Oxidant-induced cell death in lymphocytes

– mechanisms of induction and resistance

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Abstract

Reactive oxygen species (oxidants, oxygen radicals) produced by the phagocytic NADPH oxidase have pivotal roles in immunity. Patients lacking a functional NADPH oxidase suffer from chronic granulomatous disease, which is characterized by recurring bacterial infections and thus manifesting the importance of reactive oxygen species in host defense against bacteria. However, NADPH oxidase-derived radicals also efficiently inhibit lymphocyte-mediated immunity. Oxidant-induced inactivation of lymphocytes is reportedly a control mechanism for autoreactive lymphocytes and hence prevents autoimmunity. In malignant diseases, oxygen radicals have been proposed to contribute to the characteristic state of anergy of cytotoxic lymphocytes, which prevents immune-mediated rejection of the tumor. Studies of the mechanisms of radical-induced inactivation of lymphocytes may therefore be helpful in understanding the pathophysiology of important disease entities. The first paper in this thesis shows that oxidant-induced functional inhibition and cell death in cytotoxic lymphocytes is critically dependent on cooperation between a nuclear enzyme involved in DNA repair, PARP-1, and a mitochondrion-derived protein, AIF. The results presented in Paper II demonstrate that pharmacological inhibition of the PARP-1 enzyme not only prevents oxidant-induced cell death, but also preserves functions of cytotoxic lymphocytes, such as cytotoxicity against malignant cells, cytokine production, and proliferation. Paper III shows that subsets of natural killer (NK) cells display differential sensitivity to oxygen radicals: the cytotoxic $CD56^{dim}CD16^{+}$ NK cells were found to be highly sensitive to oxidative inactivation and apoptosis, while the immunoregulatory, cytokine-producing $CD56^{bright}CD16^{-}$ NK cells were highly resistant to the toxicity of oxidants. These data were extended in Paper IV, in which the effect of oxygen radical-producing phagocytes on the expression of the activating NK cell receptors, NKp46 and NKG2D, was investigated. The expression of both receptors was efficiently downregulated on $CD56^{dim}$ NK cells, while the expression remained intact on $CD56^{bright}$ cells. Recent data imply that reciprocal interactions between NK cells and dendritic cells (DCs) are important for the development of adaptive immunity. The results presented in Paper V demonstrate that DCs are equipped with an antioxidative system that efficiently protects cytotoxic cells from oxidant-induced inactivation.

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II. Thorén F.B., Romero A.I., Hellstrand K.
Oxidant-induced inactivation of natural killer cells and T cells: role of poly(ADP-ribose)polymerase-1
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NKp46 and NKG2D receptor expression in NK cells with $CD56^{dim}$ and $CD56^{bright}$ phenotype: regulation by histamine and reactive oxygen species.
Br J Haematol (2006) 132:91-98.

V. Thorén F.B., Betten Å., Romero A.I., Hellstrand K.
Anti-oxidative properties of myeloid dendritic cells: protection of T cells and NK cells from oxygen radical-induced inactivation and apoptosis
Submitted

Till Susanne

Table of contents

Abbreviations

ADCC	Antibody-dependent cell cytotoxicity
adMP	Adherent mononuclear phagocyte
AICD	Activation-induced cell death
AIF	Apoptosis-inducing factor
APC	Antigen-presenting cell
CGD	Chronic granulomatous disease
CICD	Caspase-independent cell death
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
FACS	Fluorescence-activated cell sorter
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GSH	Glutathione
HLA	Human leukocyte antigen
ITAM	Immunoreceptor tyrosine-based activation motifs
ITIM	Immunoreceptor tyrosine-based inhibition motifs
KIR	Killer cell Ig-like receptors
MHC	Major histocompatibility complex
MICA/B	MHC class I-related chain A/B
MOMP	Mitochondrial outer membrane permeabilization
MP	Mononuclear phagocyte
NADPH	Nicotinamide adenine dinucleotide phosphate
NCR	Natural cytotoxicity receptors
NK	Natural killer
PAMP	Pathogen-associated molecular pattern
PAR	Poly(ADP-ribose)
PARP-1	Poly(ADP-ribose)polymerase-1
PCD	Programmed cell death
PE	Phycoerythrin
PE-Cy5/7	Phycoerythrin-Cyanine-5/7
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
SSC	Side scatter
TCR	T cell receptor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
Trx	Thioredoxin
$\Delta\Psi_m$	Mitochondrial transmembrane potential

Preface

Unicellular organisms are endowed with diverse mechanisms such as antibiotics, RNA interference and antibacterial peptides to protect themselves from infectious agents (1-3). As multicellular organisms evolved, individual cells were assigned different tasks. Some cells were important throughout the life-span of the organism, while others were only needed for a shorter period of time. Specialized cells were developed to form a surveillance system that could identify damaged, infected or unwanted cells and remove them. These surveillance cells developed to the diverse system we today know as innate immunity, and it has two different roles: First, to remove damaged or unwanted cells ("altered self") and second, to recognize and destroy foreign cells ("non self" or "missing self"). As more advanced species evolved, more complex defense mechanisms were developed, but rather than replacing the previous system, the new ones were usually added as supplements. The result is the vertebrate immune system – a sophisticated network of intertwined defense mechanisms that act on all levels in the organism.

Paradoxically, despite the egoistic struggle for survival, unicellular organisms have developed programs for cell death. Thus, if a cell is infected, it can altruistically commit suicide to spare the community/progeny from wide-spread infection. This method has been maintained and is still utilized in multicellular organisms. Thus, in the defense against viral infections, infected cells can enter apoptosis to minimize the damage induced by the virus (4, 5). Accordingly, several human pathogenic viruses harbor genes that encode proteins that hamper apoptosis in the host cell (6).

Programmed cell death does not only have a role as a cellular suicide program. In more advanced organisms, cells of the immune system have acquired the ability to induce cell death in deviant or unwanted cells. Cytotoxic T cells and natural killer (NK) cells thus utilize death receptors (e.g. Fas) or toxic enzymes, known as granzymes, to trigger cell death programs in susceptible target cells (7), including deviant cells such as tumor cells or infected cells. NK cells also eradicate unwanted normal cells, e.g. dendritic cells, which has been suggested to have a role in shaping the immune response (8).

The presence of immune cells with a capacity to kill normal cells calls for efficient means of control. The activity of immune effector lymphocytes has to be kept in check to avoid chronic inflammation and autoimmune reactions. To maintain and secure homeostasis, the immune system has developed several overlapping systems to fine-tune the activity of cytotoxic lymphocytes. Thus, self-reactive T cells are forced to undergo apoptosis at an early stage of development, and a plethora of inhibitory

structures, immunosuppressive cells and cytokines constantly regulates immune reactivity. Notably, there is an inherent risk with efficient shut-down systems as they can be mimicked or hijacked by pathogens and tumor cells, which thereby evade lymphocyte-dependent elimination.

This thesis has been devoted to a lymphocyte control mechanism, which is dependent on the production and release of immunosuppressive oxygen radicals from phagocytic cells. We have identified molecular mechanisms of oxygen radical-dependent induction of apoptosis in lymphocytes (papers I and II), characterized the sensitivity of lymphocyte subsets to oxygen radical-induced toxicity (papers III and IV), and described a mechanism by which antigen-presenting dendritic cells rescue lymphocytes from oxygen radical-induced inactivation (paper V). Our studies may be helpful in understanding the pathophysiology of disease entities, such as malignancies and autoimmune diseases, in which oxygen radical-induced inactivation has been ascribed a role, and may also point towards novel therapeutic strategies to protect immune effector lymphocytes from oxidative stress.

Introduction

Innate immunity

The first line of defense against pathogens is the physical barriers that seal the interior of an organism from the environment. The epidermis, the mucus produced by mucous membranes, the cilia lining the respiratory epithelium all contribute to the fortress wall of the human body and thus continuously spare us from infections. However, despite these physical barriers pathogens often succeed in entering the body and establish an infection. In these instances, an immune response to the pathogen is rapidly initiated. The innate immunity uses a limited number of sensors that recognize certain conserved pathogen-associated molecular patterns (PAMPs), which are invariantly expressed by different classes of microbes but are not expressed by the host (9). These "non-self" sensors, known as pattern recognition receptors (PRRs), enable the innate immune system to respond to a pathogen instantly at the first encounter.

PRRs are present as soluble factors in the plasma and as intracellular and cell surface receptors on various cells (10). Among these receptors are the members of the Toll-like receptor family (TLR), which each recognize one kind of PAMP (11). Different innate cells have their own repertoire of TLRs and are thus able to respond to a distinct number of PAMPs. The presence of PRRs on cells at the site of pathogen entry, e.g. epithelial cells, enables a swift response to infection. Thus, the binding of a pathogen structure to a PRR expressed on an epithelial cell triggers an alert signal of cytokines and chemokines that rapidly recruit immune effector cells such as neutrophils to the site of infection.

Innate effector cells

Phagocytes

Phagocytes are derived from the myeloid lineage in the bone marrow and are divided into two principal categories of cells: polymorphonuclear phagocytes and mononuclear phagocytes.

Polymorphonuclear phagocytes

Polymorphonuclear phagocytes, and particularly neutrophilic granulocytes, play a key role in the innate defense against pathogens. Neutrophils are the most abundant leukocyte in peripheral blood and constitute approximately 50-70% of the total circulating leukocyte population. In response to infection and pathogen recognition, proinflammatory substances trigger endothelial cells lining the blood vessel to

upregulate adhesion molecules, which in turn promote neutrophil arrest and subsequent extravasation into the tissue. Neutrophils are guided towards the site of infection along a gradient of chemoattractants, such as bacterial products, complement factors and various cytokines. Complement factors and antibodies that are bound to the pathogen enable identification of the pathogen as non-self and mark it for destruction. The recognition triggers the process of engulfment, or phagocytosis, but also cytokine production which attracts more neutrophils and other immune cells to the site of inflammation.

Rapid recruitment of more neutrophils is an important amplification loop in the immediate response to infection. Ingested pathogens are killed using oxygen radicals and an array of other toxic substances and enzymes that are stored in preformed granules in the neutrophil cytosol. These substances are not only toxic to pathogens but also to the surrounding tissue. Hence, as much as the neutrophil inflammatory response is an invaluable asset in the immediate phase of a limited infection, massive neutrophil accumulation and activation can result in extensive damage and inflammation in systemic disease, such as sepsis, and in fact threaten the survival of the host (12).

The intracellular content of highly toxic substances in neutrophils calls for a timely and vigilant apoptotic program for neutrophils to ensure minimum leakage to the surrounding tissue and to enable resolution of inflammation (13). Consequently, neutrophils are kept under tight control and only circulate for 6-10 hours before they enter the tissues or undergo apoptosis.

Mononuclear phagocytes

Another group of important innate effector cells are mononuclear phagocytes (MPs). These cells include myeloid progenitors, circulating monocytes and resident tissue macrophages. Depending on the tissue, macrophages are denoted different names. Thus, the Kupffer cells of the liver, the microglia of the central nervous system, and osteoclasts and certain types of dendritic cells are all derived from monocytes. MPs are important for non-inflammatory house-keeping duties in the tissues. Damaged and apoptotic cells display several "altered self" or "eat me" structures that are recognized by various phagocyte receptors, such as CD14, CD36, scavenger receptors, lectins, and the phosphatidylserine receptor (14, 15).

In infected tissue, MPs form a second wave of recruited cells after the initial neutrophil response. Thus, they can play a critical role in the maintenance of inflammation. Interaction with pathogen-induced apoptotic neutrophils activates macrophages to produce large amounts of TNF-α (16). In response to activating signals such as

pathogen-associated structures, interferon-γ, TNF-α, MPs perform a wide range of activities, including but not limited to phagocytosis, cytokine and oxygen radical production. Engulfed pathogens are killed using the same effector systems used by neutrophils, i.e. toxic substances, hydrolytic enzymes and NADPH-oxidase-derived radicals. In addition, pathogens and infected cells that are opsonized by IgG antibodies are recognized by the FcγRIII receptor on MPs to initiate a cytotoxic response known as antibody-dependent cellular cytotoxicity (ADCC).

MPs are also important for the resolution of inflammation. Several lines of evidence suggest that MPs inactivate immune cells and trigger apoptosis using NADPH-derived oxygen radicals (17-20). As pathogens are cleared from the site of infection, a higher fraction of neutrophils display age-induced apoptosis rather than pathogen-induced apoptosis, resulting in a declined pro-inflammatory signal (16). Instead, the increasing number of cells expressing phosphatidylserine (PS) as a sign of apoptosis triggers a functional shift in the MPs, and they start producing anti-inflammatory cytokines, such as TGF-β and IL-10 (20-22). Interestingly, recent studies demonstrate that PS, which is exposed on the outer side of the plasma membrane of apoptotic cells, needs to be oxidized to enable efficient uptake of apoptotic cells by macrophages (23, 24). Thus, these studies imply that oxygen radical-induced apoptosis can be an anti-inflammatory event that initiates the processes leading to resolution of inflammation.

The phagocyte NADPH oxidase

A key element of the phagocytic weaponry against pathogens is the generation of oxygen radicals (reactive oxygen species; ROS) that are released into the phagosome after phagocytosis. This oxygen-dependent pathway was discovered in a series of experiments by Baldridge and Gerard showing that leukocyte oxygen consumption increased upon phagocytosis of bacteria (25). However, the term "respiratory burst" is a misnomer, since the increased oxygen consumption is not due to elevated respiration but rather to the conversion of molecular oxygen into different ROS (26).

In neutrophil as well as mononuclear phagocytes, ROS are produced by an enzyme complex known as the NADPH oxidase. The NADPH oxidase is an inducible multicomponent enzyme that consists of two membrane-bound components, $gp91^{phox}$ and $p22^{phox}$ that exist as a heterodimer called b cytochrome along with three cytosolic proteins, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ (26). Upon binding of pathogen structures, complement factors etc. to the corresponding receptor, the phagocyte is activated, resulting in phosphorylation of $p47^{phox}$. The phosphorylation favors the interaction between $p47^{phox}$ and the b cytochrome and thus, the assembly of a functional oxidase (Figure 1) (27).

