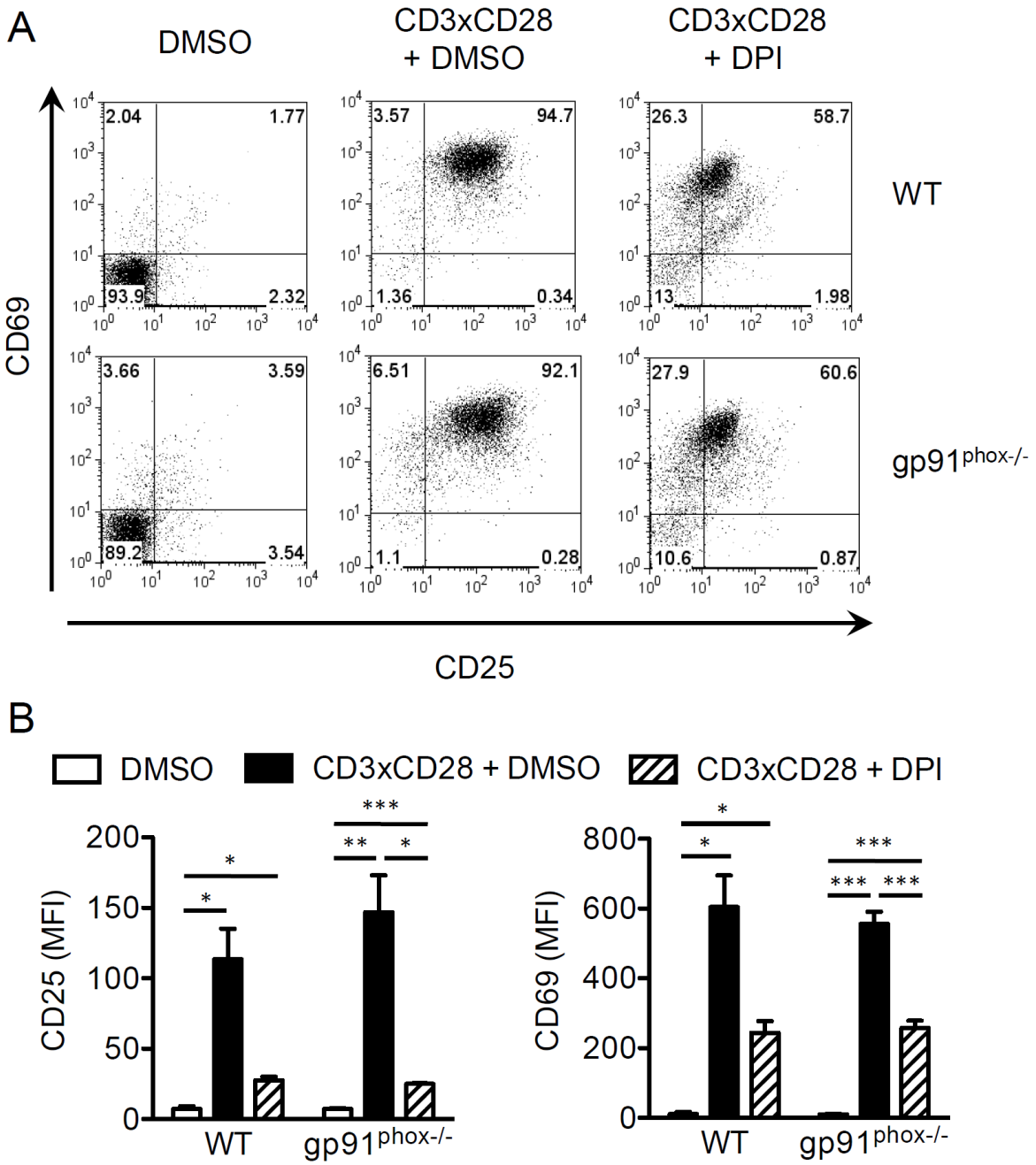

Figure 2.10 Flavin-containing proteins are crucial for the activation of mouse T cells

(A) Splenic T cells from WT (n=2) or *gp91$^{phox-/-}$* (n=3) mice were preincubated with DMSO or DPI and stimulated with CD3xCD28-coated microbeads. After 16 hours, cells were stained with CD25-FITC and CD69-PE mAbs and analyzed by FACS.

(B) Quantification of (A). The values indicate the mean fluorescence intensities. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

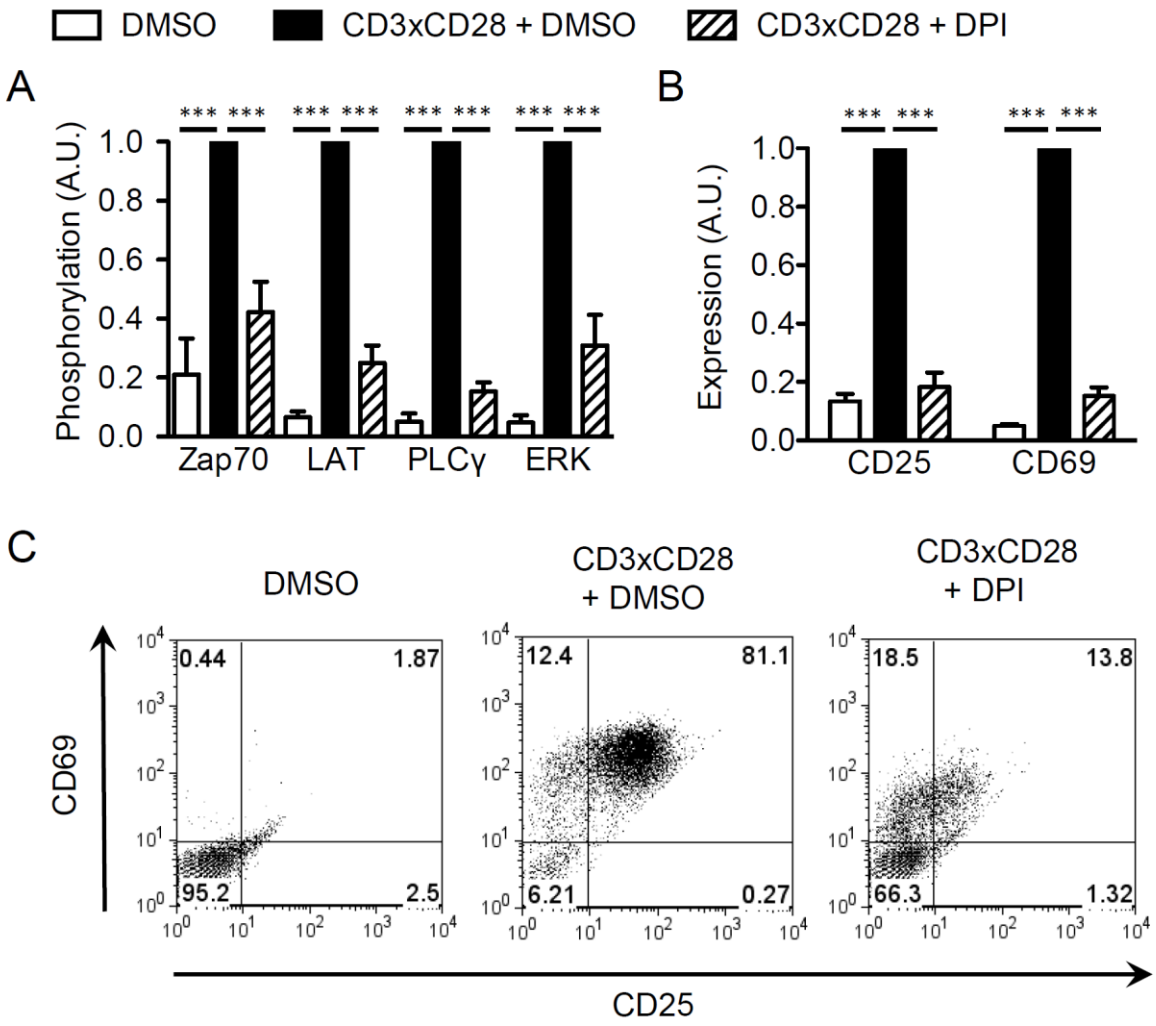

Figure 2.11 Flavin-containing proteins are crucial for the activation of human T cells

(A) Primary human T cells were preincubated with DMSO or DPI and stimulated with CD3xCD28-coated microbeads for 30 min (n=2-4). The ratios of the phosphorylated proteins to β-actin were determined by quantifying the band intensities on the western blots using ECL, scanner, and ImageQuant software. The values indicate the relative protein phosphorylation normalized to the stimulated DMSO controls. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(B) Primary human T cells were preincubated with DMSO or DPI and stimulated with CD3xCD28-coated microbeads (n=4). After 16 hours, cells were stained with CD25-FITC and CD69-PE mAbs and analyzed by FACS. The values indicate the mean fluorescence intensities normalized to the stimulated DMSO controls. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(C) Representative dot blots of the data shown in (B).

