

# Migration of Natural Killer Cells

**Matrix interaction, locomotion and regulation  
of matrix metalloproteinases (MMPs)  
by IL-2 and chemokines**

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Doctoral Thesis for the Degree of Doctor of Philosophy, Faculty of Medicine

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**Karin Edsparr**

**2009**



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Institute of Clinical Sciences  
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## ABSTRACT

Activated natural killer (NK) cells are effective anti-tumour cells. In order to reach target cells in a tumour mass they need to migrate in the extravascular space. The process of tumour localisation begins when the NK cell is attracted in the blood stream, and utilisation of matrix-degrading enzymes is crucial for passing of the basal membrane (BM) and for locomotion outside the vascular bed. NK cells express several members of the family of matrix-degrading enzymes, matrix metalloproteinases (MMPs). The aim of this study was to gain more knowledge on the regulation of NK cell migration that affects infiltration of the extracellular matrix (ECM) equivalent Matrigel. In specific; morphologically study NK cell locomotion in a matrix environment; identify the repertoire of MMPs expressed by freshly isolated human NK cells and the human NK cell lines YT and NK-92; explore the role of MMPs in NK cell migration and investigate the effect of IL-2 and chemokine stimulation as well as matrix (Matrigel) contact on NK cells' migratory ability and MMP expression.

IL-2-activated mouse A-NK cells cultured in Matrigel revealed two different patterns of matrix disintegration depending on their time in culture, and similar differences were found between two human NK cell lines. Younger ( $\leq 5$  days) mouse A-NK and NK-92 cells gave rise to a general widespread matrix reorganisation, interpreted to be due to direct release of soluble matrix-degrading enzymes. Older ( $\geq 6$  days) mouse A-NK and YT cells instead produced large excavations in the Matrigel. These cavities could be explained by a release of proteoglycan-rich material with matrix-dilating properties, combined with associated matrix-degrading proteases. The IL-2-independent NK cell line YT and freshly isolated human NK cells was used to investigate the effects of IL-2 stimulation on NK cell migration and MMP expression and production. IL-2 stimulation of the YT cell line demonstrated opposing effects related to the duration of stimulation. A rapid stimulatory response at about 2-4h on MMP production, and a later negative effect on MMP expression and MMP-9 production was seen after prolonged stimulation ( $\geq 24$ h). Both responses correspondingly affected the migratory ability. In freshly isolated NK cells, migration increased MMP-dependently in response to IL-2 and MT6-MMP expression increased. MMP-13, MT3- and MT6-MMP, previously not described in NK cells, was found to be expressed by freshly isolated human NK cells. While matrix (Matrigel) contact did not affect MMP expression in either the NK-92 or YT cell line, the chemokine CX3CL1 was found to increase NK-92 cells' MMP-9 production significantly, but had no effect on their migration.

These findings increase our understanding of how NK cell migration is regulated and provide one further step in the development of strategies to achieve greater number of tumour infiltrating NK cells.

## LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

- I**            Albertsson P, Basse PH, **Edsparr K**, Kim MH, Goldfarb RH, Kitson RP, Lennernäs B, Nannmark U, Johansson BR  
Differential locomotion of long- and short-term IL-2-activated murine natural killer cells in a model matrix environment  
*Scand J Immunol 2007 Oct; 66(4):402-9*
- II**            **Edsparr K**, Johansson BR, Goldfarb RH, Basse PH, Nannmark U, Speetjens FM, Kuppen PJK, Lennernäs B and Albertsson P  
Human NK cell lines migrate differentially *in vitro* related to matrix interaction and MMP expression  
*Immunology and Cell Biology, 2009 May 12 [Epub ahead of print]*
- III**           **Edsparr K**, Speetjens FM, Mulder-Stapel A, Goldfarb RH, Basse PH, Lennernäs B, Kuppen PJK and Albertsson P  
Effects of IL-2 on MMP expression in freshly isolated human NK cells and the IL-2-independent NK cell line YT  
*Manuscript, submitted*
- IV**           **Edsparr K**, Cullin F, Barth H, Goldfarb RH, Basse PH, Lennernäs B, Kuppen PJK and Albertsson P  
The Fractalkine (CX3CL1) chemokine stimulates NK-92 natural killer cell production of MMP-9  
*Manuscript, submitted*

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## ABBREVIATIONS

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### ABBREVIATIONS

AIT	Adoptive immunotherapy
A-NK cell	Activated and/or adherent natural killer cell
BM	Basement membrane
BRM	Biological response modifier
CCL3	Macrophage inflammatory protein 1 alpha (MIP-1 $\alpha$ ), a type $\beta$ chemokine
CmB	Cupromeronic blue
CX3CL1	Fractalkine/neurotactin, a type $\delta$ chemokine
CXCL10	Interferon gamma inducible protein 10 (IP-10), a type $\alpha$ chemokine
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IL-2	Interleukin 2
IFN- $\gamma$	Interferon gamma
MMP	Matrix metalloproteinase
MT-MMP	Membrane type matrix metalloproteinase
NK cell	Natural killer cell
NK-92	A human immature NK cell line
RT-PCR	Reverse transcriptase polymerase chain reaction
uPA	Urokinase plasminogen activator
uPAR	The receptor for urokinase plasminogen activator
YT	A human immature NK cell line



## **INTRODUCTION**

Natural killer (NK) cells were first identified and denominated based on their ability to kill malignant cells without any prior stimulation [1]. Natural killer cells are primarily found in peripheral blood, spleen, liver and lung. They constitute ~15% of human peripheral blood lymphocytes and are normally not present in lymphatic tissues but can migrate to these sites in response to chemoattractants during an inflammatory process [2]. Natural killer cells are an important part of the innate immune system and provide a major first-line defence against viral infections and possibly also malignant transformed cells. The anti-tumour activities of NK cells has been extensively studied and even been tested clinically [3].

Most NK cell-mediated killing mechanisms are dependent on a close contact between the NK cell and its target. In order to reach and infiltrate the tumour mass, the circulating NK cell needs to adhere to the endothelial cells, penetrate the basement membrane (BM) and migrate within the perivascular matrix. Matrix-degrading proteases are considered one corner stone (together with adhesion molecules and motility-promoting molecules) in this process. Matrix metalloproteinases (MMPs) are endopeptidases capable of degrading all components of the extracellular matrix (ECM) and are from rodent studies believed to be essential for the NK cells ability to extravasate and migrate within the extravascular space [4-6].

Activation of endogenous NK cells using interleukin 2 (IL-2) has been shown to increase their localisation in tumour nodules [3, 7] and adoptively transferred activated NK cells are able to find and infiltrate experimentally induced tumour nodules [8-10]. However only a small percentage reach the tumour site and many NK cells remain localised in the stroma surrounding the tumour [3, 11-17]. These findings could be part of the explanation for the limited clinical success of adoptive NK cell immunotherapy. We therefore need further knowledge about the mechanisms that affect NK cell accumulation into tumours.

## **NK cells**

### **Definition**

Natural killer cells are considered the third lineage of lymphocytes, beside T and B cells, and are defined by their morphology, surface phenotype as well as function [18]. Natural killer cells are *morphologically* classified as large granular lymphocytes (LGL) because of their high cytoplasm to nucleus ratio. These cells have discrete azurophilic granules in the cytoplasm, containing granzymes, perforin and chondroitin sulphate glycosaminoglycans, and an indented kidney shaped nucleus [19, 20].

## INTRODUCTION

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*Phenotypically*, NK cells are further characterised by their CD56<sup>+</sup>CD3<sup>-</sup>CD16<sup>+</sup> cell surface expression and lack of rearranged recognition receptors typical of T and B cells. They can be divided into many subpopulations based on differentially expressed surface molecule densities [19, 21, 22]. So far the main distinction is based on the neural cell adhesion molecule CD56 [2]. Most NK cells (~90%) express low levels of CD56 and are designated CD56<sup>dim</sup> while a minority (5-10%) express high levels and are subsequently called CD56<sup>bright</sup> NK cells. The function of CD56 on NK cells is unknown but its expression density confers unique functional properties. CD56<sup>bright</sup> NK cells are the primary source of NK cell-derived immunoregulatory cytokines, such as interferon  $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor  $\beta$  (TNF- $\beta$ ), IL-10, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF) but have a low natural cytotoxicity [23]. Conversely, CD56<sup>dim</sup> NK cells produce low levels of cytokines but are more cytotoxic.

*Functionally*, NK cells are defined by their ability to recognise and kill malignant and virally infected cells without any prior sensitisation. Natural killer cells' target recognition, activation and cytotoxicity are regulated by signals received through inhibitory as well as activating cell surface receptors. As described by the "Missing self" hypothesis, these receptors allow NK cells to distinguish between normal and transformed cells [24-26]. Ultimately, NK cell-mediated killing of target cells is decided by the net outcome of signals received from these activating and inhibitory receptors. Thus, a membrane contact between the NK cell and the target cell seems necessary for an efficient NK cell activation. In addition to their direct role in the defence against infected and tumour-transformed cells, NK cells also function as regulatory cells. They are able to influence both innate and adaptive immune responses through their interaction with other immune cells [26].

### **Cytotoxicity**

Activated NK cells can kill target cells by a range of mechanisms, one being the release of cytoplasmic granules containing perforin, which mediates cell necrosis, and granzymes which induce apoptosis by activating caspases. This is probably the main pathway used by NK cells to eliminate virally infected or malignant cells [27]. Apoptosis can in addition be induced by NK cells through expression, and possibly also secretion, of TNF-family ligands which bind to TNF-receptors on target cells [28]. Natural killer cells may further mediate antibody-dependent cellular cytotoxicity (ADCC) through binding of CD16 (Fc $\gamma$ RIII) to the Fc portion of IgG antibodies [19]. Upon activation, NK cells release various cytokines (IFN- $\gamma$ , IL-5, -10, -13) and chemokines which promote and modulate inflammatory responses. NK cells may also induce target cell killing through production of IFN- $\gamma$  which is thought to stimulate

antigen presentation by upregulating HLA class I and II, stimulate phagocytes and be involved in effector functions against tumour metastases [29, 30]. Similar to the activation process described above, this again portrays the significance of a close intercellular contact to achieve effective target cell eradication.

### **NK cell stimulatory factors**

#### ***Cytokines***

Cytokines are a large family of soluble low-molecular-weight proteins (~10-30 kDa) which mediate cellular interactions between immune cells. The biological functions of cytokines are numerous and include cellular proliferation and differentiation, induction of the inflammatory response, regulation of haematopoiesis and also the development of cellular and humoral responses. Cytokines most often exert their effects locally in an autocrine/paracrine fashion on target cells expressing the corresponding receptor [31].