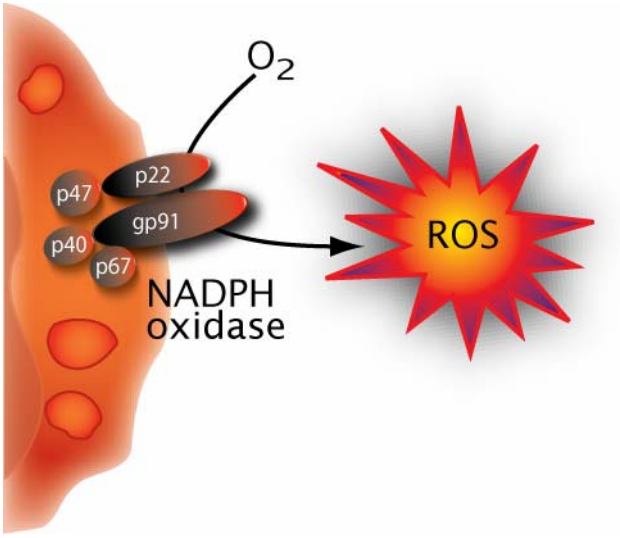

FIGURE 1. The phagocytic NADPH oxidase. Upon phagocyte activation, the NADPH oxidase is assembled. The cytosolic components $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ interact with the two membrane-bound components, $gp91^{phox}$ and $p22^{phox}$ that exist as a heterodimer which is known as the b cytochrome. The b cytochrome is present both in the plasma membrane and in the intracellular compartments. Reactive oxygen species can thus be generated intracellularly and released extracellularly.

The assembled oxidase is an electron transport chain that shuttles electrons from NADPH on the cytosolic side of the membrane to molecular oxygen on the other side of the membrane. The process leads to a one-electron reduction of molecular oxygen to the free radical superoxide anion ($O_2^{\bullet-}$). This NADPH oxidase-catalyzed reaction is only the first in a series of reactions to generate various microbiocidal compounds (Figure 2) (28, 29): Superoxide anion is a short-lived radical and it dismutases spontaneously into hydrogen peroxide (H_2O_2) – a reaction that can be catalyzed by superoxide dismutase (SOD). Hydrogen peroxide can then be further processed to form other, even more toxic, ROS. The phagocyte-encoded enzyme myeloperoxidase (MPO) converts H_2O_2 into reactive halides, such as hypochlorous acid (HOCl), and in the presence of ferrous ions, H_2O_2 can be converted into highly reactive hydroxyl radicals ($OH^{\bullet}$) via the Fenton reaction. Peroxynitrite can also be produced through the reaction between nitric oxide and superoxide anion (28, 29).

The importance of these ROS in the defense against pathogens is exemplified by an hereditary human disease, chronic granulomatous disease (CGD) (30). Patients with CGD carry a mutation in one of the genes encoding NADPH oxidase components, which results in loss of a functional oxidase. As a consequence, the patients suffer from recurrent bacterial and fungal infections. In addition, recent studies have indicated that NADPH-oxidase-derived radicals are important for the removal of apoptotic neutrophils from sites of inflammation, which would explain the presence of non-infectious granuloma in CGD patients (31, 32).

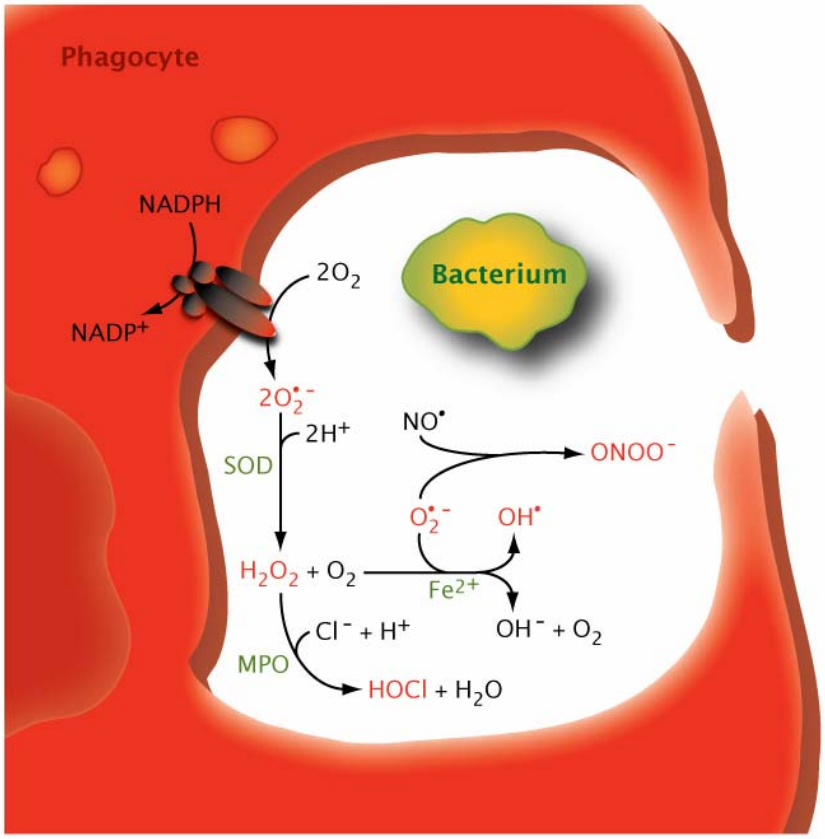

FIGURE 2. Generation of reactive oxygen species by the enzymatic machinery of phagocytes. Enzymes, except NADPH oxidase, are shown in green, ROS are shown in red.

The b cytochrome is present at two sites in the phagocyte, in the plasma membrane and in intracellular compartments. Thus, upon assembly of the NADPH oxidase, reactive oxygen species can both be generated intracellularly and/or released extracellularly (33).

Natural killer cells

A third type of innate effector cells are the natural killer (NK) cells. In contrast to phagocytes, which are of myeloid origin, NK cells are lymphoid cells. NK cells constitute approximately 10% of circulating lymphocytes and are in man phenotypically defined as $CD3^{-}CD56^{+}$ lymphocytes, i.e. lymphocytes that do not carry the prototypic T cell marker CD3, and express the CD56 antigen. The latter antigen is expressed by neural cells where it mediates homotypic attachment of neurons (CD56 to CD56), but its functional role in NK cells in unknown.

The surface chemokine receptor profile of NK cells shows a certain resemblance to that of neutrophils, and NK cells are thus rapidly recruited to sites of inflammation

(34). NK cells recognize virus-infected cells and have recently been demonstrated to express TLRs that recognize viral structures (35-37). Accordingly, NK cells are considered especially important in the defense against viral infections (38-40). The pivotal role of NK cells in viral immunity is also manifested by the multitude of evasion strategies that viruses deploy to avoid NK cells (41). NK cells have also been ascribed a role in resistance to bacteria and parasites, mainly due to their stimulatory effect on phagocytic cells through IFN-γ production, (42-45). In addition to PAMPs, NK cell activation can be achieved by several cytokines such as IL-2, IL-12, TNF-α and interferon α/β. Upon activation, NK cells display enhanced cytotoxicity and produce a wide array of proinflammatory cytokines including IFN-γ and TNF-α.

NK cell subsets

There are two major subsets of NK cells in peripheral blood, and they can be defined based on the intensity of their expression of the CD56 antigen and the FcγRIII receptor, CD16. Approximately 90 % of human NK cells are $CD56^{dim}$ and express high levels of CD16 (46). These cells produce low levels of cytokines, have limited proliferative potential, but are rich in perforin- and granzyme-containing granules and thus exert high natural and antibody-dependent cellular cytotoxicity against susceptible target cells (46). The remaining 10% of NK cells in peripheral blood display a $CD56^{bright}16^{dim/neg}$ phenotype (46). In the resting state, these cells are less cytotoxic since they contain lower levels of perforin and granzymes, and they are incapable of performing ADCC as they lack CD16 (46, 47). However, $CD56^{bright}$ NK cells express the high-affinity IL-2 receptor and strongly proliferate in the presence of low levels of IL-2 (48, 49). In response to stimulation, $CD56^{bright}$ cell produce higher amounts of IFN-γ and other NK-derived cytokines than $CD56^{dim}$ cells (47, 50).

Although $CD56^{bright}$ cells are the minor NK subset in peripheral blood, the opposite is true in secondary lymphoid tissue, where the majority of NK cells have the $CD56^{bright}16^{dim/neg}$ phenotype (51, 52). Recent data suggest that $CD56^{bright}$ cells in fact may be progenitors (53), which differentiate into cytotoxic $CD56^{dim}CD16^{+}KIR^{+}$ NK cells upon stimulation with IL-2 (52). However, these data do not preclude an immunoregulatory role for the $CD56^{bright}$ subset. As potent cytokine producers, $CD56^{bright}$ cells are believed to interact with T cells and DCs in the lymph nodes; thereby this NK cell subset contributes to the development of the adaptive immune response (51).

NK cell recognition

NK cells were first identified more than 30 years ago by Kiessling and coworkers (54). The cells were identified as non-B, non-T killer lymphocytes that spontaneously killed

leukemic cells in vitro without prior sensitization or exposure to antigen (54, 55). A first explanation as to how NK cells recognize target cells was offered by the missing self hypothesis (56, 57). Kärre and colleagues showed that a lymphoma cell line grew progressively in syngeneic mice, while an MHC class I-negative variant was efficiently eradicated by NK cells (58). The hypothesis thus postulated that NK cells screened cells for presence of MHC class I molecules and identified cells with no or low levels of MHC class I molecules as foreign. The theory also envisaged the existence of inhibitory MHC class I receptors on NK cells, which upon ligation blocked NK cytotoxicity (59, 60). Later studies have shown that NK cells not only recognize target cells that are "missing self", but by using different receptors they also utilize "altered self" and "non-self" approaches for target recognition (61). Thus, cells that do not display sufficient levels of "self" ligands to inhibitory receptors fail to generate an inhibitory signal and are killed; target cells that display ligands recognized as "altered self" or "non-self" generate an activating signal in NK cells leading to target cell lysis. Modulation of the expression levels of these different ligands on the cell surface may render targets more or less susceptible to NK cell-mediated lysis (62). Correspondingly, elevated or diminished expression of inhibitory and activating receptors on NK cells will affect their propensity to kill target cells (63, 64).

NK receptors – inhibition or activation

Inhibitory and activating receptors are common in the immune system and most receptors share certain patterns. A well-studied example of the opposing roles of activating and inhibitory receptors is Fc receptors for IgG immune complexes. The B cell receptor, FcγRIIB and the FcγRIII expressed by mononuclear phagocytes, neutrophils and NK cells have almost identical extracellular domains. However, the intracellular parts are connected to two different signaling pathways. The intracellular part of the inhibitory receptor, FcγRIIB, contains immunoreceptor tyrosine-based inhibition motifs (ITIM) that activate phosphatases resulting in dampened cellular responses (65). FcγRIII, on the other hand, has an intracellular domain that interacts with adaptor proteins with immunoreceptor tyrosine-based activation motifs (ITAM). Hence, CD16 ligation by IgG triggers kinase activation and downstream effector functions (61).

Two major classes of inhibitory receptors have been identified in NK cells. The first class comprises the killer-cell immunoglobulin-like receptors (KIR), and the second class the heterodimers CD94 and NKG2 (61).

KIRs are classified according to the number of Ig-like extracellular domains, i.e. KIR2D has two domains while KIR3D has three. The KIRs are stochastically

expressed on individual NK cells and subsets of T cells, and each individual NK cell expresses one to eight different KIRs (66). Each KIR is specific for a distinct subgroup of HLA molecules, e.g. KIR2DL2 interacts specifically with a certain HLA-C allele. Thus, the inhibitory KIRs enable NK cells to monitor virus- or tumor-induced downregulation of the expression of specific HLA molecules (61, 66).

The CD94/NKG2A heterodimer recognizes the non-classical MHC class I molecule HLA-E. This HLA molecule presents the leading peptide of most HLA-A, -B and -C molecules and HLA-G. HLA-E expression is thus dependent on expression of these other HLA-molecules, and the CD94/NKG2A heterodimer hence monitors the overall expression of HLA molecules in a cell (67, 68). In this way, NK cells are equipped with two complimentary surveillance systems, one focusing on the overall expression of HLA (CD94/NKG2A), and the other one monitoring the expression of specific HLA molecules (KIRs).

Other NK inhibitory receptors include the leukocyte immunoglobulin-like receptor-1 (LIR-1) and Siglec 7. LIR-1 senses changes in the overall transcription of HLA class I molecules, as it binds to a conserved region of almost all HLA class I molecules (69, 70). Siglec 7 is a sialic acid-binding Ig-like lectin and can upon interaction with sialated glycoproteins block activating responses in NK cells (71).

There are numerous structures that are involved in NK cell triggering, and the distinction between triggering receptors, co-receptors and adhesion molecules is not easily defined. Triggering NK cell receptors have traditionally comprised the natural cytotoxicity receptors (NCRs), including NKp30, NKp44 and NKp46; NKG2D, DNAM-1 and CD16, while other structures that stimulate NK cell responses upon ligation, such as 2B4, CD2, NKp80 etc are defined as co-receptors (8, 72, 73). However, recent studies challenge this terminology, and instead suggest that NK activation is a result of activating signals transduced by multiple synergistic receptors (74, 75).

In contrast to the inhibitory receptors, which are stochastically distributed, most activating receptors are present on all NK cells. Furthermore, while inhibitory receptors utilize one common ITIM-dependent signaling pathway, activation receptors use a number of different downstream cascades (75).

NKG2D is expressed by all NK cells and recognizes surface structures that are related to MHC class I, although most of these ligands are not able to bind peptides for T cell presentation. NK cell expression of NKG2D is inducible; its expression can be

enhanced by IL-15 or TNF-α, but is decreased by TGF-β (61, 76). NKG2D is known to ligate MHC class I chain-related A chain and B chain (MICA and MICB) and UL16-binding proteins (77-79). MIC protein expression is very restricted in normal tissue, but it is upregulated in various tumors and in response to cellular stressors, such as oxidative stress, heat shock, and bacterial infection (80-83).

Studies with blocking antibodies have indicated that the NCR family of receptors is of major importance for NK cell-mediated lysis of various tumor cell lines (84-86); in fact, simultaneous blocking of all three NCRs abolishes NK cytotoxicity (73). The NCRs are also important for NK-mediated lysis of virus-infected cells; hemag-glutinin from different influenza strains has been identified as a ligand to NKp44 and NKp46 (87, 88). However, the tumor cell ligands that trigger NK cell activation through the NCRs remain unidentified.

