Investigations on the Effect of a Platelet Derived Growth Factor-CC Secreting Breast Carcinoma on Natural Killer Cell Cytotoxicity

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## Abbreviations

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<tr>
<td>AF</td>
<td>Alexa Flour</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxy-terminal extension of amino acids</td>
</tr>
<tr>
<td>CUB</td>
<td>Complement subcomponents C1r/C1s, urchin EGF-like protein and bone morphogenetic protein-1</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>(c)DNA</td>
<td>(complementary) Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GFD</td>
<td>Growth factor domain</td>
</tr>
<tr>
<td>H-2</td>
<td>Histocompatibility-2</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic activated cell sorting</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MQ-H₂O</td>
<td>Milli-Q (deionised) H₂O</td>
</tr>
<tr>
<td>N-terminus</td>
<td>Amino-terminal extension of amino acids</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKG2D</td>
<td>Killer cell lectin-like receptor (subfamily K, member 1)</td>
</tr>
<tr>
<td>PAE cells</td>
<td>Porcine aortic endothelial cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Proprotein convertase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDGF-A/B/C/D</td>
<td>PDGF-type A/B/C/D</td>
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<tr>
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<td>PDGF Receptor type α/β</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>rm</td>
<td>recombinant murine</td>
</tr>
<tr>
<td>(m)RNA</td>
<td>(messenger) Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>tPA</td>
<td>Plasminogen activator (of PDGF-CC)</td>
</tr>
<tr>
<td>U</td>
<td>Enzyme units</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator (of PDGF-DD)</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
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**Summary**

Tumour cells are depending on the tumour stroma to support them with growth factors and also to suppress immunity. One growth factor commonly derived from the stroma is the platelet derived growth factor (PDGF) -CC, a substance known to induce tumour angiogenesis. In this thesis I present evidence that murine TUBO breast cancer cells transfected with PDGFC suppress natural killer (NK) cell cytotoxicity. These findings derive from three differently performed standard $^{51}$Cr-release assays. Purified NK cells directly incubated with TUBO/PDGF-C cells are 50 % less cytotoxic compared to NK cells incubated with normal TUBO cells. In addition, a clearly reduced cytotoxicity towards the lymphoma cell line YAC-1 is observed when purified NK cells or isolated splenocytes are previously cocultured with PDGF-CC secreting TUBO cells. By trying to reveal the mechanism of NK cell suppression, I show despite suppression, via immunofluorescence staining and by western blot experiments, that the specific PDGF-CC receptors, namely PDGFR-α and PDGFR-β, are not expressed on the NK cell surface. However, I can demonstrate by flow cytometry analysis that TUBO/PDGF-C cells exhibit up-regulated MHC class I molecules on their cell surface that could explain reduced NK cell function. Overall, my results suggest that PDGF-CC is not only a growth factor but also a modulator of innate tumour immunity.
1 Introduction

1.1 Natural killer cells

Natural killer cells (NK) were discovered due to their ability to kill various tumour cells in vitro independent of additional priming and the expression of major histocompatibility complex (MHC) class I molecules of the target cells [1]. Nowadays, NK cells are defined as a lineage of lymphocytes that mediate innate immunity against both infected or stressed cells by a direct killing mechanism and the secretion of inflammatory cytokines [2]. Notably, NK cells do not usually affect normal cells. NK cells are mainly found in the peripheral blood but also in liver, spleen and placenta [3].

1.1.1 NK cell function

The immune response of NK cells results in the secretion of cytokines or chemokines. Moreover, NK cytotoxicity against transformed cells is induced by the death receptors Fas (FasR) or tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) as well as the perforin/granzyme pathway [4, 5]. Once antibodies coat infected cells, NK cells can also bind by their fragment crystallisable receptor gamma (FcRγ) to the Fc regions of the bound antibodies and destroy these cells. The process is known as antibody-dependent cell-mediated cytotoxicity (ADCC) [2]. The major cytokine of NK cells is interferon gamma (INF-γ) since its production stimulates Th1 response, pathogen killing by macrophages and the up-regulation of antigen presenting (APC) cells. INF-γ production also exhibits an inhibitory effect of proliferating malignant or viral cells [6-8]. Fundamentally, for the production of INF-γ two signals are needed: interleukin 12 (IL-12) and cytokines such as IL-1, IL-2, IL-15 or IL-18 [9, 10]. These cytokines are released from cells of the innate immune system such as monocytes, macrophages or dendritic cells [11, 12].

1.1.2 Recognition and regulation of natural cytotoxicity

The “missing self” hypothesis proposed that NK cells become activated and lyse target cells once MHC class I expression is lost or deficient [13]. However, it is known by now that NK cytotoxicity is rather regulated by a balance between signals generated from both activating and inhibitory receptors [2]. In general, the inhibitory receptors are expressed on NK cells and recognise MHC class I molecules expressed on healthy cells, while activating receptors can be
expressed either on NK-target cells or on normal cells [2]. Importantly, once MHC class I is
detected signalling of the inhibitory receptors is dominating [2]. Many NK cell receptors are
known and they are classified into different families. However, expression and structure of
human and rodent NK cell receptors are very divergent. In the present study only murine NK
cells are investigated. Therefore, the following receptor examples refer to murine NK cells.
Inhibitory receptors, including the killer cell lectin-like receptor subfamily (Ly-49) that bind to
classical MHC class Ia ligands and CD94/NKG2A receptors that bind to non-classical MHC
class Ib ligands [14].

Their common characteristics are cytoplasmatic immunoreceptor tyrosine-based inhibition motifs
(ITIMs) which are responsible for the recruitment of intracellular inhibitory tyrosine
phosphatases [2]. The activating receptors expressed on NK cells are structurally very divergent
and only some of the ligands are known. Better-studied examples are NKG2D, which recognises
stress-inducible molecules such as MHC class I chain related protein A (MICA), or MICB and
natural cytotoxicity receptors (NCRs) that are sensitive for viral hemaglutinin or tumour
associated ligands [2, 14, 16, 17]. Non-covalently linked subunits that trigger kinases for
intracellular signalling are a common hallmark of the activation receptors [2]. This small extract
only indicates how complex the recognition process for NK cells is in order to decide whether
tolerance of the host tissue is appropriate or not.

1.1.3 NK cells and cancer
Many experimental studies have shown that NK cells can target tumour cells. For instance, it has
been reported that NK cells contribute to the eradication of experimentally induced or
spontaneously derived tumours and to the elimination of metastasising cells or small tumour
grafts [15]. Based on these facts, it has been suggested to use NK cells against human cancer. For this purpose, the overall strategy is to enhance the tumour recognition by NK cells. Proposed strategies are for example the stimulation of activating receptors via cytokines or the silencing of inhibitory receptors [15].

1.1.4 NK cell evasion by tumours
Tumour surveillance is facilitated by immune evasion mechanisms performed by tumour cells as well as cells in the tumour microenvironment [18]. Therefore, many strategies are developed by tumour cells such as the prevention of immune cell recognition, the development of resistance against apoptosis, the inhibition of immune cell development, proliferation, and maturation or the induction of immune tolerance. Well-studied protection mechanisms of tumour cells against NK cell cytotoxicity are alterations in MHC class I expression [19, 20], the down modulation of NKG2D expression due to MICA/B secretion but also the release of different growth factors inhibiting the expression of activating NK cell receptors [12, 21].

1.2 The platelet-derived growth factor family
In 1974 Ross and collaborators discovered a serum factor that was able to promote and stimulate the proliferation of smooth muscle cells to grow in culture [22]. In further investigations it was found that the growth promoting activity derived from blood platelets so that the factor was named after its origin: platelet derived growth factor (PDGF). PDGF, as one of the first identified growth factors, was then mainly characterised and purified by Heldin and colleagues over a time period of two decades (1979-1999). It has been long believed that the PDGF family only consists of two members: PDGF-A and PDGF-B [23-25]. However, in the years 2000 and 2001 a fundamental progress in understanding the impact of PDGF took place by discovering two new members: PDGF-C [26] and PDGF-D [27]. PDGFs classified in a subfamily within the PDGF/vascular endothelial growth factor (VEGF) super family. The main characteristic of the PDGF/VEGF super family is a cysteine-knot motif (growth factor domain). This motif consists of eight conserved cysteine residues that are located in the PDGF/VEGF homology regions [28, 29].

