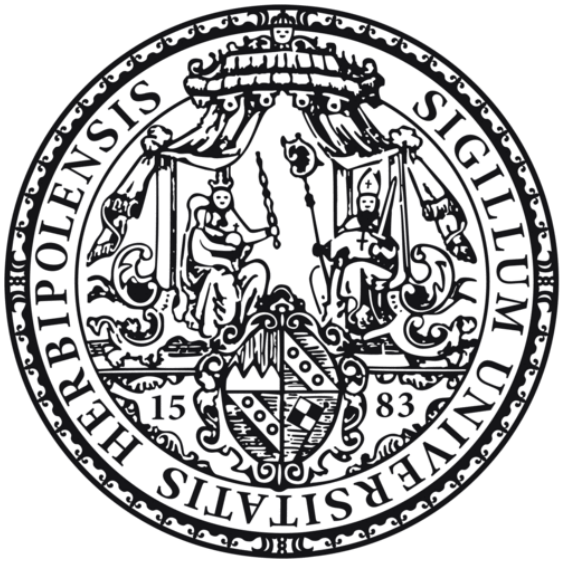
Dissertation

Influence of myeloid-derived suppressor cells and neutrophil granulocytes on natural killer cell homeostasis and function

Einfluss myeloider Suppressorzellen und neutrophiler Granulozyten auf die Homöostase und Funktion natürlicher Killerzellen



Julius-Maximilians-Universität Würzburg

Medizinische Klinik und Poliklinik II

Head of the clinic: Prof. Dr. med. Hermann Einsele

submitted by

Johanna Müller-Leisse

Doctoral thesis for a doctoral degree

at the Graduate School of Life Sciences

Submitted on:

Members of the *Promotionskomitee*:

Chairperson: **Prof. Dr. rer. nat. Thomas Hünig**

Primary Supervisor: **Prof. Dr. rer. nat. Jürgen Löffler**

Supervisor (Second): **PD Dr. med. Niklas Beyersdorf**

Supervisor (Third): **Dr. nat. med. Sabrina Brüggemann**

Supervisor (Fourth): **Dr. Maria Bouzani**

Date of Public Defense:

Date of Receipt of Certificates:

To my parents Elisabeth & Christoph

Table of contents

1 Introduction

1.1 The immune system

1.1.1 Overview

The function of the immune system is to defend the body against disease. Oftentimes, disease originates from external factors such as pathogens or toxins. As a first line of defense, natural barriers such as the skin, the mucociliary clearance of tracheal epithelial cells or the acidic milieu of the stomach prevent the entrance of pathogens. If these barriers fail, the immune system often recognizes the intruder inside the body and clears it [2]. On the other hand, disease also originates from internal processes inside the body, such as malignant transformation. Malignant transformation is also recognized and combatted by the immune system, an ability that has only been recognized in the past 50 years and is called immune surveillance [3]. However, malignant cells weaken and manipulate the immune response to their own benefit. Certain immune cells even attain immunosuppressive qualities and promote tumor growth [4]. Understanding and manipulating the immune response to cancer is essential for its management, and forms part of the concept of immunotherapy [5].

1.1.2 Components of the immune system

The immune system consists of soluble and cellular components. All immune cells derive from hematopoietic progenitors in the bone marrow and belong to either the myeloid or the lymphoid lineage [6]. Immune cells are found within the blood, lymphatic system and many other tissues, and are able to migrate to most sites of the body [7]. They have characteristic glycoproteins on their cell surface that identify and distinguish them. These are called clusters of differentiation (CD), and fulfill many different and partly unknown functions [8]. Like other body cells, all immune cells express the major-histocompatibility complex (MHC) I, which is also called human leukocyte antigen (HLA) I, in humans. MHC I labels healthy cells and distinguishes them from most diseased or foreign ones. The complex binds intracellular self-antigens and presents them on the cell surface. If the presented antigens are abnormal or if MHC I is absent, the cells are attacked by the immune system [9, 10].

Immune cells are subdivided into innate- and adaptive immune cells. Classically, innate immune cells act faster and are less specific. They are able to defend the body spontaneously, forming a crucial first line of defense against newly encountered pathologies. Innate immune cells comprise macrophages, granulocytes, dendritic cells and natural killer (NK) cells. They recognize tumor-associated antigens, pathogen- and damage-associated molecular patterns directly via certain receptors [11]. While granulocytes and macrophages are phagocytes, NK cells are non-phagocytic but kill via the release of cytotoxic molecules or activation of death receptors. They are the most studied innate immune cells in the context of cancer [2]. Adaptive immune cells like T and B cells, on the other hand, act slower on first encounter and are more specific. T cells require the presentation of pathological antigens by so-called antigen-presenting cells, before attaining their full function. Antigen-presenting cells connect the innate and adaptive immune system and include dendritic cells and macrophages. They can recognize pathological antigens, phagocytose and process them, and present them to T cells via MHC II molecules [12]. Both T and B cells form memory cells. If a pathogen is encountered for the second time, they initiate a response that is both fast and specific [3, 13].

Cells of the innate and adaptive immune system interact closely. Both secrete cytokines that shape the inflammatory micromilieu and facilitate communication. Recently, abilities classically attributed to adaptive immune cells have also been discovered in innate immune cells and vice versa, such that the strict differentiation between the two systems is not as valid as it used to be [14].

1.1.3 NK cells

1.1.3.1 Overview

NK cells form part of the innate immune system. They were first defined as large, granular, non-adherent and non-phagocytic lymphocytes. They present 10-15 % of all circulating lymphocytes in human peripheral blood, where they have a lifespan of around 10 days [15, 16]. They are also present in the lymphatic organs, skin, lung, liver, intestinal and urogenital tract [17]. They share common progenitors with T cells but in contrast to the latter, they possess the ability to exert spontaneous cytotoxicity against target cells without requirement of antigen presentation [15, 18]. NK cells are best-known for

recognizing and killing cancer cells, [19] but are also effective against microbes. The discovery that they secrete cytokines [20] has led to their recognition as regulators of the immune system [21].

1.1.3.2 Functions of NK cells

NK cells were named after their natural ability to exert cytotoxicity against target cells [18]. While under physiological conditions, transformed or virus-infected cells automatically enter programmed cell death, this self-induced apoptosis can be impaired, and immune-cell mediated cytotoxicity is a crucial alternative defense mechanism. The first NK target cells identified were leukemia cells, and the leukemia cell line K562 has since been used as a classical stimulus for NK cells in many in vitro experiments [22]. NK cells recognize neoplastic cells by their reduced MHC I expression and are further stimulated by other tumor-, pathogen- or immune cell-derived factors [23]. The importance of NK cells in the outcome of cancer in vivo was shown in several mouse models [24] and in patients with leukemia [25, 26]. NK cells are also crucial for immune surveillance, and serve to prevent the development of cancer in the first place [15]. Their counts in peripheral blood were even suggested to correlate with cancer risk [27].

NK cells kill their targets by means of soluble factors. Upon stimulation, they degranulate to release the cytolytic enzymes perforin, granzymes and granulysin [28]. These mediators induce cell death of the target cell by different mechanisms including caspase activation, proteolysis and DNA fragmentation. This NK cell killing strategy has similarities with that of cytotoxic T cells. However, T cells generate the cytotoxic granules only after sensitization, while NK cells already generate them during development and have them instantly available upon stimulation, which is essential for a rapid immune response. Death of the target cell can follow after as short as 20 minutes of time [29]. As a second killing mechanism, NK cell ligands like FasL and TRAIL can activate death receptors on target cells and induce apoptosis [30].

NK cells also produce cytokines, prominently IFNγ and TNFα, which are known for their pro-inflammatory, immunostimulatory effects, but also affect tumor cells directly [30]. TNFα can induce cancer cell death and is therefore considered as a potential therapeutic target [31]. IFNγ has inhibitory effects on fibrogenesis, angiogenesis and proliferation of tumor cells [32], but most importantly improves anti-tumor immunity. It is used clinically

to treat a variety of malignancies, despite major side effects [33]. IFNγ promotes the MCH I expression on malignant cells, which facilitates their recognition by immune cells. It also facilitates apoptosis induction by enhancing the expression of death receptors and ligands [30]. Although T cells also produce IFNγ, NK cells were the major producers in a murine tumor model [34]. NK cell-derived IFNγ was even suggested as a predictor for long-term survival in a particular tumor setting [35].

NK cells produce many other soluble factors including IL-15, GM-CSF and chemokines. Like IFNγ and TNFα, they influence most other immune cells [30]. In fact, NK cells play a complex regulatory role within the immune system. They can promote the maturation of dendritic cells [36], and the activation of dendritic cells, T cells and neutrophils [37, 38]. They can also kill other immune cells, as was demonstrated for immature dendritic cells [39], overstimulated macrophages [40], activated T cells [41] and neutrophils [42].

NK cells also show memory-like as well as antibody-dependent responses, which are abilities associated with adaptive immunity [43, 44]. The functions of NK cells within the immune system are thus various and not restricted to cytotoxicity against tumor cells. It is the particular inflammatory microenvironment that seems to determine the nature of their predominant function in each situation [37].

1.1.3.3 Markers and subsets of NK cells

NK cells can be identified by the surface expression of CD56 and lack of the T cell marker CD3 on the cell surface. CD56 is an adhesion molecule without direct cytotoxic function [15]. CD16, another NK cell marker, forms part of the NK cell activating receptors and plays a crucial role in proliferation, cytokine release and cytotoxicity [45, 46]. It presents an Fc receptor and has particularly been associated with the NK cells' ability for antibody-dependent cellular cytotoxicity [44]. Within human NK cells, subsets have been defined according to the expression of CD56 and CD16, most importantly $CD56^{dim}$ $CD16^{pos}$ and $CD56^{bright}$ $CD16^{neg}$. $CD56^{dim}$ $CD16^{pos}$ NK cells are mature and present the most abundant population in the peripheral blood. $CD56^{bright}$ NK cells, on the other hand, mostly reside in the peripheral lymphoid organs where they are believed to exert immunomodulatory functions. They are less cytotoxic but secrete high amounts of cytokines and proliferate more rapidly [47]. They can be either $CD16^{dim}$ or $CD16^{neg}$ and are pre-mature. $CD56^{bright}$ $CD16^{neg}$ NK cells develop into $CD56^{dim}$ $CD16^{pos}$ NK cells upon

maturation in the lymph nodes, $CD56^{bright}$ $CD16^{dim}$ NK cells presenting an intermediate state. On the other hand, $CD56^{dim}$ NK cells can also become $CD56^{bright}$ NK cells under certain stimuli [48]. Few NK cells are $CD56^{neg}$ $CD16^{pos}$ or $CD56^{dim}$ $CD16^{neg}$, but their exact functions are unknown [49]. The phenotypes change under the influence of certain stimuli and their functions also vary. The cytotoxic $CD56^{dim}$ NK cells can even be the major cytokine producer under certain circumstances [50, 51]. There is thus a high plasticity of NK cell phenotypes and functions, which may enable adaptation to specific conditions.

Certain NK cell receptors can serve as indicators for activation, such as CD69 and CD137. Their natural ligands remain unidentified. CD69 presents a transmembrane glycoprotein that is rapidly up-regulated on NK cells, T cells and other immune cells upon simulation [52], mediated by multiple activation pathways [53]. Its expression was shown to correlate with NK cell cytotoxicity [54]. CD137 is not expressed by resting NK cells but only upon stimulation [55, 56]. It's expression was correlated with activation and cytokine release but not cytotoxicity of NK cells [57].

1.1.3.4 Activating and inhibitory receptors on NK cells

NK cells are best-known for their spontaneous, natural cytotoxicity. The ability to release cytotoxic granules and cytokines without prior sensitization requires tight regulation of these effects. This regulation is mediated by several receptors on the NK cell surface, which transmit inhibitory and activating signals. They bind and distinguish ligands on healthy cells, stressed cells [58], transformed cells [58], or pathogens [59].

Inhibitory receptors include receptors of the KIR family (killer cell immunoglobulin-like receptors) [60]. Their most important ligands are MHC I molecules. However, they can be overruled by strong signals from activating receptors [30, 61]. Activating receptors include the immunoglobulin (Ig)-like natural cytotoxicity receptors NKp46, Nkp44 and NKp30, whose ligands are found on most tumor cells and viruses. Their expression correlates with NK cell cytotoxicity [62-64]. NKp30 signaling was shown to mediate both IFNγ release and degranulation [65, 66]. NKp30 is also involved in the regulation of dendritic cells by NK cells [67]. While NKp44 expression is only induced after stimulation, NKp46 is highly expressed by all NK cells and can be used to define them [68]. The expression of NKp30 fluctuates between individuals as well as between subsets of NK cells [65, 69].

NKp46 and NKp30 are considered essential for an efficient cytotoxicity against tumor cells, and low expressions are associated with cancer progression [70]. Engagement and down-regulation of NKp30 may be responsible for the suppressive effects of myeloid-derived suppressor cells (MDSCs) [71], viral components [72, 73] and corticosteroids on NK cell function [74]. NKG2D presents another activating receptor, which is constitutively expressed on NK cells [75]. It binds ligands on malignant and stressed cells [76] and transmits strong activating signals [77]. NKG2D can be down-regulated by tumor cells [78, 79] and in viral infection [79].

Other immune cells also contribute to the regulation of NK cells. Activating effects were observed for monocytes, macrophages and dendritic cells [80], while inhibitory effects were observed for monocytes [81], granulocytes [82] as well as monocytic and granulocytic MDSCs [71, 83] and regulatory T cells [84].

1.1.3.5 NK cell-activating cytokines

Several soluble mediators influence NK cells, including the monocyte-derived cytokine IL-15 and the T cell derived IL-2. Both enhance the activity of NK cells, and have been the subject of clinical trails with the aim to improve NK cell function in tumor patients [85-87]. Despite considerable side effects, IL-2 is regularly used to treat certain very advanced types of carcinoma [88].

The IL-2 receptor has two variants: a dimeric low affinity receptor on mature $CD56^{dim}$ NK cells (β and γ chain) and a trimeric high affinity receptor on pre-mature $CD56^{bright}$ NK cells (α, β and γ chain). The IL-15 receptor is also trimeric. It is composed of the β and γ subunit of the IL-2 receptor and an additional high affinity IL-15 receptor α chain [89]. However, IL-15 can also signal through the dimeric IL-2 receptor [90]. Both cytokines promote proliferation, cytokine release and cytotoxicity of NK cells, but they also have distinct functions. In contrast to IL-2, IL-15 was reported to improve the survival of NK cells [85, 91-94], although some authors have also observed increased apoptosis rates under the influence of high doses of IL-15 [95, 96]. IL-15 is more ubiquitous in the body than IL-2, and its receptor has a higher tissue distributions [97]. It is produced by a variety of cells including stromal cells [98], epithelial cells [99], macrophages [100] and dendritic cells. Together with other innate immune cell-derived cytokines, IL-15 is attributed a major role in the early phase of inflammation [101], while

the IL-2 secreting T cells enter the inflammatory setting at a later time point. Furthermore, the effect of IL-15 seems to be more stable compared to IL-2. IL-15 signaling causes up-regulation of cytokine receptors, while IL-2 provokes their down-regulation causing IL-2 desensitization. The effect of IL-15 is also less susceptible to other factors in the microenvironment, such as inhibitory cytokines [102]. Inhibitory cytokines include IL-12, IL-21 and TGF-β [103]. TGF-β was suggested as a mediator of NK cell suppression by MDSCs [104] and regulatory T cells [84], and was demonstrated to cause down-regulation of NKp30 in vitro [105].

1.1.3.6 NK cells as therapeutic target

In general, NK cells are considered more effective in the prevention of tumorigenesis than in the defeat of established tumors. Therefore, therapeutic approaches targeting NK cells seem especially promising as add-on. For example, the transfer of allogeneic NK cells following high-dose chemotherapy and hematopoietic stem cell transplantation can cause the desired graft-versus-leukemia effect: MHC I mismatched NK cells kill remaining leukemia cells. In contrast to T cells, they do not cause graft-versus-host disease [106]. In a clinical trial with acute myeloid leukemia patients, transplants with alloreactive NK cells led to better remission and survival rates compared to non-NK-alloreactive transplants [107]. The adoptive transfer of allogeneic NK cells has also shown some promising results in patients with solid tumors [108]. Other clinical approaches aim to boost the function of available NK cells and include the targeting of KIR receptors [109] and administration of cytokines [110]. There is evidence suggesting that some established chemotherapeutics also partially function via improvement of NK cell function [35, 111, 112]. The depletion or modulation of regulatory immune cells, such as regulatory T cells or suppressive granulocytic cells, which hamper NK cell function, may also be promising [84, 113].

1.1.4 Neutrophils

1.1.4.1 Definition

Neutrophil granulocytes, also called neutrophils, or polymorphonuclear neutrophils (PMNs), belong to the innate immune system and present approximately 60 % and thereby the most abundant type of the circulating white blood cells. Other granulocyte subsets like eosinophils, basophils and mast cells constitute a minor percentage of granulocytes. Mature neutrophils are large, segmented cells. They express several surface markers

including CD33, CD11b, CD15 and CD66b [114], the latter being most specific [115]. Mature neutrophils have a high density a fact that is used for their separation from lymphocytes [116].

1.1.4.2 Functions of neutrophils

Neutrophils are especially recognized for their function against bacteria and fungi [117]. The lack of functional neutrophils is a severe condition with high infectious risk and high severity of infection [118-120]. Neutrophils are short-lived and especially effective in acute inflammatory processes. They migrate to inflammatory sites by following chemokine gradients and oftentimes are the first immune cells to actively access the area [121]. Stimulated by cytokines, growth factors and microbial products, they effectively and directly kill extracellular pathogens via phagocytosis or extracellular trap formation.

However, neutrophils also degranulate and release cytotoxic substances such as proteases and reactive oxygen species (ROS) that are scarcely specific and can also harm healthy tissue. Cytokines are also produced and released. Those soluble factors importantly contribute to the general inflammatory micromilieu [121] and have an influence on the overall immune response, which is oftentimes beneficial but may also be detrimental, especially in chronic infection [122, 123]. Likewise, the role of neutrophils in tumor disease remains controversial. Tumor-associated neutrophils (TANs) present a key component of the tumor microenvironment [124] and can exhibit anti-tumor cytotoxicity [125, 126]. However, pro-tumor effects are increasingly ascertained [127] and seem to predominate in many cases. High neutrophil to lymphocyte ratios are associated with poor prognosis in many cancers [128], suggesting that neutrophils impair the function of lymphocytes. In fact, suppressive neutrophil subsets have been identified and are distinguished from “normal” neutrophils. They include N2 TANs [128].

1.2 Immunodeficiency

1.2.1 Consequences and Causes

Immunodeficiency is a condition where the immune system is impaired, and any type leads to an increased risk for infections. However, different parts of the immune system can be affected, and the severity is highly variable. While mild forms can be asymptomatic, severe forms lead to deadly infections within a short period of time [129,

130]. The patients are vulnerable to opportunistic infections that are often difficult to treat [131]. Especially after bone marrow transplantation, patients have a high mortality associated with opportunistic infections [132]. Non-opportunistic infections also occur more often and take more severe courses [133]. The general inflammatory response to any pathological process is impaired, and clinical signs such as fever are reduced, leading to a delayed diagnosis [134]. Immunodeficiency also favors cancer development and progression, and immunotherapy plays an increasing role in the treatment of cancer [135].

Immunodeficiency can be primary such as in congenital immunodeficiency syndromes, or secondary. The secondary immunodeficiency is often called immunosuppression. Its causes are various and include pregnancy, toxins, HIV infection and therapeutic immunosuppression. Cancer patients are prone to severe immunosuppression by both cytotoxic treatment and the tumor itself. Tumor cells manipulate and weaken the immune system by releasing multiple soluble mediators into the microenvironment and blood stream [4, 136]. This manipulation involves an accumulation and activation of regulatory immune cells, which impair the cytotoxicity of T and NK cells [137]. Their levels in the peripheral blood were shown to correlate with tumor burden [138].

Regulatory immune cells include regulatory T cells, MDSCs and tumor-associated macrophages and neutrophils [138]. Cytokines with immunomodulatory function such as IL-10 and TGF-β have also been identified [138, 139]. These regulatory components of the immune system can prevent an immune overreaction, and can be beneficial in autoimmune disorders, wound healing or graft-versus-host disease [140-142]. However, they are generally detrimental for the disease outcome in malignancies, and have therefore become a new target for immunotherapy against cancer [138].

1.2.2 Myeloid-derived suppressor cells

1.2.2.1 Overview

Around 30 years ago, a myeloid immune cell type with suppressive effects on T cells was described [143]. These cells were later named myeloid-derived suppressor cells (MDSCs) and present a heterogeneous group of immature granulocytic, monocytic and dendritic cells [144]. They have features of myeloid progenitors in common, but differ in their morphology and marker expression [145]. MDSCs expand in virtually all malignancies [146-148], but also in non-malignant inflammatory processes such as trauma

[149], autoimmunity [150] or infection [151]. This accumulation is associated with disease progression in malignancies and acute infections [152, 153], but can be beneficial in trauma, sepsis and autoimmune diseases [149, 154]. MDSCs are best-known for their inhibition of T cells, namely proliferation and IFNγ production [155], but they can also impair the function of macrophages [156] and NK cells [71].

1.2.2.2 Definition of MDSCs

In mouse models, MDSCs have been defined by the two myeloid markers CD11b and Gr-1 [157]. The myeloid differentiation marker Gr-1 comprises two epitopes, Ly6G and Ly6C, which define two subsets of MDSCs in mice: $Ly6G^{pos}$ $Ly6C^{low}$ granulocytic MDSCs and $Ly6C^{pos}$ $Ly6G^{neg}$ monocytic MDSCs. Similar suppressive effects were shown for monocytic and granulocytic MDSCs [158], although by different mechanisms of action. In most tumor models, the granulocytic subset was preferentially expanded [159]. However, some authors question the distinction between granulocytic and monocytic MDSCs as distinct subsets, arguing that they represent a heterogeneous immature myeloid cell population with high plasticity and variable features [159].

In humans, the differentiation between granulocytic and monocytic MDSCs has also been suggested, via the expression of the monocytic marker CD14. However, human MDSCs seem to lack a common specific marker equivalent to Gr-1 in mice. As a consequence, their definition is inconsistent and mainly based on their immaturity and suppressive effect on T cells [160]. Several markers have been postulated to characterize human MDSCs in combination with a low expression of HLA II or other lineage markers. These include the myeloid markers CD11b, CD33 and CD15, the granulocytic marker CD66b [145] and the monocytic marker CD14 [161]. Both human and mouse MDSCs have been distinguished from mature neutrophils via their lower density [127, 155]. Several studies have used the characteristics of low density and CD66b expression to define MDSCs and isolate them from the low-density fraction of peripheral blood mononuclear cells (PBMCs) [155, 162-167]. CD66b, which is also named CEACAM8, is expressed on neutrophils and eosinophils and belongs to the family of carcinoembryonic antigen-related cell adhesion molecules (CEACAM) [168]. CEACAM mediate various intercellular interactions including regulatory interactions [169]. However, the suppressive effects of MDSCs have so far not been linked to CD66b.

1.2.2.3 Expansion and activation of MDSCs

Only < 5 % of PBMCs in healthy humans constitute MDSCs [170]. Normally, immature myeloid cells are found within the bone marrow and rarely within peripheral blood, because they differentiate into dendritic cells, macrophages or granulocytes in the bone marrow. In state of disease, different stimuli can trigger myelopoiesis and at the same time inhibit myeloid differentiation, which increases the percentage of immature myeloid cells in the peripheral blood. Further factors can promote the immunosuppressive activity of these cells [171]. The accumulation of MDSCs was demonstrated in many different tumor entities, including melanoma [172], lung [173], breast [174], prostate [175], colon and pancreatic carcinoma [176], as well as in severe leukemia [148]. However, the degree of accumulation and the character of suppressive action are heterogeneous and situation-dependent [159]. MDSCs also accumulate in inflammatory states like autoimmunity [177] and infection [178].

Accordingly, many factors derived from malignant cells, immune cells and pathogens promote MDSCs expansion, migration and activation. For example, IFNγ contributes to in the activation of MDSCs in mice [179]. This seems contradictory, as IFNγ is an important pro-inflammatory cytokine. However, the effect requires a particular microenvironment. In vitro, only the combination of IFNγ and LPS promoted the accumulation and activation of MDSCs. This combination may reflect a cytokine environment during chronic infection or sepsis [180]. Other factors involved in the regulation of MDSCs expansion and activation include prostaglandins [181], stem-cell factor [182], VEGF [183], GM-CSF [184] and TNFα [185]. Cancer cell-derived PGE2 induces the migration of MDSCs to the tumor site by enhancing the expression of the chemokine receptor CXCR4 on MDSCs [186]. However, functional MDSCs that suppressed T cell proliferation were also detected in healthy mice [180, 187]. Likewise, there is evidence that MDSCs exist in healthy humans [82, 188]. However, a direct inhibitory effect on T cells has not been demonstrated.

1.2.2.4 Suppressive action of MDSCs

The first data on MDSCs were collected from murine tumor models and showed a suppressive effect on T cell proliferation, antigen-dependent IFNγ and IL-2 production as well as CD3ζ chain expression [189] [190]. The effects were similar for monocytic and

granulocytic MDSCs [158]. This antigen-specific inhibitory effect was linked to an antigen-presenting ability of MDSCs [189]. However, unspecific suppression of T cell activation was also reported [191]. Later, the accumulation of MDSCs, their inhibition of T cell function including proliferation, cytokine production and CD3ζ chain expression as well as an association with tumor progression were confirmed in humans in many different solid and hematologic malignancies [155, 174, 192]. A direct influence on tumor invasion, vasculogenesis and angiogenesis by MDSCs was also suggested [193].

Different, cell-cell contact-dependent mechanisms mediate the effects of MDSCs [71, 137, 171]. For example, the enzyme arginase 1 has frequently been associated with T cell suppression [192, 194]. Arginase 1 is produced by granulocytic MDSCs and metabolizes L-arginine, depriving this semi-essential amino-acid from the microenvironment [195]. Low L-arginine levels were correlated with high MDSCs counts and poor outcome in tumor patients [196]. Likewise, the enzyme NO-synthase (NOS) uses L-arginine as a substrate and suppresses T cells on various levels [197, 198]. NOS levels are high in monocytic MDSCs [159]. Other mechanisms involve the molecules IFNγ [158], ROS and peroxynitrite [199]. Additionally, the induction of regulatory T cells [200] and the release of anti-inflammatory cytokines such as IL-10 [156, 201] and TGF-β [172] are part of the repertoire of MDSCs.

It has been proposed that the mechanisms of action differ between granulocytic and monocytic- MDSCs, between antigen-specific- and non-specific effects and dependent on the location and tumor type [160, 191]. Under certain circumstances, MDSCs can also induce activation of NK cells [202, 203]. These functional differences underline the heterogeneity and plasticity of MDSCs [193].

1.2.2.5 MDSCs and NK cells

Beside the well-studied T cell suppression, MDSCs also inhibit NK cell cytotoxicity and cytokine release. This was demonstrated in several murine tumor models, and the effects were cell-cell contact-dependent [204]. Molecules involved included STAT5 [204], membrane-bound TGF-1β, NKG2D [104] and ROS [205]. In contrast to most evidence, one group has described NK cell activation following co-culture with monocytic MDSCs [203], similar to the activating effect observed on murine T cells [202].

An in vitro experiment with monocytic MDSCs derived from tumor patients confirmed the inhibitory effect on NK cells cytotoxicity and cytokine secretion in humans [71]. The effect was independent of arginase 1 and NOS but involved the natural cytotoxicity receptor NKp30 and was accompanied by its down-regulation. Down-regulation and binding of NKp30 are known mechanisms to impair NK cell cytotoxicity and cytokine production in other contexts [72, 73, 206, 207]. Very recently, the suppressive effect of in vitro generated granulocytic MDSCs on NK cell cytotoxicity (but not cytokine production) was also confirmed in humans [208].

1.2.2.6 MDSCs as therapeutic target

Because MDSCs suppress several other immune cells and accumulate in most inflammatory conditions, targeting these cells could be beneficial in many diseases. Vitamin A was demonstrated to promote the differentiation of MDSCs into mature myeloid cells, which resulted in lower MDSCs counts in the peripheral blood of cancer patients and improved T cell responses [113]. A similar effect was suggested for vitamin D3 [209]. Furthermore, factors involved in the accumulation and activation of MDSCs like SCF and VEGF have been considered as therapeutic targets [210, 211], as well as factors mediating the suppressive effects like arginase 1 and ROS [192, 212, 213], and components of intracellular signaling pathways [214]. The cytostatic drugs gemcitabine and 5-fluorouracil decreased the number of MDSCs in tumor-bearing mice and improved anti-tumor immunity [215-217]. Although most drug tests were performed in murine models or in vitro so far, further investigations are expected.

1.2.2.7 Differentiation between MDSCs, PMNs and N2 TANs

There is evidence that not only MDSCs, but also “normal” PMNs can influence the function of T and NK cells. NK cell proliferation and IFNγ production were impaired under the influence of arginase 1, an enzyme derived from PMNs [82]. Furthermore, several older publications have reported that PMNs caused impaired NK cell cytotoxicity [218-221]. The effects were partly attributed to ROS [222].

The fact that immunosuppressive effects are described for different subsets of neutrophils raises the question of how these cells are related. Suppressive neutrophils found within tumor tissues are referred to as N2 TANs (tumor-associated neutrophils) [223], while suppressive neutrophils in the spleen and peripheral blood are often called MDSCs [224].

Both subsets accumulate in cancer disease and have been considered as targets for anti-cancer therapy [113, 225]. It is possible that N2 TANs within the tumor tissue actually originate from peripheral blood MDSCs [128]. However, MDSCs are also present in healthy individuals and their influence is not limited to tumor disease.

Because they lack specific markers, the definition of MDSCs and their distinction from peripheral blood PMNs is controversial. MDSCs were suggested to exhibit an unusually low density, a fact that has been used to distinguish them from PMNs [155]. However, as mentioned above, "normal" high-density PMNs also have immunosuppressive features, and the morphology and surface marker expression of low- and high-density neutrophils do not substantially differ [114]. Some authors simply refer to low-density granulocytes instead of using the term MDSCs [127, 188, 226]. Only a couple of groups have directly compared low- and high-density granulocytes in the human system and have shown contradictory results. In both HIV patients and healthy pregnant women, it was shown that low-density granulocytes express higher levels of arginase, which may indicate a higher suppressive potential, because granulocytic arginase can impair the function of T and NK cells. However, the authors did not compare immunosuppressive effects of low- and high-density granulocytes directly [82, 114, 188]. Another group has found that neither low- nor high-density granulocytes derived from healthy donors had immunosuppressive effects, while both cell types derived from leukemia patients did [167]. It was also suggested that low-density granulocytes actually present activated neutrophils [114, 162], and that they can be derived from the latter via stimulation with certain cytokines [127, 180]. In mice, on the other hand, transcriptomic analysis have suggested that granulocytic MDSCs, PMNs and N2 TANs all substantially differ from one-another [128].

Taken together, the used definitions and nomenclature of human suppressive granulocytic subsets remain inconsistent [128]. Most recent publications on suppressive granulocytic cells address MDSCs, and they are mostly distinguished from PMNs by their density. However, it has not been proven that these cells are more suppressive than "normal" PMNs [227]. Instead, there is evidence that both high- and low-density granulocytes have immunosuppressive potential. Data comparing the two are limited and contradictory, and none involve the suppressive effects on NK cells.

1.2.3 Leukemia and immunosuppression

Immunosuppression plays an important role in the pathophysiology and progression of leukemia. It is caused by both the disease itself and chemotherapy, and goes along with the accumulation of immunoregulatory cells and cytokines [228, 229]. There is evidence for an important dysfunction of NK cells, which are usually very efficient against leukemia cells [22]. Low NK cell numbers, poor cytotoxic activity and IFNγ release [230] as well as low expressions of NKp46 and NKp30 were observed in patients with acute myeloid leukemia and correlated with a poor prognosis [231].

The term leukemia defines a heterogeneous group of hematological malignancies that are characterized by the expansion of pathological leukocytes in the blood forming tissues and their flooding of the peripheral blood. The production of functional erythrocytes, leukocytes and thrombocytes can be severely impaired and cause anemia, infection and hemorrhaging. Acute leukemias are the most common malignancies in children. They are characterized by rapid progression with proliferation of immature blasts. On the other hand, chronic slowly progressive leukemias involve mature leukocytes. They are more common in adults [232]. Dependent on the proliferating leukocyte type, leukemias are also subdivided into lymphocytic and myeloid leukemias. Especially in children, the outcome under chemotherapeutic treatment is mostly favorable [233]. However, the course of the disease is highly variable with very poor survival rates in high-risk patients (< 1 year) [234].

Allogeneic hematopoietic stem cell transplantation (HSCT) is regularly performed in leukemia (in up to 70 % of cases [235]). The procedure goes along with severe immunosuppression and high immunosuppression-associated mortality and relapse rates. Reconstitution of the immune system following HSCT is crucial for the prognosis [236]. Especially the reconstitution of NK cells was shown to correlate with relapse-free survival and has even been suggested as a prognostic marker [237]. Therefore, enhancing NK cell reconstitution and function following HSCT presents an important therapeutic ambition. Together with T cells, NK cells are the principal effector cells mediating the graft-versus-leukemia effect. In contrast to the first, they do not mediate graft-versus-host disease. The treatment with HLA-mismatched NK cells was shown to be protective against graft-versus-host disease, graft rejection and leukemia relapse [238, 239].

