universität wien

# MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„The role of CXCL5 and neutrophil extracellular trap formation in hepatocellular carcinoma“

verfasst von / submitted by

Elisa Holstein, BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2020 / Vienna, 2020

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|---|---|
| Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet: | UA 066 834 |
| Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet: | Masterstudium Molekulare Biologie |
| Betreut von / Supervisor: | Ao. Univ.-Prof. Mag. Dr. Wolfgang Mikulits |

# Acknowledgement

I hereby want to thank my supervisor Prof. Wolfgang Mikulits for offering me the possibility to work in his lab, for his guidance and for his detailed feedback during the time in the lab as well as during the writing process. Also, I am tremendously grateful for the additional skills I could acquire in his lab apart from bench work, presenting and writing: mentoring other students, assisting in designing the poster, and the possibility to participate in writing the review article “Dynamics of Axl Receptor Shedding in Hepatocellular Carcinoma and Its Implication for Theranostics” together with him and Matthias Binder. Therefore, thanks to Prof. Wolfgang Mikulits for pushing my scientific and academic career in course of this master’s thesis.

Furthermore, I want to express my deepest gratitude to Heidi Huber, who was always able to advise me on all experimental or personal concerns. I profited enormously from her technical knowledge and expertise. Also, thanks for the help with preparing the materials and splitting the cells during the time of the lab courses at the FH and at the University of Vienna.

Moreover, special thanks to Eva Řezníčková, Ph.D., who helped me in conducting experiments, especially the Sytox Green DNA release assay in parallel to the immunofluorescence analysis experiments. Without her, it would have been impossible to perform the experiments simultaneously with the same patch of neutrophils. Also, thanks for the advice and expertise while planning the experiments and data analysis. I really enjoyed working together with Eva on this NET formation project.

Additionally, I want to thank Gerald Timelthaler for his help with the confocal microscope and the image quantification using Definiens, although it did not work out as we hoped for. Thanks also to the animal housing facility for providing me with mice, and later for taking care of the mice we ordered. Thanks to all my lab colleagues, especially Heidi, Eva, Matthias, Gerhard and Teresita, not only for their scientific help, but also for the great atmosphere, and the enjoyable discussions we had.

Although he was not directly involved, I want to thank Prof. Valerio Izzi from the University Oulu, who taught me data analysis in R, which I could apply for this thesis.

Finally, I want to give thanks to my family and friends, who supported and accompanied me in course of this thesis. Special thanks to my mother Birgitt and Martin, who always listened carefully when I had troubles with experiments or data interpretation and who enabled me to pursue my passion for science. Last but not least, thanks to Tanja and Max who supported me especially during the time when I was writing this thesis.

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## Abstract

The molecular collaboration of transforming growth factor (TGF)-β signalling and the receptor tyrosine kinase Axl activated by its ligand growth-arrest specific gene (Gas) 6 is crucially involved in the development of hepatocellular carcinoma (HCC). The synergy of TGF-β and Gas6/Axl signalling upregulates various target genes modulating the tumour microenvironment. One of these is the chemokine CXCL5, which is a neutrophil attractant. Neutrophil extracellular trap (NET) formation has recently been associated with the progression and metastasis of breast, lung and renal cancer. The role of NET formation in HCC has not been sufficiently addressed so far. In this study, we showed that human hepatoma cells exposed to long-term TGF-β treatment induce the release of neutrophil DNA associated with citrullinated histones and granule proteins, such as neutrophil elastase and myeloperoxidase, resulting in membrane rupture. These markers indicate lytic NET formation, also known as NETosis referring to a specific type of neutrophil cell death. As the HCC cell line HLF upregulates CXCL5 in response to long-term TGF-β exposure, the impact of CXCL5 on NET formation was further analysed. HLF cells ectopically expressing CXCL5 as well as recombinant CXCL5 stimulated NET formation in an inverse dose response relationship. Finally, we analysed the dependency of NETs on protein arginine deiminase (PAD) 4, which mediates histone citrullination, and show a PAD4-independent NET formation induced by CXCL5. Altogether, these data provide evidence that the Axl/TGF-β mediated upregulation of CXCL5 in HCC cells stimulates PAD4-independent NET formation of murine neutrophils *in vitro*.

# Introduction

The word “cancer” derives from the Greek word *καρκίνος* (*karkínos*), as the cancerous tissue reminded Hippocrates of a moving crab (Papavramidou, Papavramidis et al. 2010). *Karkínos* referred to the swelling or formation of ulcers, now better known as tumour, while *karkínoma* defined the malignant cancer. Nowadays, cancer is the definition for hundreds of different malignancies caused by the uncontrolled growth of cells. Around 2.7 Mio. people in Europe in 2020 and 1.8 Mio. people in the United States are diagnosed with cancer (European Cancer Information System 2020, National Cancer Institute (NIH) 2020). There are three main categories to which all cancer types can be assigned to: (i) sarcomas, the cancers of the connective tissue, (ii) carcinomas, the cancers of epithelial cells, and (iii) leukaemia or lymphomas, which develop from cells derived of hematopoietic stem cells giving rise to blood and immune cells (Cooper 2000). While the vast majority of all cancers are diagnosed as carcinomas i.e. around 90%, roughly 8% are categorized as leukaemia or lymphoma, and only around 2% are defined as sarcomas being thus the rarest category of malignancy.

## Hepatocellular carcinoma

### Aetiologies

Liver cancer is globally the sixth most prevalent cancer and is the fourth most frequent cause of cancer-related death (Bray, Ferlay et al. 2018, Yang, Hainaut et al. 2019). South Eastern Asia as well as sub-Saharan Africa count the highest incidence and mortality rates of primary liver cancer due to high exposure to risk factors like hepatitis B and C virus (HBV, HCV) and aflatoxin (El-Serag 2012), and due to limited medical healthcare resources including early diagnosis as well as restricted curative treatment options (Fig. 1; (Yang, Hainaut et al. 2019). The most common form of primary liver malignancy is hepatocellular carcinoma (HCC) comprising 75 to 85% of all primary liver cancers (Wong, Jiang et al. 2017). Chronic liver diseases predispose HCC as 80% of HCC cases develop in the background of hepatic inflammation, fibrosis and cirrhosis mediated by secretion of inflammatory factors such as interleukin (IL)-34 recruiting monocytes and Kupffer cells (Al-Shaebi, Wenzhang et al. 2020). Around 50% of people with chronic HBV infection progress to chronic liver diseases as a result of hepatic inflammation (Arzumanyan, Reis et al. 2013). Approximately 20% of patients with chronic liver diseases develop cirrhosis within the following 5 to 20 years. Around 1 to 2% out of these cirrhosis patients progress to HCC each year. In 2015, around 250 million people were living with chronic HBV infection leading to more than 880 000 deaths due to development of liver cirrhosis and HCC (World Health Organization (WHO) 2020). HBV-associated HCC varies strongly depending on transmission. For instance, mother-to-child transmission of HBV is much more likely in Eastern Asia and African countries than in industrialized countries. In addition, HBV onset and duration of the hepatitis and other environmental exposures such as diet and age of the patient affect HCC development (Yang, Hainaut et al. 2019). For instance, HBV-associated HCC develops most frequently in absence of cirrhosis in Eastern Asia and African countries, yet in industrialized countries, HBV-associated HCC patients suffer from cirrhosis in the majority of cases (Chayanupatkul, Omino et al. 2017).

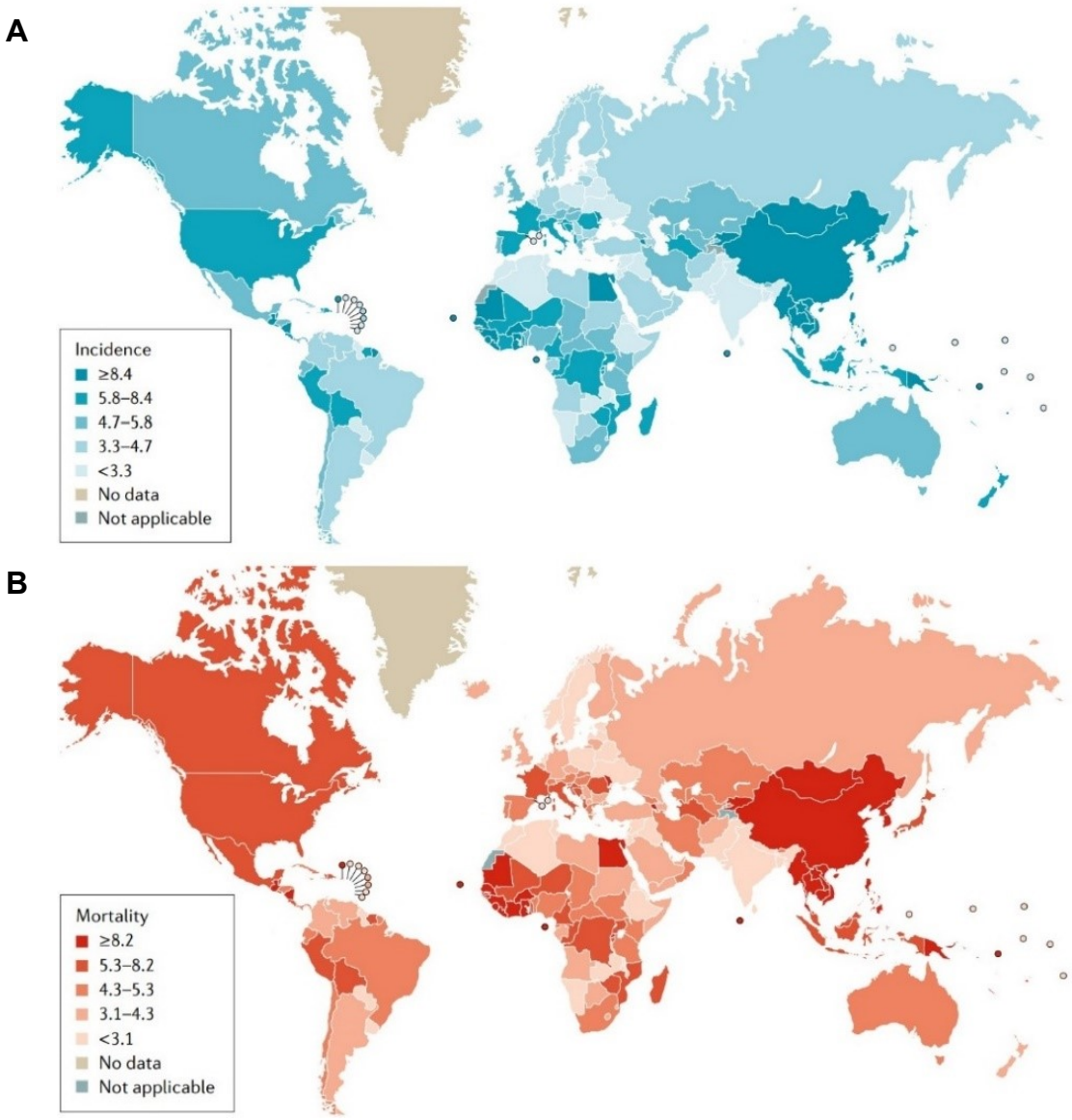



**Figure 1: Global incidence (A) and mortality (B) of primary liver cancer.** There are global variations in disease burden due to limited medical resources such as vaccination against HBV or access to treatment of HCV in East Asia and African countries compared to Western countries, where access is facilitated. Numbers are cases per 100 000 people per year. Taken from (Yang, Hainaut et al. 2019).

HBV is an enveloped partially double-stranded DNA virus, whose genome is located in the nucleus of infected hepatocytes as covalently closed circular (ccc) DNA, forming a very stable complex or is even able to integrate into the host genome (Tsai, Kuo et al. 2018). Of note, only 10% of HBV cases become chronic and this is influenced by the age of infection as infection of new-borns frequently comes along with chronic hepatitis (Farazi and DePinho 2006). In contrast, HCV infection becomes a chronic disease in 60-80% of patients. One reason is immune evasion, as HCV is an enveloped single-strand (+) RNA virus whose RNA and core proteins impair functions of dendritic cells (DCs) and thus T cell activation (Pachiadakis, Pollara et al. 2005). Cell proliferation is targeted by HCV through interaction of viral proteins with the mitogen activated kinases (MAPK) such as extracellular signal-regulated kinase (ERK), MAPK/ERK kinase (MEK), and rapidly accelerated fibrosarcoma (RAF) (Farazi and DePinho 2006).

HCV can be successfully treated with combinations of antiviral drugs such as sofosbuvir and velpatasvir (Epclusa ®) curing the disease in 90% of cases (Hong, Wright et al. 2020). On the one hand, there is a shift in epidemiology as large-scale HBV vaccination and improved access to HCV treatment were successful at reducing the chronic virus-induced liver inflammation and thus HCC in industrialized countries like Europe, Australia, Canada, Japan and the USA (Moon, Singal et al. 2019). On the other hand, diabetes, obesity as well as alcohol abuse contribute to the development of steatohepatitis, known as non-alcoholic steatohepatitis (NASH) or alcohol related liver disease (ALD), in which chronic inflammation of the liver predisposes HCC (Hartke, Johnson et al. 2017).

Alcohol-induced oxidative stress causes increased lipid peroxidation facilitating a permissive HCC microenvironment (McClain, Hill et al. 2002). Diabetes type 2 does not only increase the prevalence of non-alcoholic fatty liver disease (NAFLD), but also contributes to the progression of NAFLD to NASH (Younossi, Golabi et al. 2019). Approximately 10-20% of patients with NAFLD suffer from NASH, and about 20% of NASH patients are likely to develop liver fibrosis and eventually progress to cirrhosis and/or HCC (Itoh, Ogawa et al. 2020).

### Fibrosis and cirrhosis

Continuous liver injury and subsequent regeneration leads to dramatic changes in liver architecture (Fig. 2; (Bataller and Brenner 2005)) by the activation of resident Kupffer cells and infiltration of immune cells, which drive liver inflammation to fibrosis by scarring (Al-Shaebi, Wenzhang et al. 2020). Metabolic stress such as lipotoxicity or constant alcohol consumption causes cell death of hepatocytes, which induces inflammation to clear dead hepatocytes and to activate tissue repair (Itoh, Ogawa et al. 2020).

Stromal cells including immune cells, fibroblasts, and hepatic sinusoidal cells are the executive force mediating regeneration. Upon chronic inflammation, the activation of hepatic stellate cells (HSCs) causes fibrosis due to alterations in the extracellular matrix (ECM) composition such as deposition of collagen (Barcena, Stefanovic et al. 2015). Usually, HSCs remain in a quiescent state, yet in the presence of chronic liver damage, these cells become myofibroblast-like and migrate towards necroptotic areas where continuous cycles of hepatocyte cell death and regeneration take place (Friedman 2008). Proteins such as α-smooth muscle actin (α-SMA), collagen 1A1 (COL1A1), matrix metalloproteinase 9 (MMP9), and tissue inhibitor of metalloproteinases (TIMPs) are upregulated (Barcena, Stefanovic et al. 2015).

Furthermore, activated HSCs secrete cytokines such as transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), hepatic growth factor (HGF), and vascular endothelial growth factor (VEGF) among other cytokines to attract immune cells. The MAPK pathways are crucial modulators of the HSC-mediated fibrotic process (Bataller and Brenner 2005). The c-Jun N-terminal kinase (JNK) initiates apoptosis of hepatocytes and mediates secretion of inflammatory cytokines (Schwabe, Uchinami et al. 2004). Among others, the phosphatidylinositol-3-kinase (PI3K) and TGF-β1-activated Smad pathway stimulate hepatic fibrosis (Schnabl, Kweon et al. 2001, Bataller and Brenner 2005). In patients suffering from fibrosis in the background of viral hepatitis or NASH, natural killer (NK) cells become activated and express anti-fibrotic factors such as interferon (IFN)-γ to stop the fibrotic process by induction of HSC apoptosis (Al-Shaebi, Wenzhang et al. 2020).

IL-34 impedes that process by blocking production and secretion of IFN-γ, while also stimulating differentiation of monocytes into macrophages by binding to macrophage colony stimulating factor (M-CSF) 1 receptor (Preisser, Miot et al. 2014). Advanced fibrosis often results in development of hyperplastic nodules of regenerating hepatocytes which is a characteristic of cirrhosis.

Hepatocellular dysfunction, hepatic insufficiency and portal hypertension result from cirrhosis predisposing HCC development. Yet, HCC can also develop independently from cirrhosis. Notably, the incidence of cirrhosis has been increasing during the last 20 years (Moon, Singal et al. 2019). Despite the long-time accepted theory that cirrhotic disease is irreversible, reversibility of liver fibrosis and cirrhosis was proven for the first time in 2002 (Arthur 2002).

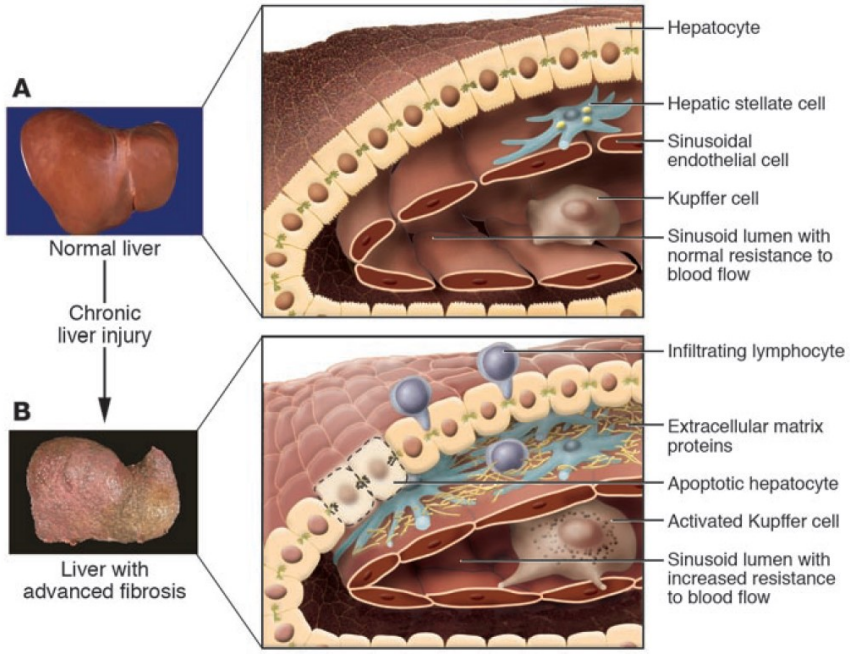



**Figure 2: Chronic liver disease causes change in liver architecture associated with liver fibrosis.** Continuous cycles of tissue damage and liver regeneration results in infiltration of inflammatory lymphocytes, apoptosis of hepatocytes, activation of Kupffer cells and of hepatic stellate cells (HSC), as well as alterations of cell-cell contacts between sinusoidal endothelial cells restricting the blood flow. Taken from (Bataller and Brenner 2005).

## Hepatocarcinogenesis

In the context of chronic HBV infection, viral genome integration into host DNA is often associated with deletions (Farazi and DePinho 2006). If genes are affected, which regulate cell cycle and growth, or protect DNA such as the tumour suppressor p53 or telomerase reverse transcriptase (TERT), the host cell becomes transformed. Additionally, the viral protein HBx alters the expression of growth-control genes and modulates src and MAPK signalling (Tarn, Lee et al. 2001, Farazi and DePinho 2006). Hyperplastic nodules caused by continuous rounds of liver damage and regeneration are considered as the first step towards hepatocarcinogenesis (Fig. 3; (Farazi and DePinho 2006). Dysplastic nodules evolve along with chronic inflammation of the liver and accumulation of mutations. Increase of genomic instability generates malignant cells, which invade the surrounding fibrous stroma, vessels and might even metastasise to distant sites. In most HCC patients, key oncogenes and tumour-suppressors, apart from the already mentioned, such as β-catenin, epidermal growth factor receptor (EGF-R), HGF receptor (c-Met), the cell cycle inhibitor $p16^{INK\ 4A}$, epithelial (E)-cadherin and cyclooxygenase 2 (COX2) are frequently deregulated (Farazi and DePinho 2006).

Furthermore, telomere shortening is a key event in the initiation of liver cancer driving genomic instability. Telomerase re-activation occurs at later stages and seems to be crucial for the malignant progression. This was experimentally shown by dysfunctional telomerase, which stopped HCC progression (Farazi, Glickman et al. 2003). Other cytogenetic features of cancer cells are dysfunctions of chromosome segregation, which is often found in HCC cells, as Aurora kinase A and its target hepatoma upregulated protein (HURP) are overexpressed (Andrews 2005).

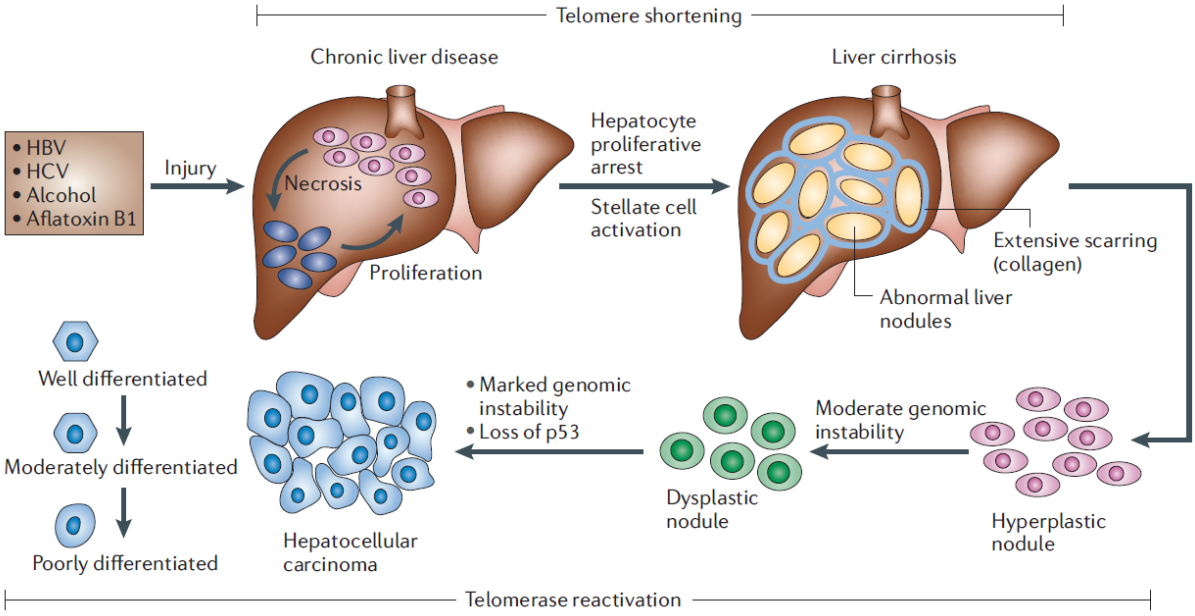



**Figure 3: Development of hepatocellular carcinoma (HCC).** There are different risk factors causing liver injury and chronic liver disease by continuous necrosis and proliferation of hepatocytes. Advanced liver fibrosis results in cirrhosis which is characterized by the abnormal liver nodule formation surrounded by collagen deposition and scarring. Hyperplastic nodules develop to dysplastic ones by accumulation of mutations, which then results in HCC upon loss of p53 and increased genomic instability. Taken from (Farazi and DePinho 2006).

## Tumour progression and metastasis

Increasing genomic instability, telomere shortening, accumulation of mutations and chromosome aberrations initiate the metastatic cascade. The first steps of metastasis are denoted with cytoskeletal rearrangements combined with changes of the epithelial phenotype, known as epithelial-to-mesenchymal transition (EMT) (Quail and Joyce 2013). EMT is crucial for embryonic development as it is not only required for the formation of the mesoderm and gastrulation, but also for organogenesis. Cells undergoing primary, secondary and tertiary EMTs give rise to various tissues and organs such as the neural crest and the heart (Thiery, Acloque et al. 2009). As a result, cells transformed to EMT become more stem cell-like in order to build e.g. the mesenchyme from the primitive streak (Zhang and Weinberg 2018). The same mechanisms including signalling pathways and regulators are used in pathological processes such as cancer progression and fibrosis. For instance, hepatocytes are able to undergo EMT during $CCl_4$ induced liver fibrosis (Zeisberg, Yang et al. 2007, Thiery, Acloque et al. 2009). During this process, tumour cells lose their epithelial markers such as E-cadherin while switching to a stem-cell-like, mesenchymal phenotype with increased migratory abilities.

Activation of Snail, Zeb, E47 and Klf8 represses the E-cadherin promoter directly, while E-cadherin transcription can also be decreased indirectly via Twist, Goosecoid, E2.2 and FOXC2 (Peinado, Olmeda et al. 2007, Thiery, Acloque et al. 2009). Furthermore, Ras and Wnt signalling cooperating with TGF-β signalling can induce EMT. Smad proteins act together with Snail to repress tight junction proteins as well as E-cadherin during the TGF-β-driven EMT (Vincent, Neve et al. 2009). In course of EMT, the cell polarity is lost, which is a key feature of epithelial cells. Canonical TGF-β signalling activates Snail and Zeb resulting in the downregulation of cell adhesion constituents, but also the noncanonical TGF-β pathway was described to initiate degradation of RhoA and leading to the re-organization of the actin cytoskeleton (Thiery, Acloque et al. 2009, Bendris, Arsic et al. 2012). Hypoxic conditions also induce EMT via Snail and Twist, which is a direct target of the hypoxia inducible factor (HIF)-1α. In order to disseminate from the primary tumour site, carcinoma cells enter new blood vessels, which were generated upon tumour neo-angiogenesis to provide sufficient blood supply, or intravasate into the lymphatic system (Fig. 4; (Chambers, Groom et al. 2002, Obenauf and Massague 2015)).