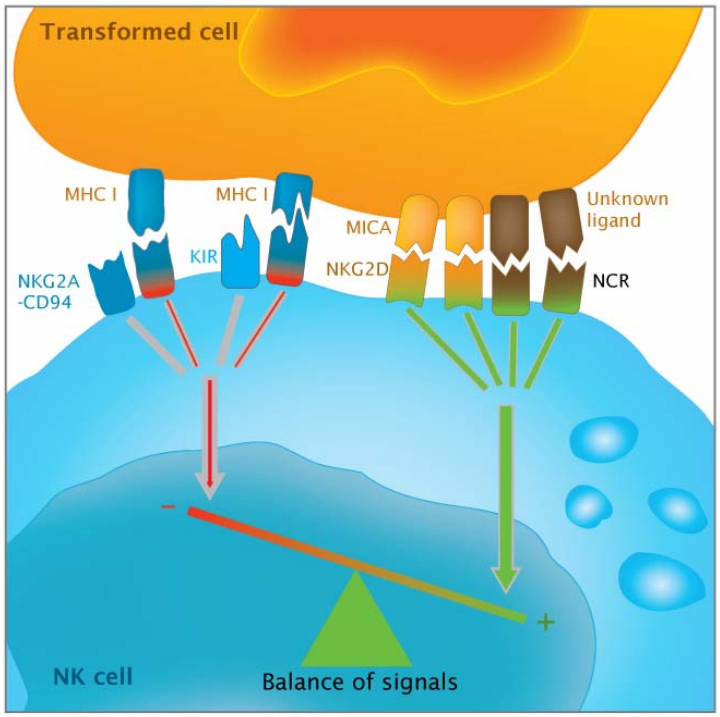

FIGURE 3. Recognition of transformed cells by NK cells. Upon encounter with another cell, activating (NKG2D and NCR) and inhibitory (NKG2A/CD94 and KIR) receptors on NK cells interact with their corresponding ligands. The outcome is determined by the balance between inhibitory (red) and activating (green) signals in the NK cell.

NKp30 and NKp46 are expressed on all NK cells, while NKp44 expression is restricted to activated NK cells, which may serve to explain why IL-2-activated NK cells display enhanced cytotoxicity (72). Although NCRs are always expressed on all NK cells, NCR expression varies both intra-individually, between NK cells, and inter-individually. There is a strict correlation between NCR expression and NK cell-mediated cytotoxicity (63); however, $MICA^{+}$ tumor cells are readily killed by NCR^{dull} NK cells owing to activation through NKG2D (64).

To summarize, NK cells recognize cells with three distinct characteristics:

i) Targets that are "missing self" – cells with low amounts of MHC class I molecules fail to induce inhibitory signals in NK cells.
ii) Targets with "altered self" – cells that express deviant proteins, such as MICA and MICB, trigger activation signals through NKG2D.
iii) Targets with "non-self" – cells that express aberrant structures, such as influenza hemagglutinins, are recognized by NCRs resulting in NK activation.

However, inhibitory and activating signals occur in parallel, and the decision whether an encountered cell is to be lysed is a complex process, integrating signals from many different inhibitory and activating receptors (Figure 3). Thus, a strong activating signal resulting from a massive expression of activating ligands on a target cell can override an inhibitory signal conveyed by MHC class I on the same cell (74, 75).

Effector mechanisms

Upon activation, NK cells and cytotoxic T lymphocytes (CTLs, see section below) kill target cells using a death receptor-dependent pathway or a perforin-dependent pathway. Historically, these mechanisms have been regarded as independent of each other, since the death ligands were believed to be exposed on the cell surface, while the second pathway depended on exocytosis of granules. However, recent data indicate that exocytosis is critically involved in both pathways (89, 90). Thus, upon encounter with a susceptible target cell, cytotoxic cells are activated and release secretory lysosomes into the tightly sealed intercellular synapse that is created between the cytotoxic cell and the target (91). The exocytosis of these lytic granules exposes death ligands, such as Fas ligand (CD95), on the cell surface of the cytotoxic cell. Fas ligand interacts with the corresponding receptor on the target cell, and triggers a caspase-dependent death program in the target cell (92), which is known as the extrinsic pathway. The details of this program are described below (cf. Cell death section).

The lytic granules also contain the toxic substance perforin and a family of serine proteases, known as granzymes (93). The exact mechanism for granzyme entry into the target cell remains unknown. A new model suggests that complexes containing perforin and granzymes enter the cell by endocytosis, and that granzymes subsequently escape from the endosome into the cytosol (94). In the cytosol of the target cell, granzymes trigger caspase-dependent and -independent cell death (7, 95).

Adaptive immunity

Dendritic cells and adaptive immunity

Adaptive cellular immunity is based on T cells, which are equipped with receptors that are generated by somatic gene rearrangement. By this mechanism a vast repertoire of cells is produced, and each of these cells expresses a unique activating antigen receptor, known as the T cell receptor (TCR). The TCR is functionally similar to the activating receptors on NK cells, such as the NCRs and NKG2D. While activating receptors on NK cells recognize target cells with non-self or altered self antigens, the TCR on cytotoxic T lymphocytes (CTL) recognizes a specific antigen/MHC class I complex presented on the surface of the target cell. Since TCRs are generated randomly, many T cells are generated that have specificities for self-antigens. These T cells are generally deleted at an early stage in the thymus in a process known as central tolerance. However, the body is constantly exposed to dietary and environmental antigens, against which the T cells are not supposed to react. Thus, in order to know how to react when they encounter a cell with their ligand, T cells need education regarding the biological context (96). This education is performed by professional antigen-presenting cells, known as dendritic cells.

The existence of leukocytes with dendritic morphology (dendritic cells; DCs) was known for more than a century before their role in immunity was discovered (97). DCs are innate phagocytic cells with a unique capacity to process antigens, present them as peptides bound to MHC molecules and initiate adaptive immunity. Thus, dendritic cells form a bridge between innate and adaptive immunity. Immature dendritic cells are distributed throughout the body, especially in the vicinity of body surfaces. According to one model for peripheral tolerance (98), they continuously sample the environment for antigens. Under steady-state conditions in the absence of infection, DCs do not upregulate co-stimulatory molecules but migrate to lymph nodes where they present self-antigens and other innocuous antigens and instruct T cells to remain tolerant (98).

In order to induce an adaptive immune response, DCs have to undergo maturation (98). This process induces an array of changes in DCs:

i) Upregulation of MHC structures on the surface (99).
ii) Increased expression of co-stimulatory molecules, such as the B7 molecules CD80 and CD86 (100).
iii) Production of proinflammatory cytokines such as IL-12 (101, 102).
iv) An altered chemokine receptor profile that enhances migration to lymphoid organs (103).

Some controversy has arisen regarding how the immune system makes the decision whether immunity or tolerance is to be induced (104, 105). According to the "danger model" proposed by Matzinger, DCs respond by maturation when sensing danger in the form of normally intracellular proteins that are released extracellulary from damaged cells (106). Janeway, on the other hand, emphasizes the signals generated by pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) such as TLRs (10). Dendritic cells express several different TLRs, and TLR stimulation with pathogen-derived stimuli readily induces DC maturation (9). However, the two models are not mutually exclusive, and recent data in fact suggest that some endogenous danger signals use TLRs to trigger DC maturation (104). Another danger signal involved in DC maturation is possibly mediated by reactive oxygen species, since exposure of DCs to H_2O_2 induced upregulation of MHC, co-stimulatory molecules and enhanced production of proinflammatory cytokines (107, 108).

When mature DCs present an antigen/MHC class I complex to a T cell with a matching TCR, and simultaneously stimulate the T cell with co-stimulatory molecules, an ITAM-mediated activating signal is triggered within the T cell. Once activated, the T cell undergoes clonal expansion which results in a large number of functional mature CTLs that all recognize the particular antigen/MHC class I complex that was presented by the dendritic cell. When these CTLs encounter a cell that displays this complex on the cell surface, the TCR binds to MHC complex and an activating signal triggers the cytotoxic machinery in the T cell. Once the infection is resolved, most of the clonally expanded CTLs undergo apoptosis, while the remainder of them differentiates to long-lived memory cells (109).

NK cells, DCs and adaptive immunity

The first observation of DC-NK interactions showed that DCs induce IFN-γ production and enhance NK activity towards tumor cells (110). Further studies have revealed that the interaction between NK cells and DCs is reciprocal and is of importance in shaping the adaptive immune response (8). In infections, DCs are activated by PAMPs that are recognized by different TLRs on the cell surface. Activated DCs form stimulatory synapses with NK cells, which induces directed IL-12 release towards the NK cell (111). It has also been proposed that DCs promote the immune response by providing a reducing microenvironment (112). NK cells are further activated by the DC expression of CD48 and CD70, which are ligands for the NK-activating co-receptors 2B4 and CD27 (113), as well as the as yet unidentified ligand to the activating receptor NKp30 (114). Activated NK cells respond by

producing IFN-γ and TNF-α, which promote DC maturation and up-regulation of co-stimulatory molecules (114-116).

Interestingly, apart from the bilateral activation, NK cells can also kill immature dendritic cells (115, 117, 118), while mature DCs are resistant as they display higher expression of HLA-E (119). Selective cytotoxicity towards immature DCs may be a mechanism to ensure that only fully matured DCs reach the lymphoid tissue. This mechanism could serve two purposes: first, to promote efficient Th1 priming and warrant that immature DCs do not induce tolerance (114, 115); second, it could constitute an inhibitory mechanism to avoid autoimmunity: In the absence of antimicrobial signals, DCs do not become fully matured and are eradicated to avoid the risk of them presenting self antigens to T cells (120). Alternatively, NK-mediated killing could be a way to regulate the amplitude of the DC response and thus the adaptive response (121).

Mostly, DC activation seems to be the initial step in NK/DC interactions. However, in malignancies, which are often poorly immunogenic, NK activation can provide the spark that drives the NK/DC crosstalk (122). NK cell interaction with activating ligands on tumor cells triggers NK cell activity, which can result in direct NK-mediated tumor rejection, but also indirectly as DCs are stimulated with cytokines and supplied with antigens from NK-lysed targets that can be used for induction of adaptive immunity (123, 124).

When the DCs reach the lymph node, they are believed to interact with $CD56^{bright}$ NK cells that are localized in T-cell areas of human lymph nodes (51, 52, 125). By releasing IFN-γ, the $CD56^{bright}$ NK cells are assumed to shape the adaptive immune response, promoting Th1 polarization (126).

Cell death

The death of cells is essential to life. In the developing fetus, selective cell death is a crucial event in organ development and formation, and in an adult human being, cell renewal and proliferation must be perfectly balanced by cell death. As cells become aged, they should die and be replaced by new cells. For example, as stated above the majority of T cells succumb at an early stage as their specificity is irrelevant or directed to self antigens (127), and newly synthesized neutrophils have a life expectancy in peripheral blood of approximately 4-10 hours, followed by apoptotic death. Cells are supposed to fulfill their duties; thereafter, their retirement is brutal. Cell death is thus a key element in maintaining cellular homeostasis. Insufficient apoptosis has been implicated in cancer and autoimmunity, while a higher propensity of apoptosis is assumed to contribute to the pathophysiology of degenerative diseases and immunodeficiency (128-130). Reflecting the importance of cell death, over 200 proteins are dedicated to the cell death machinery and lethal pathways are under tight control (95, 131).

Cell death has traditionally been divided into two forms: active cell death, apoptosis, mediated by the caspase cascade, which orchestrates the degradation of the cell without the release of toxic substances into the surrounding tissue; and passive, accidental cell death, necrosis, in which cells rapidly lose plasma membrane integrity and are degraded in an uncontrolled way. Although this simplistic model of two different principal modes of cell death holds true for insects and nematodes, it has in recent years become evident that cell death is more complex in mammals; thus, active cell death can occur without the involvement of caspases, and caspase activation is not exclusively associated with cell death (132).

The different modes of cell death have created nomenclature confusion in the cell death field. Programmed cell death (PCD) can be defined as cell death events occurring at pre-defined points during development (133), while others define PCD as all modes of cell death that follow a signaling pathway within the cell, and thus can be blocked by inhibitors targeting structures along this pathway (134). Furthermore, apoptosis is sometimes defined as cell death with the characteristic morphology associated with full-blown caspase activation (complete DNA degradation, membrane blebbing etc), while yet others use the term for describing cell death following a signaling pathway within the cell, as opposed to accidental cell death, necrosis.

Active cell death

Classical apoptosis

A significant part of today's knowledge of the apoptotic processes stems from research conducted in the nematode, *Caenorhabditis elegans*. Careful mapping of the fate of each individual cell in this roundworm revealed that 131 cells disappear during development through a regulated process and hence are not present in the adult roundworm which comprises a total of 959 cells. The genes encoding the proteins of the PCD machinery were identified in the beginning of 1990s, and the mammalian homologues were identified a few years later (135). Although the PCD phenomenon had been observed earlier (136), the term apoptosis was coined by Kerr *et al.* in 1972 and is derived from the Greek word for the process of leaves falling from trees (137).

The mammalian forms of the proteins involved in apoptosis are known as caspases, which are cysteine-dependent aspartate-directed proteases (138). In man, there are at least 7 caspases that are involved in cell death (139). These caspases are divided into two main groups: the initiator caspases (2, 8, 9 and 10) and the executioner caspases (3, 6 and 7). Caspases are transcribed as inactive zymogens, and are either activated by proteolytic cleavage or upon interaction with activating proteins (140, 141).

Two major pathways lead to caspase-dependent apoptosis, the intrinsic and the extrinsic pathway.

Intrinsic pathway

The intrinsic pathway is also known as the mitochondrial pathway or the B cell lymphoma 2 (Bcl-2)-regulated pathway (142). The Bcl-2 family comprises two groups of proteins which differ in the number of Bcl-2 Homology (BH) domains and thus have opposing roles in cell death (143, 144). The anti-apoptotic group contains proteins with three or four BH domains, e.g. Bcl-2, Bcl-x_L; the pro-apoptotic proteins can be sub-divided into two groups, where Bax-like proteins (Bax, Bak, etc) contain two or three BH domains, while the BH3-only group of proteins (Bad, Bik, Bid etc) only contain the short BH3 domain.