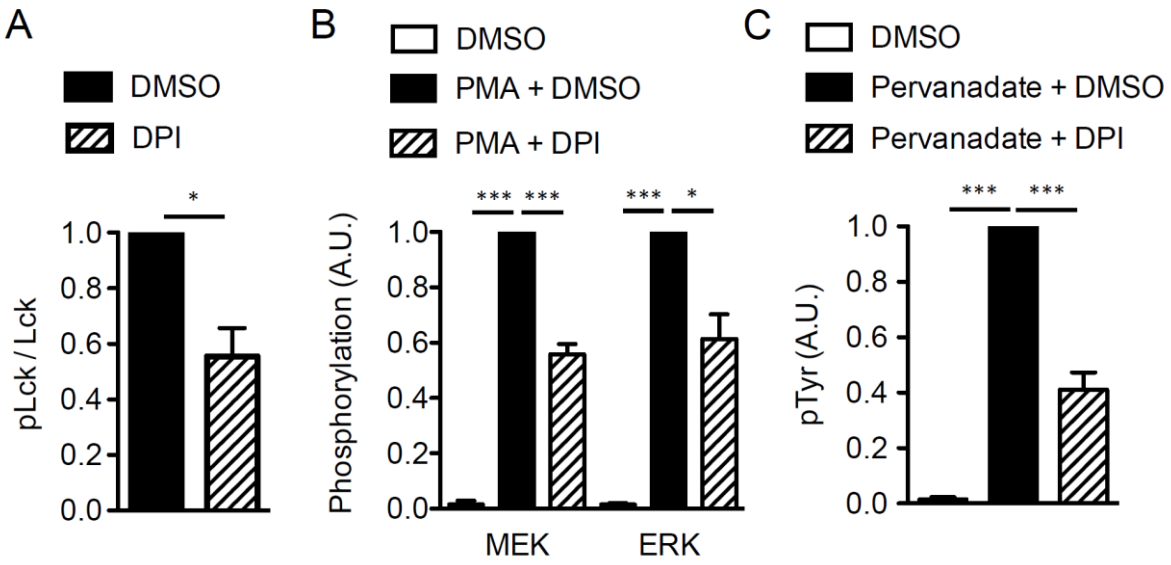

Figure 2.12 Flavin-containing proteins are required for proper TCR signaling in primary human T cells

(A) Primary human T cells were incubated with DMSO or DPI for 30 min (n=3). The ratio of phosphorylated LCK to total LCK was determined by quantifying the band intensities on the western blots using the Odyssey system. The values indicate the relative LCK phosphorylation normalized to the DMSO control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(B) Primary human T cells were preincubated with DMSO or DPI and stimulated with PMA for 30 min (n=2-3). The ratios of the phosphorylated proteins to β-actin were determined by quantifying the band intensities on the western blots using ECL, scanner, and ImageQuant software. The values indicate the relative protein phosphorylation normalized to the stimulated DMSO controls. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(C) Primary human T cells were preincubated with DMSO or DPI and stimulated with pervanadate for 2 min (n=4). The ratio of tyrosine-phosphorylated proteins to β-actin was determined by quantifying the band intensities on the western blots using ECL, scanner, and ImageQuant software. The values indicate the relative protein phosphorylation normalized to the stimulated DMSO control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

2.4.2 Mitochondrial Complex I is required for T-cell activation

Rotenone inhibits the transfer of electrons from iron-sulfur centers in mitochondrial Complex I to ubiquinone (185), thus decreasing electron flow through ETC, MMP (186), and hence ATP production and calcium uptake (187). Interestingly, rotenone has been shown to inhibit ERK phosphorylation, CD69 expression, proliferation, and TNFα production in murine $CD8^+$ T cells (169), CD95L expression and AICD in Jurkat T cells and human T-cell blasts (110), as well as IL-2 and IL-4 production in human T cells (113, 134).

In agreement with the literature, I have found that rotenone inhibits the activation of primary human T cells. It also decreases the phosphorylation of Zap70, PLCγ, LAT, and ERK (Fig. 2.13 A) and diminishes CD69 and CD25 expression (Fig. 2.13 B, C), upon stimulation with CD3xCD28-coated microbeads. Moreover, rotenone decreases the phosphorylation of LCK in unstimulated cells (Fig. 2.14 A), the phosphorylation of MEK and ERK after PMA stimulation (Fig. 2.14 B), and general tyrosine phosphorylation after pervanadate treatment (Fig. 2.14 C).

Interestingly, rotenone does not have such a dramatic effect on T-cell activation as DPI. This confirms my hypothesis that, in addition to Complex I, DPI affects other enzymes that are important for T-cell activation. Nevertheless, these data show that Complex I activity is required for the activation of primary human T cells. To further elucidate the involvement of mitochondria in T-cell activation, I have decided to utilize other inhibitors of mitochondrial processes.

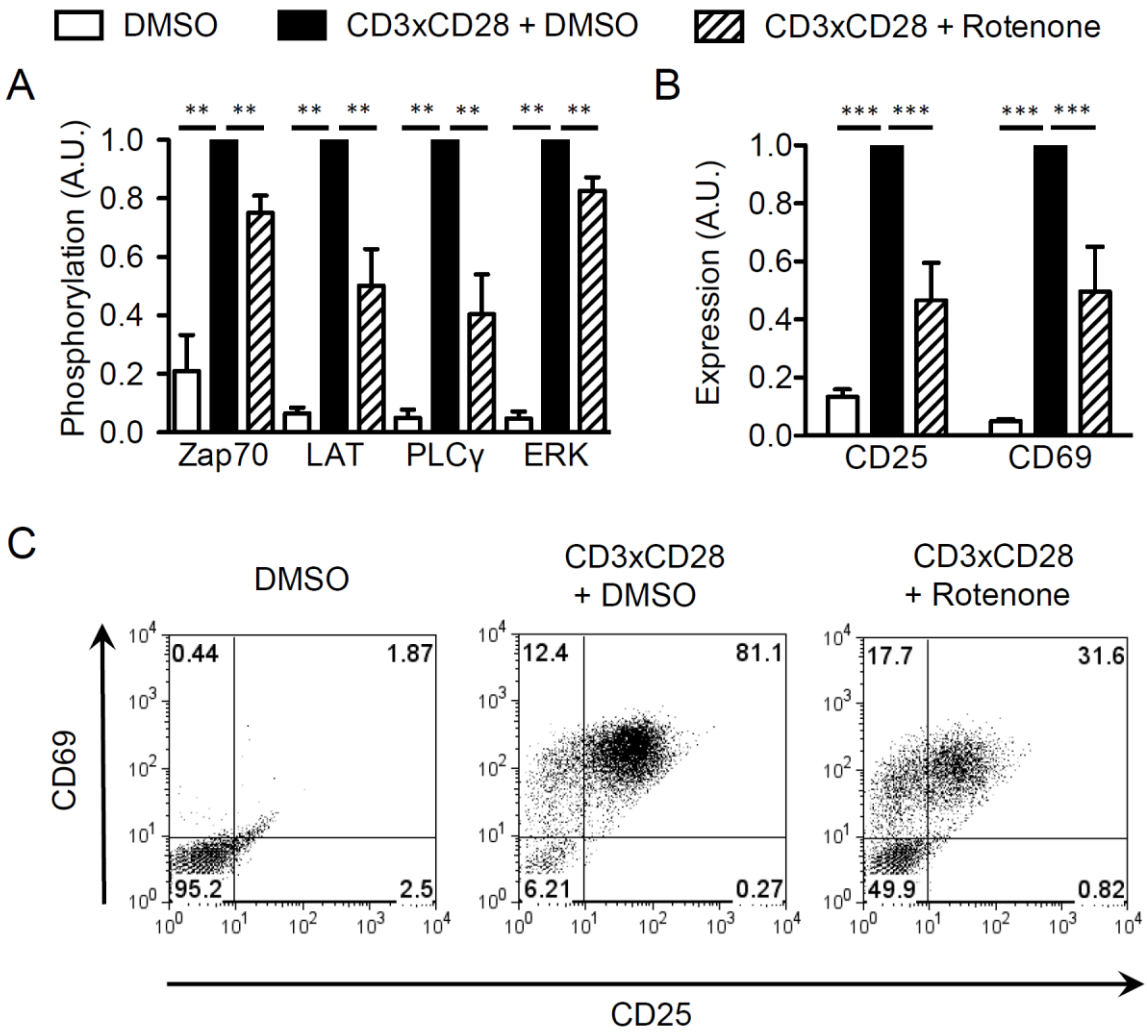

Figure 2.13 Mitochondrial Complex I is required for the activation of human T cells

(A) Primary human T cells were preincubated with DMSO or rotenone and stimulated with CD3xCD28-coated microbeads for 30 min (n=2-4). The ratios of the phosphorylated proteins to β-actin were determined by quantifying the band intensities on the western blots using ECL, scanner, and ImageQuant software. The values indicate the relative protein phosphorylation normalized to the stimulated DMSO controls. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(B) Primary human T cells were preincubated with DMSO or rotenone and stimulated with CD3xCD28-coated microbeads (n=3). After 16 hours, cells were stained with CD25-FITC and CD69-PE mAbs and analyzed by FACS. The values indicate the mean fluorescence intensities normalized to the stimulated DMSO controls. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(C) Representative dot blots of the data shown in (B).

