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**Interactions between natural killer cells
and neutrophil granulocytes**

Thesis

for
the acquisition of doctorate
at the University of Lübeck
-Medical Faculty-

presented by
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Lübeck 2006

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Day of oral examination: 12.04.2007

Granted for print. Lübeck, 12.04.2007

**Signed Prof. Dr. med. W. Solbach
-dean of the medical faculty-**

to my mother

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Abbreviations

⁵¹ Cr	Chromium-51
ADCC	Antibody-dependent cellular cytotoxicity
BHI	Brain heart infusion
BSA	Bovine serum albumin
CD	Cluster of differentiation
CEACAM1	Carcinoembryonic-antigen-related cell-adhesion molecule 1
cpm	Counts per minute
DC	Dendritic cell
DNAM	DNAX accessory molecule
DR	Death receptor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immuno sorbent assay
FACS	Fluorescence activated cell sorter
Fc	Fragment crystallisable
FcγRIII	Fc gamma receptor III
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
G-CSF	Granulocyte-colony-stimulating factor
GM-CSF	Granulocyte-macrophage-colony-stimulating factor
h	Hour
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
Her2	Human epidermal growth factor receptor-2
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer-cell immunoglobulin-like receptor
KLRG1	Killer-cell lectin-like receptor G1

<i>L. major, Lm</i>	<i>Leishmania major</i>
LDL	Low-density lipoprotein
LGL	Large granular lymphocyte
LIR	Leukocyte immunoglobulin-like receptor
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MACS [®]	Magnetic cell sorting
MHC	Major histocompatibility complex
MICA/B	MHC class I chain related antigen A/B
MIP	Macrophage inflammatory protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NCR	Natural cytotoxicity receptors
N.D.	Not detectable
NK	Natural killer
NNN	Novy-Nicolle-McNeal
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PMN	Polymorphonuclear neutrophil granulocytes
PP	Polypropylene
PRR	Pattern recognition receptor
PS	Polystyrene
PVR	Poliovirus receptor
RANTES	Regulated upon activation, normal T cell expressed and secreted
rh	Recombinant human
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
Siglec	Sialic-acid binding immunoglobulin-like lectins
SSC	Sideward scatter
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper

TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TRAIL	TNF-related apoptosis-inducing ligand
type I IFN	Universal type I interferon

1 Introduction

The health of the human body is constantly exposed to threats by invading microorganisms. In order to counteract, the body has various lines of defence. Epithelial surfaces lining the body present a physical barrier to intruding pathogens. However, once this barrier has been overcome, the immune system has to take over to prevent replication and spread of pathogens. Two arms of the immune system can be distinguished. The innate immune system is the first to respond to infections. Its effector cells are able to recognise self from non-self and recognise broad classes of pathogens. Innate immunity is quick in action and serves to contain the infection before the more specific adaptive immune response takes over. The cells of the innate immune system, namely macrophages, mast cells, dendritic cells, granulocytes and natural killer (NK) cells, are recruited to the site of infection, where they not only engage in direct elimination of the pathogen but also prime the subsequent adaptive immune response through antigen presentation and the production of cytokines and chemokines. Adaptive immunity is then able to eliminate pathogens in a specific manner and induce an immunological memory.

1.1 NK cells: Historical overview

NK cells were originally discovered in the 1970s, when researchers observed that allogenic tumour cells were lysed by lymphocytes isolated from hosts not immunized against these tumour cells (50,51,68,69,128). Studies conducted using virus-infected target cells, also suggested the presence of cytotoxic lymphocytes in the absence of prior immunisation (109).

Initially, NK cells were defined in purely functional terms, as cells being able to lyse tumour cells *in vitro* without the need for prior stimulation. Due to the lack of expression of cell surface markers characteristic of B or T lymphocytes, they were also called “null cells” (52). Morphologically, they were described as being “large granular lymphocytes” (52,133).

After their existence was questioned for quite some time, by the late 1980s their existence as a unique functional, morphological and immunophenotypical lymphocyte subset was widely accepted (108). The “missing self hypothesis” postulated the idea that NK cells are

able to recognise aberrant cells by the lack of expression of major histocompatibility complex (MHC) class I cell markers (80).

Currently, NK cells are divided into two main functional subsets: cluster of differentiation (CD)56^{dim} cells, which are largely responsible for cytotoxic effector functions and CD56^{bright} cells, that mainly secrete cytokines (27). However, NK cells are a phenotypically heterogeneous group, at least 48 different subsets can be found in peripheral blood (62).

In the past, the function and exact nature of NK cells left many questions unanswered, however they are now considered an integral part of the immune system (54).

1.2 The role of NK cells within the immune system

NK cells are lymphocytes belonging to the innate immune system, making up about 5 to 20 % of the lymphocytes within peripheral blood. They develop in the bone marrow from a common lymphoid progenitor cell, but diverge from other lymphocyte lineages at an early stage in development (26). Under normal conditions, they are found in peripheral blood, the spleen, bone marrow and the liver. In response to various inflammatory processes, they migrate to inflamed tissues, lymph nodes and lymphatic tissues, where they then engage in various effector functions (92,94). NK cells play an important role in innate host defence against infection and tumours, but also have important immune regulatory functions (27,108). Their main effector functions are cytotoxicity (allowing them to kill infected or aberrant cells) and cytokine secretion. However, they are also able to stimulate other cells via cell-cell contact (98).

Phenotypically, NK cells are classically characterised by the expression of CD56 (NKH-1) and CD16 (Fc gamma receptor RIII [FcγRIII]) and the lack of CD3 (part of the T cell antigen receptor complex) expression (59,108). They have a large granular lymphocyte (LGL) morphology, owing to numerous granules in their cytoplasm.

Natural killer cells express a number of cytokine receptors, such as those for interleukin (IL)-2 and IL-21, for the monocyte-derived cytokines (monokines) IL-1, IL-10, IL-12, IL-15, IL-18, for interferon (IFN)-α/β as well as expressing several chemokine receptors (27,98). Despite being classified as belonging to the innate immune system, NK cells seem to play an important role in bridging the adaptive and innate immune system.

1.2.1 NK cells and infection

NK cells are among the first cells recruited towards a site of infection. They play an important role in host defence against infection, especially against viruses such as herpes viruses (7,8) and intracellular pathogens such as *Leishmania (L.) major* (74). In order to prevent antigen presentation to T cells, virally infected cells downregulate the expression of MHC class I molecules on their cell membrane. This reduced MHC class I expression activates NK cells to become cytotoxic towards these cells. Cytotoxicity serves to contain the infection before antigen specific cytotoxic T cells are generated by the adaptive immune response. IFN- α and- β production in viral infection promotes NK cell cytotoxicity towards infected cells. IFN- γ , a pro-inflammatory cytokine produced by NK cells is crucial for infection control before IFN- γ production is initiated by the adaptive immune system through CD8⁺ T cells (59,98).

1.2.2 NK cells and tumours

NK cells are probably responsible for the early elimination of aberrant cells before they are able to cause a tumour. Cancerous cells downregulate their MHC class I molecule expression and upregulate stress-induced cell surface molecules leading to NK cell activation, culminating in cytotoxic lysis of the cell. In addition, IFN- γ secretion by NK cells suppresses tumour growth (138).

1.2.2.1 Clinical correlation: NK cell cancer therapy

NK cells are potentially useful for the therapy of cancer, because they are not restricted to recognizing specific antigens expressed by malignant cells, which are poorly characterised for most tumours. A variety of approaches selectively augment NK cell responses to cancer.

1. Infusion of therapeutic cytokines that act via NK cells, such as IL-2 (23).
2. Adoptive transfer of *ex vivo* expanded autologous adherent or lymphokine activated NK cells (116). The adoptive transfer of natural killer cells is implemented as a therapeutic approach to renal cell carcinoma (41).
3. Antibodies reactive with NK cells in combination with cytokines such as type I IFN, IL-2 and IL-12. Combinations of IL-2 or IL-12 with rituximab (a chimeric anti-CD20 monoclonal antibody) in the therapy of non-Hodgkin's lymphoma are being investigated (5,40). IL-2 in combination with Herceptin®, a monoclonal antibody against the human

epidermal growth factor receptor-2 (Her2) has been shown to enhance NK cell activity towards Her2/neu-positive breast cancer cells (17).

However, although a lot of progress has been made in the field of NK cell cancer therapy, the systemic administration of cytokines, especially IL-2 and IFN- α , is associated with severe adverse effects as these cytokines lead to an unspecific activation of the immune system (138).

1.3 Effector functions of NK cells

Two main effector functions of NK cells are distinguished: cytotoxicity (mediated predominantly by CD56^{dim} cells) and cytokine secretion (mediated mainly by CD56^{bright} cells) (27). The effector functions of NK cells are regulated by a complex set of activating and inhibiting receptors, which are discussed in detail under 1.4.

1.3.1 Natural cytotoxicity

NK cells are able to kill cancerous or pathogen infected cells without prior sensitization by recognizing downregulated MHC class I molecule expression or stress-induced ligands on infected or aberrant cells. The cytotoxic killing of target cells occurs via two distinct mechanisms, both of which require direct cell-cell contact and result in apoptosis of the target cell.

A) Perforin-dependent cytotoxicity: The mechanism predominantly used by NK cells is perforin-dependent cytotoxicity. Here, upon contact with the target cell, secretory lysosomes containing perforin (a membrane disrupting protein) and granzymes (a family of structurally related serine proteases) and granulysin (a member of the saposin-like protein family) are secreted by exocytosis into the immunological synapse formed between the NK cell and the target cell and induce apoptosis. Perforin functions as an essential enabler of granzyme mediated apoptosis of the target. Five different granzymes are expressed in humans (granzyme A, B, H, K and M). They trigger apoptotic cell death either directly via the mitochondria, via the activation of cellular caspases or via as yet uncharacterised caspase independent pathways (121).

B) Death receptor pathways: Natural killer cells can also kill target cells in a classical caspase dependent apoptotic pathway. Via interaction between death receptors on target cells, such as Fas/CD95, and the corresponding ligands on NK cells, such as Fas ligand, apoptosis of the target cell is induced. NK cells are able to induce Fas expression on cancer cells by secreting IFN- γ and then kill these cells in a Fas-dependent manner (113).

Another death receptor pathway mediating natural killing involves tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), expressed by natural killer cells. Binding of TRAIL to death receptor (DR)4 and DR5 on target cells results in a caspase-activating signal leading to apoptosis (121,125).

1.3.2 Antibody-dependent cellular cytotoxicity (ADCC)

While natural cytotoxicity is the mechanism predominantly used by NK cells in the killing of target cells, they are also able to destroy cells opsonized by immunoglobulin (Ig)G antibodies using antibody-dependent cellular cytotoxicity (ADCC). ADCC is mediated through the FcγRIIIa-receptor complex (CD16). IgG antibodies produced by B cells bind to the corresponding target with their antigen binding sites. The free fragment crystallisable (Fc) of the antibody can then bind to the CD 16 receptor, leading to lysis of the target cell (103,108).

1.3.3 Cytokine secretion

While cytotoxicity is mainly mediated by CD56^{dim} cells, cytokine secretion is mediated largely by CD56^{bright} natural killer cells, which make up about 10 % of NK cells (27,28,36). Cytokine secretion takes place early in the immune response in response to stimulation by monokines (38). Cytokine secretion is a function of NK cells which can induce antiviral and inflammatory responses, has a direct suppressive effect on tumour growth, regulates haematopoietic cell differentiation and primes immune effector cells crucial to subsequent antiviral responses. CD56^{bright} cells have been shown to secrete pro-inflammatory cytokines such as IFN-γ and TNF-β, anti-inflammatory cytokines such as IL-10 and IL-13, as well as the growth factor granulocyte-macrophage-colony stimulating factor (GM-CSF) (28). Early IFN-γ production by NK cells in the innate immune response activates macrophages and other antigen presenting cells before production is taken over by other cell types, such as CD8⁺ T cells. Other cytokines secreted by NK cells include TNF-α and IL-5 (30,82).

Chemokines, such as macrophage inflammatory protein (MIP)-1α and β and the chemokine “regulated upon activation, normal T cell expressed and secreted” (RANTES) are also secreted by NK cells. These chemokines may recruit other effector cells during the immune response and also appear to play an important role in NK cell responses to infected and neoplastic cells (11,32,37,106).

1.4 Regulation of NK cell effector function: inhibitory and activating receptors

NK cell effector functions are controlled through the interaction of multiple inhibiting and activating cell surface receptors on NK cells and their ligands found on target cells and other cells of the immune system. These receptors belong to different families; however, the same structural family of receptors can contain both activating and inhibiting members.

1.4.1 Inhibitory NK cell receptors recognising MHC class I molecules

The “missing self” hypothesis, formulated by Ljunggren and Kärre in 1990, postulated that cells failing to express MHC class I molecules which are expressed by almost all nucleated cells of the body would become susceptible to killing by NK cells (80). Thus, rather than recognizing a target cell as foreign, it is recognised as non-self. Indeed, tumour cells and virally infected cells, which have decreased MHC class I expression, become susceptible to killing by NK cells (65).

NK cells express numerous receptors recognizing polymorphic epitopes of MHC molecules. The killer-cell immunoglobulin-like receptor (KIR) family comprises 15 members with both activating and inhibiting properties, recognizing human leukocyte antigen (HLA)-A, HLA-B and HLA-C proteins. Inhibitory KIR include KIR2DL and KIR3DL (65,72,93). They block NK activation via protein tyrosine phosphatases that bind immunoreceptor tyrosine-based inhibitory motives (ITIM)s. CD94-NKG2A is another receptor involved in HLA recognition (76). Leukocyte immunoglobulin-like receptor (LIR)-1, which engages almost all MHC class I molecules and also binds UL18, a human cytomegalovirus class I homolog (24), also inhibits NK cell function.

1.4.2 Inhibitory receptors not recognising MHC class I molecules

Besides MHC class I molecular receptors, NK cells also express a variety of receptors that recognise cellular ligands other than MHC class I molecules. These include the killer-cell lectin-like receptor G1 (KLRG1) (29), the carcinoembryonic-antigen-related cell-adhesion molecule 1 (CEACAM1) (86) and Sialic-acid-binding immunoglobulin-like lectins (Siglec)-7 and 9 (97,144). 2B4 (CD244) which binds CD48, may function either as an inhibiting or activating receptor (15).

1.4.3 Activating receptors

Activating receptors expressed by natural killer cells include the natural cytotoxicity receptors (NCR), NKG2D and a variety of co-receptors. Activation of NK cells via these

receptors leads to the reorganisation and release of cytotoxic granules as well as the transcription of cytokine genes. Cytotoxicity is mediated largely via natural cytotoxicity receptors. The surface density of NCR on NK cells directly correlates to their cytotoxic activity against target cells (120). NKp46, NKp30, NKp44 are highly specific to NK cells (91). NKp44 is selectively expressed by activated NK cells. NCR are coupled to the intracellular signal transduction via immunoreceptor tyrosine-based activation motif (ITAM)-bearing polypeptides.

Co-receptors support the function of NCR: the 2B4 and NTB-A receptor, which can be either activating or inhibiting associate with CD48, a glycosyl-phosphatidyl inositol and NTB-A respectively (15,35). Other co-receptors include NKp80 and CD59 (85,137). NK receptors that cannot clearly be defined as co-receptors or as receptors include NKG2D and DNAX accessory molecule (DNAM)-1 (CD 226). NKG2D is a receptor for stress-inducible MHC class I chain related antigen (MIC)A and MICB or ULBP proteins (a group of altered MHC-I-complexes). DNAM-1 binds to the poliovirus receptor (PVR) (CD155) and to Nectin-2 (CD112) (12).

1.5 Induction of NK cell effector functions by cytokines

The effector functions of NK cells can be increased by a variety of cytokines, the most well-characterized being IL-2 and type I IFN. IL-2 (a product of T cells) has been shown to induce IFN- γ secretion by NK cells (4) as well as augmenting natural cytotoxicity towards target cells (49). Virus-induced type I IFNs have also been shown to increase NK cell cytotoxicity (110).

1.6 NK cell lines

Work with natural killer cells is often hindered by the fact that natural killer cells are relatively scarce in peripheral blood and that even highly purified preparations still contain cells from other lineages. This has led to the development of natural killer clones (108).

Two natural killer cell lines that were used in this study are the NKL cell line and the NK-92 cell line. The NKL cell line was derived from the blood of a patient with CD3⁻CD16⁺CD56⁺ large granular lymphocyte (LGL) leukaemia. Morphologically, NKL cells resemble normal activated NK cells. They mediate both ADCC and natural killing. NKL cells are dependent on the presence of IL-2 for sustained growth (107). The NK-92 cell line was established from the blood of a patient with rapidly progressive non-Hodgkin's

lymphoma. NK-92 cells, which are $CD56^+CD3^-CD16^-$, also effectively mediate natural killing. Like NKL cells, growth of NK-92 cells is dependent on IL-2 (44).

