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Characterization of CD4-memory T helper cell populations as a source of interleukin-22 production in murine and human breast and lung cancer models

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In dedication to my family

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1 Introduction

1.1 Biology of IL-22

1.1.1 The unusual cytokine IL-22

Interleukins describe a subgroup of cytokines first seen to be expressed by leukocytes that are crucial for the function of the immune system. Interleukin-22 (IL-22) – discovered in 2000 and originally termed IL-10-related T cell derived inducible factor – is a member of the IL-10 family which also comprises IL-10, IL-19, IL-20, IL-24, IL-26 and type III interferons (IFNλ) (Ouyang, et al. 2011). IL-22 can be regarded as an unusual interleukin since it does not regulate the function of immune cells directly (Wolk, et al. 2004). In fact, its designation as an "interleukin" can be regarded as a misnomer since the corresponding IL-22 receptor α chain 1 (IL-22-Rα1) is not expressed on leukocytes, meaning that the cytokine cannot function as a mediator between leukocytes (reviewed in: Hernandez, et al. 2018). Instead, IL-22-Rα1 is constitutively expressed on non-hematopoietic cells and facilitates cross-talk between hematopoietic and non-hematopoietic cells. IL-22 thus targets cells at external barriers, which includes mainly tissues of the skin, digestive tract and respiratory systems, but also cells of the liver, pancreas and kidney (reviewed in: Sabat, et al. 2014).

The exact functions of IL-22 seem to be dependent on the context of its induction as well as its localization. IL-22 controls a gene expression program linked to epithelial defense, regeneration and repair (reviewed in: Rutz, et al. 2013). As such, IL-22 increases proliferation, inhibits differentiation and protects its target cells against damage – effects that are not shared by other cytokines (reviewed in: Sabat, et al. 2014). In many epithelial cells IL-22 also induces the production of antibacterial proteins, acting in conjunction with cytokines such as IL-17, tumor necrosis factor (TNF) or IL-1β.

1.1.2 Cellular sources of IL-22

The precise cellular sources of IL-22 in both physiological and pathological situations are often unidentified and most likely vary depending on the context (reviewed in: Sabat, et al. 2014). It is known that IL-22 is almost exclusively produced by cells of the immune system (Wolk, et al. 2004). Though initially thought to be secreted primarily by a subset of $CD11c^+$ mononuclear phagocytes (Pickert, et al. 2009, Zheng, et al. 2008), IL-22 is now mostly regarded as a cytokine produced by $CD4^+$ T helper cells, γδ T cells, group 3 innate lymphoid cells (ILC3 and LTi), and CD1d-restricted T cells (iNKTs) (Dudakov, et al. 2015, Sanos, et al. 2009, Takatori, et al. 2009). In addition, it has been shown that under inflammatory conditions cells from the myeloid compartment can sometimes produce IL-22, namely, neutrophils, monocytes, dendritic cells, and macrophages (Kulkarni, et al. 2014, Zheng, et al. 2008, Zindl, et al. 2013).

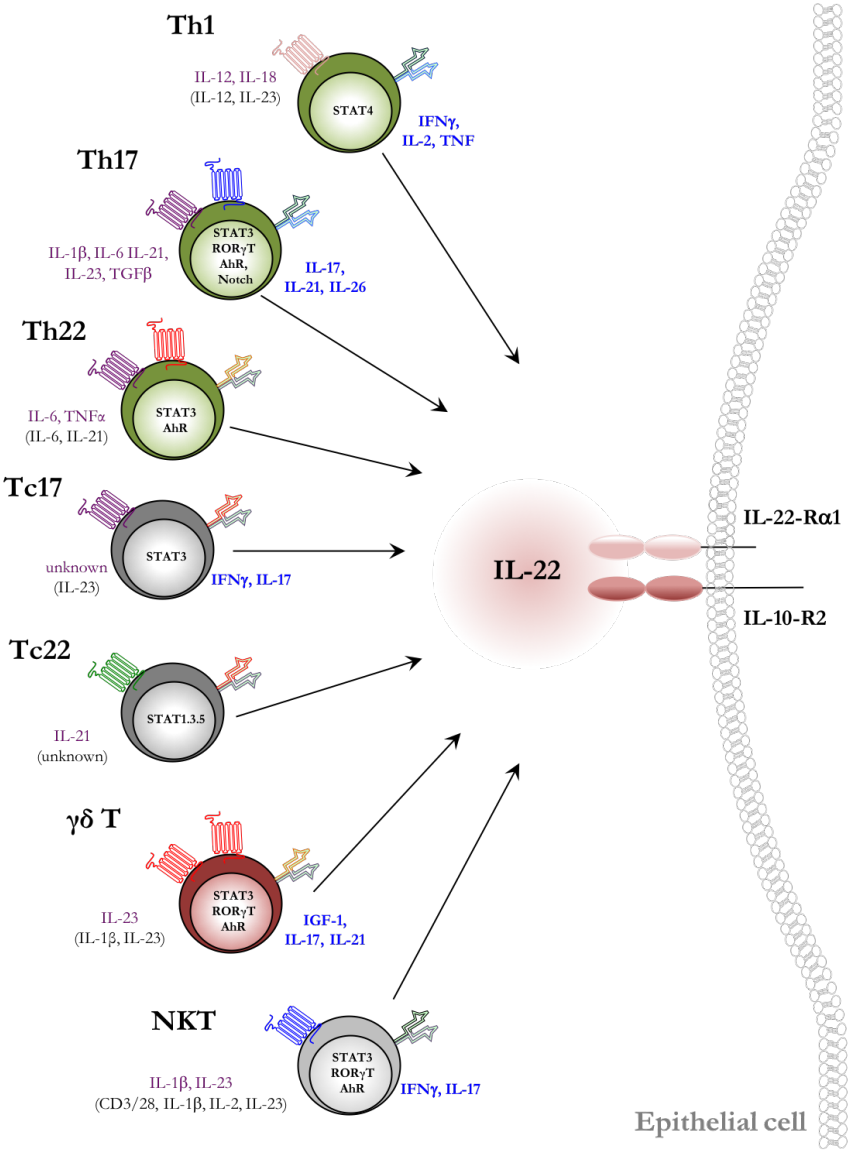

Figure 1: Cellular sources of IL-22 in humans (modified from: Lim and Savan 2014).

The first identified T helper cell subset to produce IL-22 were Th1 cells. Around 35 % of all IL-22^{+}CD4^{+} cells in peripheral human blood are estimated to be Th1 cells (Duhen, et al. 2009). This is consistent with the finding that IL-22 production in human T cells is highly correlated with IFNγ and T-bet, the Th1 master transcription factor (Volpe, et al. 2009). In human Th1 cells, IL-22 production is primarily induced by IL-12 – alone or in synergy with IL-18 (Gurney 2004). In comparison, murine IL-22 secretion from Th1 cells stems from activation by IL-12 and IL-23 (Kaczmarek, et al. 2013).

Th17 cells also produce significant amounts of IL-22 (Chung, et al. 2006, Liang, et al. 2006). While seen as the major source of IL-22 in mice, in human peripheral blood only 10-18 % of IL-22-producing CD4^{+} T cells co-express IL-17 (Duhen, et al. 2009). Given the synergistic actions of IL-17 and IL-22, this co-expression can lead to notable amplifications

of their effects. Differentiation of naïve T cells into Th17 cells can be stimulated with IL-1β, IL-6 and transforming growth factor-β (TGFβ) (Ivanov, et al. 2006), and IL-21 and IL-23 act to further maintain and stabilize Th17 cells. Interestingly, IL-1β, IL-6 and IL-23 do not only promote Th17 differentiation, but also IL-22 expression. IL-21 and TGFβ can potently inhibit IL-22 in murine Th17 cells (Zheng, et al. 2007).