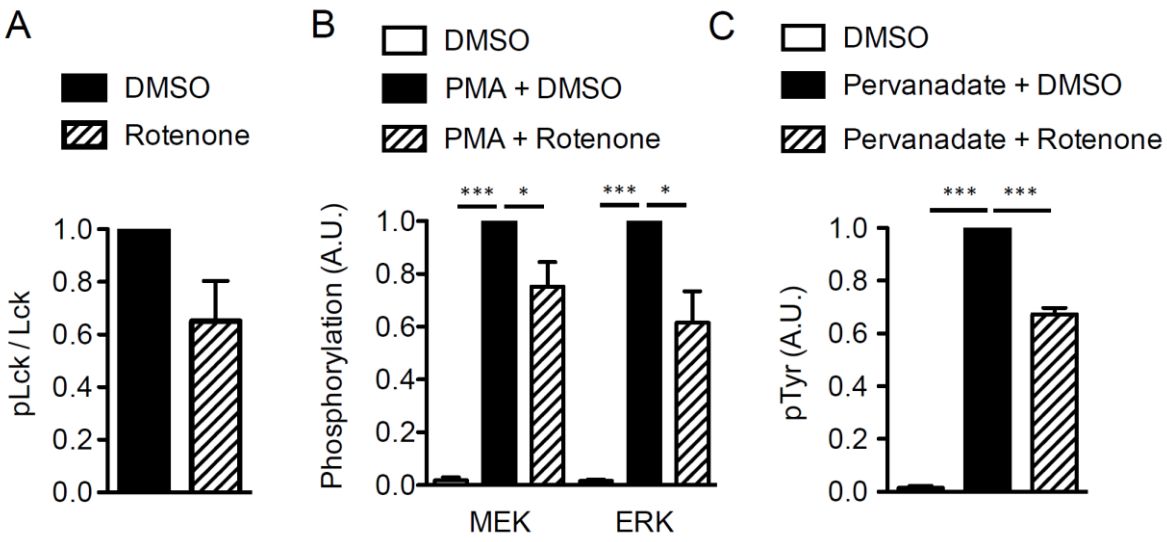

Figure 2.14 Mitochondrial Complex I is required for proper TCR signaling in primary human T cells

(A) Primary human T cells were incubated with DMSO or rotenone for 30 min (n=3). The ratio of phosphorylated LCK to total LCK was determined by quantifying the band intensities on western blots using the Odyssey system. The values indicate the relative LCK phosphorylation normalized to the DMSO control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(B) Primary human T cells were preincubated with DMSO or rotenone and stimulated with PMA for 30 min (n=2-3). The ratios of the phosphorylated proteins to β-actin were determined by quantifying the band intensities on western blots using ECL, scanner, and ImageQuant software. The values indicate the relative protein phosphorylation normalized to the stimulated DMSO controls. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(C) Primary human T cells were preincubated with DMSO or rotenone and stimulated with pervanadate for 2 min (n=4). The ratio of tyrosine-phosphorylated proteins to β-actin was determined by quantifying the band intensities on the western blots using ECL, scanner, and ImageQuant software. The values indicate the relative protein phosphorylation normalized to the stimulated DMSO control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

2.4.3 Mitochondria-derived ATP is required for T-cell activation

The inhibitor *oligomycin* specifically blocks the proton channel (F_o subunit) of the mitochondrial ATP synthase (188), thus completely preventing mitochondrial ATP synthesis (*oxidative phosphorylation*). This leads to the increase in MMP (186, 189), as protons are not consumed anymore by ATP synthase. However, as MMP is maintained and even elevated, MMP-dependent calcium uptake by mitochondria should not be blocked by oligomycin. Interestingly, oligomycin was shown to affect CRAC calcium currents through ATP depletion (190, 191) or even to directly inhibit CRAC in Jurkat T cells, independent of or in addition to its effect on ATP levels (187). Oligomycin has been recently shown to block CD25 and CD44 expression and proliferation of murine $CD4^+$ T cells (155).

Consistent with these findings, oligomycin reduces CD69 and CD25 expression upon stimulation with CD3xCD28-coated microbeads (Fig. 2.15). Interestingly, it also decreases the phosphorylation of LCK in unstimulated cells (Fig. 2.16 A) and general tyrosine phosphorylation after pervanadate treatment (Fig. 2.16 B).

Thus, these data suggest that mitochondrially produced ATP is required for the proper activation of primary human T cells. However, the effect of oligomycin on T-cell activation is weaker than that of rotenone. This could mean that, rather than mitochondrial ATP, some other factor, such as MMP, is crucial for proper T-cell activation. To address this issue, I have utilized *carbonyl cyanide m-chlorophenyl hydrazone* (CCCP).

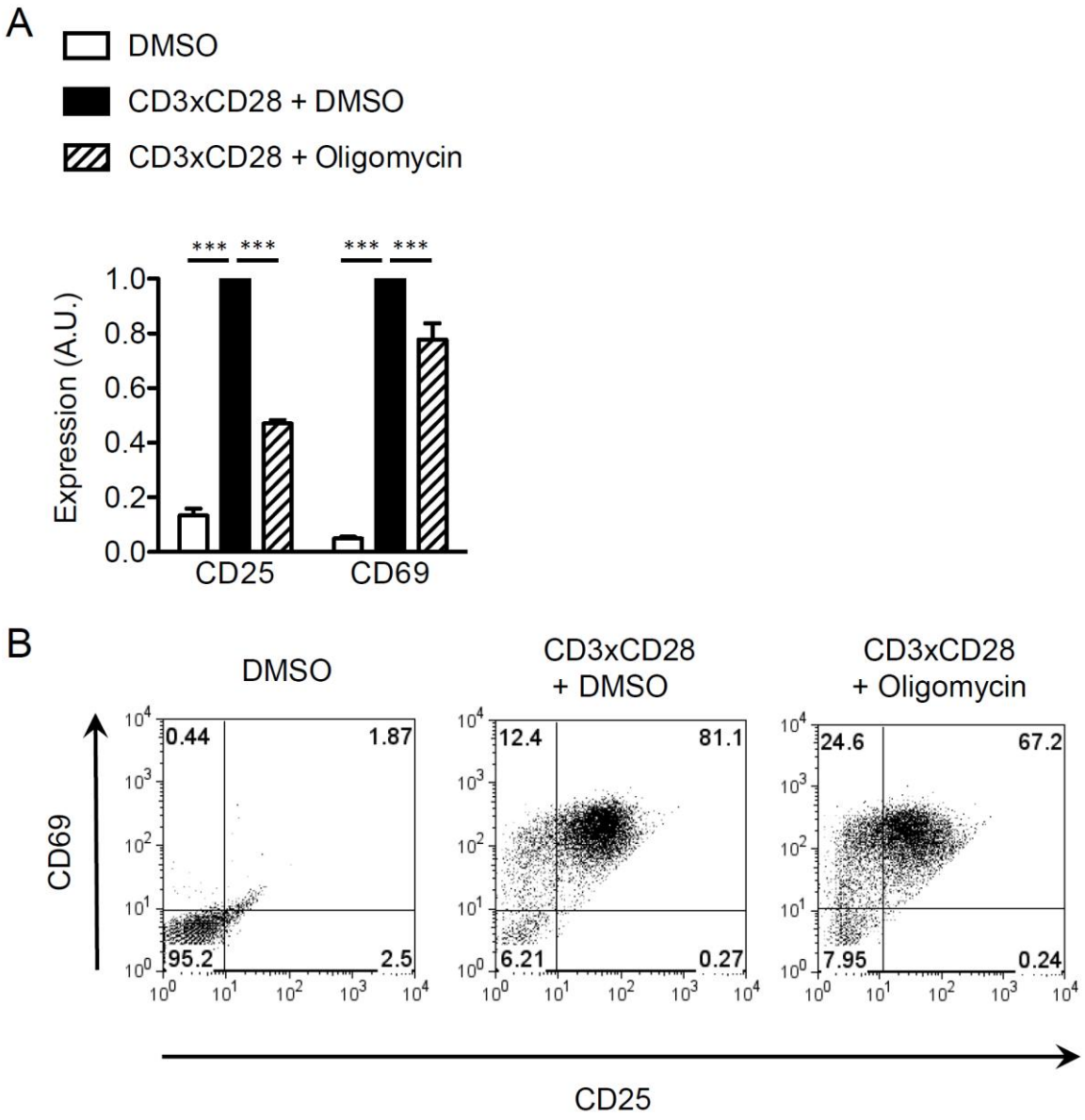

Figure 2.15 Mitochondria-derived ATP is required for the activation of human T cells

(A) Primary human T cells were preincubated with DMSO or oligomycin and stimulated with CD3xCD28-coated microbeads (n=2). After 16 hours, cells were stained with CD25-FITC and CD69-PE mAbs and analyzed by FACS. The values indicate the mean fluorescence intensities normalized to the stimulated DMSO controls. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(B) Representative dot blots of the data shown in (A).

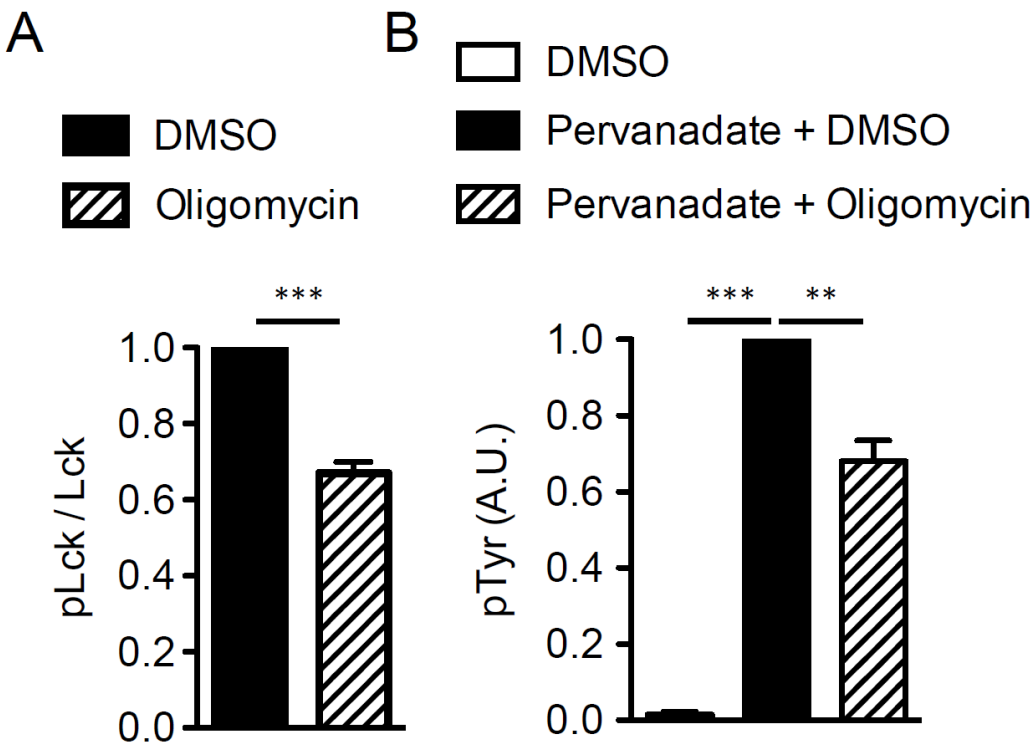

Figure 2.16 Mitochondria-derived ATP is required for proper TCR signaling in primary human T cells