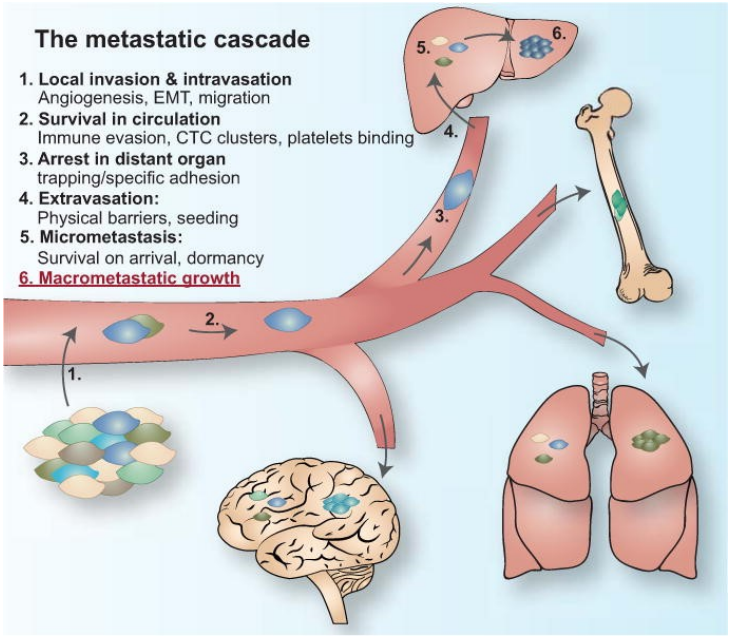



**Figure 4: The process of metastasis.** (1) Local invasion and intravasation, (2) survival in the circulation, (3) arrest at the distant site, (4) extravasation, (5) survival as micrometastasis, (6) colonization of target organs. Taken from (Obenauf and Massague 2015).

Once tumour cells survive in the circulation by evading the immune system and by platelet shielding, they can extravasate into distant organs and build micrometastases (Obenauf and Massague 2015). For that, the disseminated quasi-mesenchymal tumour cells need to change their phenotype again by undergoing the mesenchymal-to-epithelial transition (MET) (Zhang and Weinberg 2018). Reversion to the epithelial phenotype facilitates tumour metastases at distant sites. The resulting cellular phenotypes varying on the epithelial to a mesenchymal-like axis contribute to the vast tumour heterogeneity, which is an advantage for the cancer, but pose major obstacles in tumour therapy (Dagogo-Jack and Shaw 2018). Clinical studies showed that the process of metastasis is quite inefficient as even small tumours release circulating tumour cells, yet only few patients develop metastases after a period of latency (Massague and Obenauf 2016).

## Treatment

The Barcelona Clinic Liver Cancer (BCLC) system is the most commonly used staging system of HCC (Forner, Reig et al. 2018). BCLC 0 is defined as very-early stage HCC with solitary lesions. Up to 3 nodules smaller than 3 cm in diameter define the early stage (BCLC A) with preserved liver function. Patients in these groups are eligible for therapeutic options such as liver resection, transplantation or ablation with prognostic survival of more than 5 years. The intermediate stage (BCLC B) is marked by multiple nodules without vascular invasion or extrahepatic spread, and patients in this stage can benefit from transarterial chemoembolization as long as their liver function is not impaired. BCLC C is the advanced stage and patients show either vascular invasion, extrahepatic spread, or mild cancer-related symptoms. Systemic treatment with sorafenib and regorafenib, both tyrosine kinase inhibitors, are the therapies which successfully prolong survival. There are other tyrosine kinase inhibitors such as lenvatinib as well, which are nowadays applied in combinatorial therapeutic approaches. For second line treatment the VEGFR1-3, Met, and Axl inhibitor cabozantinib has been an effective therapeutic option even in sorafenib resistant patients (da Fonseca, Reig et al. 2020). Yet, the prognostic survival is about 1 year in the advanced stage. The terminal stage (BCLC D) is characterised by poor liver function and severe cancer-related symptoms (Forner, Reig et al. 2018). Transplantation is not applicable due to poor patient prognosis, therefore end stage patients are provided with supportive care. Furthermore, the albumin-bilirubin (ALBI) score is able to stratify patients across their BCLC stages according to their liver function (Johnson, Berhane et al. 2015). The α-fetoprotein serves as tumour marker as increased levels are associated with poorer prognosis.

Altogether, therapeutic options are limited, and survival prognosis is poor mostly due to late diagnosis. Advanced stage metastasis is still the most frequent cause of death in patients with solid tumours in general as well as in HCC (Acharyya and Massague 2016). Therefore, studying the process of tumour progression and metastasis results in further insights into disease mechanisms, and thus leads to innovative therapeutic strategies to combat cancer progression and relapse while increasing patient survival and patient care.

## The tumour microenvironment

As proposed by Stephan Paget in 1889, the tumour cells, to which he referred as “the seed”, metastasise to specific niches, referred to as “the soil”. This means that metastasis only results from implanting the appropriate “seed” in the suitable “soil” (Paget 1989, Langley and Fidler 2011). Nowadays, there are various studies confirming Paget’s hypothesis that the tumour microenvironment (TME) is crucial for regulating the growth of new tumour foci (Quail and Joyce 2013). Upon tumour progression, systemic signals such as inflammatory cytokines, exosomes and matrix remodelling enzymes released from the primary tumour prepare the pre-metastatic niche leading to recruitment of stromal cells even before tumour cells disseminate and arrive at these sites (Massague and Obenauf 2016). Many cancer cells secrete exosomes, which contain factors that induce vascular leakiness, inflammation and TGF-β secretion at the target organ (Costa-Silva, Aiello et al. 2015).

The hallmarks of cancer described by Hanahan and Weinberg constitute a conceptual rationale about the common characteristics for heterogenous cancer diseases (Hanahan and Weinberg 2011). Yet these commonalities depend to a large extent on stromal cells in the TME, which can be grouped into 3 categories: (i) infiltrating immune cells, (ii) cancer-associated fibroblastic cells, and (iii) angiogenic vascular cells (Fig. 5; (Hanahan and

Coussens 2012)). Despite the fact that driving oncogenic mutations are essential for the development of cancer, the TME is able to support uncontrolled growth. The mitogens secreted by the TME influence cancer cells right from the initiation of malignancy to advanced stages.

Angiogenic vascular cells such as endothelial cells and their supporting pericytes direct neovascularization. Therefore, inhibition of angiogenesis is effective at blocking hyperproliferation of cancer cells (Carmeliet and Jain 2011). Of note, not only the factors arriving with the blood stimulate proliferation of tumour cells, but trophic factors which are secreted by angiogenic vascular cells modulate the malignant phenotype of cancer cells as well. In addition, cancer-associated fibroblastic cells as well as mesenchymal stem cells can be activated to differentiate into myofibroblasts or adipocytes which express HGF, EGF, insulin-like growth factor (IGF), stromal-derived factor-1 (SDF-1/CXCL12), FGFs and importantly TGF-β (Hanahan and Coussens 2012). In contrast, “normal”, connective tissue fibroblasts even suppress the growth of cancer cells along with contact inhibition in order to regulate epithelial homeostasis (Flaberg, Markasz et al. 2011). They can also block apoptosis of cancer cells by ECM remodelling and production of ligands for anti-apoptotic integrins (Hanahan and Coussens 2012, Zeltz, Primac et al. 2020).

Tumour cells often secrete cytokines attracting immune cells of the myeloid and lymphoid lineage, referred to as infiltrating immune cells, which then directly as well as indirectly stimulate proliferation of neoplastic cells (Balkwill, Charles et al. 2005). Infiltrating immune cells secrete EGF, tumour necrosis factor-α (TNF-α), FGFs, ILs, chemokines, histamine, heparins as well as TGF-β in order to send survival signals for maintaining tissue integrity, which is exploited by tumour cells. For instance, tumour-associated macrophages (TAMs) expressing α4-integrin have been found to bind vascular cell adhesion molecule-1 (VCAM-1) on breast cancer cells. This promotes survival of the cancer cells by downstream activation of PI3K/Akt signalling (Chen, Zhang et al. 2011). Even chemoresistance of breast cancer cells was influenced by TAMs as the high levels of cathepsin expressed by the macrophages were able to protect the cancer cells from cell death (Shree, Olson et al. 2011).

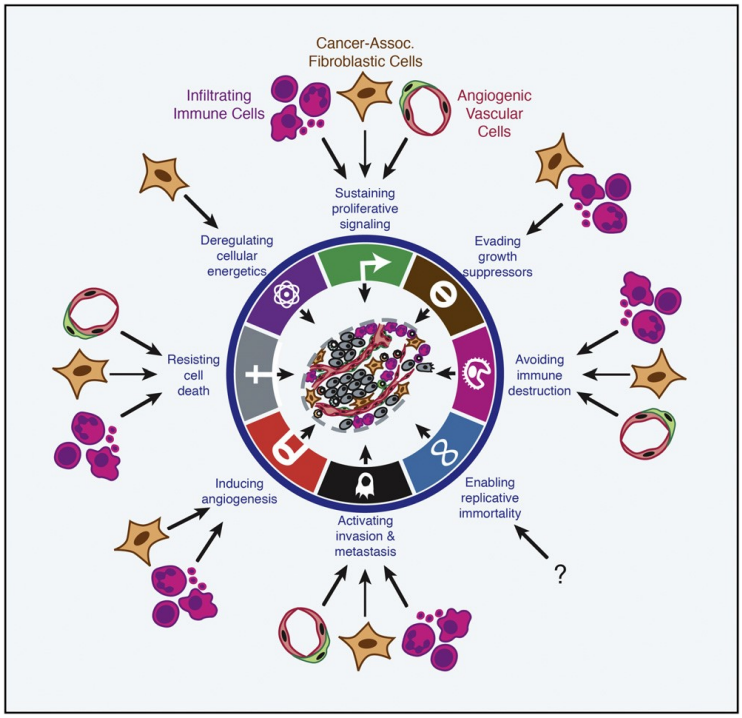



**Figure 5: Activated and recruited stromal cells are linked to the hallmarks of cancer.** There are 8 acquired hallmarks of cancer, 6 of them are core hallmarks and 2 are emerging. 7 hallmarks involve stromal cells of the TME. Taken from (Hanahan and Weinberg 2011).

Furthermore, the phenotype of infiltrating immune cells in the TME mimics the one in wound healing. First, infiltrating immune cells remodel the ECM by expressing proteolytic enzymes such as metallo-, serine or cysteine proteases to restore tissue integrity, which is also taking place in the TME (Hanahan and Coussens 2012). Additionally, these proteases are able to release membrane-bound precursor growth factors in order to activate them or other ECM ligands influencing the stoichiometry of extracellular signalling molecules which promotes proliferation. Under physiological conditions, infiltrating leukocytes have an immunosuppressive effect in order to stop tissue damage by NK cells and T cells. Again this feature is exploited by cancer cells by avoiding immune destruction (Ruffell, DeNardo et al. 2010). Regulatory T cells, immature myeloid cells and myeloid-derived suppressor cells are programmed to help cancer cells to escape cytotoxic T lymphocytes (CTLs). Yet, T cell inhibition is also mediated by macrophages, which express ligands for costimulatory receptors of T cells (Topalian, Drake et al. 2012). Leukocytes play an important role in the process of tumour progression and metastasis (Acharyya and Massague 2016). The infiltration of macrophages and neutrophils leads to the polarization from a pro-inflammatory macrophage or neutrophil type 1 (M1 or N1), usually induced by IFN-γ, towards an immunosuppressive M2 or N2 phenotype, which can be induced by IL-4, IL-13 or TGF-β (Coffelt, Wellenstein et al. 2016, Wu, Saxena et al. 2019). While M1 express pro-inflammatory cytokines including TNF-α, the M2 cells secrete anti-inflammatory factors like arginase-1/nitric oxide synthase (ARG-1/NOS) and IL-10 (Ostrand-Rosenberg 2008). The macrophage polarization is thought to be more explicit than neutrophil polarization as there are distinct markers expressed, which is not the case for N1 and N2 (Wu, Saxena et al. 2019). The M2 phenotype shares many similarities with TAMs, yet their transcriptional profile is distinguishable (Lawrence and Natoli 2011). The original study about neutrophil polarization suggests that N1 show hypersegmented nuclei, whereas N2 can be identified by banded or ring-like nuclei similar to immature neutrophils (Fridlender, Sun et al. 2009). Yet, it has not been entirely ensured that N2 are immature neutrophils (Coffelt, Wellenstein et al. 2016). Anyways, tumour-associated neutrophils (TANs) influence CTLs in order to suppress the anti-tumour response (Wu, Saxena et al. 2019). The role of neutrophils in cancer has many facets and are therefore introduced in detail.

## Neutrophils

In order to understand the role of neutrophils in cancer, their physiological role in wound healing and infection is briefly discussed (Coffelt, Wellenstein et al. 2016). Neutrophils are polymorphonuclear leukocytes derived from myeloid precursors in the bone marrow (Fig. 6). They have important functions in innate immunity, as these cells are the first ones at inflammation sites or arrive together with platelets as first response to sites of injury in wound healing (Kolaczkowska and Kubes 2013). Usually, neutrophils make up to 50-70% of leukocytes in humans and around 30-40% of leukocytes in mice (O'Connell, Mikkola et al. 2015). In the case of acute inflammation, the number of neutrophils increases, which is then downregulated by DCs and macrophages upon clearance of infection via IL-23/IL-17 suppression (Greenlee-Wacker 2016). Almost two-thirds of the space in the bone marrow is used for formation of neutrophils and monocytes under homeostasis, only 1-2% of neutrophils are located in the circulation (Borregaard 2010). Their release from the bone marrow is dependent on the interplay between 2 C-X-C motif receptors, i.e. CXCR2 promoting egress by binding its ligands CXCL1/2/5 (Fig. 6) and CXCR4 retaining neutrophils by interaction with CXCL12 (Coffelt, Wellenstein et al. 2016, Jablonska, Lang et al. 2017). Neutrophils undergo maturation resulting in sequential segmentation of the nucleus as well as formation of granules from the promyelocyte to the polymorphonuclear cell stage. The primary, also called azurophilic granules containing myeloperoxidase (MPO) mark the differentiation from the myeloblast into the promyelocyte (Galli, Borregaard et al. 2011). Subsequentially, the transition from the myelocyte to the metamyelocyte is accompanied by the development of secondary, also known as specific granules carrying lactoferrin. Last, the tertiary gelatinase granules form as the band cell matures into the segmented cell giving rise to mature neutrophils (Galli, Borregaard et al. 2011, Coffelt, Wellenstein et al. 2016).

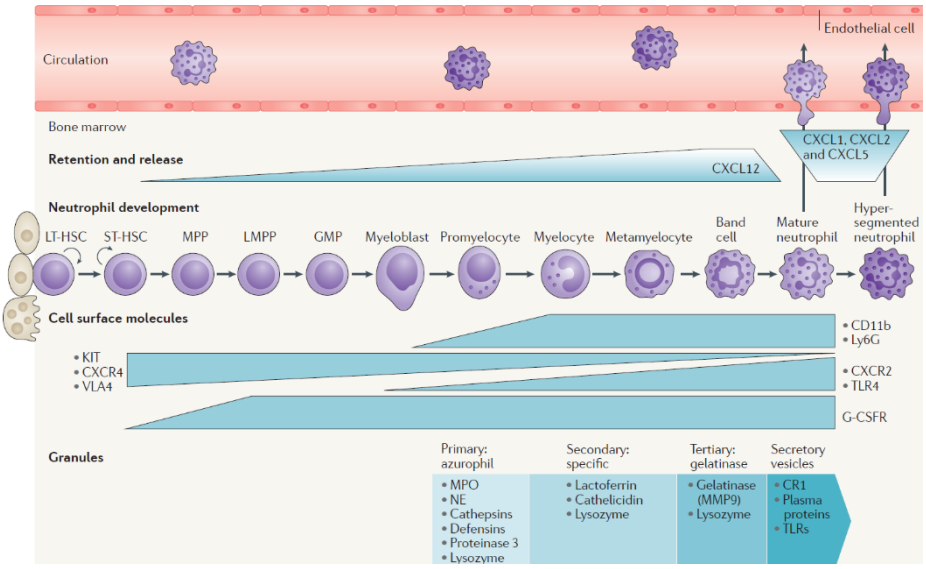



**Figure 6: Differentiation of neutrophils during homeostasis.** Neutrophil development starts in the bone marrow, where self-renewing long-term haematopoietic stem cells (LT-HSC) differentiates into a short-term haemato-poietic stem cell (ST-HSC), which then becomes a multipotent progenitor (MPP) without self-renewing capacity. MPPs transits into lymphoid-primed MPPs (LMPP), which give rise to granulocyte-monocyte pro-genitors (GMPs). These GMPs differentiate into granulocytes and as such myeloblasts in the presence of granulocyte colony stimulating factor (G-CSF) or granulocyte-macrophage colony stimulating factor (GM-CSF). Primary granules develop during the transition to myelocytes, secondary granules mark the transition from myelocytes to metamyelocyte and tertiary granules appear upon the change of the nuclear morphology from banded to segmented shape which give rise to mature neutrophils. Neutrophils can either be retained in the bone marrow or released into the circulation under homeostatic conditions. Taken from (Coffelt, Wellenstein et al. 2016).

The lifespan of neutrophils is dependent on their location, as the ones in the circulation live up to 7 hrs, while the ones residing in tissues were found to be viable for several days (Cheretakis, Leung et al. 2006).

Neutrophils have important functions in elimination of pathogens. One of these mechanisms is phagocytosis, in which microorganisms are encapsulated in phagosomes. Reactive oxygen species (ROS) or antibacterial proteins such as cathepsins, defensins, lactoferrin and lysozyme mediate killing of the pathogens either intracellularly in the phagosomes or extracellularly by releasing granule contents into the extracellular milieu (Borregaard 2010).

Another mechanism upon strong activation of neutrophils is the formation of extracellular DNA traps (NETs) (Fig. 7; (Brinkmann and Zychlinsky 2012)). The extracellular DNA is associated with granule contents such as cathepsin G, MPO and neutrophil elastase (NE) in order to immobilize and kill pathogens. The binding mechanism how microbes interact with the NETs is not entirely unravelled, yet electrostatic interactions are thought to play a major role. Furthermore, antimicrobial effects of histones have been described already in the 1950s, but these findings were brought into context only after the discovery of NET formation (Hirsch 1958). Multiple stimuli including pyrogens, chemicals like phorbol esters, ionophores, cytokines, antibody-covered beads, bacterial, and fungal fragments are able to activate the release of neutrophil DNA and granule contents (Brinkmann and Zychlinsky 2007). There are at least two pathways including lytic as well as vital NET formation (Jorch and Kubes 2017). In the lytic pathway, also called NETosis referring to this certain kind of cell death in neutrophils upon NET release, external stimuli such as bacteria, fungi, ionophores or phorbol esters can induce either NADPH-dependent or -independent formation of NETs (Ravindran, Khan et al. 2019). Stimuli such as phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS) induce NADPH-dependent NET formation through different mechanism. PMA causes calcium release from the endoplasmic reticulum (ER) thus activating protein kinase C (PKC) (Kaplan and Radic 2012). PKC phosphorylates NADPH-oxidase leading to the generation of ROS, as well as activation of RAF, MEK and ERK kinases (Fig. 7B). Although there is inconsistency in the literature whether this intracellular efflux of calcium is sufficient to activate the protein-arginine deiminase 4 (PAD4), ROS are suggested to activate PAD4 otherwise (Fig. 7C; (Ravindran, Khan et al. 2019)). PAD4 converts arginine into citrulline on histones resulting in decondensation of the chromatin (Jorch and Kubes 2017). Yet, citrullination is not an obligatory process in the NADPH-dependent pathway as NET formation takes place with and without PAD4 activation (Holmes, Shim et al. 2019). Ionophores like ionomycin and calcium ionophore A23187 rather stimulate formation of citrullinated NETs in a NADPH-independent way due to the extracellular influx of calcium, which directly activates PAD4 in the cytosol and mediates its translocation into the nucleus (Parker, Dragunow et al. 2012). Of note, none of NET formation stimuli exclusively induces citrullinated or uncitrullinated NETs, as there is always histone citrullination present, yet to different extents (Holmes, Shim et al. 2019). This renders citrullinated histone H3 a good qualitative marker of NET formation in general, yet quantitative interpretation should be conducted with precaution.

However, ionophore-induced calcium influx can activate potassium channels of small conductance and mitochondrial ROS production in neutrophils (Douda, Khan et al. 2015). ROS are mainly responsible for the release of NE and MPO into the cytosol due to disintegration of the granule membranes (Ravindran, Khan et al. 2019).

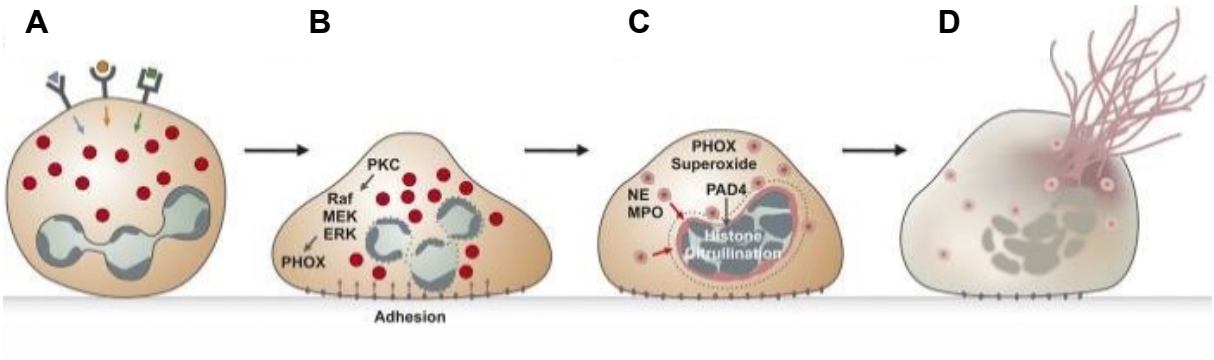



**Figure 7: Lytic neutrophil extracellular trap (NET) formation.** (A) NET formation stimuli like interleukin-8 (IL-8)/CXCL8 activate receptors for downstream signalling. (B) Protein kinase C (PKC) and mitogen activated kinases (MAPK) pathways are activated mediating neutrophil adhesion. (C) Arginine is converted into citrulline by protein-arginine deiminase 4 (PAD4) and attached to histones leading to decondensation of the chromatin, while the granule contents like neutrophil elastase (NE) and myeloperoxidase (MPO) are mobilized and mixed with the cytoplasm. (D) Membrane rupture releases nucleic acid and associated granule contents into the cell exterior. Taken from (Brinkmann and Zychlinsky 2012).

Once in the cytosol, ROS/MPO-dependent NE activation facilitates actin degradation in the cell interior (Metzler, Goosmann et al. 2014). Soluble NE and MPO translocate to the nucleus supporting further relaxation of the chromatin and delobulation by processing of histones and tight association with DNA. Thus, upon breakdown of the nuclear envelope, the nuclear content is mixed with cytosolic components and associates further with granule proteins (Fig. 7D). Therefore, NETosis can be monitored using extracellular MPO and NE as marker. The combination of citrullinated histone H3 and MPO is even described as hallmark of NET formation (Santos, Martin et al. 2018). Altogether, lytic NETosis leads to release of NETs by disruption of the plasma membrane after adhesion, delobulation of the nucleus and mixing of cytoplasmic and nuclear contents (Erpenbeck and Schon 2017).

In contrast to that, vital NET formation leads to controlled release of extracellular DNA traps while maintaining neutrophil functions in host defence such as phagocytosis (Fig. 8; (Yipp and Kubes 2013)). Sterile infections and agents such as PMA are known to induce early vital NET formation, yet PMA was also shown to stimulate suicidal NETosis. In non-sterile infections as well, vital NET formation allows maintenance of membrane integrity while imprisoning of pathogens, which makes the capture more successful as complete lysis of neutrophils might free the pathogen again (Yipp and Kubes 2013).

There are multiple hypotheses suggesting different subsets of neutrophils, which take different roles in the process of trapping and killing pathogens. While some neutrophils trap pathogens in lytic NETs, the remaining viable neutrophils kill these. Still, there is no clear evidence on this specific mechanism. Vital NET formation was suggested to rely mainly on the release of mitochondrial DNA (Yousefi, Mihalache et al. 2009). Interestingly, electron microscopy could identify even another vital mechanism of NET formation where nuclear DNA was budded of the neutrophils without rupturing the membrane (Pilsczek, Salina et al. 2010).

Apart from NET formation as mechanism to immobilize and kill pathogens, it can also activate the complement system due to the release of component 3 (C3), complement factor P, and complement factor B (Ravindran, Khan et al. 2019). C3 is cleaved into C3a and C3b followed by forming the C5 convertase, which is required for the membrane-attack complex.