1.3 The PDGF system
1.3.1 PDGF ligands
The PDGF family consists of four different polypeptide chains, which can either assemble into disulphide-bonded homo- or heterodimers. So far, there are five different isoforms described:
PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD [23, 26, 27]. The typical growth factor domain (GFD) is important for the subunit dimerisation. Furthermore, the GFD is involved in the binding and activation of receptors triggered by the growth factors [30]. PDGF-A is encoded as two isoforms due to alternative splicing. The shorter and more abundantly expressed isoform is 196 amino acids long whereas the longer isoform consists of 211 amino acids [31]. PDGF-B is 241 amino acids in length [32]. PDGF-C exists only in one isoform of 345 amino acids whereas PDGF-D is synthesised as two isoforms with a length of 364 (isoform 2) and 370 (isoform 1) amino acids, respectively [27, 30]. The main structurally difference between the so-called classical (PDGF-A/B) and novel (PDGF-C/D) PDGFs is their amino (N)-terminal extension. The classical PDGFs possess a short N-terminal extension that undergoes intracellular proteolytic processing for activation [32]. In contrast, the PDGF-C and PDGF-D chains carry a longer N-terminal CUB domain (*figure 2*) [26, 27].

**CUB** stands for the initial letters of the first three identified proteins containing this domain, namely complement subcomponents C1r/C1s, urchin EGF-like protein and bone morphogenic protein-1. The domain is an evolutionary conserved protein domain of approximately 110 residues and found on different extracellular proteins. According to the literature the CUB domain plays a role in protein-protein as well as protein-carbohydrate interactions [33, 34]. In the novel PDGFs the CUB domain is separated from the GFD via an accumulation of amino acids, a so-called hinge region [26, 27]. By now, it is known that the CUB domain blocks the binding of the growth factors to their receptors. Therefore, a proteolytic cleavage is necessary in order to produce functional PDGF-CC and PDGF-DD (further described in section 1.3). After maturation, PDGF-A and PDGF-B monomers have a molecular weight of 15 kilo Dalton (kDa) [23] and PDGF-C and PDGF-D molecules weight around 50-55 kDa. However, after their proteolytic cleavage, the novel PDGF monomers consist of a molecular weight of 20 kDa [26, 27, 30].
1.3.2 PDGF receptors

The five dimeric isoforms: PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD are capable to activate and stimulate two structurally related tyrosine kinase receptors: the PDGF receptor alpha (PDGFR-α) and the PDGF receptor beta (PDGFR-β) [35] (figure 3). The PDGFR-α is activated and bound by the isoforms PDGF-AA, PDGF-BB, PDGF-AB and PDGF-CC [36]. In contrast, only PDGF-BB and PDGF-DD bind to the PDGFR-β [27, 36]. It is notable that PDGF-AB, PDGF-BB and PDGF-CC can also trigger a heterodimeric PDGFRα/β complex [30, 37].

1.3.3 Structure and signal pathways of PDGF receptors

The polypeptide chains of the receptors contain 1089 (PDGFR-α) and 1106 (PDGFR-β) amino acids and after their maturation, the receptors are expressed on the cell surface. PDGFR-α consists of a molecular weight of 170 kDa while PDGFR-β has a molecular size of 180 kDa. Both PDGF receptors are separated into three parts: an extracellular part composed of five immunoglobulin (Ig) -like domains, a transmembrane domain and an intracellular split tyrosine kinase domain [38]. The five Ig-like domains within the extracellular part of the receptors take over different functions. The three N-terminal Ig-like domains play a role in ligand binding and the fourth Ig-like domain stabilises the receptor-receptor complex. However, the function of the fifth domain has not been discovered yet [39]. The receptor activation occurs due to ligand-induced dimerisation. In order to create a stable receptor dimer, the PDGF isoforms bind to two receptor molecules simultaneously. This is possible since all isoforms are dimeric and contain two binding epitopes, respectively. Receptor dimerisation induces a stepwise autophosphorylation of the intracellular tyrosine kinase residues. Once activated, these residues act as a binding site for different intracellular binding proteins, which initiate a cascade of many intracellular events [40, 41]. Based on the literature, members of the sarcoma (Src) family kinases, phosphatidylinositol 3’-kinase (PI3K) and phospholipase C (PLCγ) are involved in the downstream signalling of PDGF receptors [42]. These pathways trigger responses such as migration, cell proliferation and differentiation. Importantly, only the PDGFR-β seems to be involved in anti-apoptotic signalling pathways [39].
1 Introduction

Figure 3: The PDGF system. The PDGF polypeptide chains assemble to five homo- or heterodimeric ligands. PDGFR-α is bound by PDGF-AA and PDGF-CC whereas PDGF-DD signals through PDGFR-β. PDGF-BB and PDGF-AB exhibit a binding affinity for both receptors. Following receptor dimerisation, a stepwise autophosphorylation of intracellular tyrosine kinases is induced (adapted from [43]).

1.4 Regulation of PDGF activity

As previously mentioned the members of the PDGF family need to be proteolytically processed in order to mature and become functional. In many proteins proteolytic activation is an important regulatory step and performed by proteases. The PDGF family members perform their proteolytic activation in two different ways: the classical PDGFs undergo intracellular proteolytic activation whereas the novel PDGFs are extracellular activated [26, 27, 30, 32].

1.4.1 Activation of classical PDGF ligands

The intracellular proteolytic activation of PDGF-AA and PDGF-BB occurs in their N-terminus. The classical PDGFs are primarily synthesised as pro-PDGF-AA and pro-PDGF-BB. Next, the PDGF precursors dimerise in the endoplasmatic reticulum (ER) and are transferred through the Golgi apparatus towards the Golgi network. Here, the dimers are proteolytically cleaved and the activated ligands reach the cell surface via vesicles before they are released by exocytosis [32]. However, the responsible protease was long unknown. In the early 2000s, furin, a dibasic-specific proprotein convertase (PC), was associated with the processing of PDGF-AA and PDGF-BB at isoform specific amino acid residues [44, 45]. Also other members of the PC family (PC5A, PACE4, PC7) were able to mature the classical PDGFs but to a much lesser extent [43].
Figure 4: The PDGF system The classical PDGF-AA and PDGF-BB are processed by the proprotein convertase furin in the Golgi network while activation of the novel PDGFs, PDGF-CC and PDGF-DD, occurs extracellularly by tPA and uPA, respectively. The arrows indicate the ligand-specific cleavage sites (amino acid sequence of this region is illustrated after the name of each PDGF isoform) (adapted from [43]).

1.4.2 Activation of novel PDGF ligands

The extracellular processing of the novel PDGFs can be performed by the protease plasmin in vitro [37]. However, it is very likely that plasmin is not the physiologically relevant activator due to its broad substrate specificity. Instead, in 2004 and 2005, studies on the novel PDGFs identified a plasminogen activator (tPA) as an activator of PDGF-CC while PDGF-DD is activated by a urokinase plasminogen activator (uPA). The highly specific enzymes belong to the serine protease family and activate plasminogen into plasmin [37, 46]. PDGF-CC and PDGF-DD are cleaved at a growth factor specific amino acid residue within their hinge region so that functional PDGF-CC and PDGF-DD can be released [26, 27] (figure 4).