(A) Primary human T cells were incubated with DMSO or oligomycin for 30 min (n=3). The ratio of phosphorylated LCK to total LCK was determined by quantifying the band intensities on western blots using the Odyssey system. The values indicate the relative LCK phosphorylation normalized to the DMSO control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(B) Primary human T cells were preincubated with DMSO or oligomycin and stimulated with pervanadate for 2 min (n=4). The ratio of tyrosine-phosphorylated proteins to β-actin was determined by quantifying the band intensities on the western blots using ECL, scanner, and ImageQuant software. The values indicate the relative protein phosphorylation normalized to the stimulated DMSO control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

2.4.4 Mitochondrial membrane potential is crucial for T-cell activation

CCCP is a mitochondrial uncoupler, which acts as an ionophore, thus allowing protons to freely pass through the inner mitochondrial membrane. It disrupts MMP, thus preventing ATP synthesis by mitochondrial ATP synthase, as well as calcium uptake (113, 159, 160, 189, 192). Interestingly, despite the strong interference with mitochondria, CCCP by itself does not induce apoptosis (193-195). CCCP has been recently shown to affect IL-2 expression in human $CD4^+$ T cells (113).

In agreement with these observations, I have found that CCCP strongly affects the activation of primary human T cells. It decreases the phosphorylation of Zap70, PLCγ, LAT, and ERK (Fig. 2.17 A) and completely abolishes CD69 and CD25 expression (Fig. 2.17 B, C), upon stimulation with CD3xCD28-coated microbeads. Notably, it also decreases the phosphorylation of LCK in unstimulated cells (Fig. 2.18 A), the phosphorylation of MEK and ERK after PMA stimulation (Fig. 2.18 B), and general tyrosine phosphorylation after pervanadate treatment (Fig. 2.18 C).

Upon comparing the data obtained using rotenone, oligomycin, and CCCP, it becomes clear that CCCP exerts much stronger effects on T-cell activation, which are especially severe in case of the expression of the activation markers. From these data it can be concluded that, in contrast to mitochondria-derived ATP, MMP is crucial for primary T-cell activation.

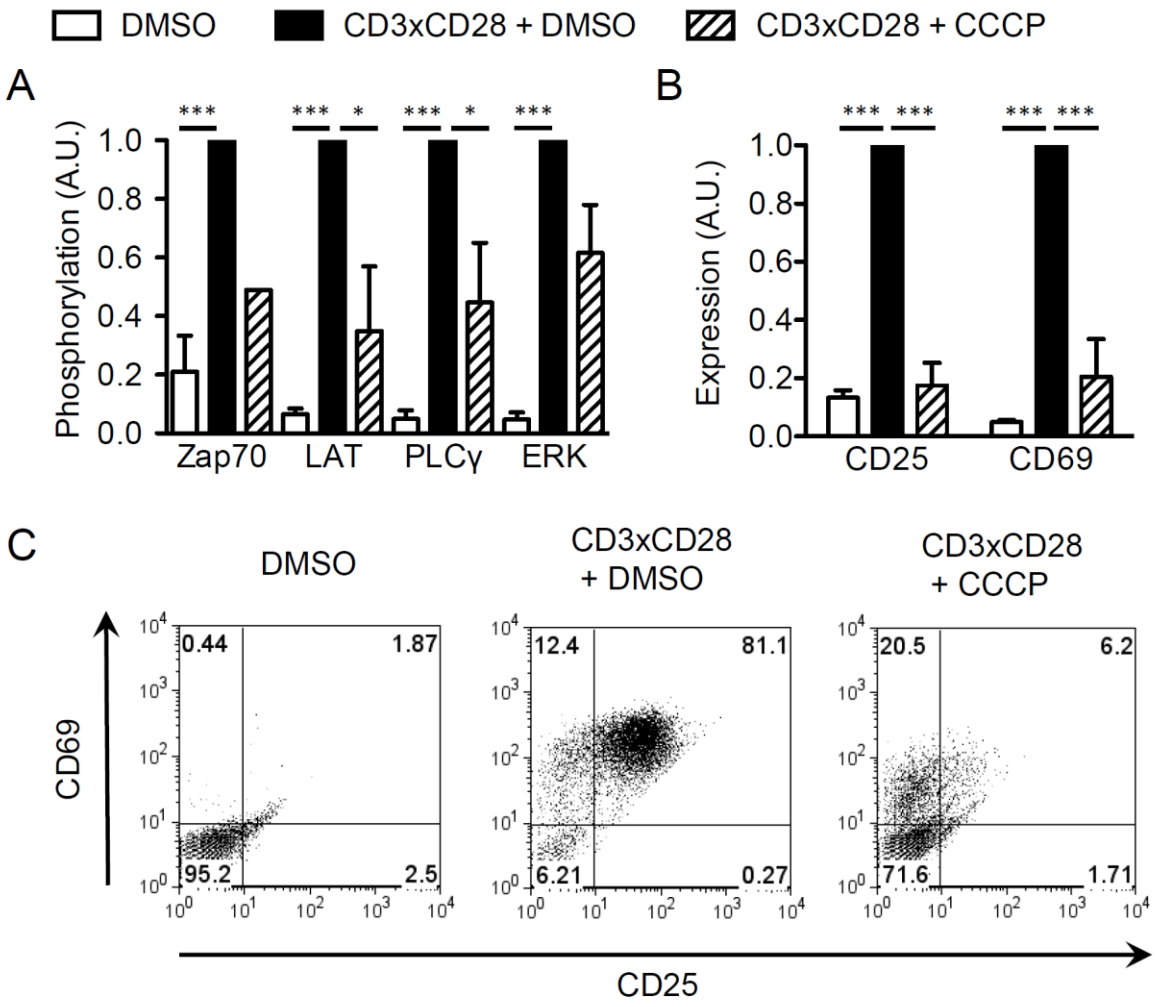

Figure 2.17 Mitochondrial membrane potential is crucial for the activation of human T cells

(A) Primary human T cells were preincubated with DMSO or CCCP and stimulated with CD3xCD28-coated microbeads for 30 min (n=1-4). The ratio of the phosphorylated proteins to β-actin was determined by quantifying the band intensities on western blots using ECL, scanner, and ImageQuant software. The values indicate the relative protein phosphorylation normalized to the stimulated DMSO controls. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(B) Primary human T cells were preincubated with DMSO or CCCP and stimulated with CD3xCD28-coated microbeads (n=2). After 16 hours, cells were stained with CD25-FITC and CD69-PE mAbs and analyzed by FACS. The values indicate the mean fluorescence intensities normalized to the stimulated DMSO controls. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(C) Representative dot blots of the data shown in (B).

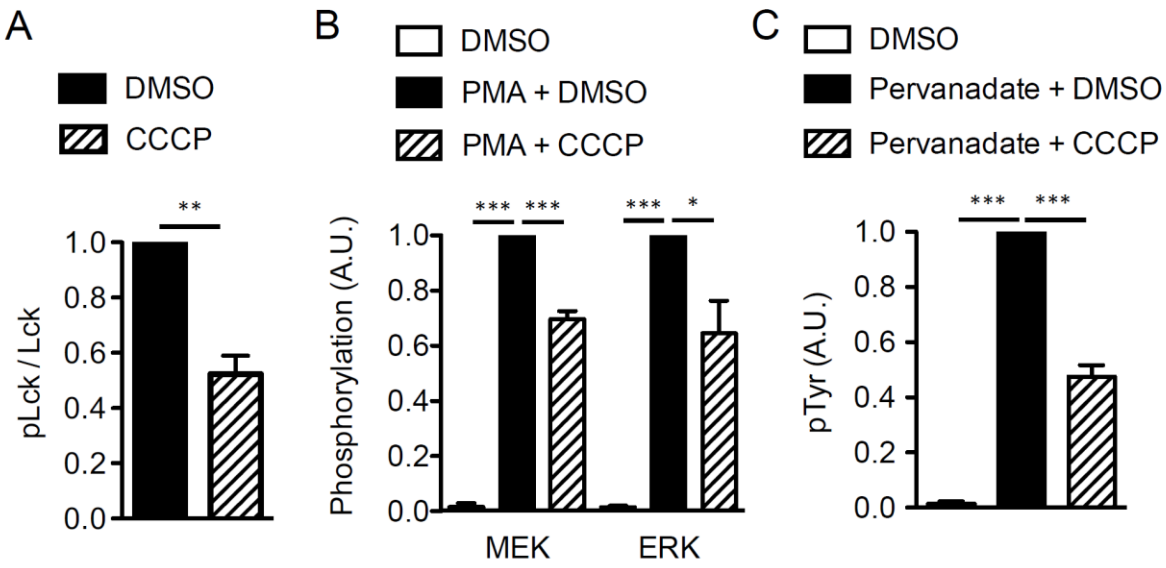

Figure 2.18 Mitochondrial membrane potential is required for proper TCR signaling in primary human T cells