However, the majority of Th cells producing IL-22 in peripheral human blood, between 37 % and 63 %, do not co-express IL-17 nor IFNγ (Duhen, et al. 2009). This separate subset of Th cells has been defined as Th22 cells. New evidence suggests that Th22 cells demonstrate plasticity toward both Th1 and Th2 cells but develop independently of the Th17 lineage (Plank, et al. 2017). Th22 differentiation is stimulated by IL-6, IL-21 and TNFα, and vitamin D leads to further increases in IL-22 production (Fujita, et al. 2009). Of note, the Th22 subpopulation does not express T-bet and only low levels of the retinoic acid-related orphan receptor γt (RORγt) (Duhen, et al. 2009, Trifari, et al. 2009). Furthermore, it has been shown that the aryl hydrocarbon receptor (AhR) and STAT3 play an important role in Th22 differentiation (Yeste, et al. 2014). Whether this separate Th22 lineage correspondingly exists in mice is still investigated (Liang, et al. 2006).

Other T cells such as $CD8^+$ T cells, γδ T cells and natural killer T (NKT) cells have also been discovered to produce IL-22, particularly upon activation by IL-23 (Witte, et al. 2010). Mirroring their $CD4^+$ counterparts, human $CD8^+$ cytotoxic T cells have been categorized into subsets secreting either IL-17 and IL-22 (Tc17) or only IL-22 (Tc22) (Rutz, et al. 2013). Differentiation from naïve $CD8^+$ T cells to Tc22 has been shown to be inducible by IL-21 via phosphorylation of STAT1, 3 and 5. Similar to their Th cell counterparts, TGFβ inhibits IL-22 but induces IL-17 secretion from Tc17 and Tc22 cells (Liu, et al. 2011).

More recently, innate T cell populations were identified to secrete IL-22 at epithelial sites. In particular, γδ T cells have been shown to produce IL-22 upon IL-23 or RORγt stimulation as well as activation of innate Toll-like receptors (TLRs) (Eken, et al. 2014, Tominaga, et al. 2013). Because of their constitutive expression of the IL-23 receptor (IL-23R) γδ T cells can instantly react to IL-23 stimulation with expression of IL-17 and IL-22 (Martin, et al. 2009). In murine settings, most γδ T cells co-express IL-17 and RORγt, underlining the correlation between IL-17 and IL-22. In humans, this link is again less pronounced and cytokine secretion in human γδ T cells is similar to that of Th cells. Another type of innate T cells, natural killer T (NKT) cells, can also produce IL-17 and IL-22. Interestingly, the transcription factor T-bet regulates the terminal differentiation and hemostasis of NKT cells (Koyama, et al. 2008). In addition, human invariant NKT (iNKT) cells have been shown to produce IL-22 via AhR in response to stimulation with IL-1β and IL-23 (Moreira-Teixeira, et al. 2011).

Group 3 ILCs are regarded as an increasingly important source of IL-22, especially at mucosal sites such as the GI tract. They are thought to be the innate counterpart of Th17 and Th22 cells and are defined by their capacity to produce IL-17 or IL-22. Sharing the common transcription factor RORγt they comprise ILC3s and lymphoid-tissue inducer cells (LTis). They express IL-23R and secretion of IL-17 and IL-22 can be stimulated by IL-1β and IL-23 (Cella, et al. 2010).

Importantly, differences between IL-22 production in humans and mice have been shown. Murine IL-22 mostly originates from Th17 cells, while mouse Th1 cells tend to express IL-22 weakly (Liang, et al. 2006). In contrast, in humans significant amounts of IL-22 are secreted by each subset, that is Th1, Th17 and Th22 cells alike (reviewed in: Ouyang, et al. 2011). Th22 cells have been shown to release IL-22 without IL-17 and IFNγ in humans, but not in mice. This difference is linked to TGFβ, which increases IL-17 but inhibits IL-22 production by human T cells.

Table 1: IL-22-producing cells in humans (murine if different)

Cell type	Specific surface markers	Other cytokines produced	Cytokines inducing IL-22	Transcription factor for IL-22 induction	References
Th1	$CD3^+CD4^+$	IFNγ, IL-2, TNF	IL-12, IL-18 (IL-12, IL-23)	STAT4	(Gurney 2004)
Th17	$CD3^+CD4^+$	IL-17, IL-21, IL-26	IL-1β, IL-6, IL-21, IL-23, TGFβ	STAT3, RORγt, Notch, AhR	(Ikeda, et al. 2014)
Th22	$CD3^+CD4^+$	-	IL-6, TNFα (IL-6, IL-21)	STAT3, AhR	(Duhen, et al. 2009), (Yeste, et al. 2014)
Tc17	$CD3^+CD8^+$	IFNγ, IL-17	unk. (IL-23)	STAT3	(Ciric, et al. 2009)
Tc22	$CD3^+CD8^+$	-	IL-21 (unk.)	STAT1, 3, 5	(Liu, et al. 2011)
γδ T	$CD3^+\gamma\delta TCR^+$	IGF-1, IL-17, IL-21	IL-23 (IL-1β, IL-23)	STAT3, AhR, RORγt	(Martin, et al. 2009), (Sutton, et al. 2009)
NK	$CD3^-CD56^+CD16^+$	IFNγ, TNF, GM-CSF	IL-2, IL-12, IL-18	STAT4	(Cella, et al. 2009)
NKT	$CD3^-$ $CD8^+CD56^+CD16^+$		IL-1β, IL-23 (CD3/28, IL-1β, IL-2, IL-23)	STAT3, AhR, RORγt	(Moreira-Teixeira, et al. 2011), (Goto, et al. 2009)
LTi	$CD117^+NKp46^-$	IL-17	IL-23	STAT3	(Takatori, et al. 2009), (Crellin, et al. 2010)
NCR^+ ILC3	$CD117^+NKp46^+$	-	IL-1β, IL-23 (IL-23)	STAT3, TBX21, Notch	(Cella, et al. 2010), (Teunissen, et al. 2014)
NCR^- ILC3	$CD117^+NKp46^-$	-	IL-1α, IL-1β, IL-23		(Hernandez, et al. 2015)

1.1.3 Stimuli required for IL-22 production

IL-22 secretion can be stimulated by several factors, including activation of the transcription factor AhR (Veldhoen, et al. 2008). AhR agonists include endogenous molecules such as kynurenine or FICZ (Mezrich, et al. 2010) and exogenous molecules such as dioxin (Ema, et al. 1994). Other stimuli include the activation of Toll-like receptors such as TLR4 (Tominaga, et al. 2013) or IL-23 binding to the IL-23 receptor (IL-23-R) (Zheng, et al. 2007). The transcription factor RORγt is necessary for Th17 differentiation in humans and mice (Ivanov, et al. 2006). IL-22 expression in Th17 is mainly regulated via the transcription factor AhR (Veldhoen, et al. 2008).