1.5 Expression pattern of PDGF ligands and receptors

The four PDGF genes are widely expressed in human and murine tissues. Interestingly, each chain displays a unique expression pattern [37]. However, there are also organs were all four PDGF mRNAs are expressed such as heart and pancreas [37]. PDGFR-α and mainly PDGFR-β expression is known on fibroblasts and smooth muscle cells. In addition, PDGFR-α is individually expressed on human platelets, liver endothelial cells and astrocytes while PDGFR-β is located on myoblasts, pericytes and macrophages [23]. Notably, PDGF ligands and their receptors are not necessarily expressed in an overlapping pattern. This leads to the conclusion that PDGFs act by both paracrine and autocrine signalling [47, 48].
1.6 Physiological and pathological functions of PDGF

Today, experimental investigations support a multifunctional effect of PDGF during development, adulthood but also pathogenesis. Studies in mice show that inactivation of single genes in the PDGF system result in severe abnormalities such as kidney deficiencies or defects in blood vessel formation [49-51]. Many abnormalities prevented a postnatal survival of mice. During adulthood PDGF has been suggested to stimulate wound healing and the maintenance of the interstitial fluid pressure [52, 53]. However, a dysregulated PDGF system is associated with disorders of excess cell proliferation such as fibrosis, atherosclerosis and certain malignancies [54-56]. In cancer, the PDGF system is associated with several tumourigenic and angiogenic processes caused by both autocrine and paracrine growth stimulation [57]. This is why the inhibition of the PDGF signalling, especially the blocking of the PDGF receptors, became a more and more interesting therapeutic target in order to combat tumour cells [58].

In 2009, a collaborating group published that murine PDGF-C transfected B16 melanoma cells grow more aggressively in vivo compared to mock-transfected B16 melanoma cells [47]. The observation has been attributed to a higher degree of vascularisation of the PDGF-CC expressing tumour. As a possible mechanism, the authors suggested that paracrine secreted PDGF-CC binds to the PDGFR-α of cancer-associated fibroblast, which leads to an increased recruitment of fibroblast into the tumour stroma. Since B16 cells are known to be rejected by NK cells in vivo [59], my group raised the hypothesis that PDGF-CC might additionally suppress functions of the innate immune system, which may further explain the findings of our collaborators. The hypothesis is supported by recent findings that melanoma-associated fibroblasts may modulate and inhibit NK cell function by secreting growth factors or hormones [60]. Furthermore, preliminary experiments in my group showed that NK cells cocultured with B16/PDGF-C were less cytotoxic compared to NK cells cocultured with normal B16 cells (H. Tufvesson, unpublished observation). This observation indicates an inhibitory effect of PDGF-CC directly on NK cells.

1.7 Aims

The present study aims to:

- verify the preliminary experiments in different experimental set-ups
- determine the mechanism behind the observed suppression of NK cell cytotoxicity
All investigations are performed *in vitro*. To further investigate and to verify the observed immunosuppressive effect of PDGF-CC on NK cells, two Balb/c mice derived breast cancer cell lines (TUBO and D2F2) are chosen. Porcine aortic endothelial (PAE) cells, stably transfected with the PDGFR-α or the PDGFR-β, are used for determining the mechanism of suppression. NK cell cytotoxicity is analysed within different $^{51}$Cr-release assays, which measure the release of radioactive chromium from target cells as a result of killer cell activity. The “gold standard” NK target is the lymphoma cell line YAC-1 that was originally used to define NK cells [1].
2 Material and Methods

2.1 Reagents
All chemicals were purchased from Sigma-Aldrich (Germany) unless otherwise specified. Recombinant murine (rm) PDGF-BB was obtained from PeproTech (United Kingdom) and recombinant human interleukin 2 (rhIL-2) from Prometheus Therapeutics and Diagnostics (USA). The antibodies were purchased from the following sources and used at the dilutions recommended by the manufactures: anti phospho-tyrosine mouse monoclonal antibody (mAb P-Tyr-100); anti PDGF receptor alpha (PDGFR-α) and anti PDGFR beta (PDGFR-β) polyclonal antibodies; anti mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP-linked); anti rabbit IgG, HRP-linked antibody (Cell Signaling Technology, USA); Alexa Fluor® (AF) 488 chicken anti rabbit IgG (H+L) (Invitrogen, USA); phycoerythrin (PE) anti mouse CD8a (Ly-2), PE mouse anti mouse H-2 Kd, PE anti mouse H-2 Dd (BD Biosciences, USA).

2.2 Media
All media (IMDM, DMEM, DMEM/F-12 (1:1) and RPMI) and their supplements were purchased from GIBCO® (Invitrogen, USA). Splenocytes were cultured in complete RPMI medium containing MEM-NEAA (100x) non-essential amino acids, 100 mM sodium pyruvate and 50 mM 2-mercaptoethanol. Complete cell culture media were supplemented with 10 % heat-inactivated (30 min, 56°C) fetal bovine serum (FBS) and penicillin (100 U/ml)/streptomycin (100 µg/ml). The complete media of transfected cell lines were additionally supplemented with Zeocin™, a copper-chelated glycopeptide antibiotic produced by Streptomyces CL990 (Invitrogen, USA).

2.3 Cell lines
All cell lines were cultured in a humidified incubator at 37°C and 5 % CO₂. Porcine aortic endothelial (PAE) cells were kindly provided by A. Östman (Karolinska Institute, Sweden) TUBO cells were provided by Guido Forni (University of Torino, Italy) and D2F2 cells by Wei-Zen Wei (Wayne State University, USA). The YAC-1 cell line was purchased from ATCC.
Table 1: Descriptions of cell lines used during project:
*1: 100 µg/ml Zeocin; *2: 150 µg/ml Zeocin; *3: 250 µg/ml Zeocin

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Background</th>
<th>Type</th>
<th>Growth properties</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2F2/mock*1</td>
<td>Balb/c</td>
<td>breast cancer cell</td>
<td>adherent</td>
<td>IMDM</td>
</tr>
<tr>
<td>D2F2-PDGF/-C*1</td>
<td>Balb/c</td>
<td>breast cancer cell</td>
<td>adherent</td>
<td>IMDM</td>
</tr>
<tr>
<td>TUBO/mock*2</td>
<td>Balb-neu T</td>
<td>breast cancer cell</td>
<td>adherent</td>
<td>IMDM</td>
</tr>
<tr>
<td>TUBO-PDGF/-C*2</td>
<td>Balb-neu T</td>
<td>breast cancer cell</td>
<td>adherent</td>
<td>IMDM</td>
</tr>
<tr>
<td>PAE alpha</td>
<td>Pig</td>
<td>aortic endothelial cell</td>
<td>adherent</td>
<td>DMEM/F-12</td>
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<tr>
<td>PAE beta</td>
<td>Pig</td>
<td>aortic endothelial cell</td>
<td>adherent</td>
<td>DMEM/F-12</td>
</tr>
<tr>
<td>YAC-1</td>
<td>C57B1/6</td>
<td>lymphoblast cell</td>
<td>suspension</td>
<td>RPMI</td>
</tr>
</tbody>
</table>

2.4 Generation of cell lines stably expressing PDGFC

The pcDNA™3.1/Zeo-PDGFC vector and control plasmid were kindly provided by K. Pietras laboratory at the Karolinska Institute, Sweden.

2.4.1 Transforming competent cells

The PDGFC expression plasmid as well as an empty control plasmid were transformed into Doug Hanahan five alpha (DH5α) E.coli host cells using a Library Efficiency® DH5® Kit (Invitrogen, USA) according to the manufacturer’s protocol. A vial of competent cells treated with phosphate buffered saline (PBS) served as negative control. Fifty µl from each transformation vial were spread out on standard Luria Bertani (LB) plates containing 50 µg/ml ampicillin. Inverted plates were incubated over night at 37°C.

2.4.2 Plasmid DNA purification

A single colony was picked from both LB plates and used to inoculate two starter cultures of 4 ml LB medium containing 50 µg/ml ampicillin. Cultures were incubated for 6 h at 37°C with vigorous shaking. Next, 2 ml of each starter culture were diluted in 500 ml selective LB medium. Bacteria were grown over night with gentle shaking at 37°C. Following incubation the bacterial cells were harvested by centrifugation at 6000 g for 15 min at 4°C. All further purification steps were performed by using a QIAGEN® EndoFree Plasmid Purification Kit according to the manufacturer’s directions. DNA was dissolved in PBS and the concentration was measured using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA).