(A) Primary human T cells were incubated with DMSO or CCCP for 30 min (n=3). The ratio of phosphorylated LCK to total LCK was determined by quantifying the band intensities on the western blots using the Odyssey system. The values indicate the relative LCK phosphorylation normalized to the DMSO control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(B) Primary human T cells were preincubated with DMSO or CCCP and stimulated with PMA for 30 min (n=2-3). The ratios of the phosphorylated proteins to β-actin were determined by quantifying the band intensities on the western blots using ECL, scanner, and ImageQuant software. The values indicate the relative protein phosphorylation normalized to the stimulated DMSO controls. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(C) Primary human T cells were preincubated with DMSO or CCCP and stimulated with pervanadate for 2 min (n=4). The ratio of tyrosine-phosphorylated proteins to β-actin was determined by quantifying the band intensities on western blots using ECL, scanner, and ImageQuant software. The values indicate the relative protein phosphorylation normalized to the stimulated DMSO control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

2.4.5 Mitochondrial ROS production does not correlate with T-cell activation

Some studies have shown that mitochondrial ROS are required for the proper activation of T cells (134, 143). Therefore, I have hypothesized that the effects of the employed mitochondrial inhibitors on T-cell activation might be due to their influence on mitochondrial ROS, rather than ATP or MMP levels. To test this hypothesis, I have measured ROS levels in primary human T cells in the presence of DPI, rotenone, oligomycin, or CCCP (Fig. 2.19 A). DPI, rotenone, and oligomycin strongly induce ROS production, whereas CCCP slightly decreases it. On the contrary, all these substances inhibit T-cell activation (Fig. 2.19 B, Fig. 2.11 – 2.18). Thus, there is no correlation between T-cell activation and the level of mitochondrial ROS.

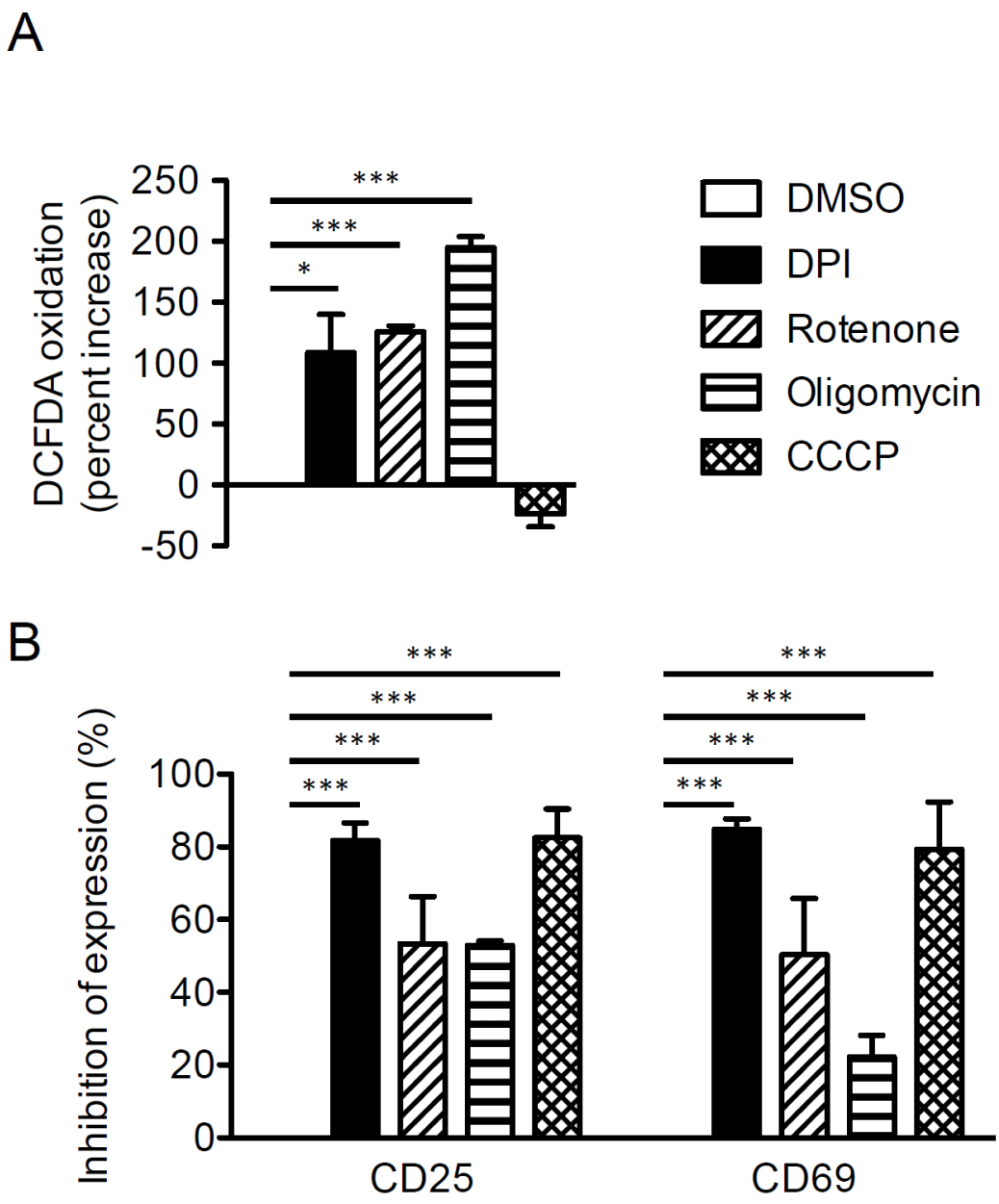

Figure 2.19 Mitochondrial ROS production does not correlate with the activation of human T cells

(A) Primary human T cells were loaded with DCFDA and subsequently treated with DMSO or mitochondrial inhibitors for 30 min (n=3). DCFDA fluorescence was measured by FACS. The values indicate the increase in the mean fluorescence intensity relative to the DMSO control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

(B) Primary human T cells were preincubated with DMSO or mitochondrial inhibitors and stimulated with CD3xCD28-coated microbeads (n=2-4). After 16 hours, cells were stained with CD25-FITC and CD69-PE mAbs and analyzed by FACS. The values indicate the inhibition of the mean fluorescence intensity relative to the stimulated DMSO control. P values were determined by two-tailed Student's t test (*P < 0.05, **P < 0.01, ***P < 0.001).

3 Discussion

In the first part of this work, I have shown that primary human and mouse T cells produce extracellular superoxide via NOX-2 upon the triggering of the TCR. However, it appears that this reactive species is not required for T-cell activation, proliferation, and cytokine production. To my knowledge, this is the first study investigating TCR-induced superoxide production and its functional requirement in primary human T cells.

During the last decades, it has been shown that TCR triggering leads to the generation of ROS. However, most of these studies were performed in preactivated T cells, such as T-cell blasts or Jurkat T cells. These cells undergo activation-induced cell death (AICD) upon restimulation (29), and the produced ROS are mostly involved in FasL upregulation. Interestingly, I have not observed any significant superoxide production in human T-cell blasts or Jurkat T cells. It has been previously shown that some Jurkat T-cell clones do not produce ROS upon stimulation (196), potentially due to the heterogeneity of Jurkat T-cell clones in different labs. I have shown that superoxide detected using the Diogenes assay was generated by NOX-2 in primary T cells (Fig. 2.7). On the other hand, it has been shown that AICD of T-cell blasts is not dependent on NOX-2 (119). Thus, it might be that the superoxide detection system that I have employed is not sensitive to ROS from other sources, such as mitochondria (108, 110, 118) and DUOX-1 (112), which play key roles in AICD.

Based on ROS measurements using DCFDA, it has been previously suggested that also *primary* human T cells can produce ROS upon CD3 and CD28 stimulations (140, 141). However, it is highly plausible that the detected ROS originated from the contaminating phagocytic cells, which are often present in preparations of primary T cells. Phagocytes can be activated by any antibody via the Fc receptors (138) or by direct contacts with activated T cells (83). The selective gating on T lymphocytes during the flow-cytometric studies does not help to discriminate the T-cell specific ROS from the ROS of phagocytic origin, as the latter is released into the extracellular environment, can reach T cells through the medium, and can oxidize a ROS-sensitive dye within T cells. Moreover, even the dye oxidized inside the phagocytes can eventually leak into the medium and can be subsequently taken up by the T cells, thus contributing to the increase in their fluorescence. To circumvent these

problems, a very high (>99%) purity of the T-cell preparation should be achieved, and isotype control antibodies should be used. Unfortunately, these conditions have never been fully met. Soluble isotype antibodies were used in one study employing primary T cells (140). However, in this study, in-house purified antibodies from an ascitic fluid were used for stimulation. It could have happened that non-TCR specific antibodies or some other substances present in the ascitic fluid triggered ROS production, either in T cells or in phagocytes. Similarly, in another study, a CD3-containing ascitic fluid was used directly and without controls (141). Additionally, one study has shown that CD3 stimulation does not induce DCFDA oxidation in human T cells (197). Thus, it is currently not clear whether primary human T cells produce ROS upon TCR stimulation, or whether the stimulatory agents trigger non-specific ROS production in phagocytes. In my study, I have detected the strong production of superoxide (measured using luminol) and ROS in general (measured using DCFDA) upon the addition of microbeads coated with isotype control antibodies. These ROS most likely originate from phagocytic cells contaminating the preparation. Nevertheless, there was a statistically significant increase in luminol (but not DCFDA) oxidation in T cells stimulated with CD3xCD28-coated beads as compared to isotype-coated beads, indicating an additional T-cell-specific superoxide production. A possibility that the activated T cells triggered a respiratory burst in the phagocytes by the direct cell-cell contact (83) is unlikely due to the rapid initiation of superoxide production. Thus, I favor the hypothesis that primary human T cells indeed produce ROS, particularly extracellular superoxide, upon TCR triggering.