These findings have important implications for diseases such as systemic lupus erythematosus, rheumatoid arthritis, and cystic fibrosis, which have been found to be NET-associated pathologies (Ravindran, Khan et al. 2019).

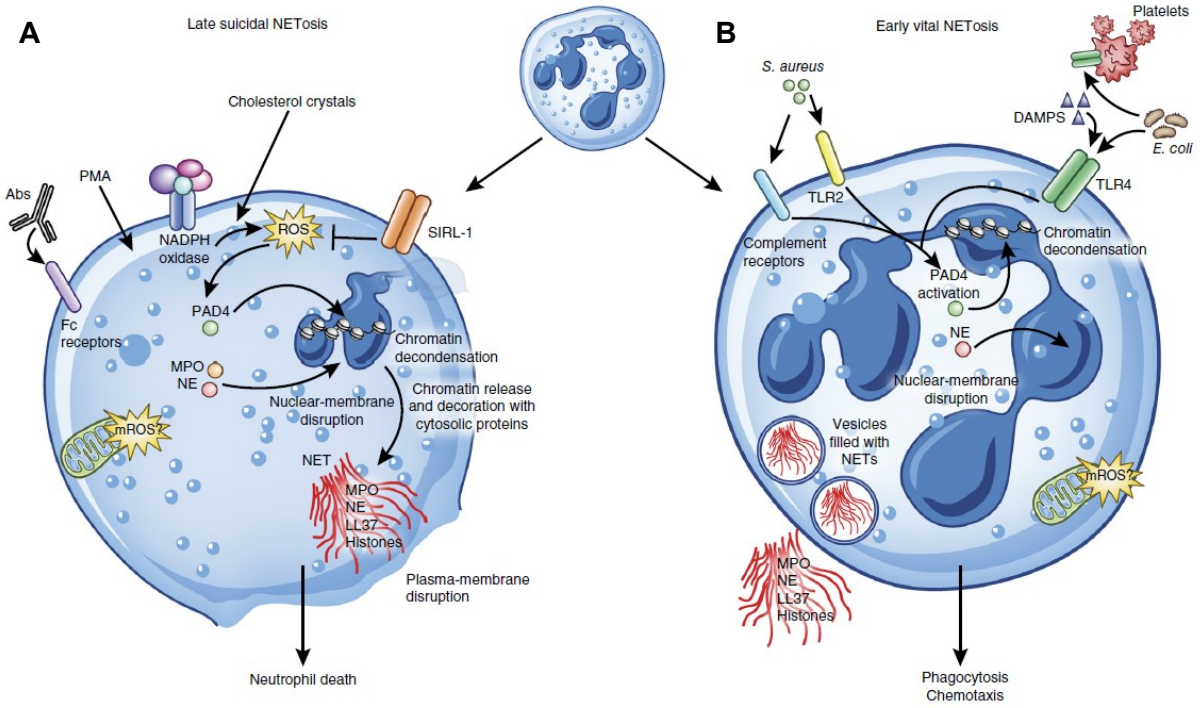



**Figure 8: Comparison of lytic and vital NET formation.** (A) Suicidal NETosis results in membrane rupture. (B) Vital NET formation occurs much earlier than the lytic one, usually within minutes compared to hours. Vesicles filled with NETs are released maintaining other neutrophil effector functions such as phagocytosis. Taken from (Jorch and Kubes 2017).

## Neutrophils and Cancer

Cancer is also conceived as a wound that never heals, therefore normal wound healing is compared to pathologic wound healing taking place in the malignant process (Singel and Segal 2016). Neutrophils and platelets, being the first at the site of injury and infection, are attracted by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), which are also released at tumour sites due to continuous necrosis and regeneration comparably to chronic inflammation (Singel and Segal 2016). Platelets are activated by binding of their P-selectins to P-selectin glycoprotein ligand (PSGL)-1 and CD24 on endothelial cells, which induces intracellular adhesion molecule (ICAM)-1 expression in endothelial cells facilitating neutrophil adhesion. Additionally, the mitochondria of activated platelets are released, which then interact with neutrophils (Singel and Segal 2016). These events together with integrin signalling and leukotriene release cause neutrophil clustering and sealing of the wound (Lammermann, Afonso et al. 2013). Normal wound healing leads to a controlled inflammatory response, where macrophages and DCs follow to the site of injury clearing neutrophil inflammation by efferocytosis, which is phagocytosis of dying cells (Greenlee-Wacker 2016). Afterwards, tissue repair is initiated by M2 macrophages and T cell suppression leading to clearance of the inflammation, regeneration and vascularization. In cancer and in pathological wound healing, disordered inflammatory responses cause persistent inflammation. Constant activation of neutrophils impedes regeneration and resolution of the injury (Singel and Segal 2016).

Angiogenesis, tissue remodelling, fibrosis, and chronic inflammation are advantages for the cancer facilitating its progression and metastasis. Furthermore, tumours often induce neutrophilia, i.e. the increased neutrophil count in the circulation and overactive granulopoiesis, i.e. the excessive generation of granulocytes, as a result of IL-23/IL-17-dependent secretion of granulocyte colony stimulating factor (G-CSF), which leads to release of immature neutrophils from the bone marrow (Coffelt, Wellenstein et al. 2016, Singel and Segal 2016).

On the one hand, activated neutrophils are able to kill tumour cells directly or indirectly. Direct mechanisms involve lysis and cytotoxicity mediated by NADPH-oxidase or the MPO-$H_2O_2$-halide system (Wu, Saxena et al. 2019). Activation of NADPH-oxidase converts molecular oxygen to superoxide anions, which is then converted into $H_2O_2$ and hydroxyl anions, and activates intracellular proteases generating NETs (Fuchs, Abed et al. 2007). Indirect tumour cell killing takes place by release of NE, which is taken up by the cancer cells. Ingestion of NE makes cancer cells more susceptible to cytotoxic T cell mediated response (Mittendorf, Alatrash et al. 2012). Additionally, neutrophils have been found to reject transplanted tumours of G-CSF producing colon carcinoma cells supporting their anti-tumour functions (Coffelt, Wellenstein et al. 2016). On the other hand, neutrophils were also found to promote all three steps in cancer – initiation, promotion and progression.

### Tumour initiation

Inflammation is one of the main drivers in carcinogenesis and this is where neutrophils as mediators of inflammation come into play. Chemical carcinogens or chronic inflammation linked to e.g. colitis-induced expression of CXCR2 ligands such as CXCL1/2/5 attract neutrophils to the site of tumour initiation (Katoh, Wang et al. 2013). Additionally, Kirsten rat sarcoma virus (Kras) oncogene-driven carcinoma show upregulation of GM-CSF and CXCL8 causing neutrophil expansion. Although it remains unclear whether all Kras-driven carcinomas depend on neutrophils, lung cancer with Kras mutations induced by chemical carcinogens such as cigarette smoke are initiated by neutrophil activation (Gong, Cumpian et al. 2013). NE, production of ROS and reactive nitrogen species (RNS) as well as angiogenic factors such as MMP9 are thought to contribute to neutrophil-mediated tumour initiation (Coffelt, Wellenstein et al. 2016).

### Tumour promotion

Due to the fact that neutrophils are promoters of angiogenesis as they remodel the ECM by secreting MMP9 and angiopoietin-1 (Neagoe, Brkovic et al. 2009), they also enhance tumour proliferation by paving the way for their oxygen and nutrient supply. HIF-1α and its downstream targets CXCL12 and VEGF are involved in neutrophil recruitment and retain neutrophils in angiogenic environments such as the TME (Jablonska, Lang et al. 2017). TAMs secrete IL-1β and this causes γδ T cell-depending expression of IL-17 raising G-CSF levels and thus neutrophil expansion (Fig. 9A; (Coffelt, Wellenstein et al. 2016)). Neutrophilia and tumour-promoting inflammation are drivers of tumour growth and progression. Additionally, neutrophil accumulation can be caused by inflammatory cytokines such as TNF-α, which induces IL-17 production in CD4+ cells mediating tumour-promoting inflammation (Charles, Kulbe et al. 2009). Yet, TNF-α signalling is strongly context-dependent as opposing findings have been published (Finisguerra, Di Conza et al. 2015). Vice versa, carcinogenic

stimuli such as ultra-violet (UV) light is able to directly act on neutrophils by inducing TNF-α secretion which stimulates cancer cells to migrate towards endothelial cells (Fig. 9B; (Coffelt, Wellenstein et al. 2016)). Signalling through the CXCR2 axis in neutrophils was found to be involved in the tumour promotion in various mouse models (Sano, Ijichi et al. 2019). General loss of CXCR2 leads to a reduction of neutrophils at tumour sites and is also involved in angiogenesis by regulating endothelial proliferation and tube formation. Furthermore, the TME can upregulate CXCL-chemokines for CXCR2 as well as CCL-chemokines, IL-1β, and TNF-α, which stimulates not only neutrophils, but also migration and invasion of cancer cells (Sano, Ijichi et al. 2019). Altogether, it is suggested that CXCR2 signalling is responsible for neutrophil recruitment, yet it does not directly activate effector functions (Coffelt, Wellenstein et al. 2016).

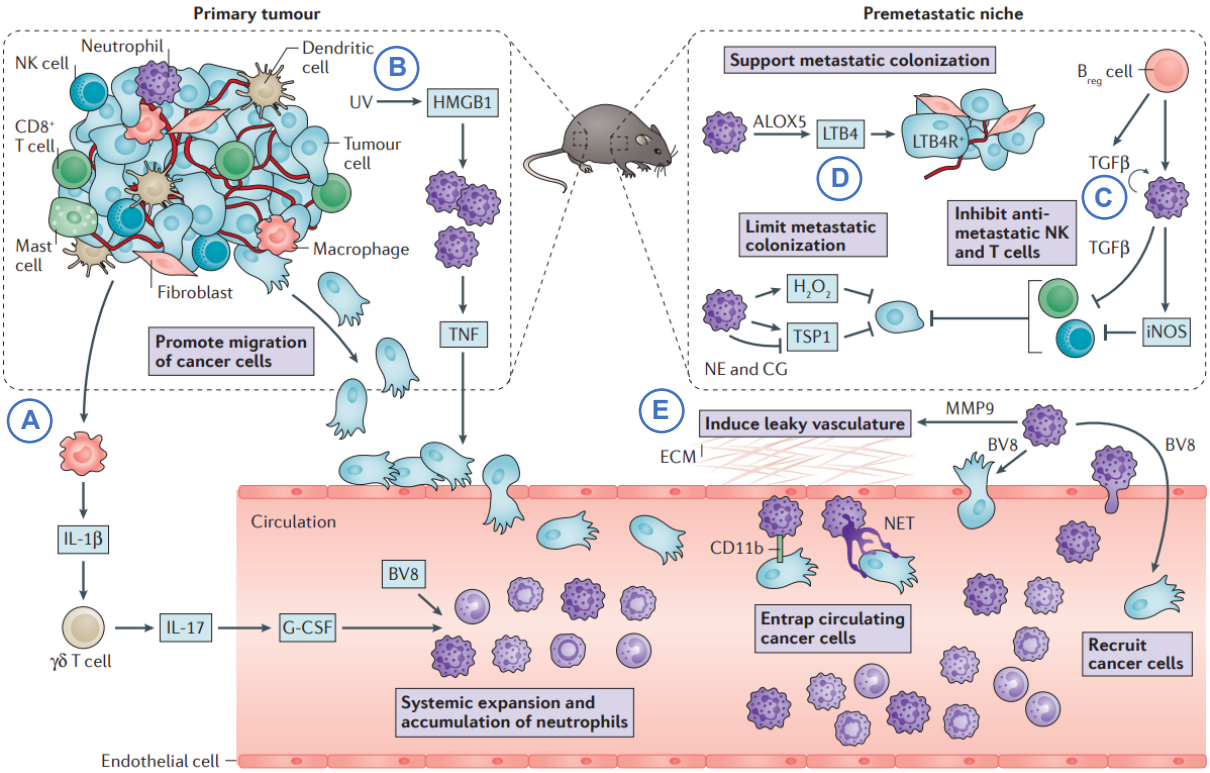



**Figure 9: Neutrophils in the metastatic process.** (A) In breast cancer, interleukin-1β (IL-1β) expressing macrophages stimulate γδ T cells to secrete IL-17, which mediates granulocyte-colony stimulating factor (G-CSF)-dependent neutrophil expansion. (B) In melanoma, ultra-violet (UV) light can recruit neutrophils by activation of high mobility group box 1 (HMGB1) in keratinocytes. These neutrophils are able to secrete tumour necrosis factor (TNF) inducing cancer cell migration towards endothelial cells. (C) In the pre-metastatic niche, regulatory B cells stimulate neutrophils to inhibit anti-tumour natural killer (NK) and T cells and to produce inducible nitric oxide synthase (iNOS), which impedes with anti-tumour CD8+ T cell responses. (D) Leukotriene production of neutrophils, e.g. leukotriene B4 (LTB4), promotes the metastatic colonization of LTB4 receptor (LTB4R)-positive cancer cells. Thrombospondin 1 (TSP1) or $H_2O_2$ have antimetastatic functions, but TSP1 is degraded by neutrophil elastase (NE) and cathepsin G during inflammation. (E) Neutrophils induce a leaky vasculature by secretion of matrix metalloprotease 9 (MMP9) to support extravasation of disseminated cancer cells and BV8, also known as prokineticin 2. Circulating tumour cells can be trapped by cell surface molecule CD11b or by neutrophil extracellular traps (NETs), which are associated with promotion of metastasis. Adapted from (Coffelt, Wellenstein et al. 2016).

### Tumour progression and metastasis

Neutrophils contribute to each step in the metastatic process – starting with establishing a pre-metastatic niche, entrapping circulating tumour cells, and resulting in supporting extravasation (Fig. 9C-E; (Coffelt, Wellenstein et al. 2016)). Activated neutrophils are able to prepare a pre-metastatic niche by secretion of ROS and tissue remodelling prior to tumour seeding (Hiratsuka, Watanabe et al. 2006, Singel and Segal 2016). In addition, TGF-β signalling in tumour-associated regulatory B cells modulates neutrophils so that these inhibit NK and cytotoxic T cells (Fig. 9C). Similar to an injury scenario, neutrophils produce leukotriene B4 (LTB4) as a lipid by-product from the arachidonate 5-lipoxygenase (ALOX5) causing swarming behaviour (Fig. 9D). LTB4 can bind to the LTB4 receptor (LTB4R) on disseminated cancer cells facilitating colonization of these cells at a new metastatic site. In addition, metastasis is promoted by activation of the chemokine ligand 9 (CCL9)/CCR1 signalling axis as these mediators recruit not only neutrophils but also immature myeloid cells (Kitamura, Fujishita et al. 2010). Neutrophils induce a leaky vasculature by expression of MMP9 and trap circulating tumour cells via CD11b in order to promote extravasation (Chen, Hajal et al. 2018). Prokineticin (BV8) is a small protein produced by granulocytes and activates the MAPK pathway upon binding to its receptor causing cancer cell migration (Fig. 9E; (Acharyya and Massague 2016)).

### NET formation in cancer

Cancer cells induce neutrophil expansion, and these peripheral neutrophils are primed for forming extracellular traps (Demers, Krause et al. 2012). Following this priming process, factors like GM-CSF and complement factor 5a (C5a) stimulate NET formation (Yousefi, Mihalache et al. 2009). Also, tumour-derived TNF-α, IL-8/CXCL8 and growth-regulated oncogene (GRO)-α/β cause NET formation (Fig. 10; (Cools-Lartigue, Spicer et al. 2014, Lee and Naora 2019, Jaillon, Ponzetta et al. 2020)). On the one hand, NETs are able to prime DC and T cells to support the anti-metastatic process (Jablonska, Lang et al. 2017). It is suggested that this interaction with DCs is mediated by toll-like receptor (TLR) 9, as the self-DNA is protected from DNase degradation by neutrophil antimicrobial peptide LL37 and high mobility group box (HMGB) 1 (Tillack, Breiden et al. 2012). Additionally, the released MPO is able to kill tumour cells (Odajima, Onishi et al. 1996). Yet, the lack of MPO was shown to promote tumour development in the Apc $^{Min/+}$ mice (Al-Salihi, Reichert et al. 2015). These mice carry a mutant allele called multiple intestinal neoplasia (Min) in the adenomatous polyposis coli (Apc) gene, giving them the name Apc $^{Min/+}$ mice, which serves as experimental model of colitis in the presence of inflammatory stimuli. Usually, colon tumours are absent in this mouse model as long as there are no inducers of inflammation. However, deletion of MPO increases the tumour incidence in these mice although inflammatory stimuli were missing (Al-Salihi, Reichert et al. 2015). Similar results were found in human patients with MPO deficiency (Lanza 1998). This suggests a protective role of MPO in the anti-tumour defence. On the other hand, NETs can help the tumour to evade the immune system by ROS-mediated immune suppression of T cells (Jablonska, Lang et al. 2017). Furthermore, upon induction of colitis in the Apc $^{Min/+}$ mouse model, MPO inhibition or deletion decreased tumour development (Al-Salihi, Reichert et al. 2015).

Also, other mouse models suggest alleviation of tumour burden by inhibition of MPO indicating a tumour-promoting role for increased MPO activity (Rymaszewski, Tate et al. 2014). NETs were able to alter the bioenergetics of cancer cells by activation of TLR4 via NE leading to increased mitochondrial biogenesis and therefore accelerated tumour growth (Yazdani, Roy et al. 2019). In cancer patients, excessive inflammation, oxidative stress and NET formation are associated with relapse after surgery, and worsened prognosis (Tohme, Yazdani et al. 2016). Additionally, citrullinated histone H3 was suggested to be a prognostic blood marker of advanced stage cancer patients predicting poor clinical outcome and increased mortality due to strong inflammatory processes (Thalin, Lundstrom et al. 2018).

There are multiple hypotheses explaining the reasons for NET formation associating with increased mortality. One explanation is that the release of nucleic acid and enzymes during the process of NET formation sequesters disseminated cancer cells for promoting adhesion at distant sites (Fig. 9E, Fig. 10; (Coffelt, Wellenstein et al. 2016)). In addition, NETs can be induced in the vasculature in experimental mouse models where they support metastatic seeding (Tohme, Yazdani et al. 2016). Microvascular NETs especially increase the metastatic burden in the lung and the liver due to trapping of circulating cancer cells (Jablonska, Lang et al. 2017). DNase I treatment successfully interferes with this process of metastatic scaffolding in mice. Interestingly, the deletion of PAD4 in mice bearing orthotopic ovarian tumours impaired NET formation and reduced metastasis, yet not primary tumour growth (Lee and Naora 2019). In addition, increased MMP9 release due to elevated NET formation facilitates extravasation of cancer cell (Cools-Lartigue, Spicer et al. 2014). Therefore, NET formation drives tumour progression and accounts as a metastasis-promoting mechanism.

Furthermore, the release of neutrophil DNA in the process of NET formation was found to activate TLR9 signalling in cancer cells (Tohme, Yazdani et al. 2016). TLR9 signalling in the TME is associated with tumour recurrence after radiotherapy due to MyD88 (myeloid differentiation primary response 88))/NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells)-dependent upregulation of IL-6 (Gao, Kozlowska et al. 2013). IL-6 activates JAK/STAT (Janus kinase/signal transducers and activators of transcription) signalling mediating proliferation, migration, and survival (Harrison 2012, Gao, Kozlowska et al. 2013). Additionally, NE and MMP9 cleave laminin 111, which activates integrin signalling in cancer cells and thus proliferation (Jaillon, Ponzetta et al. 2020). Furthermore, NET formation induces angiogenesis, as VEGF expression is stimulated by cathepsin G and TGF-β signalling (Wilson, Nannuru et al. 2010, Jaillon, Ponzetta et al. 2020).

Moreover, inflammation-driven NET formation promotes the awakening of dormant cancer cells (Albrengues, Shields et al. 2018). Dormancy is characterized by the absence of proliferation, although the cancer cells are still viable. Many patients experience relapse after surgical removal of their tumours caused by re-initiating proliferation of already disseminated cancer cells. Due to the release of ECM remodelling proteins such as NE and MMP9, tumour cell proliferation is triggered. Laminin 111 is cleaved by NE and MMP9, and the resulting fragments are suggested to induce tumour cell awakening by activating integrin β1 signalling. Outside-in-integrin signalling activates the focal adhesion kinase (FAK), ERK, and the myosin light chain 2 (MLC2) in cancer cells and inhibition of these pathways successfully abrogated tumour proliferation. Knock-down analyses revealed that NET-induced activation of integrin α3, integrin β1 as well as YAP (yes-associated protein) were crucial for the exit from dormancy in cancer cells (Albrengues, Shields et al. 2018).

Furthermore, increased risk of relapse in breast cancer patients has recently been associated with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection due to excessive NET formation (Francescangeli, De Angelis et al. 2020). COVID-19 associated lung inflammation leads to release of pro-inflammatory cytokines and DAMPs, which results in neutrophil infiltration, NET formation and consequently reactivation of dormant tumour cells that have already spread to the lung.

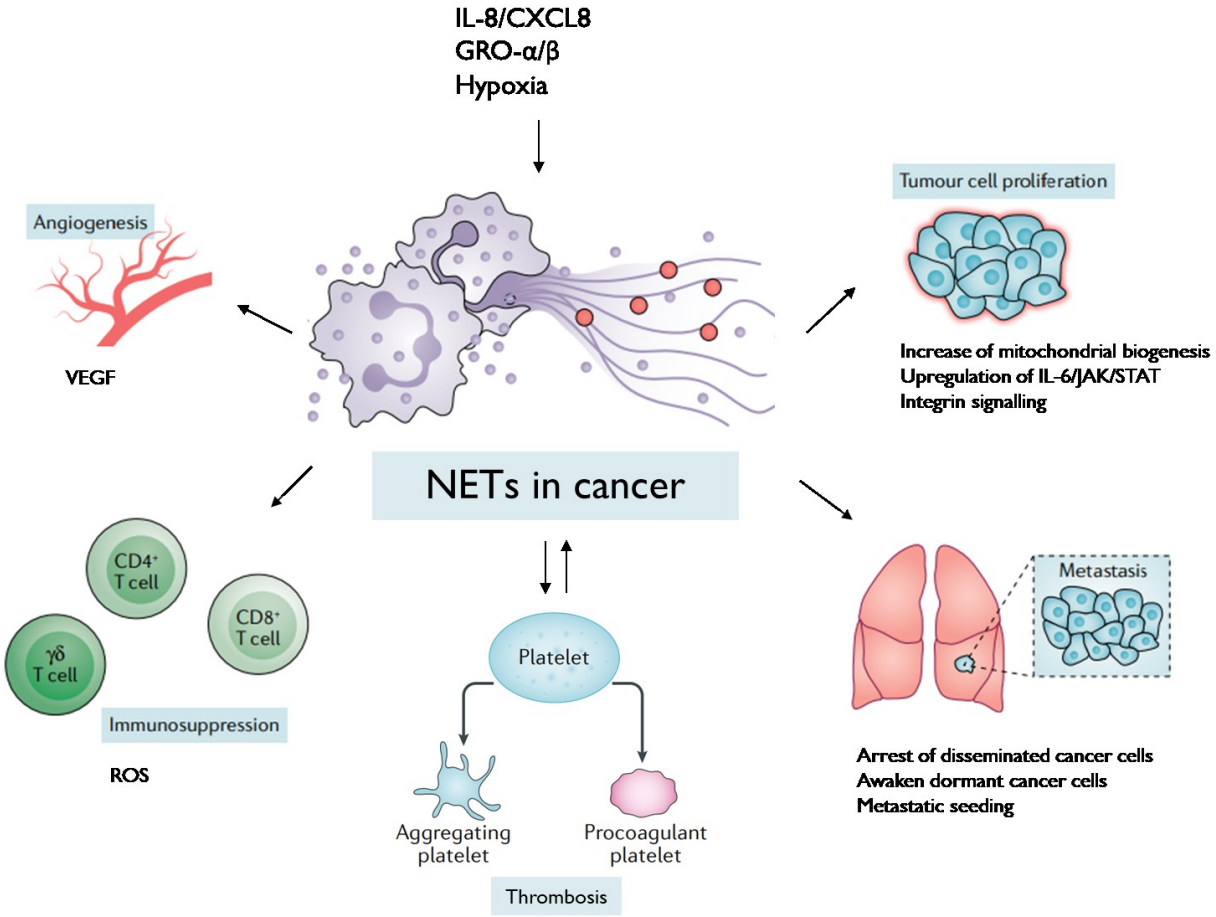



**Figure 10: Neutrophil extracellular traps and their cancer-promoting features.** Interleukin (IL)-8/CXCL8, growth-regulated oncogene (GRO)-α/β and hypoxia are just few examples for the plethora of stimuli, which induce NET formation in cancer. Cathepsin G release leads to TGF-β signalling promoting the expression of VEGF. NETs can promote tumour cell proliferation by neutrophil elastase-driven increase of mitochondrial biogenesis and by TLR-9-activation causing IL-6 upregulation and therefore JAK/STAT downstream signalling. Integrin signalling in cancer cells is activated due to laminin 111 cleavage mediated by neutrophil elastase (NE) and MMP9. Production of reactive oxygen species (ROS) inhibits T cell activation suppressing anti-tumour immune responses. Metastasis is promoted as circulating cancer cells are arrested on NETs and dormant cancer cells can be stimulated for proliferation causing metastatic seeding. The released chromatin activates platelets and thus causes pro-thrombogenic effects. Vice versa, also activated platelets can stimulate NET formation. Adapted from (Jaillon, Ponzetta et al. 2020).