Interestingly, IL-22 has also been described to induce the expression of proteins that lead to a positive feedback loop, thereby further amplifying its production and effects (reviewed in: Sabat, et al. 2014). For example, the Notch pathway leads to activation of endogenous AhR ligands, which amplifies IL-22 effects (Alam, et al. 2010).

1.1.4 Target cells of IL-22

IL-22 is secreted predominantly by immune cells, yet its receptor is restrictively expressed by non-hematopoietic cells. IL-22 transmits its cellular effects through the heterodimeric IL-22 receptor complex (IL-22-R) consisting of IL-22-Rα1 and IL-10-R2 (Kotenko, et al. 2001, Xie, et al. 2000). Since the R2 subunit of the receptor complex is expressed ubiquitously, the cellular sensitivity towards IL-22 is controlled solely by the expression of IL-22-Rα1 (Wolk, et al. 2004). In line with their lack of responsiveness to IL-22 it has been shown that hematopoietic cells do not express IL-22-Rα1 (Wolk, et al. 2002, Wolk, et al. 2004, Wolk, et al. 2008).

The principal physiological target of IL-22 seems to be epithelial cells (Boniface, et al. 2005) as cells of anatomical barriers like the skin and tissues of both gastrointestinal and respiratory tract are sensitive to IL-22. So far, the functional receptor for IL-22 has been found on the surface of bronchial epithelial cells (Aujla, et al. 2008), hepatocytes (Radaeva, et al. 2004), keratinocytes (Wolk, et al. 2004, Wolk, et al. 2006), intestinal epithelial cells (Nagalakshmi, et al. 2004), pancreatic cells (Aggarwal, et al. 2001, Shioya, et al. 2008), and thymic epithelial cells (Dudakov, et al. 2012). Other target cells include specific tissue-resident stem cells, which possibly contributes to its protective properties against damage in several tissues (Feng, et al. 2012, Hanash, et al. 2012).

The regulation of the IL-22-Rα1 remains only partially understood (reviewed in: Lim and Savan 2014). In keratinocytes, it has been shown that IFNα stimulation increases IL-22-Rα1 expression (Tohyama, et al. 2009). Similarly, IFNγ and TNF appeared to upregulate IL-22-Rα1 expression in both keratinocytes and dermal fibroblasts (Wolk, et al. 2009, Wolk,

et al. 2004). This indicates that the presence of inflammatory cytokines might intensify the effects of IL-22. Repression of IL-22-Rα1 expression has been observed in a few cases, for example via latency-associated nuclear antigen (LANA) proteins in Kaposi's sarcoma (Su, et al. 2011).

It is important to note that both subunits of the receptor complex are shared with other cytokines of the IL-10 family; only the combination of IL-10-R2 and IL-22-Rα1 specifically mediates IL-22 signaling. IL-22-Rα1 can also associate with IL-20-R2 to mediate the effects of IL-20 or IL-24. IL-10-R2, on the other hand, also mediates the effects of IL-10, IL-26 as well as IL-28 and IL-29 (reviewed in: Ouyang, et al. 2011, Sabat 2010).

In addition to the membrane-bound IL-22-Rα1 receptor, there exists a soluble receptor, IL-22 binding protein (IL-22-BP), which antagonizes the actions of IL-22 (Dumoutier, et al. 2001, Kotenko, et al. 2001). As such, IL-22 is regarded as unique among other IL-10 cytokines as it has an endogenous inhibitor. IL-22-BP binds to IL-22 at the same binding site as IL-22-Rα1, but with 1.000-fold higher affinity (Jones, et al. 2008). IL-22-BP expression has been found in lymphocytes as well as various myeloid cell types such as $CD103^+$ DCs and macrophages (Martin, et al. 2014).

1.1.5 IL-22 signaling

The binding of IL-22 to its receptor can be described as a two-step process. IL-22 first binds to its receptor subunit IL-22-Rα1 with high affinity. This association alters the conformation of the IL-22 protein and allows the cytokine to bind to the IL-10-R2 subunit (reviewed in: Sabat, et al. 2014). The attachment of IL-10-R2 to IL-22-IL-22-Rα1 stabilizes the complex and initiates intracellular signaling pathways.

Intracellularly, IL-22 primarily signals through Janus kinase (JAK) and signal transducer and activator of transcription (STAT) molecules (Lejeune, et al. 2002). The cytoplasmic part of the IL-22-Rα1 subunit is associated with the JAK1 while the IL-10-R2 subunit is linked to the tyrosine kinase 2 (TYK2). After IL-22 binds to its full receptor, both JAK1 and TYK2 become activated, resulting in phosphorylation of tyrosine residues of the STAT1, STAT3 and, to a lesser extent, STAT5 proteins (Lejeune, et al. 2002, Wolk, et al. 2007). After phosphorylation, dimerized STAT proteins translocate into the nucleus, where they are able to regulate the expression of target genes by binding to responsive elements (reviewed in: Sabat, et al. 2014).

Phosphorylation of STAT3 at the Tyr705 residue plays a major role in the IL-22-induced activation of genes (Dumoutier, et al. 2009). Strong induction of pSTAT3 through IL-22 has been demonstrated, for instance, in murine colonic epithelial cells as well as human colonic cancer cells and biopsies (Pickert, et al. 2009, Sugimoto, et al. 2008). STAT5 activation,

though rare, has been described for kidney cells and hepatocytes, with yet unknown downstream effects (Brand, et al. 2007). STAT1 activation by IL-22, while observed only in some cells (Wolk, et al. 2004), has recently been shown to have biological functions in the context of murine rotavirus infections (Hernandez, et al. 2015).

Next to the JAK-STAT signaling, activation of mitogen-activated protein kinase (MAPK) pathways have been described (Kim, et al. 2014). Other designated pathways include activation of NF-κB, AKT, phosphoinositide 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) (Kim, et al. 2012, Lejeune, et al. 2002, Mitra, et al. 2012).

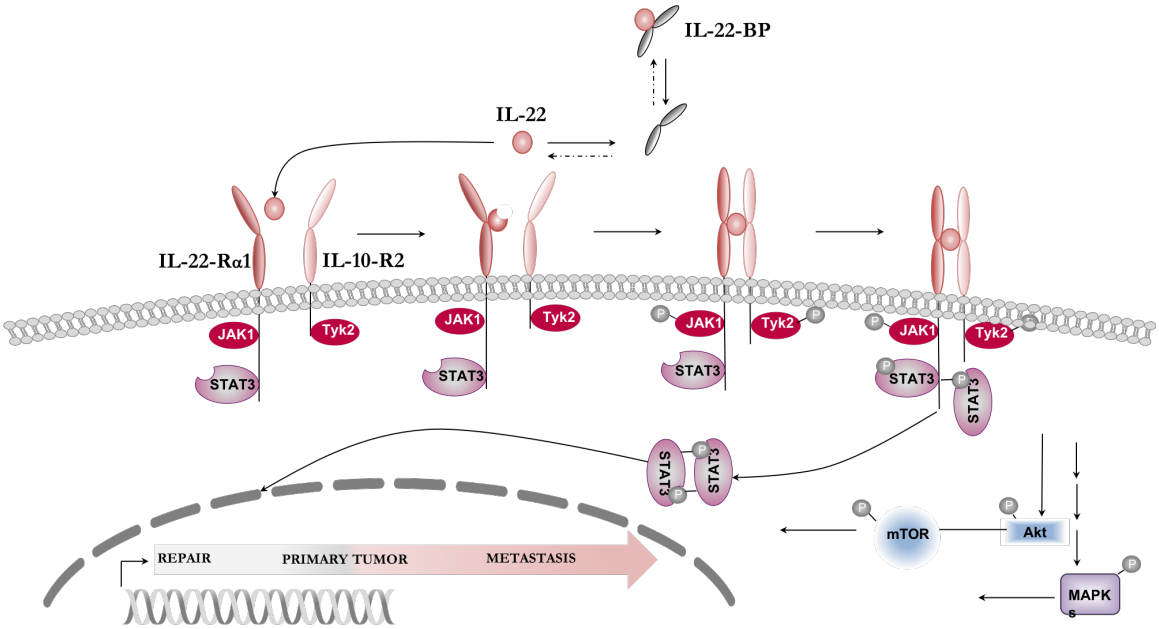

Figure 2: Signalling of IL-22 (modified from: Sabat, et al. 2014).

