Neutrophils in cancer and other inflammatory conditions

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Neutrophils in cancer and other inflammatory conditions

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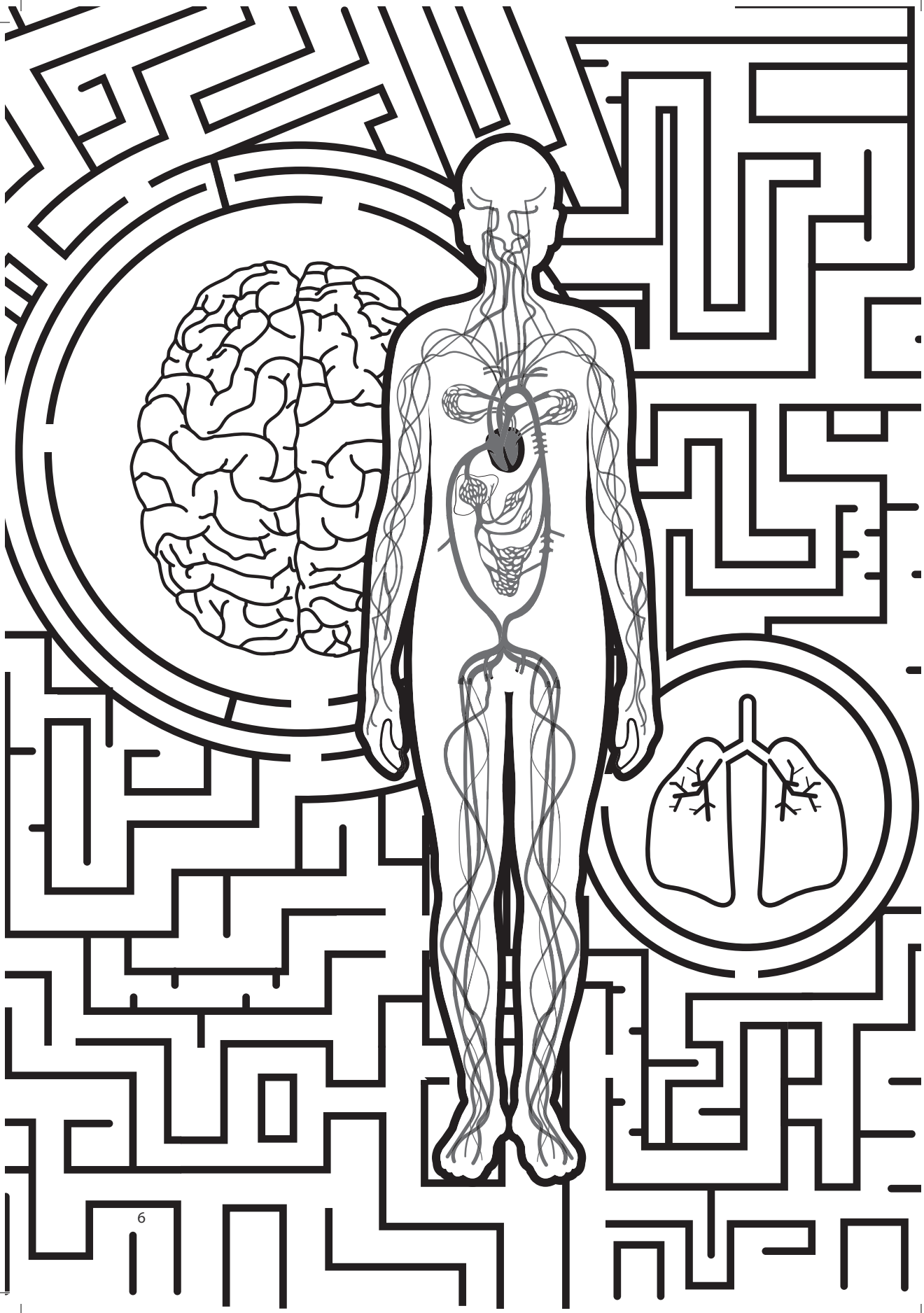

Chapter 1

General introduction

General Introduction

Neutrophils, also known as polymorphonuclear leukocytes [PMNs] are the most abundant white blood cells in the human circulation, comprising 60-70% of circulating white blood cells. They are continuously produced in the bone marrow by dividing promyelocytes and subsequent myelocytes. After the myelocyte stage cells stop dividing and differentiate into metamyelocytes, followed by immature cells with a typical banded nuclear morphology, and finally segmented polymorphonuclear mature cells [1-3]. Neutrophils are key effector cells of the innate immune system and contribute to the first line of defense against invading pathogens. However, in several clinical situations they can also contribute to immune mediated pathologies, such as sepsis, acute respiratory distress syndrome (ARDS), allergy, autoimmunity and cancer [4-10]. Nevertheless, neutrophils are essential for host defense against invasive pathogens across a wide range of species [11, 12]. The crucial role of neutrophils in the immune response against pathogens is illustrated by the severe and potentially life-threatening diseases in neutropenic individuals [13, 14].

Neutrophils as bacterial killers during infection

During inflammation, neutrophils can quickly respond to the inflammatory mediators coming from the infectious or damaged tissue site [15-17]. To respond to pathogen invasion, neutrophil recruitment involves multiple steps, including i) rolling, ii) adhesion to the surface of vascular endothelium, iii) crawling along the vessel, iv) extravasation and transmigration towards the infection site, v) elimination of foreign pathogens through phagocytosis, and vi) generation of reactive oxygen species (ROS) and release of microbicidal proteins/peptides (both intracellular and extracellular) [18]. To kill invading pathogens, neutrophils ingest them into phagosomes. Subsequently, two mechanisms are activated: 1. the activated NADPH oxidase complex produces superoxide (O_2^-) which in turn is metabolized to hydrogenperoxide (H_2O_2)and 2. fusion of granules with the phagosome to deliver antibacterial proteins [19, 20]. Neutrophils contain different types of granules: (1) primary granules, also referred as azurophilic granules; (2) secondary granules, also referred as specific granules; (3) tertiary granules; and (4) secretory vesicles [21]. The most toxic mediators such as myeloperoxidase (MPO), elastase and bactericidal/permeability-increasing protein (BPI) are stored in primary granules. MPO can react with H_2O_2 and oxidizes Cl^- to HOCl (bleach), which is a powerful microbicidal agent. The MPO-H_2O_2-chloride system plays an important role in anti-microbial activities in the neutrophil phagosome [22]. It is of interest that, while inherited MPO deficiency is rarely associated with clinical symptoms except for an increased risk for systemic

Candidiasis, defects in the NADPH oxidase complex are often life threating due to infectious complications [23]. This has raised a debate on the importance of MPO in bacterial killing. However, even though MPO might not be essentially required for bacterial killing, this mechanism might still play an important role when present. Indeed the bactericidal capacity of MPO deficient neutrophils has been found to be delayed [22, 24].

The secondary granules also contain a wide range of antimicrobial compounds including neutrophil gelatinase-associated lipocalin (NGAL), Human cathelicidin-18 (hCAP-18), lysozyme and lactoferrin which can disrupt bacterial membranes and interfere with the iron-dependent metabolic pathways of bacteria [25, 26]. The tertiary granules contain few antimicrobials, but they store some metalloproteases, such as leukolysin and gelatinase which are believed to be important for neutrophil extravasation and migration [27]. The secretory vesicles are formed through endocytosis. Their membrane contains plasma-derived proteins, such as albumin, and they can serve as a storage for some important membrane-bound molecules employed during neutrophil migration [21, 28]. Thus, the neutrophils contain a wide armament of different microbicidal componentswhich can both be released extracellularly and into pathogen containing phagosomes. These different mechanisms likely evolved to conquer many different types of pathogens and additionally to combat evolving pathogens.

The antibacterial effect of neutrophils is not restricted to phagocytosis and release of antibacterial proteins to the phagosome or the extracellular milieu. Fairly recently, studies are focusing on neutrophil extracellular traps (NETs) as another antibacterial mechanism [29]. NETs are composed of DNA/histon elements bound tocytotoxic enzymes and/or proteins (such as, MPO, elastase, lactoferrin and cathepsins) which are released from neutrophil granules [29]. Since they are released extracellularly, pathogens can be trapped and immobilized. They are also believed to kill pathogens directly by antimicrobial histones and proteases [15]. Besides their antibacterial role, NETS have also been negatively implicated in the development of auto-immune diseases such as systemic lupus erythematosus and rheumatoid arthritis [30, 31].

Neutrophil heterogeneity

In recent years, the concept of neutrophil heterogeneity has emerged with an increasing number of studies finding neutrophil populations with distinct functions under both steady state and pathological conditions [32-34]. It is an ongoing discussion whether these different populations represent bona fide subsets

or simply activation or polarization states in response to the stimuli. Different strategies have been used to identify neutrophil populations, including distinct cell surface markers, density, maturation and function. In this thesis, different populations characterized by distinct functions and specific nuclear morphology and/or cell surface characteristics will be referred to as neutrophil subsets. It is worth emphasizing that cells with similar cell surface marker expression may nevertheless show heterogeneity in their function. For instance, Hellebrekers et al showed that all neutrophils from healthy donors can phagocytose bacteria, but there appears to be a population of highly functional cells that preferentially phagocytoses and outcompetes the other neutrophils [35].

Neutrophil heterogeneity in acute inflammation

Our group discovered that there are two additional extra neutrophil subsets appearing during acute inflammation. This can be achieved by a systemic LPS challenge in healthy volunteers. During this a model of acute inflammation three neutrophil subsets are found in the peripheral blood: immature neutrophils ($CD16^{low}$/$CD62L^{high}$) with a banded nucleus, conventional mature neutrophils ($CD16^{high}$/$CD62L^{high}$) with segmented nuclei and hypersegmented neutrophils ($CD16^{high}$/$CD62L^{low}$) with a higher number of nuclear lobes [36]. These three subsets were found to have different functions; banded neutrophils show superior anti-bacteria abilities whereas hypersegmented neutrophils exhibit suppression of T cell proliferation. In fact, hypersegmented neutrophils were very poor bacterial killers. Leliefeld et al showed that the defect in bacterial containment by hypersegmented neutrophils could not be attributed to differences in survival, nor phagocytosis, or protease activity. Hypersegmented neutrophils displayed increased H_2O_2 and less phagosomal HOCL compared to the mature subset despite an increased abundance of cellular MPO, suggesting decreased fusion of granules containing MPO in hypersegmented neutrophils [37]. Pillay et al showed the hypersegmented neutrophils suppress T cell proliferation via local production of H_2O_2 in a Mac-1-dependent (CD11b/CD18-dependent) manner [36].

Before I will describe heterogeneity in the neutrophil compartment in cancer and other inflammatory conditions it is helpful to first get an overview on the reported pro- and anti-tumor effects of neutrophils.

Pro- and anti-tumor effects of neutrophils

It has been suggested that the tumor microenvironment that is characterized by chronic inflammation, is composed of stromal cells, growing blood vessels and infiltrating inflammatory cells. All these cells are known to play important roles

in cancer progression and behavior [38-40]. Neutrophils make up a big portion of infiltrating inflammatory cells in various murine tumor models and human cancers [41-44]. The cross talk between tumor cells and immune cells that leads to immune cell phenotypic alterations has been defined as immunosculpting or immunoediting [45].

Anti-tumor effects of neutrophils

Neutrophils can kill tumor cells via different mechanisms. They are able to induce tumor cell death by their potent antimicrobial killing mechanism, for example, the hydrogen peroxide produced by neutrophils is cytotoxic for tumor cells. This cytotoxic potential was absent in control neutrophils and induced by CCL2 production by the tumor [46]. In addition, interferon (IFN) activated neutrophils are able to release functional soluble TRAIL/APO2 ligand (tumor necrosis factor-related apoptosis-inducing ligand) which induces selective apoptosis of tumor cells and exerts immunoregulatory functions on activated T lymphocytes [47, 48]. CD4+ and CD8+ T cells proliferation and their cytotoxic abilities can also be boosted by neutrophils via upregulation of co-stimulatory molecules on neutrophils and by their production of chemokines and pro-inflammatory cytokines [49-51].

Pro-tumor effects of neutrophils

Although neutrophils were traditionally considered as important effector cells the context of a defensive immune response (see previous section), there is increasing evidence supporting the concept that neutrophils can promote tumor growth, angiogenesis, progression and metastasis [52, 53]. This hypothesis is supported by several findings in the literature. For instance, a high neutrophil to lymphocyte ratio (NLR) is considered as a negative prognostic indicator in patients bearing cancers such as colorectal cancer and non-small cell lung cancer [54-56]. Furthermore, by releasing prostaglandin E2 (PGE2) and leukotrienes, neutrophils can directly promote tumor cell proliferation since these factors can activate intracellular signaling cascades to induce tumor cell proliferation [57-59]. The generation of TGFβ by neutrophils has been shown to induce epithelial-mesenchymal transition (EMT) [60] which is important for tumor extravasation [61]. Neutrophils have also been shown to suppress the anti-immune response via a diverse array of mechanisms. For instance, neutrophils can inhibit the anti-tumor response of CD8+ T cells through arginase 1 (ARG1), nitric oxide synthase (iNOS) release and PD-L1/PD-1 signaling modulation [50, 62-67]. Apart from that, neutrophils also can suppress T cell proliferation by releasing H_2O_2. This oxidant leads to various mechanisms involved in T-cell suppressionsuch as apoptosis, decreasing NF-κB activation, downregulating TCRζ and oxidation of cofilin [68-70]. Moreover, neutrophils can inhibit NK cell

cytotoxicity, resulting in a reduced anti-tumor response and increased tumor cell extravasation and metastatic dissemination [71, 72].

Dual roles of neutrophil products in tumor biology.

Neutrophil elastase (NE) has been implicated in both anti- and pro-tumoral processes. NE promotes tumor cell proliferation through the hydrolysis of the insulin receptor substrate-1 (IRS-1). IRS-1 usually block PI3K activity and through IRS-1 degradation by NE PI3K can promote tumor cell proliferation [58]. NE is also involved in neutrophil-mediated epithelial-to-mesenchymal-transition of tumor cells, which is a process that is associated with metastasis formation [73]. In contrast, NE was shown to cleave cyclin E to a truncated isoform which is presented in context of HLA-1 and thereby promoting T lymphocyte-mediated lysis of tumor cells [74]. Furthermore, neutrophil elastase promotes the degradation of VEGF-A and bEGF preventing angiogenesis [75, 76]. Conversely, MMP9 secreted by neutrophils degrade the local extracellular matrix (ECM), triggering the release of VEGF-A which is in favor of angiogenesis and therefore tumor growth and metastasis [77].

NETs released by activated neutrophils have also been implicated in both pro- and anti-tumoral activity. MPO and histones as components of NETs have been described to be cytotoxic to tumor cells [78]. Besides, NETs are able to both activate dendritic cells and prime T cells [79, 80]. In contrast, other studies support support the hypothesis NETs promote tumor progression by degrading the extracellular matrix, thereby promoting tumor cell migration and metastatic colonization [81].

Neutrophil heterogeneity in tumor bearing hosts

Thus neutrophils exhibit both pro- and anti-tumoral properties. Some studies suggest neutrophils from early tumors are more cytotoxic toward tumor cells and produce higher levels of TNF-α, NO, and H_2O_2 whereas in established tumors, these functions are downregulated and neutrophils acquire more pro-tumoral activity [82]. Other studies attribute pro- and anti-tumoral properties to different neutrophil subsets. The most commonly used terminology to describe tumor associated neutrophils are: 1. Tumor Associated Neutrophils (TAN) to describe neutrophils found in tumor tissue, 2. N1 and N2 neutrophils and 3. Granulocyte myeloid derived suppressor cells (G-MDSC) [83-85]. The latter are sometimes further differentiated into low density granulocytes (LDG) as opposed to normal density granulocytes (NDG) found in healthy controls. Unfortunately different studies use different surface markers and isolation methods which complicates benchmarking between these studies [86, 87]. Overlapping and distinctive functions have been described between the different neutrophil subtypes described in cancer.

TAN, N1 and N2

Tumor-associated neutrophils (TAN) can be differentially polarized under the influence of tumor cells. This has also been described for tumor-associated macrophages (TAM) [50, 88]. IFN-β from non-hematopoietic origin induces neutrophils to acquire an anti-tumoral N1 phenotype [89, 90] whereas TGF-β originating from the tumor induces neutrophils to acquire a more N2 pro-tumoral phenotype [50]. N1 neutrophils express more immune-activating cytokines and chemokines, lower levels of arginase which play an important role in immune suppression [91], and a higher capability to kill tumor cells. N2 Neutrophils express higher level of VEGF, MMP-9, CXCR4 and arginase, supporting carcinogenesis, angiogenesis and immune suppression [50, 89, 92]. N1 neutrophils have a hyper-segmented nucleus compared to N2 neutrophil populations [50]. Although nuclear morphology is a hallmark of neutrophil maturation, it is unclear whether N1 and N2 neutrophils simply represent different stages of maturation, distinct activation states or bona fide subsets.

Low density granulocytes (LDG) and myeloid-derived suppressor cells (MDSC)

Mononuclear leukocytes and neutrophils can be separated by using density gradient centrifugation of peripheral blood. Under normal conditions few, if any, neutrophils are present in the mononuclear fraction from a healthy individual. Almost all neutrophils are found in the high density fraction, but a few are found in the mononuclear fraction and are, therefore, referred to as low density granulocytes (LDG). These number of these LDGs is substantially increased in patients with cancer, and acute/chronic inflammation [93-97]. Two sources have been suggested for LDG: Firstly, these neutrophils are mobilized from bone marrow as immature cells as some sort of compensation because of a high demand. They are still immature during disease and may complete maturation in the periphery when the conditions are permissive [98, 99]. Secondly, neutrophils get activated which leads to changes of their buoyant density [62, 87, 100]. Interestingly, different studies found either immature or activated mature PMN in the mononuclear fraction of cancer patients. The immature neutrophils displayed reduced CD16, CXCR1, CXCR2 expression and reduced oxidative burst activities whereas the mature neutrophils exhibited increased CD11b, CD66b expression and reduced CD62L expression in this fraction when compared with conventional, mature neutrophils of regular density [62, 101].

Myeloid-derived suppressor cells (MDSC) suppress T cell responses and have been reported in pathologies such as cancer, infection and inflammation. MDSCs contain variable proportions of granulocytic and monocytic cells, and are capable

of suppressing T-cell proliferation and responses via arginase, NOS activities and ROS [62, 100, 102-104]. Although the main hypothesis in the field is that MDSCs are found in the LDG population [98], NDG have also been found to suppress T cells under certain conditions [105-107]. Therefore, there is no consensus at present whether these T cell suppressing MDSCs/LDGs are immature or rather activated neutrophils. Marini et al propose the suppressive subset consists of mature neutrophils expressing CD10 and that these can be found in both the low and normal density fractions [108]. However, this study was performed with neutrophils isolated from G-CSF treated donors and not cancer patients,

In conclusion, it is likely that both immature and activated mature neutrophils can have aberrant densities and, therefore, can be found in the low density fraction. It would be of particular interest to establish whether CD10 can also distinguish suppressive neutrophils in cancer patients.

Neutrophil lifespan

These relatively new neutrophil subsets with different functionalities complicate potential treatment options to modulate the neutrophil response. On the other hand, if we better understand the mechanisms underlying the formation of the different subsets, this also offers a unique chance to modulate the (innate/inflammatory) immune response. For instance, we could induce neutrophil induced immune suppression in hyper-inflammatory conditions or chronic inflammation or inhibit this process to fight cancer. In this light, it is extremely surprising that fundamental knowledge on neutrophil life span in homeostasis and during inflammation has not been resolved. In the absence of inflammation, the half-life of neutrophils in blood was classically thought to be between 4-18 hours [109, 110]. However, the labels used to determine the life-span in these old studies were radio-active or toxic and therefore possibly underestimate true neutrophil life span. More recent studies, including our own, used deuterated glucose and deuterated water labeling to study neutrophil life span in vivo in an unperturbed manner. Mathematical modeling is needed to determine the life span from these labeling curves and the different studies reported a half-life of either 19 hours or 3.7 days [111, 112]. The difference in life span reported by these studies is the consequence of assumptions that the mathematical model needs regarding the kinetics of neutrophil progenitors in the bone marrow, which are currently unknown. The longer the half-life, the longer neutrophils can affect the adaptive immune response which needs days to expand. Thus, more fundamental knowledge on neutrophil life span and subsets are essential in order to harness neutrophils for treatment options in the future.

Aim and outline of this thesis

This thesis focuses on neutrophils in cancer, acute and chronic inflammation. The first part of this thesis describes how intravital microscopy (IVM) can be used in biomedical research to study dynamic processes at cellular and subcellular resolution in their environment and how different imaging windows including the abdominal imaging window (AIW), dermal imaging window (DIW) and cranial imaging window (CIW) can be implanted for facilitating the tracking of cells for different research purposes **(chapter 2)**. To investigate the role of neutrophils in biopsy-induced tumor progression, we intravitally imaged the behavior of glioblastoma cells before and after biopsy via implanted CIW in neutrophil depleted mice **(chapter 4)**. Different parameters, such as percentage and speed of migratory tumor cells, were measured to check if neutrophil play a role in biopsy-induced tumor progression. To confirm the hypothesis that neutrophil promote tumor progression, transwell assays and wound assays were also performed with human neutrophils in vitro. Although two- and three-dimensional in vitro studies of tumor cell lines are widely used and important for increasing our knowledge on tumor growth, behavior and metastasis formation, the complexity of in vivo microenvironment is not taken into consideration. Therefore, to better understand tumor cell behavior, we imaged tumors which developed from different common human and mouse breast tumor cell lines in living mice by intravital microscopy **(chapter 3)**. Cell morphology, cell-cell interaction, polarity and motility of those tumor cell lines were measured. This data can serve as a resource to instruct researchers on the appearance and migratory behavior of the widely used breast tumor cell lines and warrants caution to the use of in vitro characterization of tumor invasiveness. The more traditional role of neutrophils is to phagocytose pathogens and clear damage upon acute inflammation. During inflammation, neutrophil phenotypes appear in the blood which are not present during homeostasis and they display different bacterial containment capacities. To explain the mechanism behind this difference, we first optimized neutrophil survival in a 3D in vitro model and then performed this assay with or without bioparticle phagocytosis and in the presence or absence of an MPO inhibitor **(chapter 6)**. Apart from neutrophil heterogeneity in acute inflammation, neutrophil heterogeneity in cancer, COPD and trauma were also investigated and compared (**chapter 5**). Different maturation and activation markers were examined to further understand the similarities and differences of neutrophil populations under different conditions. Finally, in **chapter 7**, we summarize and discuss our main findings in the context of the aim of this thesis.

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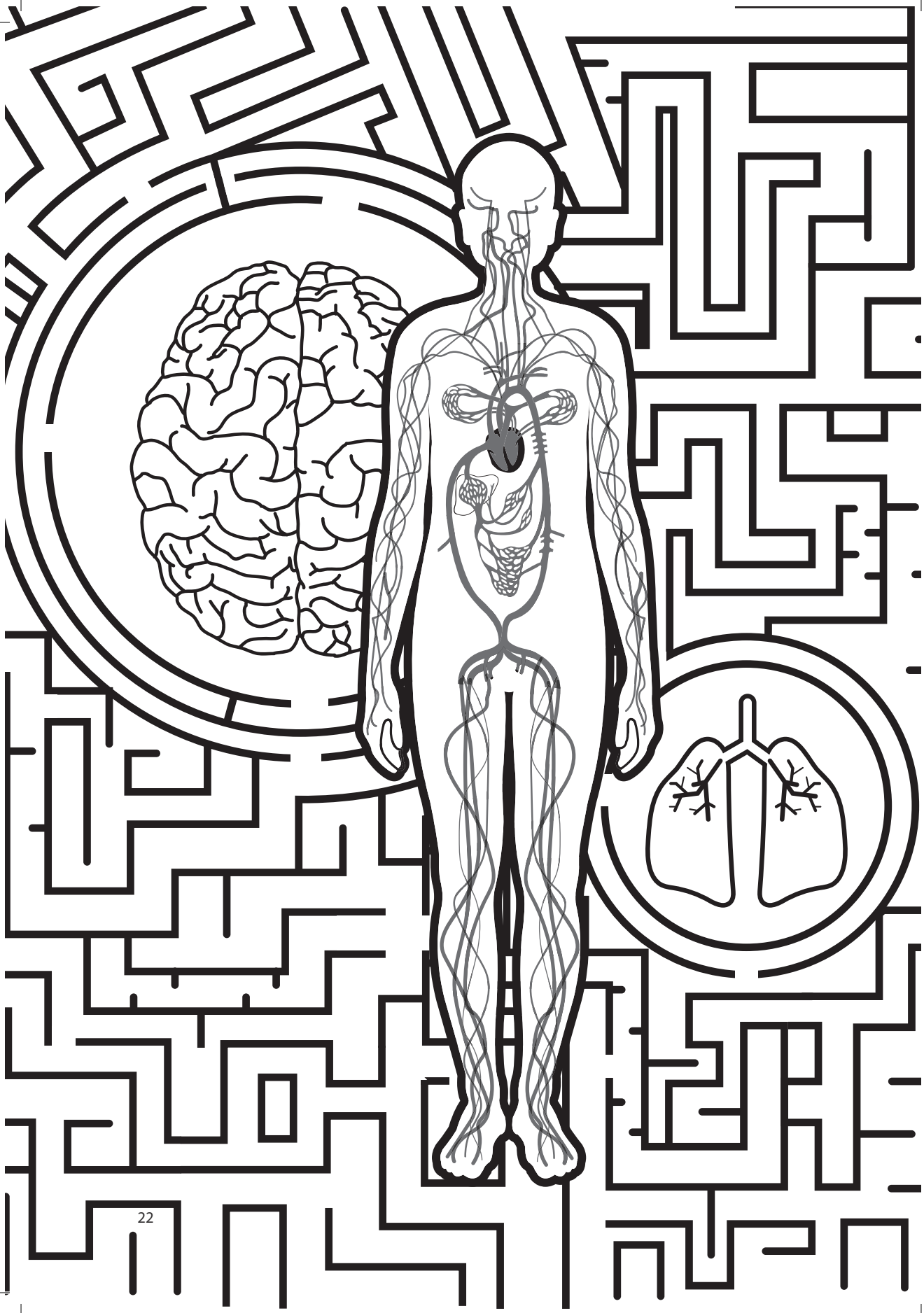

Chapter 2

Procedures and applications of long-term intravital microscopy

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Abstract

Intravital microscopy (IVM) is increasingly used in biomedical research to study dynamic processes at cellular and subcellular resolution in their natural environment. Long-term IVM especially can be applied to visualize migration and proliferation over days to months within the same animal without recurrent surgeries. Skin can be repetitively imaged without surgery. To intermittently visualize cells in other organs, such as liver, mammary gland and brain, different imaging windows including the abdominal imaging window (AIW), dermal imaging window (DIW) and cranial imaging window (CIW) have been developed. In this review, we describe the procedure of window implantation and pros and cons of each technique as well as methods to retrace a position of interest over time. In addition, different fluorescent biosensors to facilitate the tracking of cells for different purposes, such as monitoring cell migration and proliferation, are discussed. Finally, we consider new techniques and possibilities of how long-term IVM can be even further improved in the future.

Keywords

Long-term intravital microscopy, skin, dorsal skinfold chamber, dermal imaging window, abdominal imaging window, cranial imaging window.

Abbreviations

IVM: intravital microscopy, DSC: dorsal skinfold window, DIW: dermal imaging window, AIW: abdominal imaging window, CIW: cranial imaging window, CSC: cancer stem cells, SLGC: stem-like glioma cells, H2B: histone 2B

Introduction - Advantages of long-term IVM

In vitro and *ex vivo* studies are extensively used in basic research, however, they do not mimic the complex and specific microenvironment present in a living organism, which is why such studies are usually followed up by *in vivo* experiments. So far, *in vivo* experiments have mostly reflected static measurements of complex dynamic processes. In contrast, intravital microscopy (IVM) of tissues allows real-time visualization of tissues in living animals, offering an improvement over static *ex vivo* snapshot images [1]. Studies on dynamic processes, such as those involving proliferation and/or migration, especially benefit from IVM [2]. More recently, technical improvements have permitted intermittent intravital imaging over a prolonged period of time (up to weeks), termed long-term IVM. A variety of IVM techniques enable repetitive imaging. Some platforms offer whole body resolution and don't require surgical procedures. Imaging at the subcellular scale is however generally accompanied with limited tissue penetration, requiring exposure of the organ of interest. Two-photon microscopy results in less phototoxicity, less photobleaching and deeper tissue penetration compared to single-photon microscopy [3] and this technique has therefore really boosted the long-term IVM field. Several imaging windows have been developed, avoiding the need for recurrent surgeries, allowing observation of single cells within tissues and retracing the same area within living animals over multiple days [4]. This technology enables repetitive imaging in a single animal which not only reduces the number of animals needed, but also eliminates inter-animal differences to achieve more comparative data. Long-term IVM has facilitated researchers to address more advanced research questions such as visualizing the migration of slowly moving cells over several days [5] or identifying stem cell progeny over days or weeks [6]. Here, we review the different intravital imaging windows currently available to realize long-term IVM and explain how to perform surgeries, providing a guide for those interested in addressing biomedical questions with a dynamic character. Most of the surgical procedures for long-term IVM described here were developed for two-photon microscopy, however these can certainly be applied to other intravital imaging platforms. We further describe the many applications of long-term IVM with a focus on cell migration and proliferation including various useful fluorescent labeling strategies. Finally, we discuss the possibilities and opportunities of long-term IVM in the future.

Methodology – Long-term IVM

Below the different long-term IVM procedures are concisely described. Many excellent detailed protocol articles have been published, part of which are listed in Table 1. Here we merely aim to provide a short description and enable the comparison of the pros and cons of the different methods. In addition, some tips and tricks are shared which are often based on our personal experience.

Skin

Methodology

Depilate the skin area of interest by using a hair trimmer and depilation cream. When imaging the ear, depilation is not necessary, only long hairs are trimmed with scissors. When using an inverted microscope system, the desired part of the skin can be placed on top of a coverglass at the bottom of a custom stage insert or imaging box [7, 8] . The ventral side of the ear pinna can be fanned out on a thin layer of PBS and fixed using Durapore 3M tape (Fig. 1A). Alternatively, when using an upright microscope system, a clamp [9] or spatulas [10] can be used to immobilize the ear during imaging and to position a coverglass on top.

Pros

This method allows for visualization of different cell types found in healthy skin [10], immune cells recruited to an inflammation in the skin [7, 8] or cancer cells (see Table 1) without the need for surgical exposure [9]. The procedure is non-invasive and therefore avoids a wound-healing response which is inevitable in all other long-term IVM methods. Furthermore, issues such as liquid or cell accumulation, fibrosis and organ fixation are nonexistent. Thus, there are no practical problems that hamper the repetitive imaging of skin for as long as the mouse lives.

Cons

The removal of hair by chemical depilation cream might lead to skin inflammation and vascular leakage upon prolonged exposure [11]. In addition, skin harbors melanin-containing cells which are very light-sensitive. Upon death, these cells recruit immune cells and disturb homeostasis [7, 11]. Another limitation is that aside from the ear, which has relatively thin skin in mice, laser penetration through skin in other locations is limited [11].

Tips and tricks

- Since hair is strongly autofluorescent, it is essential to remove as much hair as possible in order to prevent obscuring the site of interest [11].
- Only gentle pressure can be applied to immobilize the ear to avoid obstruction of the blood circulation.
- Care should be taken with the laser power applied to the tissue to avoid phototoxicity of melanin-containing cells [11]. Alternatively, albino mice can be used to prevent melanin associated laser damage altogether.
- The anterior half of the ear pinna has the least amount of hair follicles and melanin containing cells, therefore this area is most suitable for imaging skin [7].
- General acquisition and analysis tips and tricks, for instance on locating the same regions of interest during repetitive imaging, can be found in section 3.1.

Dorsal skinfold chamber (DSC)

Methodology

To implant the DSC, start by shaving the dorsal area from nape to tail. Then fan out the dorsal skin of the animal and fix it to a C-shaped holder using 4 to 6 interrupted sutures (Fig. 1A) [12]. The front frame of the chamber is positioned on top of the skinfold and holes are punched through the skin at the place of the screw holes. Once the rear frame is positioned on the other side of the skinfold, the chamber can be fixed to the skin using screws and nuts [12, 13]. To securely tighten the chamber, additional sutures are placed through the skin and small holes in the frames. Next the C-holder is removed by cutting the 4 to 6 sutures. The last part of the procedure involves the careful removal of the upper dermis around the inner rim of the central hole in the front frame [14]. The procedure is finished by placing a cover glass on top of the removed dermis and fixing it to the frame using an O-ring [13].

Pros

This is a widely-used window model that allows visualization of various subcutaneous tumors (see Table 1) [12]. By making use of an O-ring, the coverglass can be removed to discard exudate or liquid accumulations between the tissue and coverglass. Moreover, because the skin is tightly enclosed by the frame there is no room for tissue movement. As the window extends away from the animal, fixation on the microscope is easy, preventing motion artifacts by respiration. Hence, imaging of the tissue often results in clear and crisp images [4]. In addition, the window can be fixed in a precise position by placing the bolts in a fixed frame on a microscope insert [12]. Combining this precise fixation over multiple days with a motorized stage that allows for storage of imaging positions, retracing of the same region of interest does not require internal tissue landmarks. The last advantage of

the DSC is that it can be used with both upright or inverted microscopes, providing platform flexibility.