As mentioned, the neutrophil-platelet-crosstalk plays a major role in cancer development and progression. NETs also have pro-thrombogenic effects as a result of the released tissue factor, an important component on the coagulation cascade (Singel and Segal 2016). Neutrophils primed for DNA release, e.g. by DAMPs, HIF and pro-inflammatory cytokines, also predispose lung thrombosis *in vivo* due to increased NET formation (Demers, Krause et al. 2012). Interestingly, PAD4 required for citrullination of histones is also crucially involved in thrombosis (Martinod, Demers et al. 2013).

Additionally, activated platelets are able to initiate NET formation and to promote it by binding of PSGL-1 on neutrophils via P-selectin (Singel and Segal 2016). The interaction of platelets and neutrophils is also dependent on β1 integrin and lymphocyte function-associated antigen 1 (LFA1) on neutrophils (Honda and Kubes 2018). Platelet-derived TGF-β is involved in tumour progression in at least two ways: first, as a promoter of EMT and secondly, to recruit neutrophils to tumour sites and to stimulate NET formation (Labelle, Begum et al. 2014, Jablonska, Garley et al. 2020). Therefore, the neutrophil-platelet crosstalk also causes a positive feedback loop between NET formation and thrombosis, as both events can initiate each other.

## Neutrophils and HCC

HCC mainly develops in the context of chronic inflammation, which differs from other malignant tumours (Liu, Wang et al. 2020). In patients with advanced liver cancer, myeloid-derived suppressor cells (MDSCs) including neutrophils increases, which correlate with poor prognosis (Honda and Kubes 2018). In patients with NASH, neutrophils infiltrate the liver and promote inflammation (van der Windt, Sud et al. 2018). The recruitment of neutrophils into the TME is mediated by secretion of cytokines such as IL-6 and IL-8, which are upregulated in the tumour cells (Liu, Wang et al. 2020). Additionally, platelets and γδ T cells are involved in attracting neutrophils. The resulting polarization of neutrophil populations show heterogenous phenotypes of which TANs secrete FGF2, HGF, NE, MMP9 and VEGF to promote angiogenesis, proliferation and metastasis (Gordon-Weeks, Lim et al. 2017, Liu, Wang et al. 2020). TANs from HCC patients as well as peripheral blood neutrophils, which were activated with conditioned medium from HCC cells lines, show increased expression of the chemokines CCL2 and CCL17 through activation of PI3K/Akt and MAPK pathways (Zhou, Zhou et al. 2016). These chemokines further attract CCR2 expressing monocytes and macrophages together with CCR4 expressing regulatory T cells into the TME. Yet, the interplay of neutrophils and cancer cells in HCC is poorly understood and literature is quite limited.

While the role of NETs in tumour progression and metastasis has been elaborated, their respective contribution to HCC development needs further investigation. It is already known that neutrophils and their NETs prepare a premetastatic niche and shape the TME in liver metastases (Tohme, Yazdani et al. 2016, Honda and Kubes 2018). There is some evidence that NETs play an important role in HCC. Experimental mouse models of liver inflammation mimicking NASH-induced HCC showed decreased tumour growth upon inhibition of NET formation either by DNase I treatment or PAD4 deletion (van der Windt, Sud et al. 2018). As the development of steatohepatitis was not impaired, NET formation specifically promoted the progression to HCC. Furthermore, NET formation markers such as MPO-DNA complexes in patient sera and citrullinated histone H3 in the tumour tissue were used as biomarkers for liver inflammation, which are able to predict survival of liver cancer patients after liver resection (Kaltenmeier, Yazdani et al. 2020). Inflammation is also associated with increased metastasis potential. Thus, increased NET formation in the malignant liver also predisposes metastasis similar to NET formation in breast, lung and ovary cancer (Yang, Luo et al. 2020). Activation of TLR4/9-COX2 signalling by NETs increased the metastatic potential of HCC cells. Interestingly, treatment with prostaglandin E2, a product of COX2, compensated for the loss of TLR4 or TLR9-stimulated metastatic behaviour in HCC cells.

Additionally, hydroxychloroquine, an anti-inflammatory drug blocking TLR-pathways, was able to inhibit COX2 upregulation and thus metastatic potential (Yang, Luo et al. 2020).

Unravelling the mechanisms and signalling routes leading to NET formation in liver cancer might offer alternative therapeutic approaches for interfering with tumour progression and metastasis. Additionally, understanding the interplay between neutrophils and HCC tumour cells in detail might offer targets for new therapeutic approaches.

## TGF-β and Axl in HCC

HCC development is reflected by aberrant hepatocyte proliferation and dedifferentiation. One important driver is the pro-fibrotic cytokine TGF-β (Thiery, Acloque et al. 2009). TGF-β is a small homodimeric signalling protein, which exists in 3 isoforms in mammals, namely TGF-β1/2/3 (Dash, Sahu et al. 2020). Canonical TGF-β signalling leads to dimerization of TGF-β receptor I and II, and Smad2/3 phosphorylation (Fig. 11). Phosphorylated Smad2/3 dissociates from the receptor and interacts with Smad4 in the cytoplasm for further translocation into the nucleus where this heterotrimeric complex acts as a transcriptional regulator (Lucarelli, Schilling et al. 2018). Alternatively, TGF-β activates pathways independent of the Smad complexes such as the p38, MAPK and JNK mediating either growth arrest and apoptosis or invasion and EMT (Fig. 11; (Dash, Sahu et al. 2020)). Under physiological conditions, TGF-β mediates tumour-suppressive functions regulating tissue homeostasis, proliferation and differentiation (Massague 2008). Yet, upon HCC development, cancer cells can circumvent the tumour-suppressive functions of TGF-β either by inactivation of core components such as the TGF-β receptor or by alternative downstream signalling routes. The second option leads to a TGF-β switch, where its regulatory functions are exploited for enhanced growth, invasion, production of autocrine mitogens and cytokines. Autocrine TGF-β secretion offers cancer cells the possibility to evade immune surveillance due to TGF-β-mediated immune tolerance and to induce EMT (Giannelli, Villa et al. 2014). Cancer cells undergoing the process of EMT lose their polarity and become motile mesenchymal cells which are able to metastasise. It is characterized by down-regulation of E-cadherin and upregulation of N-cadherin. Numerous signalling pathways are involved in mediating EMT including oncogenic Ras and RTK signalling. Recent studies elucidated the role of RTK Axl being involved in mediating the TGF-β switch (Calvisi 2015).

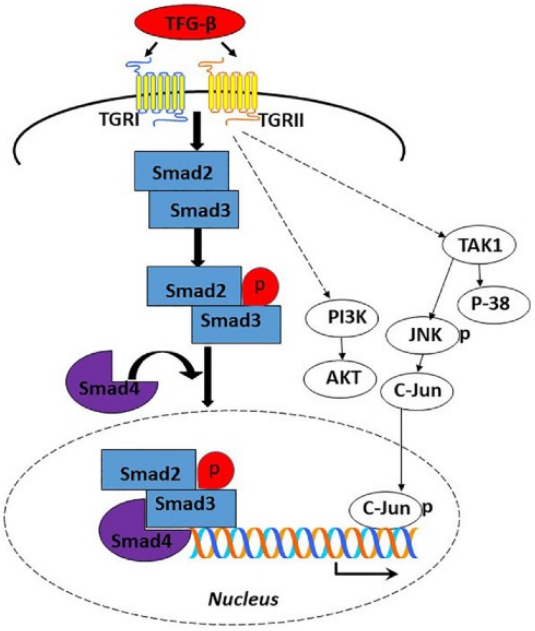



**Figure 11: The TGF-β signalling pathway.** After receptor dimerization, Smad2/3 are phosphorylated and dissociate from the receptor complex in order to bind Smad4 in the cytoplasm. The heterotrimeric complex interacts with transcription factors in the nucleus and modulates transcription of TGF-β-target genes. Alternative pathways are Smad-independent and target PI3K/Akt or JNK causing either cell death or EMT. Taken from (Dash, Sahu et al. 2020).

Axl belongs to the TAM family of RTKs together with Tyro3 and Mer (Lemke 2013). Its name derives from the Greek word *ανεξέλεγκτος* (*anexelektos*), which is translated with "uncontrolled" due to its first discovery in patients with chronic myeloid leukaemia (Zhu, Wei et al. 2019). Interestingly, ligand-independent activation of TAM RTKs occurs upon receptor overexpression due to spontaneous dimerization and auto-trans-phosphorylation. The TAM RTKs are physiologically expressed in monocytes, platelets, endothelial cells, the brain, the heart and the liver (Zhu, Wei et al. 2019). Additionally, dysregulation of TAM RTK signalling leads to inflammation and tumorigenesis (Trahtemberg and Mevorach 2017). Axl together with Tyro3 and Mer modulate immune responses as DC, NK cell and macrophage activation can be inhibited (Rothlin, Ghosh et al. 2007). Upregulation of Bcl-2 (B-cell lymphoma) and Twist due to aberrant TAM signalling mediates immune evasion through suppression of TLR signalling and decreased expression of pro-inflammatory cytokines (Zhu, Wei et al. 2019). Therefore, overexpression of RTKs and their ligands resulting in hyperactivity is associated with tumour progression and metastasis in various cancer types (Di Stasi, De Rosa et al. 2020).

Furthermore, TAM RTK activation upon binding of the ligand growth arrest specific gene 6 (Gas6), which has the highest affinity for Axl, or Protein S (ProS) are known pathways in clearance of apoptotic cells, platelet aggregation and vessel integrity by activation of PI3K and Akt (Angelillo-Scherrer, Burnier et al. 2005). PI3K and Akt phosphorylate β3-integrin which amplifies thrombus formation. Thrombosis can thus be prevented in Gas6/Axl-deficient mice by destabilization of platelet aggregates (Angelillo-Scherrer, de Frutos et al. 2001). Cancer-associated thrombosis is another important mechanism in metastasis as it is needed to shield disseminated cancer cells in the circulation, yet it is responsible for the high mortality in cancer patients due to formation of thromboembolisms.

In the liver, Axl is mainly expressed on hepatic oval cells, the precursors of hepatocytes, which differentiate and proliferate upon hepatic injury, and endothelial cells (Axelrod and Pienta 2014). Survival is mediated by Gas6/Axl activation of PI3K/Akt and NFκB pathways that inhibit apoptosis. In the TME, Gas6 is released by tumour infiltrating leukocytes, activated HSCs, sinusoidal endothelial cells and Axl-positive myofibroblasts, and thus Gas6 is heavily abundant (Fig. 12; (Loges, Schmidt et al. 2010, Holstein, Binder et al. 2018)). Due to hypoxic conditions within the TME, Axl receptor expression is upregulated by binding of HIF-1α and HIF-2α to the Axl proximal promotor (Rankin, Fuh et al. 2014). As a result, increased Gas6/Axl signalling activates MAPK/ERK pathways in malignant hepatocytes via binding to 14-3-3-ζ and recruitment of JNK in order to mediate migration, invasion and proliferation (Calvisi 2015, Reichl, Dengler et al. 2015). JNK mediates the phosphorylation of the Smad3 linker region, which results in an altered transcription program. Instead of pro-apoptotic genes, TGF-β resistance and EMT-inducing genes are activated facilitating the TGF-β switch and as such invasion and metastasis (Calvisi 2015).

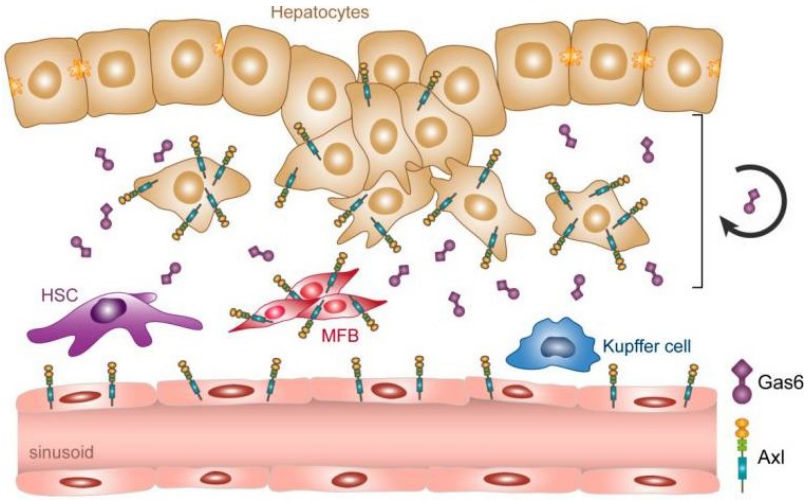



**Figure 12: Gas6 and Axl expression in the tumour microenvironment (TME) of hepatocellular carcinoma (HCC).** Malignant hepatocytes undergo EMT and upregulate Axl due to hypoxia. Gas6 is released by activated hepatic stellate cells (HSCs), myofibroblasts (MFB), and Kupffer cells. Autocrine Gas6/Axl signalling promotes proliferation, migration and invasion of HCC cells. Taken from (Holstein, Binder et al. 2018).

## CXCL5

The Axl-mediated TGF-β switch leads to expression of several target genes, and the chemokine CXCL5 has recently been identified to be one of those (Haider, Hnat et al. 2019). CXCL5, also known as neutrophil activating peptide 78 (ENA-78), is a neutrophil attractant. It activates the CXCR2 receptor in order to mediate inflammation, but its overexpression is linked to various cancer types and correlates with poor patient survival, recurrence of disease and metastasis (Zhang, Wang et al. 2020). Additionally, CXCL5 is overexpressed in HCC as well and associates with neutrophil infiltration (Zhou, Dai et al. 2012, Haider, Hnat et al. 2019). A high co-expression of CXCL5 and its receptor CXCR2 induces EMT and promotes metastasis (Zhou, Zhou et al. 2015). Furthermore, CXCL5 is able to attract neutrophils *in vitro* mediated by Axl/TGF-β signalling (Haider, Hnat et al. 2019).

## Hypothesis

The collaboration of Axl and TGF-β signalling is crucially involved in HCC progression and metastasis. In particular, the upregulation of Gas6/Axl signalling allows malignant hepatocytes to amputate anti-oncogenic functions of TGF-β which facilitate pro-oncogenic traits such as EMT, cancer cell invasion and chemoresistance (Massague 2008, Reichl, Dengler et al. 2015). The upregulation of Axl and TGF-β target genes involves the expression of certain chemokines, among them CXCL5, which attracts neutrophils (Fig. 13; (Haider, Hnat et al. 2019)). Recent studies show that increased CXCL5 secretion among other chemokines leads to neutrophil infiltration into tumour foci of HCC as well as in other cancer entities (Zhou, Dai et al. 2012). One of the executive functions of neutrophils is NET formation in breast cancer, which can be stimulated by tumour cells (Park, Wysocki et al. 2016). Although NET-associated proteins like MPO and NE have the capacity to kill cancer cells, recent findings suggest that NET formation promotes cancer cell dissemination. In HCC, the link between NET formation and HCC progression as well as the role of CXCL5 has not been addressed so far. Therefore, we hypothesise that NET formation in HCC is important in driving tumour progression and metastasis as observed in breast cancer. To elucidate this issue, we focused on the impact of HCC cells and the Axl/TGF-β/CXCL5 signalling axis on NET formation of neutrophils.

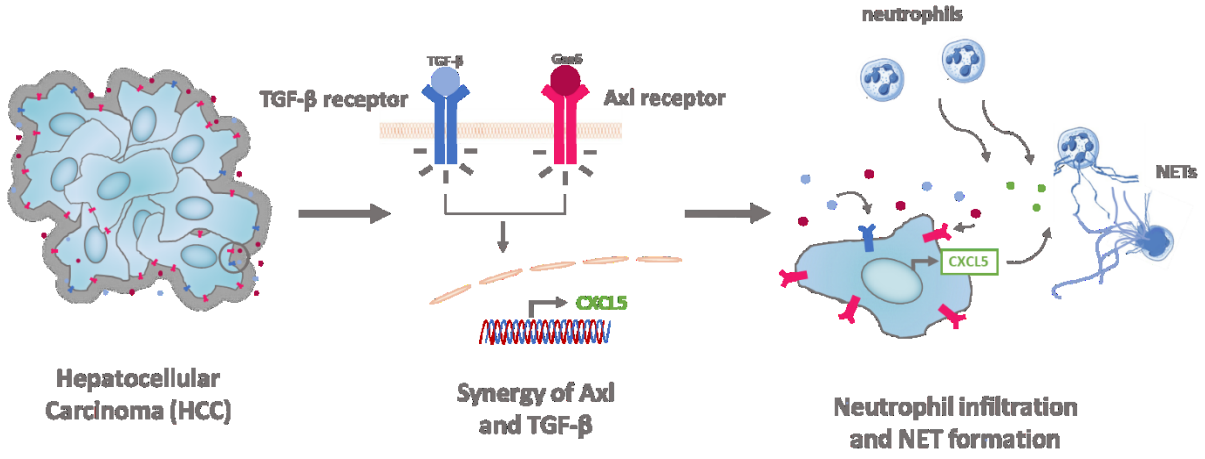



**Figure 13: Hypothesis.** The synergy of Axl and TGF-β upregulates expression of the chemokine CXCL5 leading to neutrophil infiltration. We hypothesise that neutrophils release extracellular traps (NETs) in response to CXCL5 induced by Axl/ TGF-β signalling of malignant hepatocytes. © Elisa Holstein

# Materials and Methods

## Cell culture

The human hepatoma cell line SNU449 was cultured in RPMI 1640 (Sigma-Aldrich, St.-Louis, USA) supplemented with 10% foetal calf serum (FCS, Sigma-Aldrich, St. Louis, USA) and 1% penicillin/streptomycin (P/S, Sigma-Aldrich, St. Louis, USA), referred to as R10. The human HLF hepatoma cells were propagated in DMEM (Sigma-Aldrich, St. Louis, USA) supplemented with 10% FCS and 1% P/S, referred to as D10. HCC cell lines were long-term treated with 1 ng/ml TGF-β1 (Peprotech, Rocky Hill, USA) for more than 10 days, giving rise to SNU449-T or HLF-T cells. All cells were kept at 37 °C and 5% $CO_2$ and routinely tested for the absence of mycoplasma.

## Isolation of murine neutrophils

Neutrophils were isolated from femur and tibia of 2 male C57BL/6 mice. The bones were kept in cold RPMI 1640 cell culture medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% FCS (Sigma-Aldrich, St. Louis, USA) and 1% penicillin/streptomycin (R10). The bones were sterilized with 70% ethanol for 10 seconds under sterile conditions in a tissue culture hood followed by 3 subsequent washes in ice-cold phosphate buffered saline (PBS) to remove the ethanol. After cutting off the epiphyses, 25-gauge needles were used to flush the bone marrow cells from the bone shafts using R10 supplemented with 2 mM EDTA to prevent clumping of the cells. Blanching of the bones indicated sufficient scraping of the cells. In order to avoid cell aggregates, the cell suspension was filtered with a 40 µm cell strainer. Approximately 5 ml R10 supplemented with EDTA were used per femur and 2.5 ml per tibia for sufficient rinsing of the bones. After centrifugation at 600 xg for 7 minutes (min) at 4 °C, red blood cells were lysed with a sterile hypotonic solution, consisting of 0.2% NaCl, for 20 seconds followed by addition of a hypertonic solution, consisting of 1.6% NaCl, to restore an isotonic solution. The suspension was again centrifuged at 600 xg for 7 min at 4 °C and washed with R10 supplemented with EDTA. After centrifugation at 600 xg for 7 min at 4 °C, the cell pellet was resuspended in 1 ml ice-cold PBS and put on top of a density gradient for separation of the neutrophils from the primary bone marrow cells (PBMC). The bottom layer consisted of Histopaque 1119 with the density of 1.119 g/ml (Sigma-Aldrich, St. Louis, USA), while the top layer consisted of Histopaque 1077 with the density of 1.077 g/ml (Sigma-Aldrich, St. Louis, USA). After centrifugation for 30 min at 800 xg at 25 °C without brake, the neutrophils were collected from the intermediate layer of the density gradient. The neutrophils were washed with either R10, D10 or PBS depending on the final medium used for each experiment. Finally, cells were counted, and the viability was determined using the CASY cell counter and analyser TT (Omni Life Science, Bremen, Germany).

## Immunofluorescence analysis

In order to facilitate neutrophil attachment, glass cover slips were coated with poly-L-lysine (Sigma-Aldrich, St. Louis, USA) for 1 hour (hr) at room temperature (RT) in a 24-well-plate. 4 x $10^5$ neutrophils were seeded on the coated cover slips and incubated after treatment for 4 hrs at 37 °C with 5% $CO_2$. The cells were washed with PBS followed by fixation with 4% para-formaldehyde (Histofix, Roth Lactan, Karlsruhe, Germany) for 15 min at 37 °C.

The fixed cells were stored at 4 °C in PBS and then further processed for immunofluorescence analysis. For that, cells were permeabilized with 0.25% Triton-X 100 in PBS for 5 min. Afterwards, the cells were blocked with 5% horse serum in 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, USA) in PBS, and a 1:12.5 dilution of mouse-on-mouse (M.O.M.) protein concentrate in PBS supplemented with 5% M.O.M. blocking serum from the Vectastain M.O.M.® Immunodetection Kit (BMK-2202, R&D, Minneapolis, USA) for 30 min in a humidity chamber. The primary antibodies used were α-citrullinated-histone-H3 (ab5103, Abcam, Cambridge, UK) at a dilution of 1:150 and α-neutrophil-elastase (NP57, Santa Cruz, Dallas, USA) at a dilution of 1:100 in blocking solution. After incubation for 1 hr. in a humidity chamber at RT, the slides were washed 3 times with PBS. The secondary antibodies were diluted in 1% BSA/PBS. The α-rabbit-Alexafluor-A488 visualizing the citrullinated-histone-H3 was used at a dilution of 1:300, and the α-mouse-Alexafluor-A647 visualizing the neutrophil-elastase was used at a dilution of 1:200. Nuclei were visualized with Hoechst 33342 (Sigma-Aldrich, St. Louis, USA) at a dilution of 1:1.000 in 1% BSA/PBS. After incubation for 1 hr at RT in the dark, the slides were washed 3 times with PBS. Finally, the slides were embedded in Mowiol (Sigma-Aldrich, St. Louis, USA). Imaging was performed by confocal immunofluorescence microscopy (Zeiss, Jena, Germany).

### Sytox Green DNA release assay

1 x $10^5$ neutrophils per well were seeded into a 96-well plate. The cells were either incubated with HCC tumor cell supernatants, cell culture medium or PBS. After adding the NET activating substances, 5 µM Sytox Green (Thermo Fischer Scientific, Waltham, USA) was added to the neutrophils. Sytox Green is able to intercalate with extracellular DNA without perturbing the cell membrane. The stained extracellular DNA was measured as a time course every 20 to 30 min during 4 to 8 hrs. Calcium ionophore (A23187, Sigma-Aldrich, St. Louis, USA) and phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, USA) were used as positive control for stimulating neutrophil DNA release, and the PAD4-inhibitor GSK484 (Sigma-Aldrich, St. Louis, USA) was used for inhibiting NET formation.

### Enzyme-Linked-Immunosorbent-Assay (ELISA)

In order to quantify the NET formation, the amount of released MPO was measured with a Mouse Myeloperoxidase DuoSet Kit (DY3667, R&D, Minneapolis, USA). The MPO-ELISA was performed following manufacturer's instructions. The Capture Antibody was diluted to the working concentration [800 ng/ml] in PBS (Sigma-Aldrich, St. Louis, USA), and a 96-well-microplate (Greiner Bio-One, Kremsmünster, Austria) was coated with 100 µl per well using an electric stepped multichannel pipette. After incubation over night at RT, the plate was washed 3 times with 400 µl of 0.05% Tween in PBS. Subsequently, the plate was blocked using 300 µl Reagent Diluent consisting of 1% Probumin Bovine Serum Albumin Diagnostic Grade Powder (820451, Merck-Millipore, Massachusetts, USA) in PBS for 1 hr at RT. The MPO standard was reconstituted in 0.5 ml Reagent Diluent to a concentration of 370 ng/ml and a 7-point standard curve using a 2-fold dilution series was prepared with the concentrations of 16 000 – 8 000 – 4 000 – 2 000 – 1 000 – 500 – 250 pg/ml MPO. After 3 washing steps with 0.05% Tween in PBS, 100 µl of standard or 100 µl of samples diluted 1:20 in Reagent Diluent were added per well and incubated for 2 hrs. at RT.

After 3 washing steps, 100 µl of 50 ng/ml Detection Antibody were added per well and incubated for 2 hrs at RT. After 3 washing steps, 100 µl of a 1:200 dilution Streptavidin-horseradish-peroxidase (Streptavidin-HRP) were added per well and incubated for 20 min at RT in the dark. After 3 washing steps, 100 µl of Substrate Solution (Substrate Reagent Pack, DY999, R&D, Minneapolis, USA) were added per well and incubated for 20 min at RT in the dark. Finally, 50 µl of 2 N $H_2SO_4$ were added to stop the colour reaction resulting in a colour change from turquoise to yellow. The optical density (OD) was measured with a microplate reader at 450 nm. The data were further processed with Graph Pad Prism in order to calculate the MPO concentration. The mean of the blanks was subtracted from all values, and all values were log transformed. A non-linear regression curve fit with sigmoidal dose response was performed and the samples were interpolated from the standard curves. After that, the sample values were transformed back by y = 10 ^ y and the means were calculated. The dilution factor was multiplied to the calculated concentration values.