1.7 Neutrophil granulocytes

Polymorphonuclear neutrophil granulocytes (PMN), are leukocytes belonging to the innate immune system. Being the most abundant population of cells within the immune system, they play a critical role in host defence against various pathogens including bacteria, fungi and protozoa.

Like NK cells, PMN develop in the bone marrow. However, unlike NK cells which originate from a lymphoid progenitor cell, PMN develop from the myeloid cell lineage. During their development, which takes about two weeks, they pass through a series of distinct morphological stages, starting from the myeloblast, which is followed by the promyeloblast, myelocyte and the metamyelocyte. This progenitor cell then gives rise to the basophil, eosinophil and neutrophil granulocytes, the latter making up the majority of the polymorphonuclear leukocytes (63). The mature polymorphonuclear neutrophil granulocyte takes its name from its morphologically characteristically lobulated nucleus and its cytoplasmic granules, which can be stained with both acidic and basic dyes.

The bone marrow produces about 10^{11} neutrophil granulocytes per day; production can increase to about 10^{12} per day in response to inflammation or infection. From the bone marrow, PMN are then released into the blood stream to serve as sentinels for foreign matter, including microorganisms. The life span of circulating PMN is very short, after about 6-8 hours they undergo spontaneous apoptosis (48). Apoptosis leads to a variety of characteristic cell changes including shrinkage of the cell, condensation of nuclear chromatin and loss of the multilobed shape of the nucleus (95,111). Phosphatidylserine appears on the on the surface of apoptotic PMN by a process called membrane flip-flop (56). This aids their clearance by macrophages.

1.7.1 PMN in inflammation

In response to inflammation, PMN leave the blood stream to enter the infected tissues following a chemotactic gradient, with IL-8 being the major chemokine involved (57). The subsequent extravasation takes place in different steps. First, through the expression of adhesion molecules on PMN and the endothelium lining the inside of blood vessels, PMN bind reversibly and “roll” along the endothelium. The next step involves tight binding to the vascular endothelial surface. After diapedesis through the wall of the blood vessels, the

neutrophil granulocyte can then migrate to the site of inflammation following a chemotactic gradient (59,140).

In an inflammatory environment, the life span of PMN is prolonged. Cytokines produced in response to inflammation, including IL-1, IL-2, TNF- α , IL-15, granulocyte-colony-stimulating factor (G-CSF) and GM-CSF, are able to delay the apoptosis of PMN (3). Several intracellular bacterial pathogens have been reported to delay apoptosis of PMN in order to overcome host defence (42). Infection with the protozoan parasite *L. major* has also been shown to inhibit their apoptosis (2).

PMN are able to identify microorganisms through their pattern recognition receptors (PRR)s. These receptors, to which the group of Toll-like receptors (TLR)s belongs, recognise common pathogen-associated molecular patterns (PAMP)s (129,143). Neutrophil granulocytes are then able to perform their main effector function: uptake and destruction of the pathogen: phagocytosis. After internalisation of the microorganism, a vesicle, known as a phagosome forms around it. The engulfed microorganism can then be killed by two different mechanisms, one being non-oxidative and the other oxidative. In the first pathway, the phagosome fuses with lysosomes, which are preformed granules containing enzymes and proteins, to form the phagolysosome in which killing of the pathogen then takes place (53). The other, oxidative mechanism employed, is known as oxidative burst. Here, under a burst of oxygen consumption, superoxide and a range of other reactive oxygen species such as hypochlorous acid and hydrogen peroxide are generated by a nicotinamide dinucleotide phosphate (NADPH) oxidase complex. These reactive oxidant species are able to kill the ingested pathogen (47).

1.7.2 Cytokine secretion by PMN

Besides phagocytosis, which is a well-established function of PMN, more recent research has addressed the ability of PMN to act as immunomodulatory cells through the secretion of chemokines and various pro- and anti-inflammatory cytokines. In this way, they are able to communicate with other cells of the innate immune system, among others NK cells, as well as forming a bridge between the adaptive and innate immune system (20).

Through chemokine secretion of, for example, macrophage inflammatory protein (MIP)-1 α and MIP-1 β , PMN are able to recruit other cells such as monocytes and T and B lymphocyte subpopulations to the site of inflammation (20,66,67). More PMN can be recruited to a site of inflammation by the release of IL-8, a key chemokine for PMN recruitment (6).

Pro-inflammatory cytokines secreted by PMN include TNF- α and IL-1, which are also important priming agents for PMN (31,78). IFN- α , a potent activator of natural killer cells, has also been shown to be produced by PMN (118).

Anti-inflammatory cytokines secreted by PMN include TGF- β and IL-1 receptor antagonist (IL-1ra) (46,88).

Through the secretion of various cytokines, PMN can lead the adaptive immunity towards either a T helper 1 (Th1) or T helper 2 (Th2) response. IL-4, which is crucial in the development of a Th2 response, has been shown to be stored in and secreted by PMN (14). Th1 responses can be mediated via the secretion of IL-12p70 (with the subunits p40 and p30) (22). IL-12 has been shown to increase IFN- γ production by both T and NK cells (134). In an autocrine loop, it has also been shown that PMN themselves are able to secrete IFN- γ upon stimulation by IL-12 (34). This secreted IFN- γ is then able to stimulate macrophages and prime adaptive immunity towards a Th1 response (55).

1.8 Interaction of NK cells and PMN with other cells of the immune system

1.8.1 Interaction between NK cells and other immune cells

NK cells have been shown to reciprocally communicate with cells of both the innate and the adaptive immune system. This reciprocal influence is mediated by cytokine secretion as well as by cell-cell contacts. The most relevant interactions to this study are outlined below.

1.8.1.1 Contact-dependent cross-talk between NK cells and other immune cells

NK cells are able to receive stimulatory signals or send them to other leukocytes such as dendritic cells (DC)s, B and T cells via cell-cell contacts. Co-stimulatory ligands like CD40L and OX40L expressed on NK cells provide co-stimulatory signals to B and T cells (9,145). Cell-cell contacts between dendritic cells (DC)s and NK cells result in an activation of both cell types (105,139). Activated DCs are then in turn able to co-stimulate B and T cells. In this way, NK cells serve as a bridge between innate and acquired immunity.

1.8.1.2 NK cell and PMN interaction

Investigations into the interaction between NK cells and PMN have mainly focused on the effect of PMN on natural killer cell cytotoxicity. These studies mostly date back to the 1980s, an era, where PMN had not yet evolved as an important cytokine producing source.

Taken together, the available data is equivocal, as a number of studies reported inhibition of NK cell cytotoxicity by PMN, whereas others showed that PMN are able to augment natural killing.

PMN were repeatedly described to have an inhibiting effect on NK cell cytotoxicity. This effect was attributed to reactive oxygen species (ROS) production, especially H_2O_2 , by PMN. 12-O-tetradecanoylphorbol-13-acetate (TPA) and zymosan, which stimulate release of ROS from PMN and monocytes, were found to stimulate this suppression (115). It was postulated that soluble factors of PMN mediate this suppression (99,100). Other studies suggested a PMN-mediated suppression through prostaglandins and oxidised low-density lipoprotein (LDL) (102,131). It was also reported that PMN suppress cytotoxicity by decreasing NK-target binding (131). Shau and Golub reported that the inhibiting effect on IL-2 activated NK cells was not dependent on ROS, as catalase and superoxide dismutase, both oxidant scavengers, did not block this inhibition (117). These results were in contrast to previous studies, which reported that catalase did inhibit PMN-mediated suppression of cytotoxicity (115). Patients with eosinophilia were found to have increased intrinsic NK cell activity, whereas patients with high levels of neutrophil granulocytes in peripheral blood were reported to have low activities (73).

Despite many studies suggesting inhibition of NK cell cytotoxicity by PMN, there are various other studies describing increased NK cell cytotoxicity in response to PMN. One such study reports augmentation of NK cell cytotoxicity by PMN, whereas monocytes were found to suppress cytotoxicity when cells were co-incubated in diffusion chambers (142). In another study, supernatants from serum-opsonized zymosan stimulated PMN were found to increase cytotoxicity, whereas unstimulated PMN decreased cytotoxicity. The same authors then carried out further studies suggesting that some components of azurophil granules from PMN might mediate this effect (71,79). A study testing the effect of PMN from patients with aplastic anaemia on NK cell cytotoxicity reported augmentation of cytotoxicity by granulocytes from healthy volunteers (127). Terres and Coffman reported that IL-4 treatment of mice led to splenomegaly with increased neutrophil content of the spleen and marked increase in NK activity (132), suggesting that PMN might be responsible for the observed increase in NK cell cytotoxicity.

On the whole, the existing studies leave the question whether PMN activate or inhibit NK cell cytotoxicity unanswered.

1.8.1.3 NK cell and DC interaction

Whereas the interaction between NK cells and PMN is ambiguous, mutual activation of NK cells and DCs has been reported in numerous studies. Dendritic cells are potent NK cell activators, leading to an increase in NK cytolytic activity and IFN- γ secretion through cytokine production (IL-12, IL-15, IL-18 and IFN- α/β) as well as cell-cell contact (139). In turn, DC maturation can be induced by NK cells either directly or in the presence of microbial signals. This activation is due to both cytokine production (TNF- α and IFN- γ) by NK cells as well as cell-cell contacts (43,105).

The implications of this cross-talk to immunity are: DCs activated by NK cells are able to prime T cell responses. NK cells activated by DCs produce cytokines, in particular IFN- γ , which may polarize T cells towards a Th1 response. Furthermore, the antitumour response of NK cells was, in some cases, shown to be dependent on prior activation by DCs.

1.8.2 PMN and DC interaction

It has been shown that neutrophil granulocytes are able to communicate with dendritic cells. PMN are able to induce DC maturation via the secretion of TNF- α , but cellular interactions also seem to play a role. Of particular relevance to this project is the finding that dendritic cells in turn, are able to prolong the lifespan of neutrophil granulocytes (89), showing, that PMN functions can be promoted by other cells of the immune system.

1.9 Aims of the study

In recent years, research has led to an increased understanding of the biology of natural killer cells and neutrophil granulocytes. In particular, the immunomodulatory effects of both NK cells and PMN through the secretion of various cytokines and through cellular interactions have been elucidated, allowing them to communicate with cells of the innate immune system, such as dendritic cells and cells of the adaptive immune system, such as B or T cells.

Whereas recent knowledge on the importance of NK cells and PMN for both innate and adaptive immunity has substantially increased, surprisingly little research has been conducted regarding a potential cross-talk between these cells. In infection and inflammation, NK cells and PMN are present at the same site, at the same time, whereby the potential for mutual influence is given. It is therefore justified to carry out an in-depth analysis, asking, whether bi-directional activating interactions between NK cells and PMN might exist in an infectious and non-infectious environment.

In order to provide answers to this question, this study addresses:

1. the effect of PMN on natural cytotoxicity of NK cells.
2. the effect of PMN on IFN- γ secretion of NK cells.
3. the effect of NK cells on PMN.

2 Materials and Methods

2.1 Materials

2.1.1 *Leishmania* parasites

The *L. major* isolate MHOM/IL/81/FEBNI used in this study was originally isolated from the skin biopsy of an Israeli patient and was kindly supplied by Dr. F. Ebert (Bernhard-Nocht-Institute for Tropical Medicine, Hamburg). In order to obtain a continuous pool of infectious parasites, *in vitro* cultures of promastigotes in the stationary phase were used to infect BALB/c mice. Amastigotes were then re-isolated from the spleen or footpad of the infected mice and cultured *in vitro* in biphasic Novy-Nicolle-McNeal (NNN) blood agar medium at 26 °C in a humidified atmosphere containing 5 % CO₂. Stationary phase promastigotes were collected after 7 to 10 days in culture.

2.1.2 Tumour cell lines:

K-562 (ATCC-CCL-243)	ATCC, Manassas, U.S.A.
NK-92 (ATCC-CCL-2407)	ATCC, Manassas, U.S.A.
NKL	kind supply from Dr. A. Diefenbach, Skirball Institute of Biomolecular Medicine, New York University Medical Center, New York, U.S.A.

2.1.3 Cells isolated from peripheral blood or buffy coat

PMN, peripheral blood mononuclear cells (PBMC) and NK cells were isolated from peripheral blood of healthy adult volunteers or buffy coats (kindly provided by the Institute for Immunology and Transfusion Medicine, University of Lübeck) as described under 2.2.1.

2.1.4 Culture media and buffer

Buffer for intracellular	
FACS staining	PBS + 1 % heat inactivated FCS + 0.09 % sodium azide
Complete medium	Roswell Park Memorial Institute (RPMI) 1640 medium + 50 µM 2-mercaptoethanol + 2 mM L-glutamine + 10 mM HEPES + 100 U/ml penicillin + 100 µg/ml streptomycin + 10 % low endotoxin FCS
ELISA wash buffer	PBS + 0.05 % Tween 20
FACS-buffer	PBS + 1 % human serum + 1 % BSA + 0.01 % sodium azide

NNN blood agar medium	50 ml defibrinated rabbit blood + 50 ml PBS + 100 U/ml penicillin + 100 µg/ml streptomycin+ 200 ml Brain Heart Infusion (BHI) medium (10.4 g BHI agar in 200 ml distilled water)
MACS® Buffer	PBS + 0.5 % BSA + 2 mM EDTA

2.1.5 Laboratory supplies

Cell culture flasks	Greiner bio-one, Frickenhausen
<i>L.major</i> culture ELISA plate + lid Microlon, flat bottom, medium binding for <i>Lm</i> culture	Greiner bio-one, Frickenhausen
Examination gloves Peha-Soft®, powder free	Hartmann, Heidenheim
Examination gloves Vinyl 2000 PF	Meditrade, Kiefersfelden
Finntip 300	Thermo Electron, Oy Vantaa, Finland
Microscope slides superfrost	Menzel, Braunschweig
Microtestplate + lid (96-well, V-bottom)	Sarstedt, Nümbrecht
Microtiter plates: MaxiSorb™, PolySorb™ (96-well, flat bottom)	Nunc, Wiesbaden
Mictotiter plates (96-well, round bottom)	Greiner bio-one, Frickenhausen
Millex-HA syringe driven filter unit	Millipore, Schwalbach
pH indicator paper Acilit®	Merck, Darmstadt
Pipette 5, 10, 25 ml	Greiner bio-one, Frickenhausen
Pipette filter tips	Nerbe plus, Winsen
Pipette tips (1-10 µl, 10-100 µl, 100-1000 µl)	Greiner bio-one, Frickenhausen
Plastic tubes (5 ml (PS) Falcon)	BD Biosciences, Heidelberg
Plastic tubes (15 ml (PS), 50 ml (PP))	Sarstedt, Nümbrecht
Reaction tubes (0.5, 1.5, 2 ml (PP))	Sarstedt, Nümbrecht
S-Monovette, 9 ml, lithium-heparin	Sarstedt, Nümbrecht

Tissue culture plates

(6, 12, 24, 48,

96-well, flat bottom)

Transfer pipette 3.5 ml

U-tubes for cytometry

Greiner bio-one, Frickenhausen

Sarstedt, Nümbrecht

Micronic, Lelystad, The Netherlands

2.1.6 Instruments

Analytical balance BP61S

Balance BL 150

Cell counting chambers

Centrifuges

Biofuge fresco

Centrifuge 5417R

Centrifuge 5415C

Centrifuge GF-6

Cytocentrifuge Cytospin3

Megafuge 2.0R

Microfuge 22R

Multifuge 3 S-R and 3s

CO₂ Incubator IG 150

CO₂ Incubator CO₂-AUTO-ZERO

Deep freezer, -20 °C, -70 °C

Finn pipette

Flow-cytometer FACS-Calibur[®]

Gamma Counter CS 1282

Ice machine

Laminar flow workbench

Liquid nitrogen tank LS 750

MACS[®] Multi Stand

Magnetic stirrer: Ikamag, Reo

Microplate reader Sunrise[™]

Microscopes

Axiostar 25

Axiostar plus

Sartorius, Göttingen

Sartorius, Göttingen

Neubauer, Marienfeld

Kendro (Heraeus), Langenselbold

Eppendorf, Hamburg

Eppendorf, Hamburg

MSE, London, U.K.

Shandon, Frankfurt

Kendro (Heraeus), Langenselbold

Beckmann, Munich

Kendro (Heraeus), Langenselbold

Jouan, Unterhaching

Heraeus, Langenselbold

Liebherr, Ochsenhausen

Labsystems, Helsinki Finland

Becton Dickinson, Heidelberg

LKB Wallac, Turku, Finland

Ziegra, Isernhagen

Biohit, Cologne

Taylor-Wharton, Theodore, U.S.A.