When stressors, like ionizing radiation or starvation, tip the balance towards death, the pro-apoptotic BH3-only proteins Bim and Bid are post-translationally modified and activate Bax and Bak to trigger mitochondrial outer membrane permeabilization (MOMP) (145). This permeabilization results in release of death-inducing intermembrane space proteins including cytochrome c (146). Bax and BAK can also trigger MOMP and cell death independently of the BH3 proteins. Thus the two groups of pro-apoptotic Bcl-2 family proteins both promote mitochondrial membrane leakage,

either directly (Bax-like proteins) or indirectly (BH3-only proteins). The anti-apoptotic proteins, Bcl-2 and Bcl-x_L, accordingly, act to stabilize the mitochondrial membrane (147). These pro-life proteins bind to BH3-only proteins and thus obstruct their lethal interaction with Bax and Bak (148).

The cytochrome c release from mitochondria enables a structure known as Apoptotic protease activating factor 1 (Apaf-1) to recruit procaspase-9 to form the apoptosome (149, 150). Within the apoptosome, caspase-9 is autoactivated and cleaves the executioner enzyme, caspase-3.

Along with cytochrome c, other effectors associated with caspase-independent processes (see below) are released from the mitochondria. To ensure proper caspase activation, mitochondria also release Smac/DIABLO, which interacts with a family of proteins known as inhibitors of apoptosis (IAP). IAPs have a role in regulating caspase activity in the cytosol, and Smac/DIABLO release from the mitochondria thus augments the caspase activation obtained after cytochrome c release (151).

Extrinsic pathway

The extrinsic pathway is initiated upon stimulation of extracellular death receptors of the TNF receptor superfamily, such as CD95 (Fas), the TNF receptor or the TNF-related apoptosis-inducing ligand (TRAIL) receptor. Upon binding of Fas ligand, the cytoplasmic part of the receptor recruits death domain-containing adaptor proteins, e g FADD (Fas-associated death domain), which interacts with caspase-8 to form the death-inducing signaling complex (DISC) (152). Within this complex caspase 8 becomes active and promotes cell death in two complimentary ways: it acts directly on downstream effector caspases, e.g. proteolytic cleavage of procaspase 3 to active caspase 3, and it amplifies the signal by activating the BH3-only protein, Bid, which triggers MOMP, cytochrome c release and apoptosome formation as mentioned above (153, 154). Thus, both the extrinsic and intrinsic pathways converge onto caspase 3 activation.

The activity of caspase-3 and the other executioner caspases results in the downstream morphological changes associated with apoptosis: for example, cleavage of the inhibitor of caspase-activated DNase (ICAD) induces chromatin and DNA degradation (155); cleavage of lamins causes nuclear shrinkage; cytosolic rearrangement results from degradation of cytoskeletal proteins, such as gelsolin and fodrin; and cleavage of p21-activated kinases mediates membrane blebbing (156).

Caspase-independent cell death

The importance of cell death for a multicellular organism implies that it would be dangerous to rely on one single system for the destruction and removal of unwanted cells. Accordingly, the caspase cascade is a major mechanism for mammalian cell death, but complimentary activities occur in dying cells (157). Thus, in various experimental settings, it has become evident that pan-caspase inhibitors cannot always inhibit death of cells exposed to an apoptogenic stimulus. In fact, there are even examples of cell death where the death program proceeds in the absence of caspase activation (134, 158). In these forms of caspase-independent cell death (CICD), there is evidence for a role of other proteases, such as calpains, cathepsins and proteasomal proteases in cell death (159-161).

Similarly to the processes in the intrinsic pathway, MOMP seems to be a critical step in most forms of CICD (132, 162, 163). As mentioned above, MOMP results in the release of proteins from the mitochondrial intermembrane space. In addition to cytochrome c and other proteins involved in the caspase cascade, caspase-independent effector proteins are released, including apoptosis-inducing factor (AIF) (164), endonuclease G (165), and HTRA2 (166). The regulation of mitochondrial release of different death-inducing proteins is poorly understood. It has been suggested that the proteins involved in cell death are bound in different ways within the intermembrane space of the mitochondria and thus are differentially prone to be released upon MOMP (167, 168).

It has also been speculated that MOMP can occur in alternative ways in different modes of cell death. Thus, in addition to the mechanism depending on the bcl-2 family of proteins, MOMP can occur as a consequence of mitochondrial permeability transition (169, 170). This alternative mechanism for MOMP seems to involve an upstream release of Ca^{2+} from the endoplasmic reticulum (ER). In response to a lethal signal, Ca^{2+} is released from the ER and enters the mitochondria. The mitochondrial Ca^{2+} overload is believed to trigger opening of a pore channel in the inner mitochondrial membrane. This process results in depolarization of the mitochondrial transmembrane potential ($\Delta\Psi_m$) and swelling of the mitochondrial matrix as water enters. As a consequence, the outer mitochondrial membrane ruptures allowing release of intermembrane proteins into the cytosol (171). The permeability transition pore channel is formed at the contact sites between the inner and outer mitochondrial membranes, where the adenine nucleotide translocator (ANT) interacts with the voltage-dependent anion channel (VDAC) (172), and inhibitors that target these structures can block the opening of the pore.

However, the two pathways leading to MOMP seem to be intimately intertwined. Bcl-2 proteins control the release of Ca^{2+} from ER stores (171), and bcl-2 proteins interact with the pore-forming protein, VDAC, to release mitochondrial intermembrane-space-located proteins without rupturing the outer membrane (162, 170).

Apoptosis-inducing factor

Apoptosis-inducing factor (AIF) is a mitochondrial flavoprotein which in healthy cells is confined to the mitochondrial intermembrane space (164). As mentioned above, in response to an apoptogenic stimulus, MOMP is induced and AIF can be released into the cytosol and translocate to the nucleus. In the nucleus, AIF interacts with the DNA (173), and induces chromatin condensation and large-scale degradation of DNA into 50,000 base-pair fragments (164). AIF is devoid of nuclease activity, suggesting that AIF either recruits nucleases, or that the AIF-DNA interaction renders DNA susceptible for attack by latent nucleases (174, 175).

Several lines of evidence point to a role for AIF in CICD (175). The translocation of AIF to the nucleus can occur without concomitant caspase activation (164), and it is not inhibited when key structures of the caspase cascade are knocked out (176). However, there is considerable crosstalk between AIF and the caspase system. For example, AIF is released from mitochondria upon MOMP induced by FasL through the initiator caspase-8, and conversely, AIF can trigger cytochrome c release from purified mitochondria (175). Kinetic studies have also indicated that AIF release from the mitochondria precedes cytochrome c release and thus caspase-3 activation (164, 177, 178).

Poly(ADP-ribose) polymerase-1

Poly(ADP-ribose)polymerase-1 (PARP-1) is a nuclear enzyme with diverging roles in cellular processes including DNA repair, transcription and induction of cell death (179). In response to DNA-strand breaks, PARP-1 covalently attaches polymers of ADP-ribose (PAR) to various nuclear target proteins, which results in recruitment and activation of DNA repair systems (180). However, excessive DNA damage leads to over-activation of PARP-1 and ensuing cell death.

Historically, PARP-1-dependent cell death has been associated with accidental cell death, necrosis. PARP uses NAD^+ as a cofactor in the poly(ADP-ribos)ylation reaction. Massive PARP activation depletes the cellular stores of NAD^+ and ATP, which induces necrosis (181, 182). However, recent findings suggest that PARP-1 activation induces cell death in the absence of cellular energy store depletion (183, 184). Instead, it has been shown that the enzymatic activity causes release of free PAR from the nucleus

into the cytosol. The released PAR acts on mitochondria, and induces depolarization of the mitochondrial transmembrane potential ($\Delta\Psi_m$), which triggers AIF release from the mitochondria (185, 186). As discussed above, released AIF subsequently translocates to the nucleus where it induces DNA fragmentation (164, 178).

The conflicting findings regarding depletion of energy stores in PARP-1-dependent cell death could be related to secondary AIF-caspase crosstalk. The PARP-1 enzyme is a well-known caspase target (187). Thus, when the caspase cascade is activated downstream of AIF release, energy depletion is prevented as PARP-1 is cleaved and its energy-consuming activity is blocked.

PARP-1-dependent cell death has predominantly been described in neural tissue as a consequence of ischemia-reperfusion injury, but a role for PARP-1 has also been implicated in e.g. myocardial infarction, inflammatory injury, and glutamate excitotoxicity (181, 188-190).

Antioxidative systems and oxidant-induced cell death

Several cellular processes, such as energy metabolism generate reactive oxygen species as byproducts. To function properly cells need to neutralize these oxidants in order to maintain an adequate intracellular redox balance. For this purpose, cells are endowed with a complex system to detoxify oxygen radicals. Many of the structures involved in these systems are based on cysteine-containing peptides/proteins since the sulfur-group in cysteine can exist in multiple oxidation states (191).

The antioxidative defenses can be divided into antioxidative enzymes and scavengers. The scavengers include antioxidative thiols, such as glutathione (GSH) and thioredoxin (Trx), in addition to ascorbic acid, carotenoids and α-tocopherol. The thiol-containing scavengers are also the key players in two different enzymatic antioxidative systems within the cell. In these systems, GSH is the principal reductant in reactions catalyzed by glutathione peroxidase, while Trx has the same role in peroxiredoxin-dependent reactions. The two systems operate independently and GSH seems to be more effective in reducing small disulfide molecules, while Trx predominantly reduces disulfides of proteins (191). Cells also have extracellular thiols, which are known to be important for cellular function (112, 192-194). These thiols can react with extracellular oxidants and may function as a first line of defense against oxidative challenge by neutralizing oxygen radicals (195).

Oxidants are key mediators of cell death in many cell types. Exposure of cells to exogenous hydrogen peroxide or oxidant-producing phagocytes triggers cell death, but oxidants also have a role in secondary processes resulting from a non-oxidant stimulus (18, 196-201). The exact mechanisms of ROS in these different forms of cell death remain unknown or incompletely defined. It has been proposed that thiol groups are involved in the regulation of the mitochondrial permeability transition (202). Thiol-reactive agents oxidize a key cysteine residue in ANT leading to MOMP and cell death. Accordingly, various oxidative agents, such as peroxynitrite, nitric oxide and the lipid peroxidation product, 4-hydroxynonenal, all trigger thiol oxidation/derivatization of ANT (203).

Hence, it seems clear that oxidant-induced cell death is not due to the mere exhaustion of cellular antioxidative defense systems. On the contrary, today, it is a well-established fact that ROS play important roles in cell signaling (204, 205). For example, various growth factors that signal through receptor tyrosine kinases are dependent on ROS as second messengers (206), and H_2O_2 induces increased activity in various signaling pathways (207, 208). Oxidants are thus exploited for signaling purposes – in order to stimulate proliferation or to induce cell death (209-211).

Redox signaling seems to be critically involved in lymphocyte function. Recent findings suggest that TCR stimulation in T cells triggers oxygen radical generation by a T-cell-encoded oxidase (212), and that radicals are mandatory for activation of downstream-signaling pathways (213). However, radicals are also involved in lymphocyte cell death following stimulation with superantigens and mitogens (197, 200, 214), and addition of thiols or expression of surface thiols on the lymphocyte cell surface are critical for cell proliferation and activation (193, 194, 215). Thus, there seems to be a narrow concentration window in lymphocytes where oxidants trigger stimulation, but when the concentration is elevated above this threshold cell death ensues. The apoptotic pathways involved in oxidant-mediated lymphocyte apoptosis have hitherto remained incompletely understood. Some investigators have reported a predominant role for caspases (216), while others have indicated that caspase-independent routes are important (200, 217).

Immune Escape mechanisms

In light of the complexity of the mammalian immune system, one can easily be deceived to believe that it is invincible. However, the immune system is a product of co-evolution with a wide range of infectious microorganisms, including different virus strains. Viruses are obligate intracellular parasites and are constantly under a selection pressure exerted by the immune system. A virus with mutations that result in a host cell phenotype with increased capacity to escape from immune control can survive and propagate, while less-adapted viral phenotypes disappear. In this way, viruses have serendipitously developed multiple strategies to escape from the immune system. On the other hand, the immune system has developed measures to counter-attack viral evasive phenotypes, resulting in a plethora of effector systems that act synchronously to defend the tissues against viral infections.

From the immune system's point of view, there is a certain resemblance between tumor cells and virus-infected cells: both are deviant cells that should be eradicated. Tumor cells do also have to escape from immune-mediated rejection, and new strategies to escape are constantly evolved. Cells that happen to develop an immunoevasive phenotype will survive and continue to proliferate. An important difference is that viral evasion strategies are based on inhibitory effects of viral proteins, while tumor immune evasion is achieved by disruption of normal cellular functions as a consequence of mutations in crucial genes.

The first immune response to cell transformation or virus infection is supposed to be triggered in the cell itself, i.e. programmed cell death. However, mutations in the genes encoding structures in the apoptotic machinery are common in transformed cells, and resistance to apoptosis is one of the hallmarks of cancer (218). Also virus-infected cells can evade apoptosis since several viruses encode proteins that block the apoptotic program in the host cell, for example by mimicking the anti-apoptotic activity of bcl-2 or cytosolic caspase inhibitors (6).

A deviant cell that declines to undergo apoptosis will soon attract the attention of the immune system. In tumors, the activity of the immune system is a double-edged sword. In some cases, immune effector cells manage to discover and eradicate all tumor cells; however, often only a part of the tumor cells is eliminated, and studies have shown that the remaining tumor cells are often more resistant to immune-mediated rejection (219). Thus, the immune system is protective but can at the same time drive a Darwinian selection of immunoresistant tumor cells in a process known as cancer immunoediting (220, 221).

In the editing process of the tumor cells, various tumor escape variants can occur. Analogously, viruses have evolved ingenious mechanisms to directly block immune cell recognition, as exemplified below:

i) *Downregulation of antigen presentation:* Both tumor cells and virus-infected cells avoid CTL recognition by blocking the presentation of peptides on MHC class I (222). According to the "missing self" principle presented above (56), this would lead to increased susceptibility to NK cells if other measures were not taken.

ii) *Upregulation of inhibitory ligands:* To evade NK cell recognition and lysis, viruses encode MHC class I homologues that cannot present peptides, but ligate inhibitory receptors on NK cells (41).

iii) *Downregulation of activating ligands:* Cytomegalovirus-encoded proteins sequester ligands to the activating receptor NKG2D and prevent their expression on the surface of the infected cell (223). Tumor cells can deregulate the expression of activating ligands (62), and there are tumors that shed the NKG2D ligand MICA, which results in immune cell dysfunction (224).