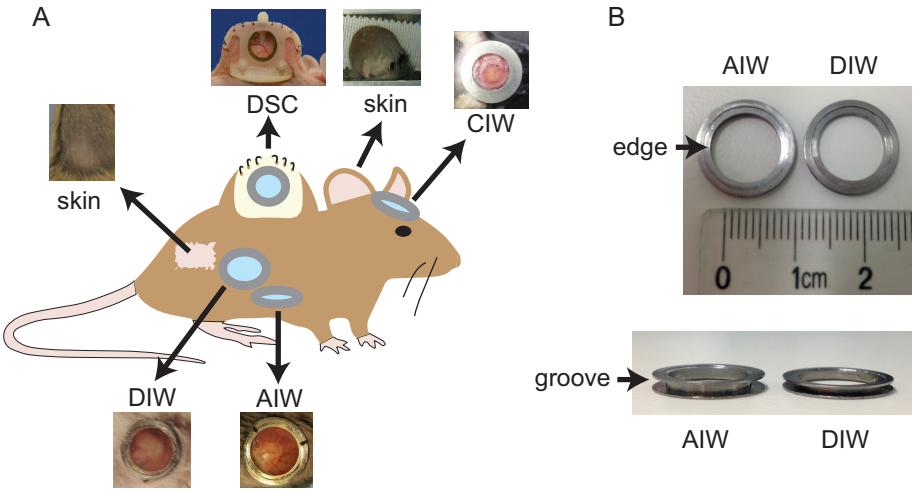

Fig.1. Schematic representation of the different long-term intravital techniques. (A) DSC (dorsal skinfold chamber) courtesy of Esther Wagena [124]. CIW (cranial imaging window) courtesy of Maria Alieva [4]. AIW (abdominal imaging window). DIW (dermal imaging window). (B) Picture of the DIW and AIW displaying the edge for the coverglass and the groove for the skin and peritoneum.

Cons

The extension of the window away from the animal's body also results in disadvantages. First, it is more difficult to retain body temperature within the chamber [15]. Moreover, if the window is not placed correctly at the midline of the animal, it can minimize the length of its use as the window can fall to one side, pull the skin and damage the tissues. Because the tissue fold is maximally 1 mm thick [16], tumors that are implanted after window placement show a characteristic growth pattern extending mostly in XY direction rather than in XY and Z direction [4, 17]. In addition, when performing tumor studies, the skin is often not the orthotopic location for most tumor types, which may influence for example the metastatic ability of tumors (Laila Ritsma, unpublished data).

Tips and tricks

- The skinfold should be symmetrical, with the fold of the skin exactly at the midline of the animal. This can be best realized by visualizing the vasculature through the skin using light from behind the animal; the vasculature is symmetrical and should overlap [18].

Table 1: Summary of the characteristics, advantages and disadvantages of the different methods used to realize long-term IVM.

	Skin	DSC	DIW	AIW	CIW
Tissue visualized	Skin	-Subcutaneous tumors -Dermal vasculature	-Mammary glands -Lymph nodes -Subcutaneous tumors	-Liver -Pancreas -Spleen -Intestine -Kidney	Brain
Examples of cells visualized	-Fibroblasts -Endothelial cells -Immune cells -Cancer cells	-Fibroblasts -Endothelial cells -Immune cells -Cancer cells	-Fibroblasts -Endothelial cells -Immune cells -Cancer cells	-Fibroblasts -Endothelial cells -Tissue specific cell type -Immune cells -Cancer cells	-Neurons -Fibroblasts -Endothelial cells -Immune cells -Cancer cells
Surgery	No	Yes (skin only)	Yes (skin only)	Yes (skin and peritoneum)	Yes (skin, periosteum and skull as well as dura mater for cranial window)
Relocation	-Photoconversion -Vascular network -Collagen network	-Marks on the window and cover-glass -Photoconversion -Vascular network	-Marks on the window and cover-glass -Photoconversion	-Marks on the window and cover-glass -Photoconversion -Vascular network -Collagen network	Marks on the window and cover-glass -Photoconversion -Vascular network -Collagen network
Microscope	Upright or inverted	Upright or inverted	Inverted preferred	Inverted preferred	Upright or inverted
Pros	-Non-invasive	-Low invasion -No motion artifacts -Replaceable coverglass	-Low invasion	-Variety of organs -Replaceable window	-Up to several months of imaging for cranial window and up to two years for thinned-skull window
Cons	-Melanin is light sensitive	-Tumor growing in XY instead of XYZ direction	-Motion artifacts	-Motion artifacts	-Blood brain barrier broken for cranial window -Bone regrowth
Example references	[7, 8, 89]	[80, 81]	[24, 57, 83, 100]	[6, 29, 66, 122]	[32-34, 39, 82, 123]
Protocol references	[9-11]	[4, 12-14, 19]	[21-23, 41]	[4, 25, 28]	[4, 13, 30, 31, 35-37, 60]

- The placement of the frame is important for the lifetime of the window; the top part and sutures should not be too close to the edge of the tissue to prevent tearing of the skin. The bottom part of the window should be high enough to prevent muscle perforation, but not too high to ensure stability and prevent the chamber from falling over to the side of the animal.
- The chamber should not be fixed too tightly using the bolts and nuts because it will cut off tissue circulation [19]. However, fixation that is too loose will increase the chance of infection [12].

- Tumors can be implanted before or after placement of the window; note that tumor growth may change when tumor cells are implanted afterwards [20].
- As the skinfold is extended away from the animal, the heat will be lost to the environment. Hence, some tumor models grow better when the animals are housed in a 33-34°C environment, in which case the temperature in the skinfold is closer to the physiological values [15].
- The window can remain fixed for about two weeks [12, 19]; if tumor growth takes longer than two weeks the cells should be implanted before surgery.
- General acquisition and analysis tips and tricks, for instance on locating the same regions of interest during repetitive imaging, can be found in section 3.1.

Dermal imaging window (DIW)

Methodology

A variety of DIWs have been developed [21-24]. Here, we describe how to implant the DIW consisting of a titanium ring with a groove to enclose the skin [24, 25]. The DIW, also known as mammary imaging window, is first prepared by gluing the coverglass to the etched inset of the DIW (Fig. 1A). The implantation is initiated by shaving the mouse in the area of interest (e.g. 4th mammary gland, lymph node, subcutaneous tumor), then an incision is made through the skin. The incision is then sutured using a purse-string suture, creating four loops and leaving the suture untightened. Then the skin is placed in the window groove (Fig. 1B), and the sutures are tightened to fix the window on top of the tissue of interest.

Pros

The advantage of the dermal imaging window is that it allows visualization ofmammary glands and lymph nodes in their orthotopic location (see Table 1). Implanting the mammary window is a standard procedure that only requires incision of the skin with low risk of infection. It has been reported that the window can be functional (no break of the coverglass or tissue damaged) up to 21 days without affecting the microenvironment or tumor growth, nor resulting in inflammation [21]. When making use of the titanium model [24], the window can be re-used, and biting and breaking the stitches are prevented, extending the period of the experiment.

Cons

Since the window does not extend from the animal's body, fixation during imaging can be challenging with a risk of motion artifacts. Hence, motion artifacts cannot always be completely eliminated. The DIW is best used on inverted microscopes, as gravity helps reduce motion artifacts after fixation on the microscope. This limits

platform applicability of the window. Nevertheless, it should be noted that efforts are made to use this window on upright microscopes (Laila Ritsma and Chloé Prunier, unpublished data). In addition, when performing tumor studies the skin is often not the orthotopic location, which may influence for example the metastatic ability of tumors [26, 27].

Tips and tricks

- The dermal window should be implanted on top of the growing tumor. According to our experience with the mammary imaging window, the best time to implant the window is once the tumor reaches 7mm in diameter.
- The incision should be large enough to accommodate the window. Incisions that are too large will hamper fixation of the window. Best is to start with a small incision, trying to fit the window, and expanding the opening if necessary [25].
- The purse-string suture should be positioned at ~3 mm from the incision edge; too far from the edge results in excess tissue in the window groove, hampering tightening of the sutures. Placement of the suture too close to the incision increases the risk of loss of the window due to tearing of the tissue [25].
- It is important to start and end the purse-string suture from the outside of the skin, to allow proper closing of the suture threads.
- If liquid accumulates underneath the window, it may be removed by aspiration with a syringe. To do so, the needle should go through the skin next to the window and reach the inner chamber. Damaging the organ or tumors with the needle can be prevented by placing the window against the coverglass, keeping the needle away from the organ of interest. Only aspirate once the needle is well positioned. Be aware that the needle needs to be small (e.g. 27g) to prevent air entry under the window.
- Another method to absorb the exudate is by making use of a biocompatible gelling alginate material that absorbs wound fluid in combination with an open DIW with removable glass coverslip [23].
- General acquisition and analysis tips and tricks, for instance on locating the same regions of interest during repetitive imaging, can be found in section 3.1.

Abdominal imaging window (AIW)

Methodology

The AIW is first prepared by gluing the coverglass to the etched inset of the AIW (Fig. 1A). The implantation is preceded by shaving the mouse in the abdominal area, after which an incision is made through the skin and peritoneum. The incision of skin and peritoneum is then sutured using a purse-string suture through both layers around the edges, creating four loops and leaving the suture untightened

[25]. Then the organ of interest is brought into position and fixed to the window using a variety of methods, depending on the organ (see tips and tricks). Ultimately, the skin and peritoneum are placed in the window groove (Fig. 1B), and the suture is tightened to fix the window into the abdomen [25].

Pros

The advantage of the AIW is the ability to visualize a variety of abdominal organs at their original location [28] such as liver, intestine, pancreas and others (see Table 1). Moreover, this allows for studying a plethora of tumors in their orthotopic location (e.g. pancreatic tumors and liver tumors) over time. As the technique is quite similar for the various abdominal organs, and the procedure can be performed within 45 minutes [25], it can be adopted for a whole range of studies. In addition, the technique can be combined with other procedures requiring abdominal surgery like tumor cell injection in the mesenteric vein or spleen [28]. It has been reported that imaging through the AIW can range between two to four weeks, depending on the location of the window [28]. The use of the replaceable window (where the coverglass can be replaced to remove exudate or liquid accumulations) [25] doubled the lifetime of the liver AIW from approximately one to two weeks.

Cons

The risk of exudate or fluid accumulation that was mentioned above is specifically present for AIWs that are placed at the midline of the animal's belly, most likely because of gravity [25]. Precise fixation of the organ to the entire window edge using glue reduces this risk [4]. Glue can also help to prevent motion artifacts caused by respiration or tissue contractions that are often observed by IVM of abdominal organs [28]. However, glue may also lead to tissue damage and immune infiltration at the place of fixation. The AIW has so far only been used on inverted microscopes, as gravity helps to prevent motion artifacts caused by respiration; hence it is more limited in terms of platform applicability.

Tips and tricks

- Placement of the initial incision is dependent on the organ of choice. If possible it is best to choose a lateral position to limit abdominal fluid accumulation in between the coverglass and tissue by gravity [4]. Thus, for intestine and pancreas use a lateral incision. For spleen, use a left lateral incision above spleen (usually visible through the skin). For kidney, use lateral incision just below the ribs where a slight bulge can be observed.
- The incision should be large enough to accommodate the window. Too large incisions will hamper fixation of the window and increase the chance of infection.

Best is to start with a small incision, trying to fit the window, and expanding it if necessary [25].

- Fixing of various organs is in all cases, except for the kidney, best done using cyanoacrylate glue. Cyanoacrylate glue is preferred over vetbond as it is more viscous, preventing the glue from covering larger areas than necessary that would hamper imaging. In case of the liver, the organ is settled in position by placing a cotton gauze in between the liver and diaphragm to move the organ caudally to the site of the window. This is made possible by cutting the falciform ligament and clamping and removing the caudal part of the xyphoid process. The liver is then fixed to the window using cyanoacrylate glue [25]. Use glue to cover the entire lower half of the interior lining of the titanium window ring to create a tight seal between the liver and the window, preventing fluid accumulation caused by gravity [4]. The cecum, pancreas and, spleen can also be fixed to the window by placing glue on the interior lining of the titanium AIW at the place where the organ will be fixed [6]. Note that because these organs can be extracorporated, the AIW can be placed upside down on the animal's skin next to the incision for more precise organ positioning. The small intestine can also be extracorporated and placed in the window, but then the mesenterium can be used for fixation to the cover glass of the window, to prevent the need of gluing the intestine directly onto the titanium ring. Direct fixation of the small intestine with glue is not advised, since this can lead to fatal obstruction within a few days. For extended AIW implantation procedures, see [25]. At last, the kidney is not fixed to the AIW by glue, but by securely fixing it in the subcutaneous space [25, 29]. For this a purse-string suture is placed along the incision of the peritoneum, and tightly fixed around the stalk of the kidney. Care should be taken when fixing the suture since too tight fixation may stop the blood flow towards the kidney. However, the suture should not be too loose, as it might result in an opening from the abdominal cavity to the subcutaneous space resulting in enhanced fibrosis and possible infection. After fixing the kidney in the subcutaneous space, a purse-string suture in the skin is used to fix the titanium AIW in the skin above the kidney. Once sutures are tightened, excess air in the subcutaneous space should be removed to reduce organ fibrosis by placing a small needle through the skin reaching underneath the window and aspirating the air with a syringe.
- The position of the purse-string suture should be ~3 mm from the incision edge; too far from the edge results in excess tissue in the window groove, hampering tightening of the sutures. Placement of the suture too close to the edge increases the risk of loss of the window due to tearing of the tissue [25].
- It is important to start and end the purse-string suture from the outside of the skin, to ensure closing of the suture threads. Skin and peritoneum are sutured

using a purse-string suture through both layers to ease the procedure.

- General acquisition and analysis tips and tricks, for instance on locating the same regions of interest during repetitive imaging, can be found in section 3.1.

Cranial imaging window (CIW)

Methodology

Two types of cranial imaging windows are used, the cranial window or the thinned-skull cranial window. To surgically implant the cranial window, depilate the skin area on top of the skull by using a hair trimmer and cut in a circular manner. The periosteum underneath the skin is scraped and a groove on the margin of the intended craniotomy is made by using a high-speed dental drill. Next, the skull can be perforated and lifted by very thin tip forceps. The exposed brain is sealed by applying silicone oil, gently pressing a cover glass on the oil and gluing the edge with cyanoacrylate glue. Dental crylic cement is applied on the entire skull surface and the rim of the coverslip to create an even surface. A stainless steel ring is glued on top of the cemented coverslip enabling fixation to the microscope (Fig. 1A) [4]. The thinned skull cranial window leaves the integrity of the periosteal dura intact. Using a dental drill, the complete outer layer of compact bone and the majority of the inner layer of cancellous bone is removed. Approximately 200 µm in diameter of the remaining skull bone is thinned to a thickness of around 20µm using a microsurgical blade [30].

Pros

The CIW allows visualization of cells in living brain under normal and pathological conditions over time (see Table 1). It is applicable on both upright and inverted microscopes [31]. Chronic cranial window application enables high-resolution imaging in various brain regions [32-34]. It also allows for repeated intracerebral injections and subsequent *in vivo* imaging of the mouse brain over several months [35]. Window clarity can be sustained for up to four months after initial window implant, or two months after subsequent window removal/replacement [18, 36].. The cranial window is preferred when imaging deep or when imaging fine morphological structures. An advantage of the thinned skull technique is that the integrity of the periosteal dura remains intact and inflammation can be avoided. Multiple imaging sessions can be carried out within five days after the initial surgery.[37].

Cons

Chronic cranial window surgery is an invasive method since part of the mouse skull needs to be removed. It is important to realize that the blood brain barrier

will be broken if removal of the dura mater is necessary, which affects the brain's microenvironment [37]. Moreover, only part of the brain can be imaged, as the imaging depth is limited to 900 µm [37]. Fibrotic scarring or periosteal dura and bone regrowth can occlude the window over time, eliminating the possibility of further imaging [38]. Surgical trauma or introduction of contaminants through the skin or bone marrow vasculature can occur with both approaches and might lead to systemic and local inflammatory responses [37].

The limitation of the thinned-skull technique is that non-uniform skull thickness may cause significant spherical aberrations and that it is difficult to distinguish fine morphological structures beyond 50 µm deep [30, 39, 40]. Over-thinning the skull beyond 15 µm carries the risk of mild cortical trauma resulting from pushing the skull downwards, leading to mild cortical inflammation. After 5 days, the thinned skull window requires re-thinning of the bone, which can be difficult for inexperienced operators. Moreover, the optical properties of the skull gradually deteriorate with repeated thinning [30, 37].

Tips and tricks

General tips

- Accuracy is critical to avoid bleeding and brain damage; it is therefore recommended to perform the whole procedure using a stereomicroscope.
- It is important not to apply pressure to the skull and brain while drilling; cold sterile cortex buffer can be used during the drilling process to prevent brain overheating and to keep the exposed cortical surface continuously hydrated during the surgical procedure.
- General acquisition and analysis tips and tricks, for instance on locating the same regions of interest during repetitive imaging, can be found in section 3.1.

Tips for the cranial imaging window [4]

- Stop the drilling when the bone flap become loose and gently lift it after applying a drop of cortex buffer to prevent dehydration and bleeding of the dura mater.
- An absorbable gelatin sponge can be applied to stop any small bleeding that occurs when the skull is removed.
- To improve imaging resolution and/ or for superficial brain injection the dura mater can be removed. Take extra care in removing the dura mater, since it is a very thin and elastic membrane that may induce massive bleeding.
- Make sure the glue seals the edges of the coverslip completely to prevent infection.

Tips for thinned-skull window [30]

- The size, dimensions and quality of the microsurgical blade are important in order to achieve maximum thinning without depression of the skull.
- To ensure that the thinned region is ~20 µm thick and relatively even in thickness, the thickness of the skull can be monitored using the two-photon microscope.

Others

One of the biggest challenges of long-term IVM is to develop surgical procedures and windows to image areas which are hard to reach due to their anatomical location. Over the last 5 years, a lot of progress has been made as demonstrated by the above-mentioned existing windows. Besides these, several other windows have been developed. Jeong and colleagues introduced a novel chronic lymph node window (CLNW) model to facilitate new discoveries in the growth and spread of lymph node metastases [41]. Additionally, the design of new windows allowing visualization of the ovaries and testes in their physiological conditions or during tumor invasion have been reported [42-45]. Moving structures are even more challenging to visualize as it results in motion distortions during imaging. The spinal cord is not only a challenging anatomic area for imaging, but additionally situated close to the heart and lungs resulting in cord motion. Impressively, Farrar and colleagues designed an adapted window where the spine is surgically attached to a metallic structure that allows the elevation of the mouse body and leads to a reduction of motion artifacts without perturbing the physiology of this tissue [46]. It has been reported that this window can be combined with other imaging techniques such as ultrasound or photoacoustic leading to multiparametric information acquisition and enrichment of the imaging data-set [47].

Acquisition and analysis

General acquisition and analysis tips

Labelling cells for IVM visualization

Many long-term IVM techniques require the cells of interest to be fluorescently labeled for detection. Transgenic animals expressing fluorescent proteins in specific cells of interest are ideal tools for IVM experiments. However, the generation of transgenic animals is expensive and time-consuming. Antibodies can be administered that label specific cells *in vivo*, but these often have depleting, activating or inhibiting functions and thereby undesirable side-effects. Alternatively, cells can either be fluorescently labeled *in vivo* or specific fluorescently labeled cells can be adoptively transferred for IVM visualization. For instance, cell lines endogenously expressing fluorescent reporters can be used, cells

can be isolated from donor mice ubiquitously expressing a fluorescent protein, or unlabeled donor cells can be stained using exogenous cell tracker dyes. Different cell tracker dyes exist that label either the membrane (e.g. PKH), the cytoplasm (e.g. succinimidyl esters) or the nucleus (e.g. Hoechst) of cells, offering many possibilities for different research questions [48-50]. The exogenous cell tracker dye carboxyfluorescein succinimidyl ester (CFSE), an intracellular covalent coupling dye, has the added value that it enables the tracking of cell divisions *in vivo* over time [51, 52]. Endogenous photoconvertible reporters are another useful tool [53-56]. Photoconverting (regions of) cells allows the retracing of certain areas, the visualization of long-term migration within a tissue and between tissues and the migration of single cells in dense fluorescent areas [21, 57, 58]. In contrast to dyes that lack specificity to label subgroups of cells, these photoconvertible reporters do allow monitoring small groups of cells in e.g. specific microenvironments.

Recommendations for optimization of window surgery and imaging

Before gluing a coverglass to the titanium window ring, the coverglass can be coated with PLL(20)-g [3.5]-PEG(2) (Poly-L-lysine-graft-poly(ethylenglycol)). This reduces cell attachment and a possible immune reaction against the coverglass [25]. The application of Seprafilm Adhesion Barrier can prevent fibrosis-induced adhesion of organs [25, 59]. During window surgery, analgesics may be administered to reduce postoperative pain and speed up the recovery [60]. After surgery it is important to house the animal solitarily to avoid window damage or detachment [14, 25]. Antibiotics can be used to prevent complications in long-term studies [61]. During imaging sessions, saline infusion (200µl for imaging up to 3 hours and 100µl/h for longer imaging) ensures proper hydration of the animal. In addition, glucose can be supplemented to further prolong the imaging period [62].

Relocation of the same position over time

To retrace the same location over multiple imaging sessions, crude tracing can be done by a) fixing the position of the tissue behind the window and/or b) aligning marks on the window and stage insert (Fig. 2A) [63] and/or c) marking of positions using a motorized stage (Fig. 2B) [10, 25] and/or d) using special coverglasses with grids (Fig. 2C) [25]. For more precise retracing we advise the use of type I collagen (Fig. 2D) [25]. It is highly stable and distinctive, and can be visualized using second harmonic generation. Characteristic patterns of fluorescence visualized using a tilescan can also be used as a landmark for proper alignment of the regions of interest. Examples include transplanted fluorescent islets of Langerhans, the vascular network, the clustering of fluorescent intestinal crypts or hair follicles in distinctive groups as well as photoconverting an area of interest (Fig. 2E) [6, 10, 29,

58]. Imaging of the blood stream using dyes and visualizing the matrix by applying second harmonic generation can also help to track cells back [28, 64]. Finally, fluorescent bead injection can be used to retrace the same region, however this does evoke an inflammatory response.

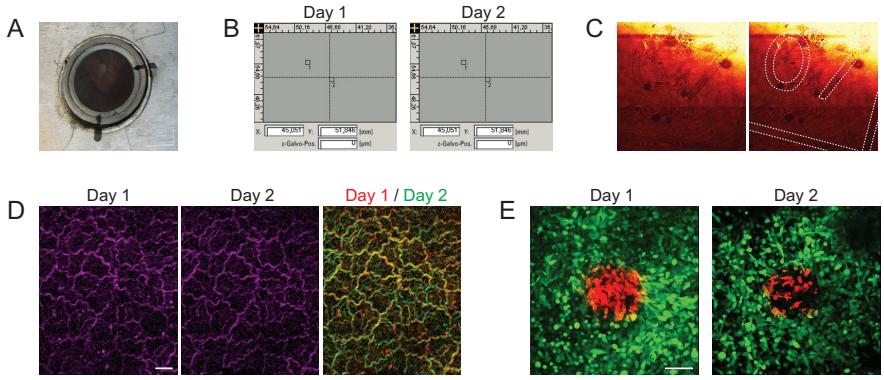

Fig.2. Retracing the same position over time. (A) Crude markings on the window and stage insert can be used to keep the window in the same position at multiple timepoints. (B) The coordinates of positions can be saved and applied at consecutive timepoints in microscopy software. Reprinted from [6] by permission from Macmillan Publishers Ltd: Nature. (C) Special coverglasses with grids can facilitate relocation. Reprinted from [25] by permission from Macmillan Publishers Ltd: Nature (D) Collagen type I fibers (purple) visualized by second harmonic generation can be used to retrace a position. Images of day 1 and day 2 were merged in false color (red or green respectively), yellow indicating colocalization. Scale bar, 20 µm. Image from [28] Reprinted with permission from AAAS. (E) Photoconverting cells in an area of interest from green to red fluorescence using special reporters such as Dendra2 in this example provides a straight-forward landmark. Scale bar, 100µm. Data courtesy of Chloé Prunier.

Correction of movies

Although animals are under anesthesia during IVM, imaging fields can drift and/or deform because of breathing and gravity, resulting in unwanted XYZ changes during imaging. Therefore, the correction of recorded images is often necessary to avoid getting biased results. Registration (movement correction) options are available in imaging software such as ImageJ, Volocity and Imaris. Generally a non-moving channel, or non-moving objects, are used to re-align all the images in XYZ dimensions over time. This of course only works if the distortion does not take place within one XY image. If during imaging set up it becomes apparent that XY distortions are occurring within one image, it is advisable to choose another position, to re-position the animal or to decrease the acquisition time (e.g. by decreasing the number of pixels).

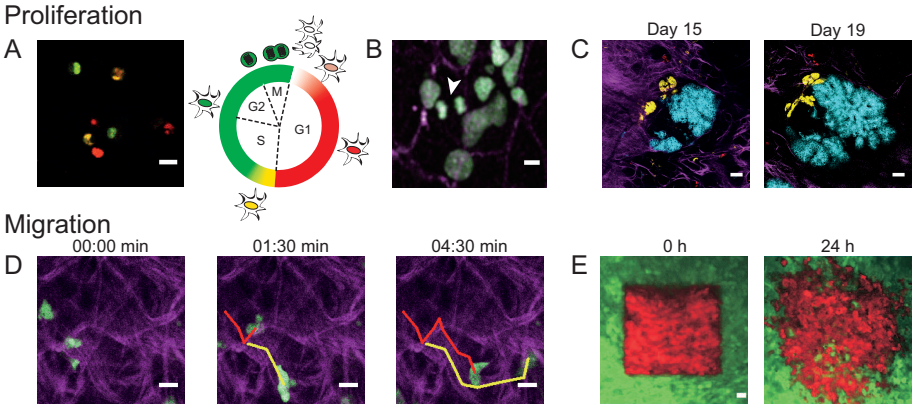

Fig.3. Monitoring proliferation and migration. (A) The FUCCI reporter allows the visualization of the cell cycle. Scale bar, 20 µm. Data courtesy of Silvia Ariotti and Nienke Vrisekoop. (B) *In vivo* visualization of mitosis using the H2B-Dendra construct. Data courtesy of Laila Ritsma. (C) The Confetti construct can be used to monitor clone size over multiple days. Scale bar, 50 µm. Data courtesy of Anoek Zomer [24]. (D) Neutrophil recruitment in the ear in response to heat-killed Staphylococcus aureus. Migration paths of individual cells are indicated in red and yellow. Scale bar, 10 µm. Data courtesy of Nienke Vrisekoop. (E) Photoconverting green fluorescent cells to red in an area of interest using the Dendra2 reporter allows the tracking of migration over multiple days. Scale bar, 30 µm. Images reprinted from [21] by permission from Macmillan Publishers Ltd: Nature Methods.

Tools to study cell proliferation

Cell proliferation can be assessed by IVM both directly and indirectly. The direct way to look at cell division is by using specific fluorescent cell cycle reporters. Among them, the fluorescence ubiquitination cell cycle indicator (FUCCI) cell cycle reporter which colour changes (from red to yellow to green) during cell-cycle progression (Fig. 3A) [51, 52] and the histone 2B (H2B) tagged with a fluorophore (Fig. 3B) [65, 66] are the most effective. Depending on the research question, different indirect measures of proliferation are also available. One of them is lineage tracing which is based on the genetic expression of a fluorophore that is inherited by all daughter cells upon cell division [6, 24, 67]. Initially lineage tracing has been used to look at clonogenicity. For instance, under Cre induction, the Cre-inducible Confetti/ Brainbow construct allows expression of one of the four confetti colors i.e. CFP, GFP, YFP and RFP [68, 69], allowing retracing of every tumor cells and their progenies (Fig. 3C). However, by counting the number of cells expressing the same fluorophore, one can also indirectly infer the number of cell divisions [51, 52]. Another tool that allows identification of dividing cells is label dilution [70, 71]. Upon division, cells initially loaded with a dye will equally divide this dye between daughter cells leading to its dilution. By measuring the intensity of the dye, one can make inferences about cell divisions. An attractive alternative to these methods is

the use of inducible systems which are based on the controlled photoconversion of a fluorescent protein such as Kaede or Dendra2 to study proliferation [21, 56]. These proteins are GFP-like fluorophores that can be photo-converted from green to red using blue light (405 nm). The advantage of this method is that specific regions of interest can be targeted. The conversion from green to red is irreversible, but red proteins are diluted as the cell divides. Both by measuring the intensity of the red fluorescence and by counting the number of red cells, one can draw conclusions about cell division [21]. Finally Fluorescence Resonance Energy Transfer (FRET), a method based on transfer of energy between two light sensitive molecules, has been used to detect apoptosis in a long-term IVM experiment [66].

Tools to study cell migration

Cell migration can be assessed by different techniques depending on the research question. In order to accurately track the displacement, velocity and directionality of single cells, the time interval should be such that the cells have moved maximally one cell diameter per time point. Tracking of single cells can be done manually or automatically using imaging software such as ImageJ, Imaris or Volocity (Fig. 3D). Although accurate migration measurements can be obtained within one imaging session and compared between imaging sessions, cells likely move more than one cell diameter between imaging sessions on different days,. A more crude measure of migration at the population level is to calculate the amount of changed pixels over time correlated to the total cell area [24]. By overlaying pictures taken at different timepoints and giving each timepoint a different color, migration at the population or individual cell level can be displayed [72, 73]. In addition, photoconversion can be used to monitor migration of cells from or into the switched region, to switch single cells and retrace them in a next imaging session (Fig. 3E) or to find cells migrating from one tissue to another [58, 74]. These techniques can be used to compare motility between conditions or between different areas over multiple days. Finally Fluorescence Recovery After Photobleaching (FRAP), a method that allow to determine the kinetics of diffusion of molecules through tissues, has also been used in long-term IVM experiments to assess protein mobilization during cancer cell migration [75].

Applications

In the following section some inspiring examples of applications of long-term IVM are reviewed with a focus on cell migration and proliferation. For easy referral we have also included the references mentioned in this section in Table 1.

Monitor proliferation and/or migration of cells from organs

Proliferation and migration within tissue often take days to weeks and therefore long-term IVM offers great opportunities to study the dynamic aspect of those processes. Angiogenesis and wound healing have already been studied by long-term-IVM in skin for decades using traditional microscopy [76, 77]. Direct imaging of the ear by two-photon microscopy, has been described to monitor the division and migration of pathogens over a couple of days [7]. More sophisticated windows have been reported to visualize cellular dynamics of other tissues using two-photon microscopy. For example, the spinal cord window chamber has been used to study microglia dynamics after a laser induced spinal cord lesion [46] and vascular changes in response to radiation [47]. Additionally, the abdominal imaging window has been placed on the intestine, spleen, liver and kidney [28]. These different types of windows can be combined with smart reporters to study the cell cycle [64] or to perform lineage tracing (sections 3.2) [68, 69].

Monitor proliferation and/or migration of tumor cells

The primary cause of cancer-related deaths is the presence of metastases [79]. The process of metastasis refers to the capability of tumor cells to spread through the body and form secondary tumors at distant sites. The metastatic process is complex and includes several steps: migration away from the primary tumor, intravasation into blood vessels, extravasation at secondary sites and formation of secondary foci. Since tumor progression and metastasis formation often are relatively slow processes, long-term IVM is an extremely helpful tool to study tumor cells *in vivo*. It has indeed been widely used to investigate primary tumor cell proliferation, migration and invasion in different organs, such as skin, mammary fat pad and even ovary [9, 21, 42, 80]. Cell cycle reporters (section 3.2) allow the study of tumor cell proliferation. The second step of the metastatic process, tumor cell intravasation, has been studied in the mammary fat pad by tracing back photoconverted Dendra2-expressing tumor cells in proximity to blood vessels. This approach revealed that tumor cell migration and blood entry take place not only at the invasive front, but also within the tumor mass [21, 58]. Using a whole body imaging system and the FUCCI reporter it was demonstrated that nearly all tumor cells attached to the inner wall of the veins are in G1 phase, while multiple phases are present when tumor cells invade and proliferate alongside the veins [64]. In addition, the Fucci reporter has been used to quantify cell-cycle effects of antimitotic cancer drugs over 8 days in HT-1080 fibrosarcoma xenografts using automated quantitative image analysis [81]. Interestingly, this uncovered that mitotic arrest was relatively low in contrast to *in vitro*. Finally, tumor cell behavior at the metastatic site has been visualized in the brain and liver by using imaging windows [28, 82].

Besides tumor cells, the microenvironment can be investigated using IVM, and more particularly its role in tumor cell proliferation, migration and metastasis formation [24, 83-85]. For example, long-term IVM was used to establish that melanoma-associated fibroblasts provide "safe havens" for melanoma cells to tolerate kinase targeted therapy [86] and to show that dormancy, a state in which tumor cells refrain from proliferation, is reversible and dependent on the niche [70]. These latter studies open up the possibility to target the niche rather than the tumor cell to overcome drug resistance.