Natural killer cells are activated and regulated by several cytokines, e.g. IL-2, IL-12, IFN- $\alpha$ , IFN- $\beta$ , IL-15 and IL-18, out of which IL-2 is generally considered to be the most important [29]. The IL-2 receptor (IL-2R) is composed of three chains;  $\alpha$  (CD25),  $\beta$  (CD122) and  $\gamma$  (CD132). The intermediate-affinity heterodimeric IL-2R $\beta\gamma$  receptor is expressed by all NK cells while the CD56<sup>bright</sup> NK cells also express the high-affinity heterotrimeric IL-2R $\alpha\beta\gamma$  [32]. The IL-2 receptor has no intrinsic kinase activity and has been found to signal through the downstream signalling pathways Jak/STAT [33], Ras-Raf [34, 35] and MKK/ERK [36]. Upon IL-2 stimulation, NK cell proliferation and cytotoxicity is increased and their production of various cytokines is amplified [3, 37-39]. High-dose IL-2 has therefore been used both experimentally as well as clinically to enhance adoptively transferred as well as endogenous NK cells anti-tumour activity [40-42].

#### ***Chemokines***

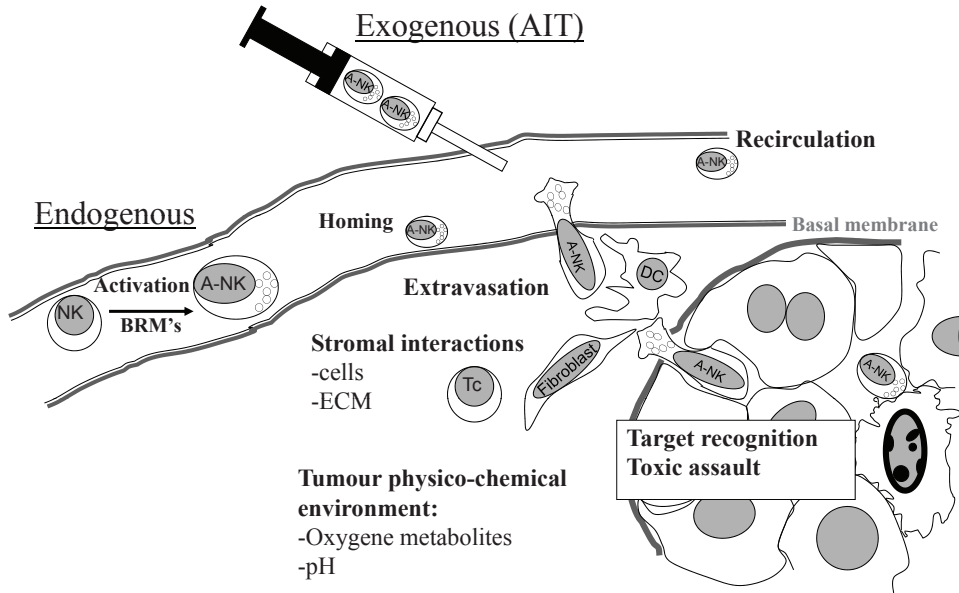
Chemokines are a cytokine subgroup consisting of small polypeptides (~10 kDa) that control the activation, adhesion and chemotaxis of many types of leukocytes. Chemokines direct leukocyte trafficking in and out of lymphoid tissue and in inflammatory processes. The family of chemokines consists of more than 40 members which can be divided into four major groups based on the presence and spacing of the first NH<sub>2</sub>-terminal cysteine residues; CXC ( $\alpha$ ), CC ( $\beta$ ), C ( $\gamma$ ) and CX3C ( $\delta$ ). Chemokine receptors are seven-transmembrane-spanning molecules and are similarly classified into four subfamilies, i.e. CXCR, CCR, CR and CX3CR.

Chemokines are known to stimulate NK cell movement as well as their activation [43]. Both resting and activated NK cells have been shown to express high levels of the CXCR1, CXCR4 and CX3CR1 receptors, and CXCR2 and CXCR3 at lower levels [44]. The chemokine receptors CCR2, CCR4, CCR5 and CCR8 have also been shown to be expressed by activated NK cells [43]. In accordance with the receptor expression studies, resting human NK cells have shown chemotactic movement in response to CCL2-5, CCL7-8, CX3CL1 and CXCL9-12 [44-46]. The chemokines CCL2, CCL3 and CCL5 have further been described to induce chemotaxis of IL-2-activated human NK cells [47].

### **Tumour localisation of NK cells**

A variety of cancer models have established a role for NK cells in tumour rejection and evidence point towards a role for NK cells in tumour surveillance *in vivo* [48]. Natural killer cells are for instance efficient eliminators of blood-borne metastases by eradication of tumour cells present in the peripheral circulation of both mice [49] and rats [50]. In the human setting there is often abnormal NK cell function and/or number in patients with advanced malignancies [51]. Collectively, the several lines of evidence strongly imply that NK cells are of importance for the defence against metastases. Despite the fact that NK cells are not found in large number in advanced human neoplasm's, their number of infiltration have been shown to have a prognostic value in several different malignancies [52-55].

Several approaches have been tried to augment the NK cell response and increase the numbers of NK cells at tumour sites. A schematic view of some of the crucial steps that needs to be successfully overcome for the NK cell to eventually meet up cell membrane to cell membrane with the tumour cell are shown in Figure 1. The two strategies for clinical therapeutic use of NK cells have been activation of endogenous NK cells and adoptive transfer of *in vitro*-activated autologous NK cells, i.e. adoptive immunotherapy (AIT) [3]. The major strategy for the activation of endogenous NK cells has been by injecting the highly potent immunostimulatory compound IL-2, used either alone or with other biological response modifiers (BRMs) such as IFNs. A clear increased accumulation of NK cells inside tumour nodules has been found in response to high-dose IL-2 injections [7]. However the toxicity, sometimes life-threatening, associated with IL-2 treatment is unfortunately a limitation of this approach [56]. Other cytokines with effects on NK cell activity that could be used for the enhancement of NK cell function in cancer therapy include IL-12, IL-15 and IL-18 [56, 57]. Both IL-15 and IL-18 have shown anti-tumour activity in mice [56, 58] and while IL-12 has shown potent anti-tumour activity in preclinical studies it has unfortunately had limited clinical effect [59].



**Figure 1.** Tumour localisation of NK cells. Activation of endogenous NK cells using biological response modifiers (BRMs) and adoptive transfer of *in vitro*-activated NK cells have been used to increase the numbers of NK cells at tumour sites. Activated NK (A-NK) cells need to extravasate and migrate through the extracellular matrix (ECM) in order to reach and eradicate the malignant cells. In the tumour microenvironment, NK cells will interact with both the ECM as well as other immune and stroma cells. They also need to cope with the harsh physico-chemical environment within the tumour tissue, e.g. low pH level and toxic reactive oxygen metabolites.

Clinical trials using *in vitro* expanded IL-2-activated NK cells (A-NK cells) started in the early/mid 1980s and despite promising preclinical studies there are only limited evidence for NK cell-based immunotherapy being clinically effective [3, 42, 48]. This has been explained in part by the fact that trials have been performed on patients with end stage disease with a large tumour burden, or widely metastasized cancer. Also, the complicated and difficult laboratory procedure with NK cell AIT has probably been taken into account when the results of AIT have been compared to traditionally chemotherapeutics or other newly developed targeted drugs.

Considering the rodent situation, adoptively transferred mouse A-NK cells are able to migrate to and infiltrate pulmonary metastases [8, 10], via the microvasculature or from venules surrounding the tumour [60]. Infiltration by A-NK cells has been shown to vary in different tumours with different morphology. For instance, certain morphologically distinct lung metastases allow infiltration while more densely growing lung metastases do not [13]. These infiltration-permissive and infiltration-

## **INTRODUCTION**

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resistant tumours differ in vessel content and ECM components [13, 15], indicating that the tumour microenvironment greatly influences tumour localisation of A-NK cells. Natural killer cells have been shown to re-circulate poorly [61]. Therefore, injection of A-NK cells directly into the vessels connected to rat hepatic metastases increase infiltration of NK cells significantly [9, 62]. Regional administration of A-NK cells have in addition been evaluated clinically [40], however with modest result.

In order to reach and infiltrate a tumour mass, circulating NK cells need activating signals which enables their slowing down in the circulation, rolling along the endothelial lining, crossing of the endothelial cell layer and degradation of the BM and migration through the ECM in the subendothelial compartment. The NK cells might eventually also need to penetrate a dense tumour capsule which sometimes surrounds solid tumours [11]. Homing of NK cells is a multi-step process with regulatory, adhesive and migratory events involving adhesion molecules, chemotactic signals and matrix-degrading enzymes [63, 64]. Expression of chemokine receptors on NK cells, as well as their activation status, influence their ability to migrate [7, 43, 65]. In the tumour microenvironment, NK cells will also interact with other immune cells that can affect their activation status [66, 67]. Furthermore, the physico-chemical environment within the tumour with for example low pH and high levels of reactive oxygen metabolites could suppress NK cell activity [68, 69]. A certain number of accumulated NK cells at the site of tumour growth are most certainly needed to obtain an efficient anti-tumour effect [3, 70]. However, previous studies have shown that while some of the NK cells which extravasate at the tumour site make direct contact with tumour cells [8-10], many remain localised in the stroma surrounding the tumour [3, 11-17], possibly dependent on the matrix content of the microenvironment [15].

### **Extracellular matrix**

Extracellular matrix (ECM) is defined as the material in a tissue that is not part of any cell. This matrix is produced and secreted mainly by epithelial, endothelial and mesenchymal cells. The ECM obviously functions as support and anchorage to cells, but its components also modulate other cell functions such as development, migration and proliferation [71, 72]. The main components of ECM are collagens, proteoglycans and hyaluronic acid, together with fibrin, elastin, fibronectins, laminins and nidogens. The ECM proteins probably function both as a substrate for NK cell migration as well as a barrier [3, 12]. It has been suggested that NK cells may possibly be triggered into a migratory state by ECM contact and that a release of collagen-feathered IL-2, as a result of ECM breakdown, could be a mechanism to sustain a local activation [73-75]. However, the tumour environment with its ECM proteins might also hinder NK cells

from reaching the tumour, which could explain the lack of NK cell infiltration and tumour cell destruction seen in the *in vivo* setting as previously discussed [3, 11-17].

Basement membranes are dense sheets of specialised extracellular matrix separating epithelial and endothelial cells from the underlying tissue [76], that NK cells need to traverse in order to reach a tumour nodule. Basement membranes are typically 50-100 nm thick. Although tumour vasculature is considered immature and sometimes lack an intact BM, also the opposite is true with multilayer's of BM [77]. The main BM components are type IV collagen and laminin but it also includes entactin, nidogen as well as heparan sulphate proteoglycans. Matrigel, a commercially available reconstituted basement membrane material extracted from the mouse Engelbreth-Holm-Swarm sarcoma [78] is often used experimentally as an ECM equivalent because of its biochemical and biological resemblance to normal BM [79, 80].