Apart from interfering with the cytotoxic lymphocyte recognition of target cells, viruses and tumors employ various mechanisms to obstruct the immune response. An important mechanism of immune escape is the production of cytokines that either suppress effector cell functions directly or induce recruitment of suppressive immune cells to the tumor or to the site of infection, and subsequent activation of these cells.

In recent years, considerable interest has been directed towards $CD4^+CD25^+$ regulatory T cells (T_{reg}), which can mediate immune tolerance by suppressing autoreactive T cells, mainly by producing TGF-β and IL-10 (225). It has been reported that T_{reg} cells also migrate to tumors, where they inhibit anti-tumor responses (226, 227). The suppressive effect of regulatory T cells is not restricted to T cells as T_{reg} cells reportedly dampen NK cell functions (228). Accordingly, depletion of regulatory T cells triggers effective immune responses against tumors in mice (229). Studies in humans and mice show that tumors and tumor-associated macrophages in fact produce chemokines and TGF-β that trigger recruitment and proliferation of regulatory T cells in the tumor tissue (226, 229).

Tumors and viruses also take advantage of the immunosuppressive properties of myeloid cells (230). Monocytes are actively recruited to the tumor as a result of tumor

cell-mediated production of various cytokines (231, 232). The presence of these tumor-associated macrophages (TAMs) is usually associated with poor prognosis in terms of tumor spread and survival in human malignant disease. In fact, high density of TAMs in tumor tissue correlates with poor survival prognosis in 80% of all studies (233). In mice, similar myeloid suppressor cells (MSCs) have been described, which have phenotypic resemblance to both granulocytes and monocytes. Several immuno-suppressive mechanisms have been proposed to mediate the actions of MSCs, including degradation of the essential amino acid L-arginine (234), production of TGF-β (235, 236), and oxygen radicals (237, 238).

As mentioned above, a dominant source of oxygen radicals is the phagocytic NADPH oxidase, and recent studies have indicated an important immunoregulatory role for this enzyme system (239, 240). Studies have shown that rodents lacking a functional NADPH-oxidase display increased susceptibility to autoimmune disease (19, 194, 239), implying a physiological role for oxygen radicals in limiting lymphocyte activity. Accordingly, numerous reports also suggest a role for oxidants in the immuno-suppression reported in human malignancies and chronic viral infections (17, 241-246). These reports are bolstered by several *in vitro*-studies showing that phagocytes inhibit lymphocyte effector functions and induce cell death (17, 247-250), and that these suppressive effects are reversed by blocking oxygen radical production (248-250), or by adding oxidant scavengers (17, 251).

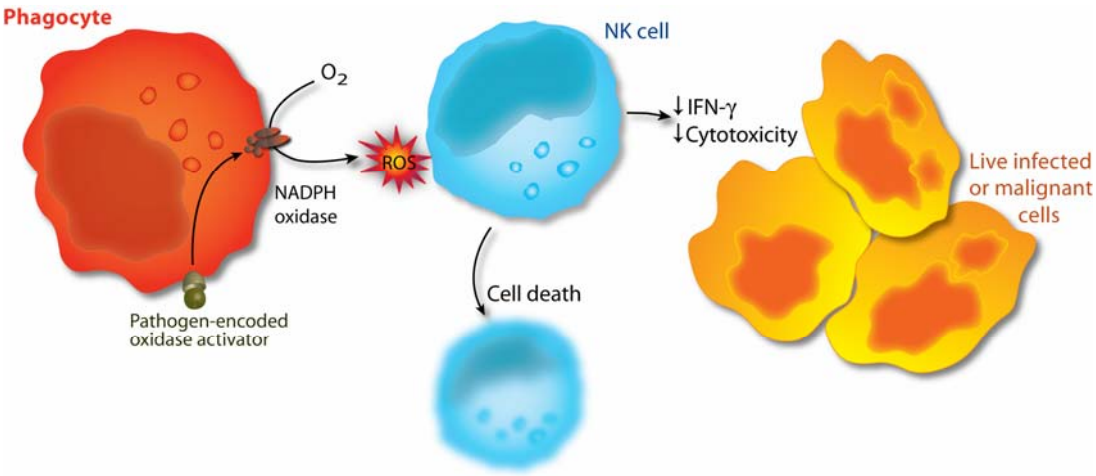

FIGURE 4. Phagocyte activation as an immunoevasive strategy.
In malignant and infected tissues, phagocytes are recruited with ensuing production of oxygen radicals. The release of oxygen radicals from phagocytes inactivates NK cells and other cytotoxic lymphocytes, enabling survival of malignant or virus-infected cells (adapted from 298).

Reflecting the importance of radicals in immune escape, several pathogens encode structures that are capable of recruiting phagocytes and of triggering oxygen radical production in these cells (252-256). These structures thus enable microorganisms to incapacitate and induce cell death in cytotoxic lymphocytes (Figure 4). Furthermore, in this way microorganisms may exploit a host feedback mechanism that limits excessive inflammatory responses: In the presence of apoptotic cells with externalized phosphatidylserine, a functional shift is triggered in macrophages, resulting in release of anti-inflammatory cytokines and resolution of inflammation (21, 22, 257).

Aims

Cellular degradation orchestrated by the family of caspases has been regarded to constitute the principal mechanism of programmed cell death. In recent years it has become clear that death-signaling is more complex than originally thought, and that there are several caspase-independent pathways leading to cell death. The aims of the first part of this thesis (papers I and II) were to investigate the molecular mechanisms underlying oxidant-induced cell death in lymphocytes, to identify potential targets for pharmacological inhibition of this pathway, and to test the potential utility of such inhibitors in *in vitro*-models of oxidant-induced immunosuppression.

The aims of the second part were to characterize the sensitivity of NK cell subsets to oxygen radical-induced toxicity (papers III and IV), and to explore the role of antigen-presenting dendritic cells under conditions of oxidative stress (paper V).

Methodological considerations

The experimental procedures are described in detail in the individual papers (I-V). In this section, considerations regarding some of the methods are discussed.

Leukocyte separation and differentiation

Separation of mononuclear and polymorphonuclear cells

Peripheral venous blood was obtained as freshly prepared leukopacks from healthy blood donors. The blood was mixed with Iscove's modification of Dulbecco's Modified Eagle's Medium (IDMEM), Dextran and acid citrate dextrose. After incubation for 15 minutes at room temperature, the supernatant was carefully layered on top of a Ficoll-Hypaque density gradient. After centrifugation, mononuclear cells were collected at the interface, while polymorphonuclear cells were recovered from the pellet.

Comments: Erythrocytes contain large amounts of catalase, and it is essential to remove these cells as efficiently as possible in studies of oxygen radical-dependent events. For this purpose we use Dextran, which increases the sedimentation rate of erythrocytes (258). Acid citrate dextrose is used as an anticoagulant (259).

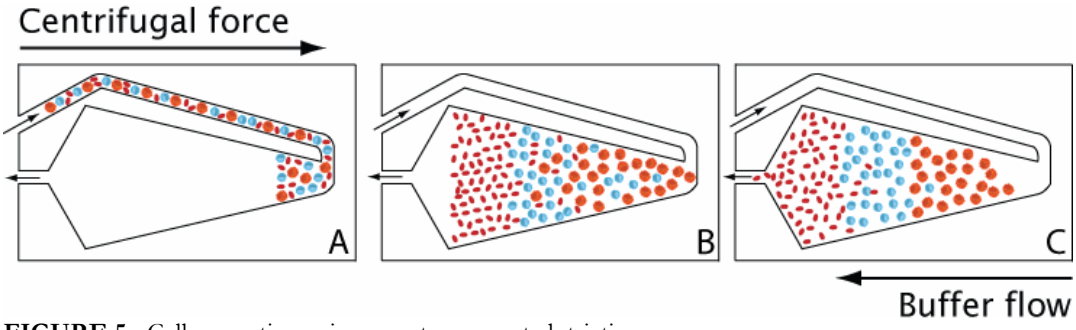

FIGURE 5. Cell separation using counter-current elutriation.
(A) Cells are fed into a spinning rotor where the centrifugal force is balanced by a counter-directed buffer flow. (B) Depending on size, cells are differentially affected by the centrifugal force and separation occurs. (C) By slowly increasing the flow rate, fractions of cells of well-defined sizes can be recovered.

Counter-current elutriation

Mononuclear cells were further separated into lymphocytes and monocytes using a counter-current centrifugal elutriation technique originally described by Yasaka and co-workers (260). Cells were resuspended in buffered NaCl, supplemented with 0.5% bovine serum albumin and 0.1% EDTA, and fed into a spinning rotor/chamber. In the chamber, the centrifugal force is balanced by a buffer stream that flows in the opposite

direction. By slowly increasing the flow rate, fractions of cells of well-defined sizes can be collected (Figure 5). From this separation procedure, a fraction with >90% mononuclear phagocytes was recovered. Lymphocyte fractions enriched for NK cells and T cells contained <3% phagocytes.

Comments: The counter-current elutriation technique allows gentle separation of different mononuclear subsets without addition of any substances or antibodies. In contrast to other methods used to generate pure preparations of mononuclear phagocytes (plastic adherence, positive selection of $CD14^+$ cells using magnetic beads), the elutriation technique does not artificially activate the phagocytes.

Cell sorting: magnetic beads and fluorescence-activated cell sorting

Elutriated lymphocytes were in some experiments enriched for NK cells using negative depletion with magnetic beads (IMag from BD Biosciences). In brief, lymphocytes or mononuclear cells were incubated with an antibody cocktail, which labels all unwanted cells but leaves the desired cells unlabeled. The labeled cells were subsequently removed using a magnetic field.

For cell sorting using a fluorescence-activated cell sorter (FACS), cell preparations were labeled with fluorochrome-conjugated antibodies directed to relevant antigens. Live lymphocytes were identified using a side scatter versus forward scatter plot (ssc vs fsc), and the different lymphocyte subsets were identified using standard bolean gating methods. The sorts were performed on a FACSAria equipped with FACSDiva software (BD Biosciences)

Comments: When studying cells in vitro, it is important to consider the inherent limitations associated with each cell separation method. Every method imposes some stress on the sorted cells, and caution is therefore warranted. Are the observed differences between two subsets in an experimental setup due to differential responses to the pharmacological treatment, or are they merely the result of differential sensitivity to the cell separation procedures? To circumvent these confounders, it is usually helpful to validate results by using different separation techniques.

Magnetic bead separation is a method to quickly obtain fairly pure populations of the cell type in question. An advantage with negative depletion techniques is that the enriched cells are not labeled with antibodies. This method can also be used as an enrichment step before FACS sorting of rare cells.

FACS sorting is an invaluable tool for obtaining preparations of highly purified lymphocyte subsets. The major disadvantages are i), that cells have to be pre-stained with fluorochrome-labeled antibodies, and ii), that they are exposed to high pressure, which can affect viability and function.

Lymphocyte cell death

In this thesis, several methods were employed to monitor different events along the oxidant-induced cell death pathway in lymphocytes. It has to be kept in mind that cell death is a dynamic process that changes over time. Most detection methods are restricted to identifying cells that are currently undergoing apoptosis. Thus, these methods can fail to identify cells that have already undergone apoptosis and lost plasma membrane integrity (indistinguishable from cells dying as a result of a necrotic process) or cells that are destined to die but have not yet obtained any visible signs of apoptosis. In experiments where the aim is to quantify cell death, it is thus advantageous to assess cell death at a late time point, to minimize the risk of failing to identify cells that seem healthy but are destined to die.

Altered light scatter

Approximately 8 hours after exposure to oxidants, dying lymphocytes start to display altered light scatter as determined by flow cytometry. Thus, end-stage apoptotic lymphocytes were identified as cells displaying a reduced forward scatter and a slightly increased side scatter.

Depolarization of the mitochondrial transmembrane potential

As discussed in detail above, MOMP is the point of no return in many cell death processes. MOMP is preceded by a depolarization of the mitochondrial transmembrane potential ($\Delta\Psi_m$). Several reagents can be used to monitor this event. In these studies, we used the mitochondrial membrane sensor kit (BD Clontech) or JC-1 (Molecular Probes). In lymphocytes with an intact $\Delta\Psi_m$, these reagents accumulate in the mitochondria and form aggregates that can be detected as orange fluorescence. In cells with altered $\Delta\Psi_m$, the reagent predominantly is in monomeric form, which emits light in the green part of the spectrum. Lymphocytes with altered $\Delta\Psi_m$ can thus be identified as cells with increased green fluorescence and slightly decreased orange fluorescence using flow cytometry.

Extracellular exposure of phosphatidylserine

In healthy cells, phospholipids are asymmetrically distributed between the two leaflets of the plasma membrane. This distribution is maintained by an enzyme known as the aminophospholipid translocase. In apoptotic cells, this enzyme is inactivated, and another enzyme, which catalyzes the randomization of phospholipids, is activated. As a consequence, phosphatidylserine is exposed on the surface of apoptotic cells (261). Annexin V specifically binds to phosphatidylserine, and fluorochrome-conjugated annexin V can be utilized to identify apoptotic cells.

Increased plasma membrane permeability

A late event in *in vitro* assays of cell death is loss of the structural integrity of the plasma membrane. Membrane-impermeable dyes with different spectral properties can be used to monitor cell integrity. In the studies included in this thesis, different stains were used depending on the other fluorochromes in the panel: ViViD (excitation: 405nm; emission: 450nm), Sytox green (488nm; 515nm), 7-AAD (488nm; 650nm) To-Pro-3 (633nm; 670nm).

Caspase activation

Lymphocytes exposed to hydrogen peroxide or oxygen radical-producing phagocytes were assayed for caspase activation using a fluorochrome-labeled inhibitor of caspases (FLICA) assay. This reagent specifically binds to the active site of active caspases, and the fluorescence detected is thus a measure of caspase activation, allowing determination of the percentage of cells with active caspase-3 using flow cytometry. Caspase-3 activation was also monitored using a substrate for caspase-3. In cells with activated caspases, the reagent is cleaved to a fluorescent molecule, and cells with active caspases can be detected using flow cytometry.

NK cell function

K562 cells, an NK-cell-sensitive cell line originating from a patient with chronic myleogenous leukemia in erythroleukemic blast crisis (262), were used as target cells for studying NK cell exocytosis of secretory lysosomes and cytotoxic function.