One of the most widespread sources of inducible superoxide are NADPH oxidases. Importantly, gp91^{phox}, the key component of NOX-2, is expressed in human and murine T-cell blasts (116, 119). Interestingly, the same studies have shown that *gp91$^{phox-/-}$* T-cell blasts are still able to produce superoxide in response to CD3 or CD3xCD28 stimulation, similarly to WT T cells. On the contrary, I have observed the complete absence of TCR-triggered superoxide production in primary T cells from gp91$^{phox-/-}$ mice. This discrepancy is mostly explained by the differences between cell types and by superoxide detection methods used. Indeed, the aforementioned studies have investigated T-cell blasts that underwent AICD, and superoxide from the apoptotic mitochondria (110) was detected by the intracellular DHE (116) or MitoSOX (119) dyes. On the other hand, I have investigated primary T cells that were physiologically stimulated and have subsequently activated NOX-2 at the plasma

membrane (in case of WT mice) or failed to do so (in case of *gp91$^{phox-/-}$* mice), and superoxide from NOX-2 was detected by the extracellular luminol dye. I have shown that, despite the defective superoxide production, *gp91$^{phox-/-}$* T cells have the normal upregulation of CD25 and CD69 and normal proliferation. This finding supports the previous observation that murine CD4$^+$ T cells from gp91$^{phox-/-}$ mice have no defect in CD25 expression or IL-2 production (144). However, T cells from that study produced less IL-4 and IL-5 but more IL-17 than the WT counterparts (144). This indicates that while ROS are not required for T-cell activation, they might be required for T-cell differentiation. Interestingly, I have not observed any significant change in cytokine production by human CD4$^+$ T cells upon the addition of antioxidants. This discrepancy might be explained by differences between human and murine T cells.

The finding that natural antioxidants do not affect the activation and proliferation of primary human T cells appeared initially to be in contrast with some previously published data, which show that antioxidants can interfere with CD25 expression (150, 151), IL-2 (143, 149) and IL-4 (133) production, and DNA synthesis (150), in human and murine T cells. Interestingly, all antioxidants used in these studies – NDGA (149, 151), BHA (150, 151), Vitamin E (133), and Mitovitamin E (143) – also specifically inhibit lipid peroxidation and/or lipoxygenase activity. SOD, catalase, and ascorbate are, on the other side, water-soluble antioxidants that do not interfere with lipid peroxidation or lipoxygenase activity. Thus, my results indicate that the reduced synthesis of biochemical products of the lipoxygenase pathway – proinflammatory molecules leukotrienes, rather than the diminished ROS levels *per se*, are most likely the reason for the previously observed impairment of T-cell activation in the presence of the lipid-soluble antioxidants.

Despite the fact that superoxide is produced upon TCR stimulation, it appears to be dispensable for T-cell activation, proliferation, and cytokine production. This raises the question: why do T cells need to produce superoxide? An intriguing possibility is that extracellularly released superoxide serves as a feedback messenger to signal the successful activation to antigen-presenting cells. Indeed, a tightly regulated crosstalk between dendritic cells and T cells has been demonstrated (92, 198). Further studies investigating T cell-APC interactions *in vitro* or *in vivo* are required to assess this hypothesis.

In addition to NOX-2, other sources of ROS exist in T cells. Mitochondria represent one of those. Although in my experimental setup no superoxide was detected in the absence of NOX-2, it is still possible that superoxide is produced in the mitochondria upon TCR stimulation. In fact, the Diogenes assay measures only extracellular superoxide, and DCFDA is not sensitive to superoxide (173). Moreover, mitochondria could produce superoxide at late stages of T-cell activation, whereas I have focused on early events following TCR triggering.

In the second part of this study, I have measured ROS levels in primary human T cells in the presence of several mitochondrial inhibitors, including DPI, rotenone, oligomycin, and CCCP. Interestingly, there is no consistency in the literature about effects of DPI on mitochondrial ROS production. DPI has been shown to inhibit ROS production at the Complex I of isolated mitochondria during only forward (199), only reverse (200), or both forward and reverse electron transport (181), or even to increase ROS levels during reverse transport (199). In addition, it has been shown that DPI inhibits (201) or induces ROS production in the whole cell (182, 183, 202). It seems that the effect of DPI on mitochondrial ROS production depends on the cell type and the physiological state of the cells. In my experimental setup, I have observed an increase in ROS production in the presence of DPI. Rotenone has been shown to induce ROS generation at the level of Complex I during forward electron transfer (199, 202-204) but to reduce it during reverse transfer (181). As forward electron transport is what is normally observed in cells, my observation that ROS production is increased in the presence of rotenone is in agreement with the published data. By increasing MMP (186, 189), oligomycin stimulates ROS production through the leakage of electrons from the over-reduced respiratory chain to oxygen (189, 202, 205). On the contrary, by decreasing MMP, CCCP reduces the leakage of electrons from respiratory chain to oxygen and thus reduces mitochondrial ROS production (189, 205-207). My data are also in complete agreement with these observations.

Interestingly, I have observed no correlation between T-cell activation and the level of ROS in the mitochondria, as both the substances inducing ROS production (DPI, oligomycin, rotenone) and the substances decreasing ROS levels (CCCP) had an inhibitory effect on T cells. Thus, the question arises whether mitochondrial ROS play any role in T-cell activation. It has been shown that mitochondrial ROS are

produced during AICD. CD3- and PMAxIono-induced DCFDA oxidation, FasL expression, and cell death were all dependent on mitochondrial complex I in Jurkat T cells and human T-cell blasts (110). CD3xCD28 stimulation induced MitoSOX oxidation and apoptosis in murine T-cell blasts (119). This is consistent with the well-known role of ROS as executors of apoptosis, but it does not implicate ROS in T-cell activation. A recent study suggests that mitochondrial ROS are involved in T-cell activation (143). The authors have shown that CD3 and CD3xCD28 stimulations of murine $CD4^+$ T cells induce the oxidation of mito-roGFP that is dependent on the mitochondrial calcium uptake. They also show that CD3xCD28 and PMAxIono stimulations induce the oxidation of MitoSOX that is dependent on the mitochondrial Complex III subunit RISP. Finally, they have attempted to show that mitochondrial ROS are required for T-cell activation, by studying RISP-deficient T cells. However, such a severe defect in the crucial mitochondrial ETC complex necessarily affects multiple processes, including the mitochondrial electron transport, respiration, and ATP synthesis, besides ROS production. Thus, RISP-deficient cells cannot be used to assess the role of mitochondrial ROS in T-cell activation.

By using mitochondrial inhibitors, I have also shown that mitochondrial membrane potential (MMP), but not mitochondria-derived ROS or ATP, is crucial for the activation of primary human T cells. Several articles support my hypothesis. Murine $CD8^+$ T cells recently activated *in vivo* by LCMV have the elevated MMP, which then decreases during the contraction phase of the immune response (167). The mitochondrial biogenesis and MMP increase after the stimulation of murine T cells (168). Interestingly, in human $CD4^+$ T cells the MMP drops immediately after stimulation, but is required for IL-2 expression (113). MMP is required for calcium buffering by mitochondria (165, 166). Mitochondrial calcium uptake, in turn, is necessary for sustained CRAC-mediated calcium influx and NFAT activation in T cells (113, 159, 160).

In summary, in this work I have shown for the first time that primary T cells produce extracellular superoxide via NOX-2 upon the TCR stimulation, and that mitochondrial membrane potential, but not NOX-2 or mitochondria-derived ROS or ATP, is crucial for T-cell activation.

4 Materials and methods

4.1 Cell isolation and culture

4.1.1 Ethics

Approval for these studies, involving the analysis of TCR-mediated signaling in human T cells, was obtained from the Ethics Committee of the Medical Faculty at the Otto-von-Guericke University, Magdeburg, Germany, with the permission number [107/09]. Informed consent was obtained in writing in accordance with the Declaration of Helsinki. All experiments involving mice were performed according to the guidelines of the State of Sachsen-Anhalt, Germany.

4.1.2 Human T-cell purification and culture

PBMCs were isolated by the Ficoll gradient (Biochrom AG) centrifugation of heparinized blood collected from healthy volunteers. T cells were further purified by non-T-cell depletion using human pan T-cell isolation kit and AutoMACS (all from Miltenyi Biotec). The purity of T cells, determined by FACS, was routinely more than 96%. T cells were cultured at 10^6 cells/ml, 37°C, and 5% CO_2 in RPMI 1640 medium supplemented with stable glutamine (Biochrom AG), 10% fetal calf serum (PAN Biotech), and 2 µg/ml ciprobay (Bayer).