The ECM can serve as a reservoir for biologically active molecules (as above mentioned for IL-2) and cleavage of ECM components by matrix-degrading proteases can thereby release growth factors, cytokines as well as ECM-derived peptides, i.e. matrikines, which affect cell growth, apoptosis, cell migration as well as cell-cell communication [72, 81].

### **Matrix metalloproteinases**

Proteinases are matrix-degrading enzymes that are used in tissue remodelling in various physiological and pathological situations. The matrix metalloproteinases (MMPs) are, together with the urokinase plasminogen activator (uPA) system, implicated in ECM degradation and BM passage by lymphocytes [72, 82]. The uPA system with uPA and its receptor uPAR contributes to ECM degradation by converting plasminogen into active plasmin. This thesis has specific focus on the large family of MMPs, consisting of conserved zinc-dependent endopeptidases that are characterised by their ability to degrade all components of the ECM and BM. Members of the MMP family are utilised in both normal and pathological processes, i.e. cancer, arthritis, cardiovascular disease, skin ulceration and gastric ulcer [83].

The family of MMPs consists of at least 25 members all encoded by different genes. A majority of these can be divided into four subgroups based on their substrate specificity and structural similarities, although there is substantial overlap. The subgroup of *interstitial collagenases* includes *MMP-1* (collagenase-1 or fibroblast collagenase), *MMP-8* (collagenase-2), *MMP-13* (collagenase-3), and *MMP-18* (collagenase-4). The members of this group are substrate-specific and degrade fibrillar collagen types I-III and X. [82]. Interstitial/fibrillar collagen is composed of three

## INTRODUCTION

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polypeptide chains arranged in a triple helical formation and it is resistant to most endoproteases except for the collagenase members of the MMP family, as well as MMP-2 and MT1-MMP [84]. Degradation of collagen results in fragments that spontaneously denature into coiled gelatine peptides, which can be cleaved by most MMPs, i.e. the ones with gelatinase activity. The subgroup of pure *gelatinases*, *MMP-2* (gelatinase A/72 kDa gelatinase) and *MMP-9* (gelatinase B/92 kDa gelatinase), are considered to be the most widely distributed MMPs and are expressed in all lymphocytes [85]. The gelatinases degrade gelatine but they, more importantly, have the almost unique ability to degrade non-fibrillar collagens type IV and V. Thus, the gelatinases have been extensively studied for their role in cell invasion due to their capacity to degrade collagen IV, the main component of BMs. The *stromelysins* include *MMP-3* (stromelysin-1), *MMP-10* (stromelysin-2), *MMP-11* (stromelysin-3), *MMP-7* (matrilysin), *MMP-26* (matrilysin-2) and *MMP-27*. The members of this subgroup have a relatively broad substrate specificity which includes collagens type III, V and IX, gelatine, fibronectin, laminin and proteoglycans [81]. **Membrane type MMPs (MT-MMPs)** is a growing group (with the last member added in 1999) that now consists of six members designated *MT1-4* (*MMP-14-17*), *MT5-* (*MMP-24*) and *MT6-MMP* (*MMP-25*). The membrane bound MMPs are attached to the cell surface either through a single pass C-terminal transmembrane domain (as for MT1-, MT2-, MT3-, and MT5-MMP) or a glycosyl phosphatidylinositol (GPI) anchor (MT4- and MT6-MMP). In addition to their ability to degrade multiple ECM components, the MT-MMPs are also involved in the pericellular activation of other MMP family members, including MMP-2 and MMP-13 [86].

Most MMPs are either expressed at low levels or not at all during resting state [87]. It is assumed that MMPs are released directly following formation. However, neutrophils have been described to store MMP-8 and MMP-9 in granules that are released upon activation [88-90]. It has in addition been proposed that pre-synthesised MT1-MMP is stored transiently in trans-Golgi network/endosomes where it is available for trafficking to the cell membrane [91]. Furthermore, retention of inactive MMPs at the cell surface or in association to proteoglycans may also be considered as storage awaiting activation [92-95].

The regulation of MMPs occurs at many different levels both transcriptionally and post-transcriptionally. Factors such as hormones, cytokines, oncogenes (cellular transformation) and growth factors, have been shown to induce the expression of MMPs [96]. Studies have shown that the AP-1 and the PEA3 binding site is involved in the transcriptional activation and repression of MMP promoters [96]. Cell-matrix and cell-cell interactions have further been shown to influence the expression of MMPs [83]. The post-transcriptional regulation of MMPs includes proteolytic activation of the latent form and inhibition of the active enzyme by natural inhibitors.



Most MMPs are produced as inactive zymogens that require proteolytical activation by serine proteases or other MMPs [97]. Once activated, MMPs are controlled by endogenous inhibitors, with the main physiological inhibitors being the tissue inhibitors of metalloproteinases (TIMPs). Four homologs, TIMP1-4, have been identified so far and they each bind several MMPs [98].

### **MMPs in NK cells**

In human NK cells, to date, the gelatinases MMP-2 and -9, the collagenase MMP-1 and the membrane-type MT1- and MT2-MMP have been described to be expressed [99-101]. In addition, the stromelysins MMP-3, -10 and -11 and the collagenase MMP-13 have been found to be expressed in rodent NK cells [4-6]. Rodent NK cell migration has moreover been shown to mainly depend on MMPs as shown by MMP inhibition studies [4-6]. Natural killer cells also express uPA/uPAR [102] and a simultaneous inhibition of both the uPA and MMP system using aprotinin and BB94 decreased rat NK cell Matrigel invasion more effectively than each inhibitor alone [103], indicating that rat NK cells may employ the uPA system in cooperation with MMPs.

In general, quantitative data on MMP secretion is lacking. There is in addition scarce information available on the regulation of MMPs in NK cells. It has however been shown that NK cells up-regulate their expression of MMP-2 upon cross-linking of the activating receptor 2B4 [104] and the prostaglandin PGE<sub>2</sub> has further been shown to enhance NK cell secretion of MMP-1 and MMP-3 and thereby facilitate their migration through Matrigel filters [6, 104]. Two studies, on each chemokine and cytokine stimulation, has also been found to positively affect NK cell migration and MMP production. The chemokine CXCL12 enhanced human NK cell invasion into type I collagen MMP-dependently [100] and IL-18 was able to increase human NK cell migration through Matrigel and to enhance secretion of MMP-2 and -9 as well as expression of MT1-MMP, as assessed by gelatine zymography, western blot and RT-PCR analysis [101].

The role of IL-2 in NK cell migration and MMP production is mainly unclear, but data suggesting an IL-2-dependent NK cell migration has been presented [99, 105]. It is therefore possible that IL-2 influences the migratory ability of NK cells through effects on their MMP expression. Up to now, no clear data are available on the effects of IL-2 on MMP-dependent migration, clearly this is the result of the difficulties to study the isolated effect of IL-2 using freshly isolated NK cells since they require IL-2 for their propagation in culture, and because even small “survival” doses of IL-2 could be enough to affect NK cell functions.

### AIMS OF THE THESIS

We need a better understanding of how NK cells infiltrate tumour, their migration in and interactions with the ECM components of the tumour and its surroundings, in order to obtain NK cells that are more efficiently located within tumour tissue *in vivo*. The general aim of this study was to gain more knowledge on the regulation of NK cell migration that affects infiltration of the ECM equivalent Matrigel.

The specific aims were to:

- Investigate NK cell morphology during locomotion in a 3-dimensional matrix environment.
- Identify novel MMPs expressed by freshly isolated human NK cells as well as describe the MMP repertoire expressed by the NK cell lines YT and NK-92.
- Establish the function of MMPs in NK cell migration.
- Investigate the effect of the cytokine IL-2, the chemokines CCL3, CX3CL1 and CXCL10 as well as increasing degree of matrix (Matrigel) contact on NK cells' MMP expression and production and migratory ability.

## MATERIALS AND METHODS

### Primary NK cells

#### Human NK cells

Human NK cells were isolated from buffy coats obtained from healthy donors. NK cells used in the semi-quantitative RT-PCR analysis (Paper III) were isolated as previously described by Jonges *et al.* [21]. Peripheral blood mononuclear cells were isolated using Ficoll-Hypaque centrifugation and monocytes depleted by nylon wool incubation. Natural killer cells were then obtained by immunomagnetic purifications using the MACS NK cell isolation kit from Miltenyi Biotech (Bergisch Gladbach, Germany) where non-NK cells are removed by antibody-conjugated colloidal superparamagnetic MACS Microbeads recognizing CD3, CD4, CD19 and CD33 positive cells. The purity of the eluted NK cells was determined by fluorescence activated cell sorting (FACS) using a FACScalibur flow cytometer (Becton-Dickinson, Mountain View, USA) and the amount of CD56<sup>+</sup> cells was always determined to be > 90% and CD3<sup>+</sup> cells < 1%.

Natural killer cells used for quantitative real-time RT-PCR analysis and migration assays in Paper III were isolated from buffy coats using Ficoll-Hypaque centrifugation followed by the NK cell isolation kit from Miltenyi. A high purity (> 99% CD56<sup>+</sup> and < 0.1% CD3<sup>+</sup> cells) was obtained through fluorescence activated cell sorting (FACS). Isolated human NK cells were either immediately prepared or further cultured for 24 or 48h in 37°C with 5% CO<sub>2</sub> humidified air using medium supplemented with 10, 100 or 1000 Cetus Units (U)/mL of recombinant human IL-2 (Proleukin®, Novartis Vaccines & Diagnostics, Emeryville, California).

#### Mouse NK cells

Mouse NK cells were isolated as previously described by Gunji *et al.* [106]. Splenocytes harvested from male C57BL/6 mice were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), 55 µM 2-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin sulphate, 2 mM L-glutamine, 0.1 mM minimum essential medium (MEM) non-essential amino acids plus 1000 U/mL IL-2. After two days of culture, non-adherent cells and cells not firmly attached to the plastic surface were removed. Adherent cells were cultured for an additional 5-9 days in complete medium supplemented with IL-2. The mouse A-NK cell preparations were determined to contain > 90% DX5<sup>+</sup> cells and < 5% CD4<sup>-</sup>, CD8<sup>-</sup> and CD19<sup>-</sup> cells by flow cytometric analysis.

### Human NK cell lines

#### NK-92

The human immature NK cell line NK-92 was established from a 50 year old Caucasian male with rapidly progressive non-Hodgkin's lymphoma and has both phenotypic as well as functional characteristics resembling activated NK cells [107]. These cells have a CD2<sup>+</sup>, CD3<sup>-</sup>, CD4<sup>-</sup>, CD8<sup>-</sup> and CD56<sup>bright</sup> phenotype but unlike normal NK cells, NK-92 cells lack the Fc receptor CD16. The NK-92 cell line is highly cytotoxic against a broad range of malignant target cells [107, 108]. Furthermore, NK-92 is dependent on exogenous IL-2 for viability, expansion and cytotoxicity. Because of its features, the NK-92 cell line is a suitable candidate as immunotherapeutic agent and is the only NK cell line that actually has entered clinical trials [109]. The NK-92 cell line was kindly provided by Dr. Torsten Tonn and grown in X-vivo 10 medium (BioWhittaker™, Cambrex Bioscience, Verviers, Belgium) supplemented with 2 mM L-glutamine, 5% human fresh frozen AB-plasma (heat inactivated at 60°C for 45 minutes) and 100 U/mL of IL-2.