2.4.3 Transfection of TUBO cells and D2F2 cells using Lipofectamine™ 2000

For the generation of stably expressing cell lines, D2F2 and TUBO cells were transfected with pcDNA™3.1/Zeo-PDGFC plasmid (D2F2/PDGF-C; TUBO/PDGF-C) or pcDNA™3.1/Zeo-
empty vector (D2F2/mock; TUBO/mock) using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer’s directions. Pre transfection, four 10 cm cell culture plates were coated with 5 ml of 0.02 % gelantine. Next, 2×10⁶ cells of each cell line were seeded out and cultured in antibiotic-free IMDM medium. After 24 h, cells were washed with PBS and then incubated for 3 h at 37°C with 4.5 ml Opti-MEM®-I (GIBCO®, Invitrogen, USA) medium. Following incubation, TUBO cells were transfected with 4 µg PDGF-C plasmid DNA and empty vector DNA, respectively. The transfection of D2F2 cells was performed with 8 µg PDGF-C plasmid DNA and empty vector DNA, respectively. In both assays the DNA was diluted in 3 ml Opti-MEM®-I medium containing 60 µl Lipofectamine™ 2000 and incubated for 3 h at 37°C. Finally, 7.5 ml of IMDM containing 20 % FBS were added to all transfection assays. Post transfection (24 h) 100µg/ml and 150µg/ml Zeocin were added to the D2F2 and TUBO cultures, respectively, for selection of resistant cells. The mass cultures were tested for PDGF-CC expression via PCR and western blot.

2.4.4 Zeocin™ Selection in Mammalian cells
Two hundred thousand TUBO and D2F2 cells were plated out in two 6-well plates, respectively. After 24 hours the cell medium was removed and new medium with varying concentrations of Zeocin™ (0, 50, 100, 150, 200, 250, 500, 1000, 1500 and 2000 µg/ml) were added to each well. The selective medium was replenished every 3-4 days and the percentage of surviving cells was observed over time.

2.5 Verification of PDGFC gene expression: PCR
2.5.1 RNA isolation
The expression of PDGFC was verified by PCR. Total RNA was extracted from whole cell lysates of D2F2/PDGF-C and D2F2/mock as well as TUBO/PDGF-C and TUBO/mock using an RNeasy® Mini Kit (Qiagen, Sweden). Around 1×10⁶ cells of each cell line were harvested from 6-well plates, transferred to 1.5 ml RNase free microcentrifuge tubes (Eppendorf, Germany) and centrifuged at 400 g for 4 min. The medium was aspirated and the cells were washed with 500 µl PBS. All further steps were performed following the manufacturer’s directions. RNA samples were resuspended in 30 µl RNase free water (Qiagen, Sweden) and the RNA concentration was measured using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA).
2.5.2 DNA digestion using Deoxyribonuclease I (DNase I)
All RNA samples (1 µg) were treated with DNase I, Amplification Grade (Invitrogen, USA) in order to eliminate single-and double stranded DNA. The digestion was done according to the manufacturer’s protocol.

2.5.3 First cDNA synthesis
For the synthesis of first strand cDNA from all total RNA templates a First Strand cDNA Synthesis Kit (Fermentas, Canada) was used following the manufacturer’s directions. The synthesis was performed on 1 µg of each DNase treated RNA sample with random hexamer primers. The cDNA concentration was measured using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA).

2.5.4 Polymerase chain reaction (PCR)
PCR was performed using a primer set specific for PDGF-C (sense 5’ -AGC TGA CAT TTG ATG AGA GAT- 3’, antisense 5’ -AGTAGG TGA AAT AAG AGG TGA ACA- 3’). The primer set was purchased from Invitrogen, USA. A total of 4 µg of cDNA were applied to 5 µl DreamTaq Green Master Mix (Fermentas, Canada) and 0.5 µl (20 µM) of sense and antisense primer, respectively. Finally, all PCR samples were adjusted to a total volume of 10 µl by using purified and deionised water. The amplification of the cDNA template samples was then performed with a conventional programmable thermal cycler (BIO-RAD, USA): denaturation for 5 min at 95°C, followed by 35 PCR cycles of denaturation of 95°C for 30 sec, annealing at 56°C for 30 sec and extension at 72 ºC for 45 sec. The PCR was terminated at 72°C for 5 min and finally forever (∞) at 10°C.

2.5.5 Agarose gel electrophoresis
The analysis of the PCR products was performed via agarose gel electrophoresis using a MINI-SUB® Cell GT System (BIO-RAD, USA). Therefore, 2 % Agarose Type I were diluted in Tris-acetate-EDTA buffer (40 mM Tris acetate, 1 mM EDTA, pH 8) and stained by 0.001 % ethidium bromide. The gel ran for 45 min at 70 V and bands were visualised in a Gel Doc EZ® (BIO-RAD). The size of the PCR products was verified by TrackIt™ 1 Kb DNA ladder (Invitrogen, USA).
2.6 In vitro proliferation assay
Two hundred thousand TUBO/PDGF-C or TUBO/mock cells were seeded out in triplicates in 6-well plates. Cell numbers were subsequently estimated using a Countess™ automated cell counter (Invitrogen, USA) 24, 48 and 72 h after seeding.

2.7 Western blot for PDGF-CC secretion in transfected cell lines
To monitor PDGF-CC secretion receptor-activation experiments were performed using PAE cells with stable expression of PDGFR-α (PAE/PDGFR-α).

2.7.1 Protein isolation and detection of protein concentration
PAE cells were cultured with serum-free medium over night in six 10 cm culture plates. Further, cells were stimulated with 10 ml conditional medium from D2F2/PDGF-C and D2F2/mock as well as TUBO/PDGF-C and TUBO/mock cells for 10 min at 37°C, respectively. Recombinant murine PDGF-BB (50 ng/ml) and PBS were used as positive and negative controls. The cells were washed with 5 ml PBS and lysed with 700 µl cell lyses buffer (Cell Lytic M containing 1 % protease inhibitor cocktail, 1 % phosphatase inhibitor cocktail II) for 25 min on ice. In order to remove cell debris, the cells were transferred to 1.5 ml Eppendorf tubes and centrifuged for 15 min (20000 g) at 4°C. The supernatant was transferred to new Eppendorf tubes and the total protein concentration was measured using a BCA™ Protein Assay Kit (Thermo Scientific, USA) according to the manufacturer’s directions. Total protein (250 µg) of each sample was incubated for 1 hour with lectin-conjugated agarose beads from Triticum vulgaris with gentle rotation at 4°C. Afterwards, beads were centrifuged for 1 min (15000 g) and 3 times washed with ice-cold PBS to remove unbound proteins. Finally, all samples were collected in 10 µl sample buffer (5 µl NuPage® LDS sample buffer (4x stock), 2 µl NuPage® Sample reducing agent (10x stock), 3 µl MQ-H₂O) (Invitrogen, USA) and protein denaturation was performed at 95°C for 5 min.

2.7.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)
The denaturated samples were cooled down to room temperature (RT) and loaded (10 µl) to a 4-12 % NuPage® Bis-Tris Gel (Invitrogen, USA). Protein size was detected using 3 µl of Spectra™ Multicolour Broad Range Protein Ladder (Fermentas, USA). Proteins were separated in a SDS chamber (XCell Sure Lock™) (Invitrogen, USA) filled with 1x NuPage® MOPS SDS Running Buffer for 60 min at 180-200 V.
2.7.3 Western blot analysis
Following electrophoresis, proteins were transferred via a “wet” blot transfer technique from the gel to a 0.45 μm PVDF membrane (Millipore, USA). Therefore, the western blot transfer apparatus (XCell Sure Lock™) (Invitrogen, USA) was assembled (2 sponges, filter paper, gel, PVDF membrane, filter paper, 3 sponges) and filled with transfer buffer (85 % MQ-H2O, 5 % 20x NuPage® Transfer Buffer, 10 % methanol, 1 % NuPage® Antioxidant). Previously, the PVDF membrane was activated for 30 sec in methanol. Blotting was performed at 40 V for 3-5 h.

2.7.4 Detection
The nonspecific binding of the detection antibodies was prevented by blocking the membrane with 2 % dry milk powder solution diluted in wash buffer (10 % PBS, 90 % MQ-H2O, 0.05 % Tween20) for 30 min at RT. The membrane was incubated with the primary antibody against phospho-tyrosine over night (ON) at 4°C (1:1000 diluted in 2 % milk powder solution). Following incubation, the membrane was washed at least twice for 10 min with wash buffer. The anti mouse IgG secondary antibody (1:2000 diluted in 2 % milk powder solution) was applied for 1 h at RT. Finally, the membrane was three times washed and incubated with 1 ml Pierce® ECL western blotting Substrate (Thermo Scientific, USA). Chemiluminescent images were analysed in a LAS-1000 system (Fujifilm, Japan).