1.2 Effects of IL-22

1.2.1 Physiological effects of IL-22

IL-22 is thought to be a homeostatic cytokine that preserves the integrity of boundary tissues and organs, but a clear understanding of the mechanism through which IL-22 may protect epithelial cells against damage and stimulate tissue repair is still missing (reviewed in: Hernandez, et al. 2018). In the skin, IL-22 stimulation has been shown to cause proliferation of keratinocytes and fibroblasts (Mitra, et al. 2012), thereby promoting wound healing. IL-22 can induce antimicrobial proteins like defensins and pro-inflammatory molecules of the S100 family in epithelial cells (Liang, et al. 2006). Further, IL-22 stimulation upregulates matrix metalloproteinases to induce cell migration (Boniface, et al. 2005).

In the gut, IL-22 has been shown to stimulate epithelial repair after physical, chemical or microbial damage (Monteleone, et al. 2011, Zheng, et al. 2008). IL-22 enhances intestinal epithelial cell migration (Brand, et al. 2006), improves healing of wounded mucosa (Pickert, et al. 2009) and increases expression of mucus-associated proteins (Mizoguchi 2012). H. pylori infections have been shown to be controlled by IL-22 (Moyat, et al. 2017). Similarly, IL-22 deficient mice failed to control rotavirus infections in a STAT1-dependent manner (Hernandez, et al. 2015). In the liver, IL-22 stimulation also increased acute phase proteins such as haptoglobin or serum amyloid A (Liang, et al. 2010).

In the lung, physiological effects of IL-22 primarily include modulating immunity (Whittington, et al. 2004, Wolk, et al. 2004). IL-22 seems to play a protective role in the context of viral, bacterial and parasitic infections by promoting antimicrobial activity and proliferation of lung epithelium (Aujla, et al. 2008). In addition, IL-22 can protect cells in the lung from inflammation-induced pulmonary fibrosis through increased airway epithelial cell proliferation as well as transepithelial resistance to injury (Simonian, et al. 2009).

1.2.2 Pathological effects of IL-22

In general, IL-22 seems to play a protective role in the acute context of injury and inflammation. In chronic conditions, however, IL-22 can promote diseases through proliferation and anti-apoptosis (Eyerich, et al. 2009).

In the skin, higher numbers of IL-22-producing T cells in lesions correlate with disease activity in patients with psoriasis or atopic dermatitis (Hijnen, et al. 2013). Elevated IL-22-Rα1 expression in epidermal keratinocytes has been shown to play a role in the pathogenesis of psoriasis by enhancing the coordinated effects of IL-17 and IL-22 (Tohyama, et al. 2009). Progression from acute to chronic phases of atopic dermatitis are also associated with a progressive activation of IL-22-producing lymphocytes (Gittler, et al. 2012). A human anti-IL-22-mAb (fezakinumab) is currently tested as a treatment for atopic dermatitis, with promising preliminary results (Guttman-Yassky, et al. 2018).

In inflammatory bowel diseases (IBDs), the role of IL-22 seems to be time dependent. IL-22 was observed to be a protective factor in a murine model of colitis during acute inflammation (Zenewicz, et al. 2007). Recently, it was suggested that development of IBDs correlates with high levels of IL-22-BP and that successful anti-TNFα treatment may act in part by suppressing IL-22-BP (Pelczar, et al. 2016). Contrastingly, IL-22 can be disease-promoting in chronic states of IBDs, as IL-22 blockage at a later stage has been shown to protect mice from colitis (Eken, et al. 2014). Elevated IL-22 expression in primary tissue was also closely linked to the severity of chronic colitis and associated with progression of disease (Yu, et al. 2013).

In the lung, patients with chronic obstructive pulmonary disease (COPD) have been shown to have higher numbers of IL-22 positive immune cells like Th17 cells (Di Stefano, et al. 2009). Compared to non-smoking controls, IL-22 levels and IL-22 positive T cell populations were significantly higher in the peripheral blood and sputum of smokers and patients with COPD (Paats, et al. 2012, Zhang, et al. 2013).

1.3 IL-22 and cancer

1.3.1 Relationship between IL-22 and malignancies

So far, the participation of IL-22 in tumorigenesis paints a complex picture and its effects differ not only based on the tissue of origin, but also on the stage of disease (reviewed in: Lim and Savan 2014). In hematological malignancies aberrantly expressing the IL-22 receptor complex, IL-22 can act as an autocrine factor to enhance cancer cell survival (Bard, et al. 2008). Overexpression of IL-22-Rα1 has been observed in several types of lymphomas (Bard, et al. 2008, Ciccia, et al. 2015, Gelebart, et al. 2011, Ito, et al. 2014). However, the exact mechanisms for such aberrant expression remains unclear.

In cancers of non-hematopoietic origin, IL-22 has been proposed to either play an important role within the tumor microenvironment or to act directly on IL-22-Rα1 bearing cells. IL-22 signaling induces the expression of several genes downstream of STAT3, which mediate pro-inflammatory, pro-survival or anti-apoptotic effects (reviewed in: Lim and Savan 2014). Genetic studies support these findings, as IL-22 single nucleotide polymorphisms (SNPs) were correlated with the risk of developing lung (Liu, et al. 2014), gastric (Qin, et al. 2015), and bladder cancer (Zhao, et al. 2015).

In terms of outcomes, higher expression of IL-22 or IL-22-Rα1 have been shown to correlate with progression of disease and lower overall survival in colorectal (Wu, et al. 2013), gastric (Zhuang, et al. 2012), and pancreatic cancer (Wen, et al. 2014). The presence of IL-22 secreting cells is also linked to a more aggressive phenotype in breast (Kim, et al. 2014), lung (Kobold, et al. 2013, Zhang, et al. 2008), and skin (Nardinocchi, et al. 2015, Zhang, et al. 2013) cancers. Protection from chemotherapy through IL-22 can play a role in colorectal (Wu, et al. 2014) and lung (Kobold, et al. 2013) cancer. Interestingly, it was also observed that recurrent non-small cell lung cancer (NSCLC) tissue had elevated levels of IL-22 and IL-22-Rα1 expression in comparison with the primary tumor (Bi, et al. 2016).