## Harvesting of hepatoma cell supernatants

1 x $10^6$ HLF, HLF-T and HLF-CXCL5 or SNU449, SNU449-T and SNU449-CXCL5 cells were seeded on 60 mm cell culture dishes. After 24 hrs, the cell culture medium was removed, and the cells were washed 2 times with PBS. Then, either 3 ml serum-free cell culture medium or cell culture medium supplemented with varying concentrations of 0.1 – 10% FCS were added, depending on the cell type. After incubation for 24 hrs, the supernatants were freshly harvested and directly used for neutrophil treatment.

## Concentration of hepatoma cell supernatants

After harvesting of hepatoma cell supernatants, remaining cells were removed by centrifugation at 400 xg at RT for 5 min followed by transfer of the supernatants to a new tube. Next, the supernatants were loaded on 50 kDa centricons (Merck-Millipore, Massachusetts, USA) and centrifuged at 4 000 xg for 10 min at RT to get rid of all proteins bigger than CXCL5, which has the size of 8 kDa. Subsequently, the flow-through was transferred to a 3 kDa centricon (Merck-Millipore, Massachusetts, USA) and centrifuged at 4 000 xg for 20 min at RT for concentration until the end volume reached approximately 1.5 ml.

## Wound healing assay

For studying cell migration in a scratch wound assay, 1 x $10^6$ cells per well were seeded on 6-well plates. The cells were incubated for 24 hrs at 37 °C with 5% $CO_2$ in cell culture medium supplemented with 10% FCS and 1% P/S. Artificial wounds were inflicted to the cell layer by scratching with sterile 1000 µl pipette tips. The medium was changed and 6 photos per scratch were taken immediately using the phase contrast microscope (Nikon, Tokyo, Japan) at 4 x magnification. The cells were again incubated for 24 hrs at 37 °C with 5% $CO_2$ prior imaging. The migrated area of cells into the wound was quantified with ImageJ software (https://imagej.nih.gov/ij/).

## Quantification of immunofluorescence images

For quantification of immunofluorescence images, the Fiji software (ImageJ) was used. The images in lsm format were split and the green channel was analysed, which indicated the Alexa Fluor A488 (Thermo Fischer Scientific, Waltham, USA) stained citrullinated histone H3 (ab5103, Abcam, Cambridge, UK) antibody.

The images in czi format were imported as hyperstack into Fiji and the green channel was analysed indicating histone citrullination. The mean fluorescence intensity was exported into txt files, and the data was evaluated with R.

## Statistical analysis

Data were expressed as means ± standard deviation (SD) or ± standard error (SE). Means, SD and SE was calculated using R including the packages dplyr, plotrix and data.table. Outliers were removed after identification via Grubbs test and analysis of boxplots using the R packages outliers and ggplot2 with the function geom_boxplot(). The statistical significance of differences between 2 groups was evaluated using Mann-Whitney-U tests for unpaired, non-parametric data as most of the data were not normally distributed. Normal distribution of parametric data was tested with Shapiro-Wilk tests. In order to compare the statistical difference between more than 2 groups, Kruskal-Wallis rank tests were performed for unpaired, non-parametric data as most of the data were not normally distributed. Significant differences between experimental groups were * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

# Results

## Phenotypical alterations of HLF cells upon long-term TGF-β treatment

The hepatoma cell lines HLF and SNU449 are known to respond differently to long-term TGF-β treatment, which results in changes of migratory behaviour after treating cells with TGF-β [1 ng/ml] for more than 10 days (Haider, Hnat et al. 2019). In order to confirm and prove TGF-β-dependent transformation of HLF and SNU449 cells, cell motility has been evaluated regularly in wound healing assays (Fig. 14). Long-term TGF-β treatment transformed HLF cells towards an increased migratory phenotype of so called HLF-T cells. In contrast to that, TGF-β treatment of SNU449 (SNU449-T) cells resulted in decreased cell motility. As the synergy of Axl and TGF-β induces the expression of CXCL5, HLF and SNU449 cells ectopically expressing CXCL5, referred to as HLF-CXCL5 and SNU449-CXCL5, were used as control (Haider, Hnat et al. 2019). Interestingly, the overexpression of CXCL5 alone was not sufficient to induce changes in cell migration. These data suggest that long-term TGF-β treatment has a crucial impact on the migratory behaviour of HLF and SNU449 cells, showing a higher motility of HLF cells.

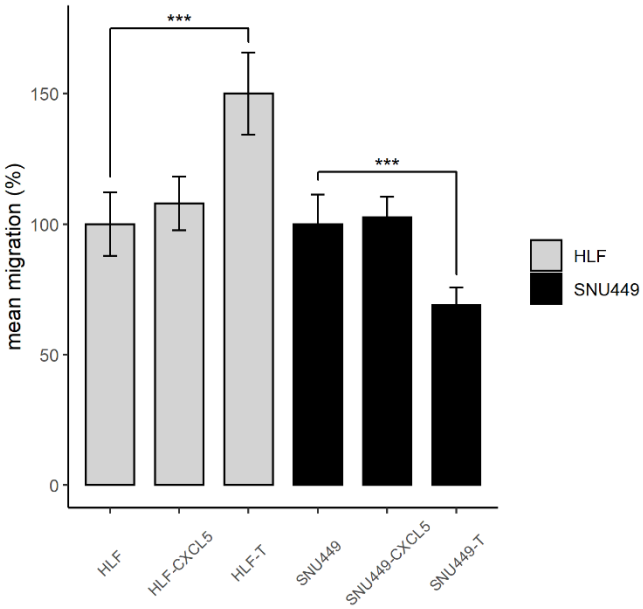



**Figure 14: TGF-β-dependent transformation of HLF cells increases cell motility**. Grey: Migrated areas of long-term TGF-β-treated HLF cells (HLF-T) and HLF cells ectopically expressing CXCL5 (HLF-CXCL5) relative to parental HLF cells as analysed by wound healing assays. Black: Migrated areas of long-term TGF-β-treated SNU449 cells (SNU449-T) and SNU449 cells ectopically expressing CXCL5 (SNU449-CXCL5) relative to parental SNU449 cells as analysed by wound healing assays. The migration of parental HLF and SNU449 cells was set to 100%. Data are expressed as mean ± SD. Error bars depict SD from 5 individual experiments. P-values were calculated with Mann-Whitney-U tests for 2 groups, *** $P < 0.001$.

## Low neutrophil density causes spontaneous murine NET formation

In order to optimise the *in vitro* NET assay setup, the impact of the murine neutrophil density on spontaneous DNA release was tested (Fig. 15). Treatment with the calcium ionophore A23187 induced murine NET formation. As expected, the higher the neutrophil density seeded per well, the more citrullinated histone (citH3) was detectable (Fig. 15A-B). Of note, the total signal for citH3 increased with the neutrophil density. When the citH3 intensities were normalised to the cell count, it was visible that the amount of citH3 per cell was higher in low neutrophil densities (Fig. 15B, right panel). Therefore, high neutrophil densities decreased spontaneous murine NET formation. Interestingly, high neutrophil densities even suppressed the citH3 signal normalised to the cell count. As a result, the optimal ratio between untreated and A23187-treated neutrophils was obtained with the neutrophil density of 400 000 cells per well.

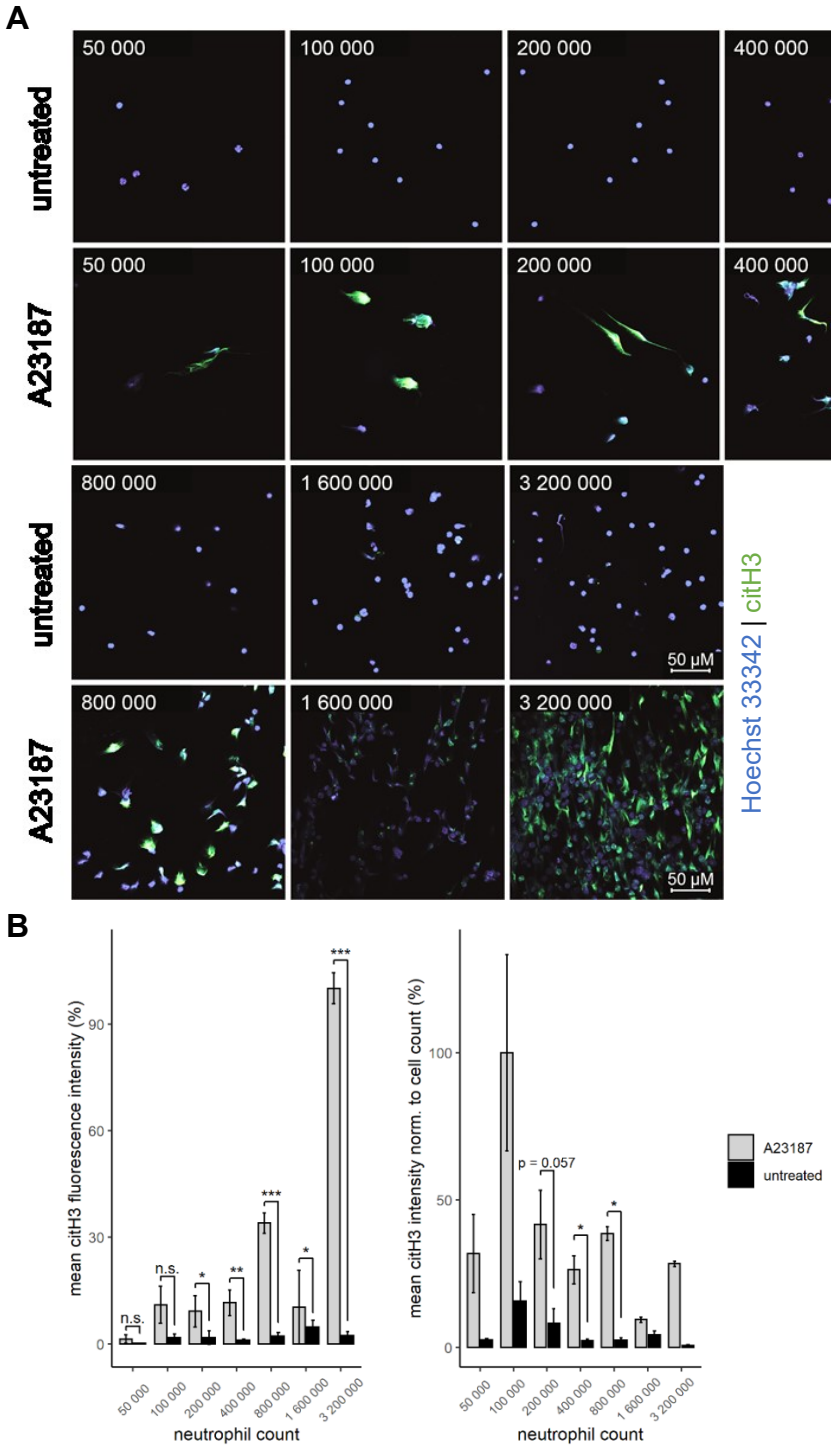



**Figure 15: *In vitro* NET formation depends on neutrophil counts.** (A) Representative pictures of confocal immunofluorescence analysis showing NET formation in murine neutrophils seeded at different cell numbers ranging from 50 000 to 3 200 000 neutrophils per well Neutrophils were plated on poly-L-lysine-coated glass slides and were incubated with DMEM plus 10% FCS (D10) in the presence of the calcium ionophore A23187 [10 µM] for 4 hours or were left untreated in D10. Citrullinated histones are indicated in green, and the DNA was counterstained with Hoechst 33342 indicated in blue. (B) Quantification of immunofluorescence analysis. Data are expressed as mean ± SD. Error bars depict SD from 2 individual experiments. P-values were calculated with Kruskal-Wallis rank sum tests for more than 2 groups, *** P < 0.001.

## Serum decreases spontaneous NET formation

In order to optimise *in vitro* culture conditions of murine neutrophils, the effect of serum on NET formation was investigated (Fig. 16). Untreated neutrophils cultured in DMEM supplemented with 10% FCS showed decreased levels of spontaneous NET formation compared to neutrophils cultured in FCS-free medium (Fig. 16A-B). Of note, even the neutrophils stimulated with the calcium ionophore A23187 used as positive controls for NET formation were rather protected in the presence of serum. The presence of serum reduced the variance of the data and decreased the total range of measured citH3 intensities compared to the data derived from neutrophils that were serum-starved, as indicated by the

distribution of data (Fig. 16C). These data were confirmed by fluorometric DNA release assays, where the released extracellular DNA was stained with Sytox Green which facilitated monitoring of NET formation over several hours (Fig. 16D-E). Sytox Green is able to intercalate with free DNA without perturbing the intact cell membrane. During the process of neutrophil DNA release, the plasma membrane ruptures facilitate the entry of Sytox Green which is visible in neutrophils treated with calcium ionophore. Of note, neutrophils released less DNA in the presence of 10% FCS as compared to serum-starved neutrophils (Fig. 16E). Therefore, NET formation should be examined in the presence of serum to reduce spontaneous DNA release in murine neutrophils.

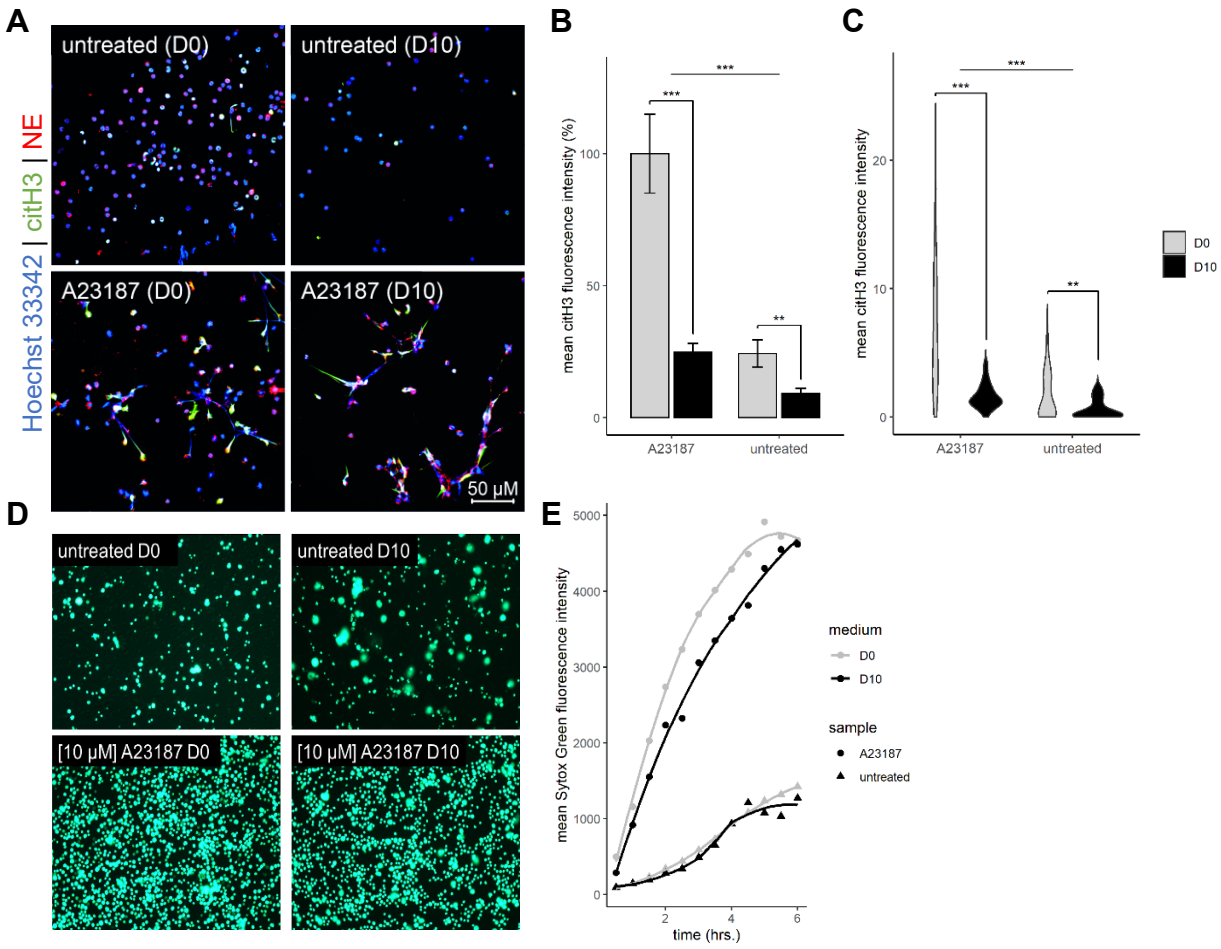



**Figure 16: Serum starvation induces DNA release of murine neutrophils.** (A) Representative pictures of confocal immunofluorescence analysis showing NET formation of murine neutrophils incubated with serum-free DMEM (D0) or with DMEM supplemented with 10% FCS (D10) and treated with A23187 calcium ionophore [10 µM] for 4 hours as positive control. Citrullinated histones (citH3) are indicated in green, neutrophil elastase (NE) is indicated in red, and the DNA was counterstained with Hoechst 33342 indicated in blue. (B) Quantification of immunofluorescence analysis. Data are expressed as mean ± SE. Error bars depict SE from 5 individual experiments. Mean of neutrophils treated with A23187 in D0 were set to 100%. (C) Distribution of data in form of violin-plots depicted in (B). (D) Representative images of neutrophils incubated for 4 hours with Sytox Green. (E) Sytox Green DNA release assay over time. Data are expressed as mean, which were calculated from 2 individual experiments. P-values were calculated with Kruskal-Wallis rank sum tests for more than 2 groups and Mann-Whitney-U tests for 2 groups. **P < 0.01, ***P < 0.001.

## Differential response of serum-starved neutrophils co-cultured with hepatoma cells

In various cancer types, NET formation is able to promote metastasis and to awaken dormant cancer cells via ECM remodelling (Park, Wysocki et al. 2016, Bordon 2018). NET formation in HCC has been recently studied revealing that NETs promote the development of HCC in NASH (van der Windt, Sud et al. 2018) and that they can be used as a biomarker for survival of patients with primary liver malignancies (Kaltenmeier, Yazdani et al. 2020). Additionally, one study showed that increased NET formation promoted the metastasis potential of HCC via tumour inflammation (Yang, Luo et al. 2020). To examine whether hepatoma cells are themselves able to induce metastasis-promoting NET formation, HCC cell lines mimicking different stages of EMT-like phenotypes were co-cultured with murine neutrophils in Transwell assays. We found that soluble factors secreted by HLF and HLF-T lead to a differential response of neutrophils depending on the presence of serum (Fig. 17A-C). Serum starvation induced spontaneous DNA release in neutrophils (Fig. 16), yet all hepatoma cells were able to stimulate NET formation to significantly elevated levels as compared to untreated controls upon serum-starvation (Fig. 17A, upper panel). Interestingly, in the presence of serum, hepatoma cells induced differential responses in neutrophils, as HLF-CXCL5 cells induced 1.3-fold more signal for citrullinated histone H3 than untreated neutrophils, and HLF-T cells even caused a 2.3-fold higher signal compared to untreated neutrophils (Fig. 17B). Upon serum starvation, HLF cells were also able to stimulate NET formation as indicated by a 2.1-fold increased level of citrullinated histones. In line, HLF-T cells caused a 2.5-fold higher and HLF-CXCL5 cells even 2.6-fold higher histone citrullination than untreated controls. This differential response might be attributed to the fact of serum-starvation in the hepatoma cells, which also impacts gene expression and thus alters the composition of the secreted soluble factors. Of note, the level of citrullinated histone H3 staining was remarkably elevated in all serum-starved conditions compared to the intensities of citrullinated histone H3 in the presence of serum (Fig. 17C). Altogether, co-culture of HLF-T cells with neutrophils induced significantly more citrullination of histones and as such NETs in presence as well as in absence of serum. In line, HLF-CXCL5 cells secrete soluble factors which leads to citrullination of histones and release of DNA in neutrophils, whose effect is amplified upon serum starvation. Co-cultures of HLF cells with neutrophils showed an opposing effect depending on the presence of serum, as serum starvation lead to NET formation, while the presence of serum impeded that effect.

Transwell experiments provided preliminary data on NET formation upon stimulation with soluble factors secreted by hepatoma cells, especially by HLF-T and to a lesser extent by HLF-CXCL5 cells. Next, these experiments were evaluated with the question whether hepatoma supernatants on their own were sufficient for NET formation. Therefore, HLF, HLF-T and HLF-CXCL5 were either serum-starved for 24 hrs or cultivated with serum in order to harvest their secretome, which was tested for its ability to induce NETs in murine neutrophils. Again, a difference between serum-starved conditions and neutrophils cultured with serum-containing supernatants was detectable (Fig. 18A, C). Additionally, the response to the HLF-T secretome confirmed the Transwell experiments as strong citrullination of histones was measurable, especially in the presence of serum. Upon serum-starvation, the effect was less obvious due to the high intensities of citrullinated histones in untreated controls (Fig. 18A). HLF-CXCL5 supernatants induced NET formation independently of the presence of serum as these conditions show significantly elevated levels of citrullinated histones than untreated controls (Fig. 18B).

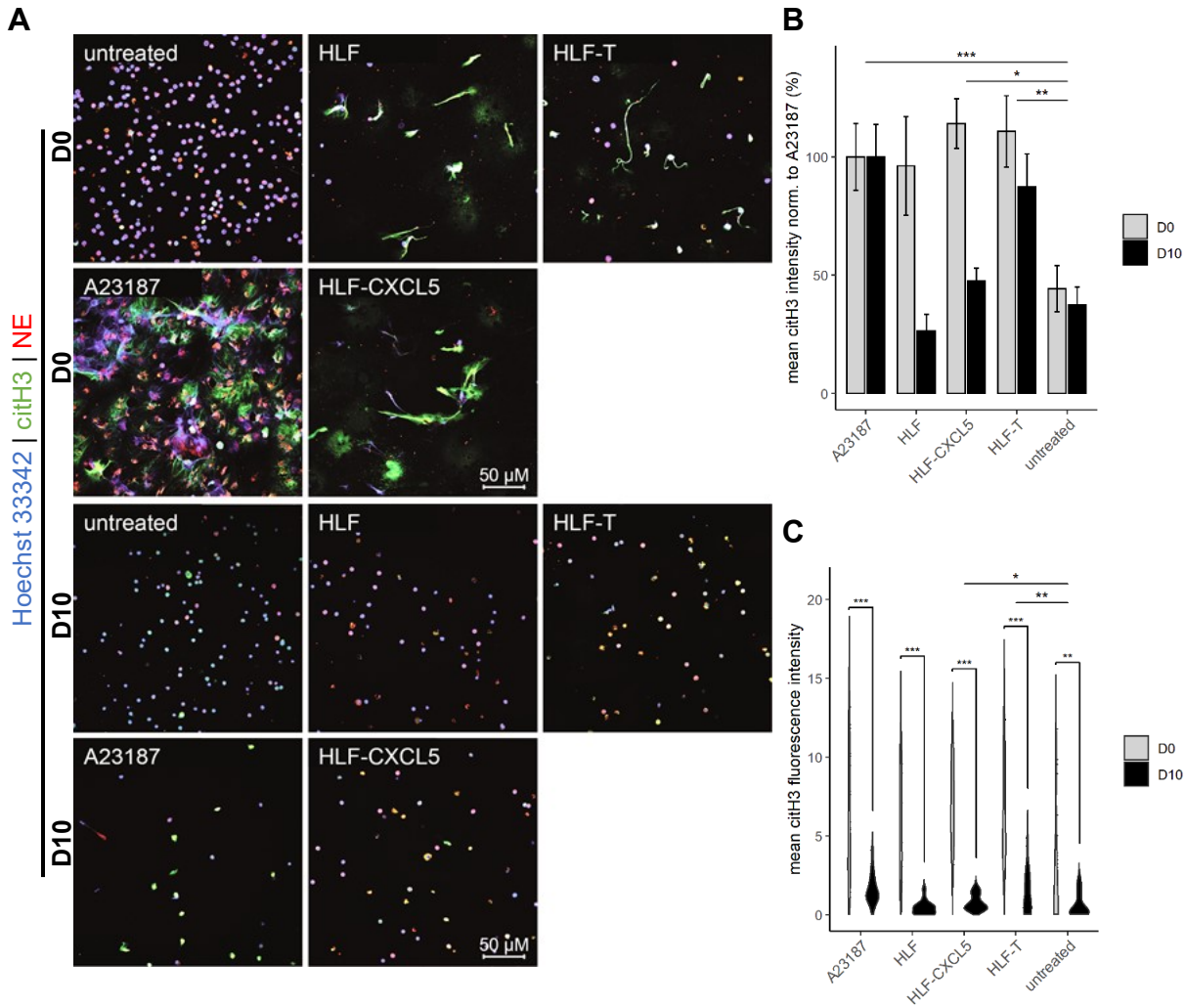



**Figure 17: Co-culture of neutrophils and human hepatoma cells induces NET formation.** (A) Representative pictures of confocal immunofluorescence analysis showing NET formation of murine neutrophils incubated with serum-free DMEM (D0) or with DMEM supplemented with 10% FCS (D10). The neutrophils were co-cultured with HLF, HLF-T and HLF-CXCL5 cells for 4 hrs or treated with A23187 calcium ionophore [10 µM] as positive control. Citrullinated histones (citH3) are indicated in green, neutrophil elastase (NE) is indicated in red, and the DNA was counterstained with Hoechst 33342 indicated in blue. (B) Quantification of immunofluorescence analysis. Data are expressed as mean ± SE. Error bars depict SE from 6 individual experiments. (C) Distribution of data depicted in (B). P-values were calculated with Kruskal-Wallis rank sum tests for more than 2 groups and Mann-Whitney-U tests for 2 groups. *P < 0.05, **P < 0.01, ***P < 0.001.