Miltenyi Biotech, Bergisch Gladbach

IKA[®] Labortechnik, Staufen

Tecan, Crailsheim

Carl Zeiss, Jena

Carl Zeiss, Jena

Standard 20	Carl Zeiss, Jena
Multichannel pipette	Eppendorf, Hamburg
pH-meter inolab	WTW GmbH, Weilheim
Pipettes	Eppendorf, Hamburg
Refrigerators	Liebherr, Ochsenhausen
Shaker KS 250 basic	Janke & Kunkel IKA® Labortechnik, Staufen
Shaker Vibrofix VF1 Electronic	Janke & Kunkel IKA® Labortechnik, Staufen
Transfer pipette	Brand, Wertheim
Vario MACS®	Miltenyi Biotech, Bergisch Gladbach
Water bath	Köttermann, Uetze (Hänigsen)

2.1.7 Chemicals and other laboratory reagents

Brain Heart Infusion (BHI)	Becton Dickinson, Heidelberg
Bovine serum albumin (BSA)	Sigma, Deisenhofen
Brefeldin A GolgiPlug®	BD Biosciences, Heidelberg
Crystal violet staining solution	Sigma, Deisenhofen
EDTA	Sigma, Deisenhofen
Fetal calf serum (FCS)	Gibco, Karlsruhe
Giemsa staining solution, modified	Sigma, Deisenhofen
L-Glutamine	Biochrom, Berlin
HEPES	Biochrom, Berlin
Histopaque® 1119	Sigma, Deisenhofen
IL-2, recombinant human	PeproTech, Offenbach
IL-15, recombinant human	PeproTech, Offenbach
IFN- γ , recombinant human	PeproTech, Offenbach
IFN, universal type I	PBL Biomedical Laboratories, Piscataway, USA
Immersion oil	Carl Zeiss, Jena
Lipopolysaccharide	
<i>Escherichia coli</i> 0111:B4	Sigma, Deisenhofen
Lymphocyte separation medium 1077	PAA, Pasching, Austria
2-Mercaptoethanol	Sigma, Deisenhofen
Methanol	J.T. Baker, Deventer, The Netherlands
Paraformaldehyde	Sigma, Deisenhofen

PBS (1 ×) sterile solution	Pharmacy of the University of Lübeck, Lübeck
PBS (10 ×) sterile solution	Gibco, Karlsruhe
Penicillin/streptomycin	Biochrom, Berlin
Percoll [®]	Pharmacia, Uppsala, Sweden
Rabbit blood	Elocin-lab GmbH, Mülheim
RPMI 1640 medium	Sigma, Deisenhofen
Sodium azide	Merck, Darmstadt
Sulphuric acid	Merck, Darmstadt
Trypan blue solution 0.4 %	Sigma, Deisenhofen
Tween 20 for molecular biology	Sigma, Deisenhofen

2.1.8 Radiochemicals

[γ - ⁵¹ Cr]Sodiumchromate	Hartmann Analytic, Braunschweig
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2.1.9 Monoclonal anti-human antibodies

Mouse IgG1 isotype control (PE, FITC, clone DAK-GO1)	Dako, Hamburg
Mouse IgG1 isotype control (PE, clone MOPC-21)	BD Biosciences, Heidelberg
Mouse anti-CD56 FITC (IgG2b, clone NCAM 16.2)	BD Biosciences, Heidelberg
Mouse anti-CD3 PE (IgG1, clone UCTH1)	Dako, Hamburg
Mouse anti-IFN- γ PE (IgG1, clone B 27)	BD Biosciences, Heidelberg

2.1.10 Ready-to-use kits

Cytofix/Cytoperm [™] plus kit	BD Biosciences, Heidelberg
NK Cell Isolation Kit	Miltenyi Biotec, Bergisch Gladbach
NK Cell Isolation Kit II	Miltenyi Biotec, Bergisch Gladbach
OptEIA [™] Set Human IFN- γ	BD Biosciences, Heidelberg

2.1.11 Software

GraphpadPrism [®] , Version 4.01	San Diego, CA, U.S.A
CellQuest [®] (cytometry)	Becton Dickinson, Heidelberg

Magellan [®] (ELISA)	Tecan, Crailsheim
Microsoft [®] Office	Microsoft Corporation, Mountain View, CA, U.S.A.
Microsoft [®] Internet Explorer	Microsoft Corporation, Mountain View, CA, U.S.A.
Win MDI 2.8	J. Trotter, The Scripps Research Institute, San Diego, California

2.2 Methods

2.2.1 Isolation of cells from human peripheral blood or buffy coat

(Approved by the ethics committee of the University of Lübeck on the 26.07.2005, reference number 05-124)

2.2.1.1 Isolation of PMN

Peripheral blood was collected by venipuncture from healthy adult volunteers using lithium-heparin S-Monovettes. Buffy coats (the resulting leukocyte rich fraction obtained from peripheral blood after preparation of erythrocyte concentrates and plasma for transfusion purposes) were kindly provided by the Institute of Immunology and Transfusion Medicine, University of Lübeck.

The heparinized blood or buffy coat (diluted 1:6 with PBS) was then layered onto a two-layer density gradient consisting of lymphocyte separation medium 1077 (upper layer) and Histopaque[®] 1119 (bottom layer) and centrifuged for 5 minutes at $300 \times g$, followed by 20 minutes at $800 \times g$. The plasma and the lymphocyte separation medium 1077 layer consisting mainly of lymphocytes and monocytes were discarded. The granulocyte-rich Histopaque[®] 1119 layer was collected, leaving the erythrocyte pellet at the bottom of the tube. Granulocytes were washed once in PBS, resuspended in complete medium and further fractionated on a discontinuous Percoll[®] gradient consisting of layers with densities of 1.105 g/ml (85 %), 1.100 g/ml (80 %), 1.087 g/ml (70 %), and 1.081 g/ml (65 %). After centrifugation for 20 minutes at $800 \times g$, the interface between the 80 and 70 % Percoll[®] layers was collected, washed once in PBS and resuspended in complete medium. All procedures were carried out at room temperature. Cell viability was $> 99 \%$, as determined by trypan blue exclusion. Cells were counted after staining with crystal violet solution.

2.2.1.1.1 Purity analysis of isolated PMN

The purity of isolated PMN was determined by microscopic examination of Giemsa stained cytocentrifuge slides. These were prepared by suspending 100,000 PMN in 100 μ l PBS, which were then spun on slides in a cytocentrifuge at $400 \times g$ for 5 minutes.

Air dried slides were fixed in methanol for 5 minutes, followed by a 30 minute staining in Giemsa solution. At least 200 cells were counted and classified as neutrophils, eosinophils, monocytes and lymphocytes by morphological examination. The cell preparations contained $> 99 \%$ granulocytes. The amount of eosinophil granulocytes ranged between 0.5 to 15 %, depending on the donor.

2.2.1.2 Isolation of PBMC

As described for the isolation of PMN above, peripheral blood or buffy coat diluted 1:6 with PBS, was layered onto a two layer density gradient. After centrifugation at 300 x g for 5 minutes, followed by 20 minutes at 800 x g, the plasma layer at the top was carefully collected until shortly above the PBMC ring (consisting mainly of lymphocytes and monocytes) and discarded. The PBMC-rich ring was then aspirated and washed three times in PBS at 140 x g at 4 °C and resuspended in complete medium. Cell viability was >99 %, as determined by trypan blue exclusion.

2.2.1.3 Isolation of NK cells

NK cells were purified from the PBMC fraction using the magnetic cell sorting (MACS[®]) NK Cell Isolation Kit or MACS[®] NK Cell Isolation Kit II for the isolation of untouched NK cells. Using this method, NK cells are isolated by depletion of non-NK cells from the PBMC suspension (negative selection). Non-NK cells were magnetically labelled with a cocktail of hapten conjugated monoclonal antibodies (mAb)s binding CD3, CD14, CD19, CD36 and IgE (NK Cell Isolation Kit) or biotin conjugated mAbs binding CD3, CD4, CD14, CD15, CD36, CD123 and Glycophorin A (NK Cell Isolation Kit II) as primary labelling reagent. Anti-hapten or anti-biotin mAbs conjugated to Micro Beads were used as the secondary labelling reagent. The magnetically labelled non-NK cells were then depleted by retaining them on a MACS[®] column in the magnetic field of a MACS[®] separator, while the unlabelled NK cells passed through the column. After isolation, cells were counted. Cell viability was >99 %, as determined by trypan blue exclusion.

2.2.1.3.1 Purity analysis of isolated NK cells: flow cytometry

The purity of isolated NK cells was evaluated using flow cytometry (FACS, fluorescence activated cell sorter) by staining cells with antibodies against the NK cell marker CD56 and the T cell marker CD3. $2-5 \times 10^5$ NK cells were resuspended in FACS-buffer in a V-bottom 96-well plate. The cells were washed with FACS buffer and subsequently stained with fluorescein isothiocyanate (FITC)-conjugated mAb against CD56 and phycoerythrin (PE)-conjugated mAb against CD3 in FACS-buffer for 20 minutes on ice in the dark. FITC- and PE-conjugated mouse IgG1 antibodies were used as isotype controls. After washing twice, the cells were fixed with paraformaldehyde (1 % in phosphate buffered saline, PBS) and analysed with a FACSCalibur[®] flow cytometer using CellQuest[®] software. The purity of NK isolates was >90 %. Cell viability was >99 %, as determined by trypan blue exclusion.

2.2.2 Tumour cell line cultures

2.2.2.1 K-562 cells

The tumour cell line K-562, a cell line highly sensitive to NK cell cytotoxicity, established from a patient with chronic myelogenous leukaemia in blast crisis (83), was cultured in complete medium in a humidified atmosphere containing 5 % CO₂ at 37 °C. The cells were seeded out in cell culture flasks at a concentration of approximately 0.1 x 10⁶ cells/ml. The culture was split every 2 to 3 days.

2.2.2.2 NK-92 cells

The tumour cell line NK-92, an IL-2 dependent NK cell line derived from the PBMC of a patient with rapidly progressive non-Hodgkin's lymphoma (44), was cultured in complete medium containing 10 ng/ml (100 U/ml) IL-2 and 10 ng/ml (20 U/ml) IL-15 (as a growth enhancer) in a humidified atmosphere containing 5 % CO₂ at 37 °C. The cells were seeded out in cell culture flasks at a concentration of approximately 0.5 x 10⁶ cells/ml. The culture was split every 2 days.

2.2.2.3 NKL cells

The tumour cell line NKL, an IL-2 dependent NK cell line derived from the blood of a patient with CD3⁻ CD16⁺ CD56⁺ LGL leukaemia (107), was cultured in complete medium containing 30 U/ml IL-2 in a humidified atmosphere containing 5 % CO₂ at 37 °C. The cells were seeded out in cell culture flasks at a concentration of approximately 0.3 x 10⁶ cells/ml. The culture was split every 2 to 3 days.

Cell lines were stored in liquid nitrogen. For initiation of a permanent cell culture, frozen cells were thawed rapidly, washed and resuspended in complete medium, transferred to a cell culture flask and incubated in a humidified atmosphere containing 5 % CO₂ at 37 °C. Cell line cultures were handled under strictly sterile conditions and tested for *Mycoplasma* contamination at regular intervals using polymerase chain reaction (PCR). Morphology of the tumour cell lines was visualized using an invert microscope. Before use in experiments, all cell lines were washed once in complete medium at room temperature and viability determined by trypan blue exclusion.

2.2.3 NK cell cytotoxicity assay

NK cell-mediated cytotoxicity was measured in a 4h ⁵¹Cr-release cytotoxicity assay. Effector cells used were either human NK cells, isolated from peripheral blood using

MACS[®] or the human NK cell lines NK-92 and NKL. K-562 cells were used as target cells.

K-562 target cells were radioactively labelled with ⁵¹Cr by adding physiological saline containing 100 µCi Na₂⁵¹CrO₄ to 1-5 million K-562 cells in 100 µl complete medium and incubating the cell suspension for one hour at 37 °C in a humidified atmosphere containing 5 % CO₂.

Any chromium not taken up by the cells was then washed off by washing thrice with complete medium (without FCS) at 140 x g for 5 minutes. The cells were then resuspended in 5 ml of complete medium and counted, after which they were brought to a concentration of 1 x 10⁵ cells/ml. A 96 well round bottom micotiter plate was prepared by adding 100 µl of complete medium to each well. The effector cells were plated out in the top row by adding 100 µl of a 10 x 10⁶ cell suspension. After mixing well, a serial dilution from top to bottom was carried out. The bottom row was spared. Then 100 µl of the K-562 suspension (the target cells) was added to each well. By this process, a ratio of effector to target cells ranging from 50:1 in the top row to 0.75:1 in the bottom row was achieved. The bottom row containing only K-562 cells served as a control.

To assess the effect of neutrophil granulocytes on NK cell cytotoxic activity, granulocytes were added at granulocyte to NK cell ratios ranging from 10:1, 3:1 to 1:1, respectively.

To assess the effect of PMN supernatants on NK cell cytotoxicity, supernatants were generated by incubating PMN at a concentration of 10 x 10⁶ cells/ml in complete medium at 37 °C in a humidified atmosphere containing 5 % CO₂ for 3-4 hours. The supernatants were then added to the NK cells at the start of the assay.

Cytokines known to augment NK cell cytotoxicity were added to some assays. Universal type I IFN (type I IFN), an alpha interferon hybrid constructed from recombinant human (rh) IFN-αA and rh human IFN-αD, was added in a concentration of 1000 U/ml. IL-2 was added in a concentration of 200 U/ml. The total volume in each well was always kept at 200 µl. Each test sample in all experiments was plated out in triplicate.

The plate was then centrifuged for 5 minutes at 50 x g and incubated at 37 °C and 5 % CO₂ for 4 hours. 100 µl of supernatant was then aspirated from each well, taking care not to aspirate any cells and transferred to plastic tubes for analysis in a gamma counter. The radiation emitted by 100 µl of K-562 suspension served as a value for the total uptake of chromium.

The amount of gamma radiation emitted by the different samples was measured in the gamma counter as counts per minute (cpm).

An average value was calculated from the 3 different readings. An average of the readings for total uptake and the background radiation was also taken.

NK cell cytotoxicity was calculated as cell lysis in percent according to the following formula:

$$\text{specific lysis (\%)} = \frac{\text{experimental release (cpm)} - \text{spontaneous release (cpm)}}{\text{total uptake (cpm)} - \text{spontaneous release (cpm)}} \times 100$$

The lysis of K-562 cells for each sample in percent was then plotted against the ratio of effector to target cells. The degree of lysis of K-562 cells was assumed to correlate directly to the degree of NK cell cytotoxicity.

2.2.3.1 NK cell cytotoxicity against different target cells

In order to investigate whether NK cells might be cytotoxic towards *L. major* infected granulocytes, granulocytes and *L. major* infected granulocytes were used as target cells in a cytotoxicity assay. PMN were infected with stationary phase *L. major* promastigotes collected from *in vitro* cultures in biphasic NNN blood agar medium by co-incubating *L. major* and PMN in a parasite : PMN ratio of 5:1 for 2 hours in a humidified atmosphere containing 5 % CO₂. Any remaining extracellular *L. major* were removed by washing the suspension twice in complete medium at 300 x g for 8 minutes. PMN were counted and infection rate determined by Giemsa staining. The infected and non-infected PMN, as well as K-562 cells, which served as a control, were then radioactively labelled with 100 µCi Na₂⁵¹CrO₄ as described above. Cytotoxicity of NK-92 cells against these different target cells was then determined in a standard 4 h ⁵¹Cr-release assay as described above.

2.2.4 Co-incubation of NK-92 cells, PMN and K-562 cells for analysis of IFN-γ production by NK-92 cells

To assess the amount of IFN-γ production by the natural killer cell line NK-92 under different conditions, overnight co-incubations at 37 °C in a humidified atmosphere containing 5 % CO₂ were carried out in round bottom 96-well plates.

PMN were isolated from peripheral blood as described previously. NK-92 and K-562 cells were taken from the permanent cell culture. All cells were washed once in complete medium before use. Viability was always >98 %, as determined by trypan blue exclusion.

The cells were co-incubated in the following ratios: NK-92 : K-562 in the ratio of 6:1, NK-92 : PMN at a ratio of 1:10, respectively.

Type I IFN was added at a concentration of 200 U/ml.

The cell free supernatant was collected after overnight incubation (20-24 hours) and stored at -20 °C until IFN- γ determination by enzyme linked immuno sorbent assay (ELISA) as described below.

2.2.5 Cytokine assays

2.2.5.1 ELISA for analysis of IFN- γ production

Cell free supernatants of NK-92 cell cultures were collected after overnight incubation and stored at -20 °C until cytokine determination. The IFN- γ content of the supernatants was measured using the OptEIA™ Human IFN- γ Set according to the manufacturer's instructions. A standard curve was made with rh IFN- γ at concentrations ranging from 600 pg/ml to 9.4 pg/ml. The absorption was measured at 450 nm. The detection limit of the ELISA was 75 pg/ml.