1.3.2 Potential mechanisms of IL-22 driven pro-tumoral phenotypes

IL-22 does not seem to promote oncogenesis or mutagenesis under physiological circumstances, but rather accelerates the development of induced or nascent tumors. IL-22

overproduction in adipose tissue did not favor spontaneous tumorigenesis in a transgenic mouse model with normal diet, but led to liposarcoma after several months when mice were fed with a high-fat diet (Wang, et al. 2011). In the liver, overexpression of IL-22 in transgenic mice did not cause significantly higher spontaneous tumor incidence, but a higher incidence of carcinogen-induced hepatocellular carcinoma (Park, et al. 2011). In accordance with these findings, fewer induced liver tumors were observed in IL-22 deficient mice (Jiang, et al. 2011).

IL-22 may also play a role in the transformation or implantation of tumor cells and activate epithelial-mesenchymal transition (EMT). For example, IL-22 enhanced the migration of A549 lung cancer cells (Ye, et al. 2012) and increased the invasiveness of pancreatic cancer cells by inducing matrix metalloproteinases (Wen, et al. 2014). More recently, it has been demonstrated that invasiveness of papillary thyroid cancer was caused by IL-22 via higher miR-595 expression (Mei, et al. 2016). Closely linked to the metastatic capacity is the ability of cancer cells to attract new blood vessels. IL-22 seems to induce angiogenesis in a feed-forward paracrine loop through induction of growth factors and chemotaxis of endothelial cells (reviewed in: Lim and Savan 2014). For example, pancreatic carcinoma cells have been shown to secrete the vascular endothelial growth factor (VEGF) after IL-22 stimulation (Curd, et al. 2012). Similarly, IL-22 secreted from tumor infiltrating lymphocytes in colorectal cancer patients increased expression of VEGF in mice with patient derived xenografts (Jiang, et al. 2013).

In addition to the pro-tumor inflammatory microenvironment with neoangiogenic and proliferative properties, IL-22 has been linked to the induction of stemness (Hanash, et al. 2012). IL-22-KO mice were found to have a significant reduction in expression of stemness genes such as SOX2 or NANOG (Khosravi, et al. 2016). For colorectal cancer, IL-22 induced the expression of stem cell markers via STAT3 activation and promoted sphere formation in *in vitro* surrogate assays for stemness (Kryczek, et al. 2014). Recently, it has been shown that IL-22 can also promote stemness in pancreatic ductal adenocarcinoma (PDAC) and that worse prognosis of patients with high IL-22-Rα1 expression can be linked to higher stemness potential (He, et al. 2018, Lanfranca, et al. 2017).

Nonetheless, some studies argue that IL-22 hinders tumor progression, for instance via STAT1 pathways. In renal cell carcinoma IL-22 decreased growth of tumor cells *in vitro* and in a murine xenograft model through a dose-dependent increase of pSTAT1 (Zhang, et al. 2011). Similarly, IL-22 inhibited growth of murine breast cancer cells by inducing cell cycle arrest (Weber, et al. 2006) and prolonged survival in a model of murine colon cancer (Nagakawa, et al. 2004). IL-22 was also shown to inhibit viral and alcohol-induced hepatocellular carcinoma (Saalim, et al. 2016). However, anti-growth effects of IL-22

appear to work through a distinct non-canonical signaling pathway linked to chronic fibrotic diseases (reviewed in: Lim and Savan 2014). It is also important to stress that the number of articles arguing in the direction of IL-22 as a tumor-controlling agent are many times outnumbered by those showing its pro-tumoral effects. Such considerations tend into the direction that the anti-tumoral activity of IL-22 might be very restricted to distinct situations or cell systems rather than a general mechanism.

In summary, the role of IL-22 in tumor development can be described as a double-edged sword. Initially, IL-22 diminishes epithelial damage and accelerates repair, thereby reducing the duration and extent of inflammatory signals and counteracting tumorigenic signals. However, IL-22 can support the transition from pre-existing inflammation to cancers and induces the maintenance of established tumors, as best described for the case of colon cancer.

1.3.3 IL-22 blockade as a potential cancer therapy

The IL-22-IL-22-R axis can be an important novel target in cancer immunology research. Several existing therapies already approved by regulatory agencies such as the FDA indirectly affect expression of IL-22 in patients. For example, anti-TNFα treatment blocks Th22 cell differentiation, thereby transiently decreasing IL-22 production. A new neutralizing antibody targeting IL-12p40 (ustekinumab), thereby inhibiting IL-12 and IL-23, has been shown to inhibit differentiation of Th1, Th17 and Th22 cells alike (Koutruba, et al. 2010). However, global inhibition of TNFα or IL-12 carry the risk of severe side effects, especially in cancer types where tumor eradication is dependent upon a functioning immune system. STAT3-targeted therapies have been developed, however with disappointing results. Side effects were linked to the loss of its homeostatic functions when inhibiting STAT3 globally (Furqan, et al. 2013). Clinical trials with anakinra, a soluble IL-1R antagonist, in patients with pancreatic and breast cancer are currently being performed, showing promising preliminary data (O'Shaughnessy, et al. 2016, Whiteley, et al. 2016). Along the same lines, a recent study showed that anti-inflammatory therapy targeting IL-1β significantly reduced lung cancer incidence and mortality (Ridker, et al. 2017).

1.3.4 IL-22-producing immune cells in cancer

For several types of cancer increased levels of IL-22-producing immune cells have been observed in both the tumor and the blood. So far, the intratumoral presence of IL-22-producing cells has been shown mainly in gastric, colonic, hepatocellular, pancreatic, and lung carcinomas (Jiang, et al. 2011, Jiang, et al. 2013, Kobold, et al. 2013, Zhuang, et al. 2012).

In human lung carcinomas, IL-22$^+$NCR$^+$ ILC3 cells were found in NSCLC tissue, with significantly higher frequencies in stages I or II compared to more advanced stages (Carrega, et al. 2015). IL-22 was also higher in malignant pleural effusions than in patients with tuberculous effusions (Zhang, et al. 2008), and elevated Th1, Th17 and Th22 cell counts have been observed in such malignant pleural effusions (Ye, et al. 2012).

In the case of colorectal cancer patients, levels of both IL-17 and IL-22 correlated positively with tumor staging (Petanidis, et al. 2013) and dysplastic regions displayed higher concentrations of intratumoral IL-22$^+$ ILCs (Kirchberger, et al. 2013). It has been suggested that Th22 cells which accumulate in colorectal cancers are associated with the chemotactic effect of the tumor microenvironment and amplified tumor growth via STAT3 activation (Huang, et al. 2015). CD3$^+$CD4$^+$CD8$^-$ memory but not naïve T cells were identified as major producers in colon cancer, with 30 % co-secreting IL-17 and less than 5 % IFNγ (Kryczek, et al. 2014).

Increased counts of IL-22$^+$CD4$^+$ T cells were found in gastric tumors compared to peritumoral tissue and non-tumor tissue (Zhuang, et al. 2012). Compared to healthy controls, gastric cancer patients also had increased counts of IL-17- and IL-22-producing T cells in the blood (Liu, et al. 2012). In pancreatic cancer, intratumoral frequency of both IL-22$^+$CD4$^+$ T cells and IL-22 levels were higher than in the peripheral blood of both patients and healthy donors (Xu, et al. 2014). For pancreatic ductal adenocarcinoma (PDAC), FACS analysis was recently used to identify ILC3 and Th22 cells as the main source of IL-22 in both human and murine specimens (Lanfranca, et al. 2017). Tumor-infiltrating lymphocytes in hepatocellular carcinoma also exhibited a higher IL-22$^+$ cell fraction and increased serum levels of IL-22 represent a negative prognostic markers in virus-associated hepatocellular carcinoma (Waidmann, et al. 2014).