Exocytosis of lytic granules

As mentioned above, the lytic granules in NK cells are specialized secretory lysosomes. Thus, the membrane of these granules contain lysosome-associated membrane protein

1 (LAMP-1 or CD107a), which is exposed on the cell surface upon degranulation. Using fluorochrome-conjugated antibodies to CD107a, NK cell degranulation in response to target cell encounter can be monitored (263).

Cytotoxicity

For assays of NK cell-mediated cytotoxicity, K562 cells were labeled with the membrane dye PKH-26 (Sigma-Aldrich), and incubated with NK cells for four hours. Before flow cytometry analysis a membrane-impermeable dye was added (e.g. To-Pro-3; TP3), which specifically labels lysed cells. Killed target cells were identified as double-positive, $PKH\text{-}26^+$, $TP3^+$ cells, while live K562 cells displayed PKH-26-staining, but remained unstained by TP3. In some experiments, cytotoxicity was determined using a traditional chromium-release assay. For these experiments, K562 cells were labeled with ^{51}Cr. After incubation with NK cells, supernatant fluids were assayed for radioactivity in a γ-counter.

Comments: These two assays allow monitoring of the cytotoxic activity of NK cells in response to susceptible target cells. The advantage with the degranulation assay is that responses in individual NK cells can be detected. Extracellularly exposed CD107a is promptly re-circulated, and the fluorochrome-conjugated antibody to CD107a must thus be present throughout the incubation. To avoid degradation of internalized fluorochromes in acidified lysosomes, the vesicular traffic inhibitor, monensin, is also added to the cells. Addition of this inhibitor thus increases the fluorescence intensity on the effector cells; however, it cannot be excluded that monensin also negatively affect the process of exocytosis.

Cytotoxicity does not automatically follow from exocytosis of secretory lysosomes, and it is thus mandatory to monitor cytotoxic function. In most studies in this thesis, we have replaced the traditional radioactive ^{51}Cr-assay with a flow-cytometric-based assay using PKH-26 and membrane-impermeable dyes. This novel assay is more sensitive, and it allows analysis of parallel responses in target and effector cells.

Results and discussion

PARP-1 and AIF as mediators of oxidant-induced cell death

Our studies on the mechanisms of cell death in lymphocytes were inspired by previous reports demonstrating that lymphocytes in the tumor microenvironment and in the peripheral blood of subjects with solid or hematological cancers frequently display signs of functional impairment and apoptosis (264-267). The poor function of lymphocytes within and surrounding tumors suggests that malignant cell expansion somehow creates an immunosuppressive environment that counteracts an appropriate immune response against malignant cells.

A wide variety of mechanisms have been proposed to contribute to the phenomenon of cancer-associated immunosuppression. As discussed in a previous section, several of these mechanisms involve macrophages, which are frequently found in the inflammatory infiltrate adjacent to malignant expansion (233). Thus, TAMs have been proposed to dampen lymphocyte-dependent anti-tumor responses by producing immunosuppressive substances including – but not limited to – IL-10, prostaglandins and TGF-β, in addition to facilitating tumor growth by promoting angiogenesis (230). One of the immunosuppressive mechanisms ascribed to TAMs is related to their production of toxic oxygen radicals, which efficiently induce lymphocyte dysfunction and cell death (17, 18, 242, 254, 268). Therefore, we sought to characterize the intracellular events associated with oxygen radical-induced cell death in lymphocytes with antineoplastic functions such as NK-cells and cytotoxic T cells with a focus on their interaction with oxygen radical-producing phagocytes.

Despite the advances in the area of cell death, the molecular mechanisms of oxidant-induced cell death in lymphocytes have remained incompletely understood. One of our initial experiments on the mechanisms underlying oxidant-induced cell death yielded an unexpected and contradictory finding: Lymphocytes that were exposed to oxygen radical-producing phagocytes or exogenous H_2O_2 displayed caspase activation after over-night culture. However, pre-treatment with caspase inhibitors failed to protect them from oxidant-induced cell death, suggesting that cell death proceeded by caspase-independent mechanisms.

In neural models of ischemia-reperfusion injury, which is characterized by a massive formation of oxygen radicals (181), several studies point to a role for poly(ADP-ribose)polymerase-1 (PARP-1) in the process of neural cell death (178, 184, 188, 190,

269, 270). To investigate whether the PARP-1 enzyme was of importance in oxidant-induced cell death in cytotoxic lymphocytes we pre-incubated lymphocytes with two different PARP-1 inhibitors before exposing them to oxidants. As shown in paper I pretreatment with PARP-1 inhibitors, PJ34 or DPQ, almost completely protected lymphocytes against oxidant-induced cell death, regardless of whether the oxidants were produced by adjacent phagocytes or delivered in the form of exogenous hydrogen peroxide.

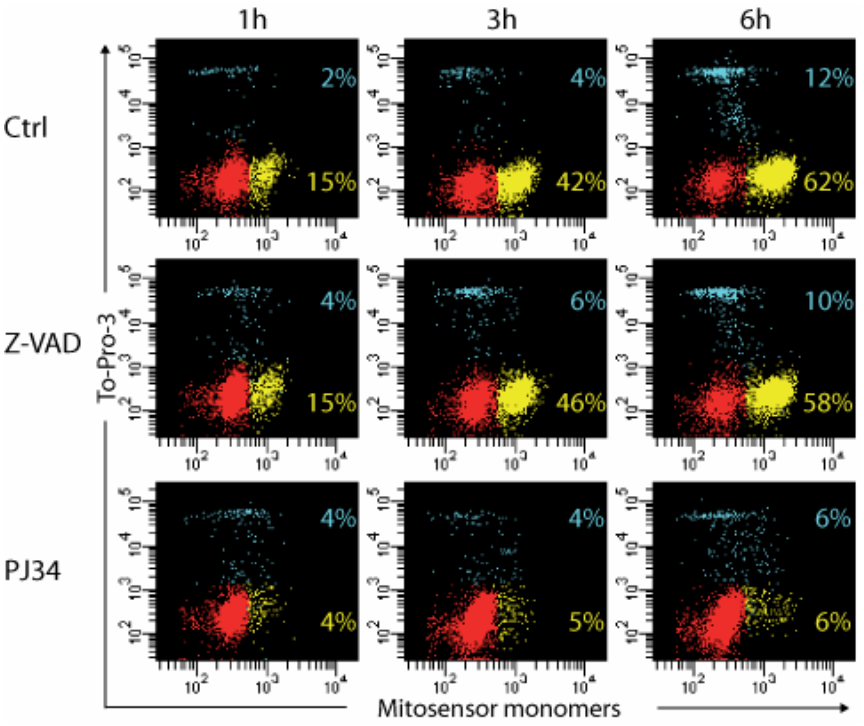

FIGURE 6. Depolarization of the mitochondrial membrane potential after oxygen radical exposure. Depolarization of the Ψ_m is seen as an increase in green fluorescence (mitosensor monomers). Cells with normal light scatter are shown in red, apoptotic cells with altered Ψ_m are shown in yellow, and end-stage apoptotic cells are shown in blue. Lymphocytes treated with H_2O_2 displayed altered Ψ_m already after 1 hour. With time, more cells obtained depolarized mitochondrial membranes, acquired modified scattering properties and became increasingly stained by the membrane-impermeable stain To-Pro-3. The PARP-1 inhibitor, PJ34, protected lymphocytes from oxidant-induced alterations of Ψ_m, while the caspase inhibitor, Z-VAD.fmk, failed to display any protective effect.

In a series of experiments, we studied the effects of PARP-1-inhibitors on downstream events of oxidant-induced cell death. Pretreatment with PJ34 prevented early events, such as depolarization of the mitochondrial transmembrane potential ($\Delta\Psi_m$), as well as later events, including extracellular exposure of phosphatidylserine, and loss of plasma membrane integrity seen in end-stage-apoptotic cells (Figure 6). Additional experiments showed that PARP-1 inhibition also blocked the observed caspase activation in oxidant-induced cell death. Together these results indicated that PARP-1 activation was

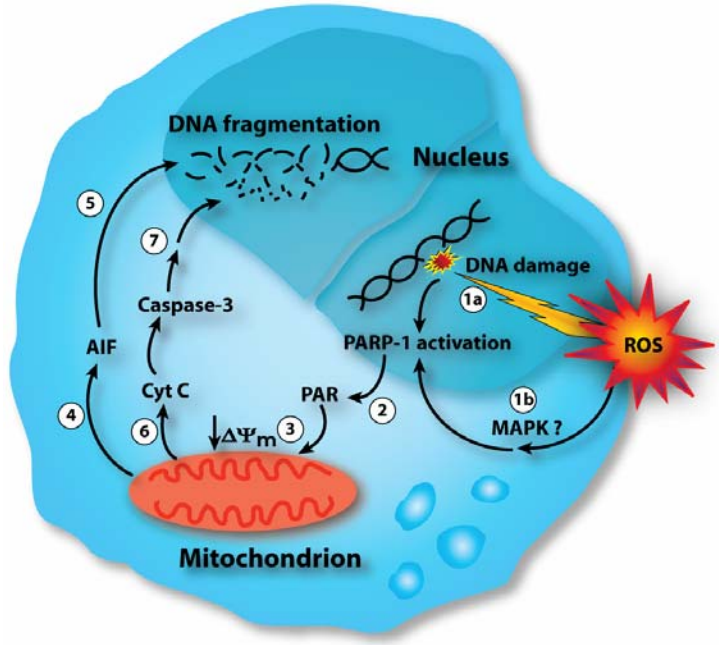

FIGURE 7. Oxidant-induced cell death in lymphocytes. PARP-1 is activated by DNA strand breaks (step 1a). MAP kinases have been identified as additional upstream events of PARP-1 (step 1b). Overactivation of PARP-1 results in the release of free polymers of ADP-ribose (PAR) into the cytosol (step 2). PAR targets the mitochondria, where it induces a depolarization of the mitochondrial transmembrane potential ($\Delta\Psi_m$) (step 3). Subsequently, apoptosis-inducing factor (AIF) is released into the cytosol (step 4). AIF translocates to the nucleus (step 5) where it induces fragmentation of DNA into larger (50 kbp) fragments. However, apart from AIF, cytochrome c is also released from the mitochondria (step 6). The cytochrome c release triggers apoptosome formation and activation of caspase 3. Caspase 3 activates the caspase-activated DNAase (CAD) (step 7), which cuts DNA into shorter fragments.

an early target in the signaling pathway leading to cell death, while caspase activation was a secondary downstream event and not critically involved in the initiation of cell death.

It was recently reported that PARP-dependent cell death and the ensuing MOMP results in release of AIF into the cytosol (178). The released AIF translocates to the nucleus where it induces DNA degradation into large (50,000 bp) fragments (164). To investigate if these downstream events occurred in oxidant-induced cell death in lymphocytes, we performed experiments in which we isolated nuclear extracts from H_2O_2-exposed lymphocytes. By use of immunoblotting, we were able to show an accumulation of AIF in the nucleus of lymphocytes that had been exposed to radicals, and analysis of lymphocyte DNA with pulsed-field gel electrophoresis revealed the presence of characteristic large DNA fragments, suggesting AIF-dependent fragmentation (Paper I). Interestingly, we found that lymphocytes pretreated with caspase inhibitors contained more large DNA fragments. This finding suggests that the observed secondary caspase activation results in activation of caspase-activated DNAse

(CAD) (155), which induces further processing of the DNA into smaller fragments (180 bp); thus, in the presence of caspase inhibitors, the secondary DNA fragmentation is blocked and large fragments accumulate (Figure 7).

The suppressed state of lymphocytes in the malignant microenvironment not only limits immune-mediated elimination of cancer cells, but may also have implications for therapeutic attempts to activate immune effector mechanisms in cancer. Alleviating tumor-induced immunosuppression may therefore be considered as an adjunct to cancer treatment with IL-2, IL-12 or other lymphocyte-activating agents. Analogously, compounds that counteract immunosuppressive signals may be useful in chronic infections; for example, a significant part of the benefit of treatment with interferon-α in chronic hepatitis C is dependent on induction of immune-mediated clearance of infected cells (271). The finding that PARP-1 inhibitors protected cytotoxic lymphocytes from oxidant-induced cell death therefore suggests a possible use of PARP-1 inhibitors in immunotherapy; the preservation of lymphocyte function by use of a PARP-1 inhibitor could be of benefit during treatment with, for example, IL-2 or IFN-α for improved treatment efficiency. However, to be useful as adjuvants, PARP-1 inhibitors should not only maintain the viability of cytotoxic lymphocytes, but surviving cells also have to remain functional.

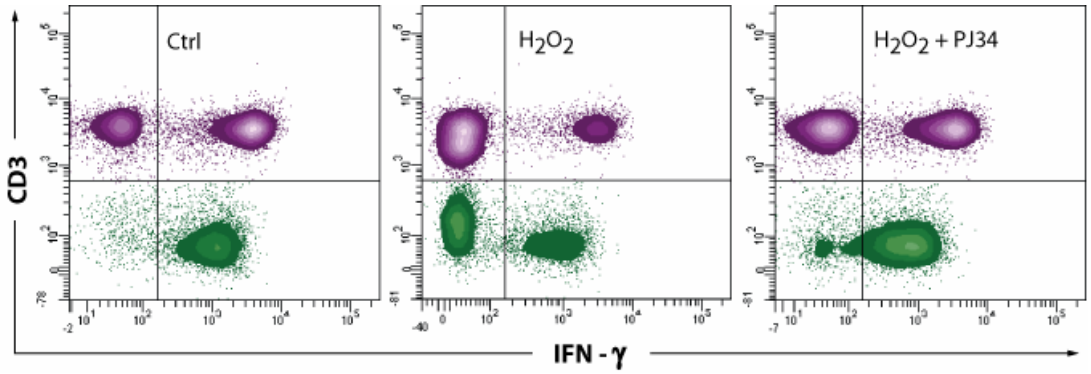

FIGURE 8. Lymphocyte responsiveness to stimulation: role of PARP-1. $CD3^+CD8^+$ Tcells (purple) and $CD3^-CD56^+$ NK cells (green) were less responsive to PMA/ionomycin stimulation in the presence of H_2O_2. Pre-treatment with PARP-1 inhibitor protected lymphocytes and preserved their responsiveness, as measured by intracellular accumulation of IFN-γ.