2.2.5.2 Intracellular cytokine staining

To determine intracellular production of IFN- γ by NK-92 cells, these were cultured at a concentration of 1×10^6 /ml in complete medium under different conditions. PMN were added in a PMN : NK-92 ratio of 10:1, type I IFN was added at a concentration of 200 U/ml. Brefeldin A (100 μ g/ml) was then added and the cultures incubated for 11.5 hours at 37 °C in a humidified atmosphere containing 5 % CO₂. This was followed by permeabilisation of the cells and intracellular staining of IFN- γ using the Cytofix/Cytoperm™ plus kit and PE-labeled anti- IFN- γ mAb, respectively. PE-labeled mouse IgG1 was used as isotype control. The cells were analysed with a FACSCalibur® flow cytometer using CellQuest® software.

2.2.6 Co-incubation of PMN with NKL cells under different conditions for assessment of PMN apoptosis

PMN were cultured at a concentration of 5×10^6 cells per ml in complete medium at 37 °C in a humidified atmosphere containing 5 % CO₂ in tissue culture plates.

The natural killer cell line NKL, K-562 cells and LPS were added in the following concentrations:

NKL cells: 2.5×10^6 cells per ml

K-562 cells: 0.5×10^6 cells per ml

LPS: 100 ng/ml

The cells were then incubated overnight for 16 hours, after which cytocentrifuge slides were prepared in order to assess PMN apoptosis as described below.

2.2.7 Assessment of PMN apoptosis

In apoptotic PMN, morphological changes are manifested by separation of nuclear lobes and darkly stained pyknotic nuclei (101,124). Nuclear morphology was assessed on Giemsa-stained cytocentrifuge slides. Cell morphology was examined under oil immersion light microscopy. A minimum of 200 cells/slide were examined and graded as apoptotic/nonapoptotic.

3 Results

3.1 The effect of PMN on NK cell cytotoxicity

In this work, the interaction between natural killer cells and neutrophil granulocytes was to be investigated. To assess the effect of PMN on NK cell cytotoxicity, ^{51}Cr -release cytotoxicity assays were carried out.

3.1.1 Establishment of cytotoxicity assays: PBMC are cytotoxic against K-562 cells

To establish the performance of cytotoxicity assays, preliminary work was performed with PBMC (as effector cells) isolated from buffy coat or peripheral blood. PBMC contain about 10 % NK cells. ^{51}Cr -labelled K-562 cells were used as target cells. K-562 cells were lysed by PBMC, as expected. Lysis of K-562 cells was directly proportional to the ratio of PBMC to K-562 cells (Fig. 1A).

As a positive control, PBMC were prestimulated with 1000 U/ml type I IFN, which is known to activate NK cell cytotoxicity (110), for 1 hour before utilisation in the cytotoxicity assay. A marked increase in cytotoxicity of stimulated PBMC could be observed when compared to unstimulated PBMC (Fig. 1B).

Thus, the required methods had been established.

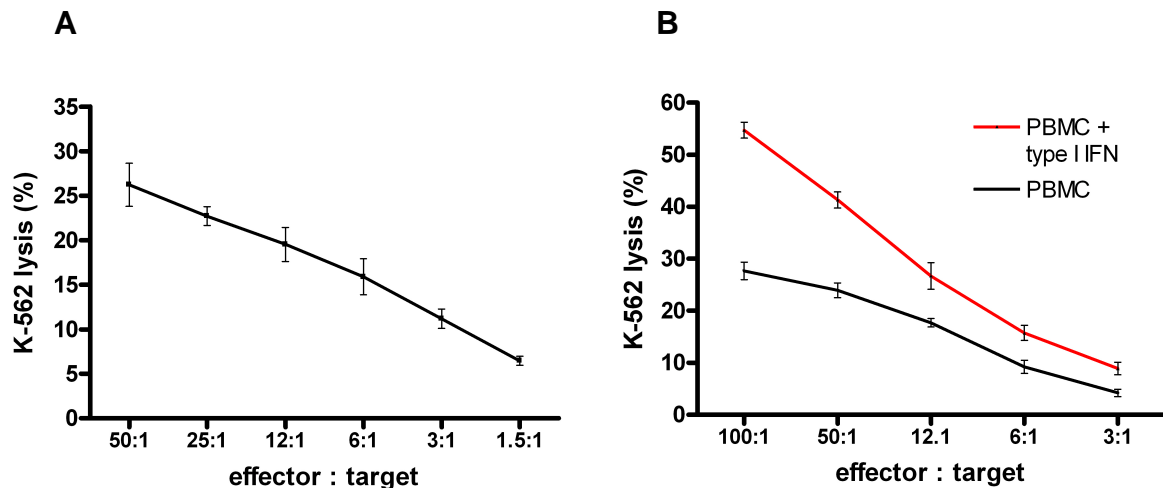


Fig. 1 PBMC are cytotoxic against K-562 cells; pre-stimulation with type I IFN increases cytotoxicity. NK cell cytotoxicity of PBMC was measured against ^{51}Cr -labelled K-562 target cells in a 4-h chromium-release assay at various effector-target cell ratios. A) Cytotoxic activity of unstimulated PMBC. The result of one experiment is shown. B) Cytotoxic activity of type I IFN pre-stimulated PBMC compared to unstimulated PBMC. PBMC isolated from peripheral blood were incubated for one hour in medium with 1000 U/ml type I IFN (red line) or medium without IFN (black line) in a humidified atmosphere containing 5 % CO_2 prior to use in the assay. The result of one experiment is shown. Values given are mean \pm SD from triplicate determinations.

3.1.2 Effect of PMN on the cytotoxicity of PBMC: do PMN activate or suppress natural killing?

In order to assess the effect of PMN on natural killer cell cytotoxicity, cytotoxicity assays were carried out in which PMN isolated from peripheral blood or buffy coat were added to PBMC in the ratio of 1 PMN to 1 PBMC. The effect of PMN on cytotoxicity could not be made out clearly in these assays. While in three separate experiments, PMN appeared to decrease the cytotoxicity of NK cells (a representative experiment is shown in Fig. 2A); in one other experiment PMN appeared to increase the cytotoxicity of NK cells (Fig. 2B).

To ensure that the PMN did not directly lyse K-562 cells, but mediated their effects through NK cells, PMN were added to K-562 cells in the absence of NK cells. PMN alone had no effect on K-562 lysis, which is valid for every cytotoxicity experiment shown in this work (Fig. 2A).

On the whole, these experiments were inconclusive as both activation and inhibition of NK cell cytotoxicity by PMN was observed. As PBMC only contain about 10 % NK cells, other cells within the PBMC, such as monocytes, might have interfered with NK-PMN interactions. Therefore, it was decided to further investigate the effect of PMN on NK cell cytotoxicity using highly purified NK cells in subsequent experiments.

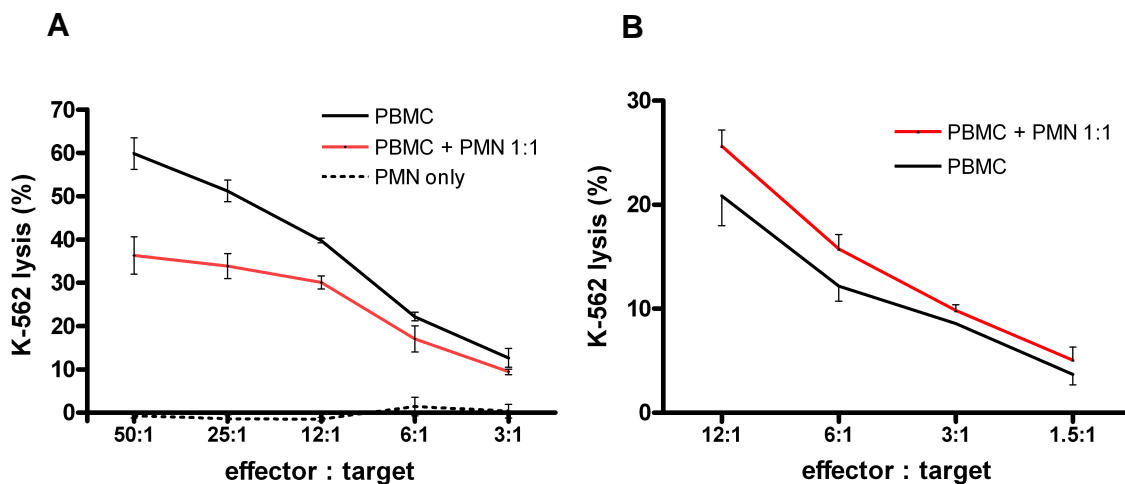


Fig. 2 Effect of PMN on NK cell cytotoxicity of PBMC: activation and inhibition. NK cell cytotoxic activity of PBMC isolated from peripheral blood or buffy coat was measured against ^{51}Cr -labelled K-562 target cells in a 4-h chromium-release assay at various effector-target cell ratios. Freshly isolated PMN were added to the assay in the ratio of 1PMN to 1 PBMC (red line). Values given are mean \pm SD from triplicate determinations. A) One experiment, representative for three experiments carried out, is shown. B) One experiment carried out is shown.

3.1.3 Increased cytotoxicity of highly purified NK cells as compared to PBMC

As experiments using the heterogeneous PBMC population to test the effect of PMN on NK cell cytotoxicity provided conflicting data, it was decided to purify NK cells from PBMC, to directly study the interaction between NK cells and PMN. NK cells were isolated from PBMC using MACS[®]. Hereby, NK cells were purified by depletion of unwanted cells from PBMC, leading to the isolation of untouched (unlabelled) NK cells. The purity of the NK cells isolated was analysed using flow cytometry, the NK cell population in this experiment was 92 % pure, as shown by positivity for the NK cell specific marker CD56 (Fig. 3A). The two peaks within the CD56 positive population (continuous line) represent the different subsets of NK cells, that is CD56^{dim} cells (mainly killer cells) and CD56^{bright} cells (mainly cytokine producers) (27).

As expected, cytotoxicity of the purified NK population was much higher than that of PBMC; a marked cytotoxic activity could still be measured at effector : target ratios as low as 1.5:1 (Fig. 3B). From this point on, NK cells used in cytotoxicity assays were purified from PBMC using MACS[®].

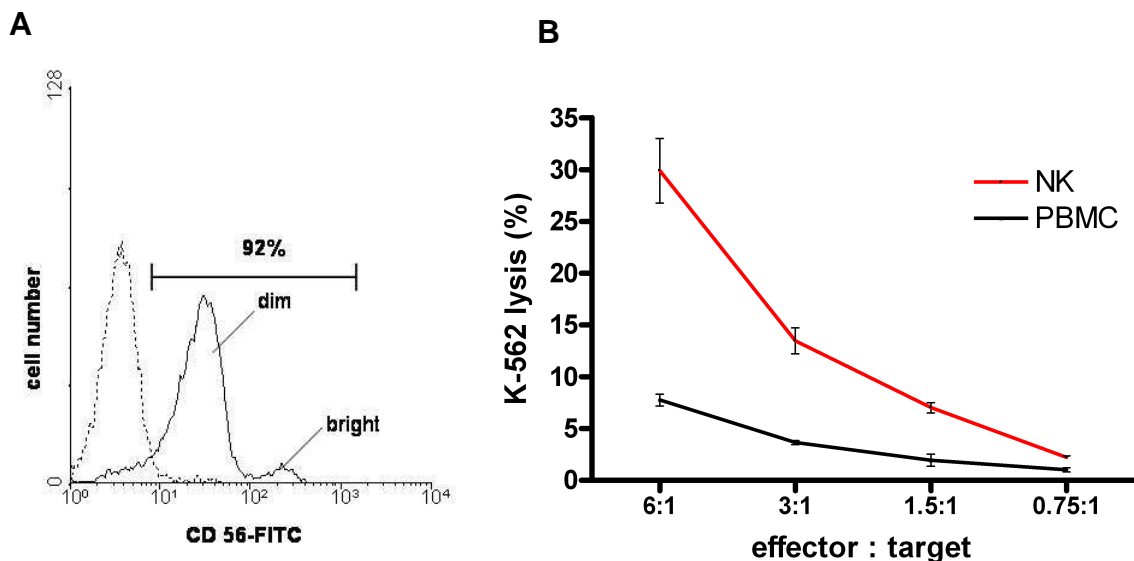


Fig. 3 Highly purified NK cells are more cytotoxic than PBMC, purity of isolated NK cells is >90 %. A) Purity of NK cells isolated by MACS[®] determined by FACS analysis. NK cells were stained a FITC-conjugated antibody against the NK cell marker CD56 (continuous line) and an isotype control antibody (broken line) and analysed by flow cytometry. B) Cytotoxicity assay comparing PBMC (black line) and purified NK cells (red line) isolated from peripheral blood as effector cells. Cytotoxicity was measured against ⁵¹Cr-labelled K-562 target cells in a 4-h chromium-release assay at various effector-target cell ratios. One experiment, representative for three experiments carried out, is shown. Values given are mean \pm SD from triplicate determinations.

3.1.4 PMN enhance the cytotoxicity of purified NK cells

After it had been shown that purified NK cells were able to mediate natural cytotoxicity against K-562 cells effectively, experiments were set up to clarify the effect of PMN on NK cell cytotoxicity.

In 8 experiments using highly purified NK cells isolated from 4 different donors, addition of PMN to NK cells resulted in a marked increase in cytotoxicity. PMN on their own did not lyse K-562 cells. In Fig. 4 the results of three experiments are shown.

During the course of experimentation, one donor was identified, where addition of PMN to NK cells resulted in a decrease in cytotoxicity in each of two separate experiments (data not shown). This finding was not further investigated, as subsequent experiments focused on the upregulation of NK cell cytotoxicity by PMN, which was the more frequently observed effect of PMN on NK cells.

Thus, the majority of experiments clearly showed enhancement of NK cell cytotoxicity by PMN.

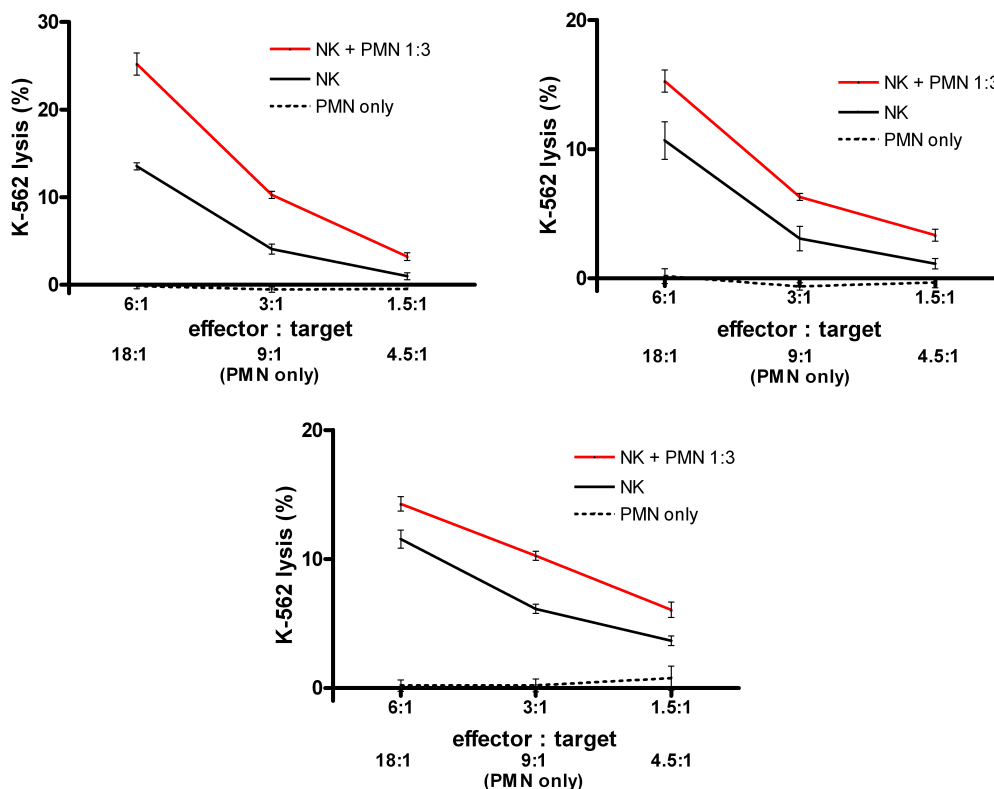


Fig. 4 PMN increase the cytotoxic activity of purified NK cells. NK cells were purified from PBMC isolated from peripheral blood using MACS[®]. Purity of NK cells evaluated using FACS analysis was >90 %. Cytotoxicity was measured against ⁵¹Cr-labelled K-562 target cells in a 4-h chromium-release assay at various effector-target cell (NK : K-562) ratios. Freshly isolated PMN were added to the assay in the ratio of 3 PMN : 1NK cell (red line). As a control, PMN were added to K-562 cells in the absence of NK cells (dotted line). Three experiments, representative for eight experiments carried out, are shown. Values given are mean \pm SD from triplicate determinations.