2 Aim of thesis

Natural killer (NK) cells are potent effector cells of the innate immune system and influence the outcome of various diseases, in particular hematologic malignancies. However, NK cells are less functional after hematopoietic stem cell transplantation (HSCT), and in tumor disease in general, which worsens the outcome [230]. Partly, this may be due to a suppression of NK cell function by granulocytic cells. An accumulation of suppressive granulocytic cells was observed in many malignancies including severe leukemia. Although these cells are best-known for their suppression of T cells, it was recently suggested that they impair the function of NK cells as well [208]. Since NK cells and granulocytes are among the first immune cells to reconstitute after HSCT, their interaction is likely to influence the cytotoxicity of graft NK cells against remaining leukemia cells [236]. Investigating and targeting this interaction could be a tool to enhance NK cell reconstitution and the graft-versus leukemia effect after HSCT.

However, there is hardly data available on the interaction between NK cells and suppressive granulocytic cells in humans. Furthermore, the few publications available have considered different subsets of granulocytic cells. Their definitions and nomenclature are inconsistent, which promotes contradictory results and delays research progress on the topic. In particular, the distinction between so-called myeloid-derived suppressor cells (MDSCs) and mature polymorphonuclear neutrophils (PMNs) is controversial. The aim of this thesis was to isolate human MDSCs and PMNs from healthy donors and examine their effects on NK cells. I co-cultured NK cells and granulocytic cells under different conditions and then evaluated the cells by flow cytometry. In particular, I assessed NK cell activation, cytotoxicity and cytokine release, using the leukemia cell line K562 as a target.

3 Materials & Methods

3.1 Materials

3.1.1 Cells

All human immune cells were isolated from the fresh blood of healthy volunteer donors. The leukemia cell line K562 was kindly provided by PD Dr. med. Ruth Seggewiss and was originally purchased from ATCC.

3.1.2 Chemicals and solutions

3.1.2.1 Antibodies for flow cytometry

Antibody	Company	Application per 100 µl
Anti-CCR7-APC	Miltenyi Biotec, Bergisch Gladbach, DE	5 µl
Anti-CD11b-PE	Miltenyi Biotec	2 µl
Anti-CD137-APC	Miltenyi Biotec	5 µl
Anti-CD14-FITC	BD Biosciences, Heidelberg, DE	2 µl
Anti-CD16-FITC	Miltenyi Biotec	2,5 µl
Anti-CD19-PerCP	BioLegend	2 µl
Anti-CD25-FITC	BD Biosciences	1 µl
Anti-CD3-PerCP	BD Biosciences	3,5 µl
Anti-CD56-APC	BD Biosciences	5 µl
Anti-CD56-FITC	BD Biosciences	2,5 µl
Anti-CD56-PerCP-Cy5.5	BD Biosciences	5 µl
Anti-CD66b-FITC	BioLegend, Fell, DE	2,5 µl
Anti-CD69-APC	Miltenyi Biotec	5 µl
Anti-CD69-FITC	BD Biosciences	2,5 µl
Anti-CD80-PE	BD Biosciences	2 µl
Anti-CD83-PE	BD Biosciences	6 µl
Anti-Dectin1-PE	R&D Systems, Wiesbaden, DE	2,5 µl
Anti-HLA I-PE	BD Biosciences	7 µl
Anti-HLA II-PE	BD Biosciences	4 µl
Anti-IFNγ-FITC	BioLegend	5 µl
Anti-NKG2D-PE	Miltenyi Biotec	2,5 µl
Anti-NKp30-PE	BioLegend	5 µl
Anti-NKp44-PE	BD Biosciences	2,5 µl
Anti-NKp46-PE	BD Biosciences	2,5 µl
Anti-TLR2-PE	BioLegend	2,5 µl
Anti-TLR4-PE	BioLegend	2,5 µl
Anti-TNFα-APC	Miltenyi Biotec	5 µl
AntiCD94-PE	BioLegend	2,5 µl

Mouse Isotype controls

Anti IgGM-FITC	Miltenyi Biotec
Anti-IgG1-APC	BD Biosciences
Anti-IgG1-FITC	BD Biosciences
Anti-IgG1-PE	BD Biosciences
Anti-IgG1-PerCP	BD Biosciences
Anti-IgG2a-FITC	BD Biosciences
Anti-IgG2a-PE	BD Biosciences
Anti-IgG2b-PE	BD Biosciences

3.1.2.2 Kits, microbeads and cytokines

Annexin V-FITC Apoptosis Detection Kit	BD Biosciences, Heidelberg, DE
Anti CD3 MicroBeads human	Miltenyi Biotec
Anti-FITC MicroBeads	Miltenyi Biotec, Bergisch Gladbach, DE
Cell Trace CFSE Cell Proliferation Kit	Life technologies, Darmstadt, DE
Dynabeads Human T-Activator CD3/CD28	Life technologies, Darmstadt, DE
NK-Cell Isolation Kit	Miltenyi Biotec
Pan T Cell Isolation Kit human	Miltenyi Biotec
Recombinant Human Interleukin-15	ImmunoTools, Friesoythe, DE

3.1.2.3 Buffers, medium and others

Buffer used if not otherwise stated

Hank's Balanced Salt solution Modified	Sigma-Aldrich, Steinheim, DE
1 % Fetal Bovine Serum non-USA origin, sterile-filtered	Sigma-Aldrich
2 mM Ethylenediaminetetraacetic acid disodium salt solution (EDTA) for molecular biology (0,5 M in H_2O)	Sigma-Aldrich

Medium used if not otherwise stated

RPMI 1640 Medium, GlutaMAX Supplement	Life technologies, Darmstadt, DE
10 % Fetal Bovine Serum non-USA origin, sterile-filtered	Sigma-Aldrich, Steinheim, DE
120 µg/ml Refobacin (Gentamicinsulfat), 80 mg Injektionslösung	Merck, Darmstadt, DE

Materials for ELISA

Aqua B. Braun Ecotainer	B. Braun Melsungen, Melsungen, DE
Biotinylated anti-human IL-10 antibody	R&D Systems
Blocking solution	1 % BSA, 5 % sucrose, 0,05 % NaN_3 in 1 x PBS
Bovine serum albumin (BSA) Fraktion V	Carl Roth, Karlsruhe, DE
Detection buffer	150 mM NaCl, 20 mM Trizma base, 0,1 % BSA in H_2O

Monoclonal anti-human IL-10 antibody	R&D Systems
Phosphate-buffered saline (PBS) 10 x	80 g NaCl, 11,6 g Na_2HPO_4, 2 g KH_2PO_4, 2 g KCL in 1 l H_2O
Recombinant human IL-10	R&D Systems
Standard buffer	0,1 % BSA in 1 x PBS
Stop solution	1 M H_2SO_4 in H_2O
Streptavidin buffer	1 % BSA in 1 x PBS
Streptavidin-Horserashish peroxidase (HRP)	R&D Systems
Substrate Reagent Pack	R&D Systems, Wiesbaden, DE
Trizma base	Sigma-Aldrich, Steinheim, DE
Tween 20	Sigma-Aldrich, Steinheim, DE
Washing buffer	0,05 % Tween 20 in 1 x PBS; pH 7,4

Others

Biocoll (Polysucrose 1,077 g/ml)	Biochrom, Berlin, DE
Brefeldin A	Sigma-Aldrich
Cytofix/Cytoperm	BD Biosciences
Erythrocyte lysis buffer	Quiagen, Hilden, DE
Ethanol 70%	Otto Fischar, Saarbrücken, DE
FACS Clean Solution	BD Biosciences, Heidelberg, DE
FACS Flow Sheath Fluid	BD Biosciences
FACS Rinse Solution	BD Biosciences
Trypan Blue Solution 0,4 %, for microscopy	Sigma-Aldrich, Steinheim, DE

3.1.3 Apparatus

BD FACSCalibur	BD Biosciences, Heidelberg, DE
CO_2-incubator HERAcell 240	Thermo Fisher Scientific, Schwerte, DE
Cryogenic storage system K series	Taylor-Wharton, Mildstedt, DE
Fridge and freezer CUP 3021, HERAfreeze	Liebherr, Bulle, CH
GENios Microplate Reader	Tecan
Heraeus Multifuge 3S und 3S-R	Thermo Fisher Scientific, Schwerte, DE
HERAfreeze (-80 °C)	Herareus, Hanau, DE
HERAsafe KS 9 Biological Safety Cabinet	Thermo Fisher Scientific
hydroFLEX microplate washer for 96-well	Tecan, Crailshaim, DE
IKA MS1 Minishaker	IKA-Werke, Staufen, DE
MACS MultiStand	Miltenyi Biotec, Bergisch Gladbach, DE
Microcentrifuge 5415R	Eppendorf, Hamburg, DE
Microscope Eclipse 50i und TS100	Nikon, Düsseldorf, DE
MiniMACS Separator	Miltenyi Biotec
Neubauer improved counting chamber	Paul Marienfeld, Lauda-Königshofen, DE
Pipette filler pipetus	Hirschmann, Eberstadt, DE
Pipettes Eppendorf Reference 10/100/1000 µl	Eppendorf, Hamburg, DE
Quadro MACS Separator	Miltenyi Biotec
Vortex-Genie 2	Scientific Industries, Schwerte, DE

VWR Galaxy Mini centrifuge	VWR, Darmstadt, DE
Waterbath Memmert	Memmert, Schwabach, DE

3.1.4 Other laboratory equipment

Acetate foil for 96 well plate	Sarstedt, Nümbrecht, DE
Biosphere Filter Tips 20 µl und 100 µl	Sarstedt, Nümbrecht, DE
Cell scraper 16 cm 2-position blade	Sarstedt, Nümbrecht, DE
CELLSTAR Cell Culture Flasks 75 cm^2	Greiner Bio-One
CELLSTAR Serological Pipettes 5/10/25 ml	Greiner Bio-One
CELLSTAR Tubes, 15/50 ml	Greiner Bio-One, Frickenhausen, DE
Falcon Polystyrene round-bottom tube, 5 ml, 12 x 75 mm style	Corning, Kaiserlslautern, DE
LS Columns, MS Columns	Miltenyi Biotec, Bergisch Gladbach, DE
Microplate 96 well PS-F Bottom	Greiner Bio-One, Frickenhausen, DE
Microtubes 1,2ml	StarLab, Hamburg, DE
Pipette tip 20 µl, neutral	Sarstedt
S-Monovette blood collection 9 ml K3EDTA	Sarstedt
SafeCeal Micro Tubes 1,5/2 ml	Sarstedt
TipOne 1000 µl Blue, Graduated Tip	StarLab, Hamburg, DE
TipOne 1000 µl XL Graduated Filter Tip	StarLab, Hamburg, DE
TipOne 200 µl Yellow, Bevelled Tip	StarLab
Tissue Culture plate Falcon Flat Bottom With Low Evaporation Lid 6/12/24/96 well	Corning, Kaiserslautern, DE
Transfer pipette 3,5 ml	Sarstedt

3.1.5 Software

- CellQuest Pro (BD Biosciences)
- Flowjo Single Cell Analysis software (TreeStar)
- Microsoft Excel
- Xfluor 4 with Sunrise reader (Tecan)

3.2 Methods

3.2.1 Cell isolations

All immune cells were isolated from healthy volunteer donors. HBSS buffer supplemented with 1 % inactivated FCS and 2 mM EDTA was used for cell isolations if not otherwise stated. The FCS was heated before use to inactivate complement. It was stored at 55 °C for 30 minutes. For washing steps, if not otherwise stated, tubes were filled up with buffer and centrifuged at 300 x g for 10 minutes at maximal acceleration and deceleration. For the magnetic bead separation procedures, all reagents were cooled. Otherwise, buffers were kept at RT. A Neubauer chamber was used to count cells.

3.2.1.1 Generation of PBMCs

Peripheral Blood Mononuclear Cells (PBMCs) in healthy individuals contain around 70 % T cells, 10 % B cells, 10 % monocytes, 1 % dendritic cells and 1-15 % natural killer (NK) cells [240, 241]. If not otherwise stated, PBMCs were generated from the fresh peripheral blood of healthy volunteer donors. 50-150 ml whole blood were drawn into blood collection tubes with EDTA anticoagulant. The blood was collected in 50 ml tubes and diluted 1:1-2:1 with pre-warmed buffer. Each 25 ml of diluted blood were then layered onto 25 ml separating solution in new 50 ml tubes for density gradient centrifugation (2000 rpm (120 x g), 20 minutes, minimal acceleration/deceleration). The separating solution contains a polymer with a molecular weight of 400 kDa, resulting in a density of 1,077 g/ml. The density of plasma is lower (around 1,025 g/ml) [242], the density of the cellular components differs between cells (Figure 1-A). During centrifugation, the cells sink down according to their density. The PBMCs, containing monocytes and lymphocytes, form a thin layer between the plasma and the separating solution, while neutrophils and erythrocytes sink to the bottom (Figure 1-B). After separation, the PBMCs were collected into 50 ml tubes using a transfer pipette (PBMCs from two tubes were pooled into one). The PBMCs were washed three times with buffer to remove platelets (120 G, 15 minutes, minimal acceleration/deceleration) and then all pooled into one tube. The PBMCs were then counted, washed and re-suspended in fresh buffer at a concentration of 1×10^6 cells/ml for direct FACS analysis or 1×10^7/80 µl for CD3 depletion according to the MDSCs isolation protocol (3.2.1.3).

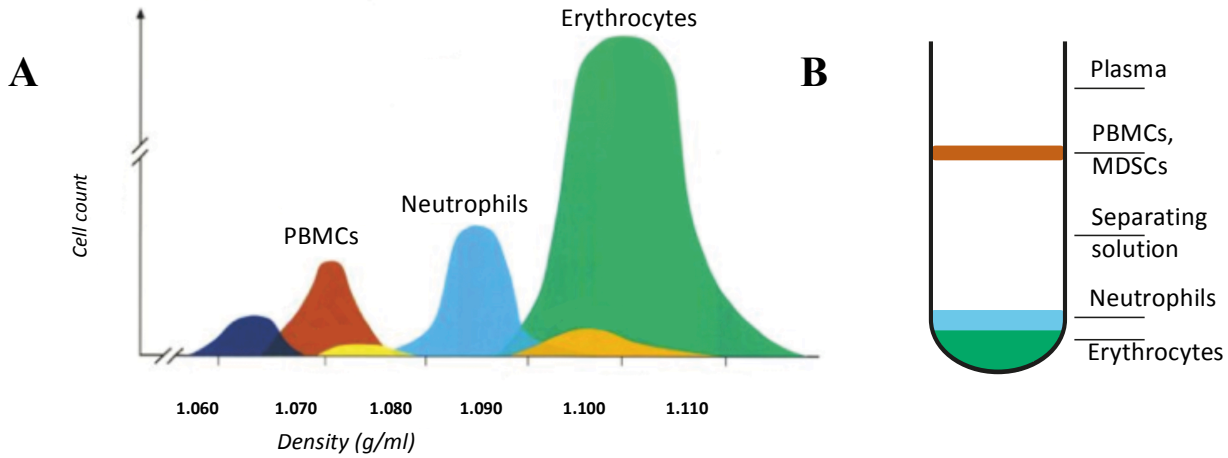


Figure 1: Density gradient centrifugation. A shows densities and counts of different cell populations in the peripheral blood (source: www.biochrom.de). **B** shows layers after density gradient centrifugation.

Only PBMCs derived from fresh whole blood contained MDSCs

Many authors have used the feature of a low density to isolate $CD66b^{pos}$ human myeloid-derived suppressor cells (MDSCs) from peripheral blood mononuclear cells (PBMCs) [155, 162-167]. I considered two sources for human PBMCs: fresh whole blood and leucocyte concentrates, both derived from healthy donors. The concentrates were derived from left-overs (from the filter used for platelet apharesis) of platelet donations. They were produced by leukocyte reduction system chambers (LRSC), which are a validated source for human lymphocytes, monocytes and dendritic cells [243, 244]. However, granulocytic cells are more fragile and are known for their short lifespan. To find out whether the concentrates are suitable to use for MDSCs isolation, I generated PBMCs from both LRSC and whole blood by density gradient centrifugation, and compared the contained cell populations by flow cytometry (for staining protocol see 3.2.3.2).

LRSC-derived PBMCs presented as two populations in the forward- and side-scatter (FSC-SSC), distinguishable by size and granularity: the population of larger and more granulated cells co-expressed CD14, HLA II and HLA I, representing monocytic cells. The population of smaller and less granulated cells represented lymphocytes. Most of them were $CD3^{pos}$ HLA II^{neg} HLA I^{pos} T cells, and a very variable fraction comprised $CD3^{neg}$ $CD56^{pos}$ NK cells (data know shown). T cells and NK cells could not be distinguished in the FSC-SSC alone. To visualize MDSCs, I scanned the PBMCs for CD66b expression. I detected no $CD66b^{pos}$ cells within the LRSC-derived PBMCs (Figure 2-A). PBMCs generated from fresh blood, on the other hand, contained a small

population of CD66b^{pos} cells. The cells were larger and more granulated compared to monocytes or lymphocytes, representing granulocytic MDSCs (Figure 2-B). Thus, only fresh blood but not leucocyte concentrates contained MDSCs, supposedly because of the limited viability of granulocytic cells. I therefore used fresh blood to isolate MDSCs. 100 ml of fresh blood yielded around 2 x 10^8 PBMCs in average (SEM = 1,4 x 10^7; n = 34). The percentage of MDSCs extracted from PBMCs varied between 0,2 % and 10 % and was 2 % in average (SEM = 0,3 %; n = 23), which is in accordance with previous observations [245].

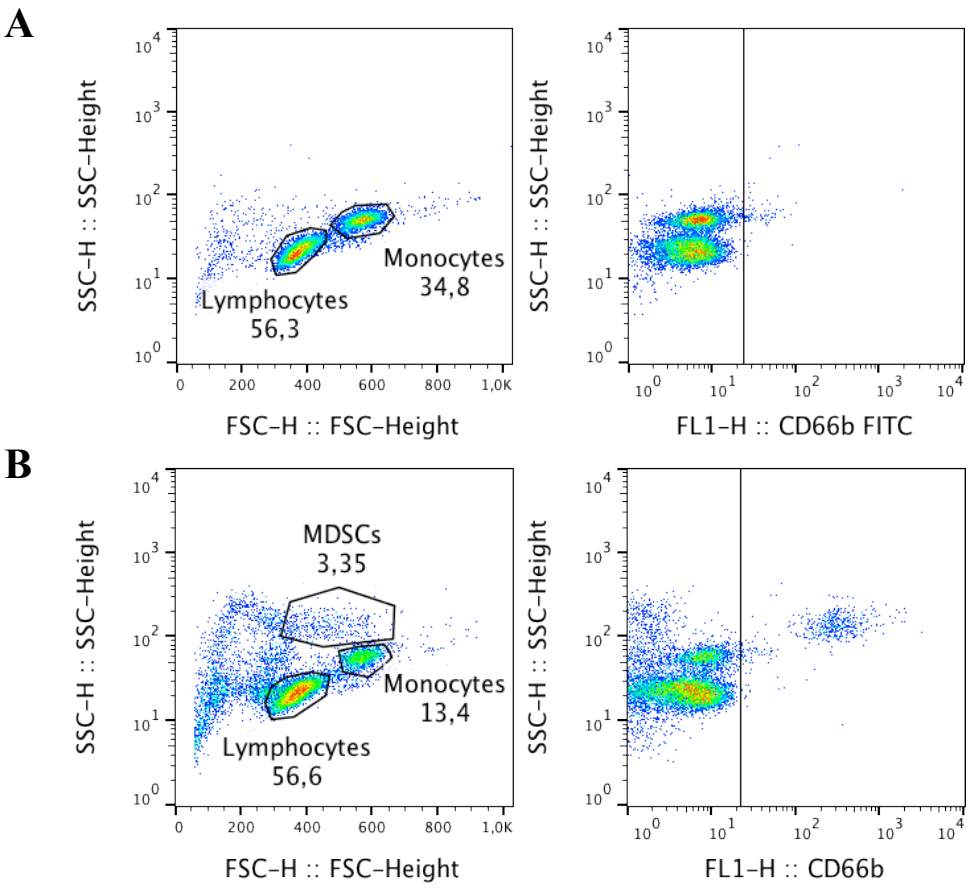


Figure 2: Only PBMCs derived from fresh blood but not from leucocyte concentrates contained MDSCs. PBMCs from either leucocyte concentrates (**A**) or fresh blood (**B**) were generated by density gradient centrifugation and stained with anti-CD66b antibody followed by flow cytometric analysis. Figures show forward scatter (FSC), side scatter (SSC) and CD66b expression of PBMCs for representative donors. Gates on the right indicate CD66b-positive cells normalized to isotype. Gated cell frequencies are indicated in %.

3.2.1.2 Principles of magnetic cell isolation

The procedure of magnetic-activated cell sorting (MACS) can be used to separate certain cell populations within a suspension from others, according to their specific surface antigens. Magnetic nanoparticles called microbeads are linked to antibodies that are

directed against these antigens. In a first step, these microbeads are added to the cell suspension and specifically attach to the cells that are to be selected. In a second step, the suspension is passed through a column exposed to a strong magnetic field, which separates labeled from non-labeled cells (Figure 3). In a positive selection, the labeled cells are the desired cell population. The rest of the suspension passes through the column and is discarded. The labeled cells are then harvested from the magnet by using mechanic pressure. In a negative selection, the desired cells are the only non-labeled cell population and are therefore purified in the flow-through. The cells bound to the column are discarded. Instead of microbeads directed against surface antigens directly, microbeads directed against fluorochromes can also be used. In this case, the specific antigens on the cell surface are labeled with fluorochromes beforehand.

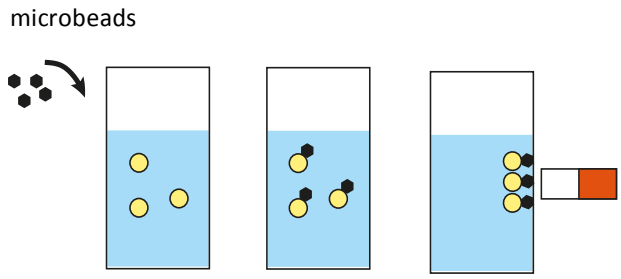


Figure 3: Principle of magnetic-activated cell sorting (MACS). Cells to be isolated (positive selection) or depleted (negative selection) are labeled with magnetic microbeads directed against specific surface antigens. The cell suspension is then passed through a column that is adjusted to a magnet, letting non-labeled cells pass while labeled cells remain in the column.

3.2.1.3 Isolation of MDSCs

MDSCs were isolated from PBMCs (3.2.1.1) by CD66b positive selection, as it has been described [155, 162-167]. While mature granulocytes are high in density, MDSCs exhibit a low density and form part of the PBMC layer after density gradient centrifugation (Figure 1-B). To separate MDSCs from PBMCs, I stained the cells with FITC fluorochrome-labeled antibodies directed against the surface protein CD66b, which is a granulocyte marker. After FITC-staining, I added anti-FITC magnetic microbeads to label $CD66b^{pos}$ cells. $CD66b^{pos}$ cells were then separated using MACS separator (positive selection) following the manufacturer´s protocol.

I then analyzed the MDSCs by flow cytometry (for staining protocol see 3.2.3.2). In the FSC-SSC, three populations were distinguishable. The majority of cells, consisting of two distinct populations, were $CD66b^{pos}$. However, a third population was $CD66b^{neg}$. These cells within the lymphocyte gate of the FSC-SSC expressed CD3, and thus represented T cells. The population varied in size between 5 and 15 % in different donors (Figure 4-A). To eliminate this population for the experiments, PBMCs were depleted of

$CD3^{pos}$ cells using anti-CD3 microbeads prior to MDSCs isolation. Less than half the amount of PBMCs remained after CD3-depletion, which is in line with the usual percentage of T cells within PBMCs [246]. I verified the success of the depletion by flow cytometry. Using this protocol, I could improve the purity of MDSCs (Figure 4-B). The steps in detail are described below, for a cell count of 10^7 PBMCs.

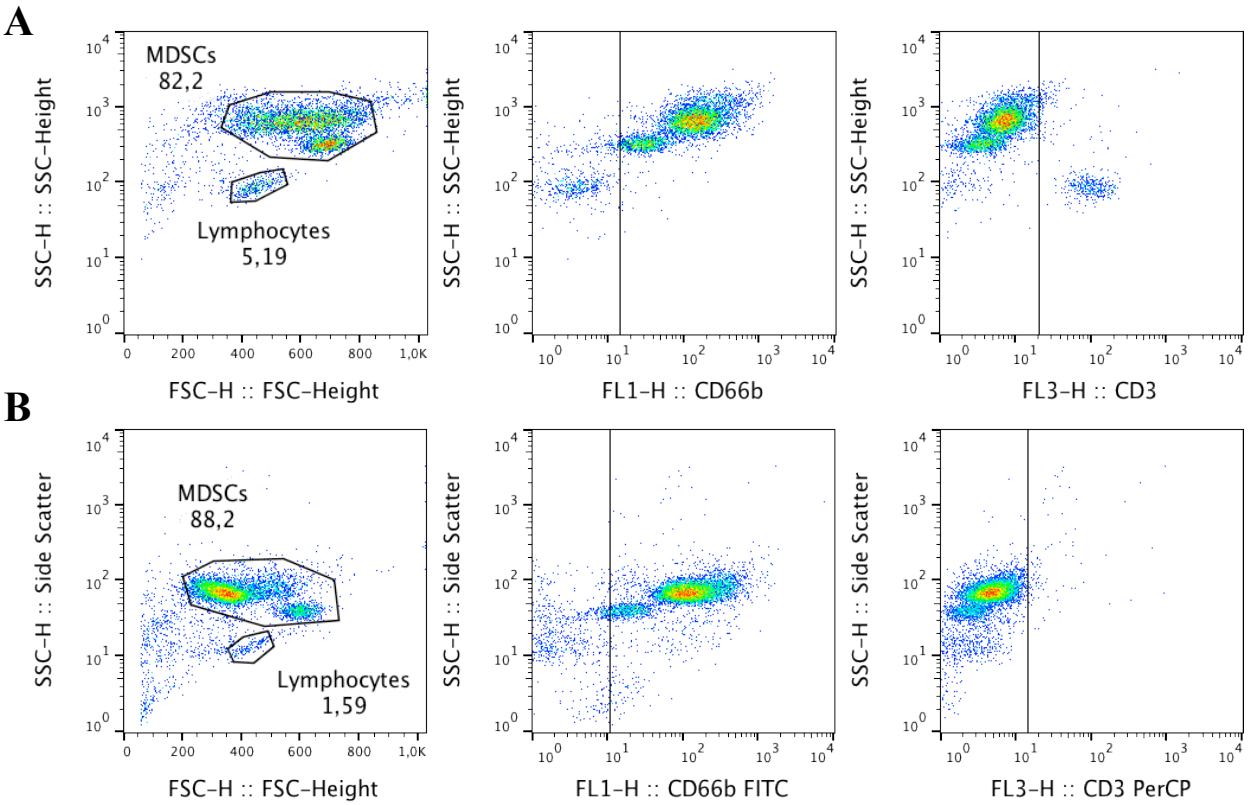


Figure 4: Cell populations after MDSCs isolation. MDSCs were isolated from PBMCs by CD66b positive selection alone (**A**) or by CD3-depletion and CD66b positive selection (**B**). Cells were then stained with antibodies against CD66b and CD3 and analyzed by flow cytometry. Figures show forward scatter (FSC), side scatter (SSC), and expressions of CD66b and CD3. Gates on the right show marker-positive cells normalized to isotypes. Gated cell frequencies are indicated in %.

10^7 PBMCs were suspended in 80 µl buffer and 20 µl CD3 microbeads were applied. Cells were incubated at 7 °C for 15 minutes and washed. Afterwards, I suspended the cells in 500 µl buffer and applied them to magnetic LD columns. The columns were washed before and after application, according to official instructions. I collected the flow-through into a 50 ml tube and counted the cells. The success of the CD3 depletion was verified by flow cytometry. Then, I suspended each 10^7 T cell-depleted PBMCs in 100 µl buffer and added 20 µl anti-CD66b-FITC antibody. Cells were incubated 10 minutes at RT. Subsequently, I washed the cells, re–suspended them in 90 µl and added 10 µl anti-FITC microbeads. After 15 minutes of incubation on ice, cells were washed at 4 °C. I re–

suspended the pellet in 500 µl buffer and applied the suspension onto an MS magnetic column. The flow-through with $CD66b^{neg}$ cells was collected into a 50 ml tube for later NK cell isolation. The $CD66b^{pos}$ cells sticking to the column were harvested by pressure according to official instructions. I counted MDSCs and suspended them in medium at a concentration of 2 x 10^6 cells/ml. The purity was verified by flow cytometry according to CD66b expression and morphology.

3.2.1.4 Isolation of NK cells

NK cells were isolated from the remaining PBMCs after depletion of $CD3^{pos}$ and $CD66b^{pos}$ cells (3.2.1.3) with the help of a specific NK cell magnetic isolation kit (Miltenyi Biotec). The cell suspension was labeled with a cocktail of antibodies binding antigens on PBMCs other than NK cells (negative selection). In detail, each 10^7 cells were suspended in 40 µl buffer and incubated with 10 µl of NK Cell Antibody Cocktail solution at 7 °C. After 10 minutes, 30 µl buffer and 20 µl of NK Cell MicroBead Cocktail were applied for another incubation time of 15 minutes at 7 °C. This cocktail contains microbeads directed against the antibodies applied, attaching magnetic beads to all non-NK cells. I washed the cells at 4 °C and re-suspended them in 500 µl buffer for application onto an LS magnetic column. The column was washed according to official instructions and the flow-through with negatively selected NK cells was collected in a tube. NK cells were counted, washed and suspended in medium at a concentration of 2 x 10^6 cells/ml.

The NK cell yield from PBMCs varied between 1 % and 10 % in different donors and was 4 % in average (SED = 0,5 %; N = 23), which is a bit lower than the expected fraction of NK cells within human PBMCs, supposedly due to cell losses during the isolation steps [247]. The purity after isolation was verified by flow cytometry (for staining protocol see 3.2.3.2). As lymphocytic cells, NK cells resemble T cells in size and granularity and cannot be distinguished from T cells in the FSC-SSC. They are commonly defined as $CD56^{pos}$ $CD3^{neg}$ cells [15] or $NKp46^{pos}$ $CD3^{neg}$ [248]. These characteristics applied to 90-100 % of cells after isolation (Figure 5). Because $CD3^{pos}$ cells were depleted before the NK cell isolation, and the number of remaining $CD3^{pos}$ cells was marginal, I limited the definition of NK cells to CD56 positivity or NKp46 positivity in the experiments. $CD56^{pos}$ and/or $NKp46^{pos}$ cells were considered as NK cells.

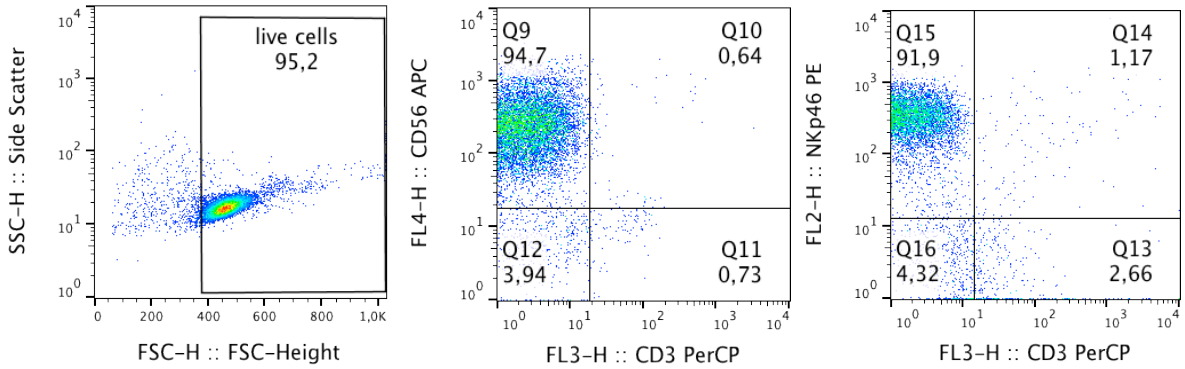


Figure 5: Purity of NK cells. NK cells were isolated from PBMCs by CD3 and CD66b depletion and a specific NK cell magnetic isolation kit. NK cells were stained for CD56, NKp46 and CD3 and analyzed by flow cytometry. Figure shows forward scatter (FSC), side scatter (SSC) and the expressions of CD3, CD56 and NKp46 for a representative donor. Quadrants indicate marker-positive- and -negative cells normalized to isotypes. Gated cell frequencies are indicated in %.

Depletion of MDSCs/PMNs from co-culture

In some experiments, NK cells were co-cultured with MDSCs or PMNs, and the latter were depleted from the cell suspension to re-purify NK cells afterwards. The same NK cell isolation kit used as for the isolation of NK cells from PBMCs (3.2.1.4). The success of the depletion was verified by flow cytometry (for staining protocol see 3.2.3.2).

While this procedure allowed good depletion of MDSCs (Figure 6-A), PMNs could not be depleted entirely (Figure 6-B). Therefore, I performed an additional CD66b negative selection step using FITC-coated anti-CD66b antibodies and anti-FITC microbeads (3.2.1.3). Depletion of both MDSCs and PMNs was successful using this protocol and a good NK cell purity could be achieved (Figure 6-C).