Monitor stem cells and competition of cells from organs

Somatic stem cells are at the base of tissue homeostasis and regeneration. They are undifferentiated cells that can, through proliferation, generate differentiated specialized cells of a tissue [87, 88]. Stem cell proliferation is essential during development and also for normal tissue turnover and tissue regeneration processes like wound healing [87]. Understanding their basic function is fundamental for improving tissue regeneration efforts, but also for curing diseases like cancer that may have hijacked properties of these stem cells. Long-term IVM has been successfully adopted to study stem cells of the hair follicle, epidermis, small intestine and spermatogenic compartment. For studies of the hair follicle, epidermis and small intestine lineage tracing was used to visualize stem cell proliferation over multiple days, and this was combined with short-term IVM to study stem cell fate, migration and/or the plane of cell division [6, 89, 90]. Interestingly, two of those studies independently observed that cell fate of small intestinal and hair follicle stem cells is dependent on the spatial organization within the niche [6, 89]. Both studies combined lineage tracing with ablation of the stem cell compartment to demonstrate this. Germ line stem cell division and longitudinal migration have been studied in the testes using long-term IVM, revealing rapid stem cell migration between niche regions (intertubular vessels), and functional hierarchy between and within subpopulations of spermatogonia [43-45]. Taken together, long-term IVM has been vital in identifying the hierarchy and population dynamics of stem cell and tissue maintenance.

Monitor stem cells and competition of tumor cells

The cancer stem cell (CSC) paradigm originates from 1994 when a transplantation experiment of hematopoietic cells from patients with leukaemia showed that only a group of cells had the ability to generate leukaemia in xenografts and that those cells had stem cell properties [91-93]. Since then, it has been widely debated whether solid tumors could also recapitulate such a hierarchical organization i.e. CSCs capable of maintaining and providing growth of solid tumors. Lineage tracing

(section 3.2), recently showed the existence of CSCs that maintain and provide growth of squamous skin tumors [94] and intestinal adenomas [95]. However, those results are derived from static images of CSCs and this approach lacks the ability to study their possible plastic properties. CSCs have also been studied using long-term IVM in mammary adenocarcinoma. In an unperturbed model of mammary tumors, Zomer and colleagues have used lineage tracing coupled to long-term IVMto look at the plasticity of CSCs [24]. Using the genetic mouse model MMTV-PyMT expressing the Cre-inducible confetti construct (see section 3.2) combined with the implantation of a mammary imaging window, they retraced tumor cells and their progeny over 3 weeks showing for the first time that cancer stem cell state is reversible and can be acquired or lost during cancer progression. In 2010, Campos and colleagues have used the long-term intravital imaging technique to study stem-like glioma cells (SLGC) and their effect on angiogenesis, invasive growth and chemoresistance [96]. Using the cranial imaging window over 9 days, they have monitored GFP-transfected SLGC spheroids expansion, migration and angiogenesis. Pre-treatment with a differentiation-inducing drug leads to anti-migratory, growth inhibiting and antiangiogenics effects in SLGCs.

Monitor therapy of tumor cells

So far, scientists have identified signaling pathways involved in the deregulation of proliferation and migration acquired by tumor cells during cancer progression and designed new therapeutics to target them. Even if highly potent *in vitro*, efficiency of drugs in patients can differ greatly. This might be due to a) *in vitro* differences in the mechanism of action [3], b) patient related differences [97] or c) the development of resistance by the cancer cells [98]. Therefore, it is important to have a clear understanding on drug mechanisms *in vivo* and which patients are more likely to respond. IVM allows visualization of cell mitosis and motility during tumor progression and has therefore been used for this purpose.

Using cell cycle reporters (section 3.2), the H2B-Dendra2 fluorescent protein [65, 66], fluorescent proteins [28], or a FRET-sensor for apoptosis (section 3.2), the effect of anti-proliferative drugs have been studied using long-term IVM. For instance, it has been shown that anti-mitotic compounds such as paclitaxel [65] or docetaxel [66] do not induce apoptosis through mitotic arrest as claimed for years, but through another non-mitotic mechanism of action. Hereafter, other supposedly anti-mitotic drugs have been investigated using a recently developed integrated workflow that allows quantitative *in vivo* cell cycling profiling also revealing discrepancies [81].

Not only anti-proliferative activity of drugs can be monitored using IVM but also anti-migratory activity. Using fluorescently labelled cancer cells, clinically used [75, 99] or newly discovered compounds [100] have been studied for their potential anti-migratory activity. For instance, using an E-cadherin mouse reporter, Erami and colleagues used intravital FRAP imaging (section 3.3) to assess the role of E-cadherin in pancreatic cancer cell mobility in response to the Src-inhibitor Dasatinib [75].

Finally, the development of a new generation of fluorescent probes that do not affect the activity of drugs, now allows for the visualization and monitoring of drugs *in vivo* using IVM [101, 102]. Altogether, these approaches demonstrate how potent long-term IVM helps to better understand the therapeutic activity of anticancer therapies *in vivo,* and how this technique could improve their clinical usefulness.

Monitor immune responses

The cells of the immune system originate in the bone marrow and subsequently get distributed throughout the body. Some immune cells continuously circulate in blood and/ or lymph in anticipation of pathogens breaching the skin barriers, after which they can be efficiently recruited into the tissue. Other immune cells are tissue-resident, waiting for pathogens at the tissue site. Most immune cells require activation and differentiation before becoming full effector cell types. Real-time visualization of those dynamic processes are enabled by IVM. As immune cells migrate notoriously fast compared to tumor and solid tissue cell migration, studies involving IVM and immune cells primarily and extensively use short-term IVM. Repetitive long-term IVM has been performed mainly in skin [7]. Additionally, an abdominal imaging window has been implemented to study long-term CD8 T cell expansion and migration in the spleen [28]. Fluorescent sensors to monitor T cell activation and proliferation have so far not been combined with long-term IVM. Reporters of the cell cycle (section 3.2) were, however, used to study T cell proliferation in explanted lymph nodes [103]. Furthermore, *in vivo* T cell activation has been studied in short-term IVM by using a combination of the fluorescent transcription factor NFAT (nuclear factor of activated T cells) and fluorescent H2B. Upon T cell activation NFAT moves from the cytosol to the nucleus where it colocalizes with the fluorescent H2B-sensor [104]. Both the FUCCI and the NFAT-H2B sensors can be helpful tools to study immune cell activation and proliferation in long-term IVM in the future.

Conclusion and Future perspective

In this review we have described how long-term IVM on the two-photon microscope enables the visualization of dynamic processes at cellular and subcellular resolution over multiple imaging sessions spanning several weeks. Combined with the development of several imaging windows and the application of fluorescent biosensors, long-term IVM provides new exciting opportunities for studying dynamic aspects of extended physiological and pathological processes. Continuous improvements are being implemented to even further prolong the imaging period and to enhance the quality of the acquired data. In order to trace back the same region over multiple imaging sessions, landmarks which completely avoid tissue damage and concomitant immune responses would be helpful. The immotile anatomical landmarks used by Vladymyrov and colleagues might serve as a reference to retrace the imaged area of interest [105]. Moreover, deeper tissue penetration to allow the visualization of processes which occur beyond the superficial layers of organs is needed and now possible by stably fixing GRIN lenses [106], or by using optical parametric oscillators [107]. However, to visualize interactions between cells deep in the tissue by using longer wavelengths, more cell tracker dyes and/or fluorophores in the far-red spectrum need to be developed, as currently few options are available. Also, the expansion of available reporters that can visualize cell signaling at subcellular resolution together with improvements on high-resolution imaging can still expand research possibilities of long-term IVM to a great extent.

Another challenge that remains is the dynamic imaging of moving organs, such as lungs and heart. Both the thorax movements caused by breathing and heartbeat and the invasive surgeries required to visualize these organs pose problems for long-term imaging. Progress has been made on the correction of lung and heart movements based on fixation methods and special gating strategies [108, 109], however, currently these methods still require invasive surgery and thus do not permit repetitive imaging. Similarly, although the morphogenesis in developing embryos has been intravitally imaged in Drosophila and zebrafish [110, 111], the observation of morphogenesis in developing mouse embryos has been challenging and would be greatly facilitated by a suitable long-term intravital imaging solution.

To get stable images, animals are generally anesthetized during imaging. However, Ghosh and colleagues demonstrated the feasibility of *in vivo* imaging of awake animals using a miniature version of a conventional epifluorescence microscope mounted onto the CIW [112]. Sasportas and colleagues also monitored blood

vessels in awake mice in an experimental model of metastasis by using a miniature mountable IVM setup [113]. This opens up possibilities for mobile assays, such as imaging the dynamics of hundreds of individual neurons in behaving animals.

Other high-resolution (<100 μm) intravital imaging platforms or microscopy techniques besides multi-photon include OFDI [114], ultrafast ultrasound [115], whole body imaging [116], spinning disk confocal [117], side-view endomicroscopy [118], and photoacoustic imaging [119, 120]. Each of these platforms brings their own advantages and disadvantages. Combining some of these methods in the same animal (multi-modal imaging) can be a powerful way to integrate the strength of each modality, while overcoming their limitations, and at the same time reduce the number of animals. For example, Schafer and colleagues used a rapid prototyping printer technology to develop a new plastic mammary window chamber that is compatible with MRI, nuclear imaging and optical imaging [121]. We expect more multi-modal imaging developments in the near future.

In conclusion, long-term intravital imaging is a powerful tool to study dynamic processes of individual cells or even molecules as well as interactions between different cells over multiple imaging sessions. Innovations in microscopy, improvements in the methods and expansion of the existing biosensors and fluorophores will further advance this technology in the coming years.

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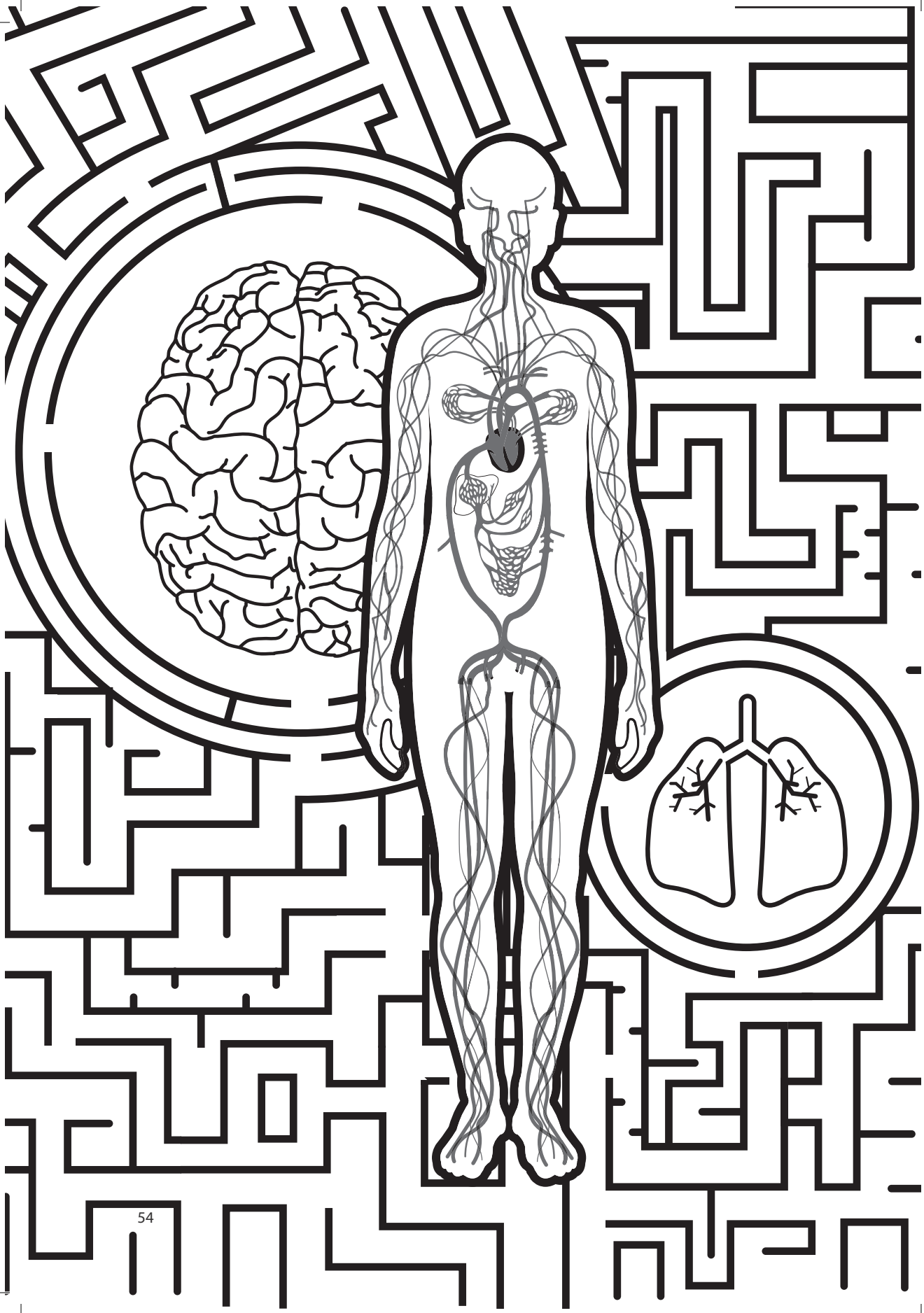

Chapter 3

In vivo characteristics of human and mouse breast tumor cell lines

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In revision

Abstract

Although two- and three-dimensional in vitro studies of breast tumor cell lines have increased our knowledge on tumor growth and metastasis formation, the complex in vivo microenvironment is not taken into consideration. The goal of our study was to illustrate the in vivo morphology and motility of widely used breast tumor cell lines. Intravital microscopy allows real-time visualization of individual cells inside tissues of living animals. We used this technique to study breast cancer migration in the complex orthotopic microenvironment. More specifically, we characterized cell morphology, cell-cell interactions, polarity and motility of mouse tumor cell lines 4T1 and mILC-1 and human tumor cell lines MDA-MB-231 and T47D. Almost all measured parameters were remarkably heterogeneous even between positions within the same tumor. Migrating tumor cells were circular in all tumor models, indicating predominantly amoeboid motility. This overview of the in vivo characteristics of mouse and human breast tumor cell lines illustrates their heterogeneity and complexity in real life, and additionally exemplifies caution should be taken to extrapolate in vitro assays of tumor invasiveness.

Keywords

Breast tumor cell lines, intravital microscopy, morphology, interaction, motility

Abbreviations

MDA: MDA-MB-231, IVM: Intravital microscopy, NSG: NOD scid gamma

Introduction

Breast cancer is the most common cancer in women worldwide and difficult to treat once spread systemically. To study the metastatic process of breast cancer, many different tumor cell lines have been generated [1-4]. Two typical human breast cell lines, MDA and T47D, grow adenocarcinoma and ductal carcinoma respectively [1]. Estrogen receptor (ER)-negative/ mesenchymal-like MDA-MB-231 (MDA) [5], a highly metastatic human breast tumor cell line, has been reported metastasize primarily to bone but occasionally to brain, ovary, and adrenal glands [6-8]. While T47D is an ER-positive/ luminal epithelial-like and non-aggressive tumor cell line [9, 10]. The 4T1 cell line, originally derived from a BALB/c mouse by Fred Miller and coworkers at the Karmanos Cancer Institute [11, 12], has been increasingly used in recent years due to its high propensity to metastasize to lung, liver and bone, sites known to be affected in human breast cancer [2, 13]. Another mouse tumor cell line, mILC-1, generated by Derksen and colleagues, mimics multiple characteristics of invasive lobular carcinoma of human breast [3] and has been used to study the function of mammary specific inactivation of E-cadherin and p53 [3, 14]. Although often studied *in vitro*, culturing breast tumor cell lines in two-dimensions noticeably alters cell morphology, cell-cell interactions and cell polarity when compared to the *in vivo* situation [15, 16]. These differences in morphology can be overcome by three-dimensional culturing which, however, still does not fully mimic the complex micro-environment in living animals [16].

Intravital microscopy (IVM) has revolutionized biomedical research during the last two decades and has been used extensively to study dynamic process in intact tissues of living animals [17-19]. It provides unique insights into cancer biology and has been particularly useful for research into the full complexity of breast tumors in an in vivo situation [20-22].

Here, we use IVM to characterize tumor cell morphology, interactions, polarity and motility in the above-mentioned mouse and human breast tumor cell lines in living mice.

Materials and Methods

Cell culture, DNA constructs and generation of stable cell lines

MDA-MB-231 cells (a kind gift from Dr. Jeffrey Segall) [23] and 4T1 cells (obtained from Dr. Eric Daanen) [24-27] were cultured in Dulbecco's Modified Eagle's Medium + GlutaMAX (DMEM; GIBCO, Invitrogen Life Technologies, Paisley, UK) supplemented

with 10% (v/v) or 5% (v/v) fetal bovine serum (Sigma, St. Louis, MO, USA), 100 µg/ml streptomycin, and 100 U/ml penicillin (Invitrogen Life Technologies, Paisley, UK). T47D cells (a kind gift from Dr. Jacco van Rheenen) [28] were cultured in DMEM/F12 + GlutaMAX (GIBCO, Invitrogen Life Technologies, Paisley, UK) supplemented with 10% (v/v) fetal bovine serum, 100 µg/ml streptomycin, and 100 U/ml penicillin. mILC-1 tumor cells (a kind gift from Dr. Patrick Derksen) were derived from mILC tumors of K14cre;Cdh1F/F;Trp53F/F mice as described [3]. They were cultured in DMEM/F12 + GlutaMAX supplemented with 5% (v/v) fetal bovine serum, 5 ng/ml insulin, 5 ng/ml epidermal growth factor, 100 µg/ml streptomycin and 100 U/ml penicillin. All cells were kept at 37°C in a humidified atmosphere containing 5% CO_2.

The cDNAs of CFP, floxed-DsRed-floxed-eGFP, Dendra2 or mTurquoise were cloned into a pLV.CMV construct and fluorescent protein-expressing cell lines were generated using a standard lentiviral transduction or Lipofectamine 2000tm (Invitrogen Life Technologies, Paisley, UK) protocol. MDA-MB-231 and T47D cells were transduced with floxed-DsRed-floxed-eGFP [28], 4T1 cells with Dendra2 or CFP [29] and mILC-1 cells with Dendra2 or mTurquoise [30]. Afterwards, cells were sorted by flow cytometry, cultured as a polyclonal population and kept under selection using puromycin (Gibco Life Technologies, Paisley, UK).

In vitro imaging

Single tumor cells (2500) were seeded into 96-well plate which was kept in an Incucyte S3 Live-Cell Analysis System at 37°C in a humidified atmosphere containing 5% CO2. Cells were imaged every 1hr for at least 24hrs.

Mice

Balb/c mice, 29P2/OlaHsd and FVB/n mice were purchased from Jackson Laboratory, Sacramento, CA, USA. 29P2/OlaHsd and FVB/n mice were crossed to obtain female 129P2/OlaHsd;FVB/n mice. Immune-competent mice were housed under standard laboratory conditions and NOD scid gamma (NSG) (own breeding) mice were housed in individualized ventilating cages. Mice (8-20 weeks old) received food and water ad libitum. Mice were shaved and all surgeries and imaging sessions were performed under anesthesia using isoflurane in air. Mice were euthanized by cervical dislocation under isoflurane sedation after the last imaging session.

Tumor cell injection

One week before injection of T47D tumor cells, mice were ovariectomized and implanted with an estrogen pellet (0.72 mg/pellet, 60-day release, Innovative Research of America, Sarasota, FL, USA), as per the manufacturer's instructions. For injections that did not include T47D tumor cells, mice were not overiectomized. For tumor growth, $0.5x10^6$ MDA-MB-231 DsRed$^+$ cells or $1x10^6$ T47D DsRed$^+$ cells were injected in the fourth and/or ninth mammary fat pad of NSG mice; $1x10^5$ 4T1 Dendra2$^+$ cells or $0.5x10^6$ 4T1 CFP$^+$ cells were injected in the fourth and/or ninth mammary fat pad of Balb/c mice; $0.5x10^6$ mILC-1 Dendra2$^+$ or mturquoise$^+$ cells were injected to 129P2/OlaHsd;FVB/n mice. Tumors were imaged when the size reached at least 2.5 mm.

Intravital Imaging

Mice were sedated using isoflurane inhalation anesthesia (1.5% to 2% isoflurane). The imaging window was implanted or the tumor was surgically exposed and the mouse was placed with its head in a facemask within a custom-designed imaging box [31] . The isoflurane was introduced through the facemask and ventilated by an outlet. The imaging box and microscope were kept between 32 and 36.5°C by using a climate chamber. Imaging was performed on an inverted Leica TCS SP5 AOBS multi-photon microscope (Mannheim, Germany) with a chameleon Ti: Sapphire laser (Coherent Inc.). Images were collected for 2 hours.

Table I: Basic information of imaged tumor-bearing mice

Cell line	Mouse No.	IVM	Injected cell No.	Mouse strain	Days post tumor injection
4T1	1	window	$1x10^5$	BALB/c	35
	2	skinflap	$0.5x10^6$	BALB/c	25
	3	skinflap	$0.5x10^6$	BALB/c	11
mILC-1	1	skinflap	$0.5x10^6$	129P2/OlaHsd;FVB/n	71
	2	skinflap	$0.5x10^6$	129P2/OlaHsd;FVB/n	53
	3	skinflap	$0.5x10^6$	129P2/OlaHsd;FVB/n	48
MDA-MB-231	1	skinflap	$0.5x10^6$	NSG	42
	2	skinflap	$0.5x10^6$	NSG	44
T47D	1	skinflap	$1x10^6$	NSG	54
	2	skinflap	$1x10^6$	NSG	67
	3	skinflap	$1x10^6$	NSG	65

Software and analysis

In vitro

Images were analyzed by Incucyte analysis software, the number of all cells and clusters (filter: 500um^2) per image were calculated and the area of clusters was measured.

In vivo

XYZ drift in recorded images was corrected with a custom-designed Visual Basic software program and imaging software Volocity. Up to 10 cells per image (z), 3 (superficial, middle and deeper) images per position, 2 positions per mouse, up to 3 mice for each tumor cell line were tracked manually with ImageJ software (NIH, Bethesda, MD, www.nih.gov). The distance between the tracked cells and their nearest neighboring cell as well as the circularity of the tracked cells were measured with ImageJ. Based on the tracks, the vector speed was calculated. Tracks were imported in "MotilityLab" (http://motilitylab.net) and velocity and mean turning angles were computed. All tracked cells were scored based on their motility (non-migrating cell, non-migrating cell with protrusion(s), cell migrating less than 1 cell diameter, cell migrating more than 1 cell diameter but less than 2 cell diameters, cell migrating more than 2 cell diameters).

Statistics

Results are shown as median with range. For all normal distributed measurements the Student's t test (when two groups were compared) or one-way ANOVA (when more than two groups were compared) test were used, and for all others Kruskal-Wallis-test was used for unpaired analysis and Friedman test was used for paired anlysis. Differences were considered significant when $p^{*} \leq 0.05$, $p^{**} \leq 0.01$ or $P^{***} \leq 0.001$.

Results

In vitro appearance of tumors

Representative images of the different tumor cell lines in *vitro* are shown (Fig. 1A). MDA, T47D and mILC-1 were single cells when they were seeded while 4T1 already formed small clusters (Fig. 1A T0 and Fig. 1B T0). After 24hrs, the % of MDA tumor cells in clusters didn't change compared to T0 (Fig. 1C), cells remained as single loose cells (Fig. 1A T1 and Fig. 1B T1). However, the other tumor cell lines formed significantly more clusters when comparing T1 to T0. In line, the average cluster area significantly increased with time in these cell lines, especially for 4T1 tumor cells.

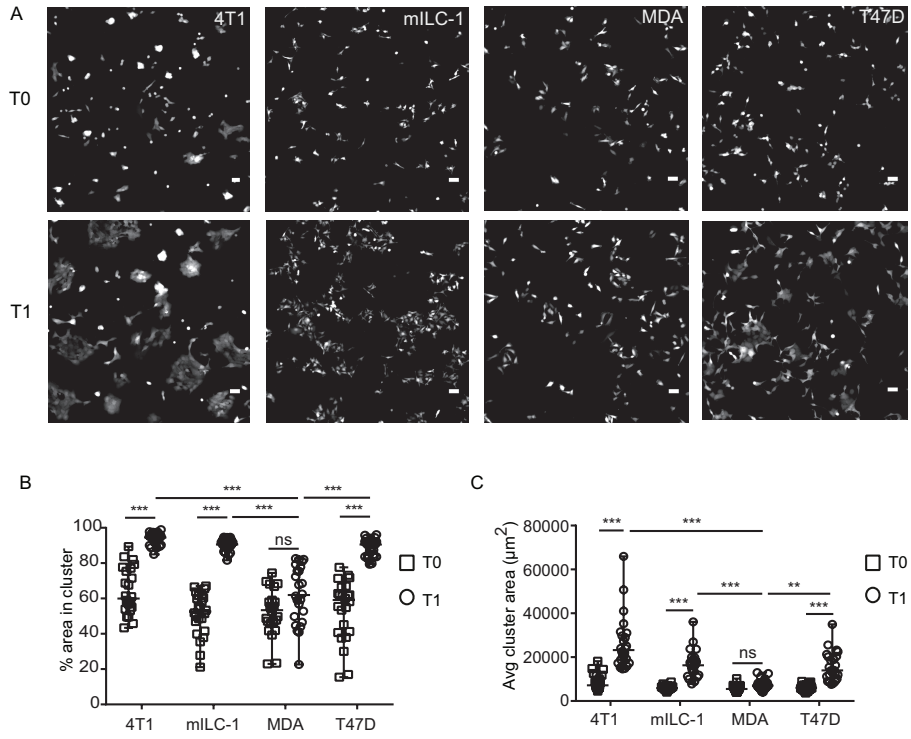

Fig 1. *In vitro* appearance of tumors. (A) Representative images of the different tumor cell lines *in vitro*. Scale bar = 50 µm. T0: 0hr, T1: 24hrs. (B) The percentage of cells in clusters. T0: 0hr, T1: 24hrs. Median with range is shown. (C) Average cluster area. T0: 0hr, T1: 24hrs. Median with range is shown.

In vivo appearance of tumors

Next we characterized these different tumor cell lines growing tumors at their orthotopic location in mouse mammary glands. Representative images and zoomed in areas (yellow square) of the different tumors in living mice are shown (Fig. 2A). As loss of cell adhesion is thought to correlate with tumor cell motility, invasion and metastasis [32], we first determined the percentage of cells attached to neighboring tumor cells (Fig. 2B). In line with the high percentage of cells in clusters and big average cluster area in vitro, the tumor cell line 4T1 had a median of 80% cells attached to neighboring tumor cells in vivo. Conversely, mILC-1 had a median of 70% and T47D of only 45% cells attached to neighboring tumor cells in vivo, whereas both cell lines formed many clusters in vitro. Interestingly, while MDA formed few and small clusters in vitro, in vivo a median of 75% of the cells was attached. Importantly, for all tumor cell lines a big range in percentages was measured between images of the same tumor model. This heterogeneity could not be ascribed to differences between mice (Fig. 2C) nor to differences between the depths of the imaging site (Fig. 2D).

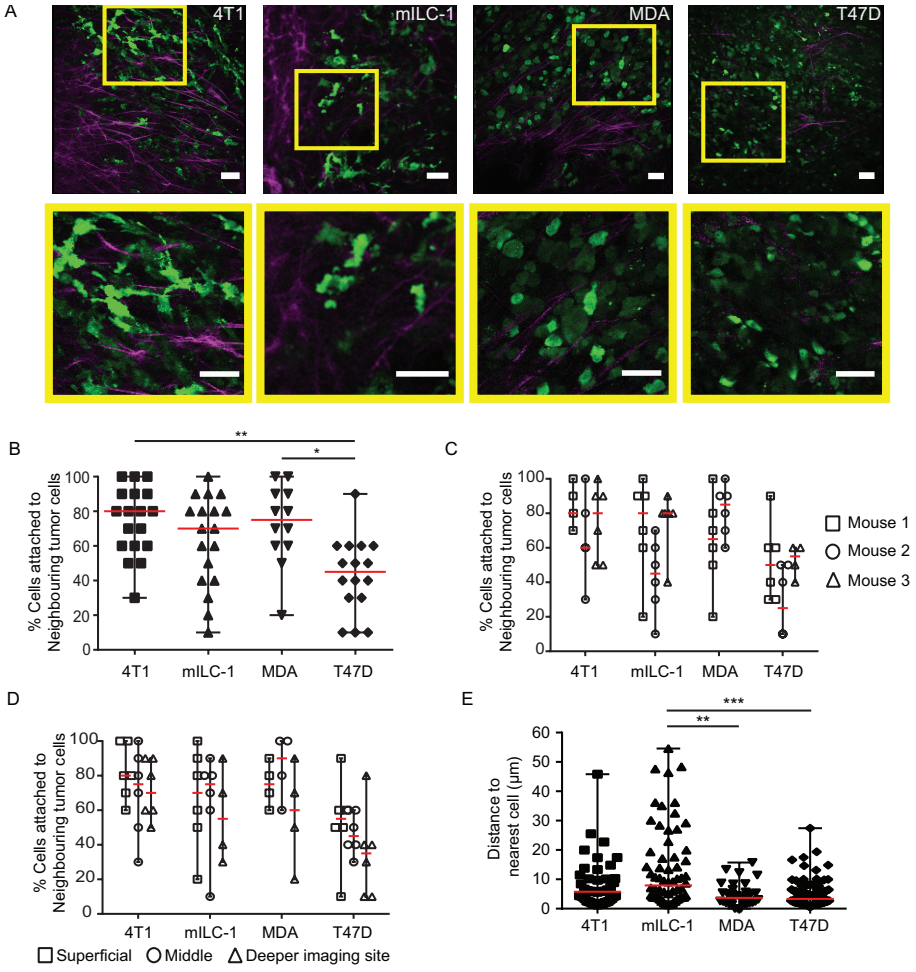

Fig 2. *In vivo* appearance of tumors. (A) Representative images and zoomed in areas (yellow square) of the different tumors in living mice. Green: tumor cells, Magenta: collagen fibers. Scale bar = 50 µm. (B) The percentage of cells attached to their neighboring tumor cells was calculated. Median with range is shown. (C) The percentage of cells attached to their neighboring tumor cells was calculated for each mouse. Median with range is shown. (D) The percentage of cells attached to their neighboring tumor cells was calculated for imaging sites at 3 different depths. Median with range is shown. (E) Distance to the nearest neighbor of sole tumor cells. Median with range is shown.

To quantify the density of the tumors in vivo, we calculated the distance to the nearest neighbor of sole tumor cells (Fig. 2E) which was relatively low for MDA and 4T1 tumor cells and in line with the relatively high percentage of tumor cells attached to each other in vivo for these cell lines. The mILC-1 tumors displayed

relatively large distances between sole tumor cells and their nearest neighbor, together with low percentages of interacting tumor cells implying loose tumor tissue. This is in stark contrast to their in vitro growth characteristics (Fig. 1). Although T47D had the lowest percentages of attached cells in vivo, the distance to the nearest neighbor of sole cells was low for this tumor type.

In vivo tumor cell motility

The first steps of the metastatic cascade are cell detachment and local invasion [33]. Therefore we used IVM to monitor tumor cell motility in living mice. A representative movie for each tumor type is shown in files S1-4. A lot of heterogeneity between the cells in terms of motility could be noted. Non-migrating cells could show the formation of 1 or more protrusions, or none at all. Only few cells were found to migrate. Some cells were migrating < 1 cell diameter with or without protrusion(s), whereas other migrated 1-2 cell diameters or even > 2 cell diameters. Hence, all tracked tumor cells were scored based on their migratory behavior into one of these 4 categories and the percentage of cells in different categories was calculated (Fig. 3 A and B). We additionally calculated the percentages of cells moving more than 1 diameter (Fig. 3B, red and blue category, Fig. 3C) for each imaging site separately. In the remaining cells, % protruding cells were calculated (Fig. 3D). The percentages moving cells and protruding cells varied widely even between positions within the same mouse. Heterogeneity in motility was not attributed to differences between mice, imaging positions nor to the depth of the imaging site. The percentages of cells migrating more than diameter were not significantly different between the tumor cell lines, but T47D showed a somewhat higher trend in line with the relatively low percentage of attached cells (Fig. 2). Interestingly, however, non-moving T47D tumor cells displayed a significantly lower percentage of protruding cells.

In vivo characterization of migrating tumor cells

Since tumor cells display many modes of migration from single isolated cells with a round or elongated phenotype to loosely-/non-adherent 'streams' of cells and collective migration of cell strands and sheets [20, 34], we characterized these modes of migration in the different tumor types. All imaging positions were re-evaluated and cells that moved more than one cell diameter within the imaging sessions were followed (files S1-4). All MDA tumor cells migrated individually, 95% of the mILC-1 tumor cells migrated individually and only 5% migrated in single cell streams. Around 70% of 4T1 and T47D migrated individually (71% and 73% respectively); the remaining cells migrated as single cell streams (Fig. 4A). We did not observe collective migration in any of the tumor models.