#### YT

The YT cell line is an IL-2-independent human immature NK cell line established from an Asian boy with thymus lymphoma [110]. YT cells have a CD16<sup>-</sup>, CD3<sup>-</sup>, CD4<sup>-</sup>, CD8<sup>-</sup> phenotype and express the intermediate affinity IL-2 receptor IL-2Rβγ. YT cells were originally described as CD56<sup>+</sup> [110] but in a more recent study by Matsuo and Drexler, the amount of CD56 expressed on YT cells was found to be extremely low [111]. The YT cell line was obtained from DSMZ (Braunschweig, Germany) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and antibiotics (all obtained from GIBCO®, Paisley, UK).

Both the YT and the NK-92 cell lines were propagated at 37°C with 5% CO<sub>2</sub> in humidified air and all cell culturing was performed under sterile conditions. Cell cultures were regularly screened for mycoplasma contamination using PCR technique.

### Cytokine stimulation

YT cells cultured in complete medium were washed twice in phosphate buffered saline (PBS) before being resuspended in opti-MEM medium (2 mM L-glutamine, antibiotics) (GIBCO®, Paisley, UK) and cultured with or without IL-2 (1, 10, 100 or 1000 U/mL) for definite times. Culture supernatants were centrifuged and particulates removed before samples were aliquoted and stored at -80°C until ELISA and gelatine zymography analysis.

## **Chemokine stimulation**

NK-92 cells cultured in complete medium were washed twice in PBS before being resuspended and cultured in opti-MEM medium (2 mM L-glutamine, antibiotics) supplemented with 100 U/mL IL-2 and 5 ng/mL of CCL3 (MIP-1 $\alpha$ ), CX3CL1 (fractalkine) or CXCL10 (IP-10) (R&D Systems Europe Ltd., Abingdon, UK). Culture supernatants were prepared as described above and stored at -80°C until ELISA and gelatine zymography analysis.

## **Morphology**

### **Co-incubations of mouse A-NK cells and melanoma cells in Matrigel**

A morphological approach was chosen for the study of NK cell matrix interaction and locomotion. For co-culture of mouse A-NK cells and melanoma cells in Matrigel the syngenic B16F10 melanoma cell line was used. The B16F10 cell line was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin sulphate, 2 mM L-glutamine, 0.1 mM minimum essential medium (MEM) non-essential amino acids, and sub-cultivated upon confluence.

Mouse A-NK cells (500,000), propagated 5-9 days, were enclosed with 500,000 melanoma cells in 15-30  $\mu$ l Matrigel droplets (one part cell suspension and two parts Matrigel) and cultured for 10 minutes, 2, 6 and 24h with or without inhibitors. Aprotinin (1  $\mu$ g/mL) and benzamidine (100  $\mu$ M) were used to inhibit serine proteases while BB94 (10  $\mu$ M) was used for the inhibition of MMPs. Glycosylation was inhibited by tunicamycin and castanospermine while the ionophore monensin was used to inhibit secretion of extracellular matrix proteins (all used in non-toxic doses). Presence of sulphated glucosaminoglycans in Matrigel droplets were visualized by overnight cytochemical staining using 0.05% Cupromeronic blue (CmB), a highly coloured cationic dye developed specifically for electron microscopic localisation of proteoglycans and sulphated glycosaminoglycans, according to the critical electrolyte concentration technique [112].

In order to study the development of pericellular clearings, fixed sheep erythrocytes were allowed to sediment on top of A-NK cells cultured for various times adherent to the plastic in 24-well plates. Micrographs were taken in random and in a blinded fashion, the particle free area was scored using a three-graded scale (no pericellular clearings, small pericellular clearings and large pericellular clearings) and data presented as the percentage of cells with different pericellular clearings.

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Matrigel droplets, containing mouse A-NK cells and melanoma cells, prepared for fluorescence and confocal microscopy were placed in ice-cold methanol before blocked in PBS (pH 7.2) containing 0.3% BSA and 0.1% goat serum. The main matrix constituents were fluorescently detected using anti-laminin or anti-collagen IV primary antibodies and a FITC-conjugated goat anti-rabbit secondary antibody. Preparations were examined in a Nikon Diaphot inverted photomicroscope. Droplets prepared for electron microscopy were instead fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M Na cacodylate buffer (pH 7.2) diluted 1:1 with PBS, and post-fixed using 1% OsO<sub>4</sub> before dehydrated and embedded in resin. A Reichert Ultracut E microtome with a diamond knife was used to cut ultrathin sections that were contrasted with uranyl acetate and lead citrate before being examined in a Zeiss CEM 902 electron microscope.

### **Culture of YT and NK-92 cells in Matrigel**

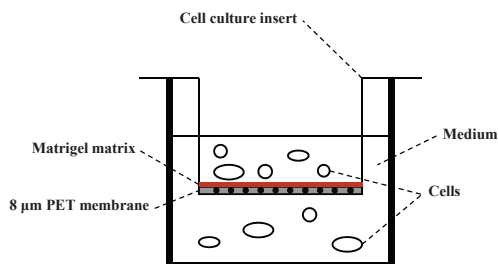
The YT and NK-92 cells were separately cultured in opti-MEM medium (2 mM L-glutamine and antibiotics) on Matrigel coated plastic (Matrigel diluted 1:10 in sterile filtered RPMI-1640 medium), mixed 3:1 with Matrigel creating Matrigel droplets or on regular plastic, in an attempt to analyse the effects of increasing complexity of Matrigel culture on MMP expression. The cell lines were cultured for 30h with 100 U IL-2 (NK-92 cells) or with or without 100 U IL-2 (YT cells) before RNA isolation and real-time RT-PCR analysis.

The NK-92 and YT cell lines' migratory phenotypes when cultured in the ECM equivalent Matrigel regarding invadopodias/podosomes, signs of matrix alteration, pericellular spaces and migratory patterns, were studied. Suspensions of YT or NK-92 cells were enclosed in gelling droplets of Matrigel (100 µl cell suspensions and 150 µl Matrigel). The droplets were covered with opti-MEM medium and cultured with or without 100 U IL-2 for 10 minutes, 2, 6 and 24h. Specimens were prepared as described above, treated with Richardson's stain and examined in a Nikon FXA Photomicroscope. Random micrographs were taken at 40x magnification and analysed blinded.

### **Matrigel invasion assay**

The migratory ability of freshly isolated human NK cells, as well as the NK-92 and YT cell line, was measured using BD Biocoat Matrigel invasion chambers with 8 micron pore size polycarbonate membranes (BD Biosciences, Bedford, MA, USA). The Matrigel layer occludes the membrane pores, preventing non-invasive cells from migrating through. By contrast, invasive cells can migrate through the Matrigel layer

(Figure 2). A total of  $5 \times 10^5$  cells in 0.3 mL medium were loaded into each insert well and 1.5 mL medium added to the lower chambers. For stimulation studies 100 U IL-2 (YT); 10, 100 and 1000 U IL-2 (fresh NK cells); or 5 ng/mL of either CX3CL1 or CXCL10 (NK-92) were added to both insert wells and bottom chambers. For inhibition studies, 10  $\mu$ M of the broad-spectra MMP inhibitor GM6001 (Calbiochem, Nottingham, UK) was added to inserts and wells. An equal amount of DMSO-vehicle alone was used in control wells. GM6001 is a hydroxamic acid dipeptide analogue that inhibits metalloproteinase activity by binding the zinc atom in the active site. The plates were incubated at 37°C for 24 or 48h before transmigrated cells present in the lower chamber were labelled using alamarBlue (Serotec, Oxford, UK). Plates were read on a SPECTRAmax® Gemini spectrophotometer (Molecular Devices Sunnyvale, CA, USA) using an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Standards with a known concentration of cells were run simultaneously and the number of invading cells was calculated by plotting the obtained fluorescence intensity on a standard curve. Invasion data is presented as: (i) the number of invaded cells with or without inhibitor (Paper II); (ii) as percent invasion through Matrigel-coated inserts compared to uncoated control inserts (Paper II); (iii) as percent invasion through Matrigel with or without IL-2 and/or inhibitor (Paper III); or (iv) as percent invasion through Matrigel with or without chemokine (Paper IV).



**Figure 2.** Schematic drawing of a Matrigel invasion chamber.

## RNA analyses

### Semi-quantitative reverse transcriptase (RT)-PCR

Reverse transcriptase (RT) PCR is a technique based on an end-point detection of a specific DNA or RNA region, using cDNA converted from RNA. After a set number of amplification cycles the cDNA reaction products are separated on an agarose gel, stained with ethidium bromide, and analysed using semi-quantitative image analysis.

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In the study of the effect of IL-2 stimulation on human freshly isolated NK cells (Paper III) expression of MMP-2 and MMP-9 semi-quantitative reverse transcriptase (RT)-PCR was used (primers listed in Table 1). Firstly, RNA was extracted using TRIzol reagent (Life Technologies, NY, USA) according to the manufacturer's instructions and cDNA was synthesised from total RNA using murine Moloney leukaemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA) and random hexamer or oligo (dT)16 primers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control.