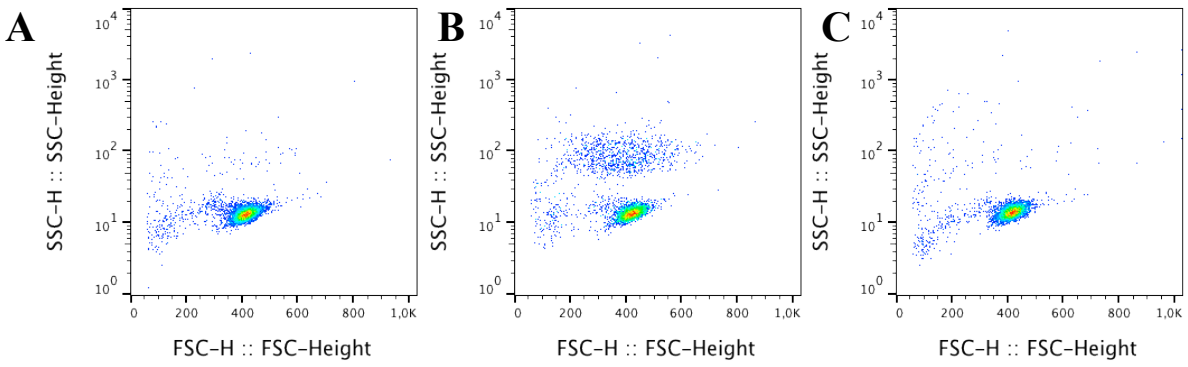


Figure 6: Successful re-purification of NK cells after co-culture. NK cells were re-purified after 16 h of co-culture with MDSCs and PMNs using an NK cell kit and subsequent CD66b depletion. Cells were then analyzed by flow cytometry. Figures show NK cells in forward scatter (FSC) and side scatter (SSC) for a representative donor. **A** shows NK cells after co-culture with MDSCs and subsequent re-purification using NK cell kit. **B** shows NK cells after co-culture with PMNs and re-purification using NK cell kit. **C** shows NK cells after co-culture with PMNs and re-purification using NK cell kit and CD66b depletion.

3.2.1.5 Isolation of T cells

T cells were isolated from PBMCs derived from leukocyte concentrates. The concentrates were derived from platelet donations at the Universitaetsklinikum Wuerzburg, Institut fuer Transfusionsmedizin und Haemotherapie, where leucocytes are removed with the help of leukocyte reduction system chambers (LRSC). The advantage of these "leftovers" is that no separate blood donations are necessary, and they are a validated source for human lymphocytes, monocytes and dendritic cells [243, 244]. The concentrates were poured into 50 ml tubes and filled up with buffer. From here, PBMCs were generated as described above (3.2.1.1). PBMCs were suspended in medium 5 x 10^7/ ml and stored at -80 °C for a couple of days for later T cell isolation.

T cells were isolated from frozen PBMCs by magnetic negative selection. Although the CD3 molecule can be used to define and isolate T cells by positive selection, as it is expressed by all T cell subsets and no other immune cells [249], binding of CD3 would activate the cells. Therefore, T cells were isolated from PBMCs by magnetic negative selection, with the help of a specific Pan T Cell Isolation Kit (Miltenyi Biotec) containing an antibody cocktail directed against non-T cell populations, and a microbead cocktail with secondary, magnetic antibodies. The isolation was performed according to the manufacturer's instructions. In brief, frozen PBMCs were suspended in warm medium to thaw. They were then washed with buffer, counted and suspended in buffer at a concentration of 1 x 10^7 cells/40 µl. 10 µl of the antibody cocktail were added and cells were incubated at 4 °C for 5 minutes. 30 µl of buffer and 20 µl of microbead cocktail were added and cells were incubated at 4 °C for 10 minutes. Afterwards, buffer was added to a total volume of 200 µl followed by magnetic negative selection with the MACS separator using an LD column. After isolation, the T cells were counted and suspended in buffer without supplements at a concentration of 4 x 10^7 cells/ml for CFSE staining (3.2.5).

3.2.1.6 Isolation of PMNs

Polymorphonuclear neutrophils (PMNs) were generated from fresh blood by density gradient centrifugation (3.2.1.1) [116]. During density gradient centrifugation, mature PMNs migrate to the bottom of the tube to form a thin leucocyte layer on top of the erythrocytes (Figure 1-B). Cells from this layer were collected into a 50 ml tube (around

5 ml volume) with a transfer pipette. Erythrocytes were eliminated by application of 40 ml of erythrocyte lysis buffer and incubation of 25 minutes at RT. After three washing steps, PMNs were counted and re-suspended in fresh medium at a concentration of 2 x 10^6 cells/ml. The purity of isolated PMNs was verified by flow cytometry (for staining protocol see 3.2.3.2). PMNs presented as large, granulated cells in the FSC-SSC. The granulocyte marker CD66b served as a purity control and was expressed by > 95 % of the cells (Figure 7).

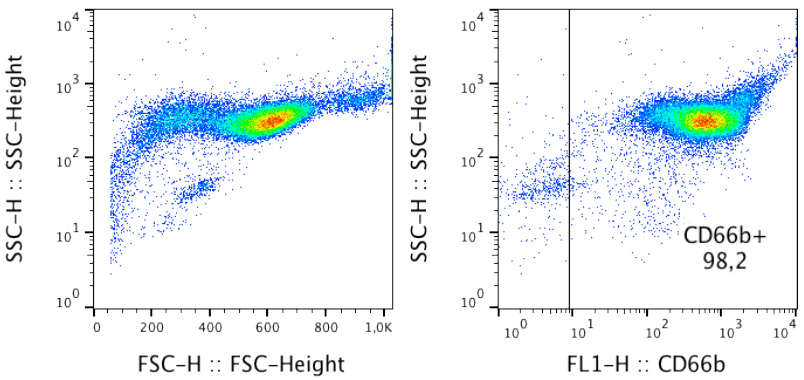


Figure 7: Purity of PMNs after isolation. PMNs were isolated from fresh blood using density gradient centrifugation and erythrocyte lysis. Figure shows forward scatter (FSC), side scatter (SSC) and CD66b expression of all cells for a representative donor. Gate on the right indicates CD66b-positive cells normalized to isotype in %.

3.2.2 Cell culture

For cell culture, I used RPMI medium supplemented with gentamicin 120 µg/ml and 10 % heat inactivated FCS. The medium was pre-warmed to 37 °C in a water bath. Cells were cultured in cell culture plates at a density of 1 x 10^6 cells/ml and were kept at 37 °C in a CO_2 incubator (5 % CO_2, 90 % humidity).

3.2.2.1 Co-culture of lymphocytes with granulocytic cells

NK cells were cultured together with MDSCs or PMNs over 3 h, 5 h or 16 h at different ratios (1:2, 1:1 or 2:1). The concentration of NK cells in the co-culture was 1 x 10^6 cells/ml. NK cells incubated in medium served as negative control, NK cells incubated in medium supplemented with IL-15 1000 IU/ml as a positive control. T cells were cultured together with MDSCs or PMNs 1:1 over 72 h. The concentration of T cells in the co-culture was 5 x 10^5 cells/ml.

3.2.2.2 Co-culture of NK cells with target

The cell line K562 is often used as NK cell target in experimental settings [22]. The cells are undifferentiated, highly proliferative myeloid blasts, which were derived from a 53 year old female patient with end stage chronic myeloid leukemia in a blast crisis in 1970 [250]. Because they do not express MHC I, K562 cells are highly immunogenic to

NK cells. They have indefinite proliferation potential and lack genetic variability. Therefore, they are cheap, easy to handle and show a consistent behavior. However, their characteristics may substantially differ from that of primary cells in vivo. K562 cells were cultured in 25 ml medium (at a concentration of 1 x 10^6 cells/ml) in 75 cm^2 culture flasks and were split every two days. To evaluate the function of NK cells, the latter were incubated with K562 cells at an effector-to-target ratio of 10:1 for 5 h at 37 °C. The concentration of NK cells in the culture was 1 x 10^6 cells/ml.

3.2.3 Flow cytometry

3.2.3.1 Principles of flow cytometry

Flow cytometry can serve to assess morphological and chemical characteristics of cells. The procedure is sometimes referred to as FACS (fluorescent activated cell sorting), although this term actually describes a specialized form of flow cytometry. Inside a cytometer, cells are passed through a narrow channel one by one and get hit by different lasers. This results in changes in the wavelength of the light, which are measured and analyzed. The size and granularity of cells correlate with diffraction of the light. The detector assessing the size of the cells detects light in the forward direction along the axis of the incident light (FSC). The side scatter (SSC) detects light that is refracted to the side, at a 90 degree angle from the incident light. The amount of refracted light is related to the granulation of the cells.

Beside the cell morphology, flow cytometry can identify immune cell subtypes and evaluate functional characteristics of cells by assessing specific cell surface antigens (for example the surface antigen CD69 indicating activation of lymphocytes). For this purpose, specific antibodies directed against these antigens are used, which interfere with the laser light. Antibodies, or immunoglobulins, are glycoproteins consisting of two heavy and two light chains connected via disulfide bonds that form a constant and a variable domain, the latter enabling specific antigen binding. Antibodies can be divided into 5 classes (and several subclasses) according to their heavy chains: IgG, IgA, IgM, IgD and IgE. IgG is used most commonly in flow cytometry. Antibodies can be monoclonal (meaning all antibody molecules are identical and recognize the same epitope on an antigen) or polyclonal (molecules recognize different epitopes). While polyclonal antibodies can provide higher signals and are less expensive in production, the advantage of monoclonal

antibodies is their higher specificity. The antibodies used in my experiments were monoclonal. Since antibodies themselves do not interfere with the laser, they are linked to fluorochromes. Fluorochromes absorb light of a specific wavelength (WL) from a laser and emit light of a longer, less energetic wavelength. The amount of emission correlates with the amount of fluorochrome and thus the amount of antigen on the cell surface. The wavelength of the laser does not equal the measured wavelength in this case. Different fluorochromes exhibit different excitation and emission ranges. In my experiments, FITC, PE, PerCP, PerCP-Cy 5.5 and APC were used. The FACS machine is provided with two lasers: blue (WL 488) and red (WL 635). FITC, PE and PerCP-Cy5.5 can be excited by the blue laser. APC requires excitation by the red laser. A cell can be stained with several fluorochromes, enabling the analysis of co-expressions of antigens. The fluorescence emitted by the 4 different fluorochromes is measured in 4 detection channels (FL1-4) (Figure 8-A).

Compensation

The detectors are equipped with different filters, to measure the fluorescence emitted by a certain fluorochrome at a specific WL. For example, FL1 detects light at a WL of 530, which is emitted mainly by FITC. FL2 detects light at a WL of 585, which is emitted mainly by PE. However, the fluorochromes do not exclusively emit light of these specific WLs. FITC-derived signals are not only detected in FL1, but to also in FL2. A signal derived from FITC might thus resemble a positive signal for PE, causing false-positive results (Figure 8-B). This phenomenon affects the combinations FL1-FL2, FL2-FL3 and FL3-FL4, and correlates with signal intensity. When using several fluorochromes, this spectral overlap of emitted light must be compensated. For this purpose, cells are stained with each antibody separately (4 compensation probes for 4 fluorochromes). With the help of a special circuitry in the cytometer, the overlapping signals from other fluorochromes can be assessed and subtracted from the specific signal. Since the overlap correlates with the signal intensity, the condition with the strongest expected signal should be chosen for compensation (for example interleukin-stimulated cells if detecting the expression of an activation marker).

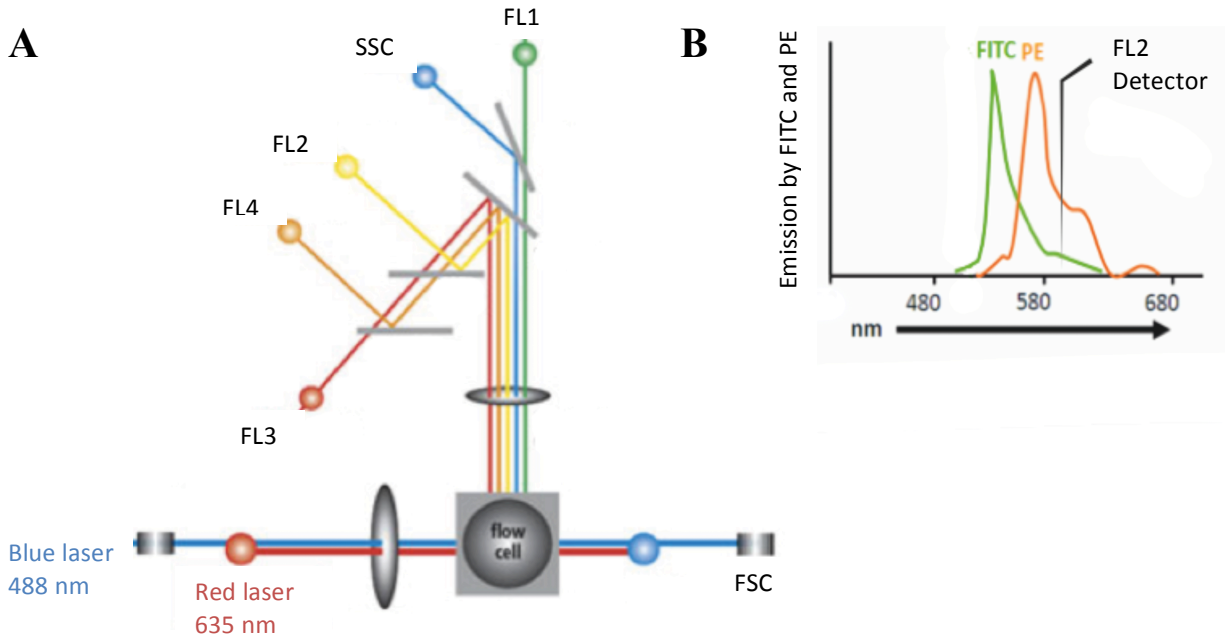


Figure 8: Principles of FACS. A: The cell surface labeled with fluorochromes is hit by 2 lasers, red and blue. Emitted light is detected in 6 detection channels: FSC (forward scatter), SSC (side scatter), FL1, FL2, FL3 and FL4. Source: www.bdbiosciences.com. **B**: Emission of the fluorochromes FITC and PE after excitation with blue laser. FL2 detector detects mainly PE-emission but also FITC-emission. Source: Blizard Institute of Cell and Molecular Science.

Isotypes

Although the strongest binding occurs between an antibody's specific Fab fragment and the according antigen, other, unspecific bindings must be taken into account (Figure 9). For this purpose, the cells are stained with isotype control antibodies. These exhibit the same characteristics as the specific antibodies, including the fluorochrome labeling, but except for the Fab fragment. During the analysis of FACS data, the unspecific signals derived from isotype control antibodies are subtracted from the specific signals.

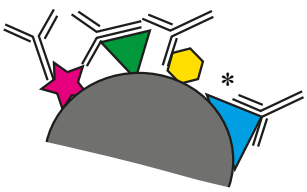


Figure 9: Unspecific vs. specific antibody binding sites. Antibodies bind antigens specifically via Fab (asterisk). Other, unspecific binding must be taken into account when analyzing FACS data. Signals derived from unspecific binding are measured with isotype control antibodies and subtracted during analysis.

3.2.3.2 Staining of surface and intracellular proteins

Surface staining

For flow cytometric analysis of surface markers, cells were suspended in buffer at a concentration of 1 x 10^6 cells/ml. 50 µl of the suspension was transferred to each of 5 ml-tube (5 x 10^4 cells), 50 µl buffer were added to achieve a volume of 100 µl per tube. Beside the different conditions, samples for unstained probes, isotype stainings and

stainings for compensations were prepared. Previously titrated staining antibodies and isotype control antibodies were applied and cells were incubated at 4 °C for 15 minutes. Tubes were washed twice with 2 ml buffer afterwards (centrifugation 5 minutes at 300 x g). The supernatant was discarded and the cells re-suspended in 200 µl. The probes were then measured with FACSCalibur.

Intracellular staining

Antigens that are not expressed on the cell surface can be assessed in the intracellular space. The cells must be lysed during the staining procedure to allow intracellular binding of the antibodies. Beforehand, if the target is a protein produced by the cell (like the cytokines IFNγ and TNFα), a substance inhibiting secretion of the produced protein must be added to the cultures to allow intracellular accumulation. Brefeldin A inhibits the transport of proteins from the endoplasmatic reticulum to the Golgi apparatus, an indispensable step for secretion (Lippincott-Schwartz, Klausner 1989). Brefeldin A was applied at a concentration of 10 µg/ml to NK cell cultures 9 h before harvesting.

First, surface stainings were performed (see above). Afterwards, cells were washed, re-suspended in 200 µl buffer and incubated with 250 µl Cytofix/Cytoperm for 20 minutes at 4 °C. This reagent induces both fixation and permeabilization of the cells. Then, cells were washed twice and re-suspended in 100 µl buffer. Intracellular antibodies and isotype control antibodies were added to incubate 30 minutes in the dark at RT. After another washing step, the samples were measured with FACSCalibur.

3.2.3.3 Staining of apoptotic cells

Flow cytometry also allows assessment of apoptotic cells. Annexin V identifies cells undergoing apoptosis by binding phosphatidylserines. These are translocated from the inner to the outer leaflet of the plasma membrane in the early phase of apoptosis, allowing for annexin V binding in the presence of calcium (Figure 10). Annexin V staining can be combined with Propidium iodide (PI) staining. PI intercalates into the DNA and RNA of dead cells where the nucleic acids are exposed. I used FITC-labeled annexin V. PI is a fluorochrome itself and its emission is detected by the FL3 channel. Cells positive for both annexin V and PI are either dead, in the end stage of apoptosis or undergoing necrosis. Cells that are annexin V^{pos} but PI^{neg} are at an earlier stage of apoptosis.

For annexin V/PI staining, a specific kit was used (BD Biosciences). The cells were washed twice with annexin V binding buffer containing Ca^{2+}. Cells were re-suspended in 100 µl and annexin V-FITC was applied. PI and anti-NKp46-PE antibody were added in some experiments. In this case, samples for compensations and isotype controls were also prepared. All samples were incubated for 15 minutes at RT. Before measurement, 400 µl of annexin V binding buffer were applied to the tubes.

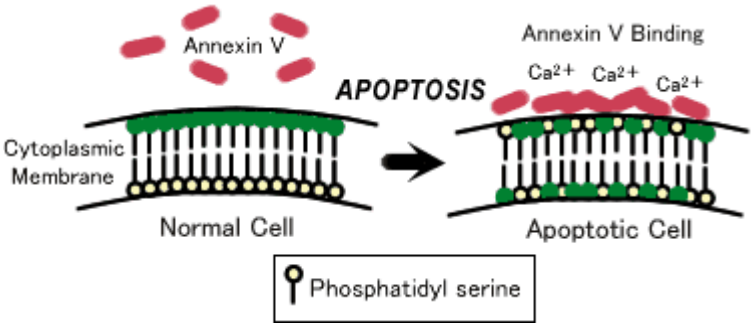


Figure 10: Staining with annexin V to assess apoptosis. Annexin V binds phosphatidylserines on the cell surface of apoptotic cells in the presence of calcium. Source: biocat.com.

3.2.3.4 Analysis of FACS data

After signal acquisition, the FACS data were analyzed using the software FlowJo. The morphology of the cells was evaluated in the FSC and SSC. Because apoptotic and dead NK cells change their morphology, the viability of NK cells was also evaluated using the FSC and SSC [251-253]. Analyzing the expression of specific markers, cells were considered positive only when the signal exceeded the signals from isotype controls, or in some cases from unstained cells (CFSE, annexin V and PI). Either the percentage of marker-positive cells or the geometric mean of the fluorescence intensity in the overall cell population was compared between the conditions. For the T cell proliferation assay (3.2.5), the division index was used, which represents the average number of cell divisions per cell (reductions in CFSE fluorescence intensity by half). NK cells, T cells and granulocytic cells were defined by their morphology as well as surface marker expressions. $CD3^{neg}CD56^{pos}$ or $CD3^{neg}NKp46^{pos}$ cells were considered NK cells, $CD3^{pos}$ cells were considered T cells, $CD66b^{pos}$ cells were considered granulocytic cells. For statistical analysis, a paired samples student's *t*-test was used. The *p*-values were labeled as follows: *<0,1, **<0,05, ***<0,01, ****<0,001.

3.2.4 NK cell cytotoxicity assay

NK cells kill tumor cells via the secretion of cytotoxic granules, which contain perforin and proteolytic enzymes. The process can be assessed by extracellular measurement of the

protein CD107a, which resides within the granule membrane and becomes exposed upon degranulation (Figure 11). Its expression correlates with the cytotoxic function of NK cells [254]. I used APC-labeled antibodies directed against CD107a for the cytotoxicity assay. Because CD107a can become re-internalized, staining at the end of the co-culture would not be an accurate measurement for cytotoxicity. For that reason, the staining antibody was added at the beginning of co-culture.

NK cells were co-cultured with K562 cells at an effector-to-target ratio of 10:1 for 5 h in 5 ml FACS tubes, 2 x 10^5 NK cells in 200 µl. Antibodies directed against CD107a or corresponding isotype control antibodies were added at the beginning of the culture. After the incubation time, cells were washed, suspended in 100 µl buffer and anti-NKp46-PE antibody was added in order to identify NK cells. The surface staining procedure was performed as mentioned above (3.2.3.2). Only cells exhibiting high signals for CD107a (high degree of degranulation) were considered as cytotoxic [255].

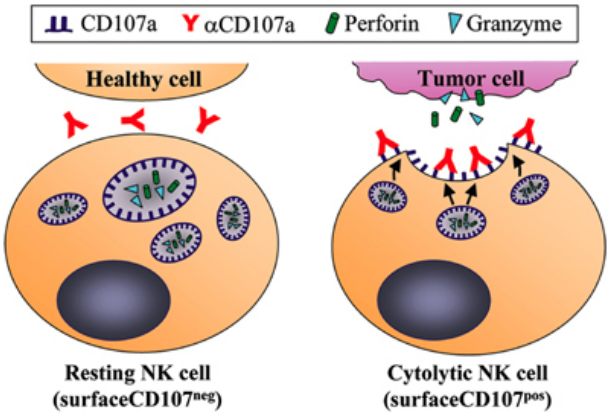


Figure 11: Staining for CD107a to assess degranulation of NK cells. Upon degranulation in response to a target, the protein CD107a, which forms part of the granule membrane, is exposed on the cell surface of NK cells. The process can be measured with the help of staining antibodies and flow cytometry. Source: Leukemia [1].

3.2.5 T cell proliferation assay

T cells are characterized by their specific T cell receptor, which mediates activation signaling. It recognizes tumor- or pathogen-associated antigens, which are presented on MHC II molecules of antigen-presenting cells. However, the T cell receptor itself does not mediate intracellular signaling. Instead, it forms a complex with chains of the CD3 molecule and the ζ chain, which contain intracellular activation motifs and are essential for an activation via the T cell receptor [256]. CD28 presents an important co-stimulatory receptor on the T cell surface, which binds ligands on activated antigen-presenting cells [257]. The engagement of either CD3 or CD28 (if stimulated by superagonistic antibodies) alone can be sufficient to stimulate T cells in vitro [140, 258]. However, the combination of both anti-CD28 and anti-CD3 antibodies is an established approach to mimic the

physiologic stimulation by antigen-presenting cells [259]. To measure T cell proliferation with the help of a CFSE-based cell proliferation assay, I stimulated T cells with Dynabeads binding CD28 and CD3 [260]. I stained the T cells with the fluorescent cell staining dye CellTrace CFSE before culture according to the manufacturer's instructions. The dye binds intracellular amines. By each cell division, the CFSE molecules per cell decrease. The staining thereby enables the visualization of multiple cell generations (reductions of fluorescence intensity by half) in flow cytometry (Figure 12).

After isolation (3.2.1.5), T cells were suspended in buffer without FCS at a concentration of 4 x 10^6 cells/ml and incubated with 2,5 µM CFSE for 10 minutes at RT in the dark (25 µl of the cell suspension were left unstained as a reference). 1 ml pre-warmed FCS was then applied to stop the staining. Cells were incubated for 2 minutes with FCS, then 5 ml of medium were added and cells were washed in medium twice. T cells were then counted again and suspended in medium at a concentration of 1 x 10^6 cells/ml for culture. They were incubated for 72 h in medium alone, with MDSCs or PMNs (1:1 and 10:1), each time with and without application of 1,5 µl Dynabeads. MDSCs sand PMNs were also incubated alone, to show that they do not express CD3. After 72 h, the cells were transferred into 1,2 ml tubes and stained with anti-CD3-PerCP to identify T cells. Samples with MDSCs and PMNs alone were stained with anti-CD66b-FITC according to the surface staining protocol (3.2.3.2). Cells were then analysed in flow cytometry. T cell proliferation was quantified by calculation of the division index, which is the average number of cell divisions (= reductions of CFSE fluorescence intensity by half) that a cell in the original population has undergone.

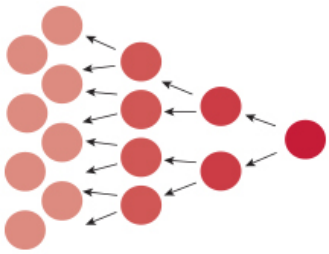


Figure 12: Visualization of cell divisions using CFSE dye. CFSE binds intracellular amines and is diluted by cell divisions. FITC-labeled CFSE antibodies serve to assess multiple cell generations. Reductions of the FITC fluorescence intensity by half correlate with cell divisions. Source: lifetechnologies.com.

3.2.6 IL-10 ELISA

The enzyme-linked-immunosorbent assay (ELISA) is a technique that determines the amount of a certain antigen in a sample. In a Sandwich ELISA, the antigen is captured between two antibodies: the capture antibody provides fixation onto a microtiter plate,

while the detection antibody allows color reaction. Major steps in the protocol are: coating of the ELISA plate with capture antibodies, blocking of remaining protein-binding sites on the wells, application of samples and standard preparations (antigen binding), binding of biotin-labeled detection antibodies, application of a biotin-binding protein conjugated to an enzyme, and application of a chromogenic substrate to induce an enzymatic reaction (Figure 13).

Supernatants from the cell cultures were harvested and stored at -20 °C for later IL-10 ELISA. The protocol for the ELISA is described in the following. During all incubation steps, the ELISA plate was sealed in order to prevent evaporation or spilling. The plate was incubated under continuous agitation at 600 rpm. Between all incubation steps, the plate was washed 4 times with PBS supplemented with 0,05 % Tween (polysorbate 20) using a microplate washer. As a first step, the wells of a 96-well-microtiter plate were coated with 100 µl monoclonal anti-human IL-10 antibody diluted in PBS 2 µg/ml. The plate was sealed and stored at 4 °C over night. The next day, 200 µl of blocking solution were applied per well for 1 h at RT while shaking. The standards were prepared by diluting recombinant human IL-10 in standard buffer 40 ng/ml - 0,0195 ng/ml. Afterwards, samples and diluted standards were applied onto wells in duplicates. The plate was incubated for 2 h while shaking to allow binding of the antigens to capture antibodies. In the following, diluted biotinylated anti-human IL-10 detection antibodies were added for 1 h. Afterwards, the enzyme Horseradish-Peroxidase (HRP) conjugated to the biotin-binding protein Streptavidin was applied to allow attachment of the enzyme to the biotin-coated detection antibody. After 30 minutes, the substrate (reagent A = stabilized hydrogen peroxide + reagent B = stabilized tetramethylbenzidine (TMB) 1:1) was added. The plate was placed in the dark at RT until a color change was visible in all standard dilutions. 100µl 1 M sulfuric acid (H_2SO_4) was applied to stop the reaction. Then, an ELISA reader measured the optical density of the samples. It presents the absorbance of light for a given wavelength and correlates with the amount of converted substrate and thus antigen in the sample. The reading wavelength is determined by the substrate used. A dual wavelength measurement compensates for optical interferences caused by instrument fluctuations, scratches, fingerprints or other matter that may absorb light. For this purpose, the absorbance at the reference WL of 620 is subtracted from the absorbance at the specific reading WL of 450.

The ELISA data were analyzed with Microsoft Excel. The mean absorption values were calculated from duplicates and the blank value was subtracted. A standard curve was defined with the standard values and was used to determine the protein concentration in each sample. For statistical analysis, a paired samples student's *t*-test was used. The *p*-values were labeled as follows: *<0,1, **<0,05, ***<0,01, ****<0,001.

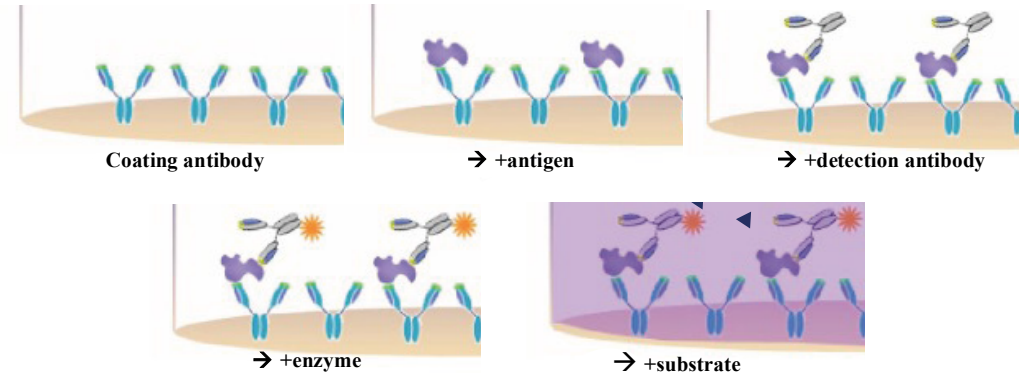


Figure 13: Principle of Sandwich ELISA. The antigen to be detected is captured between 2 antibodies. The second, called detection antibody, binds an enzyme, which allows a color reaction after substrate application. Source: www.biolegend.com.

4 Results

4.1 Characteristics of isolated MDSCs and PMNs

4.1.1 Isolated MDSCs consisted of a granulocytic and a monocytic population

The isolated MDSCs presented as two populations in the FSC-SSC, both of which were $CD66b^{pos}$. The major population were large, granulated cells within the granulocyte gate in the FSC-SSC. The cells of the second population were slightly larger but less granulated. This morphology is typical for monocytes [261]. I evaluated the monocytic population using different surface antibodies. The cells were $CD14^{pos}$ $CD66b^{dim}$ HLA II^{dim} HLA I^{pos} $CD11b^{pos}$ (**Figure 14**). This phenotype is not typical for mature monocytes, which do not express CD66b and express higher levels of HLA II. Indeed, the features $CD14^{pos}$ HLA-II^{dim} characterize monocytic MDSCs [161]. The CD66b CD14 co-expression further supports the assumption that these cells are MDSCs. As immature myeloid cells, they can share monocytic and granulocytic features [262-266]. Like other authors, I decided not to take any further isolation steps to deplete the monocytic MDSCs population [104, 189, 267]. MDSCs are heterogeneous. According to some, they should not strictly be subdivided into two subsets [268]. In my experiments, the $CD14^{pos}$ phenotype was absent in many donors and scarcely exceeded 10 %.

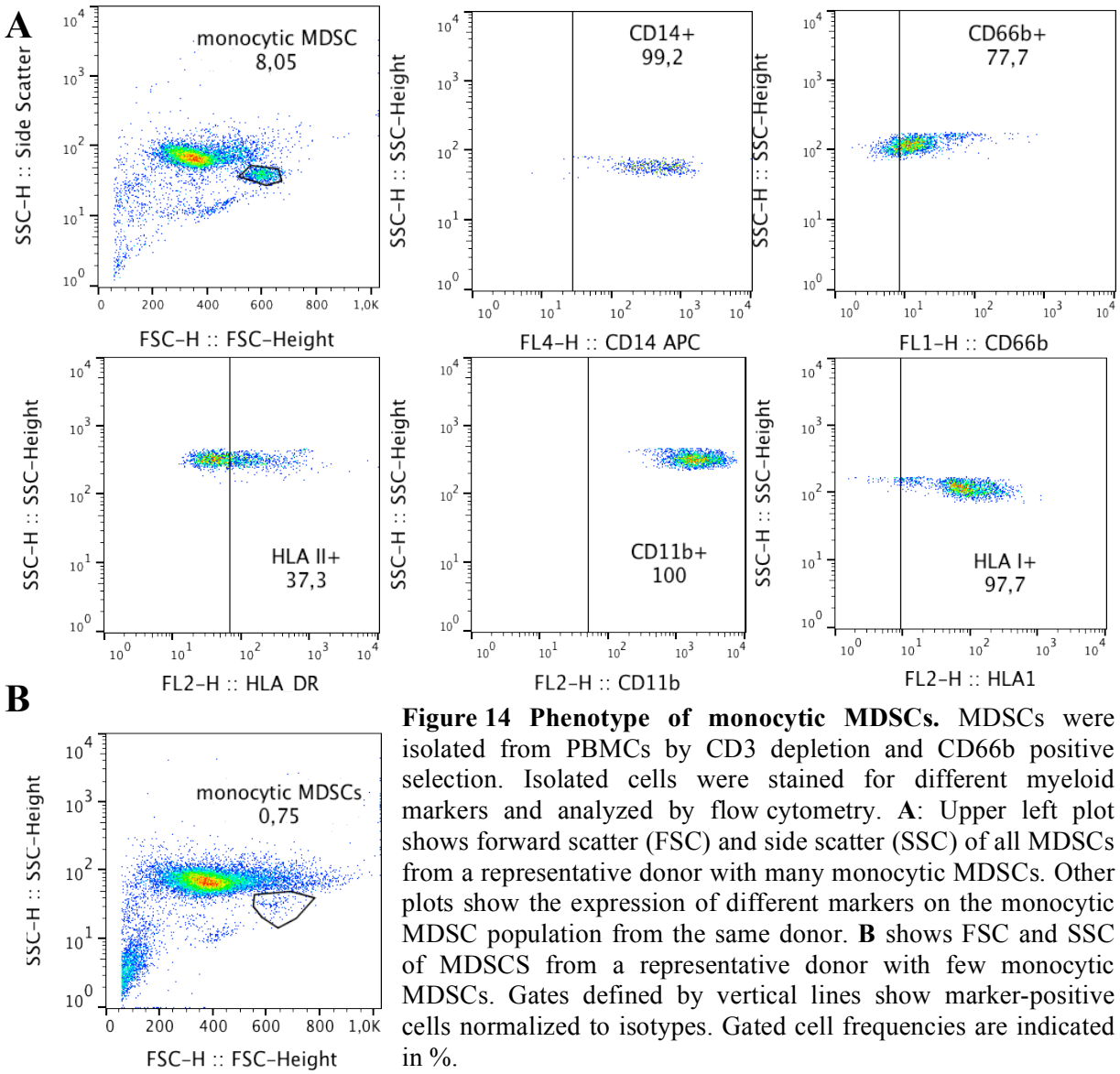


Figure 14 Phenotype of monocytic MDSCs. MDSCs were isolated from PBMCs by CD3 depletion and CD66b positive selection. Isolated cells were stained for different myeloid markers and analyzed by flow cytometry. **A**: Upper left plot shows forward scatter (FSC) and side scatter (SSC) of all MDSCs from a representative donor with many monocytic MDSCs. Other plots show the expression of different markers on the monocytic MDSC population from the same donor. **B** shows FSC and SSC of MDSCS from a representative donor with few monocytic MDSCs. Gates defined by vertical lines show marker-positive cells normalized to isotypes. Gated cell frequencies are indicated in %.