Neutrophils treated with the secretome of HLF cells did not display significant differences from untreated controls. In order to measure the amount of extracellular DNA released by the neutrophils stimulated with hepatoma supernatants, fluorometric Sytox Green DNA release assays were performed, in which only extracellular DNA was detected. Unfortunately, in the absence of serum, all conditions except the ones treated with calcium ionophore exhibited similar levels of extracellular DNA (Fig. 18D, left panel). In the presence of serum, differences were detectable, especially after 5 hrs, as neutrophils treated with hepatoma supernatants released more DNA than untreated controls (Fig. 18D, right panel). In addition, the amount of released MPO was quantified by using a MPO-ELISA for comparison of neutrophils co-cultured with HLF, HLF-T and HLF-CXCL5 cells in Transwell assays to neutrophils solely treated with hepatoma supernatants (Fig. 18E). We found significant differences in the amount of released MPO between Transwell co-cultures and supernatant conditions among all hepatoma cells.

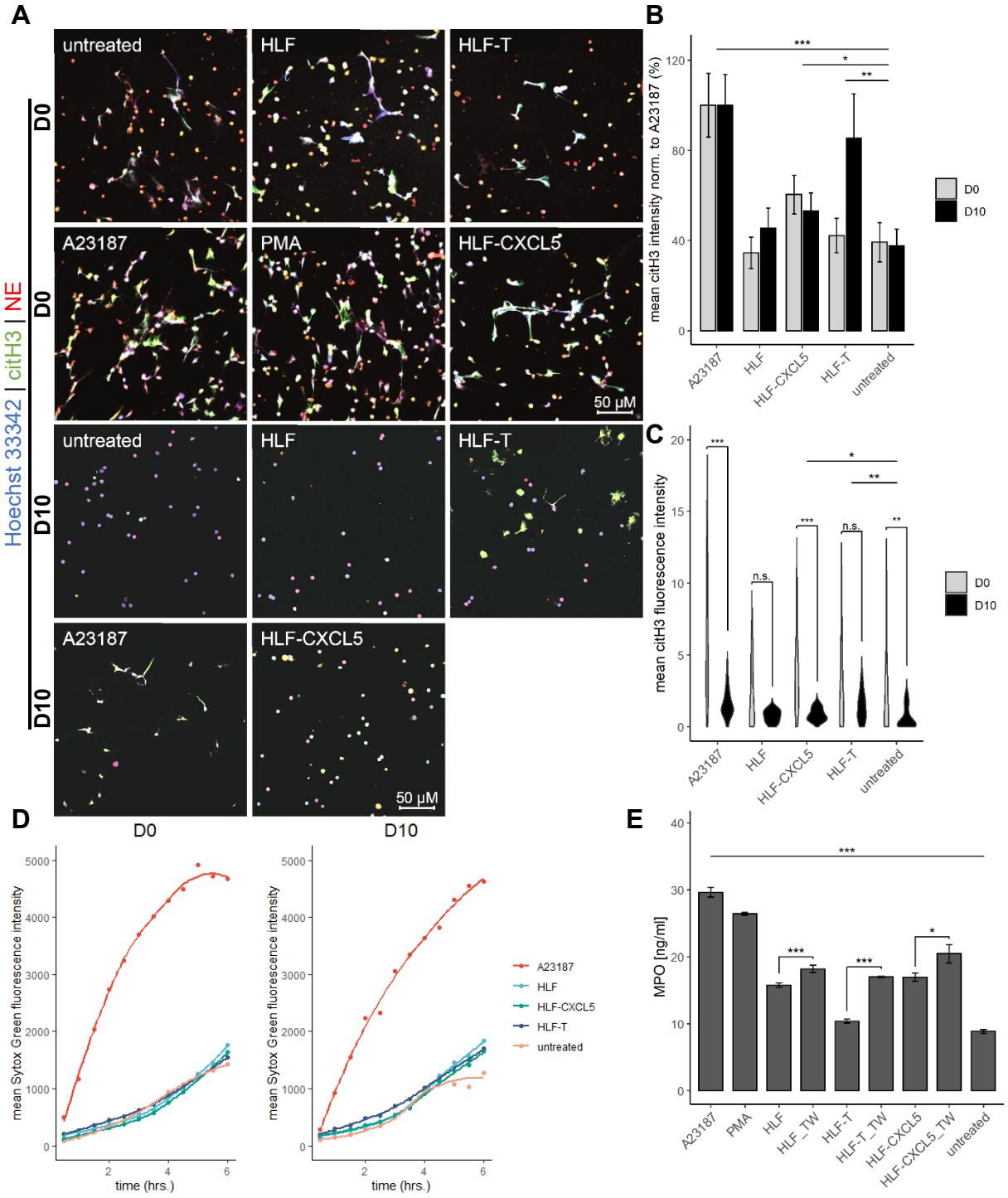



**Figure 18: Stimulation of neutrophils with supernatants of hepatoma cells.** (A) Representative pictures of confocal immunofluorescence analysis showing NET formation in murine neutrophils treated with supernatants of HLF, HLF-T and HLF-CXCL5 cells for 4 hrs or treated with A23187 calcium ionophore [10 µM] or PMA [50 nM] as positive control. The hepatoma supernatants were harvested prior after 24 hrs cultivation in either serum-free DMEM (D0) or DMEM supplemented with 10% FCS (D10). Citrullinated histones (citH3) are indicated in green, neutrophil elastase (NE) is indicated in red, and the DNA was counterstained with Hoechst 33342 indicated in blue. (B) Quantification of immunofluorescence analysis. Data are expressed as mean ± SE. Error bars depict SE from 7 individual experiments. (C) Distribution of data depicted in (B). (D) Sytox Green DNA release assay of neutrophils incubated with hepatoma supernatants in either D0 or D10. Data are expressed as mean. (E) Quantification of myeloperoxidase (MPO) via enzyme-linked immunosorbent assay (ELISA) released by neutrophils stimulated either by co-culturing with hepatoma cells in Transwells (TW) or with hepatoma supernatants for 4 hrs. Data are expressed as mean ± SD. P-values were calculated with Kruskal-Wallis rank sum tests for more than 2 groups and Mann-Whitney-U tests for 2 groups. *P < 0.05, **P < 0.01, ***P < 0.001.

Neutrophils treated with calcium ionophore A23187 show a 3.4-fold higher amount of MPO compared to untreated neutrophils, while PMA released 3-fold more MPO. HLF supernatants lead to 1.8-fold more release of MPO, while HLF co-culture even resulted in a 2.1-fold increase. Neutrophils treated with HLF-T supernatants display a 1.2-fold increase of released MPO, and neutrophils co-cultured with HLF-T cells release 1.9 more MPO than untreated neutrophils. HLF-CXCL5 cells showed the highest amount of released MPO as their supernatants lead to a 1.9-fold increase as well as Transwell co-culture released 2.3-fold more MPO than in untreated neutrophils. All conditions had to be tested in serum-free medium, as serum impeded quantification of MPO (Table 1 in annex). These data confirm that TGF-β-transformed and CXCL5-overexpressing hepatoma cells (HLF-T, HLF-CXCL5) induce significantly increased citrullination of histones and release of MPO, while parental HLF cells cause a differential response of neutrophils depending on the presence of serum.

### Differential response of neutrophils to SNU449 cell lines

As HLF and SNU449 cell lines respond differently to long-term TGF-β treatment (Fig. 14), we investigated whether long-term TGF-β treatment of SNU449 cells also leads to a different response in neutrophil DNA release. Histone citrullination is one of the first steps in initiation of NET formation, so this marker alone cannot distinguish between vital or lytic NET formation in the absence of membrane rupture. Lytic NET formation includes membrane rupture, therefore it is known as NETosis referring to this special kind of cell death in neutrophils (Yipp and Kubes 2013). If the membrane stays intact, the process of NET formation is vital. This facilitates other neutrophil effector functions. Treatment of neutrophils with SNU449-CXCL5, HLF-T and HLF-CXCL5 supernatants induced NETosis including membrane rupture comparably to the positive control calcium ionophore A23187, which is known to stimulate lytic NET formation (Fig. 19A). In contrast to HLF-T cells, SNU449-T cells were not able to induce histone citrullination (Fig. 19A-B). Surprisingly, the citrullination of histones was significantly increased upon treatment of neutrophils with parental SNU449 supernatants compared to untreated. Yet, the membranes of these neutrophils were still intact, which indicates early steps in NET formation either resulting in vital NET formation or initiation of NETosis. In line, released MPO levels were significantly elevated in neutrophils incubated with parental SNU449 supernatants (Fig. 19C). Of note, soluble proteins secreted by SNU449-T as well as SNU449-CXCL5 lead to increased MPO levels of neutrophils, although to a minor extent than HLF, HLF-T and HLF-CXCL5 cells. In conclusion, comparison of neutrophils treated with factors secreted by HLF cells and neutrophils treated with those secreted by SNU449 cells resulted in opposing levels of histone citrullination. HLF-T cells induced histone citrullination, whereas SNU449-T cells failed to do so. In contrast, MPO release was elevated in all conditions with even increased levels after incubation of neutrophils with HLF cell lines compared to untreated neutrophils. NET formation relies on many different pathways, and some of those do not require citrullination, therefore the shape of the nuclei should be taken into account as NETs are defined by extracellular DNA. Together, these data suggest that HLF-T, HLF-CXCL5 and SNU449-CXCL5 are successfully inducing NET formation including membrane rupture, while HLF, SNU449 and SNU449-T either stimulate alternative pathways resulting in vital NET formation independent of citrullination, similar to PMA, or simply lead to degranulation of neutrophils indicated by the high levels of released MPO.

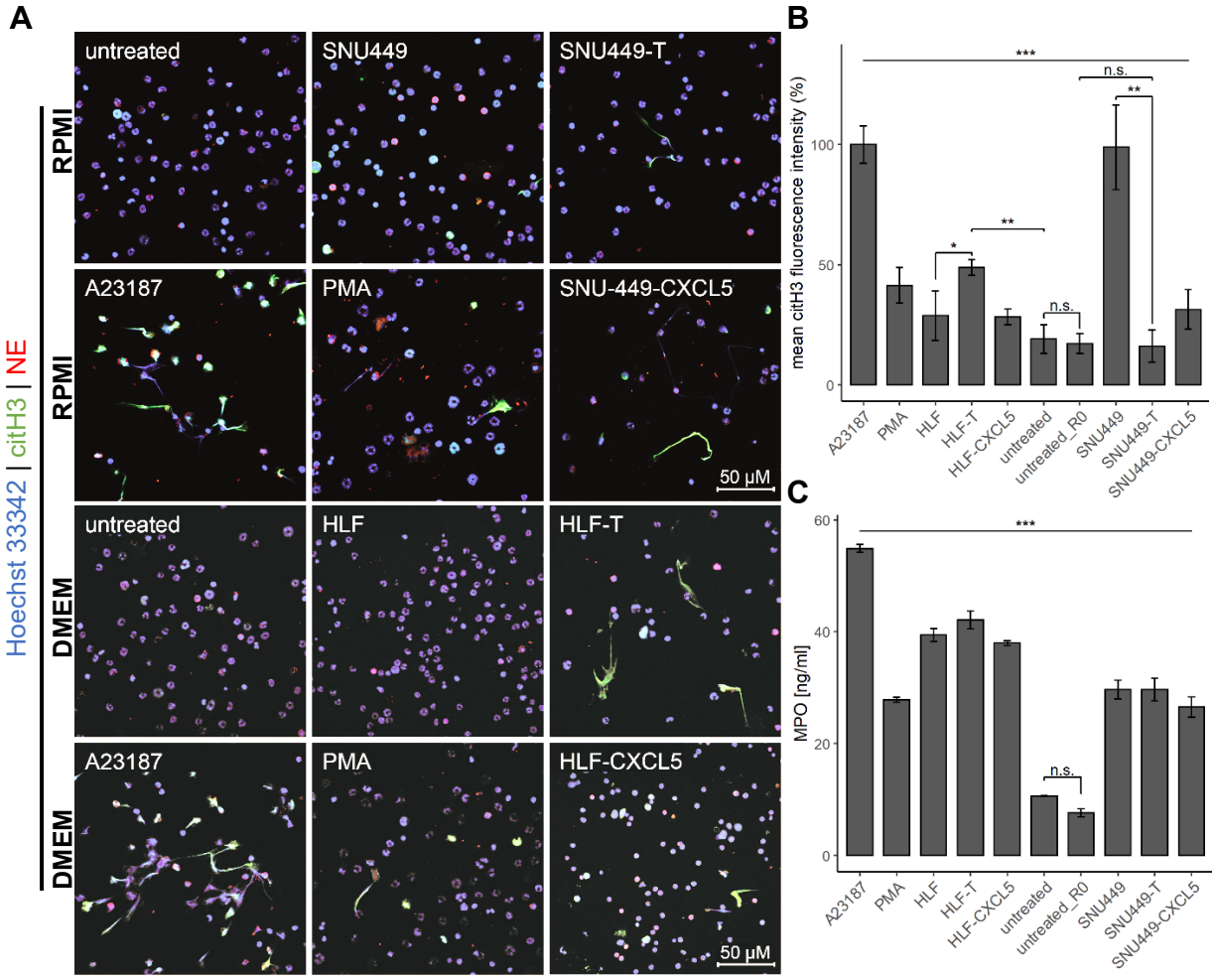



**Figure 19: Neutrophils respond differently to SNU449 cells.** (A) Representative pictures of confocal immunofluorescence analysis showing NET formation of murine neutrophils treated with supernatants of either SNU449, SNU449-T and SNU449-CXCL5 or HLF, HLF-T and HLF-CXCL5 cells for 4 hrs or treated with A23187 calcium ionophore [10 µM] as positive control. The hepatoma supernatants were harvested after cultivation for 24 hrs in serum-free DMEM (D0) or RPMI (R0). Citrullinated histones (citH3) are indicated in green, neutrophil elastase (NE) is indicated in red, and the DNA was counterstained with Hoechst 33342 indicated in blue. (B) Quantification of immunofluorescence analysis. Data are expressed as mean ± SE. Error bars depict SE from 2 individual experiments. (C) Quantification of myeloperoxidase (MPO) released by neutrophils after stimulation with hepatoma supernatants of SNU449 and HLF cell lines as depicted in (A) for 4 hrs. Data are expressed as mean ± SD. P-values were calculated with Kruskal-Wallis rank sum tests for more than 2 groups and Mann-Whitney-U tests for 2 groups. *P < 0.05, **P < 0.01, ***P < 0.001.

## Recombinant CXCL5 induces NET formation

The synergy of Axl and TGF-β signalling induces various target genes involved in EMT of cancer cells. One of these target genes is CXCL5. Ectopic expression of CXCL5 in HLF and SNU449 cells already indicated citrullination of histones and release of MPO, both being markers of NET formation. Next, recombinant CXCL5 alone was investigated for its ability to induce NETs in murine neutrophils. Quantification of immunofluorescence analysis revealed that low concentrations of recombinant CXCL5 [5 ng/ml] were more efficient than higher concentrations [>100 ng/ml] in inducing citrullination of histones and as such formation of NETs (Fig. 20A-B). Sytox Green DNA release assays did not indicate significant differences neither between different concentration of recombinant CXCL5 nor relative to untreated neutrophils due to high levels of spontaneous NET formation even though serum was present (Fig. 20C).

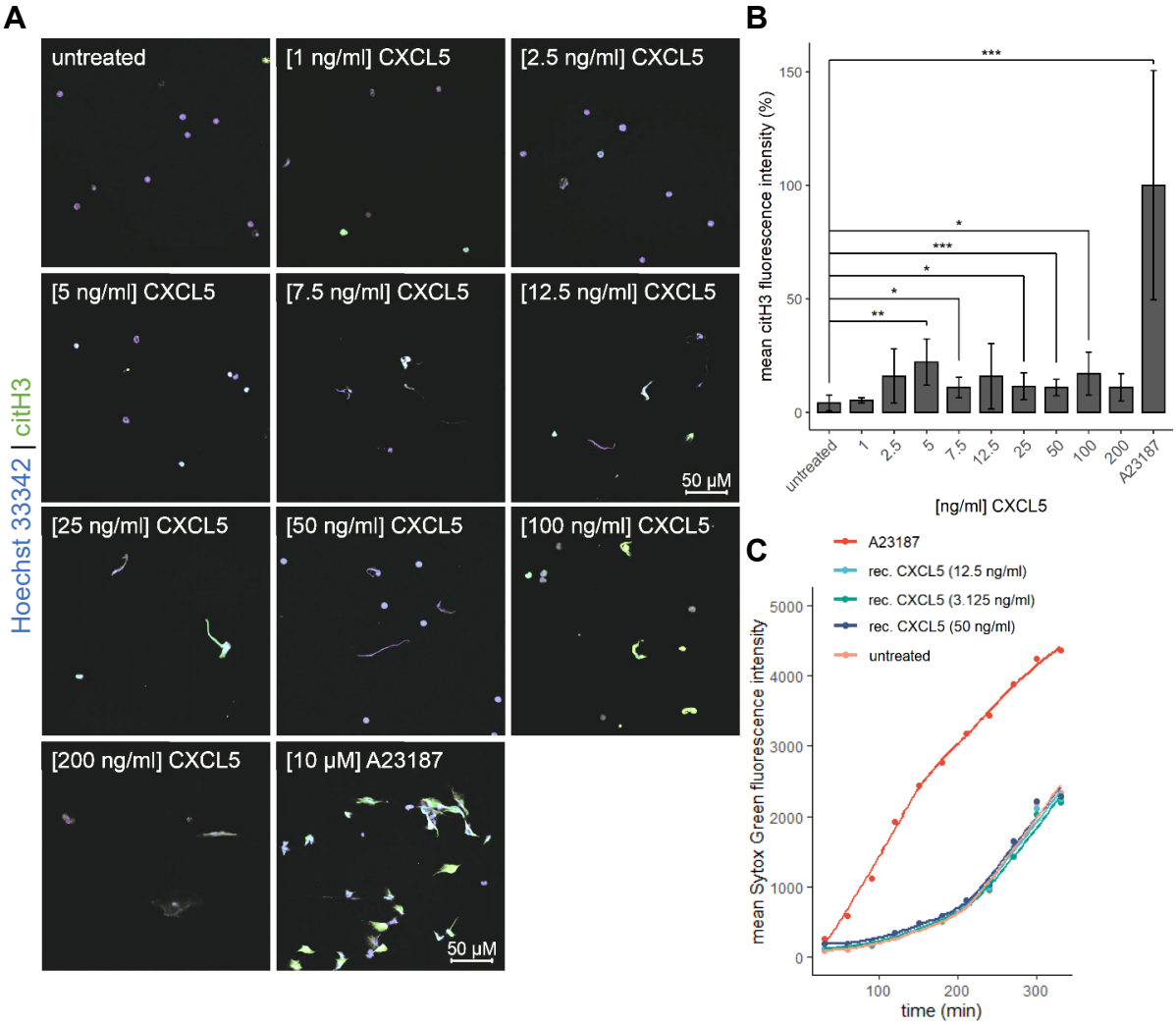



**Figure 20: Serial dilution of recombinant CXCL5.** (A) Representative pictures of confocal immunofluorescence analysis showing murine neutrophils incubated with different concentrations of recombinant CXCL5 ranging from [1 ng/ml] to [200 ng/ml] or [10 µM] A23187 in DMEM supplemented with 10% FCS (D10) for 4 hrs. Citrullinated histones (citH3) are indicated in green, and the DNA was counterstained with Hoechst 33342 indicated in blue. (B) Quantification of immunofluorescence analysis. Data are expressed as mean ± SD. Error bars depict SD from 2 individual experiments. (C) Sytox Green DNA release assay of neutrophils incubated with different concentrations of recombinant CXCL5: 3.125 – 12.5 – 50 [ng/ml] in D10. Data are expressed as mean. P-values were calculated with Kruskal-Wallis rank sum tests for more than 2 groups and Mann-Whitney-U tests for 2 groups. *P < 0.05, **P < 0.01, ***P < 0.001.

Next, the effect of recombinant CXCL5 was directly compared with the soluble factors secreted by HLF, HLF-T and HLF-CXCL5 cells. Due to the fact that neutrophils stimulated with low concentrations of recombinant CXCL5 [5 ng/ml] exhibited higher NET formation compared to neutrophils stimulated with higher concentrations, these low concentrations were tested against hepatoma supernatants first in presence and absence of serum (Fig. 21A). Quantification of citrullination of histones revealed that there is a significant difference between serum-starved and serum-supplemented conditions in HLF-CXCL5 and recombinant CXCL5, yet these did not affect their significant difference to respective untreated controls (Fig. 21B). Independently of serum-starvation, 5 ng/ml recombinant CXCL5 and secreted factors in HLF-CXCL5 supernatants were able to induce citrullination of histones in 6 independent experiments to a significantly higher extent than untreated neutrophils.

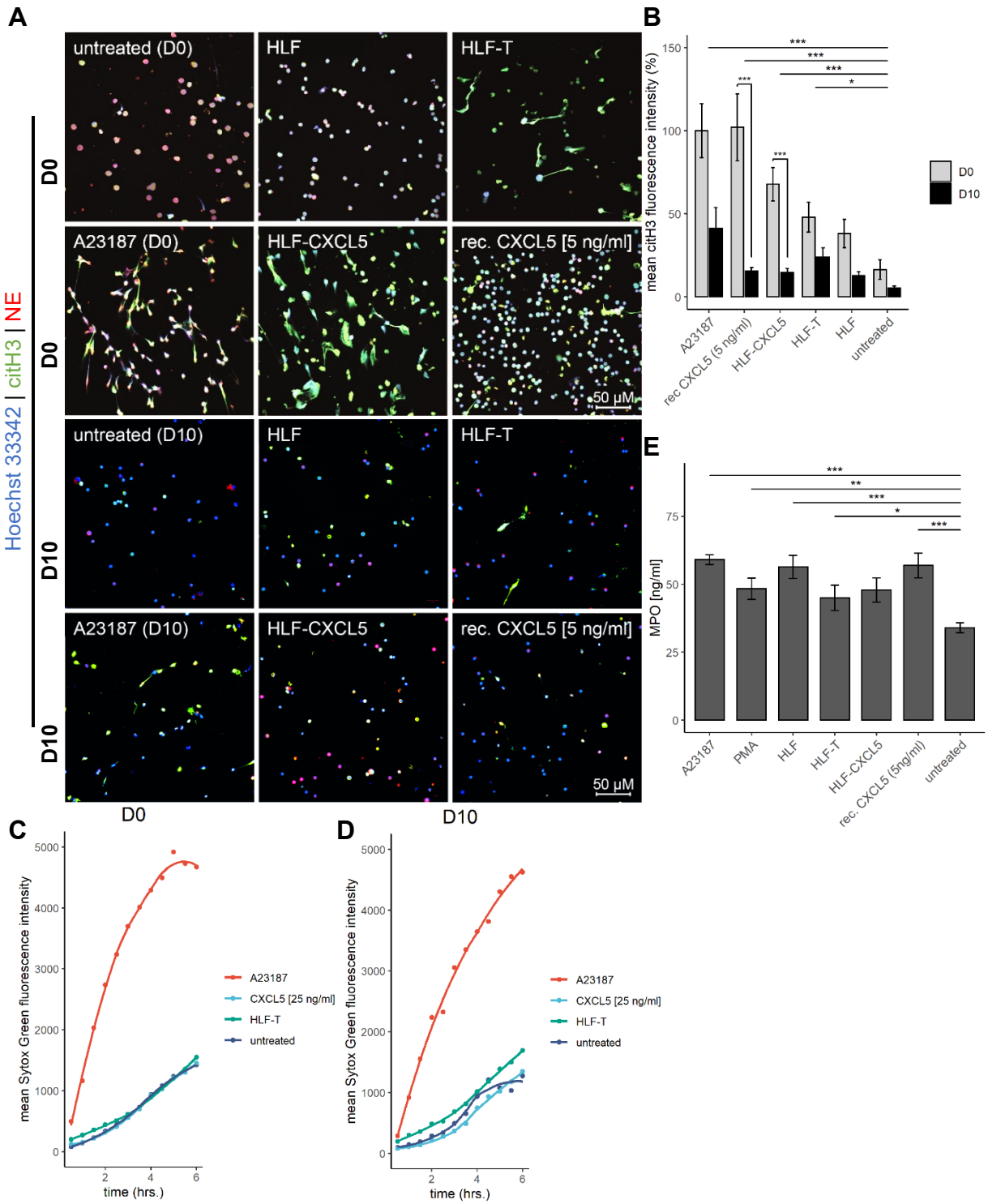



**Figure 21: Comparison of effects of hepatoma supernatants with recombinant CXCL5 on NET formation.** (A) Representative pictures of confocal immunofluorescence analysis showing murine neutrophils incubated with either hepatoma supernatants or recombinant CXCL5 [5 ng/ml] in serum-free DMEM (D0) or in DMEM supplemented with 10% FCS (D10). Citrullinated histones (citH3) are indicated in green, neutrophil elastase (NE) is indicated in red, and the DNA was counterstained with Hoechst 33342 indicated in blue. (B) Quantification of immunofluorescence analysis. All values were normalized to A23187 treated neutrophils in D0 (100%). Data are expressed as mean ± SE. Error bars depict SE from 6 individual experiments. (C) and (D) Sytox Green DNA release assay of neutrophils incubated with either hepatoma supernatants or recombinant CXCL5 [25 ng/ml] in D0 or D10. Data are expressed as mean from 2 independent experiments. (E) Quantification of released MPO with ELISA in D0 corresponding to the conditions depicted in (A). P-values were calculated with Kruskal-Wallis rank sum tests for more than 2 groups and Mann-Whitney-U tests for 2 groups. *P < 0.05, **P < 0.01, ***P < 0.001.