**Table 1.** Primer and probe sequences used in Paper II and Paper III.

<b>Real-time RT-PCR primers and probes ( Paper II, III)</b>			
<b>Gene</b>	<b>Forward primer (5'-3')</b>	<b>Reverse primer (5'-3')</b>	<b>Probe (5'-3')</b>
<b>MMP-2</b>	TCATCGGCTACACACCTGATCT	GTCCTGCTCCATCATGGATT	FAM-CCAAGTCTGGAGCGATGTGACCC-TAMRA
<b>MMP-9</b>	GGCCACTACTGTGCCTTTGAG	GATGGCGTCGAAGATGTTAC	FAM-TTGAGGCATCGTCCACCGG-TAMRA
<b>MMP-13</b>	TCCTACAAATCTCGCGGAAAT	GCAATTTCTCGGAGCCTCTCA	FAM-CATGGAGCTGTGTCATTCTCCTCAG-TAMRA
<b>MT1-MMP</b>	TGGAGGAGACACCCACTTTGA	GCCACCAGGAAGATGTCATTT	FAM-CCTGACAGTCCAAGGCTCGGCAGA-TAMRA
<b>MT3-MMP</b>	GGCTCGTGTGGAAATGGTA	AGAACTCTTCCCCTCAAGTG	FAM-ACAGCTGGCTCTACTTCCCATGGC-BHQ1
<b>MT6-MMP</b>	CATTCTGTGGCTCTGCAGTGT	GAAGTGGCCCTAAGTGACTCTAC	FAM-CCTCCACCCAGGACCAATATGTTCA-BHQ1
<b>RT-PCR primers (Paper III)</b>			
<b>Gene</b>	<b>Forward primer (5'-3')</b>	<b>Reverse primer (5'-3')</b>	
<b>MMP-2</b>	GAGGACTACGACCGGACAA	CCAAATGAACCGGTCCTTGA	
<b>MMP-9</b>	TTGACAGCGACAAGTGG	CCTGATGTGGGTGAATACAA	

### Quantitative gene expression analysis by Real-time RT-PCR

Real-time RT-PCR is a specific and sensitive method that measures the expression of a specific gene sequence at a particular time in a cell population. It can be used to determine whether or not a specific gene sequence is expressed in a sample and if the expression changes in response to stimulation. Initially, the expression of MMP-1, -2, -3, -7, -8, -9, -12, -13 as well as MT1-, MT2-, MT3-, MT4-, MT5- and MT6-MMP was analysed in a preliminary screening of the NK-92 cell line using real-time RT-PCR. Detectable expression was found for MMP-2, -3, -7, -9, -13, MT1-, MT3-, MT5- and MT6-MMP. In Paper II and III, the expression of the gelatinases MMP-2, and -9, the membrane type MT1-, MT3- and MT6-MMP and the collagenase MMP-13 was analysed in freshly isolated human NK cells and the YT and NK-92 cell lines. Further,



the effect of IL-2 (Paper III) and matrix contact (Paper II and III) on MMP expression was determined.

The RNeasy® Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA) was used to isolate RNA according to the manufacturer's instructions and RNA concentrations were measured using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) before RNA from each sample was synthesised to cDNA using Cloned AMV First-strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Real-time RT-PCR was performed according to the Taqman method of Applied Biosystems (Nieuwerkerk aan de IJssel, The Netherlands) as previously described [113], with primers and probes listed in Table 1. Expression levels were normalised against GAPDH expression. The obtained  $\Delta$ Ct values, where a lower Ct value indicates a higher expression, were used to describe MMP expression.

## Protein analyses

### Flow cytometry

Changes in cell-associated MMP-2 and MMP-9 levels on NK-92 cells in response to 24h chemokine stimulation were examined with flow cytometry using a FACScalibur flow cytometer (Becton, Dickinson & Co., Mountain View, USA). Levels of MMP-2 and -9 on the NK-92 cell surface was analysed using cells washed and stained in PBS with 0.1% BSA in 4°C, while intracellular levels were measured using paraformaldehyde-fixed cells that were washed and stained in PBS containing 0.2% saponin. The NK-92 cells were labelled using primary monoclonal antibodies directed against MMP-2 or MMP-9 (both obtained from from Oncogene Research products, Cambridge, MA, USA). An irrelevant isotype-matched antibody was used as a negative control. Secondary staining was performed using FITC- or PE-conjugated anti-mouse IgG1 antibodies (SBA, Birmingham, AL, USA) and the obtained flow cytometric data was analysed using multigraph analysing software.

### ELISA

Quantitative determinations of MMPs produced by NK-92 and YT cells, and IL-2 and IFN- $\gamma$  produced by YT cells were performed using commercial kits employing the sandwich enzyme immunoassay technique ELISA. This is a sensitive method with high specificity and low intra- and inter-variability that can be used to detect and quantify specific molecules in biological samples. In ELISA the sample to be analysed is added to wells with an immobilised antibody together with an enzyme-linked antibody and a substrate. This results in a coloured reaction product, in proportion to

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the amount of antigen present in the analysed sample, which can be measured using a spectrophotometer. The secretion of the protein of interest was measured using ELISA kits measuring total MMP-2 (zymogen and active form), zymogen but not active MMP-9 (both obtained from Calbiochem, La Jolla, CA, USA), total MMP-13 (Anaspec, San Jose, CA, USA) and IFN- $\gamma$  or IL-2 (BD Biosciences, San Diego, CA, USA).

### **Gelatine zymography**

The gelatinolytic activity in supernatants obtained from the YT (in Paper II) and NK-92 cells (in Paper II and IV) was measured using gelatine zymography. In this technique proteins in concentrated samples are separated by electrophoresis on sodium dodecyl sulphate (SDS) polyacrylamide gels containing gelatine under denatured but in non-reducing conditions. After removal of SDS by washing in Triton X-100 containing buffer (50 mM Tris, pH 7.5, 5 mM CaCl<sub>2</sub>, 1  $\mu$ M ZnCl<sub>2</sub>, 2.5% Triton X-100), zymograms containing renatured gelatinases were incubated in developing buffer (50 mM Tris, pH 7.5, 5 mM CaCl<sub>2</sub>, 1  $\mu$ M ZnCl<sub>2</sub> and 1% Triton X-100) to induce proteolysis. By staining the proteins in the gels with Coomassie Blue, areas of gelatinolysis can be seen as clear bands against the dark blue background (unaffected gelatine stained by the Coomassie Blue). Metalloproteinase activity was verified by incubating gels in 10  $\mu$ M EDTA, 20  $\mu$ M GM6001 or 250 mM 1,10-phenanthroline. The identity of the bands was based on their size and by comparison to the positive control, supernatant from the cell line HT1080 known to produce large quantities of MMP-2 and MMP-9.

### **Statistics**

Statistical significance was assessed by unpaired Student's t-test with the exception of the real-time RT-PCR data concerning YT cells in Paper III which were analysed using factorial ANOVA and Welch Modified Two-Sample t-test.

## **RESULTS AND DISCUSSION**

### **NK cell-dependent Matrigel reorganisation**

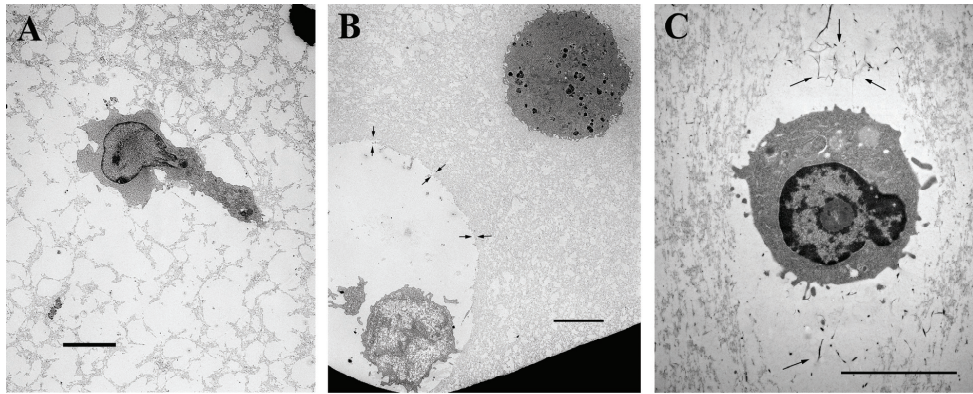
The ability of NK cells to extravasate and migrate in the extracellular space is most certainly influenced by their interactions with the ECM [12, 73, 74]. This in turn can affect the amount of NK cells that ultimately reach the necessary target cell conjugation [15]. To highlight the NK cell-matrix interaction a morphological approach was considered suitable. Therefore electron microscopy, light, confocal and immunofluorescence microscopy was used to study first long- and short-term IL-2-activated mouse A-NK cells when co-cultured with melanoma cells in Matrigel (Paper I) and later the human NK-92 and YT cell lines interaction with Matrigel (Paper II). In both investigations two distinct patterns of matrix disintegration was observed.

### **General matrix disintegration**

It has previously been shown that IL-2 culture affect mouse A-NK cells both morphologically and functionally with time [114]. While murine A-NK cells cultured for 1-6 days are highly cytotoxic, A-NK cells cultured for more than 6 days become increasingly larger, accumulate large glycogen storages, secrete mucus, retain the capacity to invade tumour aggregates but shows a decreased cytotoxicity [114-116]. To determine if the culture time would also affect the NK cell-matrix interaction and target cell conjugation, the mouse NK cells were cultured in medium containing IL-2 for 5-10 days prior to their enclosure together with tumour cells in Matrigel (Paper I).

The ultrastructural analysis demonstrated strikingly different Matrigel appearances which correlated to the A-NK cells culture time. Culture of A-NK cells cultured for < 6 days resulted in a reorganisation of the Matrigel where the initially homogenous matrix transformed into a more loose structure with a meshwork of thin strands (Figure 3A). The general matrix dissolution was further evident after immunostaining of laminin and collagen IV. Lymphocytes have previously been found to be influenced by the duration of IL-2 culture with an increase in the percentage of motile cells in both Matrigel and collagen type I gels, after a pre-culture period for up to 5 days, whereas prolonged IL-2 culture (7-12 days) decreased lymphocyte motility [117]. Prior to this study, using 5 days old A-NK cells, partly dissolved Matrigel droplets were found after 24h of co-culture with melanoma cells [73]. In addition, short-term cultured mouse A-NK cells have been shown to infiltrate B16 tumours more efficiently compared to long-term cultured mouse A-NK cells [118], which could be correlated to a more efficient ability of the younger A-NK cells to degrade the ECM.

## RESULTS AND DISCUSSION

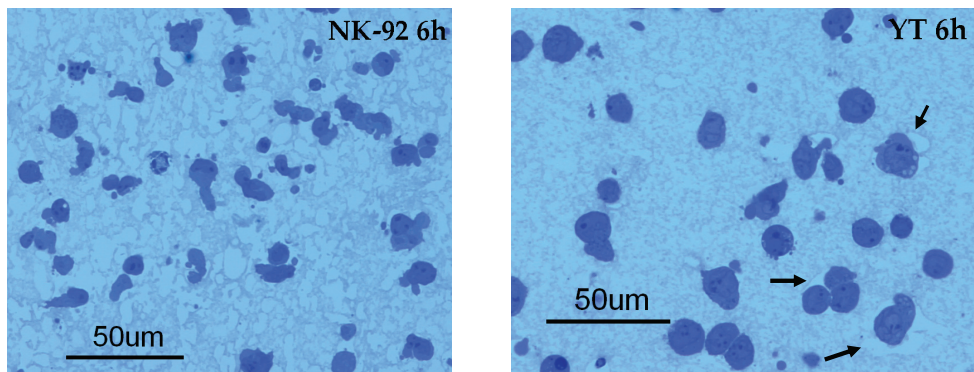


**Figure 3.** Structural alterations of short- and long-term IL-2-activated mouse A-NK cells co-cultured for 6h (A, B) or 10 minutes (C) with B16 melanoma cells in Matrigel droplets. A) Electron micrograph showing a migrating young A-NK cell (cultured < 6 days) surrounded by Matrigel. The initially homogenous Matrigel structure (seen in B) transformed into a loose filamentous meshwork. Bar = 10  $\mu$ m. B) Older mouse A-NK cell (cultured for > 6 days) and melanoma cell in Matrigel. The A-NK cell is surrounded by a large cavity while the melanoma cell is enclosed in homogenous matrix. At the cavity border a more dense Matrigel structure can be seen (between arrows). Bar = 10  $\mu$ m. C) Micrograph of a A-NK cell 10 minutes after Matrigel enclosure with CmB-positive precipitates (arrows). Bar = 4  $\mu$ m.