A growing body of data exists as to which cells produce IL-22 in the cancer environment and what clinical consequences their presence might have. However, it is still very unclear if and how cancer cells regulate IL-22 production. A greater understanding thereof would have direct consequences for designing therapeutic interventions targeting the pathway.

1.4 Research objectives

IL-22 has been described as a tumor promoting cytokine. Aberrant activation of the IL-22-IL-22-R axis has been associated with different cancer entities including breast and lung, but also colon, gastric and pancreatic cancers. The main cellular sources of IL-22 are thought to be T cells and innate lymphoid cells. However, neither the exact mechanism of induction nor the exact cellular sources have been characterized for lung and breast cancers. A previous doctoral student in the laboratory (Dr. Cornelia Voigt) showed that

cancer cells can directly induce IL-22 production from murine and human immune cells. Her research further indicated that this process is driven by IL-1α in murine and IL-1β in human immune cells. She could show that these cytokines primarily acted on $CD3^+$ T cells and mediated IL-22 production via the transcription factors AhR and RORγt. Based on these previous observations, the objective of this thesis is to further elucidate the following:

- to identify IL-22-producing T cell subtypes in murine and human breast and lung cancer models
- to characterize the factors and expose the exact mechanism by which IL-22 production is induced in these T cell subtypes
- to characterize IL-22-producing tumor infiltrating lymphocytes in human breast cancer tissue.

2 Materials

2.1 Technical equipment

Analytical balance	CPA1003S	Sartorius Laboratory, Göttingen, DE
Autoclave	Varioklav 500E	HP Medizintechnik, München, DE
Cell culture incubator	BBD 6220	Heraeus, Hanau, DE
Centrifuges	3L-R Multifuge	Heraeus, Hanau, DE
	Centrifuge 5318R	Eppendorf, Hamburg, DE
	Rotina 420R	Hettich GmbH, Tuttlingen, DE
FACS	Canto II	BD Biosciences, Franklin Lakes, USA
Heating block	Thermomixer 5436	Eppendorf, Hamburg, DE
Laminar flow hoods	HeraSAFE KS	Heraeus, Hanau, DE
MACS separators	QuadroMACS	Miltenyi Biotec, Bergisch Gladbach, DE
Microscope	Axiovert 40C	Zeiss, Jena, DE
	Axiovert HAL 100	Zeiss, Jena, DE
Multilabel plate reader	Mithras LB 940	Berthold, Bad Wildbad, DE
Photometer	NanoDrop 2000c	Thermo Fisher, Waltham, USA
pH-Meter	inoLab pH 720	WTW GmbH, Weilheim, DE
Vortex mixer	RS-VA 10	Phoenix, Garbsen, DE
Water bath	Unitherm-HB	uni equip, München, DE

2.2 Materials

Cell culture flasks (T25 to T175)	Costar Corning, New York, USA
Cell culture plates (6- to 96-well)	BD Medical, Franklin Lakes, USA
Cryotubes	greiner bio-one, Frickenhausen, DE
Disposable scalpels (No. 10)	FEATHER, Osaka, JP
ELISA microplates (96-well)	Costar Corning, New York, USA
Eppendorf tubes (0.5 ml; 1.5 ml; 2.0 ml)	Sarstedt, Nürnbrecht, DE
FACS tubes	BD Biosciences, Franklin Lakes, USA
Nylon filter SmartStrainers (100 µm, 30 µm)	Miltenyi Biotec, Bergisch Gladbach, DE
Petri dishes	BD Medical, Franklin Lakes, USA
Pipetboy	Hirschmann Laborgeräte, Eberstadt, DE
Pipettes	Eppendorf, Hamburg, DE
Polypropylene round bottom tubes	BD Biosciences, Franklin Lakes, USA
Serological pipettes	Costar Corning, New York, USA
Syringes with Luer-Lok-Tip (50 ml)	BD Medical, Franklin Lakes, USA
Syringes (2 ml, 10 ml)	BD Medical, Franklin Lakes, USA

2.3 Chemicals and reagents

Albumin Fraction V (BSA)	Sigma-Aldrich, St. Louis, USA
BD Pharm Lyse™ Lysing Buffer (10x)	BD Biosciences, Franklin Lakes, USA
Biocoll Separating Solution (d = 1,077 g/ml)	Biochrom Merck Millipore, Darmstadt, DE
Brefeldin A	Sigma-Aldrich, St. Louis, USA
Collagenase D	Sigma-Aldrich, St. Louis, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, USA
DNase I	Roche, Mannheim, DE
Ethanol 96-100 %	Sigma-Aldrich, St. Louis, USA
FACSFlow	BD Biosciences, Franklin Lakes, USA
Heparin-Sodium (25,000 I.U./ 5 ml)	Ratiopharm, Ulm, DE
Ionomycin calcium salt	Sigma-Aldrich, St. Louis, USA
Isoflurane	CP Pharma, Burgdorf, DE
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, St. Louis, USA
Sulfuric acid (2 N)	Pharmacy of LMU, München, DE
Trypan blue	Sigma-Aldrich, St. Louis, USA
Tween-20	Roth, Karlsruhe, DE

2.4 Assays

Bio-Plex Cell Lysis Kit	Bio-Rad, San Diego, USA
CountBright™ Absolute Counting Beads	Invitrogen, Carlsbad, USA
Cytofix/Cytoperm	BD Biosciences, Franklin Lakes, USA
DC™ Protein Assay	Bio-Rad, San Diego, USA
IC Fixation/Permeabilzation Kit	eBioscience, San Diego, USA
IL-22 ELISA (human)	R&D Systems, Minneapolis, USA
IL-22 ELISA (murine)	Antigenix America, Huntington State, USA
MACS Pan T cell isolation kit, human	Miltenyi Biotec, Bergisch Gladbach, DE
MACS Pan T cell isolation kit II, mouse	Miltenyi Biotec, Bergisch Gladbach, DE
MACS naive CD4+ T cell isolation kit, mouse	Miltenyi Biotec, Bergisch Gladbach, DE
MACS naive CD4+ T cell isolation kit II, human	Miltenyi Biotec, Bergisch Gladbach, DE
Zombie NIR Fixable Viability kit	BioLegend, San Diego, USA

2.5 Cytokines and antagonists

CRID3 sodium salt	Tocris Bioscience, Bristol, UK
InVivoMAb anti-mouse IL-1R (clone: JAMA-147)	BioXCell, West Lebanon, USA
InVivoMAb polyclonal Armenian hamster IgG	BioXCell, West Lebanon, USA
Kineret® (anakinra) (100 mg/0.67 ml)	Sobi, Stockholm, SE
Recombinant IL-1α (human)	PeproTech, Rocky Hill, USA

Recombinant IL-1α (murine)	BioLegend, San Diego, USA
Recombinant IL-1β (human)	PeproTech, Rocky Hill, USA
Recombinant IL-23 (murine)	BioLegend, San Diego, USA