2.8 Western blot for PDGFR-α expression and activation
The induction of PDGFR-α phosphorylation and detection of PDGFR-α were carried out in TUBO/PDGF-C and TUBO/mock cells. Duplicates of 2×10^5 cells of each group were seeded out in 6-well plates and cultured over night in serum-free medium. One of each duplicate was treated by rmPDGF-BB (50ng/ml) or PBS for 5 min at 37°C. All further steps were performed as described in 2.7. In the first instance, the membrane was incubated with the antibody against phospho-tyrosine. For re-probing the same membrane with the antibody against PDGFR-α (1:1000 diluted in 2 % milk powder solution), the membrane was stripped with 0.4 M sodium hydroxide (NaOH) for 10 min at RT, washed three times with wash buffer and blocked with 2 % dry milk powder solution as previously described. Anti rabbit IgG was applied as secondary antibody (1:2000 diluted in 2 % milk powder solution).
2.9 Western blot for PDGFR-α activation on NK cells

Murine (C57B1/6) NK cells were purified as described below (2.10 and 2.11) and either activated with 500 U/ml IL-2 for 24 h or only cultured in complete splenocyte medium supplemented with 20 U/ml IL-2. For the western blot, NK cells were collected in Eppendorf tubes. Activated and non-activated NK cells were divided into two groups whereof one was incubated with rmPDGF-BB (50ng/ml) or with PBS for 5 min at 37°C, respectively. The suspension cells were centrifuged (400 g, 4 min) and the pellet was washed with 1 ml PBS. All further steps were performed as described in 2.7. The membrane was incubated with the antibody against phospho-tyrosine.

2.10 Isolation of murine splenocytes

C57B1/6 or Balb/c mice were sacrificed by CO₂ asphyxia. Spleens were extracted and a single cell suspension was prepared. One to two spleens were grinded with a sterile 1 ml syringe plunger through a 70 µm cell strainer into a 50 ml tube. The strainer was rinsed with 15-20 ml of complete RPMI medium specific for splenocytes. After the first washing step (centrifugation at 400 g, 4 min) red blood cells were lysed within 5 min using 3 ml 1x BD Pharm Lyse™ Lysing Buffer (BD Bioscience, USA). The lysis was stopped with 30 ml splenocyte medium. The remaining splenocytes were washed in two additional steps (centrifugation at 400 g, 4 min) and finally resuspended in 5 ml splenocyte medium. The cell number was determined by staining the cells in 0.4 % trypan blue (Invitrogen, USA) using Fast Read 102™ disposable counting slides (Immune Systems, United Kingdom).

2.11 Immunomagnetic bead purification of NK cells

NK cells were purified from fresh splenocytes by negative selection using a magnetic activated cell sorting (MACS) NK-cell isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions. To this end, 1×10⁷ splenocytes were resuspended in 40 µl MACS buffer (PBS, 0.5 % bovine serum albumin (BSA), 2 mM ethylenediamintetraacetic acid (EDTA), pH 7.2). Non NK-cells such as T cells, dendritic cells, B cells, or macrophages were indirectly labelled by using a cocktail of biotin-conjugated antibodies (10 µl per 1×10⁷ cells) against CD4, CD8a, CD5 or CD19 and incubated for 10 min at 4°C. Afterwards, 30 µl of MACS buffer and 20 µl Anti-Biotin MicroBeads per 1×10⁷ were added and incubated for another 15 min at 4°C. Cells were washed by adding 1 ml of MACS buffer per 10⁷ cells and centrifuged for 4 min at 400 g. Up to 10⁹ cells were resuspended in 500 µl MACS buffer and applied to a pre-cooled and pre-washed (3 ml
MACS buffer) column that was previously placed in a magnetic field. The entire effluent (mainly NK-cells) was collected. Cells were centrifuged (400 g, 4 min) and resuspended in complete RPMI medium (1 ml per 1×10⁶).

2.12 Coculture experiments and rmPDGF-BB treatment of NK cells

2.12.1 Splenocyte/NK-cell and tumour cell coculture

Isolated splenocytes or purified NK cells (2×10⁶ cells/1 ml complete medium) were stimulated with 500 U/ml IL-2 for 24 h. Thereafter, splenocytes or NK-cells and tumour cells (TUBO/PDGF-C or TUBO/mock) were cocultured (ratio 6:1, 2:1, respectively) in complete splenocyte medium supplemented with 20 U/ml IL-2. After 16 hours, splenocytes or NK cells were separated and tested for cytotoxicity against YAC-1 cells.

2.12.2 Recombinant mPDGF-BB treatment of NK cells

Purified NK-cells were stimulated as described above. After 24 h cells were cocultured with four different rmPDGF-BB concentrations (0, 25, 50 and 100 ng/ml), respectively. After 16 hours, NK cells were tested for cytotoxicity against YAC-1 cells.

2.13 Cytotoxicity assay

2.13.1 Cytotoxicity of splenocytes/NK cells after coculture with tumour cells or rmPDGF-BB

Splenocyte and NK cell cytotoxicity was measured by using a standard chromium (⁵¹Cr)-release assay. YAC-1 target cells (1×10⁶ cells/ 200 µl RPMI) were incubated in a 15 ml falcon tube for 1 h at 37 °C with 100 µCi of ⁵¹Cr (Perkin Elmer, USA). The labelled cells were then washed three times with complete medium and adjusted to a concentration of 8×10⁴ cells/ml. During the incubation of the target cells, effector splenocytes or NK-cells were purified from the cancer cell coculture by transferring the complete medium containing splenocytes or NK-cells into 15 ml falcon tubes. NK-cells cocultured with rmPDGF-BB were simply transferred to 15 ml falcon tubes. Thereafter, cells were counted, pelleted (400 g, 4 min) and diluted in complete splenocyte medium in order to get ratios of 200:1, 100:1 and 50:1 (spleocytes) and 40:1, 20:1 and 10:1 (NK-cells), respectively. Triplicates of serial dilutions of effector cells (50 µl/well) were then applied to a 96-well V-bottomed culture plate. Aliquots of ⁵¹Cr-labeled target cells (50 µl/well) were dispensed in wells containing effector cells. The plates were incubated for 4.5 h at 37 °C. After the incubation, the plates were centrifuged (400 g, 4 min) and 25 µl aliquots of the
supernatants from each well were transferred to Luma Plates™-96 (Perkin Elmer, USA). Radioactivity was measured using a 1450 MicroBeta® TriLux Microplate Scintillation and Luminescence Counter (Perkin Elmer, USA). The spontaneous release was detected by incubating the target cells with 50 μl complete medium and the total release was determined by incubating the target cells with 0.5 % Triton X-100. The specific cytotoxicity was calculated as (experimental release – spontaneous release)/(total release – spontaneous release) × 100.

2.13.2 Cytotoxicity of NK cells using TUBO/mock or TUBO/PDGF-C as targets
TUBO/mock or TUBO/PDGF-C targets were labelled as described in 2.13.1. After being diluted as 4×10^4 cells/ml, triplicates of cells were dispensed as 100 μl per well into U-bottomed 96-well plates. TUBO/mock and TUBO/PDGF-C targets were allowed to adhere for 1-2 h at 37 °C. Activated (500 U/ml IL-2 for 24 h) effector NK cells were then added (ratios: 40:1, 20:1, 10:1; 100 μl/well) and coincubated with the targets overnight. YAC-1 cells were used as positive controls. Data analysis was performed as described in 2.13.1.