Mouse A-NK cells are known to express MMPs [4] and a secretion of matrix-degrading proteases could be responsible for the demonstrated alteration in Matrigel structure. Protease inhibitors were indeed able to delay the reorganisation but they could not fully prevent it, and by 24h the effect of protease inhibitors was no longer visible. It is thereby possible that the MMP inhibition was incomplete by the inhibitors used, or that other proteases could be operating besides MMPs (both of which will be discussed in more detail below).

A morphological analysis of NK-92 and YT cells incorporated in Matrigel was furthermore performed to analyse their morphology in the 3-dimensional environment and their impact on the surrounding matrix (Paper II). In this study, the NK-92 cells were found to have a general influence on the Matrigel appearance (Figure 4, left panel), much similar to that observed for the short-term cultured mouse A-NK cells in Paper I. The NK-92 cells, in addition to causing a disintegration of the Matrigel, were also found to interact more extensively with the surrounding matrix compared to YT cells. Podosomes and invadopodia are actin-rich extrusions that the cells use to establish contact with a substratum, mostly found in invasive cell types. They have been found to be involved in matrix degradation and subsequently in migratory processes [119]. In Paper II, the number of NK-92 and YT cells with invadopodias/podosomes was used as a measure of moving/migrating cells. The percentage of YT cells with these features was initially low (8%), increased

significantly after 2h incubation (37%) and remained at the same percentage for the following 24h. In contrast, a high percentage of NK-92 cells with invadopodia/podosomes was seen already after a 10 minute incubation period (78%), increased slightly within the following 6h to 85% but after 24h it had decreased to 40%. The extensive disintegration of the surrounding Matrigel seen after 24h incubation, with a resulting lack of matrix molecules for the NK-92 cells to interact with, could thereby explain the observed drop in invadopodia/podosome expressing NK-92 cells. In conjunction to secreted soluble MMPs, membrane-associated MMPs shown to be localised on cellular extensions [120], are likely a part of the explanation for the demonstrated extensive NK-92 related Matrigel degradation.



**Figure 4.** The two NK cell lines, NK-92 and YT, were incorporated in Matrigel and morphologically analysed. The NK-92 cell line was found to have a higher percentage of cells with cellular extensions compared to YT cells. NK-92 cells caused a general disintegration of the Matrigel while pericellular excavations (arrows) were formed around single and multiple YT cells.

Interestingly, preliminary data from a comparison of YT and NK-92 membrane fractions by 2D gel electrophoresis and mass spectrometry indicate a differential expression of cytoskeletal proteins which are of importance for cell motility. For example, Coronin 1A, a crucial component of the cytoskeleton involved in actin assembly and thereby in forming protrusions and cell motility, were only detected in the motile NK-92 cells (data not shown).

### Pericellular cavities

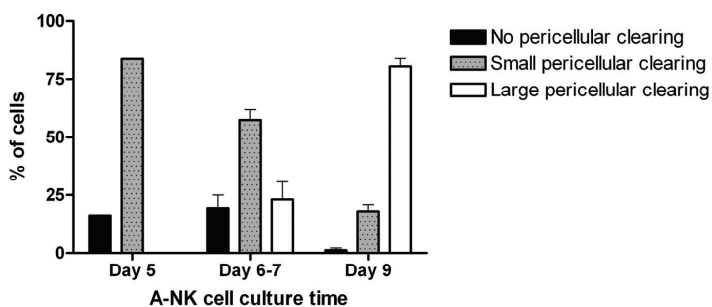
Mouse A-NK cells cultured for > 6 days change their morphological phenotype into large, glycogen-laden and mucus-secreting cells [114, 115]. These long-term cultured

## RESULTS AND DISCUSSION

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mouse A-NK cells, gave rise to a different structural change of the Matrigel compared to the younger A-NK cells (Paper I), pericellular cavitations were instead formed around these A-NK cells (Figure 3B). Importantly, a higher density of the matrix adjacent to the cavity border in the otherwise unchanged Matrigel was evident both with electron and immuno-confocal microscopy. It has previously been shown that mouse A-NK cells cultured for more than 6 days release a proteoglycan-rich mucoid material [115]. In accordance, presence of proteoglycans was visualised in the cavitations and also on the cell surface using the proteoglycan dye CmB in our electron microscopy study (Figure 3C). The observed compression zones around the cavities are thereby suggested to be explained by a release of hydrophilic proteoglycan-containing material having matrix-dilating properties. Comparable tissue-loosening effects have previously been demonstrated by hyaluronate during mesenchymal cell migration [121], and it has further been proposed as an invasion facilitating mechanism for certain malignant cells [122, 123].

To quantify the proteoglycan secretion and the development of pericellular clearings from cells of different ages the erythrocyte exclusion assay was used, which verified a change in A-NK cell phenotype by day 6 when large cavitations around the cells began to appear (Figure 5). A statistically significant increase in the percentage of A-NK cells with large pericellular clearings, and a concomitant decrease for small or no pericellular clearings was found between day 6-7 and day 9 cells.



**Figure 5.** The formation of pericellular clearings around A-NK cells cultured for different time points in IL-2 was analysed using the erythrocyte exclusion assay. The size of the pericellular clearings increased with culture time and the percentage of A-NK cells cultured for 6-7 days with no, small and large pericellular clearings all differed significantly from A-NK cells cultured for 9 days.

Rather unexpectedly, these pericellular excavations were also revealed around single and multiple YT cells when enclosed in Matrigel (Paper II), indicated by arrows in the left panel of Figure 4. The observed cavitations surrounding both long-term cultured mouse A-NK cells and YT cells cultured in Matrigel could be explained by a combination of the above described secretion of a hydrophilic proteoglycan-rich material with localised matrix degradation. Although MMPs are normally produced and released on demand, containment of proteolytically active MMPs on the cell surface will optimise matrix degradation at the invasive front as is the case for membrane bound MMPs. However, also soluble MMPs has been shown to be localised to the cell surface of malignant cells through association with proteoglycans and with the proteoglycan-binding membrane receptor CD44 and the integrin  $\alpha\beta 3$  [92-95, 124]. Such a tethering of MMPs to secreted proteoglycans, ensuring locally restricted matrix degradation in the pericellular region where the degradation also would be in control by the cell through secretion of TIMPs, is considered a feasible explanation for the observed cavities. Formation of these pericellular cavitations around long-term cultured mouse A-NK cells could however not be stopped by several used endoprotease inhibitors (BB94, aprotinin and benzamidine) or by glycosylation inhibitors (tunicamycin and castanospermine). It could be that a deficient MMP inhibition by BB94 is due to steric reasons of the MMP-proteoglycan association, which in such case would also slow down the turnover rate. It is furthermore possible that a locally high concentration of proteases confined in a small space between the cell and the substrate is able to function despite presence of a molar excess of protease inhibitors. The lack of effect with glycosylation inhibition could indicate that other glycosylation patterns than the ones inhibited (core N-glycosylation and processing of N-linked carbohydrates) are functional and control experiments confirming the success of glycosylation inhibition is admittedly absent. It is also feasible that longer incubation times are needed for an efficient inhibition. The observation of pericellular excavations already at 10 minutes suggest that the A-NK cells excrete mucus during their time in culture, as seen in the erythrocyte exclusion assay, thus likely explaining the lack of effect observed after only 6h incubation with monensin.

In summary, the same two divergent Matrigel appearances as seen after short- (< 6 days) and long-term (> 6 days) cultured mouse A-NK cells in Matrigel (Paper I), were also observed after culture of NK-92 and YT cells in Matrigel (Paper II). It is proposed that these different morphological patterns indicate two, seemingly distinct matrix-interactive strategies that these cells use to degrade and penetrate ECM. These patterns could furthermore be linked to the phenotypic properties of the mouse A-NK cells which are affected by the duration of IL-2 culture.

We have shown that the NK-92 and YT cell differ in their expression of a variety of MMPs (discussed below). The NK-92 cell line has a higher MMP expression which

## RESULTS AND DISCUSSION

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could explain this cell lines more extensive loosening of the Matrigel. To compare any quantitative amounts of secreted, and possibly also membrane-associated MMPs, between the short- and long-term cultured mouse A-NK cells, would shed more light on this matter.

Thus, the described morphological findings suggest that depending on developmental stage/phenotype there are different modes of interaction with the surrounding matrix which could also reflect different functions *in vivo*. Depending on the morphology of the encountered microenvironment the invading cell population could benefit from a sequential use of such discrete strategies, i.e. general matrix dissolution by ECM-degrading proteases and a tissue loosening to wedge the malignant cells apart by use of the dilating properties of a secreted hydrophilic matrix at the advancing front. Further, if they were to operate simultaneous a possible synergistic effect could be outlined with enhanced breaking of tight tumour masses.

### MMP expression and production

In previous studies a large amount of different MMPs have been detected in rodent NK cells (i.e. MMP-1, -2, -3, -7, -9, -10, -11, -13, MT1- and MT2-MMP) [4, 5, 125], mainly by RT-PCR. However, in human NK cells the MMPs described to date are MMP-1, -2, -9, MT1- and MT2-MMP [99-101]. Expression of MMPs has in addition been described for several other leukocyte populations. For example, T cells produce MMP-9 constitutively while MMP-2 is produced in response to IL-2 stimulation or upon VCAM-1-dependent adhesion [126, 127]. Quantitative data on the secretion of MMPs are scarce but measurements of MMP-2 and MMP-9 using semi-quantitative gelatine zymography has shown that NK cells seemingly produce lower amounts of these MMPs compared to polymorphonuclear leukocytes and T cells [128]. However no clear description on the culture quality of these NK cells is given and the value of this study may therefore be somewhat questioned. Nevertheless, through their expression of a large variety of MMPs the NK cell is still well equipped to efficiently degrade a wide range of ECM structures. In addition to their matrix degrading ability, MMPs have also been given an immuno-modulating role [81] and the MMPs expressed in human NK cells might thereby have multiple functions in the *in vivo* setting.