2.6 Antibodies

Table 2: FACS antibodies

Antibody	Reactivity	Clone	Manufacturer
Anti-CD3ε-APC	Human	OKT3	BioLegend
Anti-CD4-FITC	Human	A161A1	BioLegend
Anti-CD8-APC-Cy7	Human	HIT8a	BioLegend
Anti-CD11b-APC	Human	ICRF44	BioLegend
Anti-IFNγ-PE-Cy7	Human	4S.B3	BioLegend
Anti-IL-17-FITC	Human	BL168	BioLegend
Anti-IL-22-PE	Human	22URTI	eBioscience
Anti-CD3ε-APC	Murine	145-2C11	BioLegend
Anti-CD4-PaqBlue	Murine	GK1.5	BioLegend
Anti-CD8-APC-Cy7	Murine	53-6.1	BioLegend
Anti-IL-22-PE	Murine	140301	R&D
Anti-CD45-PerCp5.5	Murine	30-F11	BioLegend
TruStain FcX	Human	-	BioLegend
TruStain FcX (anti-CD16/32)	Murine	93	BioLegend
Mouse IgG1κ-PE isotype control	-	MOPC-21	BioLegend
Rat IgG2A-PE isotype control	-	54447	R&D

2.7 Cell lines, supplements and media

2.7.1 Cell lines

Table 3: Cell lines

Cell line	Species	Description	Medium	Reference
4T1	Murine	Breast cancer (BALB/c)	$DMEM^{+++}$	(Dexter, et al. 1978)
A549	Human	Lung adenocarcinoma	$DMEM^{+++}$	ATCC: CCL185
CAMA1	Human	Breast cancer	$DMEM^{+++}$	ATCC: HTB-21
E0771	Murine	Breast cancer	$RPMI^{+++}$	CH3 BioSystems, USA
H1339	Human	Small cell lung cancer	$RPMI^{+++}$	ATCC: CRL-5979
HCC827	Human	Lung adenocarcinoma	$RPMI^{+++}$	ATCC: CRL-2868
MCF-7	Human	Breast cancer	$DMEM^{+++}$	ATCC: HTB-22
MD-AMB-231	Human	Breast cancer	$DMEM^{+++}$	ATCC: HTB-26

* all human cell lines were identified through STR profiling

2.7.2 Supplements

Dulbecco's modified Eagle's medium (DMEM)	PAA Laboratories, Pasching, AT
Fetal bovine serum (FBS) – heat inactivated	Gibco, Carlsbad, USA
Human serum	Sigma-Aldrich, St. Louis, USA
L-Glutamine (200 mM)	PAA Laboratories, Pasching, AT
Non-essential amino acids (NEAA)	Gibco, Carlsbad, USA
Penicillin/Streptomycin (100x)	PAA Laboratories, Pasching, AT
Phosphate-buffered Saline (PBS)	PAA Laboratories, Pasching, AT
Roswell Park Memory Institute (RPMI) 1640	PAA Laboratories, Pasching, AT
Sodium pyruvate (100 mM)	PAA Laboratories, Pasching, AT
VLE-RPMI 1640	Biochrom, Berlin, DE

2.7.3 Media

Complete DMEM medium ($DMEM^{+++}$)		*Complete RPMI medium ($RPMI^{+++}$)*	
DMEM full medium	500 ml	RPMI 1640 full medium	500 ml
FBS	50 ml	FBS	50 ml
Penicillin	1 IU/ml	Penicillin	1 IU/ml
Streptomycin	100 µg/ml	Streptomycin	100 µg/ml
L-Glutamine	2 mM	L-Glutamine	2 mM

PBMC medium	
VLE-RPMI 1640	500 ml
Human serum	50 ml
Penicillin	1 IU/ml
Streptomycin	100 µg/ml
L-Glutamine	2 mM
Sodium pyruvate	1 mM
NEAA (100 %)	1 %

Digestion medium	
RPMI 1640	1 ml
Collagenase D	1 mg/ml
DNase I	0.05 mg/ml

FACS buffer	
PBS	500 ml
FBS	10 ml

Cryopreservation medium	
FBS	400 µl
DMSO	100 µl
Medium with cells	500 µl

2.8 Software

Adobe Creative Suite CS6	Adobe Systems, San Jose, USA
BD FACSDiva	BD Biosciences, Franklin Lakes, USA
EndNote X7	Thomson Reuters, Carlsbad, USA
FlowJo 8.7	Tree Star, Ashland, USA
GraphPad Prism Version 5.0	GraphPad Software, La Jolla, USA
Microsoft Office 2016	Microsoft, Redmond, USA

3 Methods

3.1 Cell biological methods

3.1.1 General cell culture conditions

All cell lines were cultured in cell culture flasks in incubators at 37 °C, 5 % CO_2 and 95 % humidity. Cell manipulations were performed under sterile cell culture conditions under a laminar flow hood. Adherent cells were detached by incubating the cells with a Trypsin solution for 5 minutes at 37 °C or by using sterile cell scrapers. Detached cells were spun down at 400 g for 5 min and resuspended in full growth medium. Cells were supplemented with new medium three times per week and divided at 80 % confluence. Neubauer hemocytometers were used to determine number and viability of Trypan blue stained cells.

3.1.2 Generation of cell-free tumor supernatants

Tumor cells were trypsinized, centrifuged and counted. 0.5 x 10^6 cells per ml were plated in 2 ml fresh medium in 6 wells and kept in the incubator for four to six days. Tumor cell-free supernatants were collected by centrifuging twice at 400 g for 5 min at 4 °C in canonical 50 ml Falcon tubes.

3.1.3 Isolation of splenocytes

WT BALB/c mice were killed by cervical dislocation and spleens were taken into 1.5 ml tubes containing complete RPMI medium. Attached fat tissue was removed and a single cell suspension was generated by mashing the organs through a 100 µm strainer attached to a 50 ml conical tube under sterile conditions, using the plunger end of a syringe. Cells were washed through the strainer with full medium and filtered through an additional 30 µm strainer. Cells were centrifuged at 400 g for 5 minutes at 20 °C. Red blood cell lysis was performed using 2 ml of BD Pharm Lyse™ buffer according to the manufacturer's protocol. After 2 min, 10 m of complete RPMI medium was added to stop the lysis. Cells were centrifuged and washed with full medium. Finally, the splenocytes were counted using Neubauer hemocytometers and used for stimulation assays.

To examine specific subpopulations, MACS enrichment was used according to the manufacturer's protocol. MACS purities were measured with flow cytometry using the appropriate FACS antibodies as described in section 3.2.2.

3.1.4 Isolation of cells from lung tissue

Lungs were removed from sacrificed mice and placed into 1.5 ml tubes containing complete RPMI medium. The lungs were homogenized with scalpels and incubated in digestion

medium at 37 °C for 30 min on a shaking heating block. Next, single cell suspensions were generated as described in section 3.1.2.

3.1.5 Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated using a density gradient with Biocoll (d = 1,077 g/ml). 30 – 50 ml of blood was drawn from donors using 50 ml syringes covered with 5,000 I.U. Heparin-Sodium. The blood was diluted 1:1 with PBS. 20 ml of the diluted blood was carefully layered over 10 ml Biocoll in a 50 ml Falcon conical tube and centrifuged at 1,000 g at 20 °C in a swinging-bucket rotor without brake. Next, the interphase ring containing PBMCs was carefully aspirated, transferred to a new 50 ml tube and resuspended in 50 ml PBS. After centrifugation (10 min, 500 g, 20 °C), all PBMC pellets of one donor were collected and washed once more with PBS (10 min, 400 g, 4 °C) before being resuspended in PBMC growth medium containing VLE RPMI and used in stimulation assays.