2.14 Immunofluorescence (IF) staining for PDGFR-α/β expression on NK cells
Purified and activated (500 U/ml IL-2 for 24 h) NK-cells (1×10^6 cells/ml) were fixed with 4 % formaldehyde for 20 min at RT. Following incubation, cells were centrifuged (400 g, 4 min) and resuspended in 1 ml MQ-H_2O. After another centrifugation step (400 g, 4 min) cells were collected in 200 μl MQ-H_2O. Five μl of the cell suspension were added to gelantin-coated (7.5 % in MQ-H_2O) object slides (MENZEL-GLÄSER, Germany) and the remaining liquid was evaporated by heat. NK cells were then encircled with a Dako Pen S2002 (DAKO, Denmark). PDGFR-α/β expressing PAE cells were used as positive controls. PAE cells (5×10^4/500 ml) were seeded out in BD Falcon™ Culture Slides (BD Bioscience, Belgium) and cultured over night in serum-free medium. Cell fixation was performed with 4 % paraformaldehyde for 15 min at RT. The fixed NK-and PDGFR-α/β expressing PAE cells were washed with PBS (2x, 5 min) and permeabilised with 0.1 % Triton X-100 for 2 min at RT. Before staining, cells were blocked with 5 % BSA (in PBS) for 1 h at RT. Primary antibodies (1:100 in 5 % BSA) against PDGFR-α or PDGFR-β were applied to all cells and incubated over night at 4°C. Following incubation, cells were rinsed in PBS (3x, 5 min) and incubated with the secondary antibody (1:100 in 5 % BSA) against rIgG-AF488 (H+L) for 1 h at RT. Finally, PBS-washed cells (2x, 5 min) were coated with Vectashield® Mounting Medium with DAPI V1005 (USA) and mounted with coverslips. Cells
were visualised by using a Zeiss (Axioplan 2 imaging) fluorescence microscope with appropriate filter sets (PDGFR-α/β by FITC green, cell nuclei by DAPI) according to the manufacturer’s directions.

2.15 Flow cytometry

Flow cytometry analysis was used to investigate the expression of murine MHC class I molecules on TUBO/mock or TUBO/PDGF-C cells. For each staining, $2.5 \times 10^4$ versene (Invitrogen, USA) detached cells were transferred into V-bottomed 96-well plates and washed with 200 µl flow cytometry buffer (PBS, 1 % FBS) for 4 min at 400 g. Supernatants were discarded and TUBO/mock or TUBO/PDGF-C cells were incubated with PE-labelled H-2 Dd and H-2 Kd specific antibodies (1:10 in flow cytometry buffer), respectively, as well as a PE-labelled IgG2a isotope control (1:10 in flow cytometry buffer) for 20 min at 4 °C. Following staining, cells were twice washed with 200 µl flow cytometry buffer. H-2 Dd and H-2 Kd expression was monitored in a BD Calibur flow cytometer combined with the corresponding BD CellQuest Pro Software (Biosciences, USA). Data analysis was performed with the flow cytometry analysis software FlowJo (6.4.2).
3 Results

The preliminary experiments of my group showed that NK cells cocultured with PDGF-CC expressing B16 melanoma cells were less cytotoxic compared to NK cells cocultured with normal B16 cells (H. Tufvesson, unpublished results). To investigate if the observed immunosuppressive effect of PDGF-CC on NK cells can be extended to other types of tumours, two Balb/c mice derived breast cancer cell lines (TUBO and D2F2) were chosen. Breast cancer is the most prevalent cancer type in women and therefore clinically highly relevant [61].

3.1 Establishment of TUBO and D2F2 cell lines expressing PDGFC

To study the effect of PDGF-CC on NK cells and splenocytes, TUBO and D2F2 cells were transfected with control plasmid or a plasmid encoding full-length PDGF-C. Following selection, mass cultures were firstly examined with regard to PDGFC gene expression. Therefore, RNA from all cells was isolated and analysed by reverse transcription-PCR. A PCR product of the expected size of 345 bp was detected in the TUBO cells (figure 5; lane 1) as well as in the D2F2 cells (figure 5; lane 3) transfected with PDGFC. TUBO and D2F2 cells transfected with the control plasmid (figure 5; lane 2,4) were found to be PDGFC negative. This indicates that both breast cancer cell lines are successfully transfected on the gene expression level.

![Figure 5: Establishment of TUBO and D2F2 cell lines expressing PDGFC. Total RNA was isolated from TUBO/PDGFC-C, TUBO/mock, D2F2/PDGFC-C and D2F2/mock cells, respectively, followed by cDNA synthesis and PCR amplification with PDGFC-specific primers. Amplification products from TUBO/PDGFC-C (lane 1), TUBO/mock (lane 2), D2F2/PDGFC-C (lane 3) and D2F2/mock cells (lane 4) were separated by agarose gel electrophoresis. A PCR product of 345 bp was detected in both PDGFC-transfected cell lines.](image)
3.2 PDGF-CC secretion of TUBO/PDGF-C and D2F2/PDGF-C transfected cells

To investigate the production of functional PDGF-CC the conditioned media of both transfected cell lines were collected and analysed pertaining to their capacity to activate the PDGFR-α of PAE cells. After stimulation, PAE cells were lysed and the phosphorylation of the PDGFR-α was investigated by performing western blot experiments using an antibody against phospho-tyrosine.

Figure 6: PDGF-CC secretion of TUBO/PDGF-C and D2F2/PDGF-C transfected cells. Serum starved PAE/PDGFR-α cells were stimulated with conditioned media of TUBO/PDGF-C (lane 3), TUBO/mock (lane 4), D2F2/PDGF-C (lane 5) and D2F2/mock (lane 6) cells. Murine rPDGF-BB (lane 1) and PBS (lane 2) were used as positive and negative control, respectively. PAE/PDGFR-α cells were lysed and proteins were separated via SDS PAGE. The phosphorylation of the PDGFR-α was determined by western blot. The conditioned medium of the TUBO/PDGF-C (lane 3) could stimulate the PDGFR-α.

As shown in figure 6, the conditioned medium of TUBO/PDGF-C (lane 3) cells induced a week but significant phosphorylation of a tyrosine kinase with a protein size of 170 kDa. The position of phosphorylation corresponded to the position of the activated tyrosine kinase of the PDGFR-α in the control cells (lane 1). In contrast, the conditioned medium of D2F2/PDGF-C cells (lane 5) caused no stimulating effect on the PDGFR-α. These results indicate that the TUBO/PDGF-C cells but not the D2F2/PDGF-C cells produce functional PDGF-CC. Since the production of functional PDGF-CC was required for the subsequent experiments, D2F2 cells were excluded from further investigations.

3.3 In vitro proliferation assay

The transfected TUBO cells were further characterised via a proliferation assay where the in vitro growth rate of the TUBO/PDGF-C cells was compared to the mock-transfected cells. This was a necessary pre-experiment before in vivo growth can be studied (ongoing experiment). Triplicates of TUBO/PDGF-C and TUBO/mock cells were seeded out and counted one, two and three days after seeding. Mean and standard deviation (SD) of the triplicates were calculated as illustrated in figure 7. The data shows that there is no difference in the in vitro growth rate between TUBO/PDGF-C and TUBO/mock cells (figure 7).
3 Results

Figure 7: *In vitro proliferation assay.* TUBO/mock (*diamonds*) and TUBO/PDGF-C (*circles*) cells were seeded out in 6-well plates. At the indicated times, cells were trypsinised and estimated with a Countess™ automated cell counter. Results are presented as mean ± SD of triplicate measurements. TUBO/PDGF-C and TUBO/mock cells show no difference in their *in vitro* growth.

3.4 TUBO/mock and TUBO/PDGF-C cells do not express PDGFR-α

The transfected cells were also analysed with regard to PDGFR-α expression. For this purpose duplicates of TUBO/mock and TUBO/PDGF-C cells were either treated with 50 ng/ml rmPDGF-BB or with PBS. PDGFR-α expressing PAE cells were used as positive control. After stimulation, western blot experiments were performed to elucidate the activation of phospho-tyrosine and the expression of PDGFR-α in TUBO/mock and TUBO/PDGF-C cells (*figure 8*).