4.1.2 Granulocytic MDSCs and PMNs exhibited similar surface marker expressions

Isolated granulocytic MDSCs and PMNs showed similar morphology in flow cytometry concerning size and granularity. Furthermore, both were positive for CD11b and CD66b and negative for HLA II, as it has been described [264, 269]. Some authors have reported slight differences in CD66b and CD11b marker expressions between MDSCs and PMNs, but their results are partly contradictory [114, 162, 167]. I did not detect any significant differences between MDSCs and PMNs concerning these markers (Figure 15-A). I also measured CXCR4 expression, because some have reported that it is enhanced on MDSCs [155, 163]. Again, MDSC and PMN showed a similar expression. On both cell types, the expression of CXCR4 increased after cultivation (Figure 15-B), which has been described

before [270]. Taken together, I detected no major phenotypic differences between granulocytic MDSCs and PMNs.

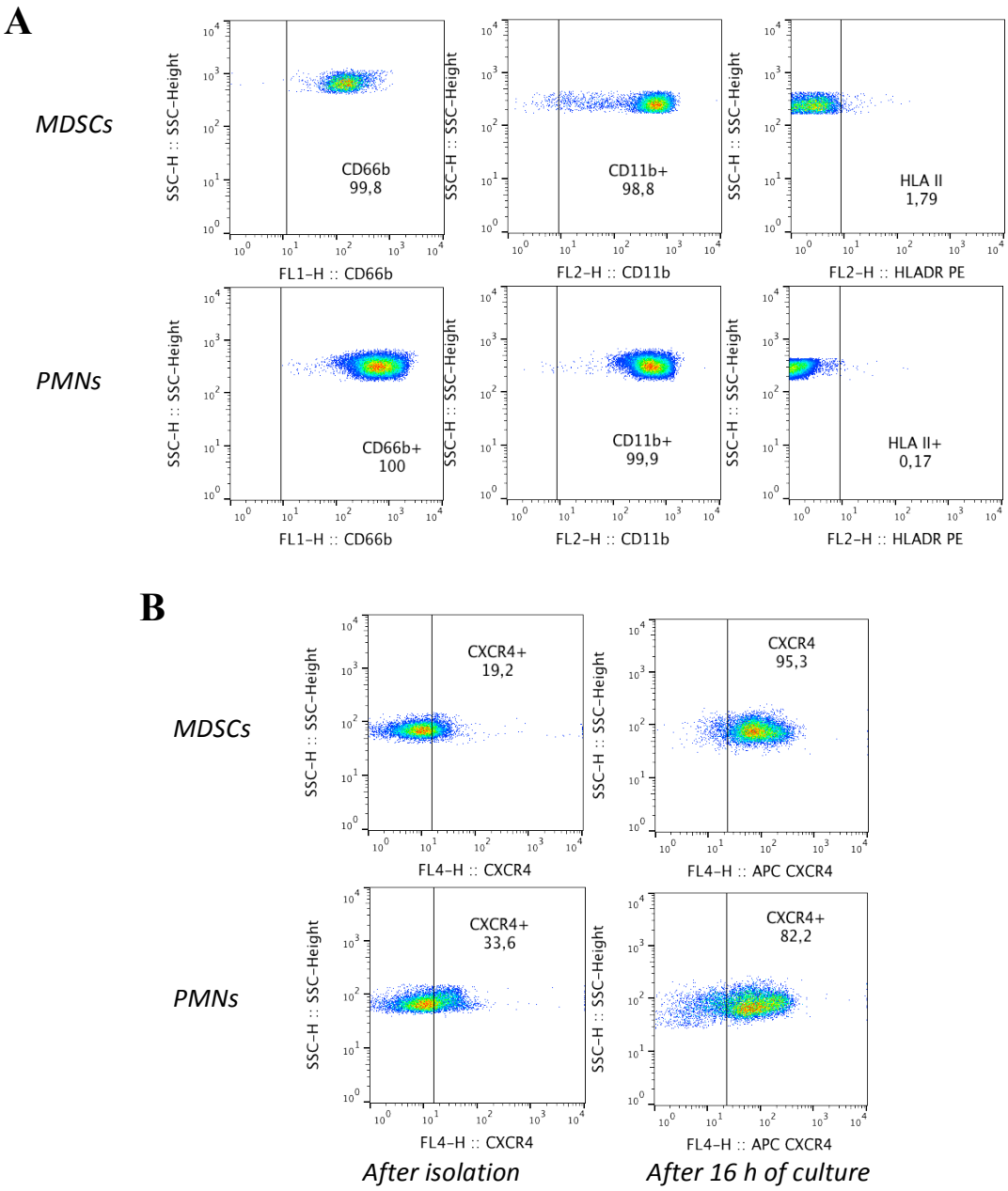


Figure 15: Similar phenotypes of MDSCs and PMNs. MDSCs and PMNs were isolated from fresh blood, stained with different antibodies and analyzed by flow cytometry. In **A**, cells were analyzed after isolation. In **B**, cells were either analyzed after isolation or after 16 h of culture in medium. Figure shows side scatter (SSC) and the expression of surface markers on MDSCs and PMNs for representative donors. Gates show marker-positive cells normalized to isotypes in %.

4.1.3 Neither MDSCs nor PMNs released IL-10 in culture

Cytokines are a group of proteins that are produced and released by cells and serve as their soluble communicators. Interleukins are a subgroup of cytokines. They are mainly released by immune cells upon stimulation and have an important influence on the overall

immune response. Many stimulatory and inhibitory interactions between immune cells are mediated via interleukins. IL-10 presents an immunomodulatory cytokine, which inhibits the function of T and dendritic cells. On the other hand, it also has activating effects on NK cells [271]. It was reported that IL-10 is released by MDSCs upon certain stimuli, and mediates part of their immunomodulatory effects [201, 272]. To find out if IL-10 might plays a role in the interaction between MDSCs and NK cells, I investigated whether IL-10 is released by MDSCs or PMNs in co-culture with NK cells. Purified granulocytic cells were either cultured in medium alone or together with NK cells at a 1:1 ratio over 16 h. NK cells were also cultured alone as a negative control (resting NK cells do not secrete relevant amounts of IL-10 [273]). Supernatants of the co-cultures were collected to perform an ELISA.

IL-10 concentrations were very low in all samples. As expected, NK cells cultured only in medium hardly secreted any IL-10. Both MDSCs and PMNs secreted low amounts of IL-10 that are probably insignificant [272], both in culture alone and in co-culture with NK cells (Figure 16).

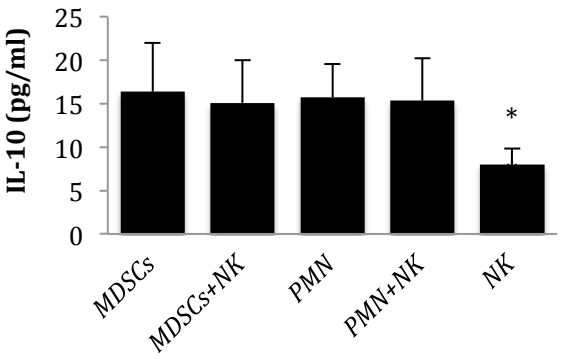


Figure 16: Neither MDSCs nor PMNs released significant amounts of IL-10. MDSCs or PMNs were incubated in medium alone or with NK cells (NK) 1:1 over 16 h. NK cells were also cultured alone. IL-10 concentration in culture supernatants was measured with ELISA. Figure shows IL-10 in culture supernatants in pg/ml. Error bars indicate SEM. Statistical analysis with Student's *t*-test (asterisk indicates significant difference to all other conditions; $*p < 0{,}1$), n = 4.

4.2 Influence of granulocytic cells on NK cell surface markers

To investigate the effects of MDSCs and PMNs on NK cells, I co-cultured the cells and analyzed phenotypic changes of NK cells afterwards. Using flow cytometry, I measured the expression of different surface proteins that are implicated in NK cell function. Similar to other groups, I co-cultured the cells 1:1 over 16 h if not otherwise stated [71, 208].

4.2.1 Granulocytic cells induced a down-regulation of NKp30 on NK cells but did not influence other activating receptors

The activity of NK cells is tightly regulated by the interplay of inhibitory and activating signaling via certain inhibitory or activating surface receptors. They receive signals from

pathogens, soluble factors or other immune cells [63]. To investigate how granulocytic cells influence NK cell activity, I analyzed changes in the expression of some of the NK cell activating receptors in the presence of MDSCs or PMNs. I considered the natural cytotoxicity receptors NKp46, NKp44 and NKp30, as well as the NKG2D and CD16 receptors. While NKp46, CD16, NKG2D and NKp30 are usually expressed on resting NK cells [65, 72], NKp44 is only expressed upon activation [68]. All of these receptors mediate activation and cytotoxic activity of NK cells. Low expressions of some of them have been associated with impaired NK cell function and correlated with cancer progression [70, 71]. Furthermore, it has been reported that monocytic MDSCs inhibit the NK cell function via engagement of the NKp30 receptor, which was associated with its down-regulation on the NK cell surface [71]. Likewise, viruses seem to modulate NK cell function via NKp30 [72, 73]. I measured the expression of NKp30 in particular to confirm an involvement of NKp30 in the interaction between granulocytic cells and NK cells. I cultured NK cells derived from healthy donors over 16 h, either in medium alone, with MDSCs or PMNs 1:1, or with IL-15. Then, I evaluated the expression of NKp46, NKp44, NKp30, NKG2D and CD16 by flow cytometry.

NKp46, CD16 and NKG2D were expressed on around 90 % of NK cells in all conditions. The geometric mean of the fluorescence intensity for NKG2D showed an increase in the positive control, indicating up-regulation of the activating receptor upon IL-15 stimulation. NKp44 was poorly expressed by resting NK cells. Upon stimulation with IL-15, the expression increased. Importantly, none of the four markers showed significant changes in expression after culture with granulocytic cells (Figure 17). The NKp30 expression on untreated NK cells showed a considerable donor-dependent variability from < 5 % to > 60 %. It was consistently reduced after 1:1 co-culture with either MDSCs or PMNs after both 3 and 16 h, but not after co-culture at a lower granulocyte to NK cell ratio of 1:2. The effect was moderate but significant after 16 h of co-culture. Incubation with IL-15 induced a considerable up-regulation of NKp30 compared to untreated cells (Figure 18).

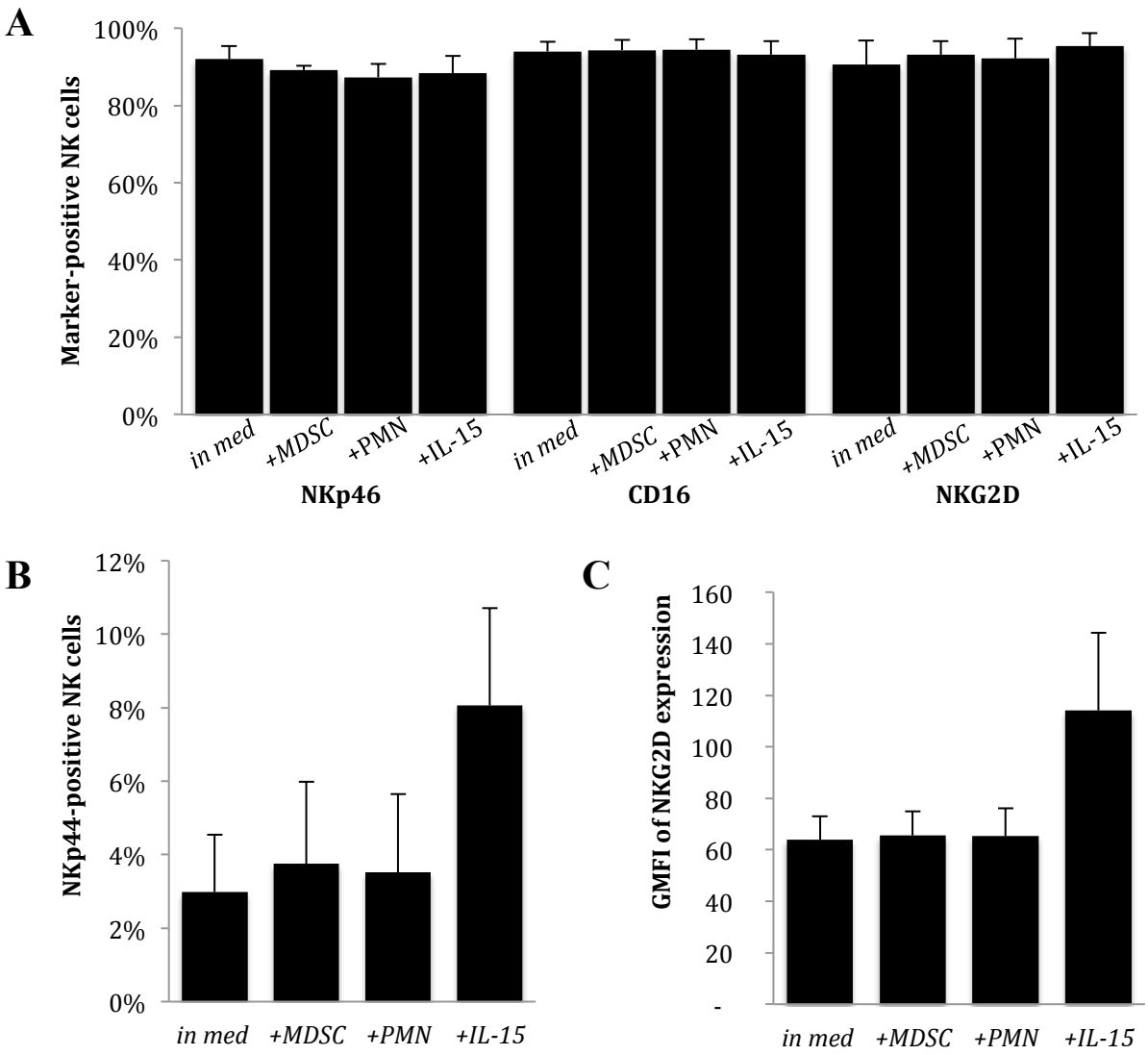


Figure 17: Neither MDSCs nor PMNs influenced the expression of NKp46, CD16, NKG2D or NKp44 on NK cells. Purified NK cells were incubated with medium alone (med), MDSCs or PMNs 1:1 or IL-15 for 16 h. Cells were stained for markers and analyzed by flow cytometry. Figure shows percentages of marker-positive cells normalized to isotypes (**A** and **B**) or the geometric mean of fluorescence intensity (GMFI) (**C**). Error bars indicate SEM, n=6 for NKp46; n=4 for NKG2D and CD16; n=3 for NKp44.

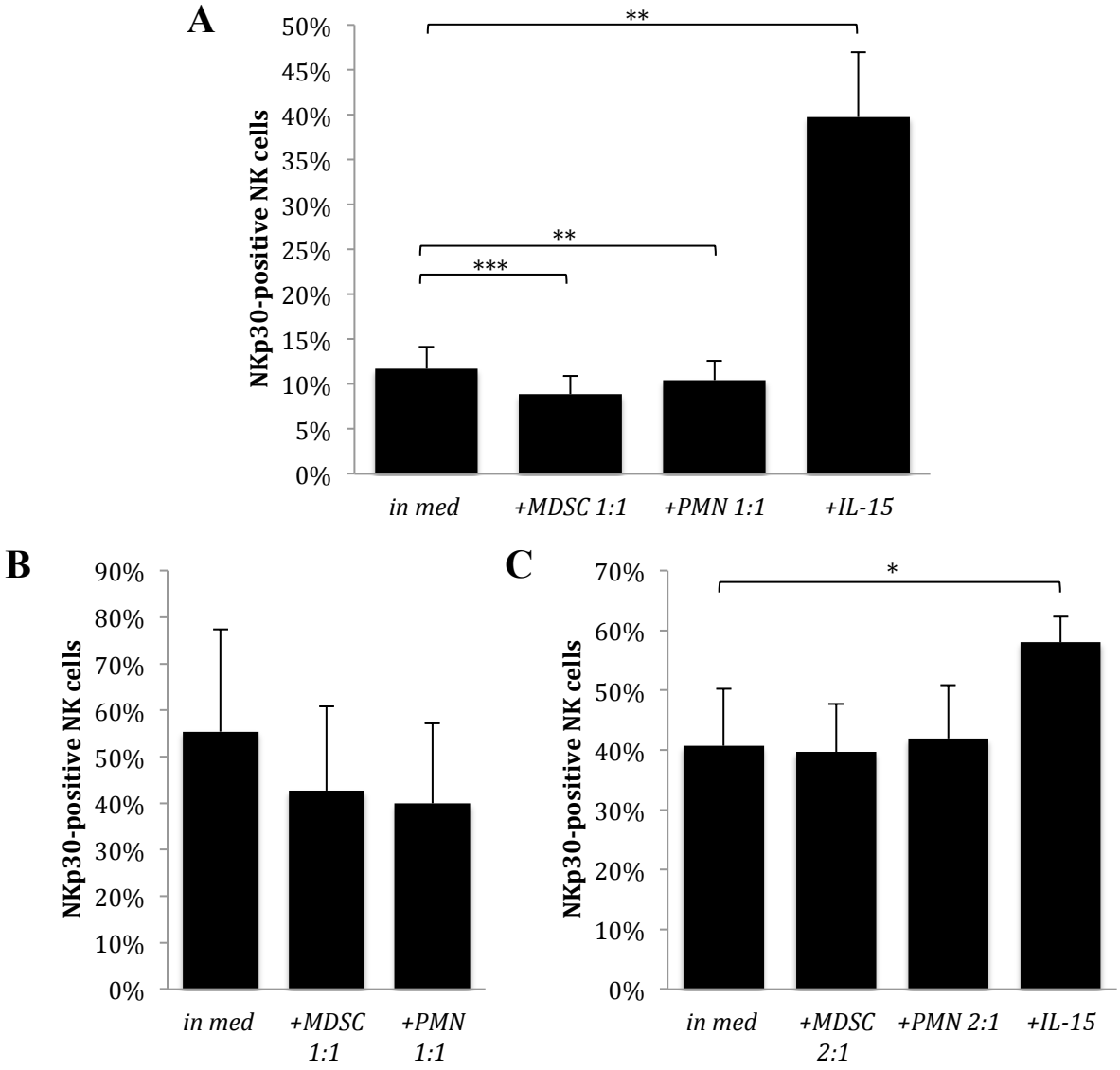


Figure 18: MDSCs and PMNs reduced NKp30 expression on NK cells. Purified NK cells were incubated with medium alone (med), with MDSCs or PMNs or with IL-15. Afterwards, cells were stained for NKp30 and analyzed by flow cytometry. Figure shows percentage of NKp30-positive NK cells normalized to isotype after 16 h of 1:1 co-culture (**A**), after 3 h of 1:1 co-culture (**B**) and after 16 h of 2:1 co-culture (**C**) of NK cells with MDSCs or PMNs. Error bars indicate SEM. Statistical analysis with Student's *t*-test (**p*<0,1 ***p*<0,05 ****p*<0,01), n=7 in A; n=3 in B; n=5 in C.

4.2.2 Granulocytic cells did not influence the expressions of HLA II, CCR7, CD25 or CD83 on NK cells

NK cells are usually HLA II negative. However, a subset of NK cells with HLA II expression and antigen-presenting abilities has been described [274]. I stained NK cells for HLA II after co-culture with MDSCs or PMNs to find out whether granulocytic cells would influence the antigen-presenting ability of NK cells. I further analyzed the surface markers CD25, CD83 and CCR7, which are markers of mature dendritic cells. They can be present on NK cells, but little is known about their functions: CD25 has been suggested

as a proliferation marker for NK cells [275]. CCR7 is a chemokine receptor promoting the migration of NK cells and its expression is influenced by dendritic or virus-infected cells [276]. CD83 is expressed on T cells upon activation. Importantly, the co-expression of CD25, CD83 and CCR7 on NK cells was reported to characterize an immunomodulatory subtype of NK cells. I measured their expression to evaluate whether MDSCs or PMNs would induce this phenotype. I incubated NK cells over 16 h in medium alone, with MDSCs/PMNs 1:1 or with IL-15. Afterwards, I measured the expression of HLA II, CD83, CCR7 and CD25 by flow cytometry.

As expected, less than 5 % of NK cells expressed HLA II, with no significant differences between the conditions. The expressions of CCR7, CD25 and CD83 were likewise unaffected by granulocytic cells. IL-15 stimulation enhanced the expression of CD83 and CD25, compatible with the activating and proliferation-promoting effect of IL-15 on NK cells (Figure 19).

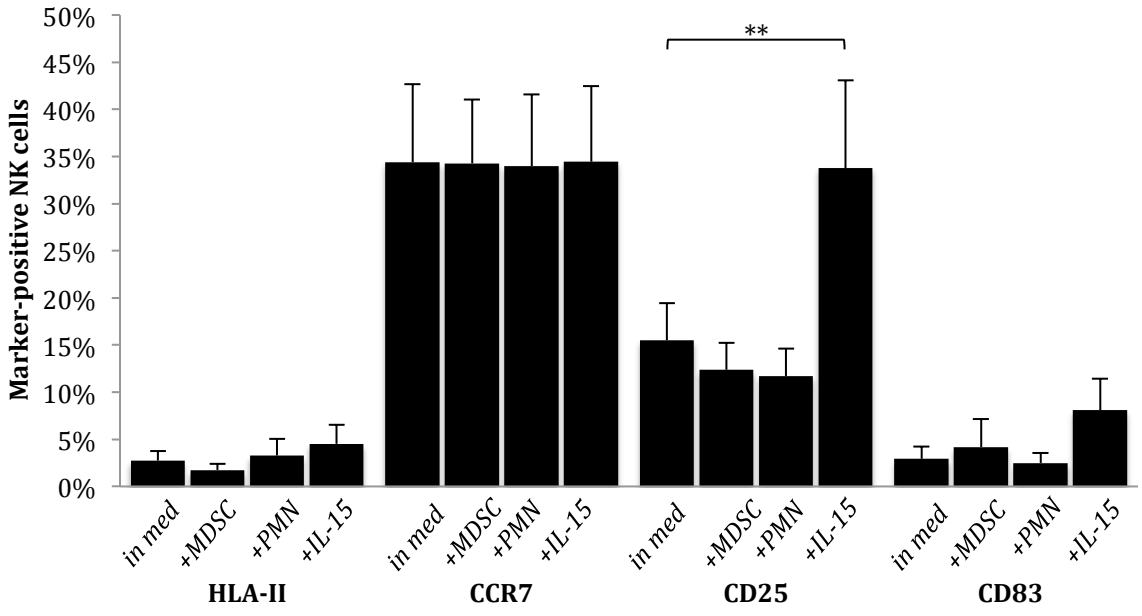


Figure 19: Neither MDSCs nor PMNs influenced the expression of HLA-II, CCR7, CD25 or CD83 on NK cells. NK cells were incubated in medium alone (med), with MDSCs or PMNs 1:1 or with IL-15 over 16 h. Cells were afterwards stained for surface markers and analyzed by flow cytometry. Figure shows percentage of marker-positive NK cells normalized to isotypes. Error bars indicate SEM. Statistical analysis with Student's *t*-test (**p<0,05), n=3 for HLA-II; n=5 for CCR7, CD25 and CD83.

4.2.3 MDSCs and PMNs induced an up-regulation of CD69 on NK cells

Certain activation-associated receptors on the NK cell surface can be used to estimate their activation status, such as CD69. Its natural ligands are unknown. However, the expression

of CD69 correlates with the cytotoxic potential of NK cells [54]. In order to evaluate and compare the effects of PMNs and MDSCs on the activation status of NK cells, I incubated NK cells over 16 h with either medium alone, PMNs, MDSCs or IL-15. Co-cultures were set at 1:1 and 1:2 ratios (NK to granulocytic cells). The expression of CD69 was analyzed by flow cytometry afterwards.

Only few naive NK cells expressed CD69. The percentage varied between 5 % and 20 %. IL-15-stimulation led to an increase in CD69 expression of 3-fold. Unexpectedly, the treatment with either MDSCs or PMNs at a 1:2 ratio also led to an increase of 2-fold and 1,5-fold respectively (Figure 20-A). However, this effect was absent after 1:1 co-cultures (Figure 20-B). These results suggest that granulocytic cells have activating effects on NK cells under certain circumstances.

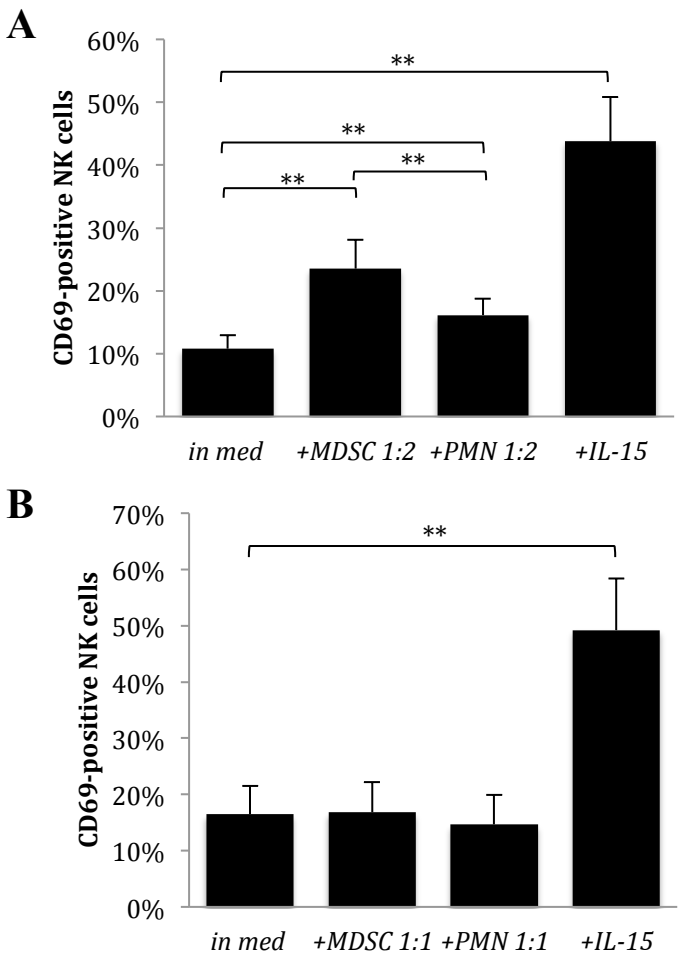


Figure 20: NK cells were activated after co-culture with MDSCs or PMNs at a 1:2 but not 1:1 ratio. NK cells were incubated with medium alone (med), with MDSCs or PMNs or with IL-15 over 16 h. Cells were stained for CD69 and analyzed by flow cytometry. Figures show percentages of CD69-positive NK cells normalized to isotypes. **A** shows CD69 expression after 1:2-, **B** after 1:1 co-culture. Error bars indicate SEM. Statistical analysis with Student's *t*-test (**p<0,05), n=5 in A; n=13 in B.

4.2.4 MDSCs and PMNs promoted degranulation of NK cells

As natural killers, NK cells have cytotoxic granules within the cytoplasm that can be rapidly released upon stimulation [29]. The protein CD107a forms part of the granule membrane and its expression on the cell surface can be used to measure degranulation [277, 278]. Although NK cells usually degranulate upon contact with tumor cells or viruses, they also degranulate to kill other immune cells [41, 279, 280]. Recently, NK cells were also shown to degranulate in the presence of PMNs. However, in in this case, degranulation did not induce killing [42]. To confirm the degranulation of NK cells upon contact with PMNs (or MDSCs), NK cells were incubated in medium alone, with MDSCs or PMNs 1:1 or with IL-15 over 16 h. As a reference, I also incubated NK cells with a classical target, the leukemia cell line K562, which is a popular target to test NK cell cytotoxicity in vitro [22]. Because of their strong stimulatory effect, K562 cells were applied at a higher effector-to-target ratio and for a shorter incubation time [69]: NK cells were incubated for 5 h in medium alone, with K562 cells 10:1 or with MDSCs/PMNs 1:1. For all conditions, after co-culture, the degranulation of NK cells was measured via the surface expression of CD107a, and apoptosis or lysis of MDSCs and PMNs was assessed by annexin V and PI staining.

After 16 h of incubation in medium, around 40 % of unstimulated NK cells were $CD107a^{pos}$, indicating degranulation. After co-culture with either MDSCs or PMNs, the percentage increased to 60 %, suggesting that granulocytic cells promoted NK cell degranulation. Likewise, the positive control with IL-15 led to an increase (Figure 21-A). Even in comparison with K562 cells, MDSCs and PMNs provoked a similar increase in $CD107a^{pos}$ NK cells (Figure 21-B). However, the degranulation in response to granulocytic cells remarkably differed from that in response to K562 cells. In the presence of K562, some NK cells showed very strong signals for CD107a, representing a high level of degranulation and cytotoxicity. On the other hand, contact with MDSCs, PMNs or IL-15 did not provoke strong degranulation. Instead, the signal intensities were weak, indicating the release of only few granules in the absence of target cells, which is unlikely to correlate with cytotoxicity [255, 281] (Figure 21-C).

Moreover, the degranulation of NK cells in the presence of granulocytic cells did not cause killing of the latter. After culture in medium alone, > 70 % of granulocytic cells were annexin V^{pos} and thus early apoptotic, confirming the known poor viability of

granulocytic cells. Only few granuloytes were PI^{pos}, representing late apoptotic or necrotic cells [282]. The co-culture with NK cells did not have an impact on annexin V or PI signals and thus did not influence viability (Figure 22).

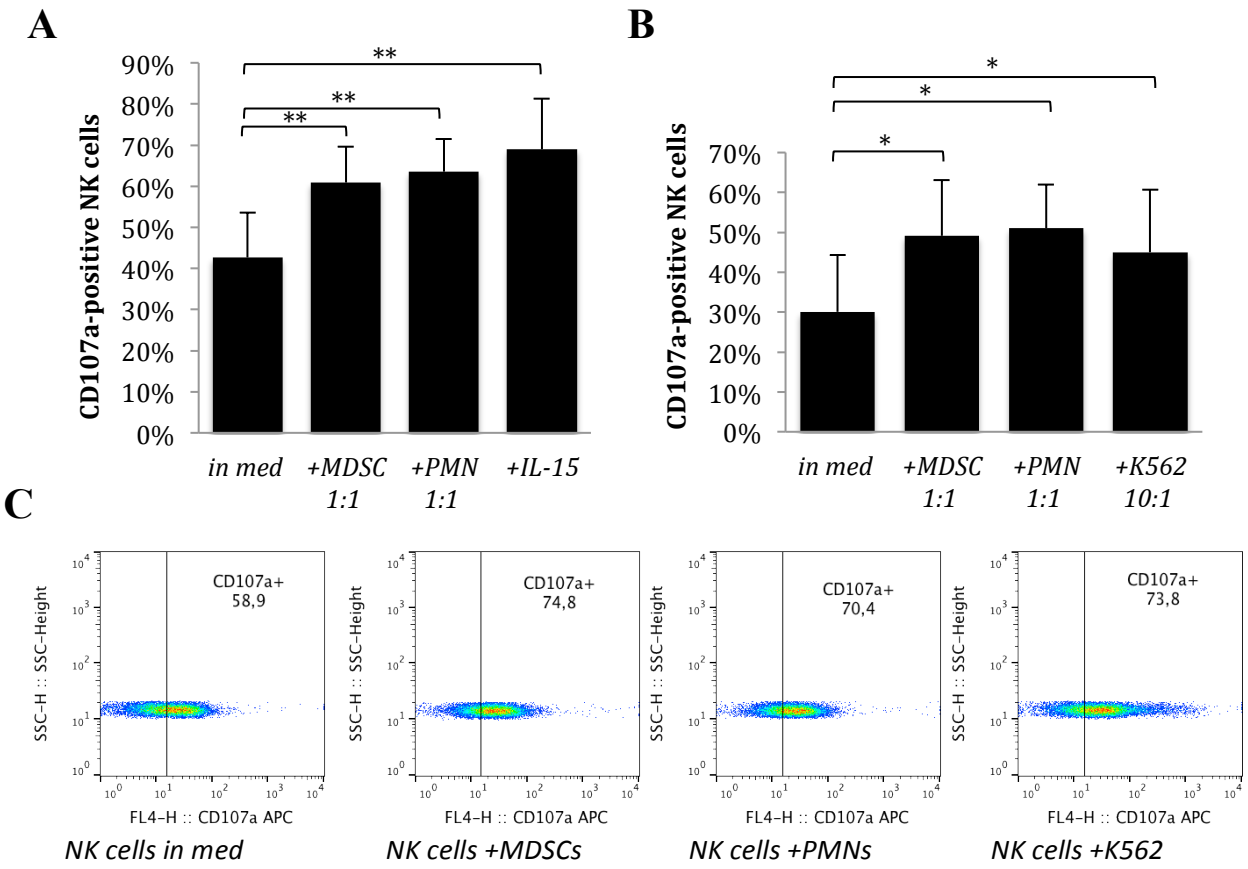


Figure 21: Granulocytic cells induced degranulation of NK cells, which was weaker compared to degranulation in response to target cells. In **A**, purified NK cells were incubated for 16 h with medium alone (med), MDSCs/PMNs 1:1 or IL-15. In **B** and **C**, they were incubated for 5 h with medium alone, MDSCs/PMNs 1:1 or K562 cells 10:1. Staining antibodies against CD107a were added at the beginning of co-cultures. Flow cytometry was performed afterwards. A and B show CD107a-positive NK cells normalized to isotype. Error bars indicate SEM. Statistical analysis with Student's *t*-test (**p*<0,1 ***p*<0,05), n=5 in A; n=3 in B. C shows side scatter (SSC) and CD107a expression of NK cells for a representative donor. Gates indicate CD107a-positive NK cells normalized to isotype in %.