3.1.5 PMN enhancement of NK cell cytotoxicity is dose-dependent

To analyse the dose-dependency of the PMN-mediated enhancement of NK cell cytotoxicity, cytotoxicity assays were set up in which PMN were added to NK cells in different concentrations. Fig. 5 shows two experiments carried out, which demonstrate that the higher the PMN : NK ratio, the greater the increase of NK cell cytotoxicity. In Fig. 5A, addition of PMN to NK cells in the ratio of 1PMN to 1NK cell led to only a slight increase in cytotoxicity, however, when PMN were added in the ratio 10 PMN to 1 NK cell, a much greater increase in cytotoxicity resulted. In Fig. 5B, addition of PMN in a PMN : NK ratio of 10:1 also resulted in a higher increase of cytotoxicity than a PMN : NK ratio of 3:1. Therefore, it could be shown that PMN enhance NK cytotoxicity in a dose-dependent manner.

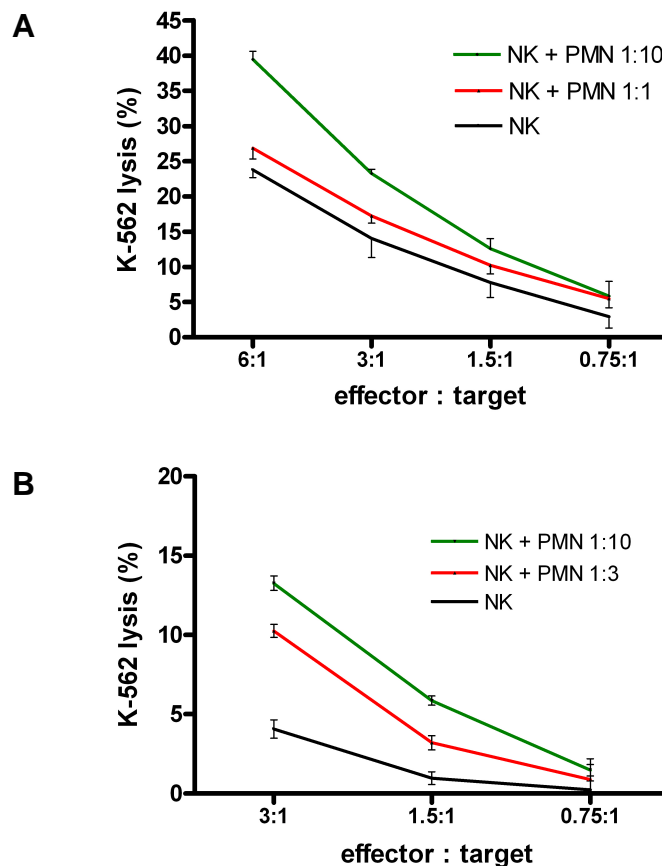


Fig. 5 Dose-dependent enhancement of NK cell activity by PMN. NK cells were purified from PBMC freshly isolated from peripheral blood using MACS®, purity of NK cells isolated was >90 %, as determined by FACS analysis. Cytotoxicity was measured against ⁵¹Cr-labelled K-562 target cells in a 4-h chromium-release assay at various effector-target cell ratios. A) PMN freshly isolated from peripheral blood were added to the assay in different PMN : NK cell ratios: 1:1 (red line) and 10:1 (green line). B) PMN freshly isolated from peripheral blood were added to the assay in PMN : NK cell ratios of 3:1 (red line) and 10:1 (green line). Values given are mean ± SD from triplicate determinations. Two experiments carried out are shown.

3.1.6 PMN-mediated enhancement of NK cell cytotoxicity does not require MHC compatibility

In previous experiments, NK cells and neutrophils were isolated from the same donor, that is, the cells were autologous.

To examine a possible influence of MHC haplotypes, NK cells were co-incubated with allogenic PMN in cytotoxicity assays. These were important preliminary studies for future experiments, in which NK cell lines were used to study the effect of PMN on NK cells. For these experiments, it was important to establish, that the effect of PMN on NK cell cytotoxicity could be mediated by PMN isolated from different donors.

In Fig. 6, one representative experiment out of two is shown. Here it can be seen, that NK cells co-incubated with both autologous and allogenic PMN in a PMN : NK ratio of 3:1 respond with increased cytotoxicity.

Thus, it could be shown that autologous and allogenic PMN were equally effective in augmenting NK cell cytotoxicity.

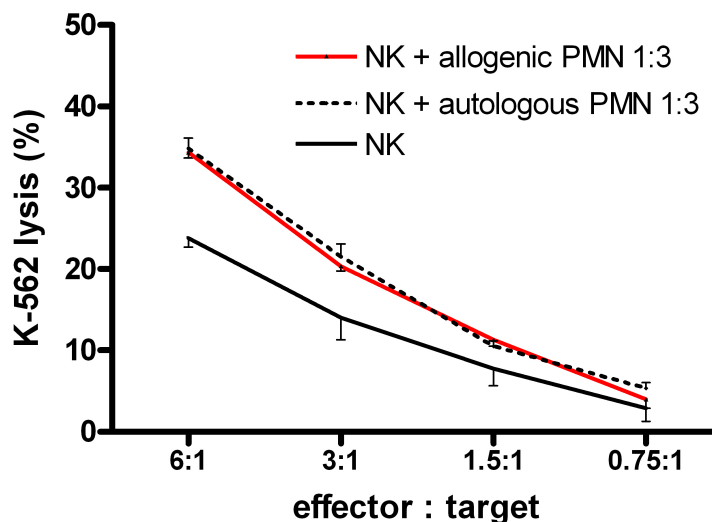


Fig. 6 Cytotoxicity of NK cells increases after addition of allogenic PMN. NK cells were purified from PBMC using MACS®. NK cell cytotoxicity was measured against ⁵¹Cr-labelled K-562 target cells in a 4-h chromium-release assay at various effector-target cell ratios. PMN freshly isolated from peripheral blood of an allogenic (red line) and the autologous donor (dotted line) were added at the start of the assay in the ratio of 3 PMN : 1NK cell. One experiment, representative for two experiments carried out, is shown. Values given are mean ± SD from triplicate determinations.

3.1.7 Supernatant of PMN increases NK cell cytotoxicity

In order to investigate whether the effect of granulocytes on NK cells might be due to soluble factors produced by PMN during the co-incubation with NK cells in the cytotoxicity assay, supernatants of PMN were produced by incubating PMN in complete

medium at a concentration of 10×10^6 PMN/ml at 37 °C in a humidified atmosphere containing 5 % CO₂ for 3-4 hours. Washed PMN (also incubated for 3-4 hours) and supernatants of PMN were then, respectively, added to NK cells in a cytotoxicity assay.

Both washed PMN (red line) and PMN supernatant (green line) were found to increase NK cell cytotoxicity. Figure 7 shows three representative experiments out of six carried out. In order to ensure that the supernatant did not lead to unspecific K-562 lysis, it was added to K-562 in the absence of NK cells. Supernatant added on its own did not mediate K-562 lysis (dotted line).

This data shows, that both PMN and supernatant of PMN are equally capable of enhancing NK cell cytotoxicity.

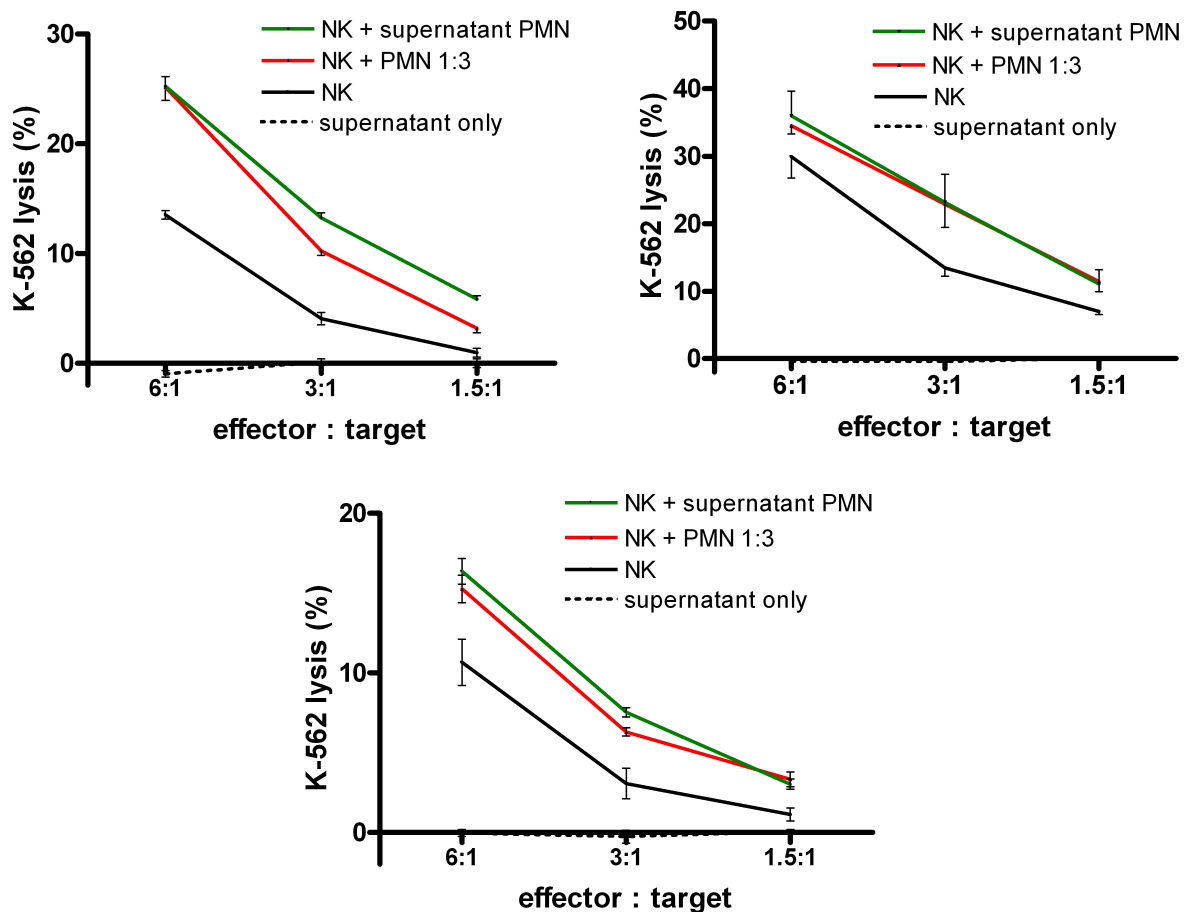


Fig. 7 Supernatant of granulocytes activates NK cells. NK cells were purified from PBMC using MACS®. Cytotoxicity was measured against ⁵¹Cr-labelled K-562 target cells in a 4-h chromium-release assay at various effector-target cell ratios. PMN freshly isolated from peripheral blood were added to the assay in the ratio of 3 PMN : 1NK cell; prior to addition to the assay, the cells were washed once in complete medium (red line). Supernatant of PMN, obtained by incubating PMN at a concentration of 10×10^6 /ml for 3-4 hours at 37 °C in a humidified atmosphere containing 5 % CO₂, was also added to the assay (green line). As a control, supernatant was added in the absence of NK cells (dotted line). Three experiments, representative for six experiments carried out, are shown. Values given are mean \pm SD from triplicate determinations.

3.2 Interferon- γ production by NK-92 cells after contact with PMN

In the experiments described so far, activation of NK cells by PMN was shown in terms of increased cytotoxicity. Cytotoxic activity is one main function of NK cells, which is mediated largely by a functional subset of NK cells called CD56^{dim} cells. However, another subset of NK cells, CD56^{bright} cells, mainly function as cytokine producers. Upon activation, this cellular subset can produce cytokines such as IFN- γ , TNF- β , IL-10, IL-13 and GM-CSF, which are known modulators of several immune functions (28). For example, IFN- γ secreted by CD56^{bright} NK cells helps to lead adaptive immunity towards a Th1 response, influences innate immunity by inducing the maturation of dendritic cells and contributes to tumour suppression.

The next experiments were therefore designed to investigate whether PMN might also be able to enhance the cytokine production of NK cells. IFN- γ was chosen as the representative cytokine to investigate.

Investigating the IFN- γ production of freshly isolated human NK cells is very cumbersome, as a relatively large number of cells are required to generate supernatants for measuring IFN- γ production by, for example, ELISA and only a small number of NK cells can be isolated from peripheral blood. In order to have a large number of NK cells readily available, further experiments investigating the effect of PMN on IFN- γ production by NK cells were carried using a natural killer cell line, NK-92, which had the additional benefit of being a homogenous cell population. The NK-92 cell line is an interleukin-2 (IL-2) dependent natural killer cell line derived from peripheral blood mononuclear cells of a 50 year old Caucasian male with rapidly progressive non-Hodgkin's lymphoma. This cell line has characteristics of activated NK cells, is cytotoxic to a wide range of malignant cells including K-562 cells and expresses CD56 at a high level (44).

3.2.1 PMN increase the cytotoxicity of NK-92 cells

The primary objective of working with NK-92 cells was to assess the effect of PMN on cytokine secretion by NK cells. However, before this was investigated, a preliminary experiment was carried out to examine whether the cytotoxic effector function of NK cells was also stimulated by PMN, as previously observed for NK cells and PMN freshly isolated from peripheral blood.

As expected, addition of PMN to NK-92 cells resulted in an augmentation of NK-92 cytotoxicity against K-562 cells in the pilot experiment carried out (Fig.8). Therefore, the results obtained with human NK cells could be reproduced for this tumour cell line.

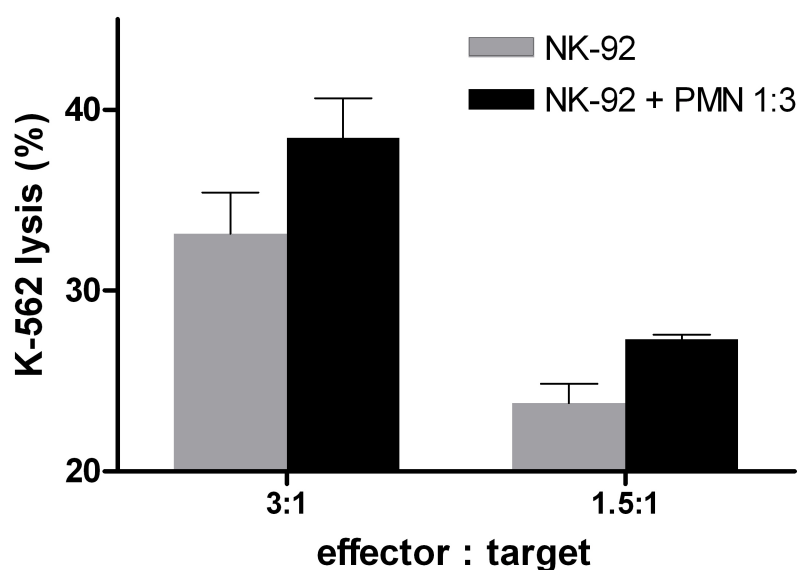


Fig. 8 NK-92 cells are cytotoxic against K-562 cells, cytotoxicity increases after addition of PMN. Cytotoxicity of NK-92 cells taken from the permanent cell culture was measured against ^{51}Cr -labelled K-562 target cells in a 4-h chromium-release assay at various effector-target cell ratios. PMN were freshly isolated from peripheral blood and added to the assay in the ratio of 3 PMN : 1 NK-92 cell. The results from one experiment are shown. Values given are mean \pm SD from triplicate determinations.

Note:

Another NK cell line, NKL, was also used in order to further investigate the effect of PMN on NK cell cytotoxicity and planned to be used to investigate the effect of PMN on cytokine production by NK cells (data not shown). However, due to a loss of cytotoxicity of these cells after 12 weeks in culture, experiments with this cell line had to be discontinued. It is known that NKL cells lose their cytotoxicity against target cells after the 40th passage (personal communication, Dr. E. Aga, Skirball Institute of Biomolecular Medicine, New York University Medical Center, New York).

3.2.2 Measurement of intracellular IFN- γ production by NK-92 cells

As NK-92 cell cytotoxicity and augmentation of cytotoxicity by PMN had now been established, experiments were set up to investigate whether PMN are capable of inducing IFN- γ secretion by NK-92 cells.

As a preliminary experiment, IFN- γ production by NK-92 cells was measured using intracellular staining followed by flow cytometric analysis. NK-92 cells were co-incubated

for 11.5 hours with PMN in the ratio of 10 PMN : 1 NK-92 cell with or without type I IFN at 37 °C in a humidified atmosphere containing 5 % CO₂.

The results of this experiment are shown in Fig. 9. Addition of type I IFN resulted in a discreet increase in IFN- γ production by NK-92 cells (Fig. 9B). Addition of PMN without (Fig. 9C), or together with type I IFN (Fig. 9D) also showed a slight upregulation of IFN- γ production of NK-92 cells. However, the observed small increase in IFN- γ production did not allow for any definite conclusions. One problem might have lain in the fact that the co-incubation period was not long enough. Incubation time was limited by the fact that cells cannot be co-incubated with Brefeldin A required for intracellular cytokine staining for longer than 12 hours. Moreover, Brefeldin A may interfere with PMN to NK interaction by inhibiting the secretion of PMN-derived soluble factors. In consequence, this method was not used further.