As shown in *figure 8*, the TUBO cell line did not possess an activated phospho-tyrosine kinase (*lane 3-6, top*) corresponding to the protein size of the phospho-tyrosine kinase of the PDGFR-α (170 kDa) (*lane 1, top*). The PDGFR-α was also not expressed on TUBO/mock and TUBO/PDGF-C cells (*lane 3-6, bottom*) when compared to the positive control (*lane 1, bottom*). This result further indicates that the expression of PDGF-CC does not affect the *in vitro* growth rate of TUBO/PDGF-C cells since autocrine growth stimulation can be excluded due to the absence of PDGFR-α on the cell surface.
The expression of PDGFR-α in TUBO/mock and TUBO/PDGF-C cells was analysed via western blot against pTyr and PDGFR-α. Duplicates of cells were seeded out in 6-well plates and cultured overnight in serum-free IMDM medium. One of each duplicate was treated by rmPDGF-BB (50 ng/ml) or vehicle (PBS). Before western blotting, cells were lysed and proteins were separated via SDS PAGE. PAE cells transfected with PDGFR-α were included as positive control (lane 1, bottom). PDGFR-α was not expressed on TUBO cells (lane 3-6, bottom).

3.5 TUBO/PDGF-C cells affect NK cell cytotoxicity

The effect of PDGF-CC expressing TUBO cells on NK cell cytotoxicity was studied in three different approaches as illustrated in figure 9.

Figure 8: **PDGFR-α expression in TUBO/mock and TUBO/PDGF-C cells.** The expression of PDGFR-α in TUBO/mock and TUBO/PDGF-C cells was analysed via western blot against pTyr and PDGFR-α. Duplicates of cells were seeded out in 6-well plates and cultured overnight in serum-free IMDM medium. One of each duplicate was treated by rmPDGF-BB (50 ng/ml) or vehicle (PBS). Before western blotting, cells were lysed and proteins were separated via SDS PAGE. PAE cells transfected with PDGFR-α were included as positive control (lane 1, bottom). PDGFR-α was not expressed on TUBO cells (lane 3-6, bottom).

**Figure 9:** Schematic illustration of different experimental set-ups.
In first set up, the direct cytotoxicity of NK cells on TUBO/PDGF-C or TUBO/mock cells was measured (A). To this end, TUBO/mock or TUBO/PDGF-C target cells were tested in a standard \(^{51}\text{Cr}\) release assay with purified and pre-activated NK effector cells overnight. YAC-1 cells were used as positive control to ensure a functional experimental set up. In the second experiment (B), purified and pre-activated NK cells were cocultured with TUBO/mock or TUBO/PDGF-C cells. After 16 h, non-adherent cells were separated from the coculture and tested in a killing assay against the NK target YAC-1 [1]. The same coculture experiment was performed with isolated and pre-activated splenocytes (C). In B and C, non-cocultured NK cells and splenocytes, respectively, were used as positive control. In all three approaches, the isolated splenocytes of Balb/c mice were syngeneic with the TUBO cell line.

3.5.1 TUBO/PDGF-C cells are less sensitive to NK killing compared to TUBO/mock cells

NK cell cytotoxicity was investigated in a direct killing assay (figure 9 A). At all tested E:T ratios, the cytotoxicity of NK cells towards TUBO/PDGF-C cells (figure 10, red squares) was about 50 % reduced compared to the cytotoxicity of NK cells towards TUBO/mock cells (figure 10, green squares). The result strongly indicates that PDGF-CC has an inhibitory effect on NK cell cytotoxicity, either by directly inhibiting NK cells or by making the tumour target cells less susceptible to NK killing.

![Figure 10: Reduced cytotoxicity of NK cells to TUBO/PDGF-C cells.](image)

Results are presented as mean (% of lysis) ± SD of triplicate measurements. NK cells coincubated with TUBO/PDGF cells (red squares) exhibited a 50 % reduced killing ability compared to NK cells coincubated with TUBO/mock cells (green squares). \(^{51}\text{Cr}\) labelled YAC-1 cells were used as positive control (black circles).
3.5.2 TUBO/PDGF-C cells inhibit cytotoxicity of purified NK cells

Next, NK cell cytotoxicity was studied according to figure 9 B. A reduced killing ability against the YAC-1 NK target cells was observed as a result of coculturing NK cells with TUBO/PDGF-C cells (figure 11, red squares) compared to TUBO/mock cells (figure 11, green squares). At a E:T ratio of 40:1, the killing efficiency of NK cells previously cocultured with TUBO/PDGF-C cells was 40 % lower compared to NK cells previously cocultured with TUBO/mock cells. A reduced killing ability was also determined at E:T ratios of 20:1 and 10:1. These results support the hypothesis that PDGF-C has an inhibitory effect on NK cells.

Figure 11 shows the inhibitory effect of TUBO/PDGF-C cells on NK cell cytotoxicity. The graph illustrates the reduced killing efficiency of NK cells when cocultured with TUBO/PDGF-C cells compared to TUBO/mock cells. The results support the hypothesis that PDGF-C has an inhibitory effect on NK cells.

3.5.3 TUBO/PDGF-C cells inhibit splenocyte NK cell function

NK cell cytotoxicity was studied according to figure 9 C. The application of isolated splenocytes simplified the experimental set up. However, by using YAC-1 target cells predictions about NK cell cytotoxicity were still possible since in non-immune spleens only NK cells are able to kill YAC-1 cells. The experiment demonstrated that splenocytes cocultured with TUBO/PDGF-C cells (figure 12, red squares) had a reduced killing capacity compared to splenocytes cocultured with TUBO/mock cells (figure 12, green squares). At the highest E:T ratio (200:1), the killing efficiency of splenocytes cocultured with TUBO/PDGF-C cells was 30 % lower compared to splenocytes cocultured with TUBO/mock cells. At E:T ratios 100:1 and 50:1 splenocytes...
cocultured with TUBO/PDGF-C cells exhibited a 50 % reduced killing ability compared to splenocytes cocultured with TUBO/mock cells.

Figure 12: TUBO/PDGF-C cells inhibit splenocyte NK cell function. Results are presented as mean (% of lysis) ± SD of triplicate measurements. Splenocytes cocultured with TUBO/PDGF cells (red squares) exhibited an up to 50 % reduced killing ability against YAC-1 cells compared to splenocytes cocultured with TUBO/mock cells (green squares). Splenocytes only (black circles) were used as positive control.

All three approaches support the hypothesis that PDGF-CC has an immunosuppressive effect on NK cell cytotoxicity. Furthermore, these results strongly indicate that the preliminary observations with the B16 melanoma line, which triggered the interest in this project, are not a melanoma-restricted phenomenon but could be extended also to mammary carcinomas.

3.6 NK cell cytotoxicity towards YAC-1 cells is not affected after rmPDGF-BB treatment

In order to study a direct effect of PDGF on NK cells, purified and pre-activated NK cells were cultured for 16 h in rmPDGF-BB concentrations varying from 0 - 100 ng/ml. Post incubation, NK cells were tested for cytotoxicity against YAC-1 cells (figure 13). Recombinant mPDGF-BB was used since rmPDGF-CC is not commercially available, so far. However, in terms of receptor activation and cell signalling, both ligands exhibit similar features [43].

Unexpectedly, NK cells (blue) treated with various concentrations of rmPDGF-BB were not functionally affected when compared to non rmPDGF-BB treated NK cells (red circles) because their cytotoxicity (%) was similar at all E:T ratios.
Figure 13: **NK cell cytotoxicity towards YAC-1 cells is not affected after rmPDGF-BB treatment.** Results are presented as mean (% of lysis) ± SD of triplicate measurements. Treated NK cells exhibited no functional difference compared to non rmPDGF-BB treated NK cells.

### 3.9 NK cells do not express PDGFR-α/β on their cell surface

Immunoflourescence (IF) microscopy was performed to investigate if the specific receptors for PDGF-CC (PDGFR-α) and PDGF-BB (PDGFR-β) are expressed on NK cells. PAE/PDGFR-α/β cells were used as positive controls. DAPI staining (blue) was applied to visualise the cell nuclei while AF-488 (green) was used to detect either PDGFR-α or PDGFR-β. The results, illustrated in **figure 14 A**, demonstrate that NK cells lack the expression of PDGFR-α (*top, right corner*) and PDGFR-β (*bottom, right corner*) on their cell surface. In contrast, PAE-α control cells (*top, left corner*) and PAE-β control cells (*bottom, left corner*) clearly express the PDGF receptors.