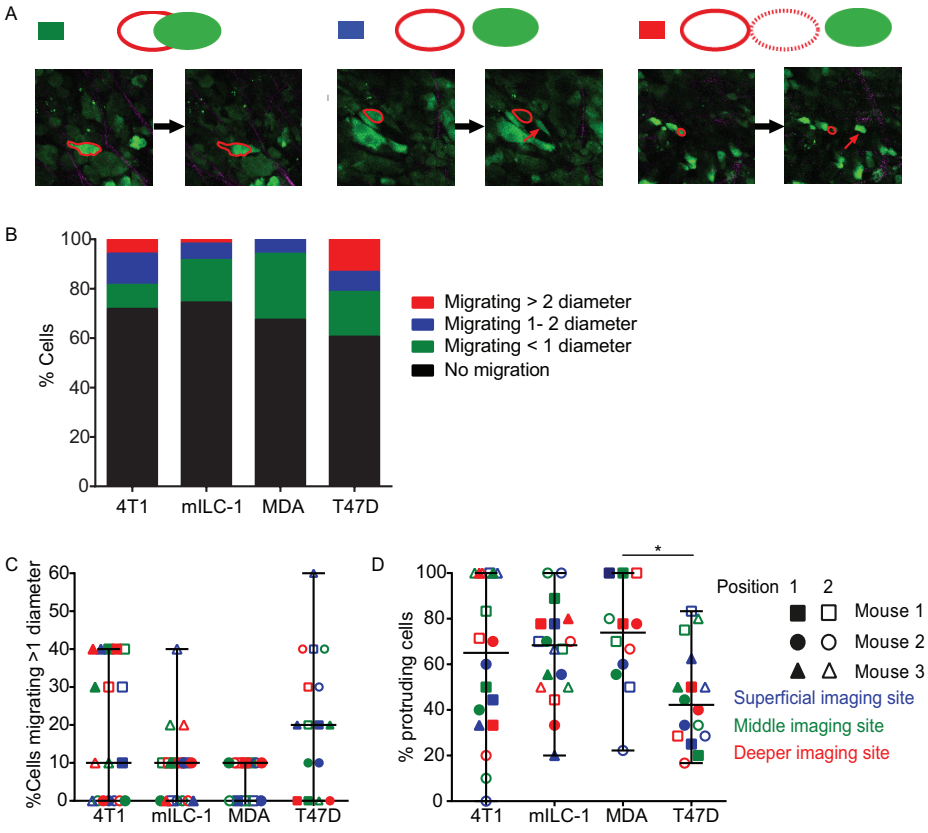

Fig 3. *In vivo* tumor cell motility. (A) Cartoon illustration and representative images of tumor cells with different migratory behavior (upper part). Green: tumor cells, Magenta: collagen fibers, Red line: tumor cell position at t=0, Red arrow: tumor cell position at t=2hr. (B) In the graph the percentage of cells with different migratory behavior of total tracked cells was quantified. (C) The percentage cells migrating more than 1 cell diameter was calculated for 3 different depths of imaging sites for each position, 2 positions for each mouse. Median with range is shown. (D) The percentage cells migrating less than 1 cell diameter with or without protrusion(s) was calculated for 3 different depths of imaging sites for each position, 2 positions for each mouse. Median with range is shown.

Next, the migrating cells were manually tracked and the velocity, vector speed and directionality were calculated (Fig. 4B and C). Remarkably, there was no big difference in the median velocity and vector speed among the tumor types, although the velocity and vector speed of 4T1 and T47D showed more variation than mILC-1 and MDA possibly due to the single cell streaming in these models. A good measure for directionality of cells moving at heterogeneous velocities is to calculate the mean turning angle as a function of time [35]. The expected mean turning angle of random migration is 90 degrees, whereas directed migration is characterized

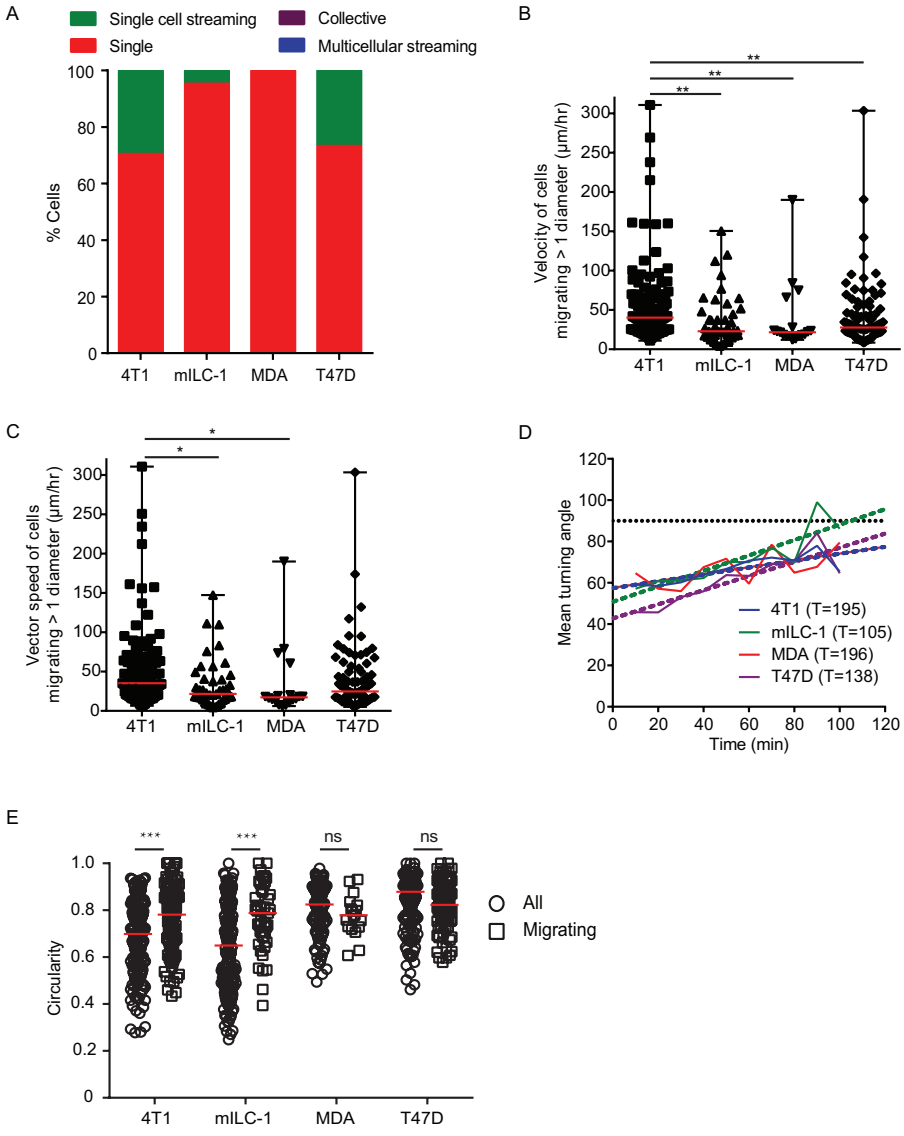

Fig 4. *In vivo* characterization of tumor cells migrating more than 1 cell diameter. (A) The percentage of cells with different migratory behaviors was quantified. (B) Velocity was calculated and median with range is shown. (C) Vector speed was calculated and median with range is shown (D) Mean turning angle as a function of time with an angle of 90 degrees representing random migration. The extrapolated time (T) when the trend line (dotted line) reaches 90 degrees is given in brackets. (E) Circularity of all cells and cells migrating more than 1 cell diameter was measured and median with range is shown.

by lower turning angles [35]. The mean turning angle of MDA, T47D and 4T1 did not reach 90 degrees, indicating these migratory tumor cells on average moved persistently for the duration of the movie. Although mILC-1 tumor cells reached a mean turning angle of 90 degrees within the duration of the movie, this was still only after 105 min. Extrapolating the observed curves of the other tumor cell lines generated a persistence of 196 min for MDA, 138 min for T47D and 195 min for 4T1 tumor cells (Fig. 4D).

To evaluate whether the migrating tumor cells in our tumor models move in a relatively slow elongated mesenchymal or a relatively fast and more rounded amoeboid manner, we measured cellular circularity of individual cells from the different tumor models, a value of 1 indicating a perfect circle (Fig. 4E). Sahai et al have previously characterized slow moving mesenchymal tumor cells to have a circularity of less than 0.5 whereas fast moving amoeboid tumor cells would have a circularity higher than this threshold [36]. Based on our total tumor cell population analysis, both 4T1 and mILC-1 displayed heterogeneous circularities above and below 0.5, whereas MDA and T47D mainly displayed a circularity higher than this threshold. Compared to the total tumor cell population, migrating tumor cells are significantly more circular for 4T1 and mILC-1 which now also predominantly display circularities above 0.5. When circularity was plotted against velocity or vector speed (S5 and S6) no distinct relatively slow mesenchymal population was found in any of the tumor models. Also no correlation was found between circularity and velocity or vector speed (S5 and S6 Figs, $R^2 < 0.1$ in all cases). This indicates that tumor cells which migrated > 1 cell diameter were predominantly amoeboid. Interestingly, single stream cells do migrate faster than single migrating cells, especially for T47D (S5 and S6).

Discussion

Using the Incucyte Live-Cell Analysis System and 2-photon microscopy we here imaged the mouse breast tumor cell lines 4T1 and mILC-1 and human breast tumor cell lines MDA and T47D in vitro and in living mice and determined characteristics of their appearance. In vitro, MDA forms significantly fewer and smaller clusters than the other tumor cell lines (Fig.1 B and C), whereas 4T1 stands out because it starts in clusters and forms clusters with the largest average area upon culture. In contrast, in vivo MDA and 4T1 have fairly similar percentages of cells attached to neighbouring tumor cells. T47D has a significantly lower percentage of cells attached to neighbouring tumor cells than both MDA and 4T1 (Fig.2B). The percentage of loose

cells did not show a relationship with the distance to the nearest neighbor of sole cells as T47D displayed relatively low values for this parameter even though it had the highest percentage of single cells. Migrating tumor cells could be detected in all tumor models. Remarkably, in vivo almost all measured parameters were fairly heterogeneous not only between mice, but also between imaging sites within the same tumor (Fig. 2C and D). When we focused on migrating tumor cells, we found these are circular in all tumor models, indicating predominantly amoeboid motility.

The fact that we found T47D breast tumor cells to be more migratory than MDA breast tumor cells in vivo might be surprising in light of the literature suggesting MDA is particularly invasive [9, 10]. One explanation could be that we found T47D displayed more single cell streams than MDA (Fig. 4A). There's no difference of single cell velocity and vector speed between MDA and T47D whereas T47D single stream cells migrate much faster than single cells (S5 and S6). Besides, it should be noted that both tumor cell lines have a metastatic origin and many studies comparing MDA and T47D invasiveness were performed *in vitro* [37-40]. In vivo MDA tumors do not form metastasis in all animals and often MDA derived metastasis are passaged further in order to create a more metastasis prone derivative cell line [16, 41, 42]. Moreover, metastasis have been found *in vivo* in T47D bearing mice upon careful inspection [42, 43].

Conclusions

Combined, our data might serve as a resource to instruct researchers on the appearance and migratory behavior of the widely-used breast tumor cell lines MDA, T47D, 4T1 and mILC-1 in living mice. An appreciation of the *in vivo* characteristics of different breast cancer cell lines not only aids our understanding of the heterogeneity and complexity of breast cancer, but additionally, our study warrants caution to the use of *in vitro* characterization of tumor invasiveness.

Acknowledgements

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Authors' contributions

NC analyzed, interpreted the data and drafted the manuscript, NV participated in manuscript writing and data interpretation. NV and LR provided images. LR critically reviewed data analyses and the manuscript. All authors read and approved the final manuscript.

Ethics approval

All experiments were carried out in accordance with the guidelines of the Animal Welfare Committee of the Royal Netherlands Academy of Arts and Sciences, The Netherlands. All mouse experiments were prospectively approved by the animal ethical committee of the Royal Netherlands Academy of Arts and Sciences (KNAW), the Netherlands, and animals were kept at the Hubrecht animal facility in Utrecht, the Netherlands. Animal welfare was monitored throughout the study and mice would be euthanized should the humane endpoint be reached before the end of the study.

Declaration of interest

The authors have no competing interests to declare.

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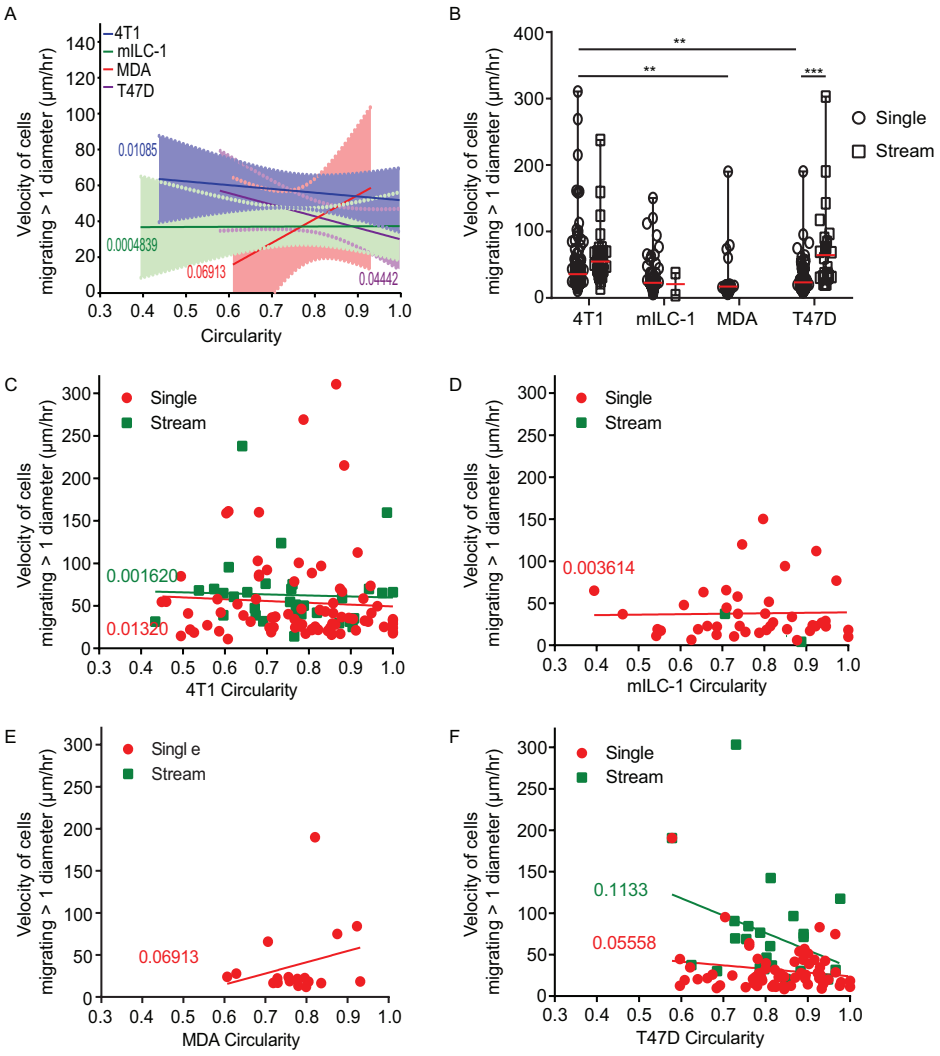
Supplemental information

S1 File. Combined stacks of 4T1 tumor cells. This movie shows two-photon time-lapse images of 4T1 tumor cells in living mice. The time-lapse covers a period of 2hrs. **Left panel:** Original movie. **Right panel:** Example of tracked migrating cells. Scale bar = 50 μm.

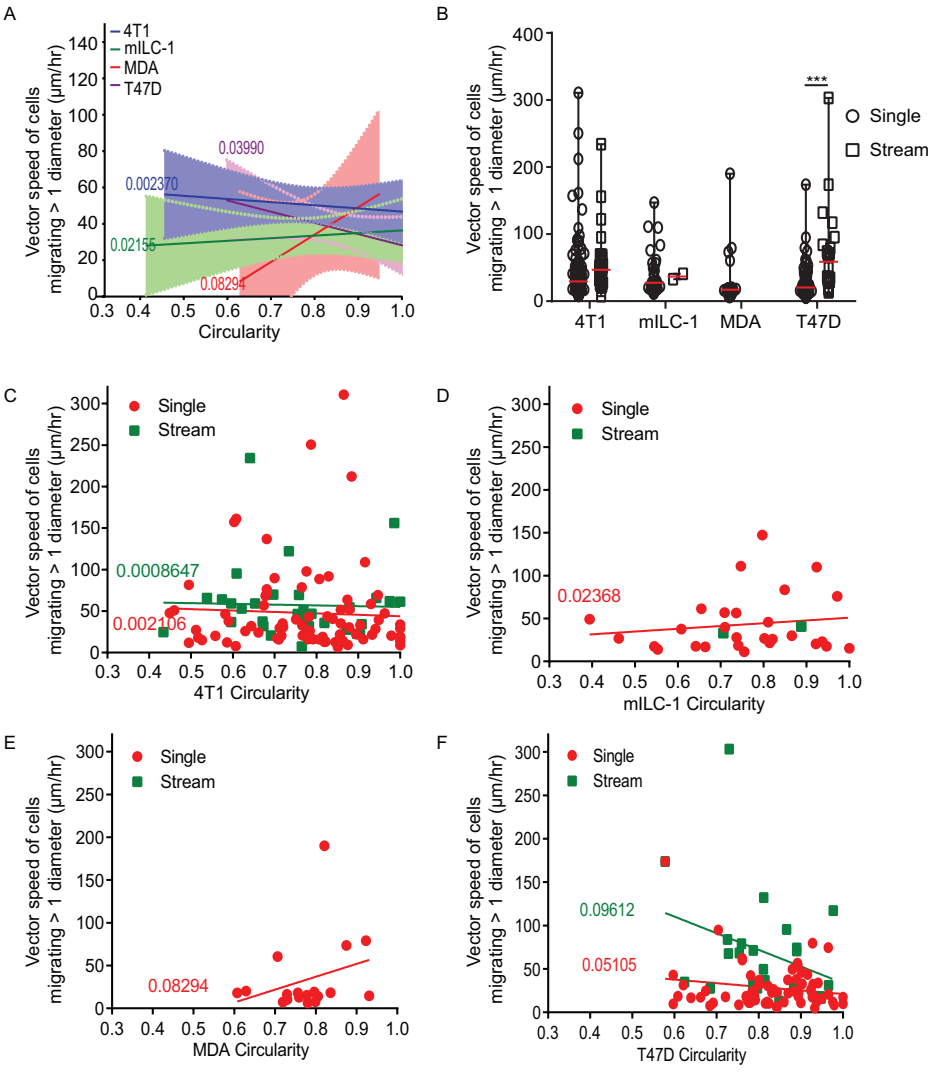
S2 File. Combined stacks of mILC-1 tumor cells. This movie shows two-photon time-lapse images of mILC-1 tumor cells in living mice. The time-lapse covers a period of 2hrs. **Left panel:** Original movie. **Right panel:** Example of tracked migrating cells. Scale bar = 50 μm.

S3 File. Combined stacks of MDA tumor cells. This movie shows two-photon time-lapse images of MDA tumor cells in living mice. The time-lapse covers a period of 2hrs. **Left panel:** Original movie. **Right panel:** Example of tracked migrating cells. Scale bar = 50 μm.

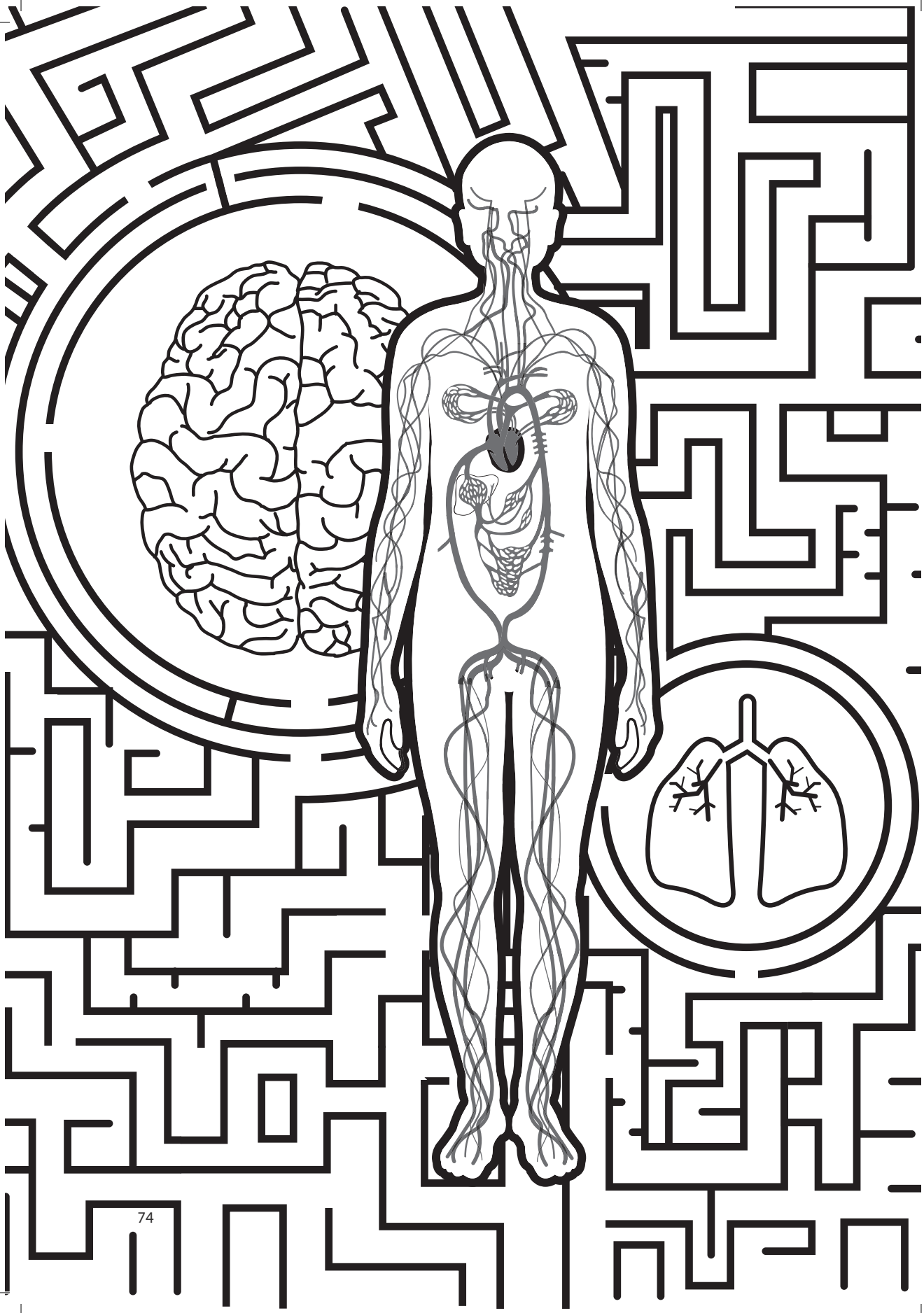
S4 File. Combined stacks of T47D tumor cells. This movie shows two-photon time-lapse images of T47D tumor cells in living mice. The time-lapse covers a period of 2hrs. **Left panel:** Original movie. **Right panel:** Example of tracked migrating cells. Scale bar = 50 μm.



S5 Fig. Correlation between velocity and circularity of cells migrating more than 1 cell diameter. (A) Correlation between velocity and circularity of different tumors. (B) Velocity was calculated for single migrating cells and cells migrating in single streams . (C, D, E, F) Correlation between velocity and circularity for single migrating cells and cells migrating in streams within one tumor type.



S6 Fig. Correlation between vector speed and circularity of cells migrating more than 1 cell diameter. (A) Correlation between vector speed and circularity of different tumors. (B) Vector speed was calculated for single migrating cells and cells migrating in single streams migrating. (C, D, E, F) Correlation between vector speed and circularity for single migrating cells and cells migrating in streams within one tumor type.



Chapter 4

Neutrophils promote glioblastoma tumor cell migration after biopsy

Na Chen, Maria Alieva, Tom van der Most, Joelle Klazen, Arabel Vollman, Peter Hau, and Nienke Vrisekoop

Submitted for publication

Abstract

Biopsies and tumor resection are currently essential procedures for the diagnosis and treatment of solid tumors. However, emerging evidence suggests that these surgical interventions may increase the risk of developing metastases. It has been hypothesized that the damage to the tumor leads to infiltration of immune cells that consequently form an environment that favors tumor cell spread. It was previously found that biopsy of a mouse glioma tumor induced migration of tumor cells *in vivo* and that recruitment of monocytes from the blood was involved in this effect. Here we study the contribution of neutrophils on the pro-migratory effect of biopsy. Using repetitive intravital microscopy, *in vivo* migration of glioma tumor cells before and after biopsy were compared in mice systemically depleted of neutrophils with Ly6G antibodies. Despite the presence of residual neutrophils, biopsy did not induce *in vivo* migration of glioma tumor cells in Ly6G antibody-injected mice. Interestingly, macrophages/microglia were almost completely depleted from the tumor, indicating neutrophils may be indirectly involved in biopsy-induced migration of glioma tumor cells through the recruitment of monocytes to the tumor. To further investigate whether neutrophils have the potential to independently promote glioblastoma tumor cell migration, we performed *in vitro* migration assays using human neutrophils. Indeed wound-healing of human primary glioblastoma tumor cell lines was promoted by human neutrophils. The pro-migratory effects of human neutrophils on glioblastoma tumor cells could also be recapitulated in transwell migration assays, indicating a soluble factor is involved. We therefore provide evidence for both an indirect and direct involvement of neutrophils in the tumor spread following biopsy of glioblastoma tumors.

Introduction

It is common practice to take biopsies of solid tumors in order to determine the correct diagnosis and treatment plan for the patient. In many cases, treatment includes the partial or complete removal of the tumor tissue. Although these surgical procedures are essential for patients and are overall prognostically beneficial, they have been associated with accelerated tumor growth and the formation of metastases [1, 2]. Experimental studies in mice similarly find that tumor biopsy or complete resection can lead to an increased number of metastases or to the promotion of proliferation and invasion of the primary tumor [3-5]. One of the hypotheses to explain the increased risk for developing metastases is that the damage to the tumor and surrounding tissue triggers an inflammatory reaction that creates a pro-metastatic environment [4-6]. Several studies have investigated how biopsy affects the immune microenvironment in different tumor types, finding increased numbers of ('alternatively activated') macrophages [5, 7, 8], regulatory T-cells [6], neutrophils [4, 5] and eosinophils [8] at the biopsy site or around the tumor. In interaction with tumors, macrophages have been thought to either adopt a pro-tumoral phenotype (alternatively activated (or M2) macrophages) or anti-tumoral phenotype (M1 macrophages), although the strict duality has recently been questioned [9]. The potential for alternatively activated macrophages to create an immunosuppressive environment promoting cancer disease progression, has been well described [9]. An increase in the number of (alternatively activated) macrophages is therefore thought to mediate the pro-metastatic response following biopsy [5, 7].

Neutrophils have received less attention in the past, as they were deemed too short-lived to affect a chronic disease such as cancer [10]. However, this view is changing in light of recent developments showing that there is profound interaction between tumor cells and neutrophils. Tumors produce factors such as G-CSF and GM-CSF to promote the release of neutrophils from the bone marrow and secrete pro-survival factors to increase their life-span [10, 11]. For several tumor types a high neutrophil-to-lymphocyte ratio (NLR) or high number of intra-tumoral neutrophils is associated with poor prognosis in patients and is used as a prognostic marker [12-17]. It has been postulated that similar to macrophages, neutrophils may be induced to have a pro-tumoral (N2 neutrophils) or anti-tumoral (N1 neutrophils) phenotype, depending on factors released by the tumor [18]. Furthermore, in inflammatory conditions including cancer, myeloid cells are found with immunosuppressive capabilities, called myeloid-derived suppressor cells (MDSC). The granulocytic subset (G-MDSC) can be considered to be neutrophils with immunosuppressive

capabilities [10, 19]. In a mouse model for breast cancer, it was hypothesised that infiltration of MDSCs in the tumor following biopsy created an immunosuppressive environment favouring metastatic spreading of the tumor to the lung [4].

Recently we investigated the effect of needle-biopsy on progression of glioblastoma tumors [5]. Glioblastoma or grade IV glioma is the most prevalent and aggressive brain tumor in adults [20]. Median survival of patients is around one year and both tumor growth and local dissemination have been found to be associated with decreased survival [21, 22]. Alieva *et al.* showed retrospectively in patients with multifocal glioblastoma (presenting with multiple tumor foci), that biopsied tumor sites increased in volume compared to non-biopsied foci [5]. This finding was recapitulated in a mouse model for glioblastoma in which it was shown that biopsy-like injury promotes local tumor cell invasion [5]. Effects of the biopsy could also be observed within the same mouse, comparing *in vivo* migration of glioma cells with intravital microscopy before and after biopsy. Biopsy of the mouse glioma tumor was found to increase the percentage of migratory tumor cells compared to before biopsy. Both macrophages and neutrophils were recruited to the tumor upon injury. Depleting monocytes using clodronate liposomes prevented the increased *in vivo* migration of tumor cell upon biopsy indicating the involvement of these cells. In addition to preventing macrophage-recruitment via blood monocyte depletion with clodronate liposomes, biopsy-induced recruitment of monocytes to the tumor could be prevented by CCL-2 blockade. CCL-2 is a chemokine secreted by tumor and stromal cells and mediates recruitment of immune cells expressing the receptor CXCR2, such as monocytes [23]. Interestingly, mice injected with this antibody showed an overall reduction in the percentage of migratory glioblastoma cells after biopsy, lower than that observed for control and clodronate liposome-treated mice. The increased effectiveness of CCL-2 antibodies over clodronate liposome treatment points toward a role for immune cells besides monocytes being recruited to the tumor via CCL-2, that consequently affect glioma tumor cell migration. Anti-CCL2 in addition to blocking recruitment of macrophages to the tumor, also prevented the recruitment of neutrophils, which express CCR2 on their surface [5, 24]. Given that neutrophils are recruited to the glioma tumor following biopsy, and to explain the increased effectiveness of CCL-2 antibodies, we attempted to further understand the inflammatory reaction following biopsy and study the role of neutrophils.

To study the *in vivo* involvement of neutrophils, we used the same mouse glioblastoma biopsy-injury model as previously [5] and injected mice with Ly6G antibodies to systemically deplete the mice of neutrophils. In direct comparison to the study by Alieva *et al.*, we observe that depletion of neutrophils prevented

the increase of *in vivo* glioma migration by biopsy. Interestingly, Ly6G antibody treatment led to a local depletion of macrophages from the tumor area, implicating a role for neutrophils in recruitment of macrophages to the tumor. To determine whether neutrophils also independently promoted wound-healing of glioma cells, *in vitro* co-culture experiments with human glioblastoma tumor cell lines and neutrophils were performed. Indeed neutrophils promoted the *in vitro* motility of human glioblastoma tumor cells. Overall, we provide evidence for a direct and indirect role of neutrophils in the promotion of glioblastoma tumor cell migration following biopsy.

Materials & Methods

Mice

C57BL/6 mice between 8-12 weeks old were used for the experiment. Mice were housed at the animal facility in the Hubrecht Institute and received food and water ad libitum. Implantation of tumor cells, cranial imaging window (CIW) placement and biopsy-like injury have been described extensively elsewhere [2, 5]. In short, on day 0 mice were sedated, the head was shaved and an incision was made on the top part of the head. A circular hole was drilled over the right parietal bone and 1 x 10^5 GL261 H2b-dendra2 glioma cells, suspended in 3µl PBS were injected at a depth of 0.5 mm. A chronic CIW was placed for intravital imaging on later days. Mice received intraperitoneal injection of 100 µg Ly6G antibodies (1A8; BioXcell) on days 8 and 11. On day 12, the CIW was replaced and mice received biopsy-like injury. The injury consisted of 4 needle punctures with a 25G needle in the tumor at a depth of 1 mm. On day 13, the mice were sacrificed. Blood was obtained via heart puncture and the tumor was harvested. These procedures were perfomed by the same researcher in the same time period as control data without Ly6G antibody depletion previously reported by Alieva et al [5].

Mouse intravital Imaging & *in vivo* cell tracking

Mice were intravitally imaged through the CIW on days 11 and 13 with a Leica TCS SP5 AOBS two-photon microscope as described before [5]. Mice were imaged for a duration of 2 hours and an image was taken every 20 min. A z-stack was made of different positions of the tumor at a maximal depth of 300 µm deep, depending on the position of the tumor, with a 3 µm interval between the z-slices. Z-drifts are corrected for using Jittering Corrector software. Three z-slices are merged to create a maximum projection of 6µm thickness. Maximum projections are corrected for deformations (e.g. caused by breathing of the animal) using the Match software.

For analysis, 3 random positions are chosen and every other maximum projection is analysed for the complete time period using the 'MtrackJ' plug-in for ImageJ. Per maximum projection, 2-50 cells were tracked and the displacement was determined adding up to a total number of 1081-3562 tracks per mouse. Vector speed is the displacement/hour. Using the cut-off value of 4μm/h vector speed to define migratory cells, the percentage of migratory cells before and after biopsy is determined. The fold change is the %migratory cells after/before biopsy. Data of the fold change in migratory cells from biopsy mice without neutrophil depletion were reused from Alieva et al [5].