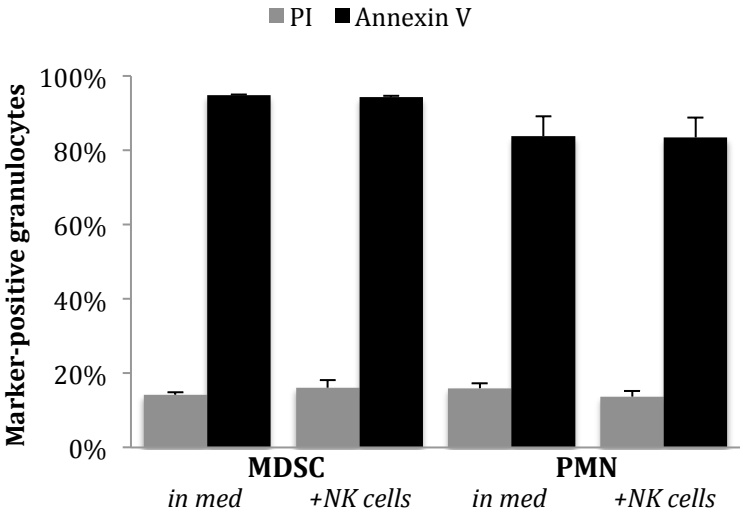


Figure 22: Co-culture with NK cells did not influence the viability of MDSCs or PMNs. Purified MDSCs and PMNs were incubated with medium alone (med) or with NK cells 1:1 over 9 h. Cells were stained with annexin V and PI and analyzed by flow cytometry. Figure shows marker-positive MDSCs or PMNs normalized to unstained samples. Error bars indicate SEM, n=3

4.3 Influence of granulocytic cells on NK cell homeostasis and function

NK cells are best known for their activity against tumor cells and are named after their ability to kill the latter spontaneously [19]. Like many authors before, I used the leukemia cell line K562 as a stimulus to analyze NK cell function in vitro [22]. NK cells were pre-treated by co-culture with MDSCs or PMNs. I analyzed NK cell viability and maturity under the influence of the granulocytic cells. Afterwards, I incubated the NK cells with K562 target cells and assessed activation, cytotoxicity and cytokine release.

4.3.1 MDSCs and PMNs improved the viability of NK cells

Although NK cells have a half-life of around 10 days in vivo [16], they are more prone to apoptosis in culture, and their in vitro viability depends on the culture conditions [283]. Dependent on the concentration, cytokines like IL-15 can have both positive and negative effects on NK cell survival [95, 96]. It has been suggested that granulocytic cells influence the viability of NK cells with contradictory results [222, 284]. Therefore, the effects of MDSCs and PMNs on NK cell survival were analyzed here. NK cells were incubated in medium alone, with MDSCs or PMNs 1:1 or with IL-15 over 16 h. NK cell viability was also analyzed in the presence of target cells: NK cells were incubated with K562 cells 10:1 over 5 h following the pre-treatment. Analysis was done by flow cytometry. Similar to previous publications, morphology changes visible in the FSC-SSC were used to identify apoptotic cells and to estimate the proportion of dead vs. viable cells [251, 253].

To confirm that morphology changes visible in the FSC-SSC are suitable to identify apoptotic cells, NK cells were stained with annexin V and PI after 16 h of culture in

medium, followed by flow cytometric analysis. In the FSC-SSC, two populations were distinguishable. One population presented as uniform, large, granulated cells, similar to the morphology of NK cells right after isolation. A second population consisted of smaller and more granular cells (lower FSC and higher SSC). Nearly all cells within this latter population were positive for both annexin V and PI, indicating dead cells. In contrast, the cells within the original NK cell gate were mostly negative for annexin V and PI, indicating vital cells (Figure 23). These staining results are similar to previously published ones, and confirm that a population of dead NK cells can be distinguished from a population of mostly vital NK cells in the FSC-SSC [252].

I measured the proportion of viable NK cells in the FSC-SSC following culture, which was < 50% after 16 h of culture in medium. Strikingly, after co-culture with either MDSCs or PMNs, the population of vital NK cells was increased almost 2-fold compared to conditions without granulocytic cells. Stimulation with IL-15 led to a slight but significant decrease in viability (Figure 24-A). The experiments with K562 cells revealed that the latter had no influence on NK cell survival. The positive effects of pre-treatment with granulocytic cells on NK-cell viability were retained (Figure 24-B).

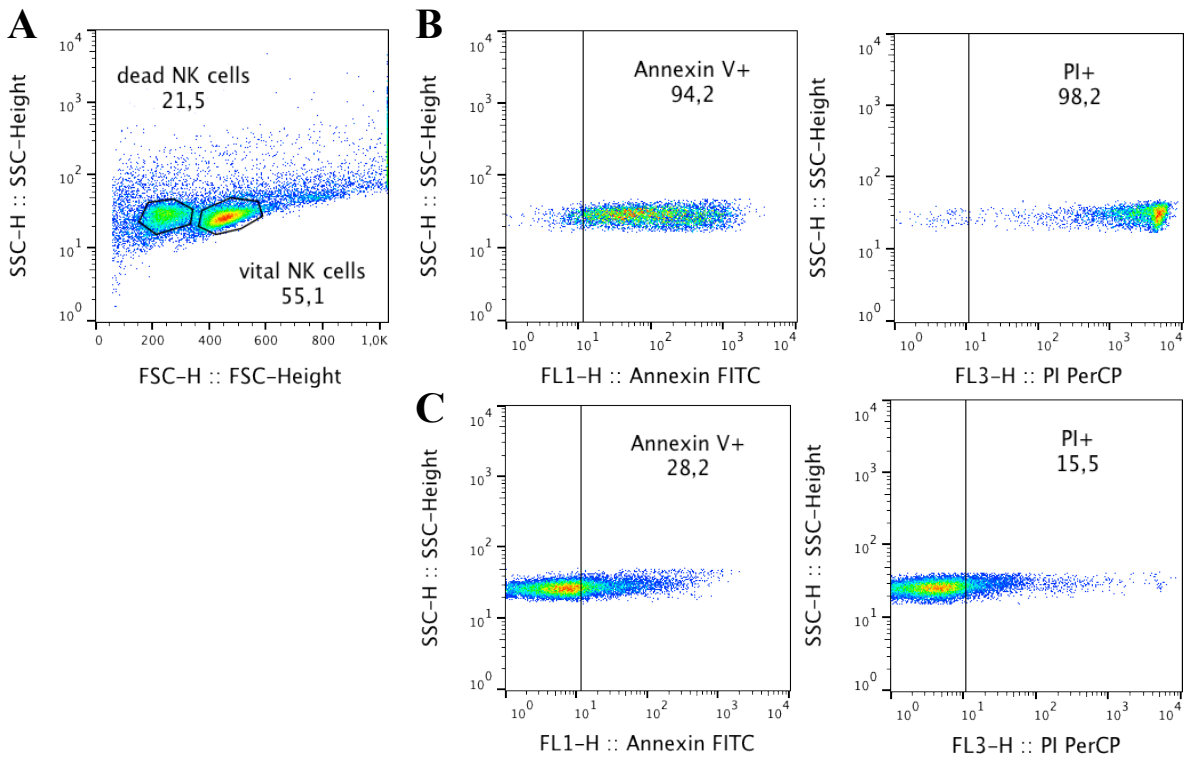


Figure 23: Dead vs. vital NK cells in the FSC-SSC. Purified NK cells were cultured in medium over 16 h and stained with annexin V and PI afterwards. Figure shows NK cell populations in the forward scatter (FSC) and side scatter (SSC) and annexin V and PI staining results. **A** shows all cells, **B** shows dead NK cell gate, **C** shows vital NK cell gate. Rectangular gates indicate marker positive cells normalized to unstained in %.

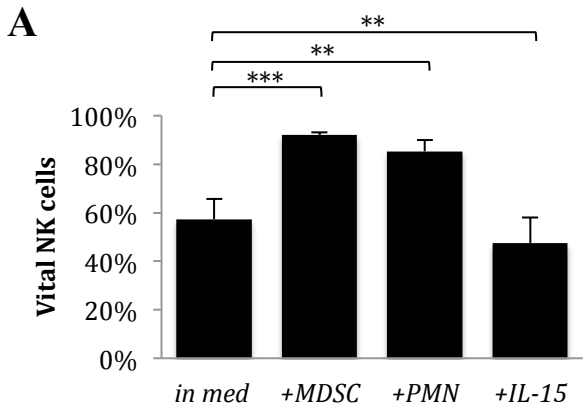
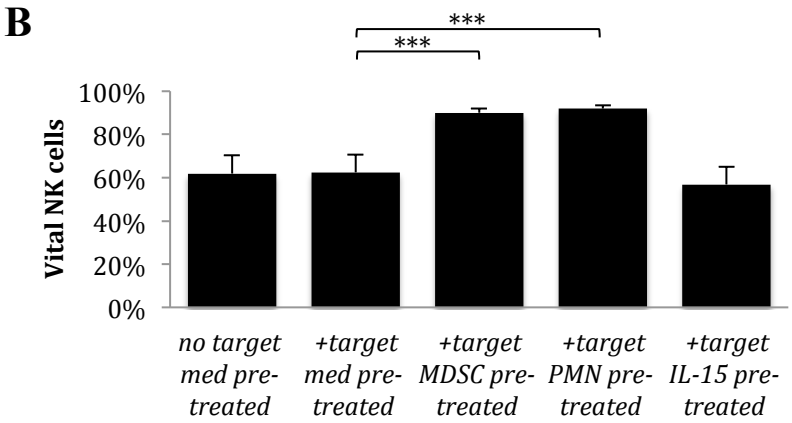


Figure 24: Co-culture with MDSCs or PMNs improved survival of NK cells. In **A**, purified NK cells were incubated for 16 h with medium alone (med), MDSCs or PMNs 1:1 or IL-15. In **B**, NK cells were incubated for another 5 h with- or without K562 target cells afterwards. Cells were then evaluated by flow cytometry, and the proportion of vital NK cells estimated using the FSC-SSC. Figure shows percentage of vital NK cells out of all NK cells. Error bars indicate SEM. Statistical analysis with Student's *t*-test (**p<0,05 ***p<0,01), n=8 in A; n=6 in B.



4.3.2 MDSCs and PMNs provoked a decrease in the CD56^{bright} NK cell population

CD56^{bright} NK cells have been described as a subset of NK cells that are less cytotoxic but secrete cytokines. They comprise a small, variable fraction (10% maximum) of NK cells in the peripheral blood. They can further be divided into CD16^{neg} and CD16^{dim} cells. They are pre-mature and are considered direct precursors of CD56^{dim} CD16^{pos} NK cells [49]. Using a mouse model of neutropenia, Jaeger et al. have suggested that the maturation of NK cells is dependent on the presence of neutrophils [284]. To confirm an influence of granulocytic cells on NK cell maturation, and compare the effects of PMNs and MDSCs, I measured the percentage of pre-mature, CD56^{bright} NK cells after co-culture with PMNs or MDSCs. I cultured NK cells over 16 h in medium alone, with MDSCs or PMNs at 1:1 co-culture ratios. Then, I stained the cells with anti-CD56 antibodies and analyzed CD56 expression by flow cytometry. In a second set of experiments, I also investigated the CD56 expression in the presence of NK target cells. After pre-treatment with MDSCs or PMNs, I incubated NK cells with K562 cells over 5 h at a 10:1 effector-to-target ratio. Again, I measured the percentage of CD56^{bright} NK cells.

The immature, CD56^{bright} NK cell population was very variable in size. In average, its proportion of all CD56^{pos} cells was around 8 %, which is consistent with observations made by others [285]. The majority of CD56^{bright} NK cells were CD16^{neg}, and most of the CD56^{dim} cells were CD16^{pos}. However, an intermediate phenotype consisting of CD56^{bright} CD16^{dim} NK cells was also distinguishable (Figure 25). Co-culture of NK cells with either MDSCs or PMNs led to a decrease in the CD56^{bright} population by 20 % in average, without affecting the total amount of CD56^{pos} cells. This suggests a transformation of immature CD56^{bright} NK cells into mature, CD56^{dim} cells [49]. The effect was preserved after incubation of NK cells with K562 target cells, although the latter had the opposite effect and provoked a slight but significant increase in pre-mature CD56^{bright} NK cells (Figure 26). Together with the published data, these findings suggest that granulocytic cells are able to induce maturation of NK cells.

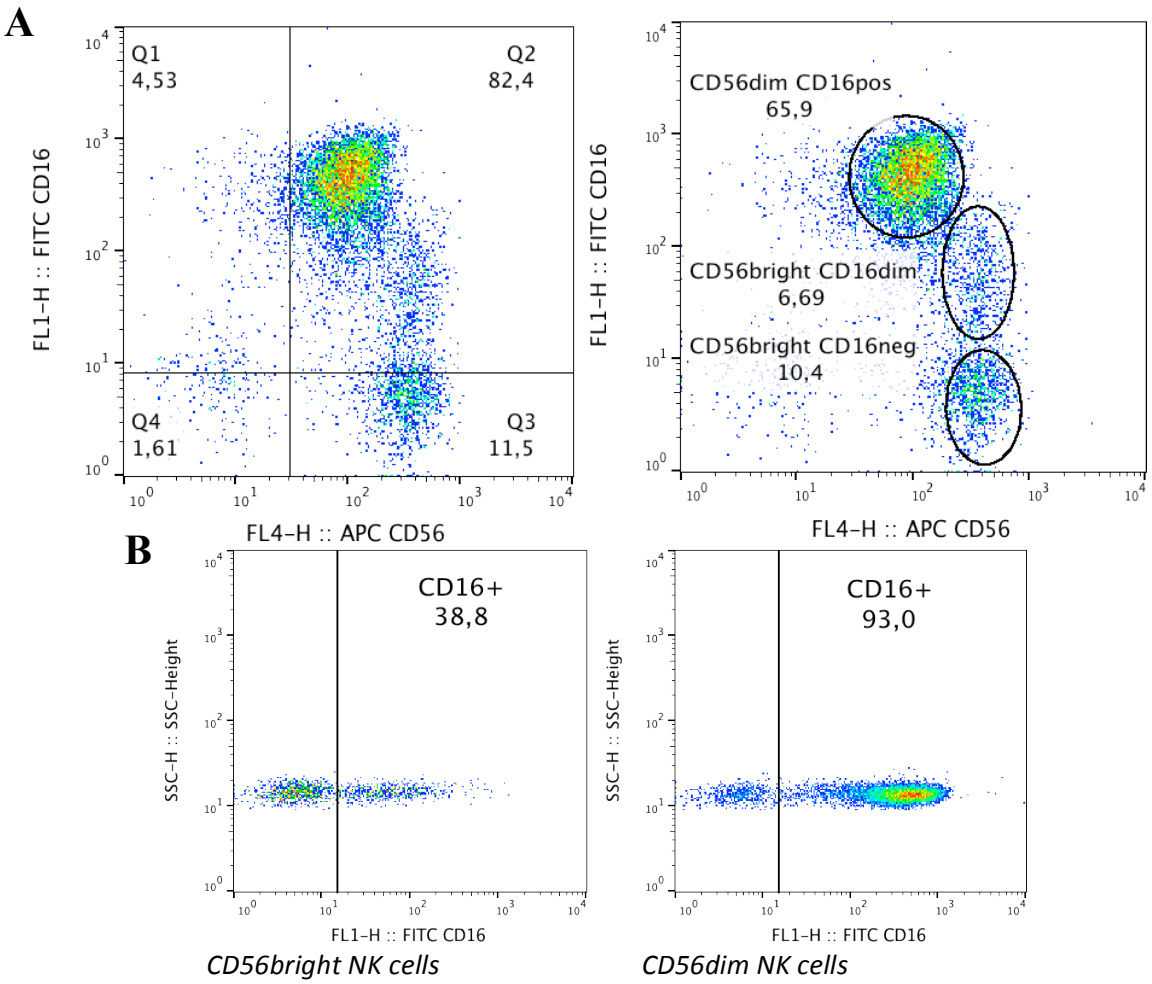


Figure 25: **Subpopulations of resting NK cells: CD56^{dim} CD16^{pos}, CD56^{bright} CD16^{neg} and CD56^{bright} CD16^{dim}.** Purified NK cells were stained for CD56 and CD16 and analyzed by flow cytometry. For better illustration, a donor with a relatively high proportion of CD56-bright NK cells is shown. **A** shows co-expression of CD16 and CD56 by all cells. Quadrants 1-4 indicate marker-positive and –negative cells normalized to isotypes in %. **B** shows CD16 expression of CD56-bright and CD56-dim cells separately. Gates indicate CD16-positive cells normalized to isotype in %.

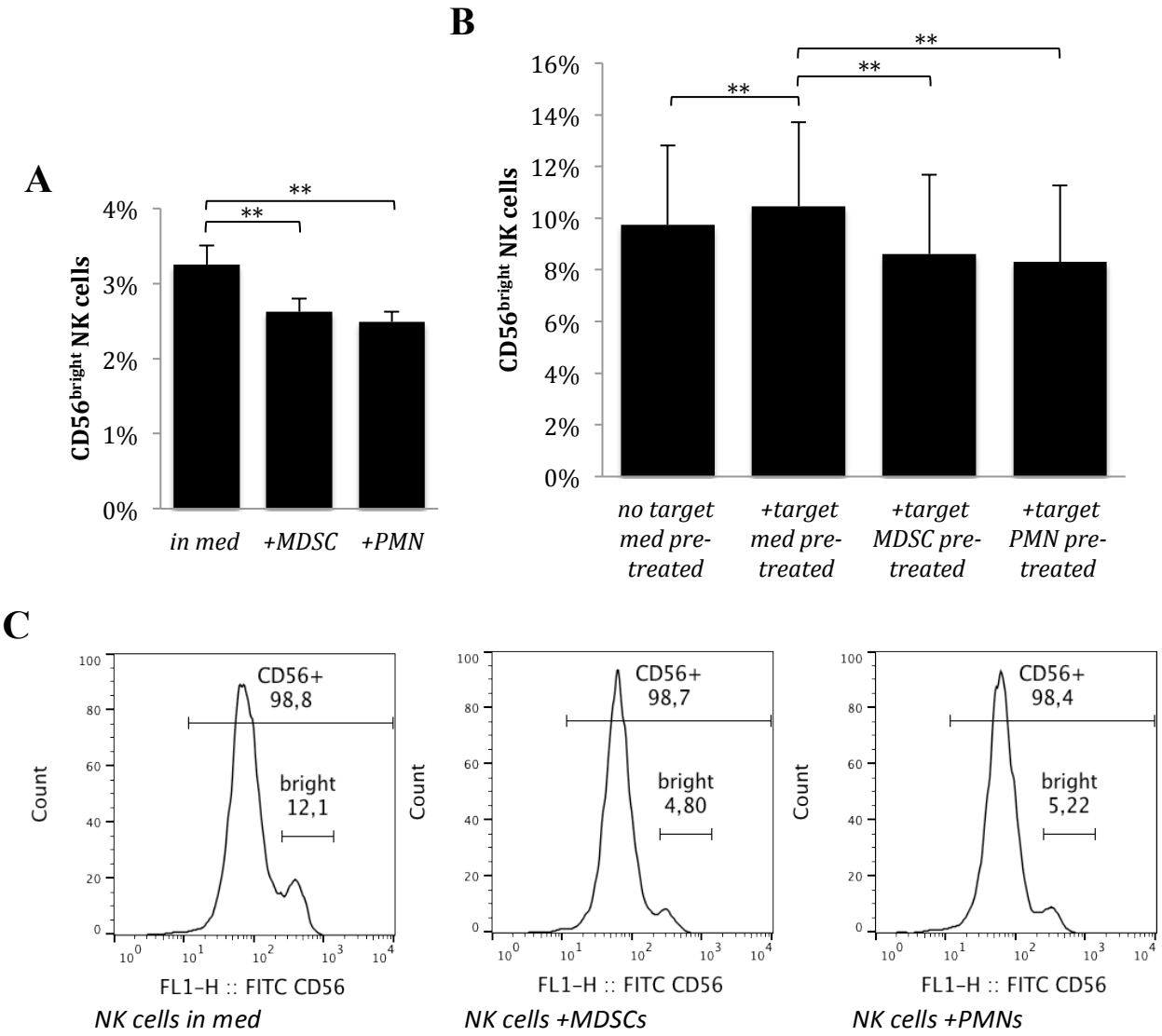


Figure 26: MDSCs and PMNs provoked a decrease in CD56^{bright} NK cells. Purified NK cells were incubated with medium alone (med), MDSCs or PMNs 1:1 over 16 h. In B, NK cells were afterwards incubated with- or without K562 cells for another 5 h. Cells were stained with anti-CD56 antibody and analyzed by flow cytometry. **A** and **B** show percentages of CD56-bright NK cells. Error bars indicate SEM. Statistical analysis with Student's *t*-test (**p<0,05), n=3 in A; n=8 in B. **C** shows histograms of CD56 expression from a representative experiment. Gates indicate CD56-bright NK cells and CD56-positive cells normalized to isotype in %.

4.3.3 MDSCs and PMNs impaired the activation of NK cells in response to target cells

As shown in 4.2.3, I have demonstrated that MDSCs and PMNs can activate NK cells under certain circumstances. Here, I investigated what kind of influence MDSCs and PMNs have on the activation of NK cells in response to a target. Purified NK cells were pre-incubated in medium alone, with IL-15, MDSCs or PMNs 1:1 over 16 h, and then incubated with K562 cells 10:1 for 5 h. To assess NK cell activation, the expressions of two activation markers were measured by flow cytometry: CD69 and CD137.

As expected, the co-culture of NK cells with K562 cells provoked an up-regulation of CD69 of more than 2-fold, indicating activation of NK cells. The pre-treatment with IL-15 induced an additional increase of 2-fold, yielding in a total up-regulation of 4-fold compared to unstimulated NK cells. However, NK cells that had been pre-treated with MDSCs or PMNs, showed a significantly diminished CD69 up-regulation in response to K562 (Figure 27-A). These results confirm an inhibitory effect of both MDSCs and PMNs on NK cell activation by target cells. Regarding CD137, granulocytic cells had no influence on the expression. The expression was enhanced by both the treatment with K562 cells and the pre-treatment with IL-15 (Figure 27-B).

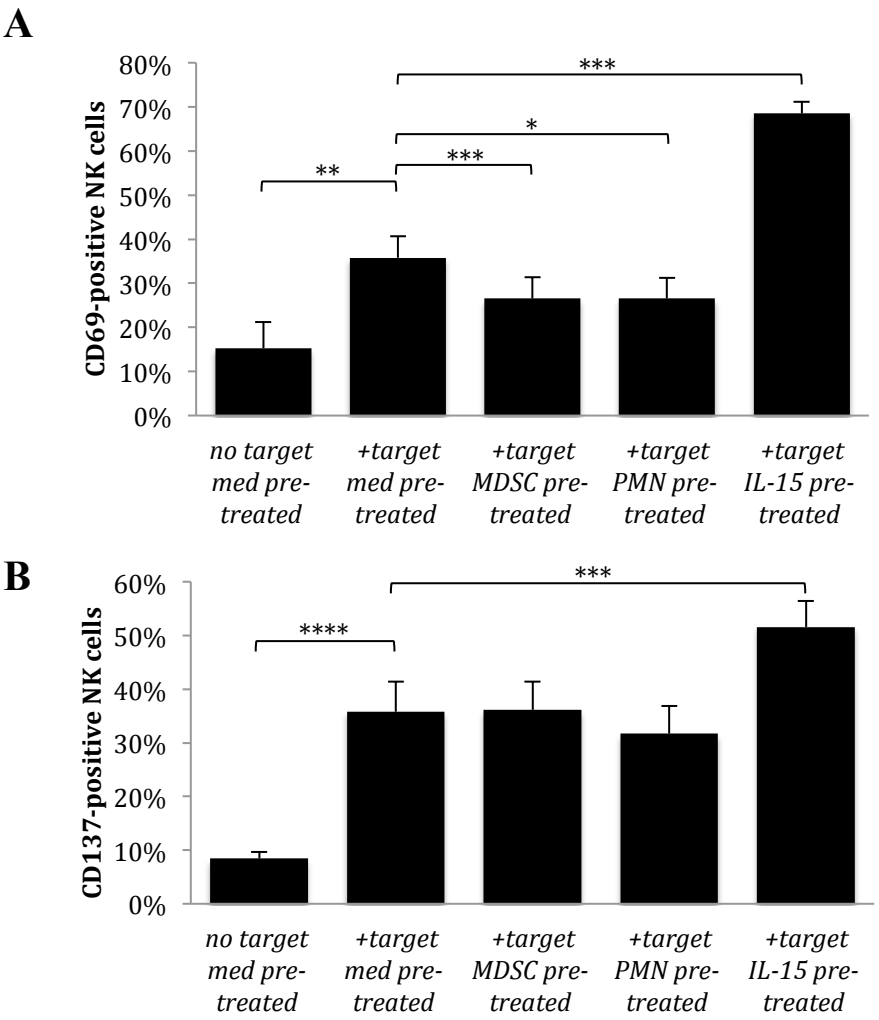


Figure 27: MDSCs and PMNs inhibited NK cell activation in response to target cells. NK cells were pre-treated in medium alone (med), with MDSCs or PMNs 1:1 or IL-15 over 16 h. NK cells were then incubated with- or without K562 target cells for another 5 h. Cells were stained for CD69 and CD137 and analyzed by flow cytometry. Figure shows percentage of CD69-positive (**A**) and CD137-positive (**B**) NK cells normalized to isotypes. Error bars indicate SEM. Statistical analysis with Student's *t*-test (**p*<0,1 ***p*<0,05 ****p*<0,01 *****p*<0,001), n=7 in A; n=11 in B.

4.3.4 MDSCs and PMNs impaired the NK cell cytotoxicity against target cells

After demonstrating that MDSCs and PMNs impair the NK cell activation by target cells, I evaluated and compared their influence on NK cell cytotoxicity towards target cells. For this purpose, I measured the expression of the degranulation marker CD107a on the NK cell surface. As I have shown in 4.2.4, the co-culture of NK cells with granulocytic cells alone enhanced CD107a expression. Here, to test NK cell cytotoxicity against K562 cells, I pre-treated NK cells with granulocytic cells and then depleted them for the cytotoxicity assay. NK cells were incubated for 16 h in medium alone, with IL-15, MDSCs or PMNs 1:1. Granulocytic cells were then depleted, and re-purified NK cells were incubated with K562 cells 10:1 over 5 h. Afterwards, the expression of CD107a was measured by flow cytometry.

Measuring the total percentage of $CD107a^{pos}$ NK cells in co-culture with K562, I could not detect any differences in CD107a expression between NK cells that had been pre-treated with granulocytic cells or in medium. In average, 30 % of naive NK cells, which had not been stimulated with K562 cells, already expressed CD107a. This percentage increased only moderately in response to K562 in all conditions and even the pre-stimulation with IL-15 showed no significant effect (Figure 28-A). In a second readout, I measured $CD107a^{high}$ NK cells only, which could easily be distinguished by their bright signal intensity (Figure 29). These $CD107a^{high}$ NK cells are highly degranulated and cytotoxic [255, 281]. While less than 0,5 % of untreated NK cells showed such strong signals, around 5 % were $CD107a^{high}$ after stimulation with K562 cells. The pre-treatment with IL-15 induced another increase of 2-fold. These results confirm that the percentage of $CD107a^{high}$ NK cells, but not the percentage of all $CD107a^{pos}$ cells, should be used to assess cytotoxicity [255, 281]. The proportion of $CD107a^{high}$ NK cells was diminished when NK cells had been pre-treated with MDSCs or PMNs (Figure 28-B), indicating that both MDSCs and PMNs can inhibit NK cell cytotoxicity against K562 cells. The decrease was stronger after pre-treatment with PMNs compared to MDSCs in most donors, but this was not statistically significant.

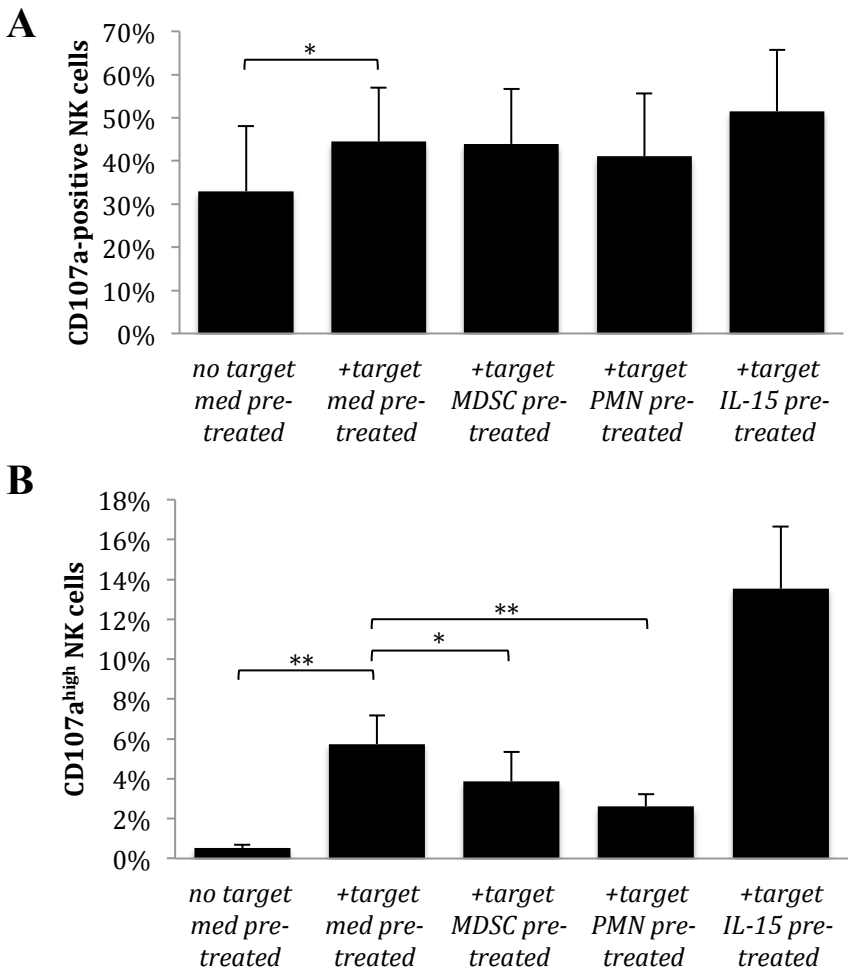


Figure 28: MDSCs and PMNs inhibited NK cell cytotoxicity against K562 target cells. NK cells were pre-treated in medium alone (med), with MDSCs or PMNs 1:1 or IL-15 over 16 h. NK cells were then re-purified and incubated with- or without K562 target cells for another 5 h. CD107a staining antibody was added to co-culture and CD107a expression was analyzed by flow cytometry afterwards. Figure shows percentage of CD107a positive NK cells normalized to isotype (**A**) or CD107a^{high} NK cells (**B**). Error bars indicate SEM. Statistical analysis with Student's *t*-test (*p<0,1 **p<0,05), n =5 in A; n=6 in B.

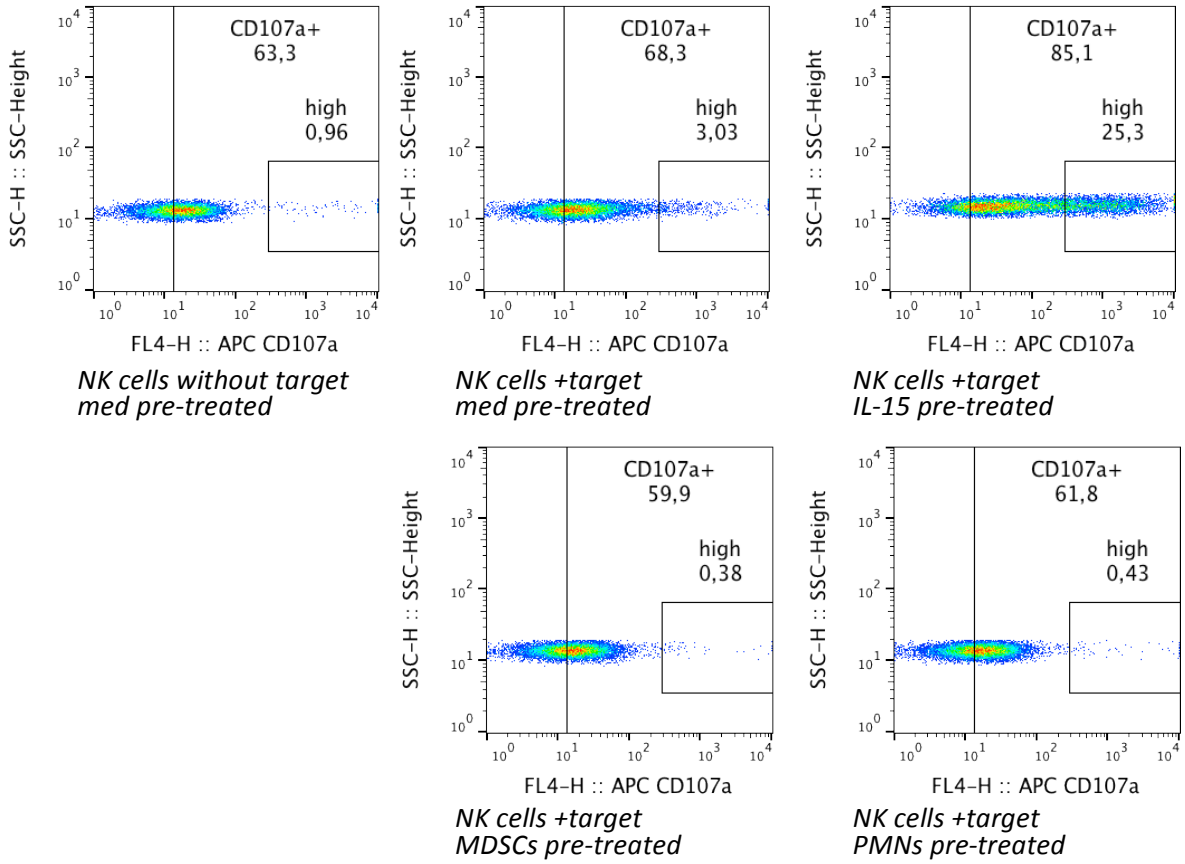


Figure 29: CD107a^{pos} vs. CD107a^{high} NK cells after stimulation with target cells. NK cells were pre-treated in medium alone (med), with MDSCs or PMNs 1:1 or IL-15 over 16 h. NK cells were re-purified and then incubated with- or without K562 target cells for 5 h. CD107a staining antibody was added to co-culture and CD107a expression was analyzed by flow cytometry afterwards. Figures show CD107a expression in different conditions from a representative experiment. Gates indicate CD107a-positive cells normalized to isotype and CD107a^{high} cells in %.