DNA quantification by Sytox Green assays again displayed only differences between samples in the presence of serum due to the high level of spontaneous DNA release in serum-starved untreated neutrophils (Fig. 21C-D). Recombinant CXCL5 did not lead to release of DNA in Sytox Green DNA release assays. Yet, it has to be considered that these assays failed to confirm immunofluorescence data in any of the experimental setups (Fig. 18, 20-21C/D). Nevertheless, quantification of released MPO from neutrophils supports the hypothesis that HLF-T and recombinant CXCL5 are able to stimulate NET formation as indicated by the significantly increased amounts of released MPO (Fig. 21E).

### Inverted dose response in NET formation to recombinant CXCL5

In order to confirm concentration-dependent effects of CXCL5 on NET formation, the soluble proteins secreted by hepatoma cells were concentrated and used to stimulate neutrophils. Additionally, different concentrations of recombinant CXCL5 were used. Despite the fact that one would assume that concentration of HLF-T secreted factors should lead to similar results as higher doses of recombinant CXCL5, these data demonstrate unconcise results (Fig. 22A-C). On the one hand, concentrated HLF and HLF-T supernatants stimulated citrullination of histones in neutrophils. On the other hand, increased concentrations of recombinant CXCL5 rather interfered with citrullination of histones, as the highest concentration of 25 ng/ml CXCL5 did even not induce significantly more citrullination than untreated controls. Nevertheless, it has to be considered that quantification of histone citrullination does not include the shape of cells and does not reflect plasma membrane rupture, which is of course the last step in NETosis.

As shown by immunofluorescence analysis, neutrophils treated with unconcentrated HLF or concentrated HLF and HLF-T supernatants mainly display intact cell membranes compared to neutrophils treated with concentrated HLF-CXCL5 or unconcentrated HLF-T and HLF-CXCL5 supernatants, which induced membrane rupture to a large extent (Fig. 22A). Quantification of citrullination of histones revealed that there is a difference between unconcentrated and concentrated HLF and HLF-T supernatants, but no difference in unconcentrated and concentrated HLF-CXCL5 supernatants (Fig. 22B). These data suggest that the level of citrullination of histones depends on the concentration of soluble factors secreted by parental or long-term TGF-β exposed hepatoma cells. Yet, final steps of NETosis indicated by ruptured cell membranes are only achieved by stimulating neutrophils with lower concentration of those soluble factors. Interestingly, histone citrullination as well as membrane rupture are independent of concentration of the soluble factors released by HLF-CXCL5 cells. Furthermore, quantification of MPO levels shows significant decrease of released MPO levels in neutrophils treated with either concentrated supernatants or higher doses of recombinant CXCL5 (Fig. 22C). These data suggest not only dose-dependent effects of NET formation to soluble factors secreted by HLF and HLF-T cells, but also an inverted dose response of NET formation to CXCL5.

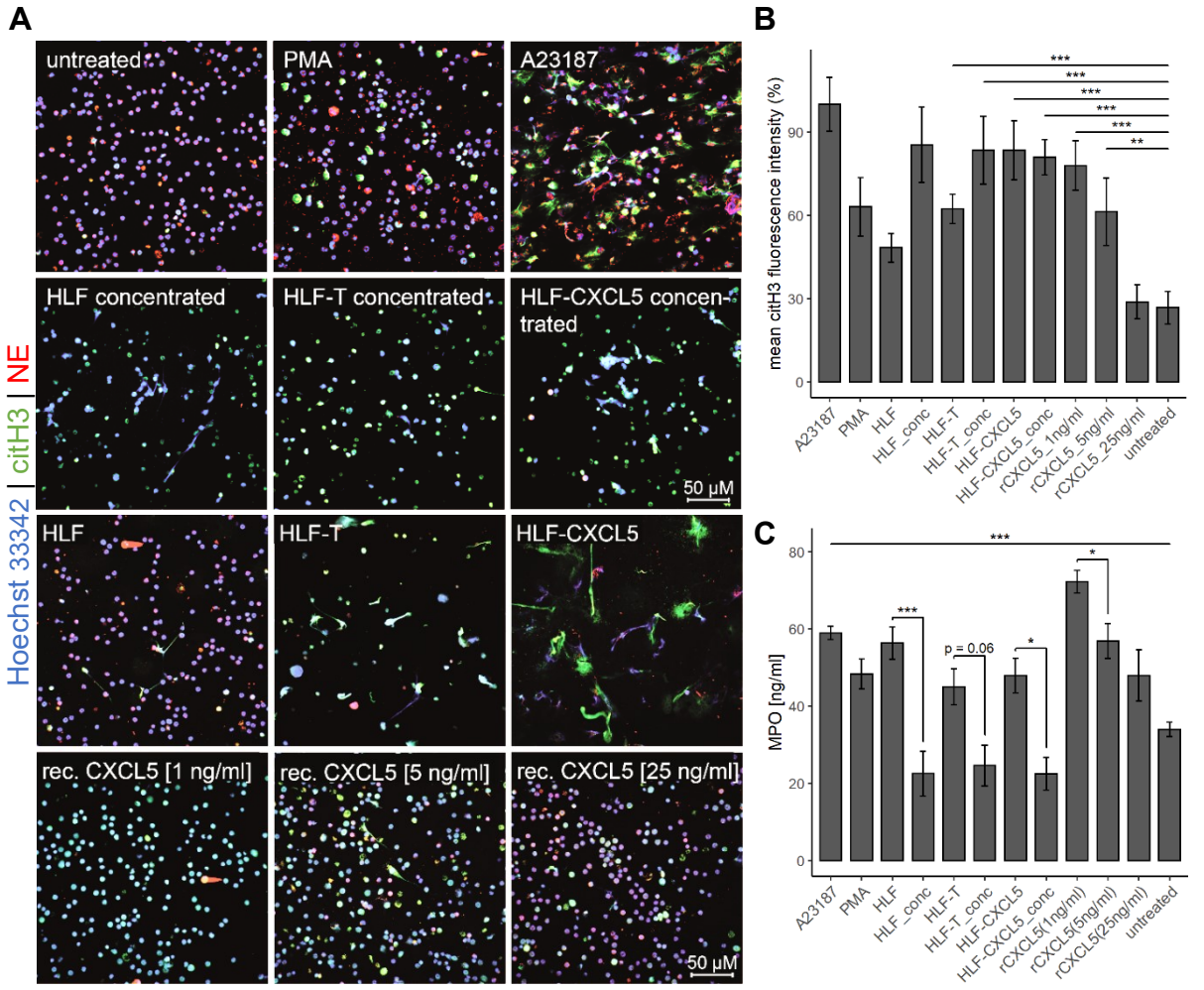



**Figure 22: Concentration of supernatants versus increasing concentrations of recombinant CXCL5.** (A) Representative pictures of confocal immunofluorescence analysis showing murine neutrophils incubated with either concentrated hepatoma supernatants using centricons or recombinant CXCL5 in serum-free DMEM (D0). Citrullinated histones (citH3) are indicated in green, neutrophil elastase (NE) is indicated in red, and the DNA was counterstained with Hoechst 33342 indicated in blue. (B) Quantification of immunofluorescence analysis. Data are expressed as mean ± SE. Error bars depict SE from 6 individual experiments. (C) Quantification of released MPO with ELISA in D0. P-values were calculated with Kruskal-Wallis rank sum tests for more than 2 groups and Mann-Whitney-U tests for 2 groups. *P < 0.05, **P < 0.01, ***P < 0.001.

## Inhibition of PAD4

There are different pathways initiating NET formation, which are dependent on or independent of NADPH oxidase and citrullination (Jorch and Kubes 2017). NADPH oxidase-dependent NET formation by e.g. stimulation with PMA does not necessarily rely on citrullination (Holmes, Shim et al. 2019). NET formation initiated by calcium ionophores such as ionomycin or A23187 are independent of NADPH oxidase but rely on calcium which further activates PAD4 mediating the conversion of arginine into citrulline (Parker, Dragunow et al. 2012). The pathways underlying CXCL5-induced NET formation are entirely unknown. Therefore, blocking of PAD4 could elucidate the dependence of CXCL5-mediated NET formation on citrullination. In order to specifically inhibit citrullination, the PAD4 inhibitor GSK484 was used at different concentrations prior to treatment with CXCL5. Surprisingly, lower concentrations of the PAD4 inhibitor than recommended [1 mM] were insufficient in blocking NET formation and even lead to increased DNA release when compared to neutrophils exclusively stimulated with recombinant CXCL5 (Fig. 23A).

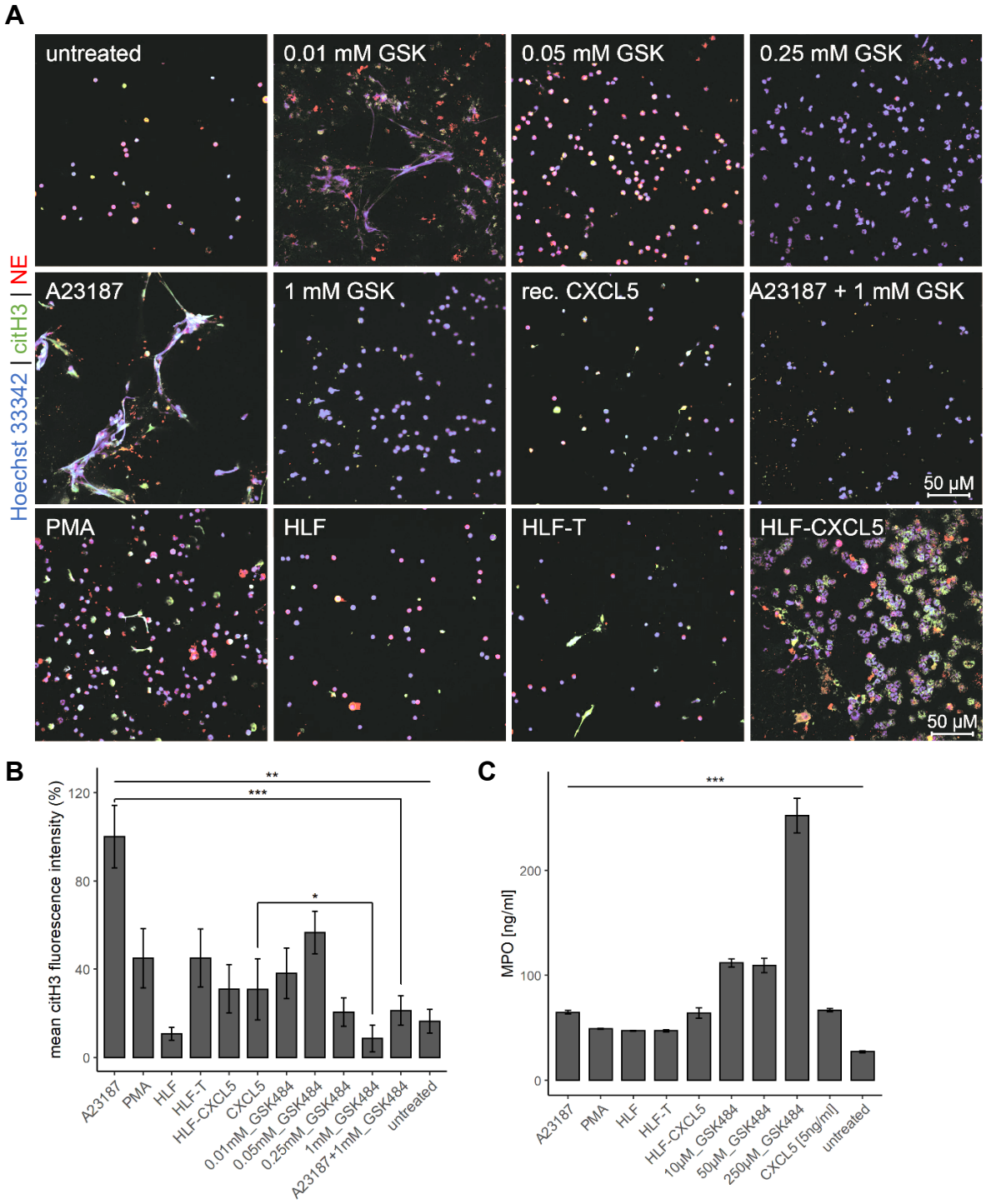



**Figure 23: Unspecific inhibition of PAD4.** (A) Representative pictures of confocal immunofluorescence analysis showing murine neutrophils incubated with different concentrations of PAD4 inhibitor GSK484 ([0.01 mM], [0.05 mM], [0.25 mM], [1 mM]) prior to stimulation with recombinant CXCL5 [5 ng/ml]. [1mM] GSK484 was added to neutrophils stimulated with calcium ionophore A23187 [10µM]. Additionally, neutrophils were incubated with hepatoma supernatants as a comparison in serum-free DMEM (D0). Citrullinated histones (citH3) are indicated in green, neutrophil elastase (NE) is indicated in red, and the DNA was counterstained with Hoechst 33342 indicated in blue. (B) Quantification of immunofluorescence analysis. Data are expressed as mean ± SE. Error bars depict SE from 3 individual experiments. (C) Quantification of released MPO with ELISA in D0. Data are expressed as mean ± SD. P-values were calculated with Kruskal-Wallis rank sum tests for more than 2 groups and Mann-Whitney-U tests for 2 groups. *P < 0.05, **P < 0.01, ***P < 0.001.

Quantification of immunofluorescence showed that higher concentrations of GSK484 (250 µM or 1 mM) decreased CXCL5- and calcium ionophore-stimulated NET formation (Fig. 23B). However, further immunofluorescence analysis revealed that histone citrullination might be decreased, and neutrophils incubated with high concentrations of GSK484 display deformation of cell nuclei and membrane rupture. Quantification of released MPO confirmed that low concentrations of the PAD4 inhibitor rather stimulated NET formation than blocking it (Fig. 23C). These findings suggest an either citrullination-independent NET formation upon CXCL5 stimulation, as it is known for PMA, or compensation of PAD4 by other enzymes such as PAD2 which could mediate citrullination and NET formation.

### Interference with Axl and TGF-β signalling

In order to examine the dependence of the soluble factors secreted by HLF-T, which are able to stimulate NET formation dependent on the Axl and TGF-β signalling pathways, chemical and genetic inhibition was used to block these receptors. The TGF-β receptor I/II was blocked with the small molecule inhibitor LY2109761, which binds to the adenosine-triphosphate (ATP) binding site of the TGF-β R1 kinase domain (Nagaraj and Datta 2010). The Axl receptor was inhibited with Dubermatinib (TP-0903), a small molecule inhibitor blocking the ATP binding site of Axl kinase domain (Mollard, Warner et al. 2011). Additionally, HLF-Axl-knock-out (KO) cell lines were used, where exon 7 was edited using the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system resulting in a premature stop in translation and thus absence of the Axl protein (Scharf, Bierbaumer et al. 2018). Quantification of citrullination of histones revealed that genetic inhibition of Axl in HLF cells blocks NET formation. Neutrophils treated with HLF-Axl-KO or HLF-Axl-KO-T supernatants did not exhibit significantly different amounts of citrullinated histone H3 from untreated controls (Fig. 24A). Although there was no significant decrease compared to neutrophils treated with HLF or HLF-T supernatants, a trend is detectable (Fig. 24B). Notably, quantification of staining intensities exhibited even increased levels of citrullinated histones in neutrophils treated with the chemical inhibitors, LY2109761 and TP-0903. These results were in line with the quantification of MPO levels, as all conditions with blocked Axl/TGF-β signalling showed elevated levels of released MPO (Fig. 24C). None of the differences within the groups, like HLF-T and HLF-T treated with LY inhibitor, were significant due to the high variance in the data (Fig. 24D-E). Yet, a certain trend of more NET induction upon Axl/TGF-β interference was detectable, which is supported by the significant increase of histone citrullination as well as by the level of released MPO compared to untreated neutrophils.

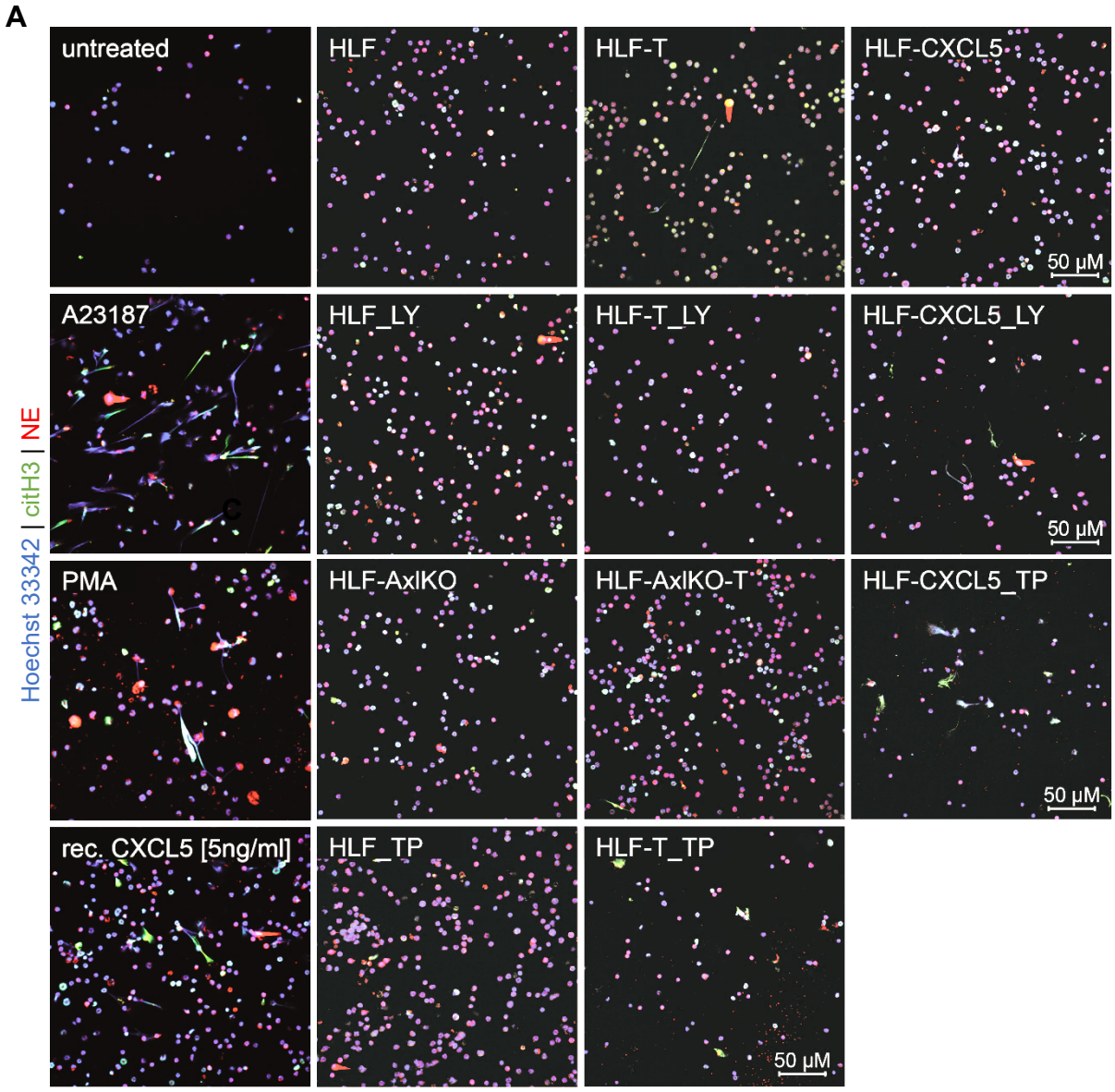
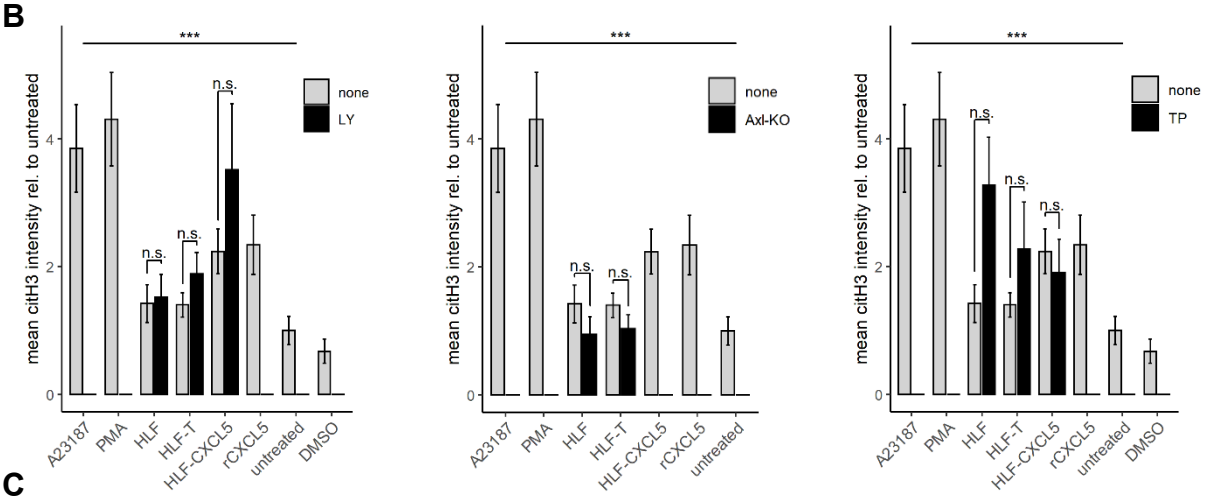



**Figure 24: Inhibition of Axl and TGF-β receptors.** (A) Representative pictures of confocal immunofluorescence analysis showing murine neutrophils incubated with supernatants of hepatoma cells treated with LY2109761 [10 µM] to inhibit TGF-β receptors I/II or TP0903 [0.5 µM] to inhibit the Axl receptor, or with supernatants of Axl-KO hepatoma cells with or without long-term TGF-β treatment cultured in serum-free DMEM (D0). Citrullinated histones (citH3) are indicated in green, neutrophil elastase (NE) is indicated in red, and the DNA was counterstained with Hoechst 33342 indicated in blue. **Next page:** (B) Quantification of immunofluorescence analysis depicted in (A). Data are expressed as mean ± SE. Error bars depict SE from 4 individual experiments. (C) Quantification of released MPO from the conditions described in (A) with ELISA in D0. (D) Table: Significant differences in histone citrullination compared to untreated neutrophils from data shown in (B). (E) Table: Significant differences in released MPO levels compared to untreated neutrophils from data shown in (C). P-values were calculated with Kruskal-Wallis rank sum tests for more than 2 groups and Mann-Whitney-U tests for 2 groups. *P < 0.05, **P < 0.01, ***P < 0.001.