A compilation of previous data and our findings on the expression of MMPs in NK cells of different species and their role in Matrigel invasion is found in Table 2. In this thesis, a preliminary screening of MMP *mRNA expression* in the NK-92 cell line, using quantitative real-time RT-PCR, demonstrated expression of MMP-2, -3, -7, -9, -13, MT1-, MT3-, MT5-, and MT6-MMP (data not shown). Out of these MMPs, we



chose to continue to analyse the expression of MMPs previously known to be expressed in human NK cells (MMP-2, -9, and MT1-MMP) as well as novel ones (MMP-13, MT3- and MT6-MMP). The collagenase MMP-13 has earlier been reported to be expressed by rodent NK cells [4, 125] but MT3- and MT6-MMP have until now not been described in NK cells of any species. Expression of all analysed MMPs (MMP-2, -9, -13, MT1-, MT3- and MT6-MMP) were found in freshly isolated human NK cells (Paper III) as well as in the NK-92 and YT cell lines (Paper II). The MMPs detected in human NK cells were found to have a moderate to low expression level indicating mostly low basal production. However, most interestingly, the MT6-MMP had a clearly higher expression in human NK cells than the other MMP members analysed. In the course of Paper III, the expression of MMP-2 and MMP-9 in freshly isolated human NK cells was in addition determined using semi-quantitative RT-PCR.

**Table 2.** MMPs expression in NK cells of different species.

	Mouse NK cells [4]	Rat NK cells [5, 6, 125]	Human NK cells [99-101] (Paper III)	NK-92 cell line (Paper II)	YT cell line [99] (Paper II, III)
<b>MMP expression</b>	MMP-2	MMP-1	MMP-1	MMP-2	MMP-2
	MMP-9	MMP-2	MMP-2	MMP-3	MMP-9
	MMP-11	MMP-3	MMP-9	MMP-7	MMP-13
	MMP-13	MMP-7	MMP-13	MMP-9	MT1-MMP
	MT1-MMP	MMP-9	MT1-MMP	MMP-13	MT3-MMP
	MT2-MMP	MMP-10	MT2-MMP	MT1-MMP	MT6-MMP
		MMP-11	MT3-MMP	MT3-MMP	
		MMP-13	MT6-MMP	MT5-MMP	
		MT1-MMP		MT6-MMP	
		MT2-MMP			
<b>Invades Matrigel</b>	Yes	Yes	Yes	Yes	Yes
MMP-dependent invasion	Yes	Yes	Yes	Yes	No
	~90%*	~50%*	~30%#	~40%#	NS

NS=Non-significant; \*=BB94 inhibition; #=GM6001 inhibition.

The gelatinases, important for ECM and BM degradation because of their superior and almost unique ability to cleave collagen IV, were furthermore analysed at the *protein level* by ELISA and gelatine zymography. In our study, no measurable levels of MMP-2 using ELISA could be found in supernatants from neither cell line while MMP-9 could be detected using both ELISA and gelatine zymography in supernatants from both cell lines. However, MMP-2 could be detected in concentrated NK-92 and YT cell supernatants using gelatine zymography. Zymography, enabling detection of as little as 10–30 pg of enzyme, is thereby a more sensitive method to detect gelatinases compared to ELISA, as been described earlier [129]. Production of MMP-13 was also analysed using ELISA but found to be extremely low and not distinguishable from background readings. Although, as recently discussed, MMP-13

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could be present but in very low quantities. In accordance to the real-time RT-PCR results the NK-92 cell line was found, by ELISA and gelatine zymography, to produce higher levels of MMP-9 compared to the YT cell line. Since findings of MMPs in human NK cells have been on the mRNA level, except for MMP-1, -2, -9, MT1-MMP [99, 100], it is of course important to confirm protein expression of the additional transcripts found to be encoded by human NK cells. Western blots have been produced with positive reaction for MT6-MMP (data not shown).

Immunolocalisation of MMPs has proven to be very difficult which could be explained by the likely low amounts of MMPs in NK cells, and so far no real evidence for a granular storage has been found despite extensive elaboration with immuno, light and electron microscopy. We did however label for intracellular and cell surface-detained MMP-2 and MMP-9 on NK-92 cells using flow cytometry (Paper IV) and while the signal was too low on each locality to distinguish the discrete MMP location we believe we have still been able to detect the “total” cell-associated MMP-2 and MMP-9. In addition, preliminary immunohistochemical data indicate a diffuse intracellular staining of MMP-2 and MMP-9 in NK-92 cells (work in progress).

### **The role of MMPs for NK cell migration**

Natural killer cell migration is a critical process that will affect the accumulation of NK cells within tumour tissues. It has experimentally been shown that while some extravasated NK cells make direct contact with tumour cells, many remain localised in the stroma surrounding the tumour [3, 11-17]. Previous *in vitro* studies using IL-2-activated NK cells isolated from mouse and rat have shown that their migration through Matrigel partly depends on MMPs [4-6]. Besides ensuring that MMPs are involved also in human NK cell Matrigel transmigration, we also sought to compare the NK-92 and YT cell lines' migratory ability and correlate the results to their MMP expression.

In Paper II, NK-92 cells were found to migrate significantly better than YT cells, with > 90% more NK-92 cells migrating through Matrigel inserts. In order to determine if their migration was MMP-dependent, the broad-spectrum MMP inhibitor GM6001 was included in the assays. While inhibition of MMPs during the migration assay decreased NK-92 cell migration by ~40% (Paper II), the inhibition of YT cell migration was in the range of 15% (Paper III).

The easiest explanation for the migratory difference found between the NK-92 and YT cell lines is the quantitative difference in their production of MMP-9 and the lower expression of all analysed MMPs in YT cells compared to NK-92 cells, indicating

lower amounts of enzyme. This furthermore correlates with the morphological findings discussed above. However, there are no quantitative data on the amount of MMPs needed for ECM degradation (or Matrigel) and NK cell migration. Of course, an efficient localisation of MMPs at the invasive front or retention of MMPs in the vicinity of cells by co-localisation with secreted proteoglycans (as proposed in Paper I) might be of more functional importance than just crude elevated amounts [120], detected in cell supernatants with ELISA.

Similar to the NK-92 cell line, inhibition of freshly isolated human NK cell migration by GM6001 was found to be approximately 30% (Paper III). The significant inhibition of freshly isolated human NK cells', as well as the NK-92 cell line', migration by GM6001 thereby shows that their invasion through Matrigel is MMP-dependent. The YT cell lines' low migratory ability, which was not significantly inhibited by GM6001, can furthermore be correlated to its low MMP expression. Previous studies on rodent NK cells have shown that inhibition of MMPs using the MMP inhibitor BB94 decreased rat A-NK cell migration by 50% while mouse A-NK cell migration was inhibited up to 90% [4, 5]. The rat NK cell line RNK-16 has also been used to study the importance for MMPs in NK cell migration and both GM6001 and BB94 inhibited their migration through Matrigel by approximately 50% [6, 103]. The data on freshly isolated human NK cells as well as the human NK-92 cell line establish a role for MMP activity also on human NK cells ability to migrate through Matrigel.

To note is that the incomplete inhibition of the migratory ability of the NK cells used in this study, of 30%-40%, is similar to other studies with an inhibition range of 25-60% when using GM6001 to inhibit NK cell migration [6, 100] and 30%-60% in inhibition of T cell migration [126, 130, 131]. It has furthermore been demonstrated that cancer cells have the ability to traverse Matrigel despite presence of GM6001 [132] and that Matrigel invasion capacity does not reflect *in vivo* invasive ability [133]. This is probably related to the lower resistance required for cell penetration of Matrigel due to its lesser degree of cross-linking compared to authentic BMs. Thus, even though Matrigel is frequently used for *in vitro* invasion studies because of its chemical and immunological similarities to BM it differs structurally. This clearly affects its strength and integrity and thus influences Matrigel relevance as a model of native BM. Moreover, the possibility of protease-independent migration have been proposed where cells can migrate in the absence of proteases by adopting an amoeboid form of movement that allow the cells to squeeze through narrow matrix gaps [134, 135]. Accordingly, NK cells have been shown to deform into extremely thin formations during extravasation and intra-tumour migration [10, 13]. Migrating NK cells seemingly use a combination of matrix degradation and shape modulation, as demonstrated here.

### **Effects of IL-2 on NK cell migration and MMP expression**

Although IL-2 can increase NK cell target recognition and tumour localisation, the more precise effects on the invasion process *per se* is still unclear and there are no previous reports describing the direct effects of IL-2 on NK cells' MMP expression and production. However, IL-2 has been shown to influence the amount of freshly isolated human NK cells that migrate through Matrigel [99], indicating that human NK cell migration might be IL-2-dependent. From other human lymphoid cells, IL-2 has been shown to regulate the production of MMP-9 in peripheral blood T cells [136], and an enhanced MMP-dependent T cell migration in response to IL-2 stimulation has also been reported [126]. At the transcriptional level, cross referencing gives an idea of a possible correlation between IL-2 and MMP expression. IL-2 stimulation of NK cells activates the MKK/ERK pathway with AP-1 as one of the downstream effectors [36]; furthermore, AP-1-mediated transcription has been reported in response to IL-2 in cultured NK cells [137]. Interestingly, the AP-1 binding site is also involved in the transcriptional regulation of several MMP promoters [138] and AP-1 could thereby be a possible connection between IL-2 stimulation and MMP expression.

In this study, we used the NK cell line YT and freshly isolated human NK cells, to exploit the effects of IL-2 on NK cell migration and MMP expression/production. The IL-2-independent YT cell line was used because of the difficulties in analysing effects of IL-2 using freshly isolated human NK cells, as previously mentioned in the introduction. Control experiments found the YT cells to lack measurable levels of endogenous IL-2 production and any significant autocrine/paracrine stimulation could thereby be excluded. Importantly, the YT cells were found to be IL-2 responsive by showing a dose-dependent secretion of IFN- $\gamma$ , verifying that that the YT cells respond to the used recombinant IL-2, a prerequisite for subsequent experiments.

### **Temporal effects of IL-2 on human YT cell invasiveness and MMP expression**

The effect of IL-2 stimulation on YT cell migration was again investigated in the Matrigel invasion assay. The number of transmigrated YT cells was shown to increase by ~50% in response to IL-2 stimulation and inhibition of MMPs with GM6001 during simultaneous IL-2 stimulation insignificantly decreased the YT cells migration by 21%. Furthermore, GM6001 was only able to marginally decrease YT cell migration in the absence of IL-2.

In order to imitate the adoptive transfer preparation procedure of freshly isolated NK cells, YT cells were pre-cultured with IL-2 preceding the invasion assay and found to migrate approximately 40% less than YT cells pre-cultured without IL-2. Thus, direct

IL-2 stimulation enhanced YT cell migration while a longer stimulation period decreased YT cell migration, which was significant using a one-tailed Student's t-test. In accordance, levels of MMP-9 in supernatant from IL-2 stimulated YT cells measured with ELISA showed a time-dependent significant increase in MMP-9 levels only within the first 2-4h. These results thereby support an early stimulatory effect of IL-2 on MMP-9 release that enhances migration in the invasion assay. In addition, in the morphological investigation, IL-2 stimulation of YT cells in Matrigel was found to increase the amount of cellular extensions after 2h culture but not at later time points (6 and 24h), which connects to the proposed rapid IL-2-induced migration.