4.1.3 Murine T-cell purification and culture

gp91$^{phox-/-}$ mice were a kind gift from Dr. Katrin Breitbach (Friedrich Loeffler Institute of Medical Microbiology, Ernst-Moritz-Arndt University, Greifswald, Germany). WT control C57BL/6J^{Bom} mice were obtained from Taconics. Mice were kept in pathogen-free conditions. In order to obtain a single-cell suspension, spleens from mice were passed through a fine mesh filter (BD Falcon). T cells were purified by non-T-cell depletion using the mouse pan T-cell isolation kit and AutoMACS (all from Miltenyi Biotec). The purity of T cells, determined by FACS, was routinely more

than 96%. The cells were cultured at 10^6 cells/ml, 37°C, and 5% CO_2 in RPMI 1640 medium supplemented with stable glutamine (Biochrom AG), 10% fetal calf serum PAN Biotech), 2 µg/ml ciprobay (Bayer), and 50 µM β-mercaptoethanol (Sigma Aldrich).

4.1.4 Jurkat T-cell culture

Jurkat T cells were maintained at 1-3x10^5 cells/ml, 37°C, and 5% CO_2 in RPMI 1640 medium supplemented with stable glutamine (Biochrom AG), 10% fetal calf serum (PAN Biotech), 100 U/ml penicillin (Biochrom AG), and 100 µg/ml streptomycin (Biochrom AG).

4.1.5 Human T-cell blast generation and culture

Purified human T cells were stimulated with 1 nM PMA and 200 ng/ml ionomycin (both from Sigma-Aldrich) and cultured at 37°C and 5% CO_2 in RPMI 1640 medium supplemented with stable glutamine (Biochrom AG), 10% fetal calf serum (PAN Biotech), and 2 µg/ml ciprobay (Bayer). After 2 days medium was replaced, and 100 U/ml interleukin 2 (PeproTech) was added. Cells were cultured for additional 24 hours before performing experiments.

4.2 Antioxidants and inhibitors

The following antioxidants or inhibitors were purchased from Sigma-Aldrich and used at the indicated concentrations: superoxide dismutase (SOD) from bovine erythrocytes at 100 U/ml, catalase from *Corynebacterium glutamicum* at 1000 U/ml, L-ascorbic acid (ascorbate) at 100 µM, L-glutathione reduced at 500 µM, diphenyleneiodonium chloride (DPI) at 10 µM, rotenone at 5 µM, oligomycin from *Streptomyces diastatochromogenes* at 1 µM, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at 10 µM. Cells were preincubated with the compounds at 37°C for 30 min before stimulation and kept in the presence of the compounds for the whole duration of the experiment. SOD, catalase, ascorbate, and

glutathione were freshly diluted in PBS. DPI, rotenone, oligomycin, and CCCP were diluted in DMSO and stored at -20°C. Appropriate solvent was used in the negative control samples.

4.3 Cell stimulation

4.3.1 Microbead stimulation

Cells were stimulated with immobilized CD3xCD28 mAbs as previously indicated (170). Briefly, SuperAvidin™-coated polystyrene microspheres (Bangs laboratories, Inc., Ø~10 µm, binding capacity: 0.02-0.04 µg biotin/mg) were coated with biotinylated CD3 (clone UCHT1, mouse, IgG1κ) and CD28 (clone CD28.2, mouse, IgG1κ) mAbs (10 µg/ml each, BioLegend) for 30 min at 37°C in PBS Dulbecco (Biochrom AG). Antibody-coated microspheres were washed three times, resuspended in PBS at 10^8 beads/ml, and incubated with cells in a 1 bead per cell ratio. Microspheres coated with 20 µg/ml biotinylated mouse IgG1κ (eBioscience) were used as the isotype control.

Mouse cells were stimulated with CD3 (clone 145-2C11, hamster, IgG1κ) and CD28 (clone 37.51, hamster, IgG2λ1) mAbs (10 µg/ml each, both from BD Pharmingen) immobilized on microspheres as described above. Microspheres coated with biotinylated hamster IgG1κ and IgG2λ1 (10 µg/ml each, both from BD Pharmingen) were used as the isotype control.

4.3.2 PMA stimulation

Cells were incubated with 1 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich).

4.3.3 Pervanadate stimulation

Cells were incubated with the 1:100 dilution of freshly prepared pervanadate mixture (10 mM sodium orthovanadate and 3% hydrogen peroxide, both from Sigma-Aldrich).

4.4 Detection of ROS

4.4.1 General oxidation assay

For general ROS detection, 2',7'-dichlorofluorescin diacetate (DCFDA, Invitrogen) was used. Cells were centrifuged, resuspended at the density of 10^7 cells/ml in a prewarmed serum-free phenol red-free RPMI 1640 medium (Gibco) containing 2 µM of DCFDA, aliquoted to microcentrifuge tubes (100 µl suspension per tube), placed on a thermoshaker (37°C, 300 rpm), and incubated for 30 min. Then cells were stimulated with microbeads for 30 min, diluted 1:5 in ice-cold PBS Dulbecco (Biochrom AG), and immediately analyzed by flow cytometry using FACSCalibur with CellQuest software or LSRFortessa with FACSDiva Software 6.1.3 (all from BD Biosciences) for data acquisition and FlowJo 7.5.5 (Tree Star, Inc.) for data analysis. During the measurement procedure, cells were protected from bright light.

4.4.2 Superoxide assay

For superoxide detection, the Diogenes Cellular Luminescence Enhancement System (National Diagnostics) was used according to the manufacturer's instructions. Briefly, cells were centrifuged, resuspended at the density of 10^7 cells/ml in a pre-warmed serum-free phenol red-free medium RPMI 1640 (Gibco), and placed into black polystyrene flat bottom 96-well plate (Costar, Corning Inc., 100 µl suspension per well). Diogenes Reagent and Diogenes Activator were freshly mixed in a 1:9 ratio and added to the samples (50 µl per well). Subsequently, T cells were stimulated with microbeads, and luminescence was repeatedly measured with 5 min intervals for the total of 60 min using TriStar LB941 multimode reader (Berthold technologies). Cells were kept at 37°C.

4.5 Western blotting

4.5.1 General procedure

T cells were centrifuged, resuspended in a serum-free RPMI 1640 medium (Biochrom AG) at the density of 10^7 cells/ml, aliquoted to microcentrifuge tubes (100 µl suspension per tube), placed on a thermoshaker (37°C, 300 rpm), and stimulated as described above. At indicated time points, reactions were terminated by adding 1 ml of ice-cold PBS Dulbecco (Biochrom AG) per sample. Cells were immediately centrifuged, and pellets were lysed in 30 µl of ice-cold buffer containing 1% Igepal CA-630 (Sigma-Aldrich), 1% lauryl maltoside (Calbiochem), 50 mM tris(hydroxymethyl)-aminomethane (pH 7.5), 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (all from Carl Roth GmbH), 10 mM NaF, 1 mM Na_3VO_4, and 1 mM phenylmethanesulfonyl fluoride (all from Sigma-Aldrich, see Appendix for detailed buffer recipes) for 20 min. Subsequently, cell nuclei were pelleted by 10 min centrifugation at 13000 x g at 4°C. Supernatants were transferred to new microcentrifuge tubes containing 7.5 µl of 5x reducing sample buffer, mixed, and heated on a thermoshaker (100°C, 600 rpm) for 5 min. Lysates were separated by SDS-PAGE system (Bio-Rad) and transferred using a semi-dry Western blotting system (Bio-Rad) onto nitrocellulose membranes (Amersham Biosciences).

4.5.2 Chemiluminescent detection

Membranes were immunoblotted using the indicated primary antibodies and the appropriate horseradish peroxidase-conjugated secondary antibodies (Dianova) and developed using the enhanced chemiluminescence detection system and X-ray film (both from Amersham GE Healthcare). The following primary antibodies were used for Western blotting in this study: anti-pY493 ZAP-70, anti-pY171 LAT, anti-pY783 PLCγ-1, anti-pS217/221 MEK-1/2, anti-pT202/Y204 ERK-1/2 (all from Cell Signaling Technology), anti-phosphotyrosine horseradish peroxidase conjugate (clone 4G10, Millipore), and anti-β-actin (clone AC-15, Sigma-Aldrich). For data analysis, film images were acquired using the Epson V700 scanner, and intensities of the detected

bands were quantified using the Kodak 1D ImageQuant software. Band intensities from phosphorylated proteins were normalized to band intensities from β-actin, before the further processing of the data.

4.5.3 Fluorescent detection

Membranes were incubated with primary rabbit anti-pY416 Src antibody (Cell Signaling Technology) and secondary anti-rabbit antibody labeled with IRDye 680LT (LI-COR Biosciences) and scanned with Odyssey Classic infrared imager (LI-COR Biosciences) in 700 nm channel. Subsequently, membranes were incubated with primary mouse anti-LCK antibody (clone 28/LCK, BD Biosciences) and secondary anti-mouse antibody labeled with IRDye 800CW (LI-COR Biosciences) and scanned in 800 nm channel. Images were acquired and quantified using the LI-COR Image Studio 2.1.15 software. Signal from phospho-LCK (lower /p56/ band of pSrc staining) was normalized to signal from total LCK, before the further processing of the data.

4.6 Cellular assays

4.6.1 Activation assay

T cells were seeded to flat bottom polystyrene 48-well plates (Costar, Corning Inc.) at 10^6 cells/ml in the volume of 500 µl per well and stimulated with microbeads. After 16 h, cells were stained with FITC- or PE-labeled mAbs against CD25 and CD69 (BD Pharmingen, BioLegend) and analyzed by flow cytometry using FACSCalibur with CellQuest software or LSRFortessa with FACSDiva Software 6.1.3 (all from BD Biosciences) for data acquisition and FlowJo 7.5.5 (Tree Star, Inc.) for data analysis. Microbeads and cell debris were always excluded from the analysis.