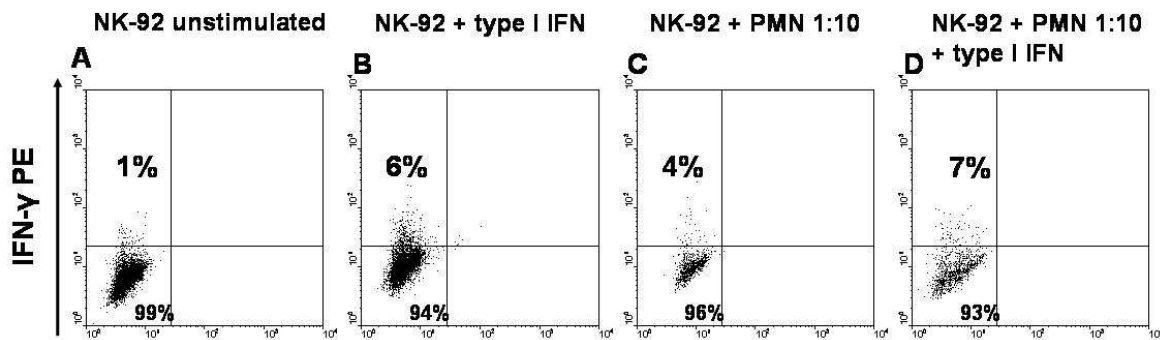


Fig. 9 Intracellular IFN- γ production by NK-92 cell is induced by different stimuli. NK-92 cells were co-incubated for 11.5 hours with PMN isolated from peripheral blood in the ratio of 1 NK cell to 10 PMN and 200 U/ml type I IFN at 37 °C in a humidified atmosphere containing 5 %CO₂. 100 μ g/ml Brefeldin A was added at the start of the incubation period, to prevent exocytosis of produced IFN- γ . IFN- γ produced by NK-92 cells was then measured by flow cytometry after permeabilisation and intracellular staining of the cells with PE-conjugated antibody against IFN- γ . The result from one experiment is shown.

3.2.3 PMN enhance IFN- γ secretion of NK-92 cells; PMN and type I IFN synergise in inducing IFN- γ secretion

As intracellular FACS staining did not appear a suitable method for determining IFN- γ production by NK-92 cells in response to PMN, it was decided to measure IFN- γ using a different method. ELISA was used to measure IFN- γ content of supernatants collected after 20-24 hour co-incubation of NK-92 cells with granulocytes under different conditions. This method had the advantage over intracellular cytokine staining, that incubation time was not limited, so that cells could be co-incubated for a longer period of time.

Figure 10 shows one representative experiment of three carried out. Unstimulated NK-92 cells produced a substantial amount of IFN- γ . Co-incubation of NK-92 cells with unstimulated PMN resulted in an increase IFN- γ production, stimulation of NK-92 with type I IFN resulted in an even higher production. The largest amount of IFN- γ was measured when NK-92 cells were co-incubated with PMN in the presence of type I IFN. PMN with or without type I IFN did not produce IFN- γ (Fig. 10).

Therefore it could be shown, that PMN induce IFN- γ secretion alone and in synergy with type I IFN.

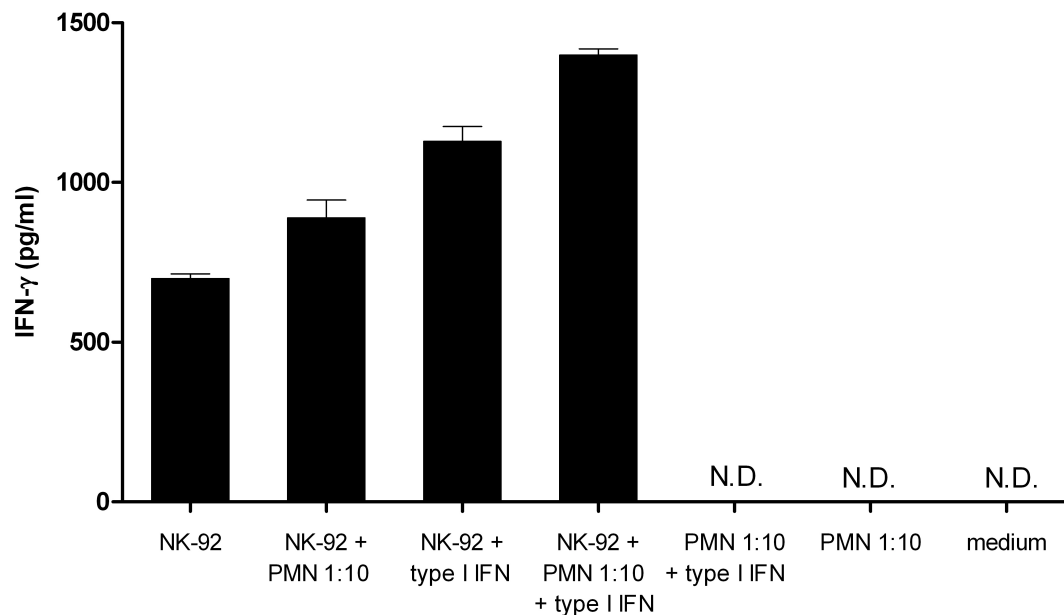


Fig. 10 Increased IFN- γ production by NK-92 cells after co-incubation with PMN and/or type I IFN. NK-92 cells were incubated overnight (20-24 h) in medium at 37 °C in a humidified atmosphere containing 5 % CO₂ at a concentration of 1×10^6 /ml. PMN freshly isolated from peripheral blood were added in the ratio of 10PMN : 1NK-92 cell. Universal type I interferon was added in a concentration of 200 U/ml. IFN- γ concentration in supernatants was determined using ELISA. The diagram shows one experiment representative for three carried out, each bar is the mean of 2 determinations \pm SD. N.D. (not detectable) indicates readings below the detection limit of the ELISA (75 pg/ml).

3.2.4 PMN and K-562 cells synergise in inducing IFN- γ secretion

The activating effect of PMN on both NK cell cytotoxicity and cytokine secretion had now been established. In addition, PMN were found to synergise with type I IFN, a known activator of NK cells in enhancing IFN- γ secretion by NK cells. The question was now put forward whether PMN would also be able to synergise with other activators of NK cell

cytotoxicity in inducing IFN- γ secretion by NK-92 cells. Tumour cells, being target cells for NK cells, activate NK cell cytotoxicity, which then leads lysis of the tumour cell. Therefore, it was investigated whether PMN might synergise with tumour cells in inducing IFN- γ secretion by NK-92 cells. The K-562 tumour cell line, previously used as target cells in cytotoxicity assays, was chosen for this purpose.

NK-92 cells were co-incubated with K-562 cells and PMN. IFN- γ content of supernatants collected after 24 hours of co-incubation was measured by ELISA. In two experiments carried out on the same day with cells from the same donor, addition of PMN or K-562 to NK-92 cells on their own resulted only in a small increase in IFN- γ production. However, when both types of cells were added to NK-92 cells together, a strong increase in IFN- γ production could be observed (Fig.11).

Thus, PMN synergise with tumour (K-562) cells in inducing IFN- γ secretion by NK cells.

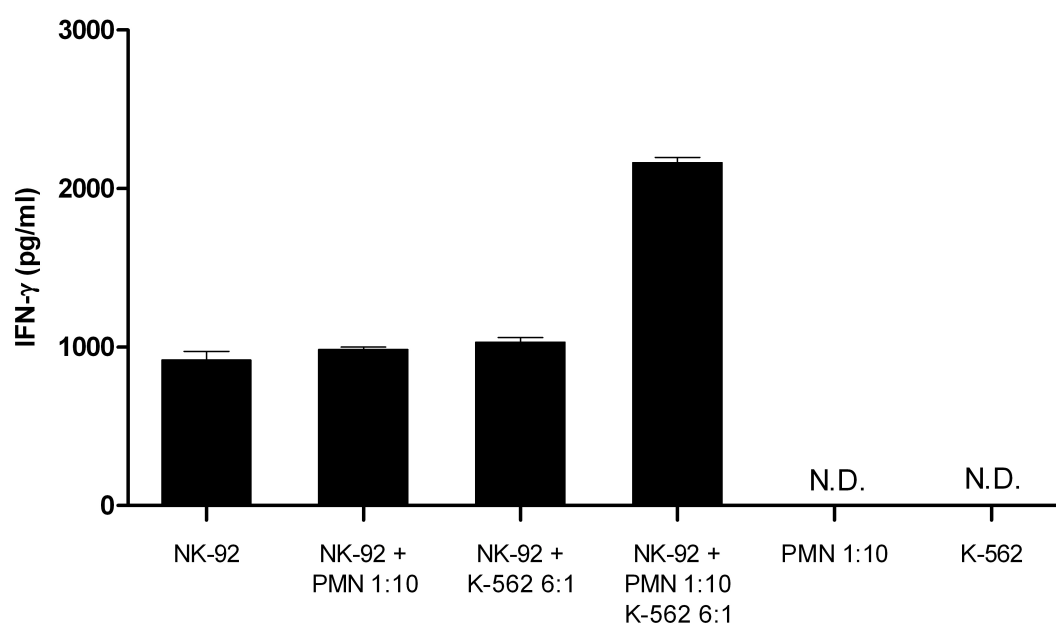


Fig. 11 IFN- γ production of NK-92 cells increases when co-incubated with the tumour cell line K-562 and PMN. NK-92 cells taken from the permanent cell culture were incubated for 24 hours in medium at a concentration of 1×10^6 /ml in a humidified atmosphere containing 5 % CO₂. PMN freshly isolated from peripheral blood were added in the ratio of 10 PMN : 1 NK-92 cell. K-562 cells taken from the permanent cell culture were added in the ratio of 1 K-562 cell : 6 NK-92 cells. IFN- γ concentration in supernatants was determined using ELISA. The diagram shows one representative experiment for two experiments carried out on the same day with cells of the same donor. Each bar is the mean of 2 determinations \pm SD. N.D. indicates readings below the detection limit of the ELISA (75 pg/ml).

3.3 The effect of NK cells on PMN

In the previous experiments, it was shown that neutrophil granulocytes are able to activate natural killer cells. To investigate, whether bi-directional activating interactions between NK cells and PMN exist and whether infected PMN might become susceptible to NK cell killing, the following experiments were set up.

3.3.1 NKL cells inhibit the spontaneous apoptosis of PMN

PMN are short-lived cells, with a life span of about 6 hours in the blood stream, at the end of which they undergo apoptosis. However, in response to inflammation, the apoptosis of PMN is delayed, allowing them to engage in their effector function in infected tissues. This delay of apoptosis correlates to the degree of activation of PMN, so that inhibition of apoptosis may be used to assess PMN activation.

To investigate whether NK cells might also have an activating effect on granulocytes, an experiment was set up in which the inhibition of the spontaneous apoptosis of PMN by cells of the NK cell line NKL was investigated. NKL cells were co-incubated overnight with PMN in the ratio of 1 NKL cell to 1 PMN. Lipopolysaccharide (LPS), as a known activator of PMN, was added in a concentration of 100 ng/ml and K-562 cells were added in the concentration of 1 K-562 cell : 5 NKL cells. After 16 h co-incubation the degree of PMN apoptosis was assessed by light microscopy of cytocentrifuge slides using typical morphological criteria of PMN apoptosis such as cell shrinkage, cytoplasmic condensation and condensation of nuclear heterochromatin (95,111).

As expected, the amount of apoptotic granulocytes nearly halved when stimulated with LPS (Fig 12). On co-incubation of PMN with NKL cells the degree of apoptosis delay was even greater than with LPS stimulation. Co-incubation of K-562 cells with granulocytes also resulted in a decrease in the amount of apoptotic PMN, suggesting that PMN are activated by this tumour cell line. Upon co-incubation of both cell lines together, apoptosis of PMN was even further reduced. When both cell lines were co-incubated together with PMN in the presence of LPS, a nearly complete inhibition of PMN apoptosis resulted.

Thus, this data suggests that NK cells are able to delay the spontaneous apoptosis of PMN and synergise with both NK cell activators (K-562 cells) and PMN activators (LPS) in inhibiting PMN apoptosis.

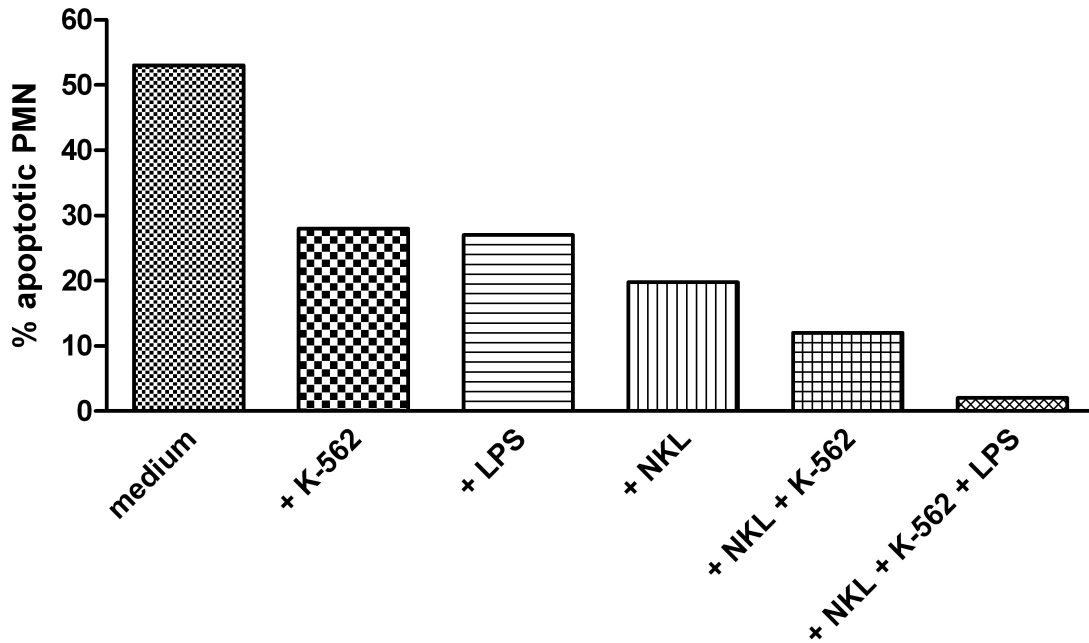


Fig. 12 Apoptosis of PMN is inhibited after co-incubation with NK cells. PMN freshly isolated from peripheral blood were incubated overnight (16h) in a concentration of 5×10^6 cells/ml in a humidified atmosphere containing 5 % CO₂. K-562 cells were added in the ratio of 1 K-562 cell : 5 NK cells, NK cells added in the ratio of 1 NK cell : 2 PMN. LPS was added in a concentration of 100 ng/ml. The results from one experiment are shown.

3.3.2 NK-92 cells are not cytotoxic against *L. major* infected PMN

NK cells are important effector cells in the fight against intracellular pathogens. NK cell cytotoxicity towards virus-infected host cells is well-established. It has also been shown, that NK cells lyse macrophages infected with mycobacteria (1,64).

Studies from our laboratory have shown an important role for NK cells in the host response to infection with the intracellular protozoan parasite *L. major*. So far, the role for NK cells in controlling *L. major* infection has chiefly been attributed to cytokine secretion. IFN- γ produced by NK cells is instrumental for parasite containment (74). Cytotoxicity of NK cells against *L. major* infected host cells, in particular against *L. major* infected PMN, has not been shown.

To investigate, whether NK cells might contribute to the resolution of *L. major* infection by the lysis of infected PMN, a cytotoxicity assay was set up in which, besides K-562 cells, granulocytes and *L. major* infected PMN were used as target cells. NK-92 cells were used as effector cells.

Figure 13 shows that K-562 cells (blue line), were highly susceptible to killing by NK-92 cells as shown in previous experiments. However, neither *L. major* infected PMN (red line) not uninfected granulocytes (black line), were lysed by NK-92 cells. (Fig. 13).

This shows that NK-92 cells are not cytotoxic against *L. major* infected PMN.