Immunostaining of mouse brain slices

Tumor cryosections of 14μm thickness were stained overnight with biotin-conjugated F4/80 (mf48015; Invitrogen). Secondary staining was performed with fluorophore-conjugated streptavidin (S-21374; Life Technologies). A Leica SPE confocal microscope was used to make tile scans and the LaSAF software was used to quantify the cells. The biopsy area could be identified based on the presence of fluorescent beads injected during biopsy-like injury. The number of $F4/80^+$ cells was determined in both the biopsy and non-biopsied area and normalized for the size of the area (mm^2). The macrophage numbers from biopsy mice without neutrophil depletion were reused from Alieva et al [5].

Flow cytometry of mouse blood cells

Blood was diluted with 1ml red blood cell lysis buffer (NH_4Cl) per 100μl blood and kept on ice for 10 min. Cells were spun down (5 min at 4°C 500 RCF), resuspended in 1 ml NH_4Cl and kept on ice for another 3 min. Afterwards, 3 ml of FACS buffer (PBS + 5% FCS + 5mM EDTA) was added and cells were spun down again (4 min at 4°C 500 RCF). The pellet was resuspended in 500 μl FACS buffer for flow cytometric analysis on a FACS Calibur. The neutrophil percentages in blood from biopsy mice without neutrophil depletion were reused from Alieva et al [5].

Tumor cell lines

MDA-MB231 (MDA) and MCF7 red-fluorescent human breast tumor cell-lines were generated by transfection with a pLV CMV vector with the cDNA construct loxP-DsRed-loxP-eGFP and were a kind gift of Jacco van Rheenen. MDA was cultured in Advanced Dulbecco's Modified Dulbecco's Eagle (Gibco; DMEM)/F12 supplemented with 10% FCS. MCF7 was cultured in DMEM(1x) + Glutamax (Gibco) supplemented with 10% FCS. Fluorescent cells were selected with 5 μg/ml puromycin treatment. Green fluorescent human glioblastoma BTIC cell lines (RAV21/27) were lentivirally transduced with a U57 pHR SFFV GFP plasmid and were kindly provided by Arabel

Vollman & Peter Hau. RAV cell-lines were cultured in RHB-A stem-cell permissive medium (Stem Cell Sciences) supplemented with 20 ng/ml human recombinant EGF/basic FGF (Peprotech). All cell-lines were cultured at 37°C with 5% CO_2. All cell-lines grew in adhesive monolayers and were disassociated from culture plates using Tryple Express (Gibco) (for MDA/MCF7) or 1:1 Accutase/PBS (for RAVs) for passaging.

Human blood neutrophil isolation

Blood was obtained by vena puncture from healthy volunteers from the University Medical Centre (UMC) Utrecht in agreement with the medical ethical committee of the UMC. Blood with sodium heparin anticoagulant was diluted 1:1 with PBS^{++} containing 40g/L Albumin and 3.2% Sodium Citrate and separated by Paque-Ficoll (GE Healthcare) density centrifugation for 20 min at RT 2000 rpm. After removal of the plasma, PBMCs and most of the Ficoll, remaining cells were suspended in red blood cell lysis buffer for 10-15 min on ice. Cells were spun down (1500 rpm 5 min 4°C), washed with red blood cell lysis buffer and subsequently with PBS^{++}. Cells were kept in culture medium on ice until further use.

Wound-healing Assay

For the wound-healing assay, silicone culture inserts (IBIDI) were used in which tumor cells are grown in two separate compartments, leaving a cell-free gap in between the tumor cells after removal of the insert. Tumor cells were seeded in both compartments of the culture insert on a culture-treated 24-well plate in their respective medium DMEM 10% FCS (for MDA/MCF7) or RHB-A (RAV21/RAV27) until confluency was reached. Subsequently, culture medium was aspirated and the culture insert removed. Tumor cells were resuspended with culture medium including $6x10^4$ human neutrophils or culture medium only. The Incucyte live cell imaging system was used to make hourly images and allowed the tumor cells to be left unperturbed in the incubator throughout the entire period. In order to quantify migration into the cell-free area, cells are masked on ImageJ, transforming the images into binary images in which cell-covered pixels are given the value 255 and background 0. By defining the cell-free area, migration of cells into the gap is represented by increases in the average pixel intensity in the cell-free area. Cells were judged confluent at the end of imaging and percentage confluency over time was determined by comparing the average pixel intensity in the present time point to that at the end of imaging.

Transwell migration assays

In the transwell migration assay, the tumor cells were seeded in a transwell with a pore size of 8µm (Corning) placed on top of wells of normal tissue culture plates. In

the transwell, $1x10^5$ tumor cells were seeded in 300 µl of their respective medium *(see 'Wound-healing Assay')* and were allowed to migrate through 8µm pores on the bottom of the transwell. On the bottom, 700 µl culture medium including $5x10^5$ human neutrophils or culture medium only was pipetted ensuring that the culture medium extended above the bottom of the transwell. Using this set-up, the only contact between the neutrophils and the tumor cells is via the culture medium, preventing contact-dependent pro-migratory effects of neutrophils. After 24 hours, the transwells were carefully removed and images were taken of the bottom of the plates using the Incucyte. The number of fluorescent tumor cells was determined by applying a mask on fluorescence and automatically counting the particles on ImageJ. To determine the average nr. of transmigrated cells, images of 5 positions within the same well were analysed.

Stastistics

Results are shown as median with range. When > 2 medians were compared, Kruskal-Wallis test were used, and when 2 medians were compared, the Mann-Whitney U-test was used. For paired comparison, two-tailed Wilcoxon matched-pairs signed rank test was used, * $p < 0.05$, ** $p < 0.01$.

Results

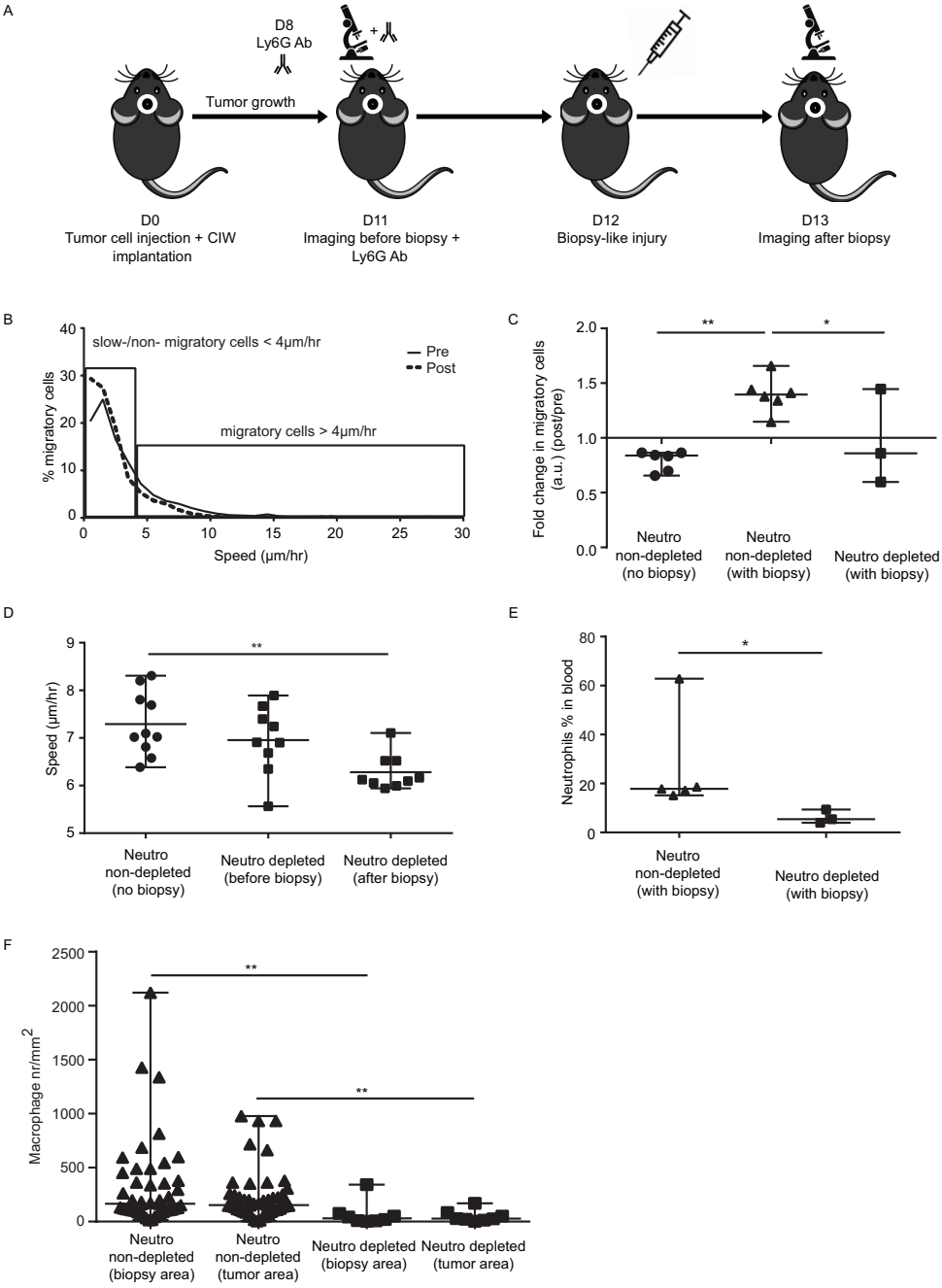
Biopsy induces a neutrophil-dependent increase in the *in vivo* motility of mouse glioma tumor cells

First, the role of neutrophils on glioblastoma tumor cell migration was evaluated in the same *in vivo* mouse biopsy-injury model in which we have previously shown blood-derived macrophages play an essential role [5]. Mirroring the approach, brain surgery was performed on C57BL/6 mice to inject GL261 mouse glioma cells expressing a nuclear fluorescent protein (H2B-Dendra2) in the brain. Consecutively, a chronic cranial imaging window was implanted on top of the injection site allowing to track the movement of glioma tumor cells *in vivo* at later time points [2, 5]. Within one week the mice develop an invasive brain tumor, in line with the aggressive nature of glioblastoma tumors in humans [5, 20]. In order to study the role of neutrophils, they were depleted by two injections with Ly6G-antibodies. The aim was to see whether in these neutrophil-depleted mice, biopsy would still lead to an increase in the percentage of migratory tumor cells. The biopsy-intervention consisted of four needle punctures in the brain and the effect of biopsy on *in vivo* tumor cell migration was determined by repeated intravital microscopy, the day before and after biopsy-like injury (Fig. 1A). On both days, mice were intravitally

imaged with a two-photon microscope for two hours through the cranial imaging window and an image was made every 20 minutes. With this approach, individual GL261 glioma cell movement could accurately be tracked in a horizontal plane at multiple depths within the tumor mass and the migration speed of 1081-3562 tracks per mouse were calculated. The same cut-off speed of 4 μm/h was previously used to define migratory cells and to determine the percentage of migratory tumor cells both before and after biopsy for three Ly6G antibody-treated mice (Fig. 1B). It was observed that although the number of migratory cells was increased after biopsy in Alieva *et al.*, this significantly decreased upon Ly6G antibody treatment (Fig. 1C). These results imply that neutrophils are involved in the biopsy-induced migration of mouse glioma cells *in vivo*. Not only did the number of migratory cells decrease after biopsy in Ly6G depleted mice, the speed of migratory cells was even significantly decreased compared to non-depleted non-biopsied mice (Fig. 1D).

Directly after intravital imaging after biopsy, the mice were sacrificed and blood was measured by flow cytometry to evaluate depletion efficacy. Since neutrophils might be coated with Ly6G depletion mAbs, we did not use this antibody to detect neutrophils. To avoid overestimating the depletion effect, residual neutrophils were identified based on forward scatter (FSC) and side scatter (SSC), and the percentage of residual neutrophils in the blood for the three mice was 3.95%, 5.42% and 9.38% (of all FSC/SSC gated events; supplementary Fig. 1). The percentage of neutrophils in non-depleted tumor bearing mice was typically about 17% of total cells (Fig. 1E). Thus neutrophil numbers in the blood were reduced but not completely depleted following Ly6G antibody treatment as has been desribed before [25, 26].

There is evidence that neutrophil depletion leads to a decrease in macrophages number in wounds [27]. In order to see what the effect of Ly6G-depletion was on the number of macrophages in the tumor, brain cryosections were stained with F4/80 to quantify the number of macrophages in biopsied and non-biopsied tumor tissue for each mouse. The biopsied area was identified by the presence of fluorescent beads in the tumor that were injected during biopsy-like injury. When comparing the numbers of macrophages in the brain of the Ly6G antibody-treated mice to the untreated biopsied mice from Alieva *et al.*, macrophages were significantly reduced in both biopsied and non-biopsied tumor tissue (Fig. 1F). Given the absence of Ly6G expression on both macrophages and monocytes, the reduction of macrophages in the tumor is unlikely to be a direct result of treatment with Ly6G antibodies. Indeed Jean M. Daley et. al provide evidence that Ly6G antibody treatment did not affect monocyte counts while blood neutrophils were depleted [27]. Instead, our data indicate that neutrophils are involved in the recruitment of macrophages


A
D8
Ly6G Ab
Tumor growth
D0
Tumor cell injection + CIW implantation
D11
Imaging before biopsy + Ly6G Ab
D12
Biopsy-like injury
D13
Imaging after biopsy
B
slow-/non- migratory cells < 4µm/hr
migratory cells > 4µm/hr
Pre
Post
% migratory cells
Speed (µm/hr)
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Fold change in migratory cells (a.u.) (post/pre)
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Speed (µm/hr)
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Neutro depleted (before biopsy)
Neutro depleted (after biopsy)
E
Neutrophils % in blood
Neutro non-depleted (with biopsy)
Neutro depleted (with biopsy)
F
Macrophage nr/mm2
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Neutro non-depleted (tumor area)
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< Figure 1. Biopsy induces a neutrophil-dependent increase in the in vivo motility of mouse glioma tumor cells. (**A**) Cartoon showing set-up of experiment to study the effect of biopsy on *in vivo* glioma tumor cell migration in mice depleted of neutrophils with Ly6G antibodies. (**B**) Graph showing an example of results from intravital tracking of glioma tumor cells before (solid line) and after (dotted line) biopsy and the distinction between migratory (vector speed > 4μm/h) and slow-/non-migratory (vector speed < 4μm/h) tumor cells. (**C**) Direct comparison of the fold changes in percentage of migratory glioma tumor cells before/after biopsy in 3 neutrophil-depleted biopsied mice to that of neutrophil non-depleted mice with or without biopsy from Alieva *et al*. * $p < 0.05$, ** $p < 0.01$ in one way ANOVA. Bars represent median values with range. (**D**) Speed of migratory cells of neutrophil depleted mice (before and after biopsy) compared to that of neutrophil non-depleted mice without biopsy from Alieva et al. (E) Neutrophil percentage in the blood of neutrophil depleted mice compare to that of neutrophil non-depleted mice from Alieva et al. (F) Enumeration of the numbers of ($F4/80^+$) macrophages/microglia by immunostaining of tumor tissue cryosections in neutrophil depleted mice and biopsied mice without depletion from Alieva *et al.* The beads area represents the biopsy site and the tumor area random non-biopsied tumor tissue.

to the tumor. Thus our data show neutrophils are involved in promoting *in vivo* glioma tumor cell migration after biopsy, and this may in part be achieved through recruitment of blood monocytes to the tumor.

Neutrophils promote in vitro wound-closure of human glioblastoma tumor cells but not human breast tumor cells

As *in vivo* neutrophil depletion results in the local depletion of macrophages from the tumor, our mouse model was unsuited for investigating independent effects of neutrophils. In order to study the independent effect of neutrophils on tumor cell motility and to extend these findings to human tumor cells and additional tumor types other than glioblastoma, the effect of human neutrophils on the *in vitro* motility of human tumor cell lines was determined. Human glioblastoma tumor cell lines were derived from high-grade glioma patients and enriched for cells with stem-cell like features, termed brain tumor initiating cells (BTICs; RAV21/RAV27) [28]. These cell-lines have been described extensively and used to investigate human glioblastoma tumor cell behaviour *in vitro* [28-30]. To obtain more information on the specificity of potential pro-migratory effects of neutrophils, two well-described human breast tumor cell lines (MDA, MCF7) were additionally studied. Wound-healing assays were performed using culture inserts with a cell-free zone, and migration into the wound area was evaluated using the Incucyte Live Cell Imaging system. In the presence of neutrophils, wound closure time was significantly reduced for both RAV21 and RAV27 glioblastoma tumor cell lines, but not for MCF7 and MDA breast tumor cell lines (Fig. 2A, B and C). Thus, the wound-healing assay shows neutrophils promoted the in vitro migration of glioblastoma cells, but this effect could not be extrapolated to all tumor types.

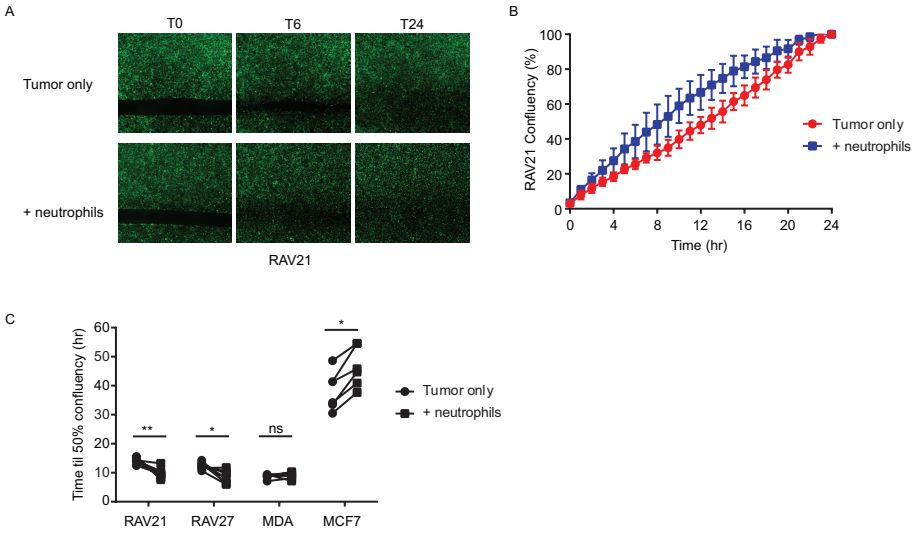

Figure 2. Neutrophils promote in vitro wound-closure of human glioblastoma tumor cells but not human breast tumor cells. (**A**) Representative images of RAV27 at 0, 6 and 24 hours after start of incubation in the wound-healing assay with or without neutrophils. Comparison of wound closure at 6 hours shows increased migration of glioblastoma cells in the presence of neutrophils. (**B**) Representative graph of image analysis of RAV27 showing the median confluency of the cell-free gap with range over time in the presence and absence of neutrophils for all donors. Confluency at 24 hours was defined as 100%. (**C**) The analysis method depicted in A and B was used to determine the time in which 50% confluency was reached in the wound-healing assay for the different glioblastoma (RAV21/RAV27) and breast (MDA/MCF7) tumor cell lines. Dots represent different neutrophil donors. * $p < 0.05$, ** $p < 0.01$ in two-tailed Wilcoxon matched-pairs signed rank test.

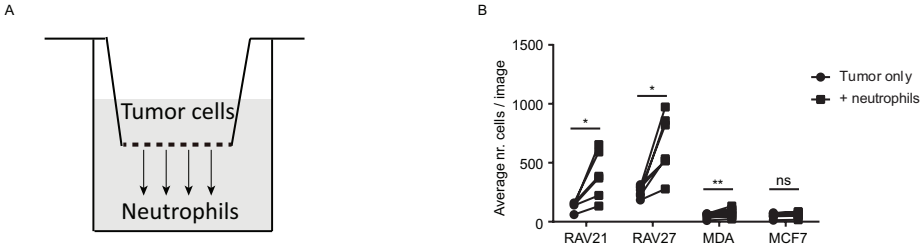

Figure 3. A soluble factor from neutrophils increases transmigration of human glioblastoma and MDA breast tumor cells. (**A**) Cartoon showing the set-up of the transwell-migration assay. Tumor cells are seeded on top of the transwell with neutrophils on the bottom of the well. (**B**) Graph comparing average nr. cells / image (of 5 images) for glioblastoma (RAV21/RAV27) and breast (MDA/MCF7) tumor cell lines transmigrated in the presence or absence of neutrophils seeded on the bottom of the well. Each dot represents a different neutrophil donor. * $p < 0.05$, ** $p < 0.01$ in two-tailed Wilcoxon matched-pairs signed rank test.

A soluble factor from neutrophils increases transmigration of human glioblastoma and MDA breast tumor cells

To establish whether the increase in tumor cell migration by neutrophil was cell-contact dependent, we performed transwell migration assays with the human glioblastoma and breast tumor cells. Culture medium with or without neutrophils was added to the bottom of the well to see whether transmigration of tumor cells was promoted by neutrophils (Fig. 3A). Tumor cells were allowed to migrate for 24 hours after which the transwell was removed and an image was made of the bottom of the culture well to quantify the number of transmigrated tumor cells. In accordance with the wound-healing assay, the presence of neutrophils on the bottom of the culture well significantly increased transmigration of RAV21 and RAV27 glioblastoma tumor cells (Fig. 3B). Interestingly, although again no effect was observed for MCF7, transmigration of MDA breast tumor cells was promoted by the presence of neutrophils, despite the absence of a pro-migratory effect for neutrophils on MDA in the wound-healing assay. The transwell-migration assay confirms a role for neutrophils in promoting *in vitro* migration of human glioblastoma tumor cells. Furthermore, as there was no direct contact between neutrophils and tumor cells in this assay, a soluble factor is involved in the promotion of tumor cell migration by neutrophils. This, however, does not exclude that neutrophils can additionally stimulate tumor cell migration through direct contact.

Discussion

Taking a biopsy is an essential procedure for the diagnosis of patients with solid tumors, but may come with an increased risk for developing metastases [2-5]. Understanding the underlying mechanism behind this side-effect would allow us to employ supplementary therapeutic strategies during biopsy for its prevention. Experimental studies in mice point towards involvement of immune cells that are attracted to the tumor due to the damage caused by the surgical procedure [4-7]. For glioblastoma tumor specifically, the prevention of macrophage recruitment from blood monocytes was found to prevent the induction of tumor cell migration by biopsy [5]. In this study we investigated the role of neutrophils in affecting migration of glioblastoma tumor cells.

In direct comparison to the study by Alieva *et al.*, we found that systemic neutrophil depletion by injection with Ly6G antibodies prevented biopsy-induced tumor cell migration. We observe a decrease in the percentage of neutrophils in the blood compared to non-depleted controls, but not a complete absence, at day 5 after

depletion. Although this could represent problems with the availability of antibody (e.g. dosing, frequency of injections), previous studies point towards biological effects preventing complete depletion of neutrophils in mice [25, 26, 31]. Moses et al., found that although blood could be effectively depleted of neutrophils by both Ly6G and Gr-1 antibodies, over time under continual antibody injections, $Ly6G^+$ cells always completely 'rebound' to normal levels and could be identified in the circulation of naïve mice starting around day 8 [25]. Furthermore, in tumor-bearing mice the reappearance of neutrophils in the blood was accelerated, and $Ly6G^+$ cells could already be detected from day 3 onwards. Insufficient antibody presence could not explain the 'rebound' of neutrophils as increased dose or application frequency did not affect this result [25]. The biological effect explaining the impact of the tumor on reappearance of neutrophils was not further investigated and may differ between tumor types.

Despite the limited effect of neutrophil depletion, we do report clear reduction in the number of macrophages/microglia in the glioblastoma as an effect of Ly6G-antibody injection. Neutrophils have been shown to contribute to monocyte recruitment to sites of inflammation [32]. Indeed, impaired monocyte recruitment has been seen in several acute and chronic models of inflammation where neutrophils were depleted with Ly6G or Gr-1 antibodies [32]. Several mechanisms are proposed to mediate this effect, including neutrophil release of CCR2 ligands (CCL-2, MIP-1α), leading to the recruitment of inflammatory monocytes expressing CCR2 [32]. Alieva *et al.* reported that the CCL-2/CCR2 axis was involved in the biopsy-induced recruitment of inflammatory monocytes to the glioblastoma tumor as CCL2 antibodies could be used to block their recruitment [5]. It could be speculated that the same mechanism mediates neutrophil-dependent monocyte recruitment to the tumor.

Finally, we show that neutrophils promote the *in vitro* migration of human glioblastoma tumor cell lines, indicating an additional direct role for neutrophils in affecting glioblastoma tumor-cell migration following biopsy. The cell-lines are enriched for brain tumor initiating cells (BTICs) as recurring malignant gliomas are believed to be derived from a population of cells with stem-cell like characteristics [33]. These cell-lines have been extensively tested and used as a model for human glioblastoma *in vitro* [28-30]. Employing two well-established migration assays, the transwell migration assay and wound-healing assay, we observe increased transmigration and faster wound-closure respectively of both RAV21 and RAV27 glioblastoma tumor cell lines in the presence of neutrophils [34]. Furthermore, these results provide some explanation to clinical observations in glioblastoma patients,

where high numbers of blood and tumor-infiltrating neutrophils are associated with glioma grade, poor prognosis and resistance to therapy [17, 35, 36].

In summary, we provide evidence for an indirect and direct involvement of neutrophils in the tumor spread following biopsy of glioblastoma tumors. Discovering a role for neutrophils may provide rationale for the development of therapeutic strategies targeting neutrophils in order to reduce the risk of developing metastases following biopsy or tumor resection.

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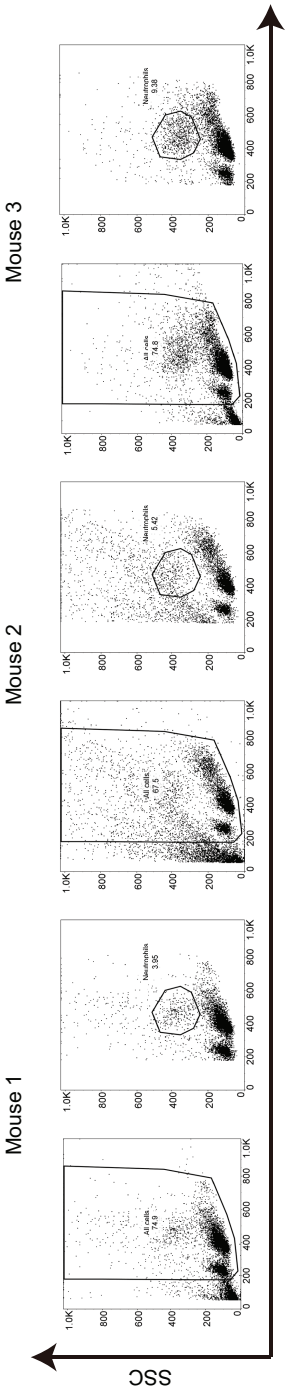

Supplementary figure
FACS plots of neutrophils in blood from neutrophil depleted mice.

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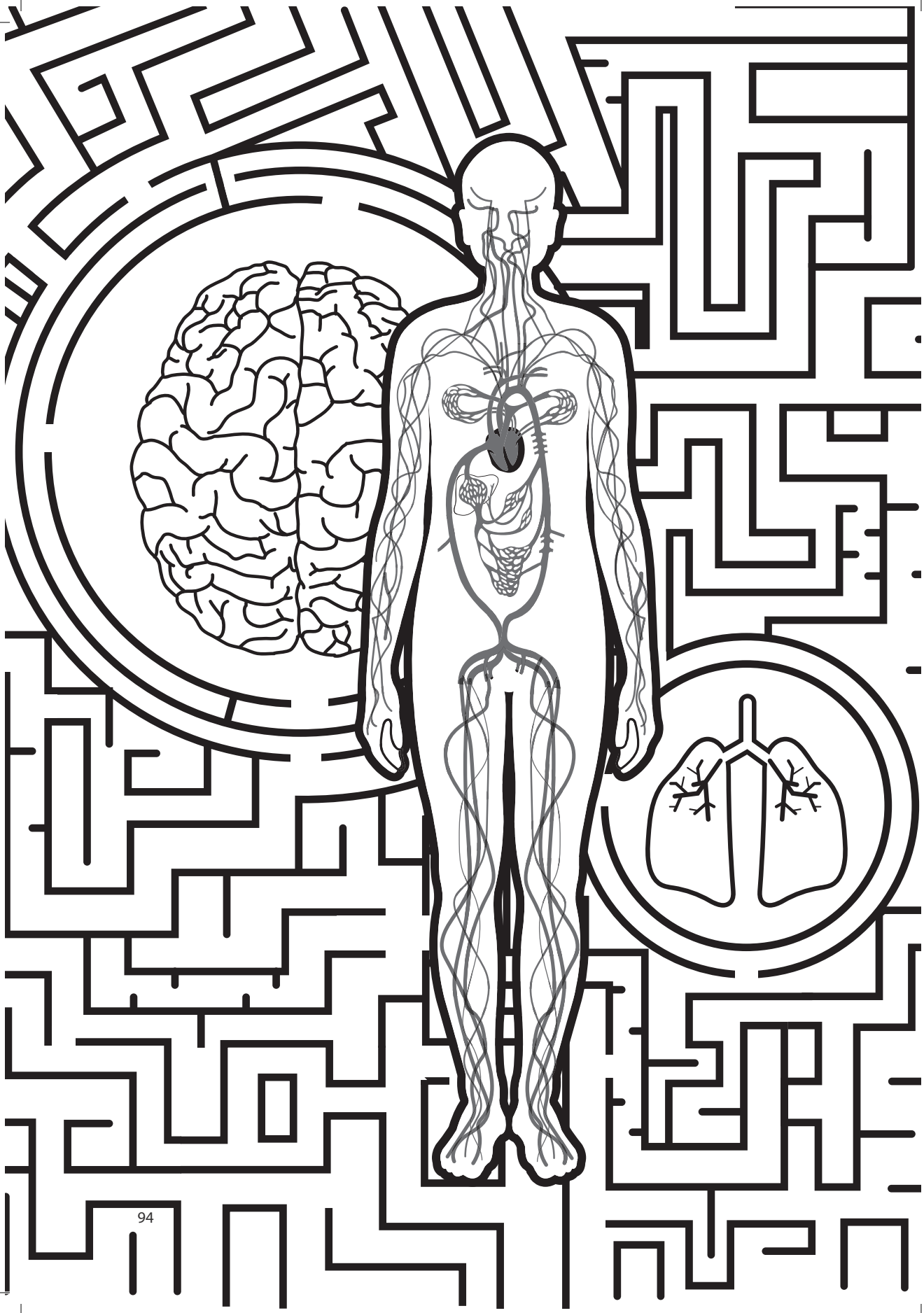

Chapter 5

Kinetics of neutrophil heterogeneity in acute and chronic inflammation

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Abstract

Increasing attention is being paid to neutrophil heterogeneity in activation, phagocytosis, bacterial killing capacity and immune modulation. Different cell surface markers and isolation methods are being used to capture neutrophil heterogeneity, which hampers direct comparisons between studies and between different inflammatory diseases. This study is a first attempt to compare neutrophil subtypes found in different inflammatory settings. The well-characterized acute inflammatory LPS model served as a point of reference. In the LPS model neutrophil subsets have been identified capable of superior bacterial killing (banded $CD16^{low}$) and immune suppression (hypersegmented $CD62L^{low}$). In this study we compared the neutrophils subsets found after LPS to a more physiological acute inflammatory state at the first day of trauma. Trauma patients were further followed in time to obtain cells under a more chronic situation and these were compared to cells from patients with chronic disease: COPD and cancer patients.

Additional neutrophil subsets appeared not only in acute inflammation, but also in the peripheral blood in patients with polytrauma, COPD and lung cancer. More specifically, $CD16^{low}$ neutrophils only appeared in the circulation during acute inflammation after LPS challenge and at the day of hospital admission after trauma. Unlike the situation found after LPS administration, $CD62L^{low}$ neutrophils were hardly detected acutely after trauma, but increased from day 3 onward following trauma. This subset was also detected in patients characterized by chronic inflammatory disease.

We hypothesize $CD62L^{low}$ cells are highly activated neutrophils involved in immune regulation. A better understanding of neutrophil subsets in different inflammatory conditions will facilitate future treatment strategies directed to inhibit or stimulate only certain neutrophil subsets.

Introduction

Neutrophils have long been recognized as essential cells of the innate immune system that eliminate invading pathogens and prevent their systemic spread [1-3]. Besides the traditional role of neutrophils as cells that phagocytose and kill pathogens, recently multiple subsets of neutrophils have been described that are able to perform additional functionalities. Several studies have reported immunosuppressive neutrophils in tumor bearing mice and humans termed granulocyte-myeloid derived suppressor cells (G-MDSC) [4-10]. In different studies the suppressive capacity of neutrophils was demonstrated in the complete pool of neutrophils of tumor bearing hosts [11], in neutrophils with decreased density [12, 13] and in neutrophils with normal density [14, 15]. To add to the confusion, the reported composition of the low density granulocytes (LDG) is contradictory, with studies reporting either immature [16, 17] or activated mature neutrophils [9, 18]. Unfortunately different studies use different surface markers and different types of suppression assays, complicating benchmarking between the data obtained in different laboratories [18, 19].