We therefore set to explore the functional status of lymphocytes after exposure to oxidants and PARP-1 inhibitors. It was found that PJ34-protected NK cells remained capable of releasing their lytic granules upon encounter with susceptible malignant cells, and that the resulting cytotoxicity largely remained intact (Papers I and II). Apart from a role in cell death, PARP-1 is reportedly involved in transcriptional events (272), and we therefore studied the synthesis and release of IFN-γ, which is a transcription-dependent event in lymphocytes. PJ34 did not inhibit *de novo*-synthesis of IFN-γ in

cytotoxic lymphocytes; in contrast, oxidant-exposed, PJ34-protected $CD8^+$ T cells and NK cells produced more IFN-γ in response to in vitro-stimulation than unprotected cells (Figure 8). Furthermore, oxidant-exposed lymphocytes proliferated significantly better if they were pre-treated with a PARP-inhibitor (Paper II).

Taken together, these data imply that PARP-1 inhibition not only protects lymphocytes from oxidant-induced cell death, but also maintains important effector functions. Our findings thus indicate that PARP-1 inhibitors could be of use as adjuvants in immunotherapy: Blocking excessive PARP-1 activity could protect cytotoxic lymphocytes under conditions of oxidative stress in malignant or infected tissues, and serve to improve the efficiency of treatment aiming at activating or expanding cytotoxic lymphocytes.

However, the multiple functions of PARP-1 in cell biology call for caution. PARP-1 has a role in maintaining genome integrity (180, 273, 274) and inhibiting PARP-1 could thus e.g. increase the risk of potentially harmful mutations. Furthermore, the first step in PARP-1-dependent cell death has traditionally been described as DNA damage (275), and blocking repair and cell death in cells with extensive DNA damage seems undoubtedly hazardous.

The question may be asked whether it is possible to pharmacologically interfere with the PARP-1/AIF axis without affecting PARP-1-dependent events such as DNA repair and transcription. Historically, PARP-dependent cell death has been described as necrotic cell death in the "suicide hypothesis" (276). According to this model, PARP-1 activation induces massive production of polymers of ADP-ribose, which results in depletion of cellular stores of energy, mitochondrial dysfunction and cell death (277). However, recent findings suggest that PARP-dependent cell death occurs in the absence of cellular energy collapse (183, 184), as reviewed in a previous section. Instead, PARP-1 activation after exposure to oxidants can trigger a signaling cascade from the nucleus to mitochondria (278). Other investigators have identified the polymers of ADP-ribose (PAR) as the signaling entity targeting the mitochondria and triggering AIF release (185, 186).

Thus, PARP-dependent cell death has moved from being regarded as necrotic cell death due to energy failure to an acknowledged complex signaling pathway leading to programmed cell death. Elucidating the transduction pathways involved in PARP-1-dependent, oxidant-induced cell death in lymphocytes could be helpful in identifying more specific targets for pharmacological inhibition of the PARP-1/AIF pathway. For example, interfering with intracellular signal transduction pathways upstream of PARP-

1 activation by oxidants could serve to protect lymphocytes and other cells from PARP-1-dependent death without impeding PARP-1-dependent DNA repair or transcription (279, 280). Likewise, targeting of the PAR-triggered AIF release from mitochondria could specifically rescue cells from PARP-1-dependent DNA fragmentation and death.

Emerging studies suggest that the PARP-1/AIF axis may not be exclusively involved in phagocyte/oxidant-induced lymphocyte cell death, but operative also in other modes of cell death. Oxidants produced within the cell have been ascribed a role in activation-induced cell death (AICD) in lymphocytes (197, 200, 214, 281). AICD occurs independently of death-receptors or caspase activity (200). Furthermore, a recent study reported that AICD in human melanoma-specific CTLs was mediated by AIF (282). It is thus possible, albeit as yet speculative, that PARP/AIF regulates also this mode of cell death in lymphocytes, which should emphasize the potential value of inhibitors of the PARP-1/AIF pathway as adjuvants to immunotherapy.

NK cell subsets, dendritic cells and oxygen radicals

The two major NK cell subsets, $CD16^{+}CD56^{dim}$ and CD16-$CD56^{bright}$ NK cells have different roles in immunity. $CD56^{dim}$ cells are highly cytotoxic, while $CD56^{bright}$ cells are potent cytokine producers. Along with DCs and NK cells, neutrophils and mononuclear phagocytes are recruited to the site of infection. Upon activation these myeloid cells produce large amounts of oxygen radicals that to some extent are released into the extracellular space. In paper III and IV, we therefore investigated how oxidative stress – in the form of phagocyte-derived or exogenously added oxidants – affected viability and function of the $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets.

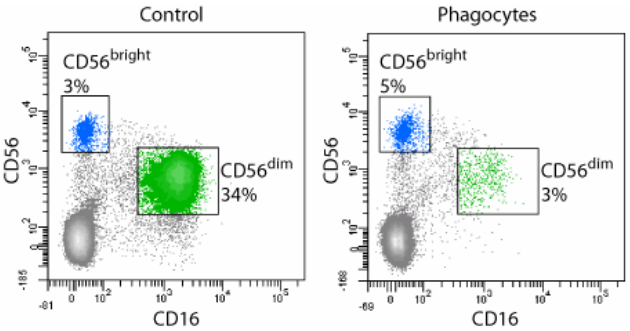

FIGURE 9. Selective cell death in $CD56^{dim}$ NK cells induced by radical-producing mononuclear phagocytes. In the presence of phagocytes, the number of live $CD56^{dim}CD16^{+}$ cells is strongly reduced, while the $CD56^{bright}CD16^{-}$ population remains intact.

As shown in figure 9, the two NK cell subsets displayed a remarkable difference in sensitivity to oxygen radicals. While the majority of $CD56^{dim}$ cells succumbed to the oxidative challenge, $CD56^{bright}$ cells remained viable. These experiments were performed with H_2O_2, neutrophils or mononuclear phagocytes as sources of oxidants, and both on mixed populations of lymphocytes and on pure FACS-sorted NK cells; all of these experimental designs yielded similar results (Paper III).

Although the $CD56^{bright}$ population of NK cells was highly resistant to oxidative stress, a fraction of these cells also died when exposed to oxygen radicals. To investigate if both $CD56^{dim}$ and $CD56^{bright}$ NK cells underwent cell death along the same PARP-1/AIF pathway (Paper I), we pretreated NK cells with PARP-1 inhibitors before exposing them to oxidants. These experiments showed that both subsets were protected to a similar extent by PARP-1 inhibitors, suggesting that the oxidant-induced cell death in the two NK cell subsets follow a common intracellular pathway (Paper III).

As discussed above, $CD56^{bright}$ NK cells are potent cytokine-producing cells (46). Hence, we investigated the cytokine-producing capacity of the two NK cell subsets under conditions of oxidative stress. In the presence of oxygen radicals, $CD56^{dim}$ NK

cells lost their ability to produce IFN-γ while the majority of $CD56^{bright}$ NK cells remained capable of cytokine production.

The resistance of the $CD56^{bright}$ subset of NK cells to oxidative stress implies that these cells are endowed with a more efficient antioxidative defense system than $CD56^{dim}$ cells. Cellular defense against oxygen radicals is a complex network of antioxidative enzymes and scavengers. Central in these systems are the two intracellular thiol-containing scavengers: glutathione and thioredoxin (191), but reduced thiols are also exposed on the cell surface. These cell surface thiols are crucial for lymphocyte function (112, 192-194), but might also act as a first line of defense against oxidants (195). Using the thiol-reactive substance maleimide conjugated to a fluorochrome, we investigated the levels of antioxidative thiols on freshly isolated NK cells. These experiments revealed that $CD56^{bright}$ NK cells express twice as many extracellular thiols as $CD56^{dim}$ NK cells. In addition, we found that $CD56^{bright}$ were significantly more efficient than $CD56^{dim}$ cells at neutralizing exogenously added hydrogen peroxide (Paper III).

The outcome of the encounter between a malignant cell and an NK cell is determined by the balance between activating and inhibitory signals in the NK cell. Thus, expression of activating receptors on the NK cell surface is crucial for NK cell activation. Low expression of activating receptors reduces the magnitude of activating signals in the NK cell upon interaction with a malignant cell, and thus the risk increases for the balance to tip towards inhibition (Figure 3). Accordingly, NK cells with low expression of the activating natural cytotoxicity receptors (NCRs) typically display low NK-cell-mediated cytotoxicity (63).

Recently, Costello and coworkers found that patients suffering from acute myeloid leukemia (AML) commonly display a deficient NCR expression, which may serve to explain why NK cells from these patients are frequently inefficient at killing leukemic blasts (283). The importance of NK cells in AML is reflected by reports showing that alloreactive NK cells from allogeneic stem cell transplants reduce the risk for leukemic relapse in AML (284, 285). In these transplantations, a benefical outcome of transplantation was observed when donor NK cells lacked inhibitory KIRs for HLA class I molecules expressed on recipient cells. Thus, due to this KIR-HLA mismatch recipient cells failed to trigger inhibitory signals in donor NK cells, and instead NK cells were activated and triggered alloreactions. These reactions induced a potent graft versus leukemia effect, which significantly reduced the risk of relapse (284, 285).

Furthermore, there are studies suggesting that functional impairment in NK cells is associated with early relapse in AML (286-288), and an inverse correlation between the

presence of NCR^{dull} NK cells and survival in AML patients has been reported (289). In a recently conducted phase III trial, immunotherapy with IL-2 and the NADPH oxidase inhibitor histamine dihydrochloride improved leukemia-free survival in adult patients with AML in complete remission (290). These beneficial effects of a combination of immunostimulatory and antioxidative treatment on leukemia-free survival, together with the correlation between NCR expression and survival in AML, incited us to investigate if expression of activating NK cell receptors is regulated by oxidants.

NK cells constitutively express the activating receptor NKG2D and the NCR NKp46, but studies on the regulation of these cell surface receptors are relatively sparse (63, 76). In paper IV, we report that mononuclear phagocytes caused a pronounced downregulation of the expression of both NKp46 and NKG2D in $CD56^{dim}$ NK cells. This effect was apparently mediated by NADPH oxidase-derived radicals since catalase, which detoxifies hydrogen peroxide, and histamine, which inhibits NADPH oxidase activity, both blocked the phagocyte-induced down-regulation. The $NKG2D^{dull}NKp46^{dull}$ phenotype was similarly induced by activated neutrophils. In contrast, $CD56^{bright}$ NK cells maintained their expression of NKp46 and NKG2D after exposure to phagocyte-derived radicals. In fact, activated neutrophils enhanced the expression of NKp46 on $CD56^{bright}$ NK cells. The mechanisms underlying this upregulation of activating NK cell receptors remain to be characterized, but it apparently occurred independently of oxidants (Paper IV).

Taken together, the results presented in papers III and IV point to a striking difference between the two major NK cell subsets regarding their sensitivity to oxygen-radical-induced inactivation and cell death. This difference may be due to the higher capacity of $CD56^{bright}$ NK cells to detoxify oxidants, which in turn may be related to the higher cell surface expression of anti-oxidative thiols on these cells.

Oxygen radical-induced inactivation of lymphocytes has recently been suggested as an important mechanism to avoid autoimmunity. Rodents expressing a non-functional phagocytic NADPH oxidase were shown to be more prone to develop arthritis than wild-type control animals; this phenomenon was explained by oxygen radical-induced inactivation of autoreactive T cells (19, 194, 239). Thus, the inactivation of $CD56^{dim}$ NK cells by oxygen radicals seen in our studies may be a protective measure to avoid harmful immune reactions. Such inhibition might be unnecessary for $CD56^{bright}$ NK cells since they display low cytotoxicity and thus are less likely to trigger autoimmune reactivity. Furthermore, the preservation of $CD56^{bright}$ cells in an environment of

oxidative stress is coherent with the view that this NK cell subset may promote the resolution of inflammation (291-293).

Another reason for the resistance of $CD56^{bright}$ NK cells to oxidants could be related to the growing evidence implying that $CD56^{bright}$ NK cells constitute a progenitor population to cytotoxic $CD56^{dim}$ NK cells (53). A recent study suggests that $CD56^{bright}$ lymph node NK cells can differentiate into cytotoxic effectors in response to IL-2. It could thus be hypothesized that the innate forces comprise a pool of ready-to-strike NK effectors that, if out of control, are easily inhibited by oxygen radicals, and a large oxygen radical-resistant pool of lymph node-situated $CD56^{bright}$ NK cells that can be activated by IL-2 and mobilized when needed.

The results in papers III and IV suggest that oxygen radicals affect cytotoxic NK cells on multiple levels: apart from inhibiting cytotoxicity, inducing cell death and blocking cytokine production, oxidants also triggered a downregulation of activating receptors on NK cells. As mentioned above, downregulation of NCRs was associated with reduced NK cytotoxicity against leukemic blasts and increased risk of relapse and death from AML (283, 289). The reason for the reduced NCR expression in AML is unknown. *In vitro* studies have shown that TGF-β can suppress the expression of NKp30 and NKG2D (294), and *in vitro* culture of NK cells with leukemic blasts from NCR^{dull} patients resulted in decreased expression of NKp30. The findings presented in paper IV, i.e. that oxygen radicals produced and released from phagocytes trigger down-modulation of NKp46 and NKG2D on cytotoxic NK cells, implies that oxidative stress may contribute to the NCR^{dull} phenotype observed in AML. Since AML blasts are myeloid cells, a theoretical source of radicals could be the blasts themselves. However, we have not been able to observe oxygen radical production in isolated blasts. The oxidase components are expressed at a rather later stage in myeloid maturation, and might thus be absent in primitive AML blasts. If the mechanism of NCR and NKG2D down-modulation described in paper IV contributes to the poor expression of these activating NK cell receptors in AML, it therefore seems more likely that adjacent, non-malignant phagocytes are the source of inhibitory radicals.

Dendritic cells are mature myeloid cells and are thus equipped with a functional NADPH oxidase (295). However, the level of expression is low, presumably reflecting the role for DC-produced radicals in antigen processing and presentation rather than in microbial killing (240, 296). In line with these studies, DCs were unable to induce cell death in co-cultured lymphocytes (Paper V). Instead, addition of DCs to lymphocytes that were exposed to oxidants, either delivered by mononuclear phagocytes or in the form of exogenous hydrogen peroxide, efficiently prevented induction of cell death in

NK cells, as well as $CD4^+$ and $CD8^+$ T cells. Investigations of NK cell function after exposure to oxidants in the presence of DCs showed that DCs efficiently maintained NK cell-mediated cytotoxicity against malignant target cells in the presence of oxygen radicals (Figure 10).