4.3.5 MDSCs and PMNs impaired the cytokine production by NK cells

Apart from cytotoxic enzymes, which are released during degranulation, NK cells also release cytokines, most importantly IFNγ and TNFα. They have direct effects on cancer cells including an impairment of proliferation, fibrogenesis and angiogenesis as well as the killing of cancer cells [31, 32]. They also enable NK cells to interact with other immune cells and play a role in the general immune response [30, 32]. To evaluate how MDSCs and PMNs would interfere with the ability of NK cells to release cytokines, I measured the presence of intracellular IFNγ and TNFα in NK cells by flow cytometry. I measured intracellular cytokines both directly after co-culture of NK cells with granulocytic cells and after another incubation with K562 cells, which are a stimulus for cytokine release [51]: First, NK cells were incubated for 16 h in medium alone, with MDSCs or PMNs 1:1 or with IL-15. In some experiments, NK cells were then incubated with K562 cells at a

10:1 ratio for 5 h. Brefeldin A was added to cultures to inhibit the secretion of proteins. After the incubation time, intracellular staining for the cytokines was performed for all conditions, followed by flow cytometric analysis.

No intracellular TNFα was detectable in unstimulated NK cells (data not shown). IFNγ was detectable in around 50 %, but the signal was weak, representing only small amounts of intracellular cytokine. The percentage of $IFN\gamma^{pos}$ NK cells was increased after stimulation with IL-15, but decreased after co-culture with either MDSCs or PMNs (Figure 30-A). The incubation with K562 target cells provoked a significant increase in intracellular cytokine levels, both TNFα and IFNγ. Again, pre-treatment with MDSCs or PMNs caused a reduction in $IFN\gamma^{pos}$ NK cells of around 50 % and 80 % respectively. The difference between the two granulocytic cell types was not statistically significant. Pre-treatment with IL-15 did not significantly enhance IFNγ secretion further (Figure 30-B). Regarding TNFα, the percentage of marker-positive NK cells was likewise reduced when NK cells had been pre-treated with MDSCs or PMNs, while pre-treated with IL-15 provoked more TNFα production. Again, the suppressive effect of PMNs was stronger compared to MDSCs in most donors, but this was not statistically significant (Figure 30-C). These results demonstrate that MDSCs and PMNs inhibit the cytokine production of NK cells, both IFNγ and TNFα.

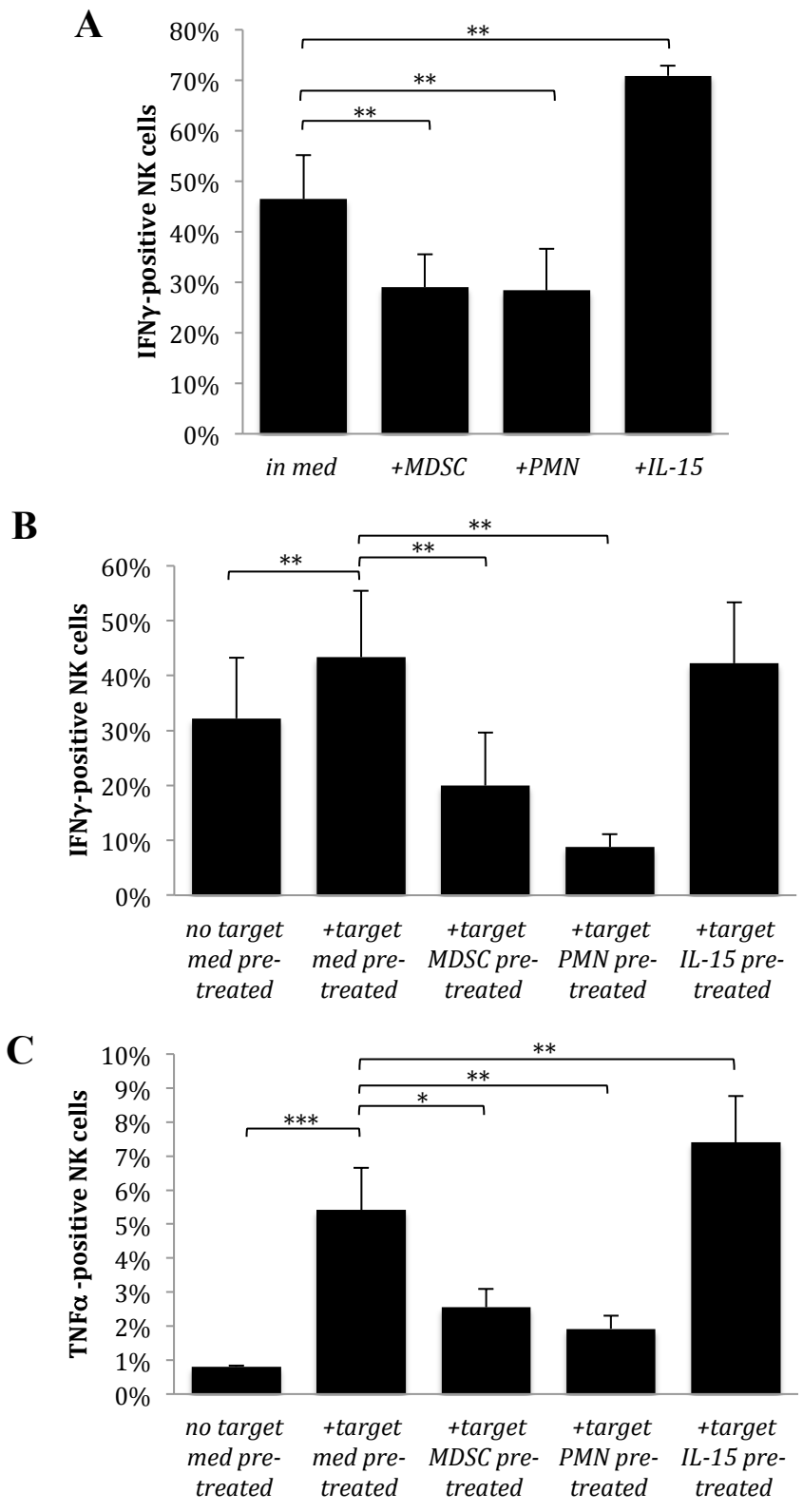


Figure 30: MDSCs and PMNs impaired IFNγ and TNFα production by NK cells. NK cells were pre-treated in medium alone (med), with MDSCs or PMNs 1:1 or IL-15 over 16 h (**A**). In **B** and **C**, NK cells were afterwards incubated with- or without K562 target cells for another 5 h. Brefeldin A was added to cultures. Afterwards, cells were lysed, fixed and stained with anti-IFNγ and anti-TNFα antibody followed by flow cytometric analysis. Figure shows percentage of marker-positive NK cells normalized to isotypes. Error bars indicate SEM. Statistical analysis with Student's *t*-test (**p<0,05 ***p<0,01), n=4 in A; n=10 in B; n=4 in C.

4.4 Influence of granulocytic cells on T cell proliferation

The definition of MDSCs has been controversial. The ability to suppress T cell proliferation is considered their most important feature, and many authors have used T cell proliferation assays to identify functional MDSCs [145, 163, 286, 287]. However, they did not show that the suppression of T cell proliferation was exclusive to MDSCs as opposed to other granulocytic cells. As a matter of fact, other authors have reported that PMNs impair T cell proliferation [288, 289]. Here, I performed T cell proliferation assays to confirm that the MDSCs isolated in this study suppressed T cell proliferation, and compared the effects of MDSCs and PMNs. I isolated T cells from healthy donors, labeled them with CFSE and incubated them in medium alone, with MDSCs or PMNs (at 1:1 and 10:1 ratios). All conditions were incubated with as well as without CD3/CD28 Dynabeads, which are a proliferation stimulus for T cells. After 72 h of culture, the cells were stained for CD3 and were analyzed by flow cytometry. Like others before, I used the division index to quantify proliferation [290]. It was calculated with the software FlowJo, and represents the average number of cell divisions (reductions of CFSE fluorescence intensity by half) per cell.

4.4.1 MDSCs and PMNs impaired T cell proliferation

In conditions without Dynabeads, all T cells exhibited a uniform, strong CFSE fluorescence intensity, indicating that no cell division had occurred. In conditions with Dynabeads, several peaks of different fluorescence intensities were distinguishable, representing distinct cell generations (Figure 31-A). The division index was around 0,8. However, if T cells were co-cultured with granulocytic cells, either MDSCs or PMNs, their division index was reduced significantly up to 75 %. These data are in line with the literature and confirm a suppressive effect of MDSCs on T cell proliferation [290]. Importantly, I show that PMNs have similar effects. The reduction was even more prominent for PMNs compared to MDSCs, and more prominent at 1:1 compared to 10:1 co-culture ratios (Figure 31-B). I demonstrate that both MDSCs and PMNs are able to impair T cell proliferation, possibly in a concentration-dependent manner.

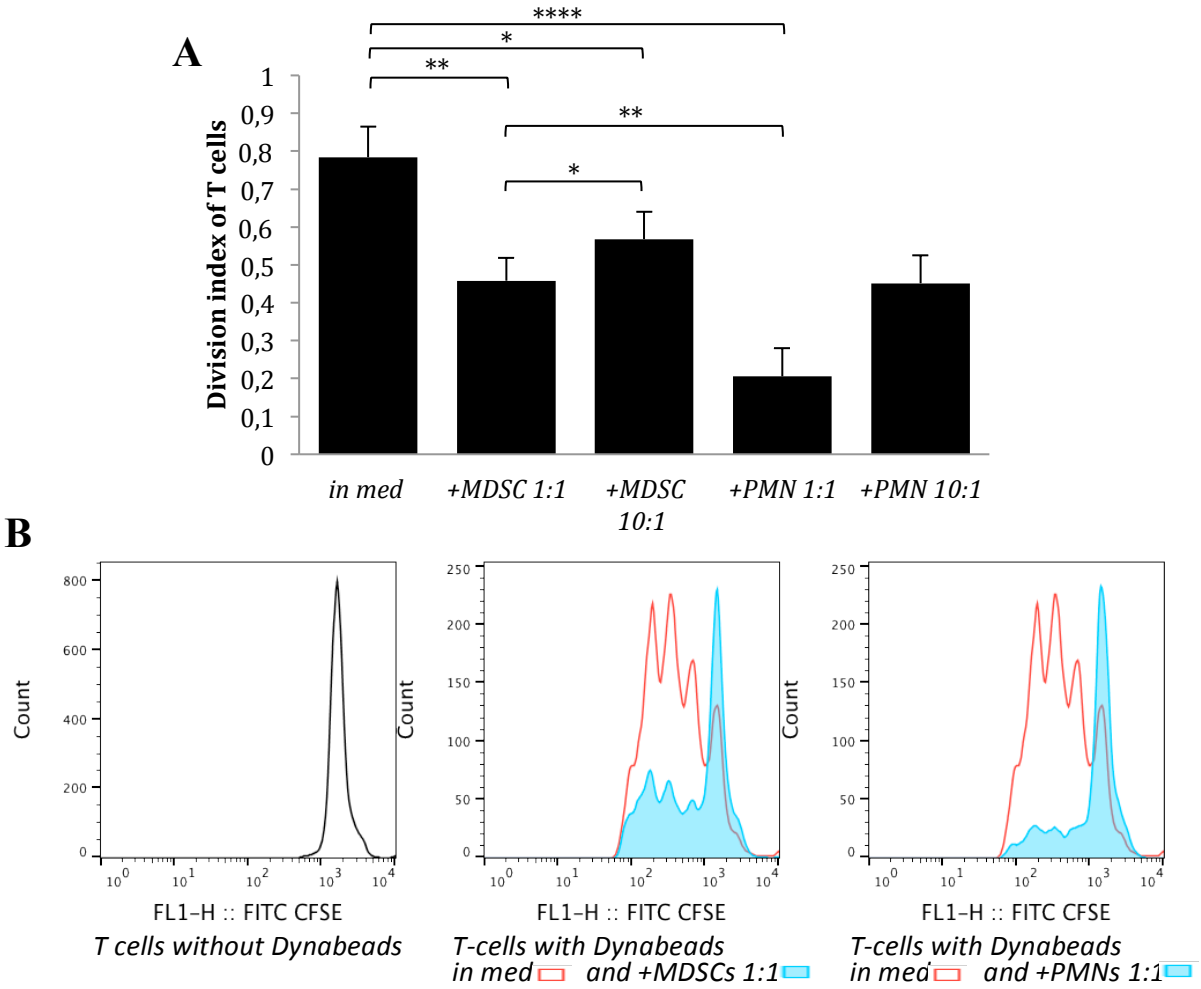


Figure 31: Both MDSCs and PMNs inhibited the proliferation of T cells. Purified T cells were cultured in medium alone (med), with MDSCs or PMNs 1:1 or 10:1. Cells were labeled with CFSE. CD3/CD28 Dynabeads were added or not for each condition. After 72 h, surface staining for CD3 was performed and CFSE staining was analyzed by flow cytometry. **A** shows division indices of T cells, which are the average numbers of divisions per cell. **B** shows histograms of CFSE-positive T cells normalized to unstained from a representative experiment. Error bars indicate SEM. Statistical analysis with Student's *t*-test (**p*<0,1 ***p*<0,05 *****p*<0,001), n=3.

5 Discussion

Granulocytic myeloid-derived suppressor cells (MDSCs) accumulate in tumor diseases and promote tumor progression. Their suppression of an efficient T cell response is considered as a major mechanism. The aim of this thesis was to examine their influence on natural killer (NK) cells, which are likewise important effector cells against cancer, and in particular against leukemia. Beside MDSCs, mature polymorphonuclear neutrophils (PMNs) were also examined, whose distinction from MDSCs is controversial. Only few previous publications have addressed the interaction between human NK cells and granulocytic cells, and none has directly compared MDSCs and PMNs. In this study, I present co-culture experiments of NK cells with either MDSCs or PMNs that demonstrate inhibitory effects of both granulocyte subsets on NK cell activation, cytotoxicity and cytokine release in response to leukemia cells.

5.1 Granulocytic cells impair NK cell activation and function

Murine tumor models have previously shown that MDSCs can suppress NK cell cytotoxicity and cytokine release [104, 204, 205]. This finding was confirmed in cancer patients, but the authors only examined monocytic and not granulocytic MDSCs [71]. Another group has recently demonstrated that human granulocytic MDSCs, which had been induced in vitro by stimulation with fungal germ tubes, could also impair NK cell cytotoxicity [208]. The results of my thesis confirm an inhibitory effect of freshly isolated granulocytic MDSCs derived from healthy human donors on NK cell activation and cytotoxicity as well as the production of IFNγ and TNFα, in response to leukemia cells. So far, a suppressive effect of MDSCs derived from healthy individuals has been described on murine T cells but not human NK cells [167, 187]. I further show that mature PMNs have similar effects as MDSCs, which is compatible with the literature: although very few authors have directly compared PMNs and MDSCs, suppressive effects on T and other immune cells have been described for both [222, 288].

5.1.1 Activation of NK cells in response to target

Rieber et al. have recently shown that the adoptive transfer of murine MDSCs into mice caused a reduced activation state of T and NK cells. The group generated MDSCs in vitro by stimulation with fungal components. Here, I demonstrate that human MDSCs and

PMNs, isolated from the fresh blood of healthy donors, had similar suppressive effects on the activation of NK cells in response to K562 leukemia cells. Like Rieber et al., I measured the expression of CD69 to assess NK cell activation. CD69 was up-regulated on the NK cell surface after stimulation with K562 cells, but this up-regulation was significantly impaired under the influence of granulocytic cells. I further analyzed the expression of CD137, which is also considered as an activation marker on NK cells. However, its role is complex and its exact function poorly understood. In other studies, the expression of CD137 correlated with general activation and cytokine release but not cytotoxicity of lymphocytes [57, 291]. Moreover, the receptor can also transmit inhibitory signaling [55]. I observed a marked increase of the CD137 expression after stimulation of NK cells with K562 cells. The pre-treatment of NK cells with granulocytic cells provoked opposing changes, which were not statistically significant. It can be concluded that granulocytic cells inhibit some, but probably not all, stimulatory signaling between NK cells and target cells.

Having shown that MDSCs and PMNs impaired the activation of NK cells in response to a target, I found that NK cells also produced less cytokines in the presence of MDSCs or PMNs. After intracellular staining, the signals for IFNγ and TNFα were weak in resting NK cells, because the cytokines are hardly produced by resting NK cells [292]. The stimulation with K562 cells provoked an increased cytokine production, but this response was diminished under the influence of granulocytic cells (> 50 %), regarding both cytokines. For IFNγ, the effect could even be demonstrated in resting NK cells. Two other publications have previously suggested that human myeloid cells inhibit the IFNγ release by NK cells: Hoechst et al. incubated NK cells with monocytic MDSCs derived from cancer patients, and demonstrated that they impaired IFNγ release via engagement of the NKp30 receptor [71]. Oberlies et al. treated NK cells with arginase 1, which was derived from PMNs, and likewise reported impaired IFNγ release [82]. My experiments confirm that both granulocytic MDSCs and PMNs, derived from healthy donors, can impair the production of IFNγ and TNFα by NK cells. These cytokines contribute to the cytotoxicity against tumor cells directly and are potent immunostimulatory cytokines [31, 32] that favor a better outcome in cancer patients [35]. The suppression of their production by granulocytic cells may therefore worsen the prognosis. Further experiments are needed to

evaluate the underlying mechanisms and find possible therapeutic targets, which may involve arginase 1 or NKp30. An engagement of the NKp30 receptor is also supported by my data (5.1.3).

5.1.2 NK cell cytotoxicity

The most recognized function of NK cells is their cytotoxicity against tumor cells. I assessed NK cell cytotoxicity by measuring highly degranulated NK cells after incubation with K562 target cells [255, 281]. I demonstrate that the cytotoxicity of NK cells is impaired by both MDSCs and PMNs. This is consistent with the recent publication of Rieber et al., who have incubated NK cells with MDSCs that had been induced in vitro by the stimulation with fungal components. The group has found that the fungus-induced MDSCs impaired NK cell cytotoxicity. However, they have not considered non-fungal-induced MDSCs or PMNs [208]. My results, on the other hand, confirm the observation in mice that MDSCs derived from healthy individuals also have suppressive potential [187]. Furthermore, they confirm earlier publications reporting that PMNs suppress NK cell cytotoxicity [218-221]. In direct comparison, I demonstrate that human MDSCs and PMNs derived from healthy individuals similarly suppressed NK cell cytotoxicity against leukemia cells in vitro. It is probable that both granulocytic cell types have even stronger suppressive effects in leukemia patients [167]. This could be detrimental in patients receiving hematopoietic stem cell transplantation, since the cytotoxic activity of NK cells is crucial for a prolonged remission [25].

5.1.3 Expression of activating receptors

The regulation of NK cell cytotoxicity is mediated via certain activating and inhibitory receptors on their cell surface. NKp30, NKp46, NKp44, NKG2D and CD16 all mediate NK cell activation in response to tumor cells and promote NK cell cytotoxicity [293-295]. Low expressions of activating receptors have been associated with tumorigenesis, and their down-regulation may represent a tumor-escape mechanism. In fact, it was demonstrated in vitro that tumor cells down-regulate NKG2D [78], and tumor-expanded monocytic MDSCs down-regulate NKp30 [71]. A ligand for NKG2D was also identified on murine MDSCs [203].

I therefore examined the expression of activating receptors under the influence of MDSCs or PMNs, and found a down-regulation of NKp30 following co-culture. Because the

expression of NKp30 on NK cells is generally variable [65, 69], the result was only statistically significant after several experiments, and the average signal reduction was weak. However, the data are in line with the work by Hoechst et al., who have observed similar effects for monocytic MDSCs. The group has demonstrated that the engagement of NKp30 was responsible for the suppression of NK cell cytotoxicity and IFNγ release [71]. Here, I show a down-regulation of NKp30 induced by granulocytic MDSCs and PMNs, which could likewise mediate their suppressive effects. In fact, the down-regulation of NKp30 has been implicated in NK cell suppression in several other contexts as well [72, 73, 206, 207]. Further experiments are necessary to verify whether NKp30 is responsible for the effects observed here, for example by using blocking antibodies.

Concerning other activating receptors, I observed no changes in expression under the influence of granulocytic cells. I could not confirm the observation in tumor-bearing mice that NKG2D is down-regulated by MDSCs [104]. However, the effect on NKG2D could be limited to a certain subset of $LyC6^{neg}$ MDSCs as opposed to the typical granulocytic $LYC6^{low}$ subset [159], as was suggested by another author [296]. Moreover, murine MDSCs differ from human MDSCs, and MDSCs in the healthy differ from MDSCs in different types of cancer [167, 193]. In line with other published data, I observed that NKp46, CD16 and NKG2D were strongly expressed by > 90 % of NK cells in all conditions, and NKG2D showed some responsiveness to cytokine stimulation [65, 297]. NKp44 expression was minimal but increased after cytokine-stimulation, which is also consistent with the literature [68].

5.2 Positive effects on NK cell activation, maturation and survival

Although I and others have demonstrated that granulocytic cells inhibit NK cell activation and function, the co-culture experiments performed here suggest that both granulocytic MDSCs and PMNs can also have positive effects on NK cell activation, maturation and survival. Few authors have previously suggested similar stimulatory effects of myeloid cells on lymphocytes in mice [202, 203, 267, 284].

5.2.1 Activation of NK cells

After co-culture of NK cells with granulocytic cells at a 1:2 ratio, I observed a significant up-regulation of the NK cell activation marker CD69. However, lower co-culture ratios of

1:1 did not influence the CD69 expression, suggesting that the effect may be concentration-dependent. Nausch et al. have previously shown a concentration-dependent activation of murine NK cells by monocytic MDSCs [203], and another group has suggested an activating effect of murine neutrophils on NK cells [298]. Furthermore, earlier publications have demonstrated that neutrophil proteases can activate human NK cells in a dose-dependent manner [299, 300]. Together with the published data, my results suggest that granulocytic cells can have activating effects on NK cells under certain circumstances.

Having demonstrated that granulocytic cells have the potential to activate NK cells, I show that they also provoke NK cell degranulation, confirming previous observations by Thoren et al. and Amano et al. [42, 298]. Amano et al. recently suggested that murine neutrophils activate NK cells in vivo and promote their CD107a surface expression. Thoren et al. have shown that human NK cells degranulate in the presence of PMNs and can induce neutrophil apoptosis via the Fas pathway. The degranulation itself did not affect the viability of PMNs. The data in my thesis confirm a degranulation of NK cells in response to MDSCs and PMNs without associated damage of the granulocytes. The degranulation pattern differed from that observed with a classical target: Incubation with K562 cells provoked very high signals for CD107a, indicating the release of many granules and strong cytotoxicity. Like Thoren et al., I show that culture with granulocytic cells leads to a much weaker degranulation, which does not correlate with cytotoxicity but may still reflect an activation of NK cells [255, 281].

Although the effects of MDSCs and PMNs on NK cell activation presented in my thesis are partly contradictory, they are consistent with the fact that granulocytic cells play a role as regulators of the immune system and show a broad functional plasticity [301]. As Bronte et al. have suggested, the particular inflammatory microenvironment may determine whether activating or inhibitory functions predominate [202]. Part of the underlying principle could be that acute, highly inflammatory situations with many neutrophils in the microenvironment provoke a positive feedback, while a less inflammatory milieu may provoke a negative feedback to prevent immune overreaction and chronic inflammation.

5.2.2 Maturation and survival

Granulocytic cells also influence the maturation and survival of NK cells. In a mouse model, Jaeger et al. have shown that neutrophils are needed for the maturation of NK cells. Here, I show that human MDSCs and PMNs induce a more mature phenotype of NK cells in vitro. The percentage of pre-mature $CD56^{bright}$ NK cells, which are considered as precursors, diminished in favor of $CD56^{dim}$ NK cells, in culture with granulocytic cells. On the other hand, CD16 expression remained stable. Although mature $CD56^{dim}$ NK cells are typically $CD16^{pos}$ and pre-mature $CD56^{bright}$ NK cells are $CD16^{neg}$, pre-mature NK cells can also be $CD56^{bright}$ and $CD16^{dim}$, representing an intermediate phenotype [49]. The maturation of NK cells does therefore not necessarily go along with an increase in $CD16^{pos}$ NK cells. As mature, $CD56^{dim}$ NK cells produce less cytokines than $CD56^{bright}$ NK cells, the maturation of NK cells may go along with a reduced cytokine production, which may partly account for my results on the IFNγ and TNFα production. Interestingly, culture with K562 target cells had the opposite effect on the NK cell phenotype, provoking an increase in the pre-mature $CD56^{bright}$ population. $CD56^{bright}$ NK cells were previously reported to accumulate in tumor tissues [302] and in the peripheral blood of advanced cancer patients, which was associated with low cytotoxic function [303]. The transformation of NK cells into a less mature and less cytotoxic phenotype by tumor cells may represent an immune escape mechanism. In my co-culture experiments, granulocytic cells induced NK cell maturation even despite the influence of K562 cells. In theory, this could affect the anti-tumor immunity in a positive way.

My data from flow cytometric analysis also suggest that granulocytic cells improve the survival of NK cells in co-culture. The viability after 16 h of culture in medium was generally poor (around 60 %), which is consistent with the viability of unstimulated NK cells observed by others [304]. Importantly, I show that the NK cell viability was improved significantly (to > 85 %) in co-culture with MDSCs or PMNs. The effects were similar when K562 cells were added to co-cultures. These findings are compatible with prior observations in a mouse model, where depletion of neutrophils led to increased NK cell apoptosis [284]. Moreover, a similar effect of granulocytes has also been described on dendritic cells [305]. On the other hand, the viability of NK cells was reduced in the presence of high-dose IL-15. Although IL-15 can have positive effects on NK cell survival, high doses have been reported to provoke apoptosis before [95, 96]. My

observations may partly be caused by changes in the proliferative behavior of NK cells, which is influenced by both IL-15 and granulocytic cells. Proliferating NK cells show reduced survival [47, 96]. IL-15 promotes NK cell proliferation, and may thereby increase apoptosis [96]. Granulocytic cells inhibit NK cell proliferation, and may thereby improve survival [82, 284].

Taken together, I demonstrate that the co-culture of NK cells with granulocytic cells has positive effects on NK cell maturation and survival, and negative effects on NK cell cytotoxicity and cytokine production. In line with other publications, my data suggest that granulocytic cells can activate NK cells under certain circumstances [42, 299, 300]. However, apart from one mouse model, there is no evidence that this is relevant in vivo [284]. Moreover, I show that the suppression of NK cell activation and function predominates when NK cells are confronted with a target.

5.3 Comparison between MDSCs and PMNs

Neutrophils present the most abundant immune cell population in the peripheral blood, and they exert various functions that are partly contradicting. These functions include phagocytosis, extracellular trap formation and the release of proteases, oxygen radicals and pro- as well as anti-inflammatory cytokines. As a consequence, the heterogeneity of neutrophils is increasingly recognized, and many authors suggest to divide them into different subsets [226]. Neutrophils in tumor tissue have been labeled as tumor-associated-neutrophils (TANs), and are further divided into pro-inflammatory N1 and anti-inflammatory N2 TANs. Another anti-inflammatory granulocyte subset was identified in the spleen and peripheral blood of tumor bearing mice and called myeloid-derived suppressor cells (MDSCs) [224]. Similar immunosuppressive cells were later identified in peripheral blood of cancer patients. In contrast to PMNs, they were considered as immature, immunosuppressive and of a low density. However, there is a lack of specific markers, and the evidence that these cells are different from PMNs is poor [268, 306]. In fact, several publications have described immunosuppressive features similar to MDSCs for regular PMNs [167, 219, 220].

Like others before, I have used the characteristic of a lower density compared to PMNs to isolate granulocytic MDSCs [155, 162-167]. However, not all authors refer to low-density granulocytes as MDSCs. Only few groups have directly compared high- and

low-density granulocytes, and only effects on T cells were considered. The data presented here support the idea of Rodriguez et al. that granulocytic MDSCs do not substantially differ from mature PMNs [162]. I observed a similar morphology and similar surface marker expressions of the cells. Although some authors have described differences in the expression of CD66b and CD11b, their results are partly contradictory and the differences reported are not major. In accordance with my results, both subsets expressed these markers to almost 100 %, differing only in signal intensity. Moreover, the authors used magnetic microbeads binding CD11b and CD66b for the isolation of MDSCs but not PMNs, which may be responsible for the differences in signal intensity [114, 162, 167].

In co-culture with NK cells, I show similar suppressive effects of MDSCs and PMNs on the NK cell function in all readouts, and also similar effects on NK cell maturation and survival. Other authors who previously compared MDSCs and PMNs have analyzed their effect on T cell proliferation. Importantly, I demonstrate that both granulocyte subsets suppress T cell proliferation. My data are contradictory to several publications on murine MDSCs, who reported that only MDSCs but not PMNs suppressed T cell proliferation [127, 180, 187]. Furthermore, they are contradicting to the data of Rieber et al., who suggested the same for human cells. However, the group worked with MDSCs generated in vitro, which most likely differed from the freshly isolated cells used in this study. Furthermore, they only showed one CFSE staining result without demonstrating statistical significance [208]. My results are consistent with other data from the human system showing that PMNs impair T cell proliferation [288, 289]. They are also partly consistent with the comparison of human MDSCs and PMNs by Giallongo et al. The group demonstrated similar suppressive effects for high- and low-density granulocytes from leukemia patients (without using the term MDSCs). In contrast to my results, they could not show the effects for cells from healthy donors [167]. However, the group used higher granulocyte- to T cell ratios. As my data on CD69 suggest, this may influence the interaction. The work presented here is the first to show that high- and low-density granulocytes, derived from healthy donors, have similar suppressive effects on both T and NK cells. Considering the plasticity of granulocytic cells, it is possible that the cells do attain distinct functions in certain pathological settings. However, together with the results of others, my data question whether density and marker expression are suitable to differentiate between suppressive and non-suppressive granulocyte subsets.

Some have suggested that granulocytic MDSCs, or low-density granulocytes, actually present activated PMNs. They have reported that in vitro stimulation of PMNs provoked a decrease in density and the acquisition of immunosuppressive abilities [127, 162]. Regarding my results, it is therefore conceivable that the PMNs were activated after isolation and became MDSCs. In fact, Costantini et al. reported that PMNs can become activated by co-culture with NK cells. However, the effect was only observed for IL-18-stimulated NK cells and is therefore unlikely to apply here [307]. Instead, my data suggest that non-activated PMNs are immunosuppressive.

In two readouts, I did demonstrate slightly stronger suppressive effects for MDSCs compared to PMNs, but in others I observed the opposite. These differences could be due to the heterogeneity of the isolated MDSC, which did not consist of a uniform granulocytic MDSCs population but contained a very variable proportion of $CD66b^{pos}$ $CD14^{pos}$ $HLA\text{-}II^{dim}$ monocytic MDSCs [161]. This population may have different effects than granulocytic MDSCs or PMNs and influence the results. For example, I show a stronger down-regulation of NKp30 and a stronger up-regulation of CD69 in 2:1 co-culture for MDSCs compared to PMNs. Both effects have previously been reported for monocytic MDSCs but not granulocytic cells. The effects could be stronger for monocytic MDSCs compared to granulocytic MDSCs or PMNs [71]. On the other hand, there is evidence suggesting that the suppression of T cell proliferation, NK cell cytotoxicity and cytokine production is stronger for granulocytic MDSCs and PMNs compared to monocytic cells [308, 309]. Consistent with this, my data show slightly stronger effects for PMNs compared to MDSCs in these readouts. The fact that the monocytic MDSCs population was very variable in size and lacked completely in some donors, could account for the poor statistical significance of these differences. The heterogeneity of MDSCs, not only between donors but especially in different pathological contexts, in combination with the lack of a uniform definition and isolation protocol, is likely to account for contradictory results when working with MDSCs, and slower research progress on the topic.

Taken together, I demonstrate that MDSCs have similar characteristics and functions as mature PMNs in the healthy individual. It is noteworthy that MDSCs are heterogeneous and show a high plasticity. They may therefore have distinct functions dependent on the microenvironment and pathological context. However, I clearly show that both MDSCs

and PMNs have the potential to suppress NK cell cytotoxicity and cytokine release. This supports the idea that granulocytic cells are immunomodulatory cells. Their impairment of NK cell function may be detrimental in the context of cancer. In fact, there is evidence that granulocytes become even more immunosuppressive in leukemia [167]. Finding potential targets for this interaction is warranted. The prospect that this concerns PMNs, and not exclusively MDSCs, will facilitate further investigations, because the latter are complex and laborious to identify and isolate [208].