Other conditions where neutrophils with different functionalities have been described are after G-CSF treatment [20] and in acute inflammation such as induced by systemic LPS challenge [21]. In the latter study, CD16 and CD62L expression was used to distinguish immature banded neutrophils ($CD16^{low}CD62L^{high}$) from normal mature neutrophils ($CD16^{high}CD62L^{high}$) and neutrophils displaying increased nuclear lobulation termed hypersegmented neutrophils ($CD16^{high}CD62L^{low}$). In our hands, the hypersegmented neutrophils suppressed T cell proliferation after LPS administration [21], while the immature banded neutrophils exhibited superior bacterial containment [22].

The new functionalities of neutrophils are of wide interest to the scientific community as these might be involved in pathological inflammatory conditions and, in addition, could be great targets for new treatment options. For instance, chronic obstructive pulmonary disease (COPD) has features of dysregulated neutrophil recruitment, activation, and survival that result in an increased release of toxic proteases and reactive oxygen species perpetuating airway inflammation and tissue injury [23-25]. A better understanding of neutrophil subsets and their behavior in COPD patients might provide potentially important insights into which subset(s) contribute to the progression of COPD. Moreover, following neutrophil subsets in trauma patients might predict which patients have an increased risk to develop sepsis [26, 27]. Since neutrophils have been described to have both a pro-

and anti-tumoral role in cancer patients it is additionally of great importance to better understand the different neutrophil subsets in this disease.

This study is a first attempt to initiate bench marking between neutrophil subtypes found in different inflammatory settings and further aims to describe the kinetics of neutrophil subsets from the acute to the chronic phase in inflammatory disease. We compare CD16 and CD62L expression on neutrophils after LPS administration as a model for acute inflammation with a more physiological acute inflammatory state that is found during the first day of traumatic injury. The neutrophil subsets of these trauma patients were followed in time and compared to neutrophils subsets found in two chronic inflammatory diseases: COPD and cancer.

Material & Methods

Experimental endotoxemia model

Blood and bone marrow samples were obtained from 10 healthy male volunteers between the age of 18–30 years participating in a human endotoxemia study (ABR NL61136.091.17). Study approval was obtained by the ethics review board of the Radboud University Medical Center in Nijmegen, the Netherlands, and written informed consent was obtained from all study participants. Subjects were healthy as determined by medical history, physical examination, electrocardiography and hematological laboratory values. Subjects taking prescription drugs were excluded from the study.

The bone marrow sampling was performed by aspiration from the posterior iliac crest under local anesthesia. A total volume of 40 ml was collected per aspiration into syringes prefilled with sodium heparin. Blood samples were drawn from an arterial catheter (radial artery), using sodium heparin as an anticoagulant. The first blood and bone marrow sample was obtained prior to LPS administration and the second blood sample 4 h after the LPS challenge. The LPS challenge was performed as published previously (Kiers et al., 2017). In short, the subjects were infused with 1.5L hydration fluid during one hour (2.5% glucose/ 0.45% saline at a continuous rate). Subsequently, the subjects received a single dose of 2 ng/kg bodyweight LPS (US standard reference Escherichia coli O:113, NIH Pharmaceutical Development Section, Bethesda, MD, USA) and were then infused with hydration fluid at a constant rate of 150 mL/h. During the challenge heart rate, blood pressure and the course of LPS-induced symptoms such as fever, muscle aches and nausea were constantly monitored.

Trauma patients

Trauma patients enrolled in this study, were part of a clinical trial performed at the University Medical Center Utrecht (UMCU, Utrecht, the Netherlands). The study was approved by the local ethics committee (ClinicalTrials.gov number NCT03489577). Written informed consent was obtained from all patients or their legal representatives in accordance with the Helsinki Declaration. Patients suffering polytrauma with an Injury Severity Score (ISS) of at least 21 and admitted to the Department of Traumatology of the University Medical Center Utrecht were included in this study. Patients analyzed had a clinical course without organ failure or sepsis. Exclusion criteria were: <18 or > 80 years old, ISS < 18, an altered immunological status and pregnancy. When patients met the inclusion criteria, the first blood sample was obtained within 12 hours after the admission. Subsequent blood samples were obtained at day 3, 6, 10 and 14-15 after trauma.

COPD Patients

Data from COPD patients was collected as part of a cross-sectional study performed by the University Medical Center Utrecht (UMCU, Utrecht, the Netherlands) and University Medical Center Groningen (UMCG, Groningen, the Netherlands). Trial register numbers are NCT00850863 and NCT00807469 (www.clinicaltrials.gov). The study design has been described elsewhere [28]. A total of 50 patients were included. Patients had a smoking history of more than ten pack years and a ratio of FEV1 to forced vital capacity of maximum 70% after bronchodilator use. GOLD stages were based on the criteria of the global initiative for chronic obstructive lung disease from 2013 (https://goldcopd.org). Patients with a history of other inflammatory diseases including asthma and active infections were excluded as were patients treated with antibiotics and/or corticosteroids up to 8 weeks before inclusion.

Cancer patients

Blood samples were obtained from 2 stadium IIIb non-small cell lung cancer (NSCLC) patients. The last chemotherapy was at least 58 days before sampling. The studies were approved by the ethics review board of the University Medical Center Utrecht, the Netherlands, and written informed consent was obtained from both study participants.

White blood cell isolation from blood and bone marrow

For cancer patients Ficoll density separation of full blood was performed in order to obtain low density (ring on top of Ficoll) and normal density fractions (pellet). For the other patient groups only full blood was analyzed. Erythrocytes in NDG

samples, full blood and bone marrow were lysed using isotonic ice-cold lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA dissolved in H_2O; pH of 7.4). Next, leukocytes were washed and resuspended in FACS staining buffer (4 mg/ml human albumin [Sanquin, Amsterdam, The Netherlands] and 0.32% (w/v) sodium citrate in PBS).

Flow cytometry

Blood and bone marrow of LPS volunteers and cancer patients were stained with a combination of 10 monoclonal antibodies, fixed in 1% PFA and measured on a BD-LSR Fortessa flow cytometer (Becton Dickenson, Mountain View, CA). The antibodies used were: CD35-FITC (clone E11), CD64-APC (clone 10,1), CBRM1/5-Alexa Fluor 700 (clone CBRM1/5), CD11b-APC-Alexa Fluor 750 (clone Bear1), CD305 (LAIR-1)-PE (clone DX26), CD14-eF450 (clone 61D3), CD16-Krome Orange (clone 3G8), CD62L-BV650 (clone DREG 56), CD49d-PECy7 (clone G9F10), CD66b-PerCPCy5.5 (clone G10F5).

Blood of the trauma and cancer patients was additionally measured on a fully automated FACS machine (Aquios, Beckman Coulter) using fluorescent antibodies against CD16-Krome Orange (clone 3G8) and CD62L-ECD (clone DREG56) from Beckman Coulter (Pasadena, CA, USA); CD11c-PeCy5.5 (clone BU15), CD66b-PerCPCy5.5 (clone G10F5) active CD11b-Alexa700 (clone CBRM1/5) from eBiosciences (San Diego, CA, USA).

COPD patient samples were stained with CD16 (clone 3G8) and CD62L (clone DREG56) and measured on a FACSCalibur (Becton Dickenson, Mountain View, CA).

The results were analyzed using FlowJo software (FlowJo, LLC, Ashland, OR, USA). Neutrophils were identified based on their specific forward- and side-ward signals and doublets were excluded from the analysis. Eosinphils were excluded either based on the distinctive expression of CD66b or CD16.

Statistics

Graphpad Prism was used to analyze data which are presented as mean +/- 95%CI or median with range based on their distribution. Comparison between groups was done with RM one-way ANOVA in case of multiple conditions, Friedman test (if data were paired) or Kruskal-Wallis test (if data were not paired). Statistical significance was accepted at $P^{*} \leq 0.05$, $P^{**} \leq 0.01$ or $P^{***} \leq 0.001$.

Results

Both CD16^{low} and CD62L^{low} neutrophils can be found in the bone marrow in addition to conventional CD16^{high}CD62L^{high} mature neutrophils

Neutrophils are continuously produced in the bone marrow by dividing promyelocytes and subsequent myelocytes. After the myelocyte stage cells stop dividing and differentiate into metamyelocytes, followed by immature neutrophils with a typical banded nuclear morphology, and finally segmented polymorphonuclear mature cells [29-31]. In healthy bone marrow, progenitors consisting of promyelocytes, myelocytes and metamyelocytes (mean: 50±5%) and CD16^{high}CD62^{high} (mean: 29±4%) neutrophils make up the biggest portion of all neutrophils. In addition, CD16^{low} cells (mean: 9±1%) and CD62L^{low} (mean: 12 ± 3%) cells also can be found in bone marrow (Fig. 1).

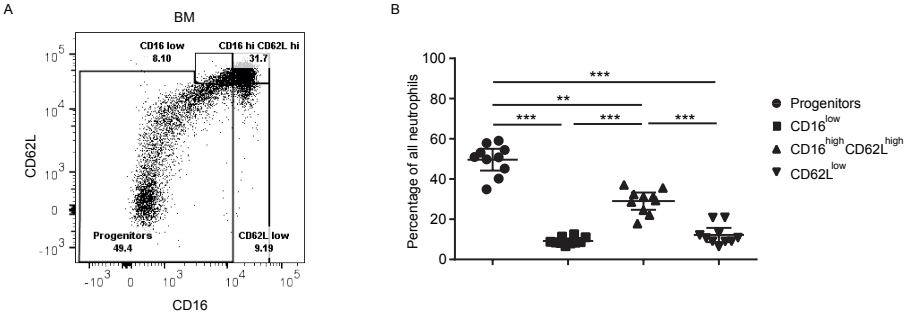

Fig. 1 In addition to mature CD16^{high}CD62L^{high} neutrophils, both CD62L^{low} and CD16^{low} neutrophils are found in bone marrow of healthy individuals

(A) Representative FACS plot of CD16 and CD62L expression of gated neutrophils in bone marrow of a healthy control. (B) Summary dot plot of the percentages progenitors, CD16^{low}, CD16^{high}CD62L^{high} and CD16^{high} CD62L^{low} neutrophils in bone marrow of a healthy controls (n=10). Dots represent different donors. * $p < 0.05$, ** $p < 0.01$ in RM one way ANOVA. Data are paired.

Acute inflammation is characterized by the presence of CD16^{low} neutrophils

As described before, circulatory neutrophils from healthy controls displayed homogeneous high expression of CD16 and CD62L (an example is shown in Fig 5A). After LPS administration as a model for acute inflammation, immature banded neutrophils (CD16^{low}CD62L^{high}) and hypersegmented neutrophils (CD16^{high}CD62L^{low}) were additionally recruited to the peripheral blood (Fig. 2A) [21]. Similar percentages of immature banded cells and hypersegmented neutrophils to that of mature neutrophils were found in the peripheral blood after LPS (~26%) (Fig. 2B). In

addition, around 22% progenitors (max: 32%, min: 15%) were present in the blood after LPS administration. These cells might be mobilized from the bone marrow as a compensatory mechanism, completing maturation in the periphery when the conditions are permissive [17].

We hypothesized the neutrophil subsets found after LPS administration would also be recruited during a clinical situation of acute systemic inflammation, such as trauma. At the day of admission, trauma patients indeed recruited $CD16^{low}$ neutrophils, median 67% (Fig. 2 C, D). In addition, hypersegmented $CD62L^{low}$ neutrophils were also recruited at the day of admission after polytrauma but to a significantly lesser extent (median 9%).

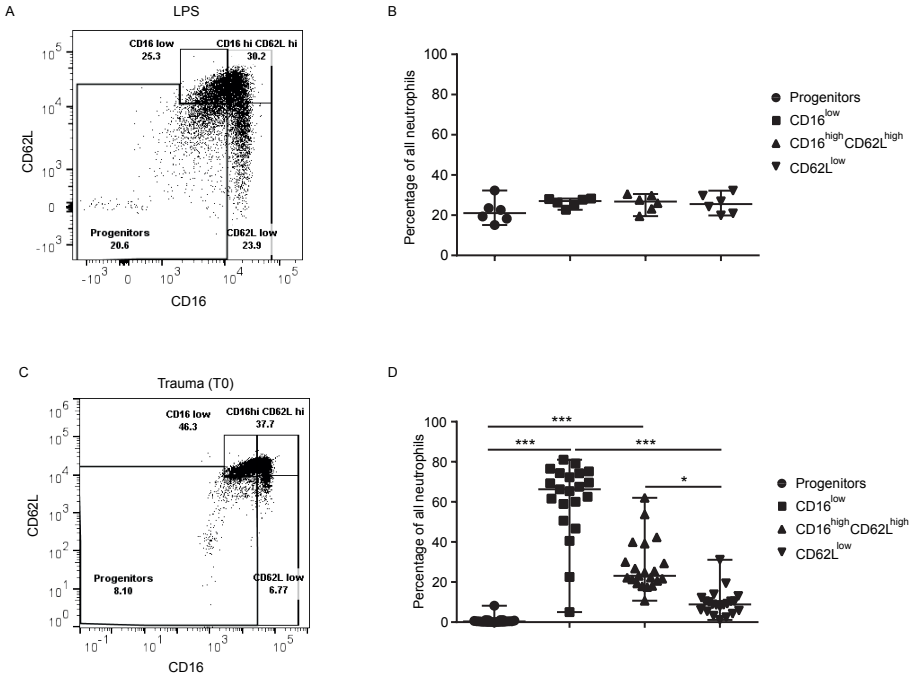

Fig. 2 Acute inflammation is characterized by the presence of $CD16^{low}$ neutrophils in the circulation

Representative FACS plot of CD16 and CD62L expression of neutrophils after LPS administration (A) or at day of admission after trauma (C). Summary dot plot of the percentages progenitors, $CD16^{low}CD62L^{high}$, $CD16^{high}CD62L^{high}$ and $CD16^{high}CD62L^{low}$ neutrophils after LPS administration (n=6) (B) or at day of admission after trauma (n=21) (D). Dots represent different donors. * $p < 0.05$, ** $p < 0.01$ in Friedman test. Data are paired.

Developing inflammation after polytrauma is characterized by the presence of CD62L^{low} and the absence of CD16^{low} neutrophils

Next we followed the kinetics of the different neutrophil subsets in developing inflammation. The same polytrauma patients were followed in time and their neutrophil subsets were determined at day 3 and day 15 after admission. Interestingly, at day 3 after polytrauma the CD16^{low} banded neutrophil population significantly decreased compared to the day of admission (median: 11%, $p<0.001$) and the CD16^{high}CD62L^{low} hypersegmented population significantly increased (median: 30%, $p<0.001$) (Fig 3A, B). A similar pattern was found 15 days after trauma (Fig. 3C, D). Thus, at the earliest time point after polytrauma banded neutrophils were predominantly recruited to the circulation, while at the later time-points this subset disappeared and hypersegmented neutrophils significantly increased.

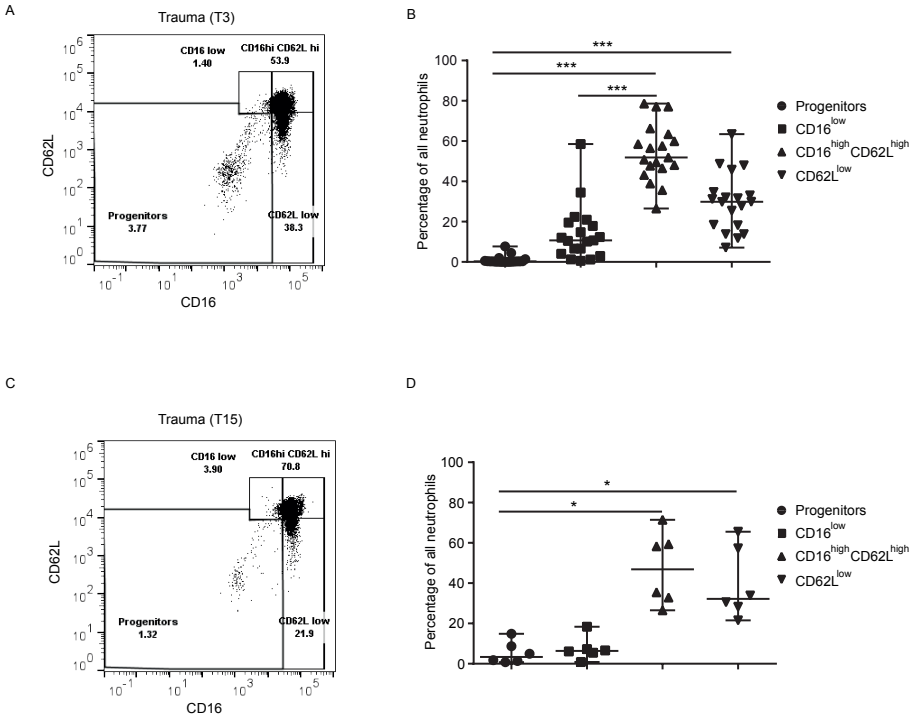

Fig. 3 Late trauma is characterized by an increase of CD62L^{low} and a decrease of CD16^{low} neutrophils

Representative FACS plots of CD16 and CD62L expression of neutrophils at day 3 (A) and 15 (C) after polytrauma. Percentages of neutrophils per subset of total neutrophils at day 3 (n=19) (B) and 15 (n=6) (D) after polytrauma. Dots represent different donors. * $p < 0.05$, ** $p < 0.01$ in Friedman test. Data are paired.

COPD as a model for chronic inflammation is characterized by the presence of CD62L^{low} and the absence of CD16^{low} neutrophils

In order to study neutrophil subsets in a truly chronic inflammation, we chose to characterize COPD patients suffering from neutrophilic inflammation [32, 33]. Similar as found during the later time-points after polytrauma hardly any immature CD16^{low} neutrophils were found in the circulation of COPD patients (median: 0.6%) (Fig. 4A and B). Also, similarly as found in later time points after trauma, CD62L^{low} neutrophils were found in the circulation of COPD patients (median: 6%). Almost no progenitors were detected in the circulation (median: 0.6%). The severity of the COPD patients ranged between GOLD I-IV, but no significant differences were found between patients in different stages (Supplementary figure).

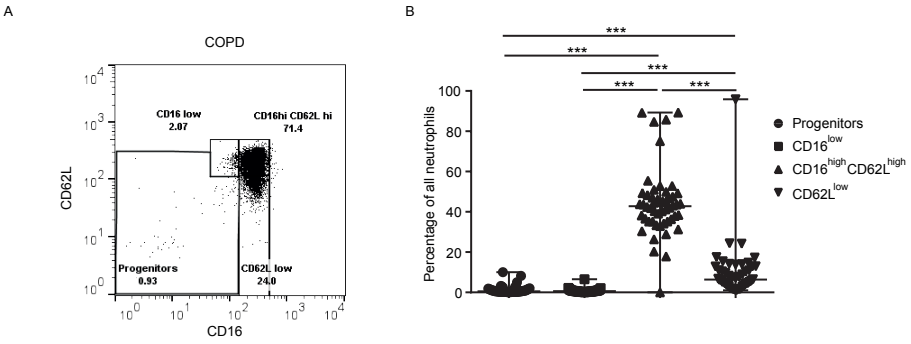

Fig. 4 COPD is characterized by the presence of CD62L^{low} neutrophils

(A) Representative FACS plot of CD16 and CD62L expression of gated neutrophils in peripheral blood of COPD patients. (B) Summary dot plot of the percentages progenitors, CD16^{low}CD62L^{high}, CD16^{high}CD62L^{high} and CD16^{high}CD62L^{low} neutrophils in blood of COPD patients (n=50). Dots represent different donors. * $p < 0.05$, ** $p < 0.01$ in Friedman test. Data are paired.

Cancer as a model for chronic inflammation is characterized by the presence of CD62L^{low} and the absence of CD16^{low} neutrophils and display a higher level of surface activation markers compared to LPS administration and late after polytrauma

Since many studies on neutrophil subsets in cancer have reported immature neutrophils in the circulation [12, 34, 35], we hypothesized the recruitment of banded CD16^{low}CD62L^{high} neutrophils in these patients. On the other hand, our own data showed an absence of CD16low immature banded neutrophils in other chronic inflammatory situations; late after polytrauma and in COPD patients. To investigate neutrophil subsets in cancer compared to other chronic inflammatory disease we had the chance to determine the CD16 and CD62L expression of neutrophils from

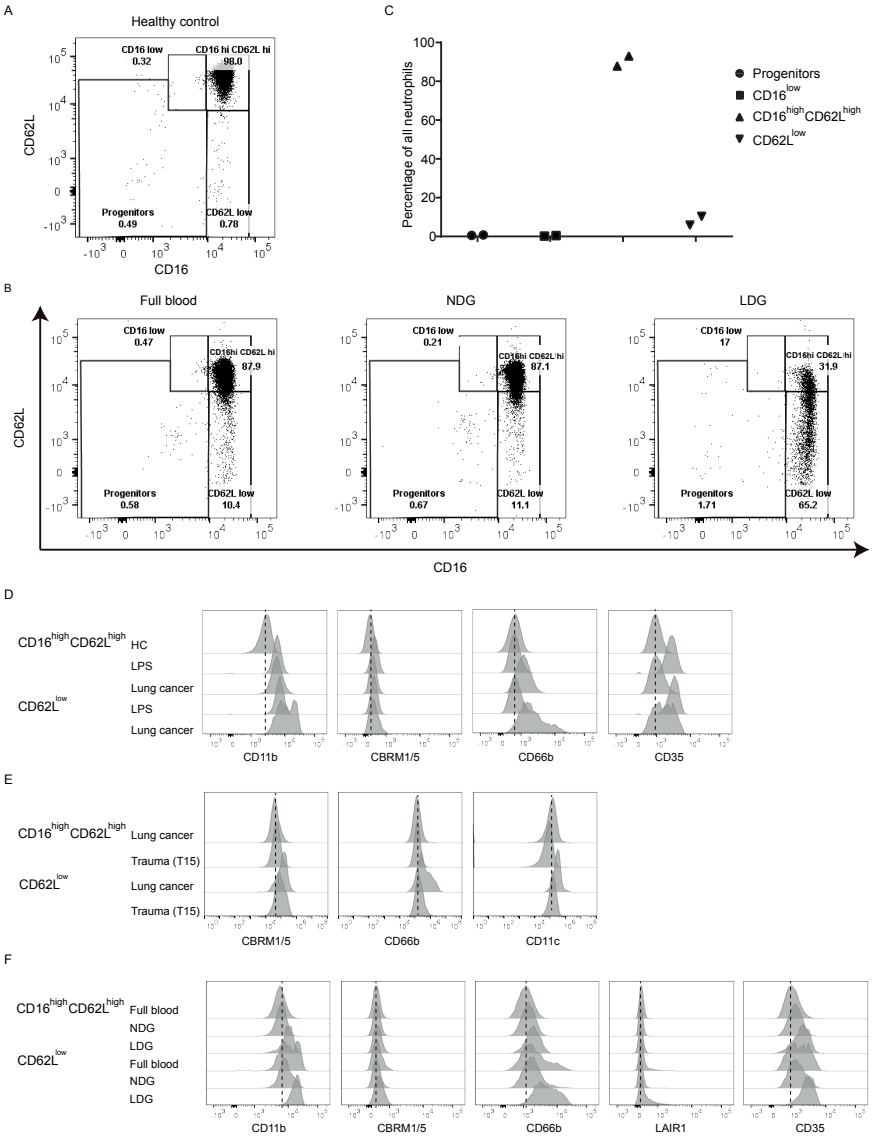

Fig. 5 Cancer is characterized by the presence of CD62L^{low} and the absence of CD16^{low} neutrophils

Representative example of CD16 and CD62L expression of neutrophils from healthy control (A) and in full blood, NDG and LDG from representative non-small cell lung cancer patient. (B). (C) Percentage of neutrophils per subset of total neutrophils (n=2). Dots represent different donors. * $p < 0.05$, ** $p < 0.01$ in Friedman test. Data are paired. (D) Expression comparison of CD11b, the active form of CD11b (CBRM1-5), CD66b and CD35 in CD16^{high}CD62L^{high} and CD16^{high}CD62L^{low} neutrophils from full blood between cancer patients and samples after LPS administration. (E) Expression comparison of the active form of CD11b (CBRM1-5), CD66b and CD11c in CD16^{high}CD62L^{high} and CD16^{high}CD62L^{low} neutrophils from full blood between cancer patients and late after trauma. (F) Expression comparison of CD11b, the active form of CD11b (CBRM1-5), CD66b, LAIR1 and CD35 in CD16^{high}CD62L^{high} and CD16^{high}CD62L^{low} neutrophils from full blood and low and normal density fractions.

2 non-small cell lung cancer (NSCLC) patients. Patients with this type of cancer have previously been reported to have elevated neutrophil to lymphocyte ratios which were found to predict disease progression [36, 37]. Because blood samples from untreated cancer patients with a relatively high tumor load are difficult to obtain, we have not been able to include more patients as of the writing of this thesis. Although the data are very preliminary we still compared these data to the other inflammatory conditions. Whereas we could find the additional population of $CD62L^{low}$ neutrophils in the peripheral blood of the lung cancer patients, $CD16^{low}$ banded cells were again absent (Fig 5A, B first panel and C).

In addition, we compared the activation status of the different subset between cancer patients and healthy controls before and after LPS administration (Fig. 5D). Mature $CD16^{high}CD62L^{high}$ neutrophils from cancer patients showed intermediate activation levels between healthy and post LPS administration. . CBRM1/5 was slightly lower in cancer patients and CD35 was expressed at similar low levels as the healthy controls. CD11b expression was similarly higher expressed than controls in LPS and cancer samples while CD66b was more highly expressed on mature neutrophils from cancer patients. Comparing the $CD62L^{low}$ population with mature neutrophils, all four activation markers increased more pronounced for lung cancer samples than after LPS administration. Lung cancer patients displayed higher levels of CD11b, CD66b and lower levels of CD35 compared to cells obtained after LPS administration. Interestingly, the activation marker pattern of $CD16^{high}CD62L^{low}$ neutrophils from cancer patients was very broad and even showed two distinctive peaks for CD11b and CD35 with the lower peak corresponding to the expression of $CD16^{high}CD62L^{high}$ mature neutrophils.

In order to further evaluate similarities between late trauma samples and cancer patients, we gated the aforementioned subsets and compared the expression of other surface markers (Fig. 5E). In both patient groups higher expression of CD66b, CD11c and the active form of CD11b (CBRM1-5) were found in $CD62L^{low}$ neutrophils compared to $CD16^{high}CD62L^{high}$ neutrophils. Interestingly, $CD62L^{low}$ neutrophils have higher expression of activation markers in lung cancer samples than polytrauma samples at day 15 (Fig. 5E).

In addition, we performed Ficoll separation of full blood from these cancer patients and compared the surface marker expression of neutrophils from full blood with normal density granulocytes (NDG) and LDG. LDG were enriched for $CD62L^{low}$ neutrophils (Fig. 5B). Mature $CD16^{high}CD62L^{high}$ LDG displayed higher expression of activation markers than NDG. Interestingly, the broad two-peaked expression

pattern of activation markers in $CD16^{high}CD62L^{low}$ neutrophils from full blood could be separated by Ficoll, with LDG displaying a marked distinctive increase in CD11b, CD66b and CD35 within this population (Fig. 5F).

Conclusion and Discussion

During the last decade neutrophils were discovered to have more influence on the immune system than merely being involved in phagocytosis and killing of pathogens. We have come to realize neutrophils are heterogeneous in activation, phagocytosis and bacterial killing capacity [22, 38]. In addition, certain conditions and separation methods have allowed the isolation of neutrophils with immune suppressive capacity [8, 12, 13, 16, 21]. It is currently unclear whether the neutrophil population is a spectrum of these different functionalities or whether *bona fide* subsets exist [39]. Different studies have used different markers and functional assays to characterize neutrophil heterogeneity hampering direct comparisons [39-42]. Neutrophil heterogeneity has mainly been studied in the context of cancer, but also in acute inflammation and trauma [21, 43-45]. Here we made a first attempt to compare neutrophil heterogeneity and kinetics from acute to chronic inflammation. To this end we compared neutrophil subsets after LPS administration as a model for acute inflammation to polytrauma patients followed in time as a model of inflammation evolving from acute to chronic inflammation. In addition we measured the occurrence of neutrophil subsets in COPD and cancer patients as models for true chronic inflammation. Interestingly, in addition to conventional mature $CD16^{high}CD62L^{high}$ neutrophils, $CD16^{low}$ immature banded neutrophils were only found in acute inflammation after LPS and at day of admission in trauma patients but not in chronic inflammation. On the other hand, $CD62L^{low}$ hypersegmented neutrophils were found in all conditions. It must be noted that when carefully examining the expression pattern of CD62L in the different conditions, $CD62L^{low}$ neutrophils at day of admission after trauma resembled more the $CD62L^{low}$ neutrophils in bone marrow, whereas the $CD62L^{low}$ expression level after LPS, from day 3 onward after trauma and the cancer patients appeared to be lower. The nuclear segmentation from $CD62L^{low}$ cells from the bone marrow resembled mature neutrophils and displayed fewer lobes than $CD62L^{low}$ hypersegmented neutrophils after LPS (data not shown). These data suggest that $CD62L^{low}$ hypersegmented neutrophils after LPS are a different subset than $CD62L^{low}$ cells found in the bone marrow. It would be interesting to FACS sort $CD62L^{low}$ neutrophils at day of admission after trauma to determine if their nuclear lobularity resembles that found in the bone marrow or the subset found in the circulation after LPS.

After LPS administration banded neutrophils were found to display superior bacterial killing [21]. Whether this is also the case for banded cells in trauma patients at the day of admission is currently under investigation. It is interesting that this banded subset has disappeared from the population from day 3 after trauma. Trauma patients have a risk of developing inflammatory and infectious complications and we are currently investigating whether subset distribution and bacterial killing capacity in fibrin gels could predict patients at risk. After trauma and LPS and in COPD patients neutrophil progenitors were occasionally detected in the blood, possibly reflecting an empty bone marrow compartment or deliberate mobilization to promote extramedullary hematopoiesis [46]. Although reported in other studies, we did not find an increase in neutrophil progenitors in the two cancer patients measured so far [47-49]. Perhaps neutrophil progenitors only appear in certain cancer types or at certain stages of cancer progression. Naturally more patients need to be included to draw firm conclusions.

The distribution patterns of progenitors, banded, mature and hypersegmented neutrophils in the different types of inflammation are interesting. Banded neutrophils, which are superior bacterial killers, are found only after acute inflammation and conspicuously absent from the circulation thereafter. We have also studied the presence of subsets in other chronic inflammatory conditions such as HIV and asthma, and banded neutrophils were also absent in these patients (data not shown). Explanations for the absence of banded neutrophils could be that these cells are simply not recruited at later time points. Alternatively, the bone marrow compartment could be devoid of these immature cells at later time points or they are being recruited but preferentially stay in the tissue. Perhaps banded neutrophils, as elite bacterial killers, are recruited only shortly after the insult and the pursuing response recruits neutrophils with a more wound-healing and/or immune suppressive phenotype [21, 22].

Neutrophils capable of suppressing T cell proliferation have been ascribed to different neutrophil subsets [7, 12, 15, 20, 21, 50, 51]. Some reports suggest the suppressive population is enriched in LDG [8, 12, 13]. Recently, it was described in G-CSF treated patients that the suppressive population consisted of CD10 expressing mature neutrophils that could be found in both the low and normal density layers. Similarly, immune stimulatory immature neutrophils which were negative for CD10 could also be found in both layers [20]. We have found CD10 on neutrophils can be upregulated upon fMLF stimulation (Hassani personal communication), further pointing to activated neutrophils as suppressors. This is also in line with studies from our own lab where we found hypersegmented CD62L^{low} neutrophils capable

of T cell suppression [21] and that these cells showed enrichment of proteins involved in immune regulation [52]. We checked whether the hypersegmented neutrophils upregulated specific proteins which have previously been described for suppressive neutrophils. MMP, elastase, collagenase and TGFbeta were all not increased in hypersegmented neutrophils as might have been expected. However, interestingly CXCL2, also termed growth-related oncogene (GRO) chemokine, was found to be elevated in hypersegmented neutrophils. In addition to a neutrophil chemoattractant, CXCL2 has been shown to be involved in cancer metastasis, angiogenesis, and wound healing [53-55]. It would be interesting to compare these proteomics data after LPS administration to proteomics data of the subsets found after trauma, COPD and cancer.

Comparing activation marker status, CD11b, CBRM1/5, CD66b and CD35 were differentially expressed on mature neutrophils from healthy controls, LPS volunteers and lung cancer patients. LPS volunteers displayed higher CBRM1/5 and CD35, whereas lung cancer patients expressed higher CD66b. CD11b was similarly higher expressed on mature neutrophils from LPS and cancer patients compared to controls. Activation of $CD62L^{low}$ neutrophils was more pronounced for CD11b and CD66b expression, and two distinctive peaks for CD11b and CD35 with the lower peak corresponding to the expression of $CD16^{high}CD62L^{high}$ mature neutrophils were found. Compared to late trauma, neutrophil activation of mature neutrophils in cancer patients was comparable, but activation in $CD62L^{low}$ neutrophils was also more pronounced in the lung cancer patients. Interestingly, double peaks in activation markers were found in the $CD62L^{low}$ population of cancer patients and the neutrophils expressing higher activation markers were found in the hypodense layer. Thus in our study LDG's comprised of highly activated degranulated neutrophils.