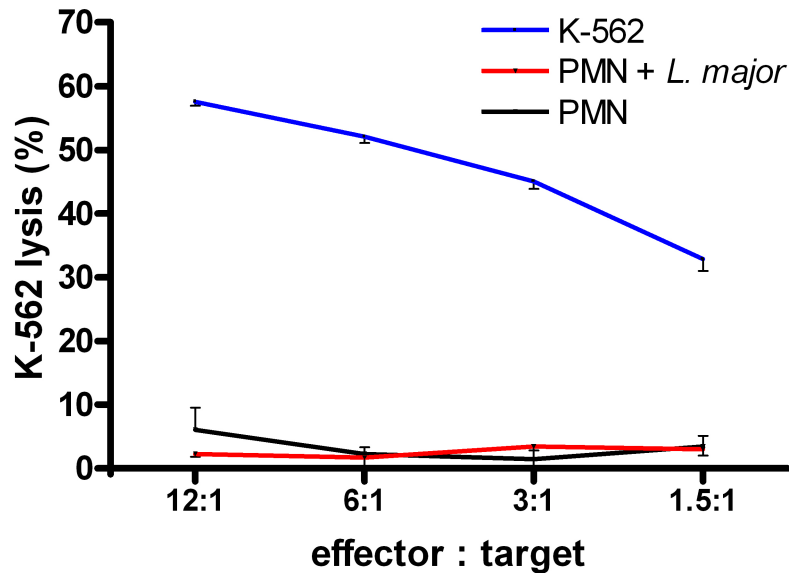


Fig. 13 *L. major* infected PMN are not susceptible to killing by NK-92 cells. Cytotoxicity of NK-92 cells against different ^{51}Cr -labelled target cells was measured in a 4-h chromium-release assay at various effector-target cell ratios. Different target cell used were: K-562 cells (blue line), *L. major* infected PMN (red line) and PMN not infected with *L. major* (black line). PMN were infected with *L. major* prior to radioactive labelling by incubating them in a concentration of 5×10^6 /ml together with *L. major* in a concentration of 50×10^6 /ml for 2 hours at 37 °C in a humidified atmosphere containing 5 % CO_2 . The infected PMN were then washed twice to remove any extracellular *L. major*. Infected PMN, not infected PMN and K-562 cells were labelled radioactively with ^{51}Cr in by adding 100 μCi $\text{Na}_2^{51}\text{CrO}_4$ and incubating the cell suspensions at 37 °C in a humidified atmosphere containing 5 % CO_2 for one hour. Any chromium not taken up by the cells was then removed by washing twice in medium. In order to activate the NK-92 cells, IL-2 was added to the assay in a concentration of 200 U/ml in each well. The results from one experiment are shown. Values given are mean \pm SD from triplicate determinations.

4 Discussion

PMN and NK cells are among the first cell types recruited to a site of inflammation, where they play an important role in host defence. Phagocytosis of microorganisms by PMN and natural cytotoxicity of NK cells are well-established effector functions of these cells, which allow them to promptly respond to infection in a non-specific manner. However in recent years, both NK and PMN have been shown to have important immunomodulatory functions. They are able to communicate with other cells of the innate immune system, as well as influencing the adaptive immune response. However, apart from inconclusive experiments regarding the effect of PMN on cytotoxicity of NK cells, little research has been carried out on the mutual influence of these two cell types.

This work investigated whether an activating cross-talk between NK cells and PMN exists. In particular, it was sought to clarify the effect of PMN on cytotoxicity of natural killer cells, as well as analysing whether PMN are able to induce cytokine secretion by NK cells in terms of IFN- γ production. Activation of PMN by NK cells was assessed in terms of delayed apoptosis of PMN.

4.1 The effect of PMN on NK cell cytotoxicity

Cytotoxicity is a well-established effector function of natural killer cells. NK cells are cytotoxic towards susceptible target cells, which include virus-infected cells and malignant cells. Natural killer cytotoxicity against target cells can be augmented by a range of cytokines, the most investigated being virus-induced type I IFNs (IFN- α and - β) (110) and IL-2 (49). Other cytokines known to upregulate NK cell cytotoxicity are IL-1, IL-7, IL-12, IFN- γ and TNF- α (108). IL-15, IL-18 and IL-21 have also been shown to augment NK cell cytotoxicity (13,108,119,141).

Besides soluble factors, cell-cell contacts have also been shown to activate NK cells in terms of increased cytotoxicity, such as interactions with T or B cells, as well as dendritic cells (9,139,145).

Previous studies investigating the effect of PMN on NK cell cytotoxicity have been inconclusive. On the one hand, experiments dating back to the 1980s, have reported that PMN decrease NK cell cytotoxicity. A role for ROS (115), PMN-derived prostaglandins (102) and LDL oxidised by PMN (131) has been implied in mediating the observed

suppression. However, other studies postulated, that PMN-mediated suppression of NK activity could not be due to reactive oxygen species, as oxidant scavengers had no effect on inhibition (117). On the other hand, there are a number of studies reporting PMN-mediated augmentation of NK cell cytotoxicity. An increase in NK cell cytotoxicity was reported after co-incubation of NK cells and PMN in diffusion chambers (142). Azurophil granule contents of PMN have been held responsible for mediating activation of NK cell cytotoxicity (79). An *in-vivo* study reported increased neutrophil content in the spleen of IL-4 treated mice to be associated with an increase in NK activity (132). A lot of controversial data has been reported regarding the effect of PMN on NK cell cytotoxicity and little research has been undertaken in this area at present, despite the existence of new methods for isolating NK cells of high purity, such as MACS® and increased understanding of both NK and PMN biology.

In the present work, experiments were set up to clarify modulation of NK cell cytotoxicity by PMN. NK (effector) cytotoxicity was measured in a standard 4h ⁵¹Cr-release assay against the target tumour-derived cell line K-562. In order to establish existing methods, peripheral blood mononuclear cells (PBMC) isolated from healthy volunteers, which contain about 10 % NK cells, were used as effector cells in the cytotoxicity assay. PBMC were found to be cytotoxic against K-562 cells and cytotoxicity was augmented by addition of type I IFN to the assay, keeping in line with literature. The method being established, the effect of PMN on NK cell cytotoxicity was then investigated by adding freshly isolated PMN to the PBMC at the start of the assay. These experiments provided controversial data. While in 3 experiments, PMN were found to decrease cytotoxicity, one experiment showed upregulation of cytotoxicity in response to PMN. How can these conflicting results be explained? One important aspect might lie in the fact that PBMC are a heterogeneous cell population, of which NK cells only make up a small portion. Therefore, other cell populations, such as monocytes, might interfere with NK-PMN interactions. Indeed, it has been reported that monocytes inhibit NK cell cytotoxicity (114). The study of Yang and Zucker-Franklin, in which soluble factors released by PMN were found to increase cytotoxicity, also reports that soluble factors produced by monocytes decreased cytotoxicity (142). They report that an *in vitro* ratio of 1 monocyte to 1000 NK cells still mediated suppression effectively. Addition of PMN to PBMC might result in PMN-monocyte interactions which induce downregulation of NK cell cytotoxicity.

These experiments lead to the conclusion, that the heterogeneous PBMC population was not a suitable model for investigating NK-PMN interactions. To eliminate interference by other cell populations, NK cells were purified from PBMC using MACS[®]. The isolated NK population was more than 90 % pure, as shown by FACS analysis of the NK-specific marker CD56. Cytotoxicity of purified NK cells towards K-562 target cells was much higher than that of PBMC, effector : target ratios of 1.5:1 still showed considerable lytic activity.

Addition of freshly isolated PMN to purified NK cells in the cytotoxicity assays resulted in upregulation of natural killer cytotoxic activity in 8 independent experiments using cells isolated from 4 different donors. The upregulation of NK cell cytotoxicity by PMN was further substantiated by the finding that addition of PMN in different PMN : NK cell ratios resulted in a dose-dependent upregulation of NK activity – a PMN : NK cell ratio of 10:1 resulted in a much higher increase of cytotoxicity than a PMN : NK cell ratio of 3:1 or 1:1. So far, NK cells and PMN used in the cytotoxicity assay were autologous, that is NK cells and PMN were isolated from the same donor. However it could also be shown, that addition of allogenic PMN to NK cells, that is PMN and NK cells isolated from different donors also resulted in increased NK cell cytotoxicity. This implies that the observed augmentation of cytotoxicity by PMN was independent of MHC molecules. These findings provided valuable information for experiments carried out at a later stage, in which a natural killer cells line was used to further investigate NK-PMN interaction, where, naturally PMN used were not from the same donor as the original donor from whose malignant cells the NK cell line was established.

During the course of the cytotoxicity experiments, one NK cell donor was identified, whose NK cells responded with decreased cytotoxicity after addition of PMN. Although this finding could not be explained, it is likely that this response is a phenomenon of the NK cells of this particular donor, as both autologous and allogenic PMN were capable of mediating this suppression. The natural killer cells of this donor had a higher intrinsic cytotoxic activity than those of most other donors, therefore augmentation of cytotoxicity might have required stronger stimuli for this donor. In addition, this donor had considerable eosinophilia, granulocytes isolated from this donor contained around 10 % eosinophil granulocytes. Because of these peculiarities, NK cells from this particular donor were not chosen for further experiments.

Although the above data showed upregulation of NK cell cytotoxicity by PMN, so far the mechanism responsible for this effect was not clear. The observed activating interaction might be mediated by a soluble factor released by PMN or cell to cell contacts between PMN and NK cells. To provide some insight into this question, experiments were carried out in which the effect of cell-free PMN supernatant added to the assay was compared to the effect of PMN washed prior to the assay, in order to remove any soluble factors. These experiments showed that supernatants of PMN were also able to mediate increased NK cell cytotoxicity. Therefore, a soluble factor seems to play a role in activation of NK cells by PMN. The involvement of cell-cell contacts could not be excluded by these experiments.

As both PMN and NK cells were present in these cytotoxicity assays, it was important to ensure that the observed increase in K-562 lysis upon addition of PMN to the assay was due to an increase in NK cell cytotoxicity towards K-562 cells and not due to PMN-mediated lysis of K-562 cells. Therefore, controls were set up in which PMN or supernatant of PMN were added to K-562 cells in the absence of NK cells. In these samples, no K-562 could be shown. This showed that NK cells must be responsible for the observed increase in K-562 lysis after addition of PMN or PMN supernatants, as opposed to unspecific K-562 lysis due to increased production of lytic substances by PMN or alteration of medium parameters such as the pH.

These findings imply that PMN are capable of upregulating NK cell cytotoxicity. One potential question regarding this finding might be why unstimulated PMN would mediate this effect. When PMN and NK cells encounter within the blood stream such an upregulation would not be necessary and might be potentially harmful. However, within the cytotoxicity assay, the tumour cell line K-562 was also present. NK cells are known to be activated by tumour cells, due to lack of MHC class I molecule expression as well as the expression of activating cell surface molecules by cancerous cells (121). Therefore it is likely that contact with K-562 cells primes NK cells for consequent activation by PMN.

If the concept is postulated that PMN activate NK cells, then how can previous studies reporting a decrease in NK cell cytotoxicity in response to PMN be explained? One important factor might have been that the cell populations used in these studies were not very pure. Other cells, such as monocytes, might have interfered with NK-PMN interactions. In fact, in the experiments where PBMC were used as effector cells, I also obtained controversial data. Only the work with highly purified NK cells led to reproducible findings.

Which mechanism lies behind the observed upregulation of NK cell cytotoxicity in response to PMN? Dendritic cells have been shown to upregulate NK cell cytotoxicity through the release of cytokines, such as IL-12, IL-15, IL-18 and IFN- α/β as well as through direct cell-cell interactions (139). The data presented suggests a role for soluble factors and does not exclude possible cell-cell contacts in PMN-mediated activation of NK cytotoxicity. PMN have been shown to release a variety of cytokines which are known activators of NK cell cytotoxicity, such as IL-12 (22) and IFN- α (118). IL-15 has also been reported to be produced by PMN (96). Secretion of these cytokines by PMN might contribute to the observed NK activation. Cell-cell contacts might also play a role in PMN-NK interactions.

Clearly, the ability of PMN to activate NK cells in terms of increased cytotoxicity is a finding which is plausible in the context of infection or neoplasia, as increased cytotoxicity would be able to contribute to the elimination of infected or transformed cells.

4.2 IFN- γ secretion by NK cells

Another important effector function of natural killer cells is their ability to act as immunomodulatory cells through the secretion of different cytokines in response to activation. Cytokine secretion is mediated largely by CD56^{bright} cells (28). One central immunoregulatory cytokine secreted by natural killer cells is IFN- γ , which is crucial in the early response to infection. IFN- γ secreted by NK cells activates macrophages and dendritic cells and is able to drive adaptive immunity toward a Th1 response. IFN- γ also contributes to the suppression of tumour growth (122). It has also been shown that IFN- γ is able to activate PMN (21).

IFN- γ secretion by NK cells can be induced by cytokines, such as IL-2 (4) and IL-12 (70). IL-1 α and IL-1 β , IL-15, IL-18 and TNF- α all synergise with IL-12 in inducing IFN- γ production by NK cells (18,58,130,135). More recently, T cell derived IL-21 has been implicated in inducing IFN- γ production by NK cells (126).

Cell-cell contacts have also been shown to play a role in inducing IFN- γ secretion by NK cells, as shown, for example by the interaction of NK cells and DCs (139).

4.2.1 Induction of IFN- γ secretion by NK cells through PMN

While the effect of PMN on the cytotoxicity of NK cells has been extensively investigated, little work has been carried out regarding the effect of PMN on cytokine secretion by NK

cells. No studies investigating whether PMN are able to induce IFN- γ secretion by NK cells have so far been carried out.

PMN have been shown to release IL-12 (22), which is known to induce IFN- γ secretion by NK cells (70). Another cytokine which might be responsible for PMN induced IFN- γ production is an IL-12 family member, IL-27. Production of IL-27 by PMN has been shown (81) and IL-27 has been suggested to induce IFN- γ secretion by NK cells (104). IFN- α , another cytokine known to induce IFN- γ secretion by NK cells, has been shown to be produced by PMN (118). Emoto and others made the assumption, that granulocytes might play a major role in IFN- γ secretion by NK cells, as they found that depletion of PMN in mice resulted in a marked reduction of IFN- γ production by NK cells (33). However, so far there are no reports in literature regarding the direct induction of IFN- γ secretion by NK cells through PMN.

As relatively large numbers of NK cells would be required to investigate the IFN- γ production of NK cells in response to PMN and only small numbers could be isolated from peripheral blood, a natural killer cell line, NK-92, was chosen for these experiments.

NK-92 cells have characteristics of activated NK cells and display CD56^{bright} expression, thus would appear to belong to the immunoregulatory subset of NK cells and be particularly suitable for studies on cytokine secretion (44,90). The secretion of IFN- γ by NK-92 cells has been shown (77). Upregulation of IFN- γ secretion by NK-92 in response to different cytokines has also been reported (87). No studies have so far been carried out regarding the effect of PMN on either cytotoxicity or cytokine secretion of this cell line.

The main objective of working with this cell line was to analyse IFN- γ secretion after contact with PMN. Before investigating the effect of PMN on cytokine secretion NK-92 cells, an experiment was set up to assess whether the cytotoxicity of NK-92 cells could be enhanced by PMN, as it had been shown for NK cells freshly isolated from peripheral blood. Augmentation of NK-92 cytotoxicity by PMN could be shown in one experiment. These findings further supported previous data, showing that, like NK cells freshly isolated from peripheral blood, this cell line also upregulates cytotoxicity in response to PMN.

After having shown the effect of PMN on NK-92 cytotoxicity, the IFN- γ production of NK-92 in response to PMN was investigated by different methods. Intracellular IFN- γ production by NK-92 cells after co-incubation with type I IFN, a known NK cell activator and PMN was assessed by intracellular cytokine staining followed by flow cytometric analysis. Although both PMN alone and PMN together with type I IFN induced a slight

upregulation of intracellular IFN- γ production by NK-92 cells, this increase was not large enough to allow for definite conclusions as to the effect of PMN on IFN- γ production by NK-92 cells. The disadvantage of intracellular cytokine staining was that incubation time was limited, as cells could not be incubated with the required transport inhibitor Brefeldin A for more than 12 hours. Given that cytokines produced by PMN would induce IFN- γ secretion by NK-92 cells, long co-incubation times might be required, as PMN only produce small amounts of cytokines (19). In addition, Brefeldin A may have inhibited the secretion of PMN cytokines which could act on NK cells.

In order to allow for longer co-incubation times of NK-92 and PMN, it was chosen to further investigate IFN- γ production by ELISA. An increase in IFN- γ production by NK-92 after 24 hour co-incubation with PMN could be shown in three separate experiments. IFN- γ production was also upregulated in response to type I IFN, which goes in line with the previous reports in literature that IFN- α induces IFN- γ mRNA production in NK-92 and NK cells (87). In addition, type I IFN and PMN synergised in their activating effects to even further increase IFN- γ production. Synergism between IFN- α and the cytokines IL-12 and IL-18 in inducing IFN- γ secretion by both NK and NK-92 cells has also been reported (87).