4.6.2 Proliferation assay

T cells were centrifuged, resuspended in 1 ml of PBS Dulbecco (Biochrom AG), and labeled with 2.5 µM CFSE (Invitrogen) for 15 min at 37°C. Then cells were

washed twice, resuspended in medium at the density of 10^6 cells/ml, seeded to flat bottom polystyrene 48-well plates (Costar, Corning Inc.) in the volume of 500 µl per well, and stimulated with microbeads. Cells were cultured for 72 h, and proliferation was assessed by CFSE dilution using FACSCalibur with CellQuest software or LSRFortessa with FACSDiva Software 6.1.3 (all from BD Biosciences) for data acquisition and FlowJo 7.5.5 (Tree Star, Inc.) for data analysis. Microbeads and cell debris were always excluded from the analysis.

4.6.3 Cytokine assay

Human $CD4^+$ T_{nai} cells were purified from PBMCs by non-T-cell depletion using human $CD4^+$ T_{nai}-cell isolation kit and AutoMACS (both from Miltenyi Biotec). T cells were resuspended in serum-free X-VIVO 15 medium supplemented with gentamycin (Lonza), seeded to flat bottom polystyrene 48-well plates (Costar, Corning Inc.) at 10^6 cells/ml in the volume of 1 ml per well, and stimulated with microbeads. After 48 h of incubation, supernatants were harvested, supplemented with 0.5% bovine serum albumin (Sigma-Aldrich), and frozen at -80°C. Later, cytokine concentrations in the supernatants were measured simultaneously using the Bio-Plex Pro assay on the Bio-Plex 200 system (Bio-Rad) according to the manufacturer's protocol. The Bio-Plex Manager software was used for acquisition and analysis.

4.7 Data visualisation and statistical analysis

The graphical visualization and statistical analysis of the data were performed using GraphPad Prism 5 (GraphPad Software, Inc.). Bars and dots on the graphs indicate the mean of values measured in several (n) independent experiments, while error bars indicate the standard error of the mean (SEM). Significance was calculated by two-tailed Student's t test (* $0.01<P\leq0.05$, ** $0.001<P\leq0.01$, *** $0<P\leq0.001$). No asterisk indicates that the difference was not statistically significant.

Western blotting recipes

Lysis buffer (1 ml)

657 µl ddH_2O

100 µl 10% Igepal CA-630 (Sigma-Aldrich)

100 µl of 10% lauryl maltoside (Calbiochem)

50 µl 1M tris(hydroxymethyl)-aminomethane, pH 7.5 (Carl Roth GmbH)

33 µl 5M NaCl (Carl Roth GmbH)

20 µl 0.5M ethylenediaminetetraacetic acid, pH 7.5 (Carl Roth GmbH)

20 µl 0.5M NaF (Sigma-Aldrich)

10 µl 0.1M Na_3VO_4 (Sigma-Aldrich)

10 µl 0.1M phenylmethanesulfonyl fluoride (Sigma-Aldrich)

5x reducing sample buffer (10 ml)

5 ml glycerol (Sigma-Aldrich)

2.5 ml 20 % sodium dodecyl sulfate (Calbiochem)

2 ml 0.5 M tris(hydroxymethyl)-aminomethane, pH 6.8 (Carl Roth GmbH)

250 µl 10% bromphenol blue (Carl Roth GmbH)

500 µl β-mercaptoethanol (Sigma-Aldrich)

SDS-PAGE resolving gel (10%, 10 ml)

4 ml ddH_2O

3.4 ml acrylamid 30% (Bio-Rad)

2.6 ml 1.5 M tris(hydroxymethyl)-aminomethane pH 8.8 (Carl Roth GmbH)

100 µl 10% sodium dodecyl sulfate (Calbiochem)

100 µl 10% ammonium persulfate (Carl Roth GmbH)

10 µl tetramethylethylenediamine (Carl Roth GmbH)

SDS-PAGE stacking gel (5%, 3.3 ml)

1.9 ml ddH_2O

0.6 ml 30% acrylamid (Bio-Rad)

0.8 ml 0,5M tris(hydroxymethyl)-aminomethane, pH 6.8 (Carl Roth GmbH)

33 µl 10% sodium dodecyl sulfate (Calbiochem)

33 µl 10% ammonium persulfate (Carl Roth GmbH)

3.3 µl tetramethylethylenediamine (Carl Roth GmbH)

Transfer buffer (1 l)

800 ml dH_2O

5.8 g tris(hydroxymethyl)-aminomethane, pH 6.8 (Carl Roth GmbH)

2.93 g glycine (Carl Roth GmbH)

3.75 ml 10% sodium dodecyl sulfate (Calbiochem)

200 ml methanol (Carl Roth GmbH)

10x TBS buffer (1 l)

1000 ml dH_2O

80 g NaCl (Carl Roth GmbH)

30 g tris(hydroxymethyl)-aminomethane pH 8 (Carl Roth GmbH)

2 g KCl (Sigma-Aldrich)

Washing buffer (1 l)

900 ml dH_2O

100 ml of 10x TBS

1 ml of Tween 20

Blocking/Antibody buffer (100 ml)

100 ml of Washing buffer

5 g of milk powder/bovine serum albumin

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Curriculum vitae of Aleksey V. Belikov

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Work experience

2014-2014 — Researcher at the Life Sciences Centre,
Moscow Institute of Physics and Technology, Moscow, Russia

Project: *Validation of in-silico model for geroprotective drug prediction on the culture of primary human fibroblasts*

2009-2013 — Research assistant/PhD student at the
Institute of Molecular and Clinical Immunology,
Otto-von-Guericke University, Magdeburg, Germany

Project 1: *The role of reactive oxygen species and mitochondria in T-cell activation*

Project 2: *The importance of post-translational oxidative modifications of cysteine residues for the function of Lck and Zap70*

Higher education

2009 — Diploma in Physiology
Lomonosov Moscow State University, Moscow, Russia

Diploma thesis: *Comparison of different cell viability determination methods for cytogerontological experiments*

2004-2009 — Biological faculty of Lomonosov Moscow State University, Moscow, Russia

Major subjects: Immunology and Microbiology

Minor subjects: Mathematics, Physics, Chemistry, Botany, Zoology, Ecology, Cytology, Histology, Physiology, Embryology, Biophysics, Biochemistry, Molecular Biology, Genetics

Specialized courses: Biotechnology and Bioenergetics

Student research project: *The influence of antibiotics, H_2O_2 and menadione on the growth and viability of E. coli cultures*

Additional education

2010-2011	German language course, Otto-von-Guericke University, Magdeburg, Germany
1994-2002	Private English language school “LinguaRex”, Moscow, Russia

Qualifications

Linguistic proficiency	English (Fluent) Russian (Native) German (Basic)
Laboratory skills	Gradient cell separation and magnetic cell sorting Mammalian cell culture (primary/transformed, adherent/suspension) Fluorescence microscopy (living and fixed cells) Flow cytometry (incl. intracellular staining and Ca^{2+} flux) Microplate measurements Western blotting (incl. Odyssey imaging) Immunoprecipitation RT-PCR Agarose gel electrophoresis siRNA and plasmid transfection Site-directed mutagenesis Bacteria transformation, culture, and plasmid purification Human and mouse T-cell isolation and activation
Computer literacy	Scientific database search (Google Scholar, PubMed, NCBI, Uniprot) Statistical analysis and graph plotting (GraphPad Prism) FACS data analysis (Tree Star FlowJo, BD CellQuest) Image analysis (Kodak 1D, Li-COR Image Studio) Mutagenesis, PCR, and sequencing primer design Nucleotide and amino acid sequence alignment Microsoft Word, Excel, PowerPoint, EndNote

Scientific publications

Belikov AV, Schraven B, Simeoni L (2015) T cells and reactive oxygen species. *Journal of Biomedical Science*, **22**:85
(doi: 10.1186/s12929-015-0194-3)

Belikov AV and Belikov VV (2015) A citation-based, author- and age-normalized, logarithmic index for evaluation of individual researchers independently of publication counts. *F1000Research*, **4**:884
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Belikov AV, Shaposhnikov MV, Moskalev AA (2014) There Are Many Ways to Fight Aging. Results of the Third International Conference “Genetics of Aging and Longevity”. *Acta Naturae*, **6**:3, 6-10

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Belikov AV, Kiselevsky DB, Kuznetsova YE, Samuilov VD (2009) The effect of antibiotics and reactive oxygen species on *Escherichia coli* cells. *Bulletin of Moscow Society of Naturalists*, **114**:2, 154-156

Conferences

2nd AMWC Eastern Europe Congress; Moscow, Russia, 31.5. – 1.6.2014 (talk)

IV International Conference "PhystechBio"; Moscow, Russia, 29 – 30.5.2014 (poster)

3d International Conference "Genetics of Aging and Longevity"; Sochi, Russia, 6 – 10.4.2014

Signal Transduction Society 17th Joint Meeting: Signal Transduction, Receptors, Mediators and Genes; Weimar, Germany, 4 – 6.11.2013 (abstract)

GRK 1167 3rd International Symposium; Magdeburg, Germany, 22 – 23.11.2012

Symposium "Immunology and Inflammation"; Magdeburg, Germany, 18.10.2012

2011 Joint Annual Meeting: Italian Society of Immunology, Clinical immunology and Allergology and German Society for Immunology; Riccione, Italy, 28.9 – 1.10.2011 (abstract, poster)

SFB-854 Meeting; Gommern, Germany, 4 – 5.11.2010

International Conference "Physiology and genetics of microorganisms in natural and experimental systems"; Moscow, Russia, 26-28.5.2009 (abstract)