An explanation for the contradicting reports on maturity of suppressing LDG might be that this population can be comprised of a mix of both progenitors and activated neutrophils. It is conceivable that both highly activated degranulated neutrophils and progenitors with lower granule content could be hypodense [9, 16]. Indeed Marini et al separated immature LDG from mature LDG using CD10 surface expression and found that only activated mature neutrophils in the hypodense fraction suppressed T cell proliferation [20]. If healthy control neutrophils are separated using a range of Percoll densities below the density of Ficoll, heterogeneous buoyant density of these control neutrophils can be found (Hassani personal communication). We therefore initially hypothesized neutrophil activation would gradually shift the spectrum towards hypodensity. However, in our

cancer patients a clear separation of a highly activated population was found and this population could be isolated in the hypodense layer. Thus the data in cancer patients are more in favor of LDG representing a truly separate population.

To summarize, immature elite killer neutrophils only appear in the circulation during acute inflammation whereas activated $CD62L^{low}$ neutrophils continue to be present in chronic inflammatory conditions. This subset might represent immunosuppressive neutrophils with wound healing characteristics and might be disadvantageous for cancer patients where these neutrophils could facilitate angiogenesis and metastasis. Furthermore, it might render trauma patients susceptible for infections and sepsis.

Acknowledgements
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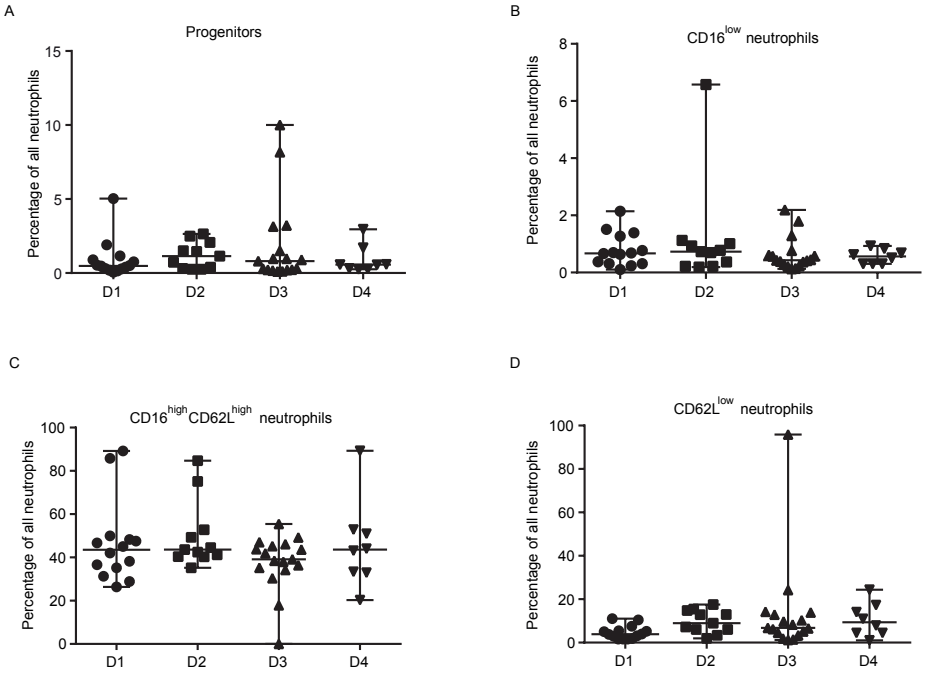

Supplementary figure

(A) Summary dot plot of the percentages progenitors of total neutrophils in different stages of COPD patients. (B) Summary dot plot of the percentages $CD16^{low}$ of total neutrophils in different stages of COPD patients. (C) Summary dot plot of the percentages $CD16^{high}CD62L^{high}$ of total neutrophils in different stages of COPD patients. (D) Summary dot plot of the percentages $CD62L^{low}$ of total neutrophils in different stages of COPD patients. Dots represent different donors in different stages of COPD. * $p < 0.05$, ** $p < 0.01$ in Kruskal-Wallis test.

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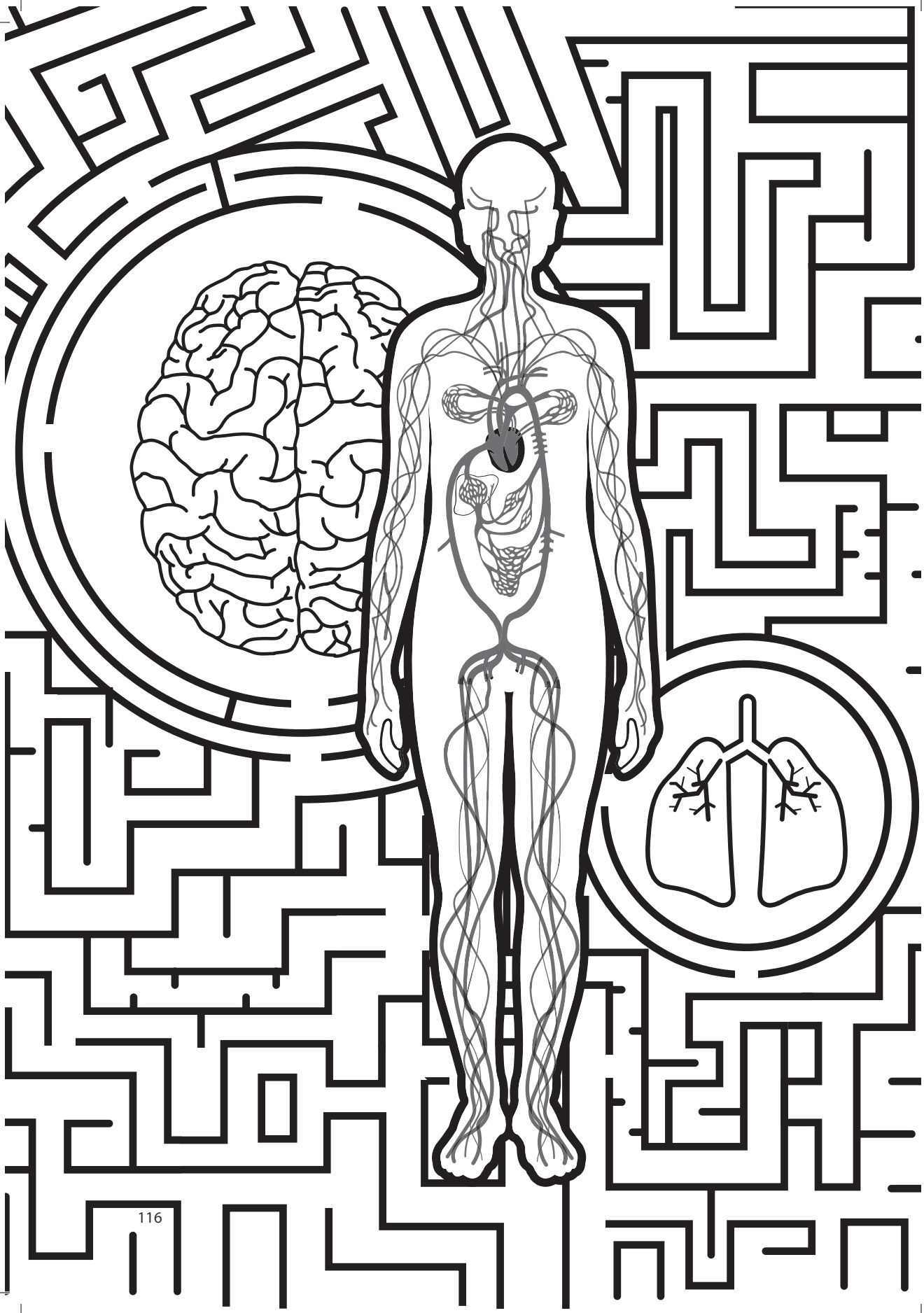

Chapter 6

The poor bacterial containment capacity of CD62L^{low} hypersegmented neutrophils in matrix scaffolds is associated with impaired survival

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Manuscript in prepration

Abstract

Neutrophils are key effector cells of the innate immune system and constitute the first line of defense against invading pathogens. During inflammation, neutrophils can quickly respond to the inflammatory mediators coming from the infectious or damaged site. Different neutrophil subsets appear in the blood after an acute LPS challenge which are not present during homeostasis and these display different bacterial containment capacities. To explain the mechanism behind this difference, we first optimized neutrophil survival in a 3D in vitro model and then studied the effect of phagocytosis on neutrophil survival. Phagocytosis decreased neutrophil survival in an MPO-dependent manner. Hypersegmented neutrophils, present in the circulation after LPS, have a relatively short in vitro lifespan which could explain their poor bacterial containment capacity. Although banded neutrophils which also appear in the blood after LPS administration live longer in vitro than normal mature neutrophils, this difference was not significant and could not fully explain their difference in bacterial containment. Thus, the mechanism of increased bacterial containment by banded neutrophils goes beyond merely increased survival.

Introduction

Neutrophils (also known as polymorphonuclear leukocytes or PMN) are the most abundant leukocytes (50-70%) in human peripheral blood. These immune cells are fast responders in the innate immune response and contribute to the first line of defense against invading microorganisms [1-3]. Upon ingestion of invading pathogens in phagosomes, antimicrobial proteins and/or enzymes released from the granules can be delivered either extracellularly by fusion with the plasma membrane or intracellularly by fusion with the phagosome. This phagosome-granule fusion is essential to contain and kill pathogens [4, 5]. Several cytotoxic mechanisms mediate the killing of these microbes and range from the de novo production of toxic reactive oxygen species by a membrane bound NADPH-oxidase to degranulation of preformed cytotoxic proteins/peptides stored in granules. The most toxic proteins/enzymes are stored in primary/ azurophilic granules, including myeloperoxidase (MPO), elastase and bactericidal/permeability-increasing protein (BPI) [6-13]

Previously, both increased and decreased survival of neutrophils upon phagocytosis have been reported [14-17]. These differences were mainly attributed to the type of pathogen where some pathogens induce neutrophil death in order to escape killing, while others extend neutrophil life span to survive and be shielded from other immune cells [15].

Apart from the mature cells that reside in the peripheral blood in homeostasis two new phenotypes are found during acute inflammation: immature banded neutrophils ($CD16^{low}/CD62L^{high}$) and hypersegmented ($CD16^{high}/CD62L^{low}$) neutrophils. Surprisingly, these different phenotypes exhibit marked differences in containment and killing capacity [18]. Interestingly, in contrast to the consensus that banded cells are mobilized as compensation by the bone marrow during inflammation and are functionally impaired it turns out that these cells contained phagocytosed bacteria best [18]. On the other hand, the hypersegmented neutrophils performed worst. This defect in bacterial containment by hypersegmented neutrophils could not be attributed to differences in survival in vitro, in phagocytosis, nor in the content of proteases in their granules. Hypersegmented neutrophils displayed increased H_2O_2 production and less phagosomal HOCL compared to the mature subset despite an increased abundance of cellular MPO, suggesting decreased fusion of granules containing MPO in hypersegmented cells [18].

The most abundant protein in neutrophils is MPO and it comprises 5% of their dry weight [19]. It catalyzes the conversion of H_2O_2 into hypochlorous acid (HOCl or bleach) by oxidation of chloride [20]. HOCl, a potent microbicidal oxidant, aids in bacterial killing and has the potential to promote tissue damage by hyperactivated neutrophils [21-24]. Defects in the phagocyte NADPH oxidase complex results in chronic granulomatous disease (CGD) which is often life threating [25] while inherited MPO deficiency is rarely associated with clinical symptoms and thus mild [26]. Although this implies MPO is not essential for bacterial killing, this mechanism might still play an important role when present. Indeed the bactericidal capacity of MPO deficient neutrophils has been found to be delayed [27-29].

Here we aimed to further investigate the difference in bacterial containment of hypersegmented neutrophils by eliminating the variable of bacterial growth using heat-killed staphylococcus aureus. In order to achieve this we first optimized neutrophil survival in a 3D *in vitro* assay.

Materials and methods

Human volunteers

Healthy volunteers with no known medical conditions donated fresh blood after signed informed consent in accordance to the Declaration of Helsinki. Blood was collected in sterile tubes with sodium heparin as anti-coagulant (Vacuette® Greiner bio-one, Kremsmünster, Austria).

Neutrophil isolation

The blood was mixed with a similar volume of phosphate buffered saline containing 40g/L Albumin and 3.2% Sodium Citrate and layered on top of Ficoll Paque (Pharmacia, Uppsala, Sweden). Tubes were spun down at 760g for 20min at room temperature. Erythrocytes pellet were lysed by cold NH_4Cl solution for 10-15 min. Cells were washed twice and resuspended in RPMI Medium 1640 (#61870010, Gibco) with penicillin or Hepes buffer (1mg $MgSO_4$, 1.2mM KH_2PO_4, 132mM NaCl, 20mM Hepes) supplemented with 0.5% (w/v) human serum albumin (HSA), 1mM CaCl2 and 5mM glucose. The absolute number of neutrophils was measured on an automatic hematology analyzer (CELL-DYN Emerald, Abbott, IL, USA) and cells were kept on ice until use. For neutrophil subsets isolation, whole blood samples were prepared as described previously [18]

Neutrophil survival in different 2D and 3D culturing system

In all experiments, $2x10^5$ neutrophils/well were seeded into 96-well tissue culture-treated plates (#353219, Falcon). In the 2D culture system, neutrophils were resuspended in RPMI Medium 1640 or Hepes buffer containing either 0.5% HSA or 40% human serum (HS, #H4522, Sigma) and seeded. In the 3D culture system, neutrophils were cultured in fibrin gels (2 mg/ml fibrinogen + 1U/ml thrombin) or collagen type I gels (#354249, Corning) at a final concentration of 3.3 mg/ml. The gels contained 0.5% HSA or 40% HS depending on the experimental conditions and the pH was adjusted to 7.4. Granulocyte-macrophage colony-stimulating factor (GM-CSF) at a final concentration of 1nM was added as a survival factor in some of the experimental conditions. Propidium iodide solution (PI, #P4864, Sigma) (2.5%) was added to measure neutrophil death. The PI signal was detected real time using a plate reader (fLUOstar or Omega, BMG Labtech). Triton X-100 (0.5%) was added at the end of the experiment when the PI signal reached the plateau to induce cell death of all potential remaining cells in order to confirm the maximal signal was reached.

Neutrophil containment capacity in 3D culture system

Neutrophils were cultured in fibrin gels mentioned above. Killed Staphylococcus aureus (Wood strain without protein A) bioparticles (#S2859, Thermo Fisher) with final multiplicities of infection (MOI) at 1 with or without myeloperoxidase (MPO) inhibitor 4-Aminobenzoichydrazide (#ab141482, Abcam) at a final concentration of 100µM, 1mM and 10mM were added to test neutrophil survival changes.

Statistics

Graphpad Prism was used to analyze data which are presented as median with range. Comparison between groups was done with RM one-way ANOVA in case of multiple conditions (data are paired). Statistical significance was accepted at $P^* \leq 0.05$ or $P^{**} \leq 0.01$.

Results

Neutrophils are notoriously difficult to isolate in an untouched/unprimed form for *in vitro* experiments. Their activation status even changes in the heparin blood tube within after 2 hrs (data not shown) making timely isolation crucial. Furthermore, adherence to plastic surfaces can immediately generate a sustained burst of superoxide (O_2^-) [30]. Therefore, we first set out experiments to develop the optimal assay to measure neutrophil viability *in vitro*.

Neutrophil viability increases in 3D culture systems

Human peripheral neutrophils were isolated within 2 hrs after venipuncture and cultured in different 2D and 3D culture systems without serum (Fig .1 A and B). In 2D culture plates neutrophils survived significantly longer in Hepes buffer (median survival of 20hrs until 50% dead, max: 32hrs, min: 18hrs) than in RPMI (median survival of 13hrs, max: 25hrs, min: 10hrs). 3D culture systems using collagen or fibrin matrics significantly increased neutrophil viability compared to the 2D cultures. Survival in fibrin reached a median of 39hrs until 50% death (max: 44hrs, min: 29hrs) and survival in collagen gels reached a similar 39hrs (max: 47hrs, min: 31hrs). Thus, neutrophil survival was not significantly different between collagen and fibrin gels. Because fibrin gels kept their gel structure even after 5 days of culturing whereas collagen gels started to liquefy at this time point (data are not shown), we chose fibrin gels for further experiments.

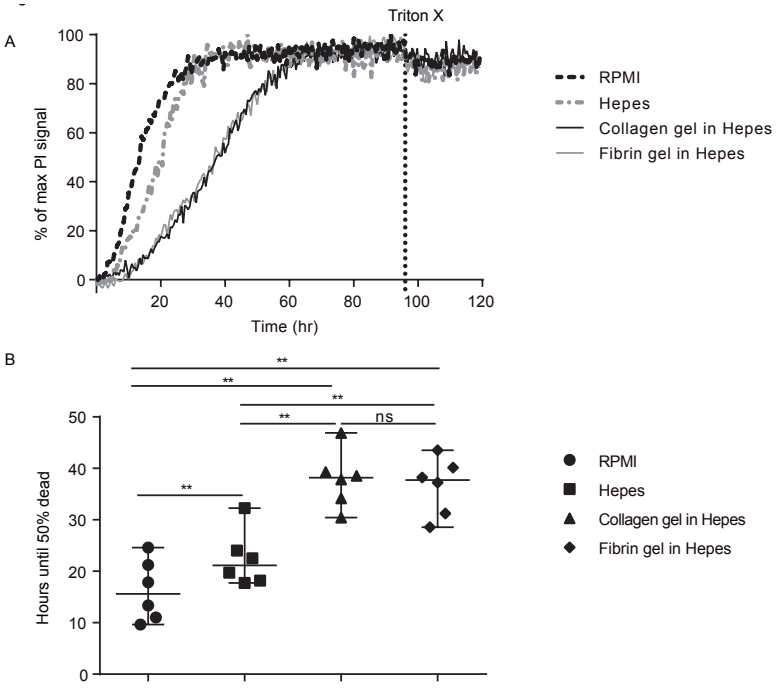

Figure 1. Neutrophil viability increases in 3D culture systems. (A) Representative example of the percentage of max PI signal over time in the different indicated culture conditions. (B) Data as depicted in A were used to calculate the hours until 50% dead for the different indicated culture conditions. Dots represent different donors. Data are paired.

Human serum and GM-CSF can prolong neutrophil survival

To further optimize neutrophil viability in the 3D culture system, we added 40% human serum to the fibrin gel (Fig. 2A and B). As expected, human serum significantly increased neutrophil survival in fibrin gels to a median 50% survival of 59hrs (max: 70hrs, min: 37hrs). When the survival factor GM-CSF was added to the cultures, neutrophil survival could even further be prolonged to a median of 68hrs (max: 67hrs, min: 37hrs). Thus, more optimal culturing conditions greatly increased *in vitro* neutrophil survival.

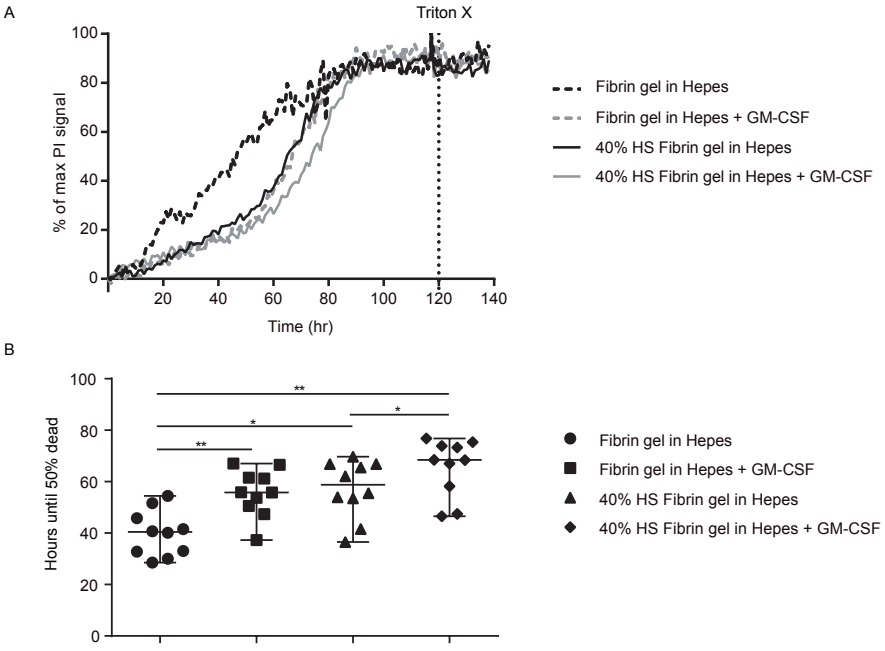

Figure 2. Human serum and GM-CSF can prolong neutrophil survival. (A) Representative example of percentage of max PI signal over time in the absence or presence of human serum and GM-CSF (B) Data as depicted in A were used to calculate the hours until 50% dead in the absence or presence of human serum and GM-CSF. Dots represent different donors. Data are paired.

Phagocytosis of bioparticles decreases neutrophil survival in an MPO-dependent matter

Both increased and decreased survival of neutrophils upon phagocytosis has been reported [14-17]. Therefore, we first tested the effect of phagocytosis in our optimized 3D assay using labeled nonviable Staphylococcus aureus bioparticles. These bioparticles cannot secrete immune evasion proteins allowing us to test the

isolated effect of phagocytosis *per se*. In the presence of bioparticles neutrophil survival was significantly decreased from a median 50% survival of 52hrs (max: 75hrs, min: 37hrs) in controls to 38hrs (max: 55hrs, min: 30hrs) (Fig. 3A and B).

Since HOCl is reported to kill a wide range of cell types [22-24, 31], we next tested whether the decreased neutrophil survival after phagocytosis could be attributed to autologous production of HOCl. in our survival assay. Indeed inhibition of MPO by 4-Aminobenzoichydrazide [32] restored neutrophil survival to control levels in a dose dependent fashion (Fig. 3A and B), suggesting that the HOCl produced by neutrophils after phagocytosis is also toxic to the neutrophils themselves.

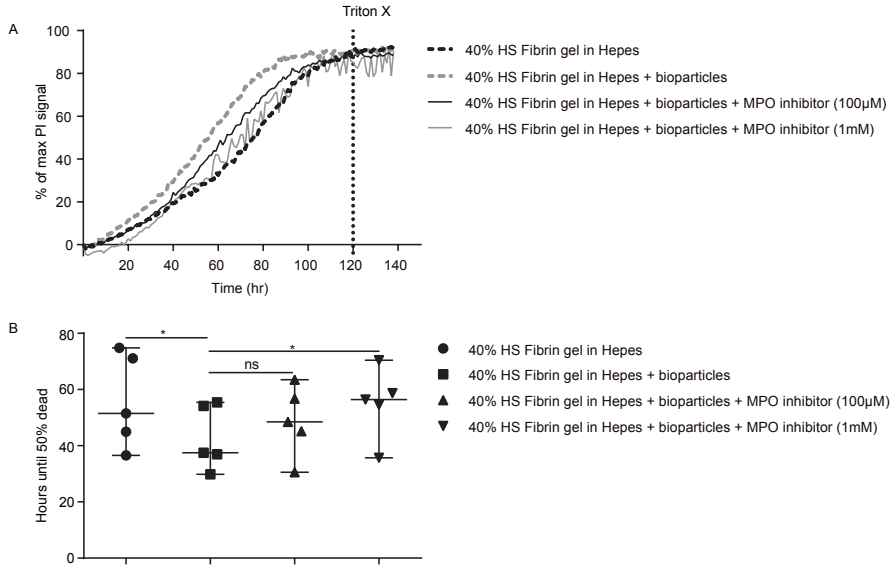

Figure 3. Phagocytosis of bioparticles decreases neutrophil survival in an MPO-dependent matter. (A) Representative example of percentage of max PI signal over time in the absence or presence of bioparticles +/- MPO inhibitor (B) Data as depicted in A were used to calculate the hours until 50% dead in the absence or presence of bioparticles +/- MPO inhibitor. Dots represent different donors. Data are paired.

The impaired bacterial containment of hypersegmented neutrophils in matrix scaffolds could be associated with a short in vitro life span

During acute inflammation two neutrophil subsets appear in the peripheral blood in addition to the mature CD16^{bright}/CD62L^{bright} neutrophils normally found in the circulation during homeostasis. These extra neutrophil subsets are CD16^{dim}/CD62L^{bright} showing reduced nuclear lobulation (banded neutrophils) or increased

nuclear lobulation concomitant with a loss of CD62L (hypersegmented neutrophils) [33]. Hypersegmented neutrophils display impaired bacterial containment *in vitro* and indirect evidence pointed out that this might be attributed to decreased MPO-containing granule fusion.

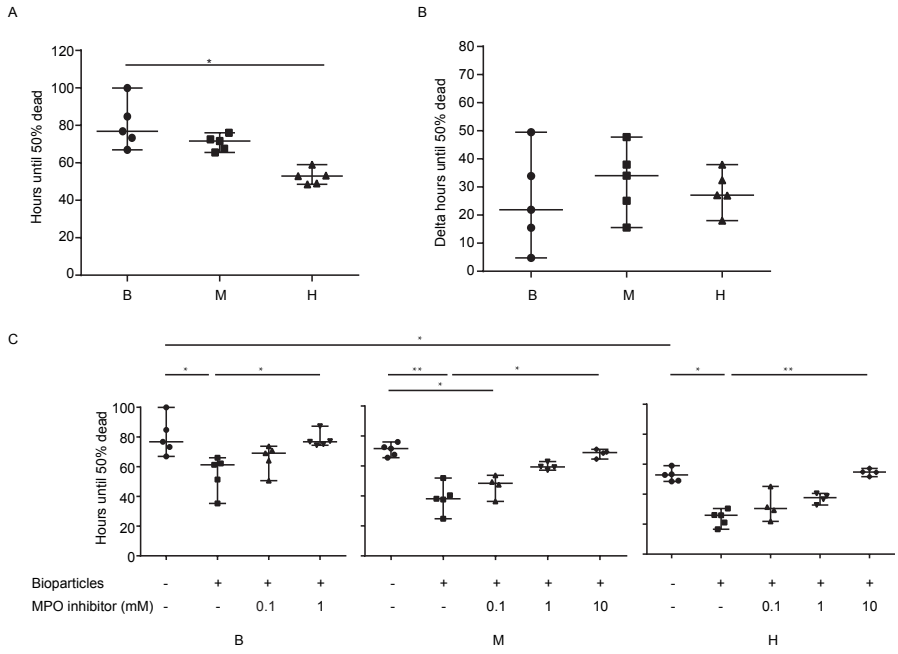

Figure 4. Differences in bacterial containment between neutrophil subset might be attributed to different in vitro life spans. (A) Hours until 50% dead comparison of banded (B), mature (M) and hypersegmented (H) neutrophils. (B) Decrease in survival (delta hours until 50% dead) after bioparticle phagocytosis forbanded, mature and hypersegmented neutrophils. (C) Hours until 50% dead of banded, mature and hypersegmented neutrophils cultured in the absence or presence of bioparticles +/- MPO inhibitor. Dots represent different donors. Data are paired.

Since phagocytosis of bioparticles led to MPO-dependent neutrophil death (Fig. 3A and B), the hypothesis was tested that hypersegmented neutrophils with impaired MPO-granule fusion would not show increased cell death after bioparticle uptake. To that end, the three aforementioned neutrophil subsets were isolated from blood by cell sorting 3hrs after intraveneuous injection of 2ng/kg Escherichia coli LPS. There is no significant difference of time to reach 50% late apoptosis between banded neutrophils (median: 77hrs, max: 100hrs, min: 67hrs) and mature neutrophils (median: 72hrs, max: 76hrs, min: 66hrs). However, hypersegmented neutrophils took a median of 53hrs to reach 50% late apoptosis (max: 60hrs, min:

49hrs) which is significantly shorter than banded neutrophils (Fig. 4). In contrast to the hypothesis, bioparticles significantly decreased the survival of hypersegmented neutrophils (Fig. 4C). In fact, no significant differences were found in the decrease in survival upon phagocytosis of bioparticles between the different neutrophil subsets (Fig. 4B). As found for mature neutrophils of healthy controls, the decreased survival could be prevented by the addition of MPO inhibitor in a concentration dependent matter for all three neutrophil subsets (Fig. 4C). Thus our experiments do not support reduced granule fusion of hypersegmented neutrophils as an explanation for their impaired bacterial containment [18]. Rather, the reported reduction in bacterial outgrowth of 19hrs between mature and hypersegmented neutrophils [18] could be explained by the reduced baseline survival of hypersegmented neutrophils in matrix scaffolds.

Discussion

Taken together, we can conclude that human peripheral blood neutrophils can be successfully cultured in 3D culture systems and their survival can be significantly improved in this system compared to traditional 2D *in vitro* culture systems. The median time of 59hrs at which 50% of healthy control neutrophils survived is of interest in light of recent discussions on neutrophil life span *in vivo*. Depending on the assumptions of the models either a relatively short half-life of 19 hr [34] or a relatively long half-life of 3.8 days [35] were reported. Our *in vitro* data is in favor of the latter as it is not expected that neutrophils, which are cells that are notoriously difficult to isolate and handle in vitro, would live shorter *in vivo*.

We found that phagocytosis of bioparticles decreased neutrophils survival and that this could be reversed by an MPO inhibitor, implying the involvement of HOCl in this process. So what might be the benefit to produce toxic levels of HOCl if it causes the death of neutrophils as well? Even though MPO deficiency is not related to severe infections [26, 36], experiments demonstrate that the bacterial killing of neutrophils of patients with MPO or other neutrophil deficiencies is delayed [26, 37]. In line, using neutrophils from CGD patients who lack NADHP and thus HOCl, we have shown that bacteria were contained but not killed [18]. Even though phagocytosis and HOCl production decrease neutrophil survival to 38hrs, this is still a relatively long time for neutrophils producing HOCl to gradually kill bacteria. On the other hand, without HOCl more neutrophils can act as Trojan horses, containing bacteria without killing them.

We have previously shown that hypersegmented neutrophils, which were recruited upon acute inflammation, displayed worse bacterial containment compared to normal mature neutrophils. In addition, we found this difference could not be attributed to differences in survival in 2D cultures and rather seemed to be the result of impaired MPO-containing granule fusion [18]. To support and confirm the hypothesis of impaired granule fusion in hypersegmented neutrophils, we tested the hypothesis whether hypersegmented neutrophils would not show the MPO dependent decrease in survival upon bioparticle uptake as found for mature neutrophils from healthy donors. Surprisingly we found the decrease in survival was comparable for all subsets, including hypersegmented neutrophils, arguing against the hypothesis that a problem in granule fusion underlies the survival phenotype of hypersegmented cells. In addition, this decreased survival could be overcome by an MPO inhibitor in all neutrophil phenotypes.

Our experiments seem in contrast to our published data [18] that suggested impaired MPO-containing granule fusion in hpersegmented neutrophils. This contradiction might be explained by the possibility that our initial study measured impaired phagolysosomal MPO, whereas this study might have detected decreased survival due to extracellular production of HOCl by MPO. This might fit with the finding that MPO content was increased at the cellular level in the hypersegmented subset [18]. Regardless of these two explanations, our *in vitro* life spans of the different neutrophil subsets at baseline significantly differed. The difference in median survival of mature and hypersegmented neutrophils of 19hrs corresponds exactly to the reduced time until unrestrained outgrowth in the bacterial containment assay reported by Leliefeld et al [18]. Thus the reduced baseline survival of hypersegmented cells could be sufficient to explain their impaired bacterial containment. A decreased survival of hypersegmented neutrophils as an explanation for their decreased bacterial containment was previously ruled out because hardly any cell death occurred at the time of unrestrained bacterial outgrowth, but survival was not measured in the presence of bacteria [18]. However, in that assay *in vitro* survival was measured relatively short and in the absence of bacteria. Our *in vitro* experiments imply that the phagocytosis of dead bacteria already greatly reduced hypersegmented neutrophil survival, yet still not to the extent that it could explain the reported extremely fast bacterial outgrowth time of 4hrs [18]. Possibly live bacteria could decrease the *in vitro* life span even further. Additionally, we are not sure how many neutrophils should be alive in order to achieve containment of bacteria in the gel and we measure neutrophil death using propidium iodide which is a late apoptotic marker. The question that remains is what influences the reduced baseline *in vitro* survival of hypersegmented

neutrophils. Banded neutrophils are immature cells and younger than the other two subsets [38], which might explain their longer life span *in vitro*. Hypersegmented cells, however, have a similar life span as mature neutrophils [38] and yet they live significantly shorter in our assays.

Immature banded neutrophils did not survive significantly longer than mature neutrophils in our in vitro gels. The median time until 50% death differed 7 hrs (max: 24hr, min: 1hr) between banded and mature neutrophils, whereas the reported difference in bacterial containment was 27hrs. Thus the superior bacterial killing of banded neutrophils in tissue scaffolds cannot merely be explained by differences in survival. Future research should focus on the mechanism of increased bacterial containment by banded cells and the reduced in vitro baseline survival of hypersegmented neutrophils. As a first step it would be of interest to compare bacterial phagocytosis and containment of the different neutrophils subsets *in vivo* using intravital microscopy where they are visualized simultaneously in space and time.