How could PMN induce IFN- γ secretion by NK cells? DCs have been shown to induce IFN- γ secretion of NK cells through both cytokine secretion (139) as well as cell-cell contact (61). These mechanisms could also play a role for PMN-NK interactions. PMN have been reported to release cytokines such as IL-12, IL-27 and IFN- α , which have been shown to induce IFN- γ secretion by NK cells. PMN might induce IFN- γ in NK cells through production of these cytokines. Indeed it might be possible that the synergism of type I IFN and PMN in inducing IFN- γ secretion by NK-92 cells might be due to PMN-produced IL-12 synergising with type I IFN, as reported in literature (87). Direct cell-cell contacts between NK cells and PMN might also play a role in IFN- γ induction.

These results suggest that PMN might play an important role in mediating immunoregulatory functions of NK cells by inducing IFN- γ production. The observed synergism of PMN and type I IFN in inducing IFN- γ production suggest that NK cells pre-activated by type I IFNs in an inflammatory environment become more responsive to stimulation by PMN.

4.2.2 Synergism of PMN and K-562 cells in inducing IFN- γ secretion by NK cells

Tumour cells are known to stimulate NK cell effector functions. Cytotoxicity is activated after the recognition of altered cell surface molecule expression, such as reduced MHC class I molecule expression. Different tumour cell lines have been shown to stimulate IFN- γ production by natural killer cells (90). Previous studies have shown that the myeloid tumour cell line K-562 cells is able to induce IFN- γ production by NK cells (60). The potential of PMN for inducing IFN- γ secretion by NK cells have been discussed previously. However, there are no reports in literature of synergism between tumour cells and PMN in inducing IFN- γ secretion by NK cells. In these experiments, it was shown that while both PMN and K-562 cells on their own had little effect on the IFN- γ production by the natural killer cell line NK-92, a strong increase in IFN- γ production was observed after co-incubation of NK-92 with both PMN and K-562. Although previous experiments showed increased upregulation of IFN- γ production in response to PMN on their own, the essential finding of synergism between PMN and K-562 cells in inducing IFN- γ production keeps in line with the previously described synergism between PMN and type I IFN and the synergism of different stimuli in inducing IFN- γ secretion reported in literature (87). The implication of this finding is that, while both PMN and K-562 have the potential to induce IFN- γ production in natural killer cells, the responsiveness of NK cells to this stimulation might be little under physiological conditions. However, the encounter with tumour cells (modelled here by K-562 cells) primes them for stimulation by PMN.

4.3 The effect of NK cells on PMN

In the first part of this study, it was shown that PMN have an activating effect on both cytotoxicity and IFN- γ secretion of natural killer cells. To analyse whether this activating interaction might be bi-directional, the effect of NK cells on PMN was investigated.

4.3.1 Inhibition of PMN apoptosis by NKL cells

PMN are inherently short-lived cells. However, in response to infection, their life span is prolonged, allowing them to engage in their effector function at the site of inflammation. Exposure to inflammatory cytokines (3), bacterial cell wall components such as LPS (75), as well as intracellular infection with bacterial pathogens (42), or protozoa, such as *L. major* (2), have all been shown to delay the apoptosis of PMN. Inhibition of PMN apoptosis has also been shown after contact with other cells of the immune system, such as DCs (84).

Cytokines known to enhance the life span of PMN, such as IFN- γ , GM-CSF and TNF- α (25) can all be produced by NK cells (28,30). However, little can be found in the literature regarding a direct effect of NK cells on PMN apoptosis.

In this study, it was shown that the natural killer cell line NKL was able to inhibit the apoptosis of PMN. Inhibition of PMN apoptosis was also observed when PMN were incubated with the myeloid-derived cell line K-562. Moreover, both cell lines synergised with LPS, a known inhibitor of apoptosis (75), to almost completely abrogate PMN apoptosis. This dramatic increase in PMN life span could be due to the fact that i) K-562 cells activated the natural killer cells and ii) that LPS primed the PMN, making them more responsive to activation by natural killer cells. Therefore, in inflamed tissues, where both NK cells and PMN are activated, NK cells are able to even further activate PMN. This activation could be mediated by a soluble factor released by NK cells, such as IFN- γ , GM-CSF or TNF- α or be due to cell contacts. Together with previously discussed results, these findings imply that a bi-directional activating cross-talk between NK cells and PMN might indeed exist.

4.3.2 *L. major* infected PMN are not lysed by NK cells

Natural killer cells are known for their ability to kill cells with altered cell surface molecule expression. These include cells of the body infected with viruses, such as herpes viruses (7) and intracellular pathogens such as *Listeria monocytogenes* (45). Pathogens are also able to infect phagocytic cells of the immune system: *L. major* infect granulocytes before being taken up by macrophages, their final host cells (136). It has been shown that NK cells play a critical role in the immune response to *L. major* infection, chiefly through cytokine production (74,112,123). However, so far, no studies have been carried out whether NK cells might also be cytotoxic towards *L. major* infected PMN.

NK cell cytotoxicity towards infected cells of the immune system has been reported. *Mycobacterium* infected macrophages are lysed by NK cells (1,64). Monocytes infected with *Legionella pneumophila* are also lysed by NK cells (10). On the other hand it was also shown, that immature DCs, which are lysed by NK cells, become resistant to lysis after infection with *Escherichia coli*, *Bacillus Calmette-Guerin* and *Leishmania infantum* (16,39).

In order to investigate whether NK cells might be toxic towards *L. major* infected PMN, a cytotoxicity assay was set up in which granulocytes infected with *L. major* were used as target cells and the NK-92 cell line was used as effector cells. No lysis of either uninfected

PMN or *L. major* infected PMN by the natural killer cell line NK-92 could be shown. Uninfected PMN were probably not lysed by NK cells as they exhibited normal self markers. One possible mechanism for the resistance of infected PMN to NK cell lysis might be that internalised *L. major* prevents cell surface molecule changes which might activate natural killer cells. This would be in line with findings from our laboratory that *L. major* is able to downregulate the expression of 200 genes induced by IFN- γ and LPS in PMN (81). It has been shown, that uptake of *L. major* infected PMN by macrophages does not result in activation of the macrophages (136). *L. major* is able to misuse PMN in order to enter the macrophages silently and thus prevent their destruction. The finding that *L. major* infected granulocytes were not lysed by NK cells suggests another possible mechanism by which *L. major* escape immune surveillance.

In conclusion, the data presented in this work suggest that a bi-directional, activating cross talk between natural killer cells and neutrophil granulocytes might exist. PMN were shown to stimulate the two main effector functions of NK cells: cytotoxicity and cytokine (IFN- γ) secretion. The finding that NK cells were able to inhibit PMN apoptosis, suggest that PMN not only activate NK cells, but are themselves responsive to activation by NK cells. As both NK cells and PMN are present at inflammatory sites and have both been shown to have important effector and immunoregulatory functions, the demonstrated mutual activation would be an important contribution in the host response to infections and tumours.

The proposed interactions between NK cells and PMN are illustrated in the diagram below.

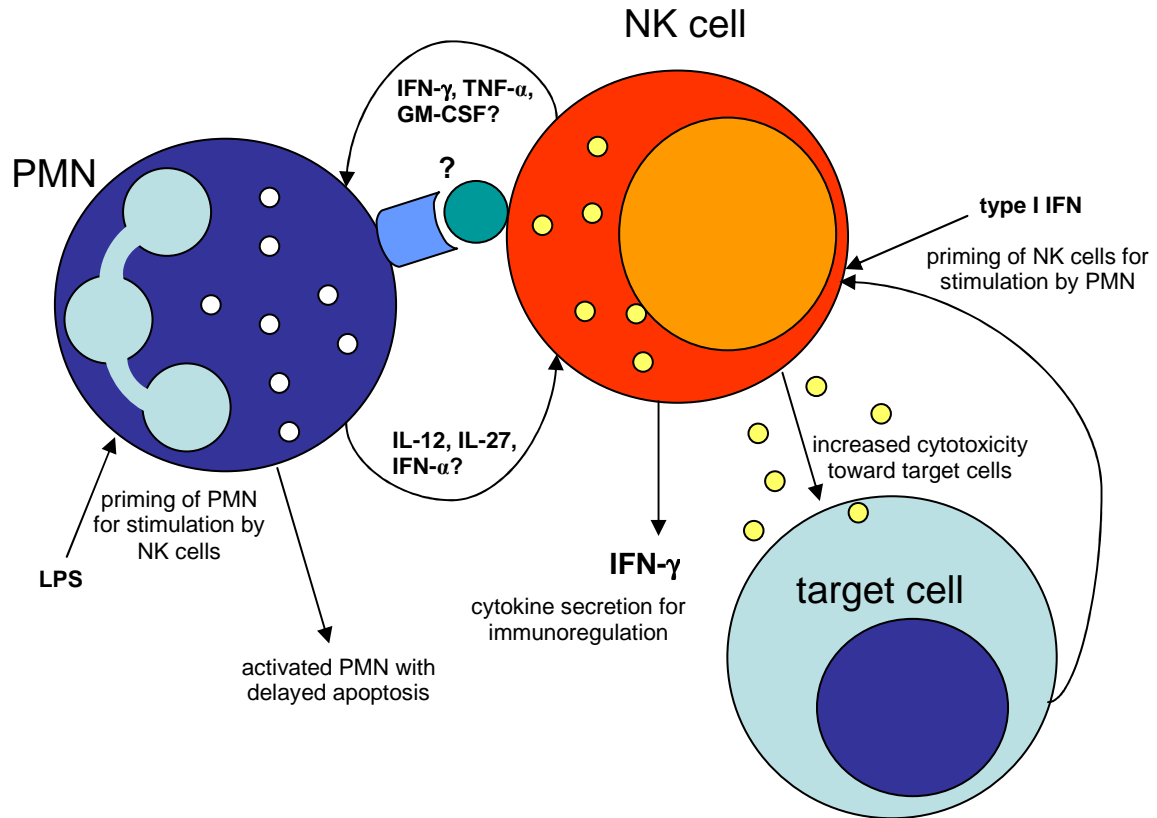


Fig. 14 Proposed interactions between NK cells and PMN. Natural killer cells are activated by PMN. NK cell cytotoxic activity against susceptible target cells is upregulated when exposed to both PMN and to cell-free supernatants of PMN, suggesting a role for PMN-derived soluble factors in mediating this activation and not excluding a possible role for direct cell-cell contacts. Secretion of the immunoregulatory cytokine IFN- γ by the NK cell line NK-92 is increased to a variable degree upon contact with PMN, PMN always synergise with both type I IFN and K-562 tumour cells in inducing IFN- γ secretion by NK-92 cells. Therefore, NK cells are clearly activated by PMN. Likewise, PMN are also activated by NK cells. PMN show a marked apoptosis delay when incubated with the NK cell line NKL. K-562 tumour cells and LPS synergise with NKL cells to mediate an even greater inhibition of PMN apoptosis. These findings show that a bi-directional cross-talk between NK cells and PMN exists, allowing them to amplify each others function in the context of inflammation.

5 Summary

Natural killer cells (NK cells) and neutrophil granulocytes (PMN) are effector cells of innate immunity. In addition, they also have an important immunomodulatory role. Little research has been conducted regarding the mutual interaction between NK cells and PMN. This work addressed the question whether an activating cross-talk between NK cells and PMN might exist. On the one hand, the effect of PMN on both cytotoxicity and IFN- γ secretion of NK cells was assessed. On the other hand, the potential of NK cells to activate PMN was investigated. Primary human NK cells, NK cell lines and purified human PMN were used to assess these interactions.

PMN were found to augment the cytotoxic activity of highly purified human NK cells. Cytotoxicity was increased by both autologous and allogenic PMN, showing that PMN-mediated induction of cytotoxicity is independent of MHC molecules. Both cell-free supernatants of PMN and washed PMN increased the cytotoxicity of NK cells, suggesting a role for PMN-derived cytokines in mediating activation of NK cell cytotoxicity.

It was shown that PMN have the capacity to induce IFN- γ secretion of the NK cell line NK-92, especially in combination with other known NK cell stimulants such as type I IFN and tumour (K-562) cells.

To analyse the effect of NK cells on PMN, inhibition of PMN apoptosis in response to the NK cell line NKL was assessed. NKL cells delayed PMN apoptosis and synergised with both NK cell and PMN activators in inducing inhibition of PMN apoptosis.

The data presented in this work shows for the first time, that bi-directional activating interactions between NK cells and PMN exist. PMN were shown to be capable of stimulating the two main effector functions of NK cells, cytotoxicity and cytokine secretion. Likewise, NK cells were found to delay PMN apoptosis. As in inflammation, NK cells and PMN are present at the same site, at the same time, the interaction between NK cells and PMN may well play an important role in the response to infection and, possibly, to tumours.

6 Zusammenfassung

Natürliche Killerzellen (NK-Zellen) und neutrophile Granulozyten (PMN) sind bekannte Effektor- und Regulatorzellen der angeborenen Immunität. Die Interaktionen zwischen NK-Zellen und PMN sind unzureichend untersucht. In der vorliegenden Arbeit wurde erforscht, ob eine bi-direktional aktivierende Wechselbeziehung zwischen NK-Zellen und PMN existiert. Einerseits wurde der Effekt von PMN auf Zytotoxizität und IFN- γ -Sekretion von NK-Zellen analysiert, andererseits die Fähigkeit von NK-Zellen, PMN zu aktivieren. Primäre NK-Zellen, NK-Zelllinien und hoch aufgereinigte PMN wurden verwendet um diese Interaktionen zu untersuchen.

Die Befunde zeigen, dass PMN die Zytotoxizität von aufgereinigten NK-Zellen gegen Zielzellen verstärken, und zwar autologe und allogene PMN gleichermaßen. Dies zeigte, dass die durch PMN vermittelte Induktion der Zytotoxizität unabhängig von MHC Molekülen ist. Sowohl zellfreie Überstände von PMN-Kulturen als auch gewaschene PMN führten zu einer Erhöhung der Zytotoxizität. Dies weist darauf hin, dass durch PMN produzierte Zytokine zu der Aktivierung der NK-Zell-Zytotoxizität maßgeblich beitragen.

In Experimenten, die die Wirkung von PMN auf die Sekretion von IFN- γ durch NK-Zellen untersuchten, konnte gezeigt werden, dass PMN die Fähigkeit besitzen, die IFN- γ -Sekretion der NK-Zelllinie NK-92 zu induzieren, vor allem in Kombination mit bekannten NK-Zell-Stimuli wie Typ I IFN und Tumorzellen (K-562 Zellen).

Um die Auswirkung von NK-Zellen auf PMN zu untersuchen, wurde die Hemmung der Apoptose von PMN durch die NK-Zelllinie NKL untersucht. NKL-Zellen verzögerten die spontane Apoptose von PMN und wirkten synergistisch mit NK-Zell-Aktivatoren sowie mit PMN-Aktivatoren in der Hemmung der Apoptose.

Zusammengefasst zeigen die Daten der vorliegenden Arbeit erstmalig, dass beiderseitig aktivierende Interaktionen zwischen NK-Zellen und PMN bestehen. Es wurde gezeigt, dass PMN die beiden Haupteffektorfunktionen von NK-Zellen, Zytotoxizität und Zytokinsekretion, stimulieren können. Ebenso wurde die Apoptose von PMN durch NK-Zellen verzögert. Da sich NK-Zellen und PMN im Rahmen von Entzündungen zur gleichen Zeit am gleichen Ort befinden, könnte die gegenseitige Aktivierung zwischen NK-Zellen und PMN einen wichtigen Beitrag zur Abwehr von Infektionen und, möglicherweise, von Tumoren leisten.

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8 Acknowledgements

This thesis has been completed with the help and encouragement of many people. I would like to take this opportunity to thank everyone who made a special contribution to this work.

First of all, I wish to express my sincere gratitude to Prof. Dr. Werner Solbach for allowing me to carry out research on this project at the Institute for Medical Microbiology and Hygiene. His constructive criticism and positive attitude towards my work have helped me a lot. The journal club meetings and Friday discussions have contributed to helping me mature in both a scientific and a personal way.

My earnest acknowledgement goes to my supervisor Prof. Dr. Tamás Laskay for his exemplary guidance, neverceeding enthusiasm and belief in my work. Many hours spent discussing and analysing my research have been instrumental in ensuring the completion of this project.

I am greatly indebted to Dr. Sonja Lotz for her guidance and help in every aspect of this project. Her continued support has helped me a lot along the way.

I am most grateful to all members of the laboratory for the friendly atmosphere in our group and their help with both technical and personal difficulties. In particular, I would like to thank Birgit Hansen for her cooperation and assistance. Former members of the laboratory to whom I am very thankful are Dr. Eresso Aga, Lars Esmann, Inga Wilde, Elisabeth Maniak, Annalena Bollinger, Meike Gabert and Beate Lembrich. I warmly thank all the present members of the laboratory for their help and support, especially Dr. Ger van Zandbergen, Uta Bußmeier, Alexander Wenzel, Christian Idel, Kirsten Broszat, Arup Sarkar, Sonja Dannenberg and Kristin Roßdeutscher.

Many thanks also to all my friends for their moral support and company.

Finally, I am indebted to my father and sister, who are always standing beside me no matter what happens.

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