5.4 Prospects

In this thesis, I demonstrate that granulocytic cells suppress NK cell activation, cytotoxicity and cytokine release against tumor cells in vitro. Several publications have reported an accumulation of suppressive granulocytic cells in cancer patients [148, 192], which has been correlated with cancer stage [174, 192]. So far, the investigations have been concentrating on T cell suppression [176, 310], but together with others, my data suggest that NK cells are likewise affected by suppressive granulocytic cells. This could be especially relevant in leukemia patients, where the functioning of NK cells importantly influences the outcome [230]. Because NK cells and granulocytes are among the first immune cells to reconstitute following hematopoietic stem cell transplantation (HSCT), it is likely that their interaction has a relevant influence on the function of graft NK cells against remaining leukemia cells [236, 311]. It would be interesting to see whether remission of leukemia after HSCT inversely correlates with the neutrophil-to-lymphocyte ratio, as has been demonstrated for several solid tumors following chemotherapy [312-314]. If a relevant NK cell suppression by granulocytic cells were confirmed in leukemia patients, inhibiting the interaction therapeutically could improve the prognosis. On the other hand, experiments in mice have suggested that MDSCs may have beneficial effects on graft-versus-host disease after HSCT by inhibiting cytotoxic T cells [315]. The relevance of this effect needs to be further evaluated and taken into account when targeting MDSCs. It would be ideal to specifically target and inhibit the interaction between granulocytic cells and NK cells, to enhance the graft-versus-leukemia effect without promoting graft-versus-host disease. In order to find targets, it is important to identify the mechanisms behind NK cell suppression. There is evidence for an involvement of IL-10 [156, 201], arginase 1 [82] and TGF-β [172]. My results do not

support an involvement of IL-10, but other mediators remain to be evaluated. In particular, TGF-β may be involved in the down-regulation of NKp30, and arginase 1 in the suppression of IFNγ production [82, 105]. Moreover, several molecules, including vitamin A, are able to modulate the function of MDSCs and could likewise be considered for therapeutic approaches [113, 192, 209, 212, 213].

6 Summary

Polymorphonuclear neutrophils (PMNs) are phagocytic cells of the innate immune system that efficiently kill bacteria. However, they also have regulatory effects on other immune cells and contribute to immunosuppression in cancer, which worsens the outcome. In particular, this has been demonstrated for a subset of granulocytic cells called myeloid-derived suppressor cells (MDSCs), but its distinction from PMNs is controversial. Most authors have explored the suppressive effects of MDSCs on T cells, but recent data suggest that NK cells are also affected. NK cells are crucial for the combat of tumor cells, in particular leukemic cells. There is hardly data available on the interaction between NK cells and suppressive granulocytic cells. Therefore, the aim of this thesis was to explore the effects of MDSCs and PMNs on the NK cell function against the leukemia cell line K562.

In co-culture experiments, I demonstrate that granulocytic MDSCs and PMNs had similar effects on NK cell function and homeostasis. On the one hand, they positively influenced the survival and maturation of NK cells. On the other, they inhibited the activation, cytotoxicity and cytokine production of NK cells, both IFNγ and TNFα, in response to K562 target cells. Furthermore, I show a down-regulation of the activating receptor NKp30 on NK cells in the presence of MDSCs or PMNs, which may form part of the underlying suppressive mechanisms.

However, there is also evidence for the involvement of other molecules. Further investigations are needed to confirm a relevant suppression of NK cells by granulocytic cells in cancer patients, and to identify therapeutic targets. The recognition that regular PMNs have similar effects on NK cells as MDSCs could simplify future experiments, since MDSCs are heterogeneous and laborious to isolate and identify.

NK cells and granulocytes are among the first immune cells to reconstitute after hematopoietic stem cell transplantation, and NK cells may be particularly exposed to suppressive effects of granulocytes this scenario. Modulating these suppressive effects of granulocytes on NK cells therapeutically may yield a better NK cell function and an improved cancer prognosis.

7 Zusammenfassung

Neutrophile Granulozyten (polymorphonuclear neutrophils, PMNs) sind Zellen des angeborenen Immunsystems, die insbesondere Bakterien durch Phagozytose unschädlich machen. Zusätzlich haben sie jedoch immunregulatorische und sogar immunsuppressive Eigenschaften. Sie tragen zur Suppression des Immunsystems im Rahmen von Tumorerkrankungen bei, was die Prognose negativ beeinflusst. Dies wurde insbesondere für einen Subtyp von Granulozyten, so genannte myeloide Suppressorzellen (myeloid-derived suppressor cells, MDSCs), gezeigt, wobei deren Unterscheidung von PMNs kontrovers diskutiert wird. Die meisten Autoren untersuchten suppressive Effekte von MDSCs auf T Zellen, doch auch NK Zellen sind betroffen. Eine funktionierende NK Zell Antwort ist entscheidend für die Abwehr von Tumorerkrankungen, insbesondere Leukämie. Über die Interaktion von NK Zellen und MDSCs ist bisher wenig bekannt. Ziel dieser Arbeit war es, den Einfluss von MDSCs und PMNs auf die NK Zell Antwort auf Leukämiezellen zu untersuchen.

In Co-Kultur Experimenten zeige ich, dass granulozytäre MDSCs und PMNs ähnliche Effekte auf die Funktionen und Homöostase von NK Zellen haben. Einerseits wirkten sie sich positiv auf das Überleben und die Reifung der NK Zellen aus, andererseits hemmten sie deren Aktivierung, Zytotoxizität und Zytokinproduktion (sowohl IFNγ als auch TNFα) als Antwort auf die Leukämie Zelllinie K562. Ferner war der Aktivierungsrezeptor NKp30 auf NK Zellen unter dem Einfluss der Granulozyten vermindert exprimiert, was mit für die inhibitorische Effekte verantwortlich sein könnte.

In weiteren Experimenten sollte die Relevanz der hier gezeigten Effekte in vivo bestätigt werden und die zugrundeliegenden Mechanismen untersucht werden. Der Hinweis, dass PMNs ähnliche hemmende Effekte auf NK Zellen ausüben wie MDSCs kann zukünftige Experimente vereinfachen, da die Isolierung der MDSCs aufwändig und uneinheitlich ist.

NK Zellen und neutrophile Granulozyten sind unter den ersten Immunzellen, die sich nach einer hämatopoietischen Stammzelltransplantation erholen. NK Zellen könnten in dieser Situation besonders den hemmenden Einflüssen der Granulozyten unterliegen. Ein therapeutisches Eingreifen in diese Interaktion könnte die NK Zell Antwort stärken und die Prognose von Leukämieerkrankungen verbessern.

8 Affidavit

I hereby confirm that my thesis entitled *Influence of myeloid-derived suppressor cells and neutrophil granulocytes on natural killer cell homeostasis and function* is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Hannover, 12.03.2016

9 Abbreviations

µl. *microliter*
µM. *mikromolar*
°C. *degrees Celcius*
APC. *allophycocyanin*
CD. *cluster of differentiation*
CEACAM. *carcinoembryonic-antigen-related cell-adhesion molecule*
CFSE. *carboxyfluorescein succinimidyl ester*
CH. *Switzerland*
DE. *Germany*
DNA. *deoxyribonucleic acid*
EDTA. *ethylenediaminetetraacetic acid*
ELISA. *enzyme-linked-immunosorbent assay*
Fab. *fragment antigen-binding*
FACS. *Fluorescence Activated Cell Sorting*
FasL. *Fas ligand*
Fc. *fragment, crystallizable reagion*
FITC. fluorescein isothiocyanate
FSC. *forward scatter*
g. *gram*
GM-CSF. *granulocyte macrophage colony-stimulating factor*
GMFI. *geometric mean of fluorescence intensity*
h. *hour(s)*
HBSS. *Hank's balanced salt solution*
HIV. *human immunodeficiency virus*
HLA. *human leukocyte antigen*
HRP. *horseradish peroxidase*
HSCT. *hematopoietic stem cell transplantation*
IFN. *interferon*
Ig. *immunoglobulin*
IL. *interleukin*
IU. *international unit(s)*, *international unit(s)*
kDa. *kilodaltons*
KIR. *killer cell immunoglobulin-like receptor*
LPS. *lipopolysaccharide*
LRSC. *leukocyte reduction system chambers*, *leukocyte reduction system chambers*
M. *molar*
MACS. *magnetic-activated cell sorting*
MDSCs. *myeloid-derived suppressor cells*
med. medium
mg. *milligram*
MHC. *major-histocompatibility complex*
ml. *milliliter*
mM. *millimolar*
n. *number of independently performed experiments*
neg. *negative*
ng. *nanogram*
NOS. *nitrit oxide synthase*
p. *p-value*
PBMCs. *peripheral blood mononuclear cells*
PE. *phycoerythrin*
PerCP. *peridinin chlorophyll*
PG. *prostaglandin*
PMNs. *polymorphonuclear neutrophils*
pos. *positive*
RNA. *ribonucleic acid*
ROS. *reactive oxygen species*
rpm. *revolutions per minute*
RPMI. Rosewell Park Memorial Institute
RT. *room temperature*
SCF. *stem cell factor*
SEM. *standard error of the mean*
SSC. *side scatter*
STAT. *signal transducers and activators of transcprition*
TAN. *tumor-associated neutrophils*
TGF. *transforming growth factor*
TMB. *tetramethylbenzidine*
TNF. *tumor necrosis factor*
TRAIL. *tumor necrosis factor related apoptosis inducing ligand*
USA. *United States of America*
VEGF. *vascular endothelial growth factor*
vs. *versus*
WL. *wavelength*
µg. *microgram*

10 References

1. Uhrberg, M., *The CD107 mobilization assay: viable isolation and immunotherapeutic potential of tumor-cytolytic NK cells.* Leukemia, 2005. **19**(5): p. 707-9.
2. Mogensen, T.H., *Pathogen recognition and inflammatory signaling in innate immune defenses.* Clin Microbiol Rev, 2009. **22**(2): p. 240-73, Table of Contents.
3. Finn, O.J., *Immuno-oncology: understanding the function and dysfunction of the immune system in cancer.* Ann Oncol, 2012. **23 Suppl 8**: p. viii6-9.
4. Yaqub, S. and E.M. Aandahl, *Inflammation versus adaptive immunity in cancer pathogenesis.* Crit Rev Oncog, 2009. **15**(1-2): p. 43-63.
5. Kitamura, T., B.Z. Qian, and J.W. Pollard, *Immune cell promotion of metastasis.* Nat Rev Immunol, 2015. **15**(2): p. 73-86.
6. Artis, D. and H. Spits, *The biology of innate lymphoid cells.* Nature, 2015. **517**(7534): p. 293-301.
7. Alberts B, A.B., Johnson A, Lewis J, et al, *Molecular Biology of the Cell*. Lymphocytes and the Cellular Basis of Adaptive Immunity. Vol. 4. 2002, New York: Garland Science.
8. Ellmark, P., et al., *The applicability of a cluster of differentiation monoclonal antibody microarray to the diagnosis of human disease.* Methods Mol Biol, 2008. **439**: p. 199-209.
9. Ljunggren, H.G. and K. Karre, *In search of the 'missing self': MHC molecules and NK cell recognition.* Immunol Today, 1990. **11**(7): p. 237-44.
10. Stadnisky, M.D., et al., *Self MHC class I-licensed NK cells enhance adaptive CD8 T-cell viral immunity.* Blood, 2011. **117**(19): p. 5133-41.
11. Perera, P.Y., et al., *The role of interleukin-15 in inflammation and immune responses to infection: implications for its therapeutic use.* Microbes Infect, 2012. **14**(3): p. 247-61.
12. Shin, J.S., et al., *Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination.* Nature, 2006. **444**(7115): p. 115-8.
13. Whitmire, J.K., B. Eam, and J.L. Whitton, *Tentative T cells: memory cells are quick to respond, but slow to divide.* PLoS Pathog, 2008. **4**(4): p. e1000041.
14. Lanier, L.L. and J.C. Sun, *Do the terms innate and adaptive immunity create conceptual barriers?* Nat Rev Immunol, 2009. **9**(5): p. 302-3.
15. Konjevic, G., et al., *Investigation of NK cell function and their modulation in different malignancies.* Immunol Res, 2012. **52**(1-2): p. 139-56.
16. Zhang, Y., et al., *In vivo kinetics of human natural killer cells: the effects of ageing and acute and chronic viral infection.* Immunology, 2007. **121**(2): p. 258-65.
17. Di Santo, J.P., *Natural killer cells: diversity in search of a niche.* Nat Immunol, 2008. **9**(5): p. 473-5.
18. Kiessling, R., et al., *"Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell.* Eur J Immunol, 1975. **5**(2): p. 117-21.
19. Lodoen, M.B. and L.L. Lanier, *Natural killer cells as an initial defense against pathogens.* Curr Opin Immunol, 2006. **18**(4): p. 391-8.
20. Chong, A.S., et al., *Phenotypic and functional analysis of lymphokine-activated killer (LAK) cell clones. Ability of CD3+, LAK cell clones to produce interferon-gamma and tumor necrosis factor upon stimulation with tumor targets.* Cancer Immunol Immunother, 1989. **29**(4): p. 270-8.
21. Vivier, E., et al., *Functions of natural killer cells.* Nat Immunol, 2008. **9**(5): p. 503-10.
22. Dunne, J., et al., *Selective expansion and partial activation of human NK cells and NK receptor-positive T cells by IL-2 and IL-15.* J Immunol, 2001. **167**(6): p. 3129-38.
23. Parham, P., *Immunogenetics of killer cell immunoglobulin-like receptors.* Mol Immunol, 2005. **42**(4): p. 459-62.
24. Cerwenka, A. and L.L. Lanier, *Natural killer cells, viruses and cancer.* Nat Rev Immunol, 2001. **1**(1): p. 41-9.
25. Lowdell, M.W., et al., *Evidence that continued remission in patients treated for acute leukaemia is dependent upon autologous natural killer cells.* Br J Haematol, 2002. **117**(4): p. 821-7.
26. Konjevic, G., et al., *Association of decreased NK cell activity and IFNgamma expression with pSTAT dysregulation in breast cancer patients.* J BUON, 2011. **16**(2): p. 219-26.
27. Imai, K., et al., *Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population.* Lancet, 2000. **356**(9244): p. 1795-9.

28. Ma, L.L., et al., *NK cells use perforin rather than granulysin for anticryptococcal activity.* J Immunol, 2004. **173**(5): p. 3357-65.
29. Cullen, S.P. and S.J. Martin, *Mechanisms of granule-dependent killing.* Cell Death Differ, 2008. **15**(2): p. 251-62.
30. Smyth, M.J., et al., *Cytokines in cancer immunity and immunotherapy.* Immunol Rev, 2004. **202**: p. 275-93.
31. Wang, X. and Y. Lin, *Tumor necrosis factor and cancer, buddies or foes?* Acta Pharmacol Sin, 2008. **29**(11): p. 1275-88.
32. Lange, F., et al., *Studies on mechanisms of interferon-gamma action in pancreatic cancer using a data-driven and model-based approach.* Mol Cancer, 2011. **10**(1): p. 13.
33. Zaidi, M.R. and G. Merlino, *The two faces of interferon-gamma in cancer.* Clin Cancer Res, 2011. **17**(19): p. 6118-24.
34. Wei, J., et al., *Critical role of dendritic cell-derived IL-27 in antitumor immunity through regulating the recruitment and activation of NK and NKT cells.* J Immunol, 2013. **191**(1): p. 500-8.
35. Menard, C., et al., *Natural killer cell IFN-gamma levels predict long-term survival with imatinib mesylate therapy in gastrointestinal stromal tumor-bearing patients.* Cancer Res, 2009. **69**(8): p. 3563-9.
36. Piccioli, D., et al., *Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells.* J Exp Med, 2002. **195**(3): p. 335-41.
37. Mailliard, R.B., et al., *IL-18-induced CD83+CCR7+ NK helper cells.* J Exp Med, 2005. **202**(7): p. 941-53.
38. Costantini, C. and M.A. Cassatella, *The defensive alliance between neutrophils and NK cells as a novel arm of innate immunity.* J Leukoc Biol, 2011. **89**(2): p. 221-33.
39. Hayakawa, Y., et al., *NK cell TRAIL eliminates immature dendritic cells in vivo and limits dendritic cell vaccination efficacy.* J Immunol, 2004. **172**(1): p. 123-9.
40. Nedvetzki, S., et al., *Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses.* Blood, 2007. **109**(9): p. 3776-85.
41. Nielsen, N., et al., *Cytotoxicity of CD56(bright) NK cells towards autologous activated CD4+ T cells is mediated through NKG2D, LFA-1 and TRAIL and dampened via CD94/NKG2A.* PLoS One, 2012. **7**(2): p. e31959.
42. Thoren, F.B., et al., *Human NK Cells induce neutrophil apoptosis via an NKp46- and Fas-dependent mechanism.* J Immunol, 2012. **188**(4): p. 1668-74.
43. Ugolini, S. and E. Vivier, *Immunology: Natural killer cells remember.* Nature, 2009. **457**(7229): p. 544-5.
44. Jia, M., et al., *Impaired natural killer cell-induced antibody-dependent cell-mediated cytotoxicity is associated with human immunodeficiency virus-1 disease progression.* Clin Exp Immunol, 2013. **171**(1): p. 107-16.
45. Lanier, L.L., et al., *The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes.* J Immunol, 1986. **136**(12): p. 4480-6.
46. Warren, H.S. and B.F. Kinnear, *Quantitative analysis of the effect of CD16 ligation on human NK cell proliferation.* J Immunol, 1999. **162**(2): p. 735-42.
47. Lutz, C.T., et al., *Human NK cells proliferate and die in vivo more rapidly than T cells in healthy young and elderly adults.* J Immunol, 2011. **186**(8): p. 4590-8.
48. Romagnani, C., et al., *CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation.* J Immunol, 2007. **178**(8): p. 4947-55.
49. Cooley, S., et al., *A subpopulation of human peripheral blood NK cells that lacks inhibitory receptors for self-MHC is developmentally immature.* Blood, 2007. **110**(2): p. 578-86.
50. De Maria, A., et al., *Revisiting human natural killer cell subset function revealed cytolytic CD56(dim)CD16+ NK cells as rapid producers of abundant IFN-gamma on activation.* Proc Natl Acad Sci U S A, 2011. **108**(2): p. 728-32.
51. Fauriat, C., et al., *Regulation of human NK-cell cytokine and chemokine production by target cell recognition.* Blood, 2010. **115**(11): p. 2167-76.
52. Ziegler, S.F., F. Ramsdell, and M.R. Alderson, *The activation antigen CD69.* Stem Cells, 1994. **12**(5): p. 456-65.
53. Marzio, R., J. Mauel, and S. Betz-Corradin, *CD69 and regulation of the immune function.* Immunopharmacol Immunotoxicol, 1999. **21**(3): p. 565-82.

54. Dons'koi, B.V., V.P. Chernyshov, and D.V. Osypchuk, *Measurement of NK activity in whole blood by the CD69 up-regulation after co-incubation with K562, comparison with NK cytotoxicity assays and CD107a degranulation assay.* J Immunol Methods, 2011. **372**(1-2): p. 187-95.
55. Baessler, T., et al., *CD137 ligand mediates opposite effects in human and mouse NK cells and impairs NK-cell reactivity against human acute myeloid leukemia cells.* Blood, 2010. **115**(15): p. 3058-69.
56. Braun, M., et al., *NK cell activation in human hantavirus infection explained by virus-induced IL-15/IL15Ralpha expression.* PLoS Pathog, 2014. **10**(11): p. e1004521.
57. Wilcox, R.A., et al., *Signaling through NK cell-associated CD137 promotes both helper function for CD8+ cytolytic T cells and responsiveness to IL-2 but not cytolytic activity.* J Immunol, 2002. **169**(8): p. 4230-6.
58. Walzer, T., et al., *Natural killer cell-dendritic cell crosstalk in the initiation of immune responses.* Expert Opin Biol Ther, 2005. **5 Suppl 1**: p. S49-59.
59. Sivori, S., et al., *CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells.* Proc Natl Acad Sci U S A, 2004. **101**(27): p. 10116-21.
60. Parham, P., *MHC class I molecules and KIRs in human history, health and survival.* Nat Rev Immunol, 2005. **5**(3): p. 201-14.
61. Fernandez-Messina, L., H.T. Reyburn, and M. Vales-Gomez, *Human NKG2D-ligands: cell biology strategies to ensure immune recognition.* Front Immunol, 2012. **3**: p. 299.
62. Sivori, S., et al., *NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells.* Eur J Immunol, 1999. **29**(5): p. 1656-66.
63. Byrd, A., et al., *Expression analysis of the ligands for the Natural Killer cell receptors NKp30 and NKp44.* PLoS One, 2007. **2**(12): p. e1339.
64. Sivori, S., et al., *Involvement of natural cytotoxicity receptors in human natural killer cell-mediated lysis of neuroblastoma and glioblastoma cell lines.* J Neuroimmunol, 2000. **107**(2): p. 220-5.
65. Hsieh, C.L., et al., *NKp30 is a functional activation receptor on a subset of rat natural killer cells.* Eur J Immunol, 2006. **36**(8): p. 2170-80.
66. Wang, H., et al., *Important role for NKp30 in synapse formation and activation of NK cells.* Immunol Invest, 2012. **41**(4): p. 367-81.
67. Ferlazzo, G., et al., *Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells.* J Exp Med, 2002. **195**(3): p. 343-51.
68. Vitale, M., et al., *NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis.* J Exp Med, 1998. **187**(12): p. 2065-72.
69. Golden-Mason, L., et al., *Increased natural killer cell cytotoxicity and NKp30 expression protects against hepatitis C virus infection in high-risk individuals and inhibits replication in vitro.* Hepatology, 2010. **52**(5): p. 1581-9.
70. Garcia-Iglesias, T., et al., *Low NKp30, NKp46 and NKG2D expression and reduced cytotoxic activity on NK cells in cervical cancer and precursor lesions.* BMC Cancer, 2009. **9**: p. 186.
71. Hoechst, B., et al., *Myeloid derived suppressor cells inhibit natural killer cells in patients with hepatocellular carcinoma via the NKp30 receptor.* Hepatology, 2009. **50**(3): p. 799-807.
72. Arnon, T.I., et al., *Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus.* Nat Immunol, 2005. **6**(5): p. 515-23.
73. Holder, K.A., et al., *Hepatitis C virus-infected cells downregulate NKp30 and inhibit ex vivo NK cell functions.* J Immunol, 2013. **191**(6): p. 3308-18.
74. Vitale, C., et al., *The corticosteroid-induced inhibitory effect on NK cell function reflects down-regulation and/or dysfunction of triggering receptors involved in natural cytotoxicity.* Eur J Immunol, 2004. **34**(11): p. 3028-38.
75. Rolstad, B., *The early days of NK cells: an example of how a phenomenon led to detection of a novel immune receptor system - lessons from a rat model.* Front Immunol, 2014. **5**: p. 283.
76. Mistry, A.R. and C.A. O'Callaghan, *Regulation of ligands for the activating receptor NKG2D.* Immunology, 2007. **121**(4): p. 439-47.

77. Cerwenka, A., J.L. Baron, and L.L. Lanier, *Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo.* Proc Natl Acad Sci U S A, 2001. **98**(20): p. 11521-6.
78. Jimenez-Perez, M.I., et al., *Cervical cancer cell lines expressing NKG2D-ligands are able to down-modulate the NKG2D receptor on NKL cells with functional implications.* BMC Immunol, 2012. **13**: p. 7.
79. Sun, C., et al., *TGF-beta1 down-regulation of NKG2D/DAP10 and 2B4/SAP expression on human NK cells contributes to HBV persistence.* PLoS Pathog, 2012. **8**(3): p. e1002594.
80. Newman, K.C. and E.M. Riley, *Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens.* Nat Rev Immunol, 2007. **7**(4): p. 279-91.
81. Hellstrand, K., et al., *Histaminergic regulation of NK cells. Role of monocyte-derived reactive oxygen metabolites.* J Immunol, 1994. **153**(11): p. 4940-7.
82. Oberlies, J., et al., *Regulation of NK cell function by human granulocyte arginase.* J Immunol, 2009. **182**(9): p. 5259-67.
83. Fortin, C., X. Huang, and Y. Yang, *NK cell response to vaccinia virus is regulated by myeloid-derived suppressor cells.* J Immunol, 2012. **189**(4): p. 1843-9.
84. Ghiringhelli, F., et al., *CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner.* J Exp Med, 2005. **202**(8): p. 1075-85.
85. Meazza, R., et al., *Role of common-gamma chain cytokines in NK cell development and function: perspectives for immunotherapy.* J Biomed Biotechnol, 2011. **2011**: p. 861920.
86. Park, J.Y., et al., *IL-15-induced IL-10 increases the cytolytic activity of human natural killer cells.* Mol Cells, 2011. **32**(3): p. 265-72.
87. Strengell, M., et al., *IL-21 in synergy with IL-15 or IL-18 enhances IFN-gamma production in human NK and T cells.* J Immunol, 2003. **170**(11): p. 5464-9.
88. Boyiadzis, M., K.A. Foon, and R.B. Herberman, *NK cells in cancer immunotherapy: three decades of discovery.* Discov Med, 2006. **6**(36): p. 243-8.
89. Carson, W.E., et al., *Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor.* J Exp Med, 1994. **180**(4): p. 1395-403.
90. Kawamura, T., et al., *Differential roles for IL-15R alpha-chain in NK cell development and Ly-49 induction.* J Immunol, 2003. **171**(10): p. 5085-90.
91. Carson, W.E., et al., *A potential role for interleukin-15 in the regulation of human natural killer cell survival.* J Clin Invest, 1997. **99**(5): p. 937-43.
92. Tan, J.T., et al., *Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells.* J Exp Med, 2002. **195**(12): p. 1523-32.
93. Cooper, M.A., et al., *In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells.* Blood, 2002. **100**(10): p. 3633-8.
94. Rodella, L., et al., *Interleukin 2 and interleukin 15 differentially predispose natural killer cells to apoptosis mediated by endothelial and tumour cells.* Br J Haematol, 2001. **115**(2): p. 442-50.
95. Ross, M.E. and M.A. Caligiuri, *Cytokine-induced apoptosis of human natural killer cells identifies a novel mechanism to regulate the innate immune response.* Blood, 1997. **89**(3): p. 910-8.
96. Zhao, Y.M. and A.R. French, *Two-compartment model of NK cell proliferation: insights from population response to IL-15 stimulation.* J Immunol, 2012. **188**(7): p. 2981-90.
97. Liu, C.C., B. Perussia, and J.D. Young, *The emerging role of IL-15 in NK-cell development.* Immunol Today, 2000. **21**(3): p. 113-6.
98. Mrozek, E., P. Anderson, and M.A. Caligiuri, *Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells.* Blood, 1996. **87**(7): p. 2632-40.
99. Reinecker, H.C., et al., *Intestinal epithelial cells both express and respond to interleukin 15.* Gastroenterology, 1996. **111**(6): p. 1706-13.
100. Carson, W.E., et al., *Endogenous production of interleukin 15 by activated human monocytes is critical for optimal production of interferon-gamma by natural killer cells in vitro.* J Clin Invest, 1995. **96**(6): p. 2578-82.
101. Warren, H.S., et al., *Analysis of the costimulatory role of IL-2 and IL-15 in initiating proliferation of resting (CD56dim) human NK cells.* J Immunol, 1996. **156**(9): p. 3254-9.
102. Pillet, A.H., J. Theze, and T. Rose, *Interleukin (IL)-2 and IL-15 have different effects on human natural killer lymphocytes.* Hum Immunol, 2011. **72**(11): p. 1013-7.

103. Muntasell, A., et al., *Inhibition of NKG2D expression in NK cells by cytokines secreted in response to human cytomegalovirus infection.* Blood, 2010. **115**(25): p. 5170-9.
104. Li, H., et al., *Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1.* J Immunol, 2009. **182**(1): p. 240-9.
105. Castriconi, R., et al., *Transforming growth factor beta 1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 4120-5.
106. Chan, C.J., D.M. Andrews, and M.J. Smyth, *Can NK cells be a therapeutic target in human cancer?* Eur J Immunol, 2008. **38**(11): p. 2964-8.
107. Ruggeri, L., et al., *Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value.* Blood, 2007. **110**(1): p. 433-40.
108. Geller, M.A., et al., *A phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer.* Cytotherapy, 2011. **13**(1): p. 98-107.
109. Sheridan, C., *First-in-class cancer therapeutic to stimulate natural killer cells.* Nat Biotechnol, 2006. **24**(6): p. 597.
110. Moga, E., et al., *NK cells stimulated with IL-15 or CpG ODN enhance rituximab-dependent cellular cytotoxicity against B-cell lymphoma.* Exp Hematol, 2008. **36**(1): p. 69-77.
111. Arnould, L., et al., *Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism?* Br J Cancer, 2006. **94**(2): p. 259-67.
112. Borg, C., et al., *Novel mode of action of c-kit tyrosine kinase inhibitors leading to NK cell-dependent antitumor effects.* J Clin Invest, 2004. **114**(3): p. 379-88.
113. Mirza, N., et al., *All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients.* Cancer Res, 2006. **66**(18): p. 9299-307.
114. Cloke, T., et al., *Characterization of a novel population of low-density granulocytes associated with disease severity in HIV-1 infection.* PLoS One, 2012. **7**(11): p. e48939.
115. Yoon, J., A. Terada, and H. Kita, *CD66b regulates adhesion and activation of human eosinophils.* J Immunol, 2007. **179**(12): p. 8454-62.
116. Boyum, A., *Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g.* Scand J Clin Lab Invest Suppl, 1968. **97**: p. 77-89.
117. Segal, A.W., *How neutrophils kill microbes.* Annu Rev Immunol, 2005. **23**: p. 197-223.
118. Demissie, D.E., et al., *Altered neutrophil counts at diagnosis of invasive meningococcal infection in children.* Pediatr Infect Dis J, 2013. **32**(10): p. 1070-2.
119. Melendez, E. and M.B. Harper, *Risk of serious bacterial infection in isolated and unsuspected neutropenia.* Acad Emerg Med, 2010. **17**(2): p. 163-7.
120. Rutella, S., et al., *Efficacy of granulocyte transfusions for neutropenia-related infections: retrospective analysis of predictive factors.* Cytotherapy, 2003. **5**(1): p. 19-30.
121. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. **13**(3): p. 159-75.
122. Lowe, D.M., et al., *Neutrophils in tuberculosis: friend or foe?* Trends Immunol, 2012. **33**(1): p. 14-25.
123. Drescher, B. and F. Bai, *Neutrophil in viral infections, friend or foe?* Virus Res, 2013. **171**(1): p. 1-7.
124. Galdiero, M.R., et al., *Tumor associated macrophages and neutrophils in cancer.* Immunobiology, 2013. **218**(11): p. 1402-10.
125. Dissemond, J., et al., *Activated neutrophils exert antitumor activity against human melanoma cells: reactive oxygen species-induced mechanisms and their modulation by granulocyte-macrophage-colony-stimulating factor.* J Invest Dermatol, 2003. **121**(4): p. 936-8.
126. Granot, Z., et al., *Tumor entrained neutrophils inhibit seeding in the premetastatic lung.* Cancer Cell, 2011. **20**(3): p. 300-14.
127. Sagiv, J.Y., et al., *Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer.* Cell Rep, 2015. **10**(4): p. 562-73.
128. Fridlender, Z.G. and S.M. Albelda, *Tumor-associated neutrophils: friend or foe?* Carcinogenesis, 2012. **33**(5): p. 949-55.
129. Fried, A.J. and F.A. Bonilla, *Pathogenesis, diagnosis, and management of primary antibody deficiencies and infections.* Clin Microbiol Rev, 2009. **22**(3): p. 396-414.