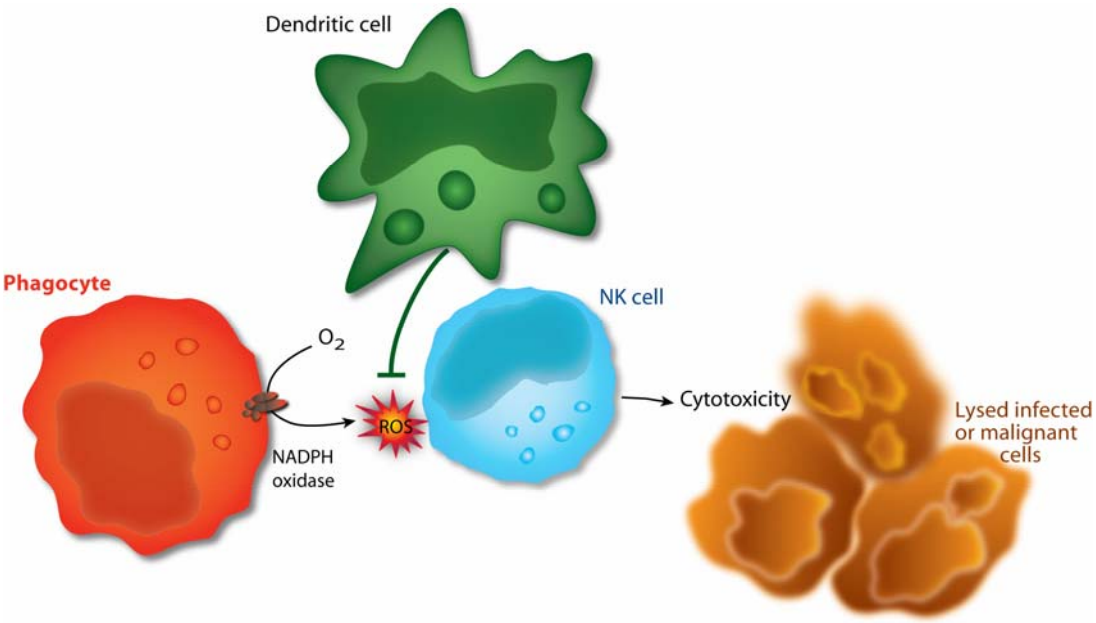

FIGURE 10. Dendritic cells preserve NK cell viability and function in the presence of oxidants. Phagocyte-derived oxygen radicals inactivate NK cells and induce cell death. Dendritic cells neutralize these oxidants and restore NK cell functions, resulting in lysis of malignant cells.

Taken together these data imply that DCs either neutralized oxygen radicals or modulated lymphocyte sensitivity to oxidants. To test the first hypothesis, we investigated the capacity of DCs to neutralize exogenous oxygen radicals. DCs or 7-day cultures of adherent mononuclear phagocytes were exposed to 30μM H_2O_2 for 15 minutes, and the remaining H_2O_2 was measured. These experiments showed that DCs were significantly more efficient than the adherent mononuclear phagocytes at neutralizing H_2O_2. An inhibitor of catalase, 3-amino-triazole, partially reduced the DC capacity to scavenge H_2O_2 and thus also reduced the protective effect of DCs when added to oxidant-exposed lymphocytes (Paper V), thus implying that DC expression of catalase may contribute to the protection of lymphocytes against oxidants.

As previously described, glutathione and thioredoxin are two essential antioxidative thiols in cells. The strong antioxidative properties of DCs incited us to assess the surface levels of thiols on both DCs as well as on lymphocytes after incubation with DCs. These experiments revealed that DCs expressed high levels of antioxidative thiols and, importantly, up-regulated thiol expression on lymphocytes. This effect was

observed already after one hour of DC/lymphocyte co-culture and was even more pronounced after over-night incubation (Paper V).

The results presented in paper V suggests that DCs are endowed with an efficient antioxidative system. By neutralizing oxygen radicals and by supplying NK cells and T cells with antioxidative thiols, DCs may be able to uphold lymphocyte functions in hostile environments, such as malignant tissue or sites of infections. Our results are coherent with a report by Angelini et al., who found that DCs can release thioredoxin upon interaction with antigen-specific T cells (112). Furthermore, proteomic analyses have revealed that several antioxidant proteins are upregulated in DCs differentiated from mononuclear phagocytes (297), thus suggesting that multiple pathways cooperate in protecting lymphocytes against oxidative stress.

Our finding that DC protects lymphocytes from oxidant stress may primarily be relevant for $CD56^{dim}$ NK cells. Recent reports of innate immune responses and the development of the adaptive immune response have highlighted the importance of bilateral DC/$CD56^{dim}$ NK cell interactions in the tumor area or at the site of infection (8, 122). Under the harsh conditions prevailing due to phagocytic NADPH oxidase activity, the presence of DCs might be a prerequisite for an appropriate function of NK cells given their high sensitive to oxygen radicals. The reducing environment offered by DCs may enable NK cells to become activated, leading to increased cytotoxicity, IFN-γ production and thus stimulation of DC maturation.

Dendritic cells that fail to mature continue to express low levels of HLA-E and can thus be eradicated by activated NK cells (117-119). In this way only appropriately mature DCs reach the secondary lymph nodes, which may secure the development of a Th1 response. The protective effect of DCs also offers a tentative additional role for elimination of DCs by NK cells. NK cytotoxicity towards DCs may provide a negative feedback mechanism for the immune response; excessive NK activity will enhance DC clearance. In this way the protective environment provided by DCs is abolished and attenuation of NK and T cell responses may follow.

Concluding remarks

The activity of the human immune system must be perfectly balanced. Immediate and powerful responses to invading pathogens are crucial to protect the host from fatal infections. Yet, a fierce immune response may also threaten the survival of the host. The same balance is needed to recognize the subtle cellular changes associated with infection or malignant transformation but still leave organs and tissues unharmed. In order to keep immune effector systems in check and avoid autoimmunity, several mechanisms of inhibition run in parallel.

A growing body of evidence implies that oxygen radicals have a regulatory role in immunity. At low concentrations, intracellularly formed radicals have a stimulating effect on T cell responses (212, 213), while higher concentrations of extracellularly released ROS following phagocyte activation triggers inactivation and cell death (17, 18, 248). The phagocyte-mediated suppression of lymphocyte function is of importance in regulating autoimmunity, as a defective NADPH oxidase renders rodents more prone to autoimmune reactions (239). However, in malignancies and chronic viral infections these immunoregulatory mechanisms may be exploited by the malignant or infected cells to evade anti-tumor and anti-viral immune responses. Thus, tumor-associated phagocytes produce oxygen radicals that inactivate and induce cell death in lymphocytes (17).

As shown in Paper V, the downregulatory effect of oxygen radicals on lymphocyte functions may be modulated by dendritic cells. DCs can neutralize oxygen radicals and thus protect NK cells from inactivation, which may be an important asset of DCs to enable the bilateral activation program between DCs and NK cells, and also may have implications for the development of adaptive immunity (8, 122).

Not all NK cells are sensitive to oxygen radicals. While, $CD56^{dim}$ NK cells were inactivated and underwent cell death, $CD56^{bright}$ NK cells remained viable and functional when exposed to oxidants, as shown in Papers III and IV. The biological significance of these findings should be the subject of further investigation, but it may speculated that the need for a ready-to-strike mechanism of inactivation is more urgently needed for the highly cytotoxic $CD56^{dim}$ cells than for their $CD56^{bright}$ counterpart, which is only weakly cytotoxic and primarily regarded as a source of immunomodulatory cytokines. Interestingly, accumulating evidence identifies $CD56^{bright}$ NK cells as a progenitor cell. Upon stimulation with IL-2 lymph node-derived $CD56^{bright}$ NK cells acquire cytotoxic functions (52) and it could thus be speculated that these cells constitute a mobilizable pool of effector cells. Further studies are warranted

to investigate if these differentiated cytolytic $CD56^{bright}$ cells remain resistant to oxidative stress.

This thesis also comprised studies on the mechanisms underlying oxidant-induced cell death in lymphocytes, and the results point towards a critical role of the PARP-1/AIF pathway. Thus, PARP-1 inhibitors efficiently protected cytotoxic lymphocytes from cell death and preserved lymphocyte effector functions (Paper I and II). These findings suggest that oxidant-induced apoptosis in lymphocytes is the result of an intriguing cooperation between mitochondria (as the source of AIF) and the cell nucleus (which harbors PARP-1). Also, the results imply that blocking of PARP-1 activity could be useful in improving lymphocyte function under conditions of oxidative stress such as in chronically infected tissue or in the malignant microenvironment.

A concern with pharmacological inhibition of PARP-1 relates to its role in DNA repair, and the use of a PARP-1 inhibitor may thus result in potentially harmful DNA mutations. Ideally, an anti-oxidative compound that targets the PARP-1/AIF axis should specifically inhibit the induction of apoptosis, without adversely affecting genomic integrity. Recently, PAR was recognized as a signaling entity between the nucleus and the mitochondria (Figure 7) (185, 186); in addition, signaling transduction pathways have been observed leading to PARP-1 activation (279, 280). These novel findings may lead to the identification of new strategies to specifically target the apoptosis-triggering function of PARP-1, thereby protecting not only lymphocytes but also other cellular histiotypes from oxidant-induced toxicity.

Populärvetenskaplig sammanfattning

Att celler dör är en förutsättning för att kroppen skall fungera. Under fostertiden bildas t.ex. fler celler än vad kroppen slutligen behöver. De överblivna cellerna aktiverar ett celldödsprogram som möjliggör en kontrollerad nedbrytning utan att omgivande celler skadas. I immunförsvaret används denna så kallade programmerade celldöd för att minimera riskerna för autoimmuna sjukdomar. Immunceller som skulle kunna skada kroppsegna celler identifieras på ett tidigt stadium och elimineras. Liknande mekanismer används också för att dämpa immunförsvaret efter en avklarad infektion. Denna grundliga kontroll av immunförsvaret är nödvändig, eftersom de immunreaktioner som initieras i samband med en infektion kan leda till angrepp på den egna vävnaden.

Det finns dock en inneboende fara med immunförsvarets noggranna reglering. Vid exempelvis virusinfektioner eller tumörsjukdomar får dessa dämpande mekanismer inte äventyra avdödningen av främmande celler. Tumörceller och virusinfekterade celler kan dock utnyttja systemen för att stänga av immunförsvaret. Detta kan leda till att immunsystemets celler misslyckas med att slå ned en infektion eller att förhindra utveckling av malign sjukdom.

Mitt avhandlingsarbete har omfattat studier av en grupp av ämnen som kroppen använder för att stänga av immunförsvaret – så kallade syreradikaler. I djurförsök har man visat att djur som inte kan producera syreradikaler löper ökad risk för att utveckla autoimmuna sjukdomar. Vid flera typer av cancersjukdom har det tvärtom visats att överproduktion av radikaler tycks kunna bidra till att immunförsvaret misslyckas med att eliminera tumörceller.

Syreradikaler finns närvarande i hög koncentration vid flera typer av immunreaktioner. Normalt utgör de en del av kroppens försvar mot mikroorganismer, men de kan även orsaka programmerad celldöd i immunförsvarets lymfocyter, såsom T-celler och naturliga mördar-celler (NK-celler, av engelskans ”natural killer”). I våra studier har vi studerat vad som sker i lymfocyter när de utsätts för syreradikaler. Vi har visat att de processer som aktiveras inte är de som traditionellt används vid programmerad celldöd i andra celler. I provrörsförsök kunde vi förhindra att immunförsvarets lymfocyter stängdes av genom att tillföra substanser – inhibitorer av enzymet PARP-1 – som blockerade celldödsprogrammet. I närvaro av dessa inhibitorer kunde således T-celler och NK-celler dels fortsätta att döda tumörceller, dels producera substanser som aktiverar andra immunceller trots närvaro av syreradikaler. Dessa fynd, som redovisas i avhandlingens arbete I och II, bidrar till ökad förståelse av de processer som sker i

lymfocyter som utsätts för radikaler, men kan också tänkas ligga till grund för utveckling av läkemedel som skyddar lymfocyter och andra celler från syreradikalers toxiska effekter.

I avhandlingens delarbeten III, IV och V studerades immuncellers förmåga att tolerera syreradikaler. Arbete III och IV omfattar studier av NK-celler, som har en unik förmåga att känna igen och avdöda främmande celler. Dessa lymfocyter förekommer i två huvudsakliga former som uppvisar olika mängder av den NK-cellstypiska strukturen CD56 på cellytan: $CD56^{dim}$-celler har få CD56-molekyler medan $CD56^{bright}$-celler har upp till 10 gånger fler. $CD56^{dim}$-cellerna är vanligt förekommande i blodbanan och dödar effektivt tumörceller och virusinfekterade celler, medan $CD56^{bright}$-celler främst återfinns i lymfkörtlar där de producerar immunreglerande substanser.

Arbete III och IV visar att dessa två celltyper drastiskt skiljer sig åt vad avser motståndskraft mot syreradikaler. $CD56^{dim}$-celler är ytterst känsliga för radikaler och inaktiveras eller dödas när de utsätts för syreradikaler i låg koncentration, medan $CD56^{bright}$-celler är påtagligt okänsliga. Denna motståndskraft tycks kunna förklaras av att $CD56^{bright}$-celler har en förmåga att neutralisera syreradikaler. Fynden talar för att kroppen kan använda radikaler för att selektivt stänga av vissa typer av immunceller i kroppen, medan andra förblir opåverkade.

I delarbete V visas att en typ av immunceller, dendritceller, effektivt skyddar lymfocyter från att inaktiveras av radikaler. Det är sedan tidigare känt att dendritceller är viktiga för immunförsvar mot såväl mikroorganismer som tumörceller, främst genom att presentera komponenter från främmande celler för T-lymfocyter (s.k. antigenpresentation). Vår upptäckt talar för att dendritceller har en tidigare okänd uppgift i immunförsvaret genom att bidra till att immunsystemets lymfocyter kan upprätthålla sina funktioner trots närvaro av höga halter av syreradikaler.

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