B

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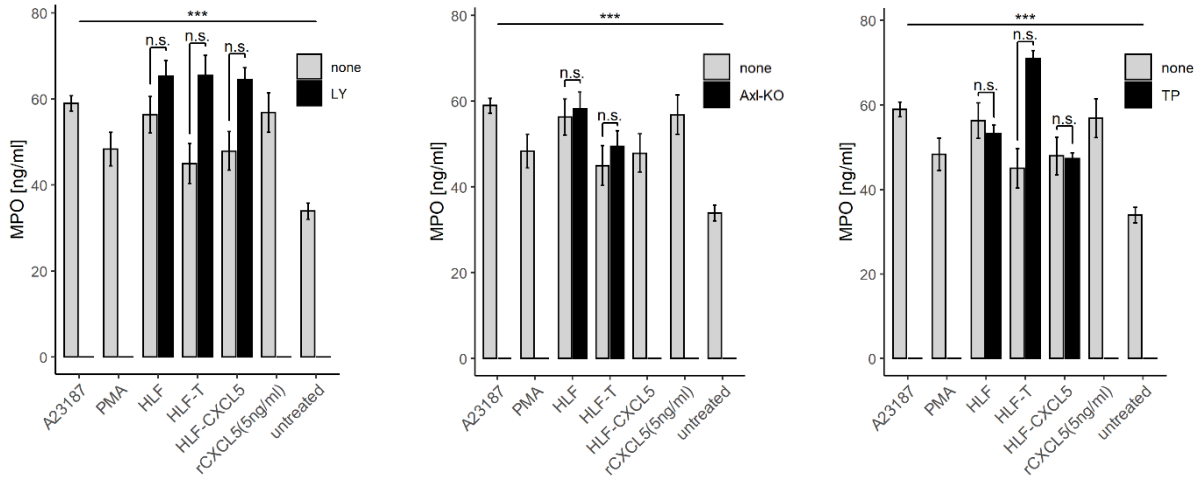


D

| sample | Mean citH3 intensity | SE | significant difference to untreated |
|---|---|---|---|
| A23187 | 9.9 | 1.8 | *** |
| HLF | 3.6 | 0.8 | . |
| HLF_LY | 3.9 | 0.9 | * |
| HLF_TP | 8.4 | 1.9 | ** |
| HLF-AxlKO | 2.4 | 0.7 | |
| HLF-AxlKO-T | 2.7 | 0.5 | |
| HLF-CXCL5 | 5.7 | 0.9 | *** |
| HLF-CXCL5_LY | 9.0 | 2.6 | ** |
| HLF-CXCL5_TP | 4.9 | 1.3 | * |
| HLF-T | 3.6 | 0.5 | ** |
| HLF-T_LY | 4.8 | 0.9 | ** |
| HLF-T_TP | 5.8 | 1.9 | * |
| PMA | 11.0 | 1.9 | *** |
| rec. CXCL5 (5ng/ml) | 6.0 | 1.2 | *** |
| untreated | 2.6 | 0.6 | |

E

| sample | mean MPO [ng/ml] | SE | significant difference to untreated |
|---|---|---|---|
| A23187 | 59.0 | 1.8 | *** |
| HLF | 56.3 | 4.2 | *** |
| HLF_LY | 65.3 | 3.7 | *** |
| HLF_TP | 53.3 | 2.0 | *** |
| HLF-AxlKO | 58.2 | 4.0 | *** |
| HLF-AxlKO-T | 49.5 | 3.6 | *** |
| HLF-CXCL5 | 47.9 | 4.4 | . |
| HLF-CXCL5_LY | 64.6 | 2.7 | *** |
| HLF-CXCL5_TP | 47.3 | 1.4 | *** |
| HLF-T | 45.0 | 4.7 | * |
| HLF-T_LY | 65.4 | 4.7 | *** |
| HLF-T_TP | 71.0 | 1.9 | *** |
| PMA | 48.3 | 3.9 | ** |
| rec. CXCL5 (5ng/ml) | 56.9 | 4.6 | *** |
| untreated | 33.9 | 1.8 | |

## Discussion

Hepatoma cells respond differently to long-term TGF-β exposure with respect to changes of their migratory behaviour as well as their secretome (Haider, Hnat et al. 2019). HLF-T cells display increased migration while SNU449-T cells exhibit decreased motility (Fig. 14). By employing this model of TGF-β-dependent cancer cell invasion, we show that NET formation can be induced *in vitro* by soluble factors secreted from hepatoma cells including the chemokine CXCL5. In particular, NET formation was observed after optimisation of culture conditions such as determination of neutrophil density and serum concentrations to avoid artefacts from spontaneous alterations of the neutrophil phenotype (Fig. 15-16). In co-cultures of neutrophils with HCC cells either exposed to long-term TGF-β (HLF-T) or ectopically expressing CXCL5 (HLF-CXCL5), we could demonstrate that neutrophils undergo histone citrullination in the presence as well as in the absence of serum *in vitro* (Fig. 17). In line, HLF-T and HLF-CXCL5 cell supernatants showed similar effects, which was confirmed by quantification of released MPO (Fig. 18). Yet, neutrophils co-cultured with hepatoma cells in Transwell assays released significantly more MPO than those stimulated by supernatants. Supernatants of SNU449 cells were also able to stimulate MPO release in neutrophils, however, histone citrullination was only detectable in neutrophils treated with supernatants derived from SNU449-CXCL5 (Fig. 19). This observation suggests that SNU449-derived soluble factors either favour degranulation of neutrophils or induce different pathways of NET formation independent of histone citrullination such as vital NET formation as the cell membranes were still intact. These data support recent findings that HLF and SNU449 cells change their secretome in response to long-term TGF-β exposure each in a different way. While the HLF-T secretome stimulated NETosis indicated by citrullination of histones linked to membrane rupture and MPO release, SNU449-T cells secreted factors only caused release of MPO due to degranulation or vital NET formation. In line with our hypothesis, recombinant CXCL5 was able to stimulate NET formation as well as the soluble factors released by HLF-T or HLF-CXCL5 cells (Fig. 20-21). While the concentration of soluble factors secreted by hepatoma cells increased the level of histone citrullination in comparison to unconcentrated supernatants, the MPO release was inversely correlated to the concentration of hepatoma supernatants and amount of recombinant CXCL5 (Fig. 22). Decreasing histone citrullination and MPO levels upon usage of rising recombinant CXCL5 concentrations implicate an inverse dose response of NET formation to CXCL5 (Fig. 22). As inhibition of PAD4 with GSK484 was only detectable at very high concentrations of 1 mM, we suggest that the stimulation of neutrophils with CXCL5 either causes PAD4-independent NET formation or uses salvage pathways if PAD4 is blocked (Fig. 23). Finally, interference with Axl/TGF-β signalling by chemical and genetical inhibition of the receptors displayed neither significant differences in histone citrullination nor MPO release (Fig. 24). We detected a trend for the chemical inhibition as quantification of histone citrullination and MPO release revealed increased levels upon interference with Axl and TGF-β receptor activation. Yet, immunofluorescence imaging revealed that there was no significant level of extracellular DNA nor extracellular citrullinated histones in general, suggesting a very early stage of histone decondensation in the process of NET formation. Genetic deletion of Axl revealed rather decreased histone citrullination, yet increased MPO release without statistical significance.

Our study employing murine neutrophils need to be confirmed by investigating the interaction of HCC cells with human neutrophils allowing a more relevant translation of data into the patient situation. Yet, it has to be considered that the extraction of murine bone marrow neutrophils depicts the status of HCC patients in a better way than using neutrophils of healthy human donors. The reason is that primary tumours cause neutrophil expansion and release of immature neutrophils into the circulation, where they can be primed for polarization towards a TAN-phenotype (Coffelt, Wellenstein et al. 2016). As bone marrow isolation of neutrophils goes along with extraction of immature neutrophils, indicated by the horse-shoe shape of the nuclei, these immature murine neutrophils behave similarly to the immature TANs in the patients. In contrast to that, neutrophils derived from the peripheral blood of healthy human donors are mature. As a result, the *in vivo* situation of patients is modelled in a better way using immature neutrophils. Ideally, these findings should be reproduced using neutrophils from HCC patients in comparison to neutrophils from healthy human donors as a control.

Detection and analysis of NETs *in vitro* came along with a couple of obstacles and technical limitations. Neutrophils are very sensitive to pyrogens increasing the risk of spontaneous NET formation in the absence of serum, which protects neutrophils from unwanted NET stimuli. Next, neutrophils are non-adhering cells, which complicates the analysis with immunofluorescence methods. Healthy, non-stimulated neutrophils show less attachment to glass slides, although these were thoroughly coated with poly-L-lysine in advance. Despite the same neutrophil count was seeded for each experiment, the densities on the plates showed high variability. Apart from that, we faced differences in neutrophil response depending on the season, which has already been reported in previous *in vitro* studies (Klink, Bednarska et al. 2012). During all experiments in spring and summer, neutrophils exhibited increased spontaneous NET formation in general and responded stronger to all stimuli compared to neutrophils in autumn and winter experiments. Prior studies confirm these findings as adhesion and ROS production in response to PMA was highest in April and Mai. Even the expression of the surface markers CD11b/CD18 varied with the season suggesting different levels of susceptibility for infections, as ROS production and CD11b expression are associated with bactericidal activity of neutrophils (Klink, Bednarska et al. 2012). Many mammal species, including humans and mice, show seasonal variations in immune cell activity, which has been linked to melatonin and the photoperiod (Silva, Rodrigues et al. 2004).

Besides, confocal microscopy facilitates imaging with low background, but if DNA, chromatin and associated proteins are not exactly located in the same plane, only one parameter per image can be analysed properly. In this case, histone citrullination turned out to be the best marker of NET formation in this experimental setup with regard to specificity as well as sensitivity. NE staining lead to different problems as at that time only two suitable antibodies were available – one of rabbit and one of mouse origin. As the antibody for detection of histone citrullination already derived from a rabbit source, the mouse-on-mouse protocol had to be used and modified for visualisation of NE on murine neutrophils. However, there was still some risk of false-positive staining, which excluded NE as quantitative marker for NET formation. Instead, NE and DNA, visualized by Hoechst 33342, were used as qualitative markers for NET formation.

Furthermore, the Sytox Green DNA release assay showed high variance in the data with high levels of spontaneous DNA release in untreated neutrophils. This resulted in almost indistinguishable levels of DNA release between hepatoma supernatant-treated neutrophils and untreated controls upon serum starvation. In the presence of serum, the spontaneous DNA release was decreased, and untreated controls exhibited lower levels of released DNA than hepatoma supernatants in Sytox Green DNA release assays. This confirms the increased levels of histone citrullination and extracellular DNA providing reliable results for NET formation upon neutrophil stimulation with hepatoma supernatants. Conversely, recombinant CXCL5 failed to stimulate significantly more DNA release than untreated controls in Sytox Green assays due to the high level of spontaneous DNA release. The reason might be that the black plates with clear bottom used for the fluorometric analysis were not pyrogen free in contrast to the ordinary cell culture plates used for immunofluorescence analysis.

Moreover, quantification of released MPO by ELISA turned out to be a reliable method for complementing histone citrullination levels in immunofluorescence analyses. The only limitation was that the presence of serum distorted the measured optical density values, so only serum-free samples could be properly quantified. Additionally, the MPO release alone does not guarantee NET formation as the cause of MPO release might also be degranulation. As a result, only the combination of histone citrullination and MPO release should be considered as quality criterium for NET formation. Together with extracellular DNA and thus membrane rupture, which is detectable by immunofluorescence analysis, the process of NETosis can be monitored. Therefore, HLF-T, HLF-CXCL5 as well as recombinant CXCL5 at lower concentrations were shown to stimulate NETosis in murine neutrophils according to these criteria.

As PAD4 is central in the process of histone citrullination, we aimed to examine the dependence of CXCL5-induced NET formation on PAD4 by using the compound GSK484. GSK484 is a competitive inhibitor which reversibly binds to the active site of the low-calcium form of PAD4 (The Structural Genomics Consortium 2020), leading to a conformational change of PAD4 and blocking the enzymatic reaction. As this inhibition is competitive, it is dependent on the substrate concentration, which partly explains why such high concentrations of GSK484 were required. Surprisingly, testing of different concentrations of GSK484 revealed that lower concentrations, i.e. below 250 µM, even potentiated the effect of CXCL5 and even increased NET formation in comparison to neutrophils treated with CXCL5 alone (Fig. 23). Interestingly, no reports about this phenomenon are available in the literature, as specific inhibition of PAD4 was described by using the concentration of 10 µM GSK484 (Lewis, Liddle et al. 2015). Calcium levels influence the affinity of GSK484 for PAD4, which lower its half maximal inhibitory concentration (IC50) to 50 nM in the absence of calcium, while high calcium concentrations such as 2 mM increases the IC50 to 250 nM (Lewis, Liddle et al. 2015). Resting calcium concentrations in the cytoplasm account for 150 nM, while receptor-mediated signalling events raise intracellular calcium concentrations to 500 nM, which is not sufficient for PAD activation as these enzymes require mM concentrations of calcium for hypercitrullination (Zhou, An et al. 2018). As such, PAD is activated by extracellular calcium influx or ROS. Previous studies suggested that other proteins binding to PAD enzymes might modulate their requirement for calcium, for instance by favouring the enzymatically active conformation (Zhou, An et al. 2018).

Therefore, a similar mechanism could lower the threshold level of PAD4 for calcium after stimulation with CXCL5. If the substrate is still in excess compared to the competitive inhibitor, only high concentrations of GSK484 will decrease citrullination. This might explain the requirement of such high dosages of inhibitor, but it does not explain the stimulating effect of low concentrations of GSK484. The second pathway of NET formation is ROS-dependent, where MPO and NE are released into the cytosol leading to nuclear fragmentation, cleavage of histones and chromatin decondensation (Ravindran, Khan et al. 2019). If CXCL5 stimulates NADPH-dependent and thus ROS-dependent NET formation, it will be independent from PAD4 citrullination and will not be blocked by GSK484 at low concentrations. Another explanation could be that CXCL5 triggers both NET formation pathways leading to histone citrullination if PAD4 is available, while favouring the NADPH-dependent pathway upon PAD4 inhibition. In order to test this alternative hypothesis, further experiments should be considered by using PAD4-deficient neutrophils in co-culture experiment with HCC cells.

Finally, inhibition of Axl and TGF-β signalling lead to controversial results. The increased MPO release in neutrophils treated with HLF-Axl-KO supernatants could be explained by degranulation in response to HLF-Axl-KO cell secreted factors. While the results of genetic inhibition of the Axl receptor can be partially explained, the neutrophil response to HLF cells treated with chemical inhibitors remains unclear. In spite of reducing histone citrullination and MPO release, these two markers were elevated upon chemical inhibition of Axl and TGF-β receptors with TP0903 and LY2109761, respectively. Their abilities to block the ATP-binding site of the kinase domains could inhibit off-target kinases (Aveic, Corallo et al. 2018). Most importantly, these compounds are known to induce apoptosis (Xu, Tabe et al. 2008, Sinha, Boysen et al. 2015, Cetin and Topcul 2019). While LY2109761 targets the cytoskeleton and causes detachment of adhering cells resulting in anoikis, i.e. anchorage-dependent apoptosis of epithelial cells (Valentijn, Zouq et al. 2004, Cetin and Topcul 2019), TP-0903 targets Aurora A and B kinases resulting in a G2/M arrest (Aveic, Corallo et al. 2018). As apoptotic cells have been shown to stimulate NET formation of adhering neutrophils (Manfredi, Covino et al. 2015), it might be possible that apoptotic bodies in the supernatants induced histone citrullination and MPO release in these treated neutrophils. Yet, these speculations require clarification in further experiments. Therefore, genetic inhibition of TGF-β receptors and knock-down of their downstream targets such as Smad proteins should be considered. Additionally, the intervention with Axl interaction partners such as 14-3-3-ζ or interference with Axl downstream signalling components such as JNK should be examined to exclude the possible apoptotic effect of the kinase inhibitors used in this study. To confirm that NET formation is solely dependent on CXCL5, neutralizing antibodies could be used before treating neutrophils with hepatoma supernatants.

In conclusion, we show that HCC cells exposed to long-term TGF-β, as well as recombinant CXCL5 are able to stimulate NET formation of murine neutrophils *in vitro*. These observations are of particular relevance and might further explain the tumour-promoting abilities of TGF-β in the HCC progression, i.e. (i) long-term exposure of TGF-β induces CXCL5 in collaboration with Axl, which (ii) attracts neutrophils into the TME of HCC (Haider, Hnat et al. 2019), and (iii) induces NET formation which might be crucial for the dissemination of HCC cells. Yet, direct evidence for an essential role of NET formation in HCC progression, as shown for breast cancer development and relapse after treatment (Cools-Lartigue, Spicer et al. 2014), is still missing.

Most notably, a recent hallmark study of breast cancer metastasis to the liver identified the transmembrane protein CCDC25 expressed in mammary tumour cells as a “NET receptor” which interacts with NET-DNA in the hepatic pre-metastatic niche and promotes integrin-like kinase/parvin-dependent cell invasion and colonization (Yang, Liu et al. 2020). We speculate that a similar mechanism of NET-driving HCC dissemination is conceivable and molecularly linked to the TGF-β/CXCL5 signalling cascade.

In order to overcome technical limitations and circumvent putative artefacts from co-culture experiments *in vitro*, further studies must focus on NET formation of HCC cells *in vivo*. Interestingly, xenotransplants of HLF-T cells show an efficient tumour formation as compared to parental HLF cells which could possibly be explained with a stronger infiltration of neutrophils into the TME that is accompanied by higher rates of NET formation. In addition, tissue samples of HCC patients must be further analysed for NET formation *in vivo* by detecting citrullinated histones together with NE and MPO. Notably, correlation of NET events with clinical patient records such as chronic HBV/HCV infection, steatosis, fibrosis/cirrhosis, BCLC stages, vessel invasion and intrahepatic metastasis might allow estimating the significance of NETs in HCC development. Moreover, stratification of HCC patients according to activated TGF-β signalling by the release of TGF-β1 into the peripheral blood is considered as beneficial for the development of novel treatment modalities along the TGF-β/Axl/CXCL5 axis. Unveiling the molecular mechanism underlying NET formation in HCC might have a high potential to get included into novel auspicious strategies to combat HCC.

# Annex

| Condition | Mean [ng/ml] | SD | Relative to untreated | Significant difference relative to untreated |
|---|---|---|---|---|
| A23187 | 29.6 | 0.7 | 3.4 | *** |
| HLF | 15.8 | 0.3 | 1.8 | *** |
| HLF_TW | 18.2 | 0.5 | 2.1 | *** |
| HLF-CXCL5 | 17 | 0.6 | 1.9 | *** |
| HLF-CXCL5_TW | 20.5 | 1.4 | 2.3 | ** |
| HLF-T | 10.4 | 0.3 | 1.2 | ** |
| HLF-T_TW | 17 | 0.1 | 1.9 | *** |
| PMA | 26.4 | 0.2 | 3.0 | *** |
| untreated | 8.8 | 0.3 | 1.0 | |

**Table 1: Data presented in Fig. 18E.** Quantification of released MPO via ELISA after 4 hrs. Incubation of neutrophils with either supernatants of hepatoma cells, Transwell-co-culture with hepatoma cells, [10 µM] calcium ionophore A23187 or [50 nM] phorbol 12-myristate 13-acetate (PMA).

## Abbreviations

| | |
|---|---|
| ALBI | albumin-bilirubin |
| ALD | alcohol related liver disease |
| ALOX5 | arachidonate 5-lipoxygenase |
| Apc | adenomatous polyposis coli |
| ARG | arginase |
| Bcl | B-cell lymphoma |
| BCLC | Barcelona Clinic Liver Cancer |
| BV8 | prokineticin |
| C3 | component 3 of the complement system |
| ccc DNA | covalently closed circular DNA |
| CCL | C-C motif chemokine ligand |
| CCR | C-C motif chemokine |
| citH3 | citrullinated histone H3 |
| COL | collagen |
| COVID-19 | corona virus disease 2019 |
| COX | cyclooxygenase |
| CTL | cytotoxic T lymphocytes |
| CXCL | C-X-C motif chemokine ligand |
| CXCR | C-X-C motif chemokine receptor |
| DAMP | damage-associated molecular pattern |
| DC | dendritic cell |
| E47 | E2A immunoglobulin enhancer-binding factor E47 |
| E-cadherin | epithelial cadherin |
| ECM | extracellular matrix |
| EGF-R | epidermal growth factor receptor |
| EMT | epithelial to mesenchymal transition |
| ER | endoplasmic reticulum |
| ERK | extracellular signal-regulated kinase |
| FAK | focal adhesion kinase |
| FGF | fibroblast growth factor |
| FOXC2 | forkhead box protein C2 |
| Gas6 | growth arrest specific gene 6 |
| G-CSF | granulocyte colony stimulating factor |
| GMP | granulocyte-monocyte progenitors |
| GRO-α/β | growth-regulated oncogene-α/β |
| HBV | hepatitis B virus |
| HCC | hepatocellular carcinoma |
| HCV | hepatitis C virus |
| HGF | hepatic growth factor |
| HIF | hypoxia inducible factor |
| HLF-CXCL5 | HLF cells with ectopic CXCL5 expression |
| HLF-T | HLF cells with long-term TGF-β treatment |
| HMGB1 | high mobility group box 1 |
| HSC | hepatic stellate cell |
| ICAM | intracellular adhesion molecule |
| IFN-γ | interferon-γ |
| IGF | insulin-like growth factor |

| | |
|---|---|
| IL | interleukin |
| JAK | Janus kinase |
| JNK | c-Jun N-terminal kinase |
| Klf | Krueppel-like factor |
| Kras | Kirsten rat sarcoma virus |
| LFA | lymphocyte function-associated antigen |
| LTB4 | leukotriene B4 |
| LTB4R | leukotriene B4 receptor |
| LT-HSC | long-term haematopoietic stem cell |
| M1/2 | macrophage type 1/2 |
| MAPK | mitogen activated kinase |
| M-CSF | macrophage colony stimulating factor |
| MDSC | myeloid-derived suppressor cell |
| MEK | mitogen-activated protein kinase/extracellular signal-regulated kinase kinase |
| Met | hepatic growth factor receptor |
| MET | mesenchymal-to-epithelial transition |
| Min | multiple intestinal neoplasia |
| MLC | myosin light chain |
| MMP | matrix metalloproteinase |
| MPO | myeloperoxidase |
| MPP | multipotent progenitor |
| MyD88 | myeloid differentiation primary response 88 |
| N1/2 | neutrophil type 1/2 |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NAFLD | non-alcoholic fatty liver disease |
| NASH | non-alcoholic steatohepatitis |
| NE | neutrophil elastase |
| NET | neutrophil extracellular trap |
| NF-κB | nuclear factor κ-light-chain-enhancer of activated B cells |
| NK cells | natural killer cells |
| NOS | nitric oxide synthase |
| PAD | protein-arginine deiminase |
| PAMP | pathogen-associated molecular pattern |
| PDGF | platelet-derived growth factor |
| PI3K | phosphatidylinositol-3-kinase |
| PKC | protein kinase C |
| PMA | phorbol 12-myristate 13-acetate |
| ProS | protein S |
| PSGL | P-selectin glycoprotein ligand |
| RAF | rapidly accelerated fibrosarcoma |
| RhoA | ras homolog family member A |
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |
| SARS-CoV-2 | severe acute respiratory syndrome coronavirus 2 |
| SDF | stromal-derived factor |
| SN | supernatant |
| src | sarcoma tyrosine kinase |

| | |
|---|---|
| STAT | signal transducers and activators of transcription |
| ST-HSC | short-term haematopoietic stem cell |
| TAM RTK | Tyro3-Axl-Mer receptor tyrosine kinase |
| TAMs | tumour-associated macrophages |
| TAN | tumour-associated neutrophil |
| TERT | telomerase reverse transcriptase |
| TGF-β | transforming growth factor-β |
| TIMP | tissue inhibitor of metalloproteinases |
| TLR | toll-like receptor |
| TME | tumour microenvironment |
| TNF-α | tumour necrosis factor-α |
| TSP | thrombospondin |
| TW | Transwell |
| UV | ultra-violet |
| VCAM | vascular cell adhesion molecule |
| VEGF | vascular endothelial growth factor |
| YAP | yes-associated protein |
| Zeb | zinc finger E-Box binding homeobox |
| α-SMA | α-smooth muscle actin |

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# Zusammenfassung

Die molekulare Interaktion der Transforming Growth Factor (TGF)-β Signalkaskade mit der Rezeptor-Tyrosinkinase Axl, die durch den Liganden Gas-6 aktiviert wird, ist entscheidend an der Entwicklung des hepatozellulären Karzinoms (HCC) beteiligt. Die Synergie dieser Signalwege bewirkt die Aktivierung von Genen, darunter das Chemokin CXCL5, die das Tumormilieu modulieren. CXCL5 ist für die Infiltration der neutrophilen Granulozyten in das Tumor-Stroma verantwortlich und fördert die Progression und Metastasierung der Tumorzellen. In kürzlich erschienenen Studien wurde die Bildung von „Neutrophil Extracellular Traps“ (NETs) mit Tumorprogression und Metastasierung in Brust-, Lungen- und Nierenkrebs in Verbindung gebracht. Diese NETs bestehen aus extrazellulärer DNA, citrullinierten Histonen und assoziierten Enzymen aus Granula-Vesikeln, wie beispielsweise neutrophiler Elastase sowie Myeloperoxidase. Bisher wurde die Rolle der NET-Bildung im HCC nur wenig untersucht. Unsere Studie zeigt, dass humane HCC-Zellen, die einer Langzeitstimulation mit TGF-β ausgesetzt wurden, die Freisetzung von neutrophiler DNA mit daran assoziierten citrullinierten Histonen und Granula-Enzymen sowie die Ruptur von Zellmembranen induzieren. Diese lytische NET-Bildung wird auch als NETose bezeichnet, da dies auf einen Zelltod der Neutrophilen Bezug nimmt. Nachdem die HCC-Zelllinie HLF als Reaktion auf eine langfristige TGF-β-Stimulation CXCL5 sezerniert, wurde der Einfluss von CXCL5 auf die Bildung von NETs analysiert. HLF-Zellen mit ektopischer CXCL5 Expression sowie rekombinantes CXCL5 zeigten einen inversen Dosis-Wirkungs-Mechanismus auf die Induktion der NETose. In einem weiteren Schritt wurde die Abhängigkeit der NET-Bildung von der Protein-Arginin-Deiminase (PAD) 4 analysiert, welche die Histon-Citrullinierung katalysiert. Zusammenfassend zeigen diese Daten, dass die Axl/TGF-β-vermittelte Expression von CXCL5 in HCC-Zellen eine PAD4-unabhängige NETose in murinen Neutrophilen *in vitro* stimuliert.