130. Carbonaro, D.A., et al., *Neonatal bone marrow transplantation of ADA-deficient SCID mice results in immunologic reconstitution despite low levels of engraftment and an absence of selective donor T lymphoid expansion.* Blood, 2008. **111**(12): p. 5745-54.
131. Thoden, J., et al., *Therapy and prophylaxis of opportunistic infections in HIV-infected patients: a guideline by the German and Austrian AIDS societies (DAIG/OAG) (AWMF 055/066).* Infection, 2013. **41 Suppl 2**: p. S91-115.
132. Hatzimichael, E. and M. Tuthill, *Hematopoietic stem cell transplantation.* Stem Cells Cloning, 2010. **3**: p. 105-17.
133. Jacobson, M.A., H. Gellermann, and H. Chambers, *Staphylococcus aureus bacteremia and recurrent staphylococcal infection in patients with acquired immunodeficiency syndrome and AIDS-related complex.* Am J Med, 1988. **85**(2): p. 172-6.
134. Fishman, J.A., *Infections in immunocompromised hosts and organ transplant recipients: essentials.* Liver Transpl, 2011. **17 Suppl 3**: p. S34-7.
135. Spurrell, E.L. and M. Lockley, *Adaptive immunity in cancer immunology and therapeutics.* Ecancermedicalscience, 2014. **8**: p. 441.
136. Sheu, B.C., et al., *Cytokine regulation networks in the cancer microenvironment.* Front Biosci, 2008. **13**: p. 6255-68.
137. Ribechini, E., et al., *Subsets, expansion and activation of myeloid-derived suppressor cells.* Med Microbiol Immunol, 2010. **199**(3): p. 273-81.
138. Lindau, D., et al., *The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells.* Immunology, 2013. **138**(2): p. 105-15.
139. Torres-Poveda, K., et al., *Role of IL-10 and TGF-beta1 in local immunosuppression in HPV-associated cervical neoplasia.* World J Clin Oncol, 2014. **5**(4): p. 753-63.
140. Beyersdorf, N., et al., *Superagonistic anti-CD28 antibodies: potent activators of regulatory T cells for the therapy of autoimmune diseases.* Ann Rheum Dis, 2005. **64 Suppl 4**: p. iv91-5.
141. Weirather, J., et al., *Foxp3+ CD4+ T cells improve healing after myocardial infarction by modulating monocyte/macrophage differentiation.* Circ Res, 2014. **115**(1): p. 55-67.
142. Beyersdorf, N., et al., *In vitro polyclonal activation of conventional T cells with a CD28 superagonist protects mice from acute graft versus host disease.* Eur J Immunol, 2015. **45**(7): p. 1997-2007.
143. Young, M.R., M. Newby, and H.T. Wepsic, *Hematopoiesis and suppressor bone marrow cells in mice bearing large metastatic Lewis lung carcinoma tumors.* Cancer Res, 1987. **47**(1): p. 100-5.
144. Gabrilovich, D.I., et al., *The terminology issue for myeloid-derived suppressor cells.* Cancer Res, 2007. **67**(1): p. 425; author reply 426.
145. Lechner, M.G., D.J. Liebertz, and A.L. Epstein, *Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells.* J Immunol, 2010. **185**(4): p. 2273-84.
146. Ostrand-Rosenberg, S., et al., *Cross-talk between myeloid-derived suppressor cells (MDSC), macrophages, and dendritic cells enhances tumor-induced immune suppression.* Semin Cancer Biol, 2012. **22**(4): p. 275-81.
147. De Veirman, K., et al., *Myeloid-derived suppressor cells as therapeutic target in hematological malignancies.* Front Oncol, 2014. **4**: p. 349.
148. Christiansson, L., et al., *Increased level of myeloid-derived suppressor cells, programmed death receptor ligand 1/programmed death receptor 1, and soluble CD25 in Sokal high risk chronic myeloid leukemia.* PLoS One, 2013. **8**(1): p. e55818.
149. Cuenca, A.G., et al., *A paradoxical role for myeloid-derived suppressor cells in sepsis and trauma.* Mol Med, 2011. **17**(3-4): p. 281-92.
150. Kerr, E.C., et al., *Analysis of retinal cellular infiltrate in experimental autoimmune uveoretinitis reveals multiple regulatory cell populations.* J Autoimmun, 2008. **31**(4): p. 354-61.
151. Drevets, D.A., et al., *The Ly-6Chigh monocyte subpopulation transports Listeria monocytogenes into the brain during systemic infection of mice.* J Immunol, 2004. **172**(7): p. 4418-24.
152. Schlecker, E., et al., *Tumor-infiltrating monocytic myeloid-derived suppressor cells mediate CCR5-dependent recruitment of regulatory T cells favoring tumor growth.* J Immunol, 2012. **189**(12): p. 5602-11.
153. Mencacci, A., et al., *CD80+Gr-1+ myeloid cells inhibit development of antifungal Th1 immunity in mice with candidiasis.* J Immunol, 2002. **169**(6): p. 3180-90.

154. Haile, L.A., et al., *Myeloid-derived suppressor cells in inflammatory bowel disease: a new immunoregulatory pathway.* Gastroenterology, 2008. **135**(3): p. 871-81, 881 e1-5.
155. Brandau, S., et al., *Myeloid-derived suppressor cells in the peripheral blood of cancer patients contain a subset of immature neutrophils with impaired migratory properties.* J Leukoc Biol, 2011. **89**(2): p. 311-7.
156. Sinha, P., et al., *Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response.* J Immunol, 2007. **179**(2): p. 977-83.
157. Bronte, V., *Myeloid-derived suppressor cells in inflammation: uncovering cell subsets with enhanced immunosuppressive functions.* Eur J Immunol, 2009. **39**(10): p. 2670-2.
158. Movahedi, K., et al., *Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity.* Blood, 2008. **111**(8): p. 4233-44.
159. Youn, J.I., et al., *Subsets of myeloid-derived suppressor cells in tumor-bearing mice.* J Immunol, 2008. **181**(8): p. 5791-802.
160. Greten, T.F., M.P. Manns, and F. Korangy, *Myeloid derived suppressor cells in human diseases.* Int Immunopharmacol, 2011. **11**(7): p. 802-7.
161. Mougiakakos, D., et al., *Immunosuppressive CD14+HLA-DRlow/neg IDO+ myeloid cells in patients following allogeneic hematopoietic stem cell transplantation.* Leukemia, 2013. **27**(2): p. 377-88.
162. Rodriguez, P.C., et al., *Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes.* Cancer Res, 2009. **69**(4): p. 1553-60.
163. Rieber, N., et al., *Flagellin induces myeloid-derived suppressor cells: implications for Pseudomonas aeruginosa infection in cystic fibrosis lung disease.* J Immunol, 2013. **190**(3): p. 1276-84.
164. Rieber, N., et al., *Neutrophilic myeloid-derived suppressor cells in cord blood modulate innate and adaptive immune responses.* Clin Exp Immunol, 2013. **174**(1): p. 45-52.
165. Rieber, N., et al., *Extracorporeal photopheresis increases neutrophilic myeloid-derived suppressor cells in patients with GvHD.* Bone Marrow Transplant, 2014. **49**(4): p. 545-52.
166. Dumitru, C.A., et al., *Neutrophils and granulocytic myeloid-derived suppressor cells: immunophenotyping, cell biology and clinical relevance in human oncology.* Cancer Immunol Immunother, 2012. **61**(8): p. 1155-67.
167. Giallongo, C., et al., *Myeloid derived suppressor cells (MDSCs) are increased and exert immunosuppressive activity together with polymorphonuclear leukocytes (PMNs) in chronic myeloid leukemia patients.* PLoS One, 2014. **9**(7): p. e101848.
168. Zhao, L., et al., *An enzyme-linked immunosorbent assay for human carcinoembryonic antigen-related cell adhesion molecule 8, a biological marker of granulocyte activities in vivo.* J Immunol Methods, 2004. **293**(1-2): p. 207-14.
169. Gray-Owen, S.D. and R.S. Blumberg, *CEACAM1: contact-dependent control of immunity.* Nat Rev Immunol, 2006. **6**(6): p. 433-46.
170. Hock B, M.K., Cross N, Taylor K, Currie M, Robinson B, Simcock J, McKenzie J, *Renal transplant recipients have elevated frequencies of circulating myeloid-derived suppressor cells.* Nephrol Dial Transplant, 2012. **27**(1): p. 402-10.
171. Gabrilovich D, N.S., *Myeloid-derived suppressor cells as regulators of the immune system.* Nat Rev Immunol., 2009. **9**(3): p. 162-74.
172. Filipazzi, P., et al., *Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine.* J Clin Oncol, 2007. **25**(18): p. 2546-53.
173. Srivastava, M.K., et al., *Lung cancer patients' CD4(+) T cells are activated in vitro by MHC II cell-based vaccines despite the presence of myeloid-derived suppressor cells.* Cancer Immunol Immunother, 2008. **57**(10): p. 1493-504.
174. Diaz-Montero, C.M., et al., *Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy.* Cancer Immunol Immunother, 2009. **58**(1): p. 49-59.
175. Vuk-Pavlovic, S., et al., *Immunosuppressive CD14+HLA-DRlow/- monocytes in prostate cancer.* Prostate, 2010. **70**(4): p. 443-55.
176. Schmielau, J. and O.J. Finn, *Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients.* Cancer Res, 2001. **61**(12): p. 4756-60.

177. Kerr E, R.B., Copland D, Dick A, Nicholson L, *Analysis of retinal cellular infiltrate in experimental autoimmune uveoretinitis reveals multiple regulatory cell populations.* J Autoimmun, 2008. **31**(4): p. 354-61.
178. Drevets D, D.M., Schawang J, Van Rooijen N, Ehrchen J, Sunderkötter C, Leenen P, *The Ly-6Chigh monocyte subpopulation transports Listeria monocytogenes into the brain during systemic infection of mice.* J Immunol, 2004. **172**(7): p. 4418-24.
179. Rossner, S., et al., *Myeloid dendritic cell precursors generated from bone marrow suppress T cell responses via cell contact and nitric oxide production in vitro.* Eur J Immunol, 2005. **35**(12): p. 3533-44.
180. Greifenberg, V., et al., *Myeloid-derived suppressor cell activation by combined LPS and IFN-gamma treatment impairs DC development.* Eur J Immunol, 2009. **39**(10): p. 2865-76.
181. Sinha P, C.V., Fulton A, Ostrand-Rosenberg S, *Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells.* Cancer Res, 2007. **67**(9): p. 4507-13.
182. Pan P, W.G., Yin B, Ozao J, Ku T, Divino C, Chen S, *Reversion of immune tolerance in advanced malignancy: modulation of myeloid-derived suppressor cell development by blockade of stem-cell factor function.* Blood, 2008. **111**(1): p. 219-228.
183. Gabrilovich D, I.T., Oyama T, Ran S, Kravtsov V, Nadaf S, Carbone D, *Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo.* Blood, 1998. **92**(11): p. 4150-66.
184. Serafini P, C.R., Noonan K, Tan G, Bronte V, Borrello I, *High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells.* Cancer Res, 2004. **64**(17): p. 6337-43.
185. Kusmartsev S, G.D., *STAT1 signaling regulates tumor-associated macrophage-mediated T cell deletion.* J Immunol, 2005. **174**(8): p. 4880-91.
186. Obermajer N, M.R., Odunsi K, Edwards R, Kalinski P, *PGE(2)-induced CXCL12 production and CXCR4 expression controls the accumulation of human MDSCs in ovarian cancer environment.* Cancer Res, 2011. **71**(24): p. 7463-70.
187. Chen S, A.S., Abe M, Hiasa Y, Onji M, *Immunosuppressive functions of hepatic myeloid-derived suppressor cells of normal mice and in a murine model of chronic hepatitis B virus.* Clin Exp Immunol, 2011. **166**(1): p. 134-42.
188. Ssemaganda, A., et al., *Characterization of neutrophil subsets in healthy human pregnancies.* PLoS One, 2014. **9**(2): p. e85696.
189. Kusmartsev, S., S. Nagaraj, and D.I. Gabrilovich, *Tumor-associated CD8+ T cell tolerance induced by bone marrow-derived immature myeloid cells.* J Immunol, 2005. **175**(7): p. 4583-92.
190. Rodriguez, P.C., et al., *Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses.* Cancer Res, 2004. **64**(16): p. 5839-49.
191. Corzo, C.A., et al., *HIF-1alpha regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment.* J Exp Med, 2010. **207**(11): p. 2439-53.
192. Zea, A.H., et al., *Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion.* Cancer Res, 2005. **65**(8): p. 3044-8.
193. Talmadge, J.E. and D.I. Gabrilovich, *History of myeloid-derived suppressor cells.* Nat Rev Cancer, 2013. **13**(10): p. 739-52.
194. Darcy, C.J., et al., *Neutrophils with myeloid derived suppressor function deplete arginine and constrain T cell function in septic shock patients.* Crit Care, 2014. **18**(4): p. R163.
195. Raber, P., A.C. Ochoa, and P.C. Rodriguez, *Metabolism of L-arginine by myeloid-derived suppressor cells in cancer: mechanisms of T cell suppression and therapeutic perspectives.* Immunol Invest, 2012. **41**(6-7): p. 614-34.
196. Gey, A., et al., *Granulocytic Myeloid-Derived Suppressor Cells inversely correlate with plasma arginine and overall survival in critically ill patients.* Clin Exp Immunol, 2014.
197. Harari, O. and J.K. Liao, *Inhibition of MHC II gene transcription by nitric oxide and antioxidants.* Curr Pharm Des, 2004. **10**(8): p. 893-8.
198. Bingisser, R.M., et al., *Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway.* J Immunol, 1998. **160**(12): p. 5729-34.
199. Nagaraj, S., et al., *Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer.* Nat Med, 2007. **13**(7): p. 828-35.

200. Huang, B., et al., *Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host.* Cancer Res, 2006. **66**(2): p. 1123-31.
201. Hart, K.M., et al., *IL-10 immunomodulation of myeloid cells regulates a murine model of ovarian cancer.* Front Immunol, 2011. **2**: p. 29.
202. Bronte, V., et al., *Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells.* Blood, 2000. **96**(12): p. 3838-46.
203. Nausch, N., et al., *Mononuclear myeloid-derived "suppressor" cells express RAE-1 and activate natural killer cells.* Blood, 2008. **112**(10): p. 4080-9.
204. Liu, C., et al., *Expansion of spleen myeloid suppressor cells represses NK cell cytotoxicity in tumor-bearing host.* Blood, 2007. **109**(10): p. 4336-42.
205. Zhu, J., X. Huang, and Y. Yang, *Myeloid-derived suppressor cells regulate natural killer cell response to adenovirus-mediated gene transfer.* J Virol, 2012. **86**(24): p. 13689-96.
206. Epling-Burnette, P.K., et al., *Reduced natural killer (NK) function associated with high-risk myelodysplastic syndrome (MDS) and reduced expression of activating NK receptors.* Blood, 2007. **109**(11): p. 4816-24.
207. Binici, J., et al., *A soluble fragment of the tumor antigen BCL2-associated athanogene 6 (BAG-6) is essential and sufficient for inhibition of NKp30 receptor-dependent cytotoxicity of natural killer cells.* J Biol Chem, 2013. **288**(48): p. 34295-303.
208. Rieber, N., et al., *Pathogenic Fungi Regulate Immunity by Inducing Neutrophilic Myeloid-Derived Suppressor Cells.* Cell Host Microbe, 2015.
209. Lathers, D.M., et al., *Phase 1B study to improve immune responses in head and neck cancer patients using escalating doses of 25-hydroxyvitamin D3.* Cancer Immunol Immunother, 2004. **53**(5): p. 422-30.
210. Pan, P.Y., et al., *Reversion of immune tolerance in advanced malignancy: modulation of myeloid-derived suppressor cell development by blockade of stem-cell factor function.* Blood, 2008. **111**(1): p. 219-28.
211. Fricke, I., et al., *Vascular endothelial growth factor-trap overcomes defects in dendritic cell differentiation but does not improve antigen-specific immune responses.* Clin Cancer Res, 2007. **13**(16): p. 4840-8.
212. Talmadge, J.E., et al., *Chemoprevention by cyclooxygenase-2 inhibition reduces immature myeloid suppressor cell expansion.* Int Immunopharmacol, 2007. **7**(2): p. 140-51.
213. De Santo, C., et al., *Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination.* Proc Natl Acad Sci U S A, 2005. **102**(11): p. 4185-90.
214. Tadmor, T., D. Attias, and A. Polliack, *Myeloid-derived suppressor cells--their role in haemato-oncological malignancies and other cancers and possible implications for therapy.* Br J Haematol, 2011. **153**(5): p. 557-67.
215. Suzuki, E., et al., *Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity.* Clin Cancer Res, 2005. **11**(18): p. 6713-21.
216. Le, H.K., et al., *Gemcitabine directly inhibits myeloid derived suppressor cells in BALB/c mice bearing 4T1 mammary carcinoma and augments expansion of T cells from tumor-bearing mice.* Int Immunopharmacol, 2009. **9**(7-8): p. 900-9.
217. Vincent, J., et al., *5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity.* Cancer Res, 2010. **70**(8): p. 3052-61.
218. Dallegri, F., et al., *Down-regulation of K cell activity by neutrophils.* Blood, 1985. **65**(3): p. 571-7.
219. Pedersen, B.K., A. Kharazmi, and M. Svenson, *Down-regulation of natural killer cell activity by autologous polymorphonuclear leucocytes. Role of indomethacin.* Allergy, 1988. **43**(1): p. 17-23.
220. Shau, H.Y. and S.H. Golub, *Inhibition of lymphokine-activated killer- and natural killer-mediated cytotoxicities by neutrophils.* J Immunol, 1989. **143**(3): p. 1066-72.
221. Shau, H.Y. and A. Kim, *Suppression of lymphokine-activated killer induction by neutrophils.* J Immunol, 1988. **141**(12): p. 4395-402.
222. Thoren, F.B., et al., *The CD16-/CD56bright subset of NK cells is resistant to oxidant-induced cell death.* J Immunol, 2007. **179**(2): p. 781-5.

223. Fridlender, Z.G., et al., *Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN.* Cancer Cell, 2009. **16**(3): p. 183-94.
224. Fridlender, Z.G., et al., *Transcriptomic analysis comparing tumor-associated neutrophils with granulocytic myeloid-derived suppressor cells and normal neutrophils.* PLoS One, 2012. **7**(2): p. e31524.
225. Gregory, A.D. and A.M. Houghton, *Tumor-associated neutrophils: new targets for cancer therapy.* Cancer Res, 2011. **71**(7): p. 2411-6.
226. Beyrau, M., J.V. Bodkin, and S. Nourshargh, *Neutrophil heterogeneity in health and disease: a revitalized avenue in inflammation and immunity.* Open Biol, 2012. **2**(11): p. 120134.
227. Pillay, J., et al., *Immune suppression by neutrophils and granulocytic myeloid-derived suppressor cells: similarities and differences.* Cell Mol Life Sci, 2013. **70**(20): p. 3813-27.
228. Winkler, B., et al., *TGFbeta and IL10 have an impact on risk group and prognosis in childhood ALL.* Pediatr Blood Cancer, 2015. **62**(1): p. 72-9.
229. Zaknoen, S.L. and N.E. Kay, *Immunoregulatory cell dysfunction in chronic B-cell leukemias.* Blood Rev, 1990. **4**(3): p. 165-74.
230. Lion, E., et al., *Natural killer cell immune escape in acute myeloid leukemia.* Leukemia, 2012. **26**(9): p. 2019-26.
231. Fauriat, C., et al., *Deficient expression of NCR in NK cells from acute myeloid leukemia: Evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction.* Blood, 2007. **109**(1): p. 323-30.
232. Motoji, T., *[Immunophenotypes on blast cells of chronic myelogenous leukemia].* Nihon Rinsho, 2001. **59**(12): p. 2342-7.
233. Pui, C.H. and W.E. Evans, *Treatment of acute lymphoblastic leukemia.* N Engl J Med, 2006. **354**(2): p. 166-78.
234. Dreger, P., et al., *Indications for allogeneic stem cell transplantation in chronic lymphocytic leukemia: the EBMT transplant consensus.* Leukemia, 2007. **21**(1): p. 12-7.
235. Mawad, R., et al., *Frequency of allogeneic hematopoietic cell transplantation among patients with high- or intermediate-risk acute myeloid leukemia in first complete remission.* J Clin Oncol, 2013. **31**(31): p. 3883-8.
236. Seggewiss, R. and H. Einsele, *Immune reconstitution after allogeneic transplantation and expanding options for immunomodulation: an update.* Blood, 2010. **115**(19): p. 3861-8.
237. Dunbar, E.M., et al., *The relationship between circulating natural killer cells after reduced intensity conditioning hematopoietic stem cell transplantation and relapse-free survival and graft-versus-host disease.* Haematologica, 2008. **93**(12): p. 1852-8.
238. Wayne, A.S., K. Baird, and R.M. Egeler, *Hematopoietic stem cell transplantation for leukemia.* Pediatr Clin North Am, 2010. **57**(1): p. 1-25.
239. Ruggeri, L., et al., *Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants.* Science, 2002. **295**(5562): p. 2097-100.
240. Hou, W., et al., *Viral infection triggers rapid differentiation of human blood monocytes into dendritic cells.* Blood, 2012. **119**(13): p. 3128-31.
241. Lunemann, S., et al., *Compromised function of natural killer cells in acute and chronic viral hepatitis.* J Infect Dis, 2014. **209**(9): p. 1362-73.
242. Massoudi, M., J. Kim, and J.F. Antaki, *Modeling and numerical simulation of blood flow using the Theory of Interacting Continua.* Int J Non Linear Mech, 2012. **47**(5): p. 506-520.
243. Neron, S., et al., *Characterization of mononuclear cells remaining in the leukoreduction system chambers of apheresis instruments after routine platelet collection: a new source of viable human blood cells.* Transfusion, 2007. **47**(6): p. 1042-9.
244. Pfeiffer, I.A., et al., *Leukoreduction system chambers are an efficient, valid, and economic source of functional monocyte-derived dendritic cells and lymphocytes.* Immunobiology, 2013. **218**(11): p. 1392-401.
245. Hock, B.D., et al., *Renal transplant recipients have elevated frequencies of circulating myeloid-derived suppressor cells.* Nephrol Dial Transplant, 2012. **27**(1): p. 402-10.
246. Seki, T., S. Yuasa, and K. Fukuda, *Generation of induced pluripotent stem cells from a small amount of human peripheral blood using a combination of activated T cells and Sendai virus.* Nat Protoc, 2012. **7**(4): p. 718-28.
247. Li, X., et al., *Expansion of NK cells from PBMCs using immobilized 4-1BBL and interleukin-21.* Int J Oncol, 2015.

248. Walzer, T., et al., *Natural killer cells: from CD3(-)NKp46(+) to post-genomics meta-analyses.* Curr Opin Immunol, 2007. **19**(3): p. 365-72.
249. Panoskaltsis, N., C.D. Reid, and S.C. Knight, *Quantification and cytokine production of circulating lymphoid and myeloid cells in acute myelogenous leukaemia.* Leukemia, 2003. **17**(4): p. 716-30.
250. Lozzio, C.B. and B.B. Lozzio, *Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome.* Blood, 1975. **45**(3): p. 321-34.
251. Mellqvist, U.H., et al., *Natural killer cell dysfunction and apoptosis induced by chronic myelogenous leukemia cells: role of reactive oxygen species and regulation by histamine.* Blood, 2000. **96**(5): p. 1961-8.
252. Perfetto, S.P., P.K. Chattopadhyay, and M. Roederer, *Seventeen-colour flow cytometry: unravelling the immune system.* Nat Rev Immunol, 2004. **4**(8): p. 648-55.
253. Kopp, L.M., et al., *Decitabine has a biphasic effect on natural killer cell viability, phenotype, and function under proliferative conditions.* Mol Immunol, 2013. **54**(3-4): p. 296-301.
254. Alter, G., J.M. Malenfant, and M. Altfeld, *CD107a as a functional marker for the identification of natural killer cell activity.* J Immunol Methods, 2004. **294**(1-2): p. 15-22.
255. Bryceson, Y.T., et al., *Defective cytotoxic lymphocyte degranulation in syntaxin-11 deficient familial hemophagocytic lymphohistiocytosis 4 (FHL4) patients.* Blood, 2007. **110**(6): p. 1906-15.
256. Love, P.E. and S.M. Hayes, *ITAM-mediated signaling by the T-cell antigen receptor.* Cold Spring Harb Perspect Biol, 2010. **2**(6): p. a002485.
257. Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan, *T cell activation.* Annu Rev Immunol, 2009. **27**: p. 591-619.
258. van Lier, R.A., et al., *Immobilized anti-CD3 monoclonal antibodies induce accessory cell-independent lymphokine production, proliferation and helper activity in human T lymphocytes.* Immunology, 1989. **68**(1): p. 45-50.
259. Trickett, A. and Y.L. Kwan, *T cell stimulation and expansion using anti-CD3/CD28 beads.* J Immunol Methods, 2003. **275**(1-2): p. 251-5.
260. Lee, R.W., et al., *CD4+CD25(int) T cells in inflammatory diseases refractory to treatment with glucocorticoids.* J Immunol, 2007. **179**(11): p. 7941-8.
261. Handin, R.I.L., S.E.; Stossel, T.P., *Blood: Principles and practice of hematology*. 2 ed. Vol. 1. 2003, Philadelphia, PA 19106 USA: LIPPINCOTT WILLIAMS & WILKINS.
262. Lachmann, N., et al., *Large-scale hematopoietic differentiation of human induced pluripotent stem cells provides granulocytes or macrophages for cell replacement therapies.* Stem Cell Reports, 2015. **4**(2): p. 282-96.
263. Huang, A., et al., *Increased CD14(+)HLA-DR (-/low) myeloid-derived suppressor cells correlate with extrathoracic metastasis and poor response to chemotherapy in non-small cell lung cancer patients.* Cancer Immunol Immunother, 2013. **62**(9): p. 1439-51.
264. Gabrilovich, D.I., S. Ostrand-Rosenberg, and V. Bronte, *Coordinated regulation of myeloid cells by tumours.* Nat Rev Immunol, 2012. **12**(4): p. 253-68.
265. Zhang, H., et al., *Fibrocytes represent a novel MDSC subset circulating in patients with metastatic cancer.* Blood, 2013. **122**(7): p. 1105-13.
266. Wesolowski, R., J. Markowitz, and W.E. Carson, 3rd, *Myeloid derived suppressor cells - a new therapeutic target in the treatment of cancer.* J Immunother Cancer, 2013. **1**: p. 10.
267. Shime, H., et al., *Myeloid-derived suppressor cells confer tumor-suppressive functions on natural killer cells via polyinosinic:polycytidylic acid treatment in mouse tumor models.* J Innate Immun, 2014. **6**(3): p. 293-305.
268. Youn, J.I. and D.I. Gabrilovich, *The biology of myeloid-derived suppressor cells: the blessing and the curse of morphological and functional heterogeneity.* Eur J Immunol, 2010. **40**(11): p. 2969-75.
269. Denny, M.F., et al., *A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs.* J Immunol, 2010. **184**(6): p. 3284-97.
270. Shiba, Y., et al., *Bone marrow CXCR4 induction by cultivation enhances therapeutic angiogenesis.* Cardiovasc Res, 2009. **81**(1): p. 169-77.
271. Mocellin, S., et al., *IL-10 stimulatory effects on human NK cells explored by gene profile analysis.* Genes Immun, 2004. **5**(8): p. 621-30.

272. Bunt, S.K., et al., *Inflammation enhances myeloid-derived suppressor cell cross-talk by signaling through Toll-like receptor 4.* J Leukoc Biol, 2009. **85**(6): p. 996-1004.
273. Mehrotra, P.T., et al., *Production of IL-10 by human natural killer cells stimulated with IL-2 and/or IL-12.* J Immunol, 1998. **160**(6): p. 2637-44.
274. Burt, B.M., et al., *Circulating HLA-DR(+) natural killer cells have potent lytic ability and weak antigen-presenting cell function.* Hum Immunol, 2008. **69**(8): p. 469-74.
275. Clausen, J., et al., *Functional significance of the activation-associated receptors CD25 and CD69 on human NK-cells and NK-like T-cells.* Immunobiology, 2003. **207**(2): p. 85-93.
276. Marcenaro, E., et al., *Uptake of CCR7 and acquisition of migratory properties by human KIR+ NK cells interacting with monocyte-derived DC or EBV cell lines: regulation by KIR/HLA-class I interaction.* Blood, 2009. **114**(19): p. 4108-16.
277. Betts, M.R., et al., *Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation.* J Immunol Methods, 2003. **281**(1-2): p. 65-78.
278. Penack, O., et al., *CD56dimCD16neg cells are responsible for natural cytotoxicity against tumor targets.* Leukemia, 2005. **19**(5): p. 835-40.
279. Wai, L.E., et al., *Distinct roles for the NK cell-activating receptors in mediating interactions with dendritic cells and tumor cells.* J Immunol, 2011. **186**(1): p. 222-9.
280. Poupot, M., J.J. Fournie, and R. Poupot, *Trogocytosis and killing of IL-4-polarized monocytes by autologous NK cells.* J Leukoc Biol, 2008. **84**(5): p. 1298-305.
281. Claudepierre, M.C., et al., *Yeast virus-derived stimulator of the innate immune system augments the efficacy of virus vector-based immunotherapy.* J Virol, 2014. **88**(10): p. 5242-55.
282. Waterhouse, N.J., et al., *Cytotoxic T lymphocyte-induced killing in the absence of granzymes A and B is unique and distinct from both apoptosis and perforin-dependent lysis.* J Cell Biol, 2006. **173**(1): p. 133-44.
283. Huang, Y., et al., *Interleukin-12 treatment down-regulates STAT4 and induces apoptosis with increasing ROS production in human natural killer cells.* J Leukoc Biol, 2011. **90**(1): p. 87-97.
284. Jaeger, B.N., et al., *Neutrophil depletion impairs natural killer cell maturation, function, and homeostasis.* J Exp Med, 2012. **209**(3): p. 565-80.
285. Johnson, T.A., et al., *Reduction of the peripheral blood CD56(bright) NK lymphocyte subset in FTY720-treated multiple sclerosis patients.* J Immunol, 2011. **187**(1): p. 570-9.
286. Tu, Z., et al., *Myeloid suppressor cells induced by retinal pigment epithelial cells inhibit autoreactive T-cell responses that lead to experimental autoimmune uveitis.* Invest Ophthalmol Vis Sci, 2012. **53**(2): p. 959-66.
287. Kostic, A.D., et al., *Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment.* Cell Host Microbe, 2013. **14**(2): p. 207-15.
288. Thewissen, M., et al., *Neutrophils and T cells: bidirectional effects and functional interferences.* Mol Immunol, 2011. **48**(15-16): p. 2094-101.
289. Hock, B.D., et al., *Effect of activated human polymorphonuclear leucocytes on T lymphocyte proliferation and viability.* Immunology, 2012. **137**(3): p. 249-58.
290. Messal, N., et al., *Differential role for CD277 as a co-regulator of the immune signal in T and NK cells.* Eur J Immunol, 2011. **41**(12): p. 3443-54.
291. Lin, W., et al., *Fc-dependent expression of CD137 on human NK cells: insights into "agonistic" effects of anti-CD137 monoclonal antibodies.* Blood, 2008. **112**(3): p. 699-707.
292. Sun, J.C. and L.L. Lanier, *NK cell development, homeostasis and function: parallels with CD8(+) T cells.* Nat Rev Immunol, 2011. **11**(10): p. 645-57.
293. Rajagopalan, S. and E.O. Long, *Found: a cellular activating ligand for NKp44.* Blood, 2013. **122**(17): p. 2921-2.
294. Koch, J., et al., *Activating natural cytotoxicity receptors of natural killer cells in cancer and infection.* Trends Immunol, 2013. **34**(4): p. 182-91.
295. Mandelboim, O., et al., *Human CD16 as a lysis receptor mediating direct natural killer cell cytotoxicity.* Proc Natl Acad Sci U S A, 1999. **96**(10): p. 5640-4.
296. Elkabets, M., et al., *IL-1beta regulates a novel myeloid-derived suppressor cell subset that impairs NK cell development and function.* Eur J Immunol, 2010. **40**(12): p. 3347-57.
297. Zhu, S., et al., *Transcription of the activating receptor NKG2D in natural killer cells is regulated by STAT3 tyrosine phosphorylation.* Blood, 2014. **124**(3): p. 403-11.
298. Amano, K., et al., *Neutrophils induced licensing of natural killer cells.* Mediators Inflamm, 2015. **2015**: p. 747680.

299. Aoki, Y., M. Sumiya, and K. Oshimi, *Medullasin enhances human natural killer cell activity.* J Clin Invest, 1982. **69**(6): p. 1223-30.
300. Lindemann, R.A., A. Lala, and K.T. Miyasaki, *The in vitro effect of human polymorphonuclear leukocyte azurophil granule components on natural killer cell cytotoxicity.* Oral Microbiol Immunol, 1994. **9**(3): p. 186-92.
301. Yamashiro, S., et al., *Phenotypic and functional change of cytokine-activated neutrophils: inflammatory neutrophils are heterogeneous and enhance adaptive immune responses.* J Leukoc Biol, 2001. **69**(5): p. 698-704.
302. Ferlazzo, G. and B. Morandi, *Cross-Talks between Natural Killer Cells and Distinct Subsets of Dendritic Cells.* Front Immunol, 2014. **5**: p. 159.
303. Mamessier, E., et al., *Peripheral blood NK cells from breast cancer patients are tumor-induced composite subsets.* J Immunol, 2013. **190**(5): p. 2424-36.
304. Hebert, P. and S.B. Pruett, *Selective loss of viability of mouse NK cells in culture is associated with decreased NK cell lytic function.* In Vitr Mol Toxicol, 2001. **14**(2): p. 71-82.
305. Micheletti, A., et al., *Neutrophils promote 6-sulfo LacNAc+ dendritic cell (slanDC) survival.* J Leukoc Biol, 2013. **94**(4): p. 705-10.
306. Muller, I., et al., *Polymorphonuclear neutrophils and T lymphocytes: strange bedfellows or brothers in arms?* Trends Immunol, 2009. **30**(11): p. 522-30.
307. Costantini, C., et al., *Neutrophil activation and survival are modulated by interaction with NK cells.* Int Immunol, 2010. **22**(10): p. 827-38.
308. Maenhout, S.K., et al., *Enhanced suppressive capacity of tumor-infiltrating myeloid-derived suppressor cells compared with their peripheral counterparts.* Int J Cancer, 2014. **134**(5): p. 1077-90.
309. Michel, T., F. Hentges, and J. Zimmer, *Consequences of the crosstalk between monocytes/macrophages and natural killer cells.* Front Immunol, 2012. **3**: p. 403.
310. Zhang, B., et al., *Circulating and tumor-infiltrating myeloid-derived suppressor cells in patients with colorectal carcinoma.* PLoS One, 2013. **8**(2): p. e57114.
311. Auletta, J.J. and H.M. Lazarus, *Immune restoration following hematopoietic stem cell transplantation: an evolving target.* Bone Marrow Transplant, 2005. **35**(9): p. 835-57.
312. Chua, W., et al., *Neutrophil/lymphocyte ratio predicts chemotherapy outcomes in patients with advanced colorectal cancer.* Br J Cancer, 2011. **104**(8): p. 1288-95.
313. Lorente, D., et al., *Baseline neutrophil-lymphocyte ratio (NLR) is associated with survival and response to treatment with second-line chemotherapy for advanced prostate cancer independent of baseline steroid use.* Ann Oncol, 2015. **26**(4): p. 750-5.
314. Rossi, L., et al., *High neutrophil-to-lymphocyte ratio persistent during first-line chemotherapy predicts poor clinical outcome in patients with advanced urothelial cancer.* Ann Surg Oncol, 2015. **22**(4): p. 1377-84.
315. Zhou, Z., et al., *Development and function of myeloid-derived suppressor cells generated from mouse embryonic and hematopoietic stem cells.* Stem Cells, 2010. **28**(3): p. 620-32.