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Competing interests

The authors declare no competing interests

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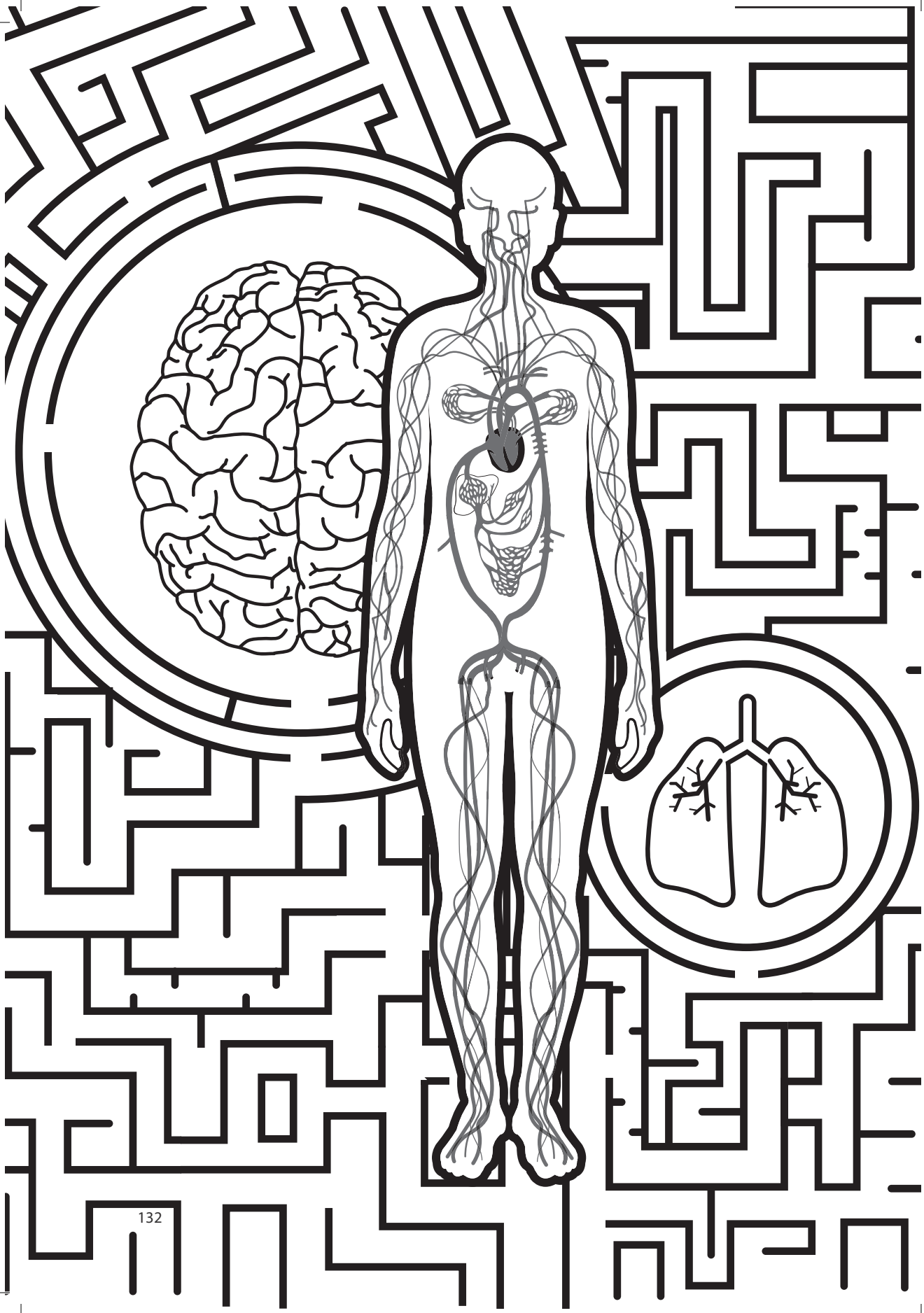

Chapter 7

General discussion

General discussion

The research presented in this thesis has been focused on the functional role of neutrophils and their subsets in the regulation of acute and chronic inflammation. Neutrophils are the most abundant white blood cells in the human circulation and are key effector cells of the innate immune system. They contribute to the first line of defense against invading pathogens. Moreover, they also make up a big proportion of infiltrating inflammatory cells to the tumor microenvironment. Their functions are not simply restricted to host defense, as neutrophils are more complex than they were initially thought to be and perform a vast array of specialized functions.

From two-dimensional and three-dimensional assays *in vitro* to intravital microscopy *in vivo*

Although two-dimensional in vitro studies are widely used and important for increasing our knowledge on immune cell and tumor cell behavior, the complexity of the in vivo microenvironment is often not taken into consideration. In my thesis I have tried to tackle these limitations by 1. developing a three dimensional in vitro environment favoring neutrophil survival and by 2. studying neutrophils and tumor cells in living mice by intravital microscopy.

Neutrophils are notoriously difficult to manage as they easily functionally change in in vitro experiments. However, using our optimized 3D culture system, neutrophil survival was significantly improved compared to traditional 2D in vitro culture systems illustrating the fact that these cells favor these conditions (chapter 6, [1, 2]). Different from the traditional idea that neutrophils have a relatively short half-life of 8 hours in vivo[3], healthy control neutrophils took a median time of 59 hours to reach 50% late apoptosis in our 3D culture system compared to 13 hours in suspension (**chapter 6**). This would mean that the survival of neutrophils in vitro is much better than the situation in vivo, which is not very likely. Indeed the concept of a short half-life (8 hrs) of neutrophils in vivo has recently been challenged by studies using non-toxic in vivo deuterated water or glucose labelling. The studies came to estimated life spans of either 19 hours or 3.8 days depending on the assumptions made for the mathematical model used to determine the kinetic values [4]. My in vitro data in 3D gels is also in favor of a relatively long neutrophil half-life (> 2-3 days).

Although three-dimensional cultures are a huge improvement, they still do not fully mimic the complex and specific microenvironment present in a living organism. Therefore, intravital microscopy (IVM) is increasingly used in biomedical research

to study dynamic processes at cellular and subcellular resolution in their natural environment. Long-term IVM with the use of tissue windows can be applied to visualize migration and proliferation over days to months within the same animal without recurrent surgeries **(chapter 2).** Tumor cell morphology and motility of different human and mouse breast tumor cell lines were imaged in living mice by intravital microscopy. These studies identified marked differences between their in vitro and in vivo behavior **(chapter 3)**. Thus caution should be taken to characterize tumor migration in vitro, especially in 2D experiments. As has been found before for leukocytes [5], the rules for migration on protein coated plastic might be very different from 3D environments. In **chapter 4** we studied the in vivo migratory behavior of glioblastoma (grade VI glioma) tumor cells. Taking clinical biopsies was previously shown to increase migration of glioblastoma cells [6] and in **chapter 4** we studied the effect of neutrophils on this biopsy-induced migration. Via intravital imaging through an implanted cranial imaging window in neutrophil depleted mice we demonstrated a role for neutrophils in biopsy induced tumor progression **(chapter 4)**.

The pro and anti-tumor functions of neutrophils

Clinical and experimental evidence suggests that local and systemic inflammation is important for cancer progression and increasing attention has been paid to the role of neutrophils in cancer-associated inflammation [7-9] . A high neutrophil to lymphocyte ratio (NLR) in peripheral blood or a high number of intra-tumoral neutrophils is associated with poor prognosis in patients and is used as a prognostic indicator for some tumor types including non-small cell lung cancer studied in **chapter 5** [10-14]. Tumors induce neutrophil expansion which, at least in part, might explain the prognostic value of neutrophils. But also a direct role of neutrophils in metastases formation has been described pointing to a causal effect of neutrophils on tumor progression [15]. Furthermore, emerging evidence suggests that biopsies and surgical interventions may increase the risk of developing metastases via infiltration of immune cells that form an environment that favors metastatic spread [16-18]. To assess the role of neutrophils in biopsy-induced tumor progression in vivo, a cranial imaging window was implanted onto neutrophil depleted mice and long-term intravital imaging was applied (**chapter 4**). Interestingly, not only the percentage, but also the speed of migratory glioma cells decreased in neutrophil depleted mice compared to that in neutrophil non-depleted mice. This implies neutrophils play a role in promoting migration of glioma cells in mice. Moreover, macrophage numbers decreased in tumor tissue in neutrophil depleted mice (**chapter 4)** which might further prevent tumor cell migration upon biopsy in vivo [6]. Combined, these data support the hypothesis that neutrophils are involved in

the recruitment of macrophages to the tumor and they may play an indirect role in promoting glioma tumor cell migration after biopsy. To investigate whether there is an independent effect of neutrophils on tumor progression and to extend these findings to human tumor cell lines, a wound closure assay and transwell assay were performed with human neutrophils in vitro. Not only human glioblastoma tumor cell lines (RAV21 and RAV27), but also human breast tumor cell lines (MDA, MCF7) were used in both assays. In the presence of neutrophils, wound closure time was significantly decreased for both RAV21 and RAV27 glioblastoma tumor cell lines, but not for MCF and MDA breast tumor cell lines, suggesting neutrophils promote the in vitro migration of glioblastoma cells but not for the two breast tumor cell lines. It should be noted that the baseline wound closure between the two breast tumor cell lines was very different. As expected, baseline wound closure in mesenchymal MDA cells was already very fast, whereas epithelial MCF7 tumor cells were extremely slow. Thus the reason why neutrophils do not promote breast tumor cell motility might be different for the two breast tumor cell lines. Neutrophils enhanced MDA wound closure in some but not all donors leading to a non-significant effect when donors were pooled. In contrast MCF7 tumor cell wound closure was not enhanced but significantly reduced by neutrophils. Because wound closure took more than 40 hours for MCF7 tumor cells it is likely neutrophils started to die in the culture probably affecting tumor cell motility in other ways than during shorter culture times of 24 hours for MDA and RAVs. Transmigration of RAV21, RAV27 and MDA was significantly promoted by the presence of neutrophils in a transwell assay, but much less pronounced for MDA compared to the glioblastoma cell lines. These data confirm a role for neutrophils in promoting tumor cell migration, especially for glioblastoma tumor cells. Since there is no direct contact between tumor cells and neutrophils in this assay, these data imply a soluble factor is involved in the promotion of tumor cell migration by neutrophils. However, the possibility of neutrophils additionally promote tumor cell migration via direct contact cannot be excluded. Combined, these results provide evidence for both macrophage dependent and independent involvement of neutrophils in the pro-metastatic response following biopsy of glioblastoma tumors. This also provides some explanation to clinical observations in glioblastoma patients where high numbers of blood and tumor-infiltrating neutrophils are associated with glioma grade, poor prognosis and resistance to therapy [19-21]. Apart from pro-tumor functions, conventional neutrophils are also able to kill tumor cells, hinder tumor growth and slow down malignant progression [22-24]. The presence and/or involvement of neutrophils may thus either be beneficial or detrimental for the patient. In part these counteracting roles of neutrophils in cancer progression might be attributed to different neutrophils subsets. Therefore, it is very important to more fully comprehend neutrophil heterogeneity in health and (malignant) disease.

Neutrophil heterogeneity in cancer and acute and chronic inflammation

Increasing evidence shows that neutrophils are heterogeneous in activation, phagocytosis and bacterial killing capacity [25, 26]. Neutrophil heterogeneity is mainly studied in the context of cancer, but also in acute inflammation and trauma [27-29]. It is still not clear whether the neutrophil population is a spectrum of cells with different functionalities or whether bona fide subsets exist. We compared neutrophil heterogeneity in healthy control individuals before and after LPS administration as an acute inflammation model. In addition, heterogeneity was studied in polytrauma patients followed over time, COPD and two lung cancer patients (**chapter 5**). Not only in acute inflammation induced by LPS challenge or initial response upon trauma, additional neutrophil subsets appear in the peripheral blood of patients with more chronic inflammatory conditions such as late after polytrauma, in COPD and in the two lung cancer patients. Apart from conventional mature $CD16^{high}CD62L^{high}$ neutrophils, $CD62L^{low}$ neutrophils can be found in all conditions. Interestingly, $CD16^{low}CD62L^{high}$ immature banded neutrophils are only found in acute inflammation such as evoked by LPS challenge and at the day of admission in polytrauma patients. $CD16^{low}$ banded neutrophils disappeared from the circulation under conditions of more chronic disease such as found from day 3 after trauma, COPD in the 2 lung cancer patients (**chapter 5)** and in other chronic inflammatory conditions such as asthma and HIV (data not shown). Thus it seems immature banded neutrophils are only recruited to the circulation during acute inflammation, whereas $CD62L^{low}$ neutrophils can be found in both acute and chronic inflammation. Multiple explanations could account for the absence of immature $CD16^{low}$ neutrophils in the circulation, among which; 1) recruitment to the tissue, 2) retainment in the bone marrow, 3) empty bone marrow, 4) faster maturation during inflammatory state. As of yet there is no evidence for the first two explanations, but a depleted bone marrow and faster hematopoietic differentiation have been described as a consequence of inflammation [30-34].

It is tempting to speculate on the kinetics of the different neutrophil subsets. Perhaps banded neutrophils which were found to display superior bacterial killing at least after LPS administration are elite killers recruited early in the response and subsequently activated $CD62L^{low}$ neutrophils (**chapter 5**) play a role in resolving inflammation by T cell suppression and initiating a wound healing response [26, 29]. We have recently found hypersegmented neutrophils release more vesicles when migrating in 3D matrices [35] and similar neutrophil vesicles have been found in healing bone fractures [36]. In addition, anti-inflammatory properties have been ascribed to these neutrophil derived vesicles by inducing TGFbeta release from macrophages [37]. However, we have not yet tested whether the subsets in trauma

and cancer patients have similar functionalities as found after LPS and this needs to be confirmed before drawing firm conclusions.

Neutrophil spectrum versus *bona fide* subsets

Although we describe additional subsets with lower CD16 or CD62L surface expression in the different inflammatory conditions, these markers are gradually changing with maturation and activation [38-40], and might also reflect a continuum as opposed to separate populations. On the other hand, the difference in T cell suppression capability and proteomic profile together with the unexpected similar maturation stage of mature and hypersegmented neutrophils point to hypersegmented neutrophils as a truly separate subset [29, 41].

Healthy control neutrophils are also not homogeneous and display a spectrum of buoyant densities. This can be visualized by using a range of Percoll densities instead of the fixed density of Ficoll (Hassani personal communication). Therefore, we initially hypothesized that neutrophil activation would gradually shift the spectrum towards hypodensity and that LDG would not represent a bona fide subset. Both progenitors with low granule content and partly activated degranulated neutrophils would be relatively hypodense. The hypothesis of different neutrophil (pheno) types with similar low buoyant density could explain the contradicting studies on maturity of T-cell suppressing LDG reporting either progenitors or activated neutrophils [42-46].

Although the data on the two lung cancer patients is very preliminary, it was exciting and unexpected to find such clear separate peaks in activation markers on $CD62L^{low}$ neutrophils in whole blood. Compared to healthy controls, neutrophils after LPS administration and neutrophils of cancer patients displayed higher levels of CD11b and CBRM1/5. In addition, neutrophils after LPS displayed higher CD35, whereas neutrophils from cancer patients were characterized by higher CD66b expression. Interestingly, comparing the activation markers between $CD62L^{low}$ neutrophils of LPS volunteers and cancer patients, CD11b and CD66b were higher on neutrophils of cancer patients and actually showed a separate highly activated peak (see Figure 5F in **chapter 5**). This discrete higher activation could be ascribed to the $CD62L^{low}$ LDG population. These data might hint that LDG in cancer represent a truly separated hyperactivated population and not the end of a continuum.

Many studies suggest that neutrophils can suppress T cell proliferation in vitro and this suppressive capacity has been implicated for neutrophils from whole blood, NDG and LDG from patients but not for cells from healthy controls [42, 44,

47, 48]. Perhaps neutrophils in all these fractions are a mix of suppressive and non-suppressive neutrophils/subsets combined with a parallel and overlapping mix of mature and immature neutrophils. Indeed, Marini et al found in the context of G-CSF treatment suppressive neutrophils could be isolated from both NDG and LDG based on the expression of the maturation marker CD10 [49]. Whether this is also true for other inflammatory conditions such as LPS administration or cancer remains to be investigated. Because samples from treatment naïve human cancer patients with a considerable tumor load are difficult to obtain, this is not a trivial experiment.

Consequences of the relatively short in vitro neutrophil life span for functional studies

In order to study the functionality of different neutrophil subsets, these have to be isolated from peripheral blood or bone marrow using Percoll, Ficoll, beads or FACS based separation. Especially functional assays in suspension should be interpreted with care, because of the more than four times lower survival in suspension compared to matrix scaffolds. The shorter in vitro survival of hypersegmented neutrophils (**chapter 6**) despite similar life spans previously measured in vivo [41] raises further concerns on functional in vitro studies with neutrophils. On the other hand, circulatory in vivo life span might not be representative for neutrophil life span in the tissues and perhaps the shorter life span of hypersegmented neutrophils in 3D gels are also found at the tissue site in vivo. Regardless, it is not inconceivable that differences in function between subsets can actually be at least in part attributed to differences in neutrophil death.

Bacterial containment

Upon inflammation, the additional neutrophil subsets that appear in the blood (as described above) display different bacterial containment capacities in 3D gels. The immature $CD16^{low}$ banded neutrophils contained bacteria better than the regular mature neutrophils. On the other hand, $CD62L^{low}$ hypersegmented neutrophils performed worst. Leliefeld et al [26] showed no survival differences between the different neutrophil phenotypes in the absence of bacteria. However, survival was only compared up to 20 hours, because the bacterial containment of the hypersegmented neutrophils was less than ten hours. When we extended the assay **(chapter 6)**, we found a significant decreased in vitro survival of hypersegmented neutrophils. Banded neutrophils took a median of 77 hours to reach 50% late apoptosis, mature neutrophils 72 hours and hypersegmented neutrophils 53 hours. The 19 hour lower baseline survival of hypersegmented neutrophils compared to mature neutrophils might already be sufficient to explain their 19 hour shorter bacterial containment. Banded neutrophils contain bacteria 27 hours longer than

mature neutrophils, whereas their difference in in vitro survival is not significant. Thus shorter survival in vitro might explain the reduced bacterial killing capacity of hypersegmented neutrophils, but the superior bacterial containment of banded neutrophils cannot be singly attributed to their in vitro survival (**chapter 6**).

Intravital microscopy of neutrophil subsets in response to a local bacterial infection could be a first step to tackle the discrepancy in life span between our in vitro 3D assay versus in vivo labelling and to translate our in vitro containment assay to an in vivo environment.

T cell suppression

We have previously shown hypersegmented neutrophils appearing in the peripheral blood after LPS are capable of suppressing T cell proliferation in suspension in vitro. This suppression could be inhibited by the H_2O_2 scavenger catalase and by adding an anti-CD11b antibody. These data imply neutrophils suppress T cell proliferation in a local H_2O_2 and Mac-1-dependent (CD11b/CD18-dependent) manner [29]. However, an alternative explanation for these data might be that neutrophil death plays a role in in vitro T cell suppression. Catalase might prevent oxidative damage to the T cells by extracellularly blocking H_2O_2 released from dying neutrophils [1] and anti-CD11b antibody has been found to reduce neutrophil apoptosis [50]. Whether neutrophil death plays a role in in vitro T cell suppression is a testable hypothesis. If so, future studies should focus on whether this mechanism also applies in vivo. It is also of interest to investigate at what place and time these two immune cells would actually meet in an in vivo environment.

Concluding remarks

In conclusion, a better understanding of neutrophil subsets and their functions in different conditions will provide an opportunity for new insights in neutrophil mediated disease processes and targeted treatment strategies. Neutrophils are double edged swords, promoting and suppressing inflammation and tumor progression. If we have true insight into whether or not these counteracting functions originate from different neutrophil subsets and how these originate, this will offer a lot of potential to tune the inflammatory response in a wide range of inflammatory conditions. Suppressive neutrophils could be elicited in chronic inflammatory diseases and acute respiratory distress, whereas elite killers could be triggered in cancer and sepsis. If, however, one functional subset can differentiate into the other subset in vivo, this would hugely impair treatment options. Because neutrophils play such an essential role in the immune system, targeting neutrophils could have major beneficial but unfortunately also disastrous consequences for the host.

7

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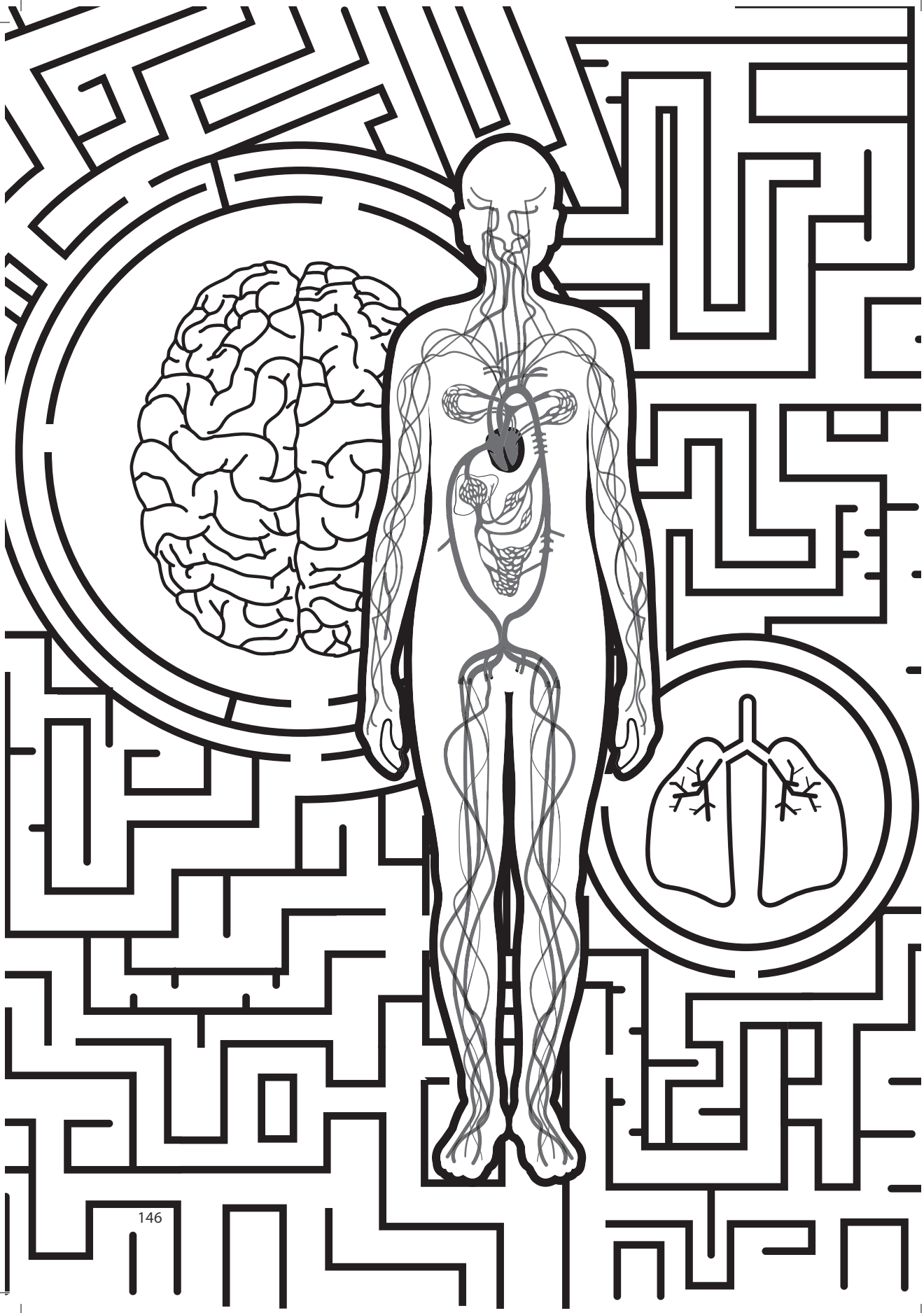

Nederlandse samenvatting

Inleiding

De meest voorkomende afweercel in humaan bloed is de neutrofiel. Neutrofielen spelen een sleutelrol in de aangeboren afweer en dragen bij aan de eerste verdediging tegen ziekteverwekkers die ons lichaam binnendringen. Echter, in sommige klinische situaties kunnen neutrofielen ook bijdragen aan afweergerelateerde ziektebeelden zoals sepsis, allergieën, auto-immuniteit en kanker. Hoewel oorspronkelijk gedacht werd dat de neutrofiel alleen ziekteverwekkers internaliseert en elimineert is er recent bewijs gevonden dat niet alle neutrofielen hetzelfde zijn. Sommige neutrofielen kunnen ziekteverwekkers beter internaliseren dan anderen. Verder is er gevonden dat neutrofielen diverse andere functies kunnen uitoefenen, waaronder het remmen van andere afweercellen. Ook is er gevonden dat er extra neutrofielensubtypes met verschillende functies gerekruteerd worden in situaties van ziekte. Men is er nog niet helemaal uit of elke neutrofiel de capaciteit heeft om alle functies uit te oefenen of dat er bona fide verschillende types bestaan.

Van tweedimensionale en driedimensionale experimenten in vitro naar intravitale microscopie in vivo

Hoewel het kweken in driedimensionale, weefselgelijkende matrices een grote verbetering is ten opzichte van het kweken in twee dimensies in plastic kweekschaaltjes, bootst dit uiteraard niet precies de complexiteit van weefsels in levende organismen na. Om die reden wordt in biomedisch onderzoek steeds vaker gebruik gemaakt van intravitale microscopie om dynamische processen op cellulair nivo in hun natuurlijke omgeving te bestuderen. Door middel van een venstertje gericht op het orgaan van interesse, kan intravitale microscopie worden toegepast om celmigratie en celdeling dagen tot maanden te bestuderen in dezelfde muis zonder dat herhaalde operaties noodzakelijk zijn **(hoofdstuk 2)**. Met behulp van deze techniek heb ik uiterlijke kenmerken (morfologie) en beweeglijkheid (motiliteit) van tumorcellen bestudeerd in tumoren gegenereerd door verschillende humane en muizen tumorcellijnen te transplanteren in levende muizen **(hoofdstuk 3)**. De morfologie en motiliteit verschilden nadrukkelijk tussen tumorcellen in kweekschaaltjes en tumorcellen in levende muizen. Er is dus voorzichtigheid geboden in het direct vertalen van tweedimensionale tumorcelkarakteristieken naar de situatie in een tumor in een levend organisme.

De pro- en anti-tumor functies van neutrofielen

Klinisch en experimenteel bewijs suggereert dat lokale en systemische ontsteking bijdragen aan kankerprogressie. Neutrofielen zijn cellen die onderdeel uitmaken van het afweer systeem en recent onderzoek laat zien dat deze cellen een rol

kunnen spelen bij kankergeassocieerde inflammatie. Als er relatief veel neutrofielen gevonden worden in het bloed of in de tumor heeft de kankerpatiënt vaak een slechtere prognose. Dit geldt bijvoorbeeld voor patiënten met niet-kleincellige longkanker waar we neutrofielen van hebben bestudeerd in **hoofdstuk 5**.

In **hoofdstuk 4** wordt met behulp van microscopie tijdreeksen de invloed van neutrofielen op tumorcelmotiliteit onderzocht nadat er een biopt is genomen van een hersentumor. Neutrofielen verhogen de motiliteit van humane hersentumorcellen in kweekschaaltjes en van muizenhersentumorcellen die we in de hersenen van levende muizen hebben bestudeerd met behulp van een venster in de schedel. Dit kan wellicht klinische observaties in hersentumorpatiënten verklaren waar hoge aantallen van bloed- en tumorneutrofielen geassocieerd waren met een slechte prognose en therapieresistentie.

Buiten functies die tumorprogressie bevorderen, kunnen neutrofielen ook tumorcellen doden en tumorgroei hinderen. Neutrofielen kunnen dus zowel goed als slecht zijn voor een kankerpatiënt. Wellicht worden deze verschillende functies door verschillende neutrofielsubtypes uitgevoerd. Het is daarom van essentieel belang om deze verschillende neutrofielensubtypes beter te begrijpen in zowel gezonde personen als patiënten.

Heterogeniteit van neutrofielen in kanker en acute en chronische inflammatie

De verschillende subtypes van neutrofielen zijn vooral in de context van kanker bestudeerd, maar ook in andere studies na acute inflammatie en trauma. Het is nog niet duidelijk of er bona fide verschillende populaties zijn. Andere mogelijkheden zijn dat er een spectrum is van verschillende functionaliteiten en dat sommige neutrofielen beter zijn in de één en andere in de andere functie of dat eenzelfde neutrofiel zich kan aanpassen aan verschillende omgevingen.

In **hoofdstuk 5** heb ik een eerste poging gedaan om neutrofielheterogeniteit te vergelijken tussen gezonde individuen voor en na een experimentele inflammatie, in patiënten gevolgd in de tijd na polytrauma, COPD patiënten en twee longkanker patiënten. Niet alleen na acute experimentele inflammatie of acuut na trauma verschijnen extra neutrofielsubpopulaties in het bloed, ook in patiënten met chronische inflammatie zoals laat na trauma, COPD en longkanker verschijnen extra neutrofielsubpopulaties. Het is interessant dat immature neutrofielen vooral gevonden worden tijdens acute inflammatie terwijl een andere neutrofielpopulatie, die de marker CD62L verliest, zowel in acute als chronische inflammatie wordt

gevonden. Verklaringen waarom de immature neutrofielen uit het bloed verdwijnen zouden kunnen zijn dat; 1) deze cellen naar de weefsels vertrekken, 2) deze cellen in latere stadia niet worden vrijgegeven vanuit het beenmerg, 3) het beenmerg leeg geraakt is, of 4) maturatie versneld is tijdens inflammatie. Voor de eerste twee verklaringen is nog geen bewijs gevonden, maar een gedepleteerd beenmerg en versnelde maturatie van voorlopers van neutrofielen als gevolg van inflammatie is wel beschreven in de literatuur.

Neutrofielheterogeniteit: een spectrum of bona fide verschillende subtypes

We kunnen de verschillende neutrofielen herkennen aan bepaalde oppervlaktemoleculen en aan de vorm van de kern. Deze verschillen zijn echter niet zwart-wit maar lopen in elkaar over. Dit pleit meer voor een continuum in plaats van echte verschillende subtypes. Aan de andere kant zijn er verschillen in functionaliteit gevonden voor de verschillende subtypes. Zo is er een subtype die andere afweercellen kan remmen en zijn de subtypes wel erg verschillend als je al hun moleculen met elkaar vergelijkt. Hoewel de data van longkankerpatiënten in **hoofdstuk 5** preliminair is, vinden we hier ook duidelijker verschillende subtypes, waar meerdere markers echt zwart-wit verschillen. Deze data suggereren dat in longkankerpatiënten er echt een separate neutrofielpopulatie te vinden zou kunnen zijn.

Consequenties van de relatief korte levensduur van neutrofielen in kweekschaaltjes voor functionele studies

Neutrofielen zijn notoir moeilijk om buiten het lichaam in leven te houden. In onze geoptimaliseerde driedimensionale kweeksystemen konden we neutrofieloverleving significant verlengen ten opzichte van traditionele tweedimensionale kweekschaaltjes

(**hoofdstuk 6**). Deze resultaten zijn ook interessant voor de interpretatie van de levensduur van neutrofielen in het menselijk lichaam. De schattingen voor de levensduur in het menselijk lichaam lopen uiteen van 8 uur tot 5 dagen. Aangezien in ons geoptimaliseerde systeem pas na 39 uur de helft van de neutrofielen dood was, lijkt dit op een relatief lange levensduur te duiden.

Aan de andere kant vinden we in onze driedimensionale systeem dat het neutrofielsubtype met een hypergesegmenteerde kern sneller dood gaat, terwijl studies in mensen laten zien dat deze cellen in het bloed even lang leven als de andere subtypes. Misschien dat de situatie in het bloed verschillend is van

driedimensionale weefsels en dat deze neutrofielen wel degelijk sneller doodgaan in de weefsels van mensen.

In vervolgstudies moet er, als er naar functionele verschillen gekeken wordt tussen neutrofielsubtypes, ook rekening worden gehouden met deze verschillen in levensduur. Er moet worden uitgesloten dat het verschil in functie alleen verklaard kan worden door verschil in overleving.

Met behulp van intravitale microscopie zouden we kunnen testen of de neutrofielen met hypergesegmenteerde kern inderdaad ook korter leven in de weefsels in levende muizen. Op die manier zouden we de discrepantie tussen de bevindingen in bloed van mensen en de driedimensionale experimentele opstelling wellicht kunnen verklaren.

Conclusie

Beter begrip van neutrofielsubtypes en hun functie in verschillende condities biedt een kans om nieuwe inzichten te genereren in neutrofielgemedieerde ziekten en bovendien om nieuwe behandelingsstrategiëen te creëren.

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Na Chen (陈娜)
Utrecht, 2019

Curriculum Vitae

Na Chen was born on February 3rd 1988 in Baotou City, Inner Mongolia Province, China. She was raised up and received primary and secondary education in the same city. After graduating from high school in 2006, she started to study Veterinary Medicine at Jilin University in the same year. After obtained Bachelor's degree in 2011, she continued with her Master's program in Pharmacology Veterinary Medicine at the same university, under the supervision of Prof. Xuming Deng and Prof. Haihua Feng. In 2014, she started her PhD project under supervision of promotor prof. Leo Koenderman and co-promotor dr. Nienke Vrisekoop. The results of this PhD research are described in this thesis. Parts of this thesis have been presented at national and international conferences. The project was financially supported by China Scholarship Council (CSC). Her research aimed to expand the knowledge regarding to the role of neutrophils in cancer and other inflammatory conditions.

List of publication

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