The expression of PDGFR-α was further studied in western blot experiments by using the antibody against phospho-tyrosine. Here, duplicates of pre-activated or freshly purified NK cells were stimulated with 50 ng/ml rmPDGF-BB or with PBS, respectively. PAE/PDGFR-α cells were used as positive control. Neither in pre-stimulated nor in freshly purified NK cells was the activation of the phospho-tyrosine kinase of the PDGFR-α (170 kDa) observable (**figure 14 B, lane 3-6**) when compared to positive control (*lane 1*). This goes in line with the results of the IF staining that NK cells do not express the specific receptor for PDGF-CC.
3.10 MHC class I up-regulation in TUBO/PDGF-C cells

In mice, MHC class I molecules are defined as Histocompatibility-2 (H-2) molecules. In the Balb/c strain H-2 D^d and H-2 K^d are most commonly expressed. The H-2 D^d and H-2 K^d expression in TUBO/mock and TUBO/PDGF-C cells was studied by using flow cytometry (figure 15). The flow cytometry analysis demonstrated that TUBO/PDGF-C cells (thin line) but not TUBO/mock cells (bold line) express H-2 K^d (A) and H-2 D^d (B). These results strongly
indicate that the PDGF-C expression is associated with the up-regulation of MHC class I molecules.

Figure 15: **MHC class I up-regulation in TUBO/PDGF-C cells.** TUBO/mock cells (*bold line*) or TUBO/PDGF-C cells (*thin line*) were incubated with PE-labelled H-2 K\(^d\) (*A*) and H-2 D\(^d\) (*B*) specific antibodies, respectively, as well as a PE-labelled IgG\(_{2a}\) isotope control (*shaded area; A, B*). Following staining, cells were washed and H-2 D\(^d\)/H-2 K\(^d\) expression was monitored in a BD flow cytometer together with the corresponding BD CellQuest Pro Software. The data was analysed with FlowJo. H-2 K\(^d\)/H-2 D\(^d\) expression of tumour cells (*x-axis*) was measured in percentage of maximal absorbance (% of Max.) (*y-axis*). A clear H-2 up-regulation was observed in TUBO/PDGF-C cells (*thin line*).
4 Discussion

In the present study the immunosuppressive role of PDGF-CC on NK cell cytotoxicity was investigated. To solve this question, different experimental set-ups were chosen whose results are discussed in the following paragraphs.

The first aim of my project was to confirm and to broaden the preliminary performed experiments of my group which showed that NK cells cocultured with PDGF-CC expressing B16 melanoma cells were less cytotoxic compared to NK cells cocultured with normal B16 cells. For this purpose, two Balb/c mice derived breast cancer cell lines (TUBO and D2F2) were transfected with PDGFC. The creation of a PDGF-CC expressing and secreting cell line succeeded with TUBO cells but failed with D2F2 cells. On the gene expression level, both cell lines express PDGFC. However, only TUBO/PDGF-C cells produce functional PDGF-CC as assessed by their ability to stimulate the PDGFR-α. Therefore, TUBO/PDGF-C and TUBO/mock cells were chosen for all further experiments. Why D2F2 cells do not secrete functional PDGF-CC can have several reasons. Most likely, D2F2 cells might secrete only an inactive form of PDGF-CC or they miss tPA, the enzyme required for ligand activation.

The next step within my project was to study the generated TUBO/PDGF-C and TUBO/mock cells with regard to their ability to suppress NK cell cytotoxicity. To this end, different 51Cr-release assays were performed. The results demonstrated that purified NK cells are found to be 50 % less cytotoxic towards TUBO/PDGF-C cells compared to TUBO/mock cells. These findings not only correlate with the preliminary results of my group but also present evidence that the immunosuppressive effect of PDGF-CC on NK cells is not only a melanoma-associated effect. A plausible explanation to these results came from the flow cytometry analysis, demonstrating that TUBO/PDGF-C cells exhibit up-regulated MHC class I molecules on their cell surface (figure 16). Since NK cells are less cytotoxic towards target cells expressing high levels of MHC class I molecules as compared to those with low-levels of MHC class I expression [13], the reduced killing ability can be explained by the natural biology of NK cells. However, if the up-regulation of MHC class I molecules and the subsequent reduction in NK cell cytotoxicity is really caused by the secretion of PDGF-CC needs to be investigated in additional experiments. One possible approach would be to equalise the level of MHC class I on the two TUBO cell lines by the treatment of INF-γ, a well known way of up-regulating MHC class I molecules [59].
Figure 16: **PDGF-CC may stimulate up-regulation of MHC class I molecules on TUBO/PDGF-C cells.** The up-regulation of MHC class I molecules on the TUBO/PDGF-C cell surface may inhibit NK cell cytotoxicity.

In addition, isolated splenocytes or purified NK cells cocultured with TUBO/PDGF-C cells were found to be clearly less cytotoxic against YAC-1 cells than splenocytes or NK-cells cocultured with TUBO/mock cells. One possible way to explain the observed phenomenon is that NK cells cocultured with TUBO/PDGF-C cells become inactivated due to their previously discussed MHC class I up-regulation and thereby less cytotoxic towards YAC-1 cells (figure 17 A). In contrast, NK cells cocultured with TUBO/mock cells remain activated and thus more efficient in their cytotoxicity towards YAC-1 cells (figure 17 B). Another conceivable mechanism of inhibition is that secreted PDGF-CC modulates the expression of receptors on NK cells so that they become less cytotoxic towards tumour cells (figure 18). The first investigations show that NK cells do neither express PDGFR-α nor PDGFR-β on their cell surface, which implicates that secreted PDGF-CC does not signal via these receptors. To what extent any of the
Discussion

known NK cell receptors, or the release of granzymes and/or perforins by NK cells, are affected by PDGF-CC was not analysed in this study. This needs to be tested in future experiments in order to reveal the inhibitory mechanism.

Figure 18: Secretion of PDGF-CC by TUBO/PDGF-C cells may directly modulate NK cell function. NK cells may be directly affected by PDGF-CC via a so far unknown mechanism.

The surprising finding that rmPDGF-BB does not affect NK cell cytotoxicity is contradicting a direct effect of PDGF on NK cells. It is possible that this could be explained by postulating that only PDGF-CC and not PDGF-BB can inhibit NK cell function, and that immunosuppression is restricted to the novel PDGFs to which PDGF-CC belongs. Differences between classical and novel PDGFs in ligand size, regulation of activity and expression pattern supports this idea but can only be verified in additional investigations. The existence of a second PDGF-CC receptor should also be considered.

Taken together, in this study I provide further evidence that PDGF-CC modulates NK cell effector functions in vitro. Notably, these findings are the first that demonstrate an effect of PDGF-CC on NK cell cytotoxicity. In addition, its expression in TUBO cells results in the up-regulation of MHC class I molecules so that cells can escape from NK cell killing. However, how modulation and the up-regulation are regulated remains to be shown and will be the aim of our future work. Also, one has to take into consideration that the up-regulation of MHC class I would make the TUBO cells more sensitive to killing by cytotoxic T cells, which needs further experiments to verify.

This study also shows that TUBO/PDGF and TUBO/mock cells exhibit no difference in their in vitro growth. Further, PDGFR-α is not expressed on their cell surface why an autocrine growth stimulation can be excluded. Based on all in vitro findings, a study in syngeneic mice was
recently initiated in order to investigate the effect of PDGF-CC on tumour development \textit{in vivo}. Therefore, three different amounts of TUBO/PDGF-C and TUBO/mock cells, respectively, were injected subcutaneously into the dorsal flank of Balb/c mice. Based on the findings of Anderberg \textit{et al.} a more aggressive tumour growth of TUBO/PDGF-C is to be expected [47]. This \textit{in vivo} study will be complemented with a similar study in NK cell depleted mice. The aim of this study is to clarify whether a tumour from PDGF-C-transfected cells is able to develop faster due to suppressed NK cell function.

In summary, I hope my thesis will be the starting point of a new and interesting project, which will hopefully reveal the role of PDGF-CC as a modulator of innate tumour immunity.
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6 References

References