Real-time RT-PCR was furthermore used to investigate the IL-2-dependent effects on the YT cell lines' MMP expression after stimulation for 30h with IL-2 alone in cell culture or in a series of experiments with increasing contact with matrix components i.e. the ECM equivalent Matrigel. The analysed MMPs were generally weakly expressed in YT cells and the overall expression (all but MT1-MMP) decreased with IL-2 culture. This is in full accordance to the above described negative effect on YT cells migratory ability seen after pre-culture with IL-2. The effect of Matrigel culture on YT cells MMP expression was furthermore investigated. Interactions with surrounding matrices have been reported to alter gene expression and result in phenotypic changes in many malignant cells [139]. Natural killer cells migratory ability have in addition been proposed to be influenced by ECM contact [73, 74]. However, the culture conditions used in our study with two modes of matrix exposure were not found to affect the expression levels of the investigated MMPs. Also the NK-92 cell line was cultured on both Matrigel coated plastic and in gelled Matrigel droplets without demonstrating a change in MMP expression (Paper II). We analyse the expression levels after 30h of Matrigel culture only and it is consequently possible that there are indeed early effects on the NK cells MMP expression that our analysis did not detect.

### **IL-2 increase freshly isolated human NK cells ability to migrate through Matrigel and MT6-MMP expression**

When the effects of IL-2 was examined using freshly isolated human NK cells, results similar to those obtained with YT cells were found, i.e. IL-2 clearly increased the ability of freshly isolated NK cells to migrate through Matrigel (68%). Again their migration was found to be partly MMP-dependent, as shown by a significant inhibition with GM6001 (~30%). The lack of complete inhibition, seen with both freshly isolated NK cells and the YT cell line, could be due to an incomplete inhibition of MMPs by GM6001 or indicate that proteases and/or mechanisms other than MMPs (as previously discussed) are part of the increased invasion. The uPA/uPAR system has

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**Table 3.** Regulation of MMPs expression/production in correlation to invasive ability in NK cells of different species.

	Mouse NK cells	Rat NK cells	Human NK cells	NK-92 cell line	YT cell line
<b>Cytokines</b>					
IL-1 $\alpha$ and $\beta$ [128]			Slight increase in MMP-9 production.		
IL-2 (Paper III)	NA	NA	Increased expression of MT6-MMP.* Increased invasion (68%).*	NA	Negative effects on global MMP expression. Increased invasion (52%) while a 48h pre-culture period with IL-2 has a negative effect on invasion.
IL-6 [128]			Slight increase in MMP-9 production.		
IL-18 [101]			Increased production of MMP-2, MMP-9 and increased expression of MT1-MMP. Enhanced invasive ability.		
<b>Chemokines</b>					
CCL2			Possible increase in MMP-9 production. [128]		
CCL3	No effect on invasion.[4]		No increase in MMP-9 production. [128]	No effect on production of MMP-9 or on invasion. (Paper IV)	
CCL5 [4]	No effect on invasion.				
CCL8 [128]			Possible increase in MMP-9 production.		
CX3CL1 (Paper IV)				Increased MMP-9 production.	
CXCL10	No effect on invasion.[4]		No effect on MMP-9 production. [128]	No effect on production of MMP-9 or on invasion. (Paper IV)	
CXCL12 [100]			Increased MMP-1 expression. Enhanced ability to invade collagen I. ~60%#		
<b>Miscellaneous</b>					
2B4 [104]					
PGI2 [6]		Increased secretion of MMP-1 and -3. Increased invasion. ~25%#			Up-regulation of MMP-2.

NA: not applicable due to obligate need for IL-2 for culture survival.\*= Survival doses of IL-2 supplemented to control cells. #=GM6001 inhibition. Invasion indicates Matrigel invasion if not otherwise mentioned.

further been shown to be involved in rat NK cell migration [103] and IL-2 has been found to up regulate uPA and uPAR in YT cells [105]. However, enhanced uPA production in response to IL-2 stimulation of the YT cell line could not be detected using ELISA (data not shown), which argues against the involvement of uPA in IL-2-stimulated YT cell migration in our study. However, as previously noted for MMP-2, the bare protein levels measured with ELISA are not sufficient to rule out possible significant enzymatic effects. Plasminogen gelatine zymography, a more sensitive method for detecting uPA, might therefore be needed. Iodinated fibrinolysis of cell supernatants in presence of plasminogen could in addition be used to amplify the uPA activity [140]. The contribution of the uPA system in IL-2-dependent migration of freshly isolated human NK cells clearly needs further investigation as well as the possible presence of other matrix degrading enzymes, e.g. heparanase, seprase.

The same general negative effect on MMP expression by IL-2, as found in YT cells, was not found in freshly isolated human NK cells. Instead a significant increase in MT6-MMP expression by both 2 and 24h in response to IL-2 was established. The expression of MT6-MMP was in addition relatively higher than the other MMP members analysed, as earlier mentioned. The expression levels of the other MMPs included in this study was not found to change following IL-2 stimulation. In agreement to the real-time RT-PCR data, no significant effect on the expression of MMP-2 and MMP-9 was seen in freshly isolated NK cell preparations after 48h of IL-2 stimulation using semi-quantitative RT-PCR. The membrane bound MT6-MMP can because of its optimal cellular location at the plasmalemma enable efficient matrix degradation at the invasive front, and its responsiveness to IL-2 stimulation makes it a very interesting MMP member for continued investigation concerning its role in NK cell migration.

Since primary NK cells are IL-2-dependent the specific effects of IL-2 are inherently difficult to study using freshly isolated human NK cells. For instance, as seen in Paper III, the expression of the MMPs investigated was indeed influenced by only 10 U IL-2, a dose that has proven to be the minimum supplement needed to ensure cell survival in culture. For the time being, we therefore have to rely mainly on data from the IL-2-independent YT cell line. Although being a malignant NK cell line and demonstrating both low MMP production and low invasive capacity, it is still regarded a reliable candidate for the study of IL-2-dependent effects on NK cells [141].

### **NK-92 cell migration and gelatinase production in response to chemokine stimulation**

Chemokines are known to promote NK cell migration but the mechanism behind this process has not been fully established. There are however a few reports regarding the effects of chemokine stimulation on NK cells' MMP expression and production, see Table 3 where previous studies on the effects of cytokine and chemokine stimulation together with our obtained results are summarised. The chemokine CXCL12 has been shown to increase human NK cell invasion into type I collagen through effects on MMP-1 [100]. However, Johnnatty *et al.* determined human NK cells to be mainly unresponsive to the chemokines included in his study, as judged by gelatine zymography [128]. Accordingly, CCL3, CCL5 and CXCL10 (also included in the study by Johnnatty) was functionally tested in Matrigel invasion assay by mouse A-NK cells but found to have no effect [4].

In Paper IV we wanted to study the influence of chemokine stimulation on NK cell gelatinase production and Matrigel invasion. We used each one members from three of the major chemokine subgroups; CCL3 (macrophage inflammatory protein 1 alpha (MIP-1 $\alpha$ )), CX3CL1 (fractalkine/neurotactin) and CXCL10 (interferon gamma inducible protein (IP-10)). Human NK cells have previously been reported to migrate in response to these potent chemoattractants [43, 46, 142-144].

### **Fractalkine stimulation increase NK-92 cells MMP-9 production**

In this study, even though stimulation with all three chemokines (CCL3, CX3CL1 and CXCL10) could demonstrate increasing levels of detected MMP-9 in supernatants in a time-dependent manner, only CX3CL1 was found to significantly increase the level of MMP-9 measured in supernatants after 24h culture compared to IL-2-stimulated control cells. An increase in gelatinase activity after 24h of CX3CL1 stimulation was also evident in zymograms. Furthermore, cell-associated levels of MMP-9 decreased in response to CX3CL1, which could indicate a release of MMP-9. However, and somewhat unexpectedly, the demonstrated increase in the NK-92 cells' production of MMP-9 in response to CX3CL1 did not translate into an enhanced NK-92 cell migration in the Matrigel invasion assay.

The effect on NK-92 cells MMP production in response to the different chemokines could in part be due to receptor expression differences. The receptor for CCL3, CCR1, has been found to be expressed on NK-92 cells but found not functional for CCL3 [145]. This was unknown to us at the time of the study but explains the lack of effect of CCL3. Human NK cells have previously shown chemotaxis in response to CXCL10 [46, 144]. The receptor for CXCL10, CXCR3, has in addition been found to be



expressed by the NK-92 cell line [145]. However, a CXCL10-dependent increase in migration could not be detected in our study, as was also the case in a previous study using mouse A-NK cells [4]. The reason for this is not clear but it is possible that a chemokine receptor is not always functionally active, even when expressed, as has been demonstrated for CCR3 on NK-92 cells.

Fractalkine (CX3CL1) stimulation has previously been reported to induce NK cell chemotaxis both *in vitro* and *in vivo* and its receptor, CX3CR1, has been detected in both freshly isolated NK cells and in NK-92 cells [142, 143, 146]. While our findings show that CX3CL1 (fractalkine) has a stimulatory influence on NK-92 cells production of MMP-9 it did not significantly affect their migratory ability. It here needs to be remembered that the *in vivo* situation is very dynamic with shear forces acting on the recruited cells and it represents a fairly rapid process when chemokines attract rolling leukocytes from the circulation. Shear forces could therefore be required in order to see the full effect of chemokine stimulation as has indeed been shown for peripheral blood lymphocytes [147, 148]. Additional studies have to be made to increase our understanding of the molecular basis for the effects of these chemokines on NK cells' migratory ability and MMP production. These include; screening using a broader repertoire of chemokines; verification that the corresponding receptors are expressed and functional; analysis of more MMP members and positive findings needs to be verified with adequate controls, and importantly using both dynamic and static culture conditions.

## CONCLUSIONS

- Morphological evidence is presented showing two different NK cell locomotive strategies during enclosure in Matrigel, i.e. general loosening of the matrix or formation of distinct pericellular excavations.
- A differential MMP expression in the NK-92 and YT cell lines coincided with different migratory phenotypes.
- The MMP members MMP-13, MT3- and MT6-MMP, previously not described in human NK cells are expressed in both freshly isolated human NK cells and the NK-92 and YT cell lines.
- Stimulation with IL-2 increased freshly isolated human NK cells expression of MT6-MMP and also increased their ability to migrate through Matrigel in a MMP-dependent manner.
- The effects of IL-2 on the IL-2-independent cell line YT included an early stimulatory effect on MMP-9 secretion, correlating with an enhanced migration through Matrigel together with later negative effects on Matrigel invasion and significant down-regulation of MMP-2, -9, -13, MT3- and MT6-MMP expression.
- Matrix (Matrigel) contact did not effect the expression (at 30h) of the MMPs included in our study, in neither the NK-92 nor YT cell line.
- Static exposure of the chemokine CX3CL1 (fractalkine) to NK-92 enhanced their secretion of MMP-9 but it did not translate into an increased Matrigel invasion.

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