3.1.6 Preparation of breast cancer tissue

Tumor tissue was homogenized with scalpels and incubated in digestion medium at 37 °C for 30 min on a shaking heating block. Subsequently, the cell suspensions were passed through a 100 µm and then a 30 µm cell strainer. To remove erythrocytes, cell suspensions were resuspended in 2 ml of BD Pharm Lyse™ buffer and incubated for 2 min at 20 °C. Erythrocytes were removed through incubation with BD Pharm Lyse™ buffer for 2 min. Cells were washed once and counted using hemocytometers. To assess the cytokine expression by tumor infiltrating lymphocytes, isolated single cell suspensions were stimulated at 37 °C for 4 h in RPMI 1640 medium containing 50 ng/ml PMA, 1 µg/ml ionomycin and 2.5 µg/ml brefeldin A (Golgi stop) at a concentration of 2 x 10^6 cells per ml.

3.1.7 Cell lysates

Approximately 1.5 mm^2 big pieces of primary tumor tissues were snap-frozen in liquid nitrogen. After homogenization, the tissues were lysed using the Bio-Plex cell lysis kit according to the manufacturer's protocol. Cell debris was removed by centrifugation at 10,000 g for 10 min. Protein concentrations of lysates were determined by the Bradford method (Detergent Compatible Protein Assay, Bio-Rad), using a microplate reader (Berthold Mithras LB940 multilabel plate reader) to detect absorbance at 750 nm.

3.1.8 Stimulation of cells

Counted cells were plated in 6- to 96-well plates at a concentration of 2 x 10^6 cells per ml. Recombinant cytokines or antagonists were added in their respective concentrations. Stimulation with tumor cell derived supernatants was achieved by replacing 50 % of the

medium with the cell-free tumor supernatant. Supernatants were harvested after four to six days, centrifuged and frozen at -20 °C for further analysis.

3.2 Immunological methods

3.2.1 Enzyme-linked immunosorbent assay

Several human and murine ELISA kits were used to measure cytokine concentrations in cell supernatants and cell lysates. Before analysis, samples were stored at -20 °C to -80 °C. ELISA and buffer preparation were performed according to the manufacturer's protocol. Absorbance at 450 nm was measured using a microplate reader (Berthold Mithras LB940 multilabel plate reader). Background correction was at 595 nm. A four parameter logistic (4PL) regression was used to calculate protein concentrations from the standard curve.

3.2.2 Flow cytometry (FACS)

Flow cytometry allows the quantification of cells according to size, granularity and expression of proteins based on fluorophore-conjugated antibodies. For FACS analysis, single cell suspensions were isolated as described in section 3.1.

After stimulation as described in section 3.1.5, cells were washed with FACS buffer and counted. 10^6 – 10^7 cells per well were plated in 96-U-well plates. To avoid unspecific binding of the antibodies, cells were incubated for 15 min at 4 °C with Fc-block reagent added at 1:100 dilution. Cell suspensions were then incubated with fluorochrome-conjugated antibodies for extracellular targets and Zombie NIR Fixable Viability Dye (BioLegend) for live/dead staining for 30 min at 4 °C in the dark. After two washing steps with FACS buffer, cells were fixed and permeabilized using the IC Fixation/Permeabilization (eBioscience) kit according to the manufacturer's protocol. Fc-block was added for 15 min at 20 °C in the dark. Cells were then stained in the permeabilization buffer for 30 min at 20 °C with antibodies directed against intracellular antigens. CD4 staining was repeated due to receptor internalization as a result of the stimulation mix. Finally, cells were centrifuged at 400 g for 5 min at 20 °C and washed twice with 200 µl permeabilization buffer and once with 200 µl FACS buffer. After centrifugation, cell pellets were resuspended in FACS buffer and analyzed by the FACS Canto II (BD Biosciences) run on FACSDiva software. For quantifying cell populations, CountBright™ beads were added to the samples prior to FACS analysis following the manufacturer's recommendations. Absolute counts were calculated according to the formula: (number of positive cells/number of bead events) × number of beads added. Data analysis was performed using FlowJo 8.8.2.

3.3 Animal experiments

3.3.1 Care of laboratory animals

Six to eight weeks old wild type (WT) female mice on BALB/c and C57/BL6 background were used for *in vivo* experiments. Mice were obtained from Janvier (Saint-Berthevin Cednex, France) and Charles River (Sulzfeld, Germany). The animals were held in the animal facility “Tierhaltung der Medizinischen Klinik Innenstadt” at least one week before the start of experiments. All animal experiments have been approved by the local authority, Regierung von Oberbayern (reference number: 55.2.1.54-2532-90/12) and adhered to the NIH guidelines for the care and use of laboratory animals.

3.3.2 Subcutaneous tumor models

For subcutaneous tumor models, 1.25 x 10^5 4T1 or 2 x 10^5 E0771 murine breast cancer cells were resuspended in 100 µl PBS and injected into the subcutaneous tissue of the right flank of young female mice. Tumor size was measured three times per week using an electronic caliper. It was defined as area in mm^2 (length in mm x width in mm). Daily scoring included tumor size and morphology, body weight, behavior and general condition.

When termination criteria were reached, mice were anesthetized with isoflurane inhalation and sacrificed via cervical dislocation. Organs were removed using forceps and scissors and kept in RPMI on ice until further processing.

3.4 Primary breast cancer patients

Breast cancer patients were recruited from the department of gynecology and obstetrics at the Klinikum Dritter Orden, Munich. Tissue samples were collected from eleven female patients. All patients had a confirmed diagnosis of mammary carcinoma and four of these were classified as triple negative breast cancer. No patient had received previous treatment in the form of (radio-)chemotherapy. Ethical approval was obtained from the Ludwig-Maximilians-Universität Ethics Committee (reference number 249-15). Written informed consent was obtained from all patients prior to collection of specimens. Patient recruitment was done in line with respective institutional policies and in accordance with the Declaration of Helsinki. Tumor specimens were only obtained from patients undergoing clinically indicated surgery. Seven patient samples were large enough to provide sufficient material for FACS analysis (see section 3.1.5 and 3.2.2). From all eleven patients, a small piece was snap-frozen in liquid nitrogen in order to measure protein concentrations with ELISA (see section 3.2.1) from cell lysates (see section 3.1.7).

Table 4: Cell lines

Characteristic	No. of patients
Union for International Cancer control stage	
0	0
I	5
IIA	1
IIB	2
IIIA	0
IIIB	0
IIIC	0
IV	3
Histology	
No specific type	11
Triple-negative breast cancer	4
Previous treatment	
Chemotherapy	0
Radio-Chemotherapy	0
Age (y)	
50-59	4
60-69	1
70-79	3
80-89	3
Gender	
Female	11
Male	0

3.5 Statistical analysis

Statistics were calculated using GraphPad Prism software 5.0. Unless stated otherwise, arithmetic mean and standard error of the mean were calculated. Statistical significance was determined using the two-sided Student's t-test for unpaired samples or for paired samples when results from the same individual human donor were compared. Mann-Whitney U test was used for significance testing comparing data points from individual mice. Tumor growth curves were analyzed by two-way ANOVA, with correction for multiple testing by the Bonferroni method.

4 Results

4.1 *In vitro* characterization of IL-22-producing cells in murine model

4.1.1 Purified CD4$^+$ T cells but not CD4-depleted splenocytes produce IL-22

Previous data from our group has shown that CD3$^+$ T cells are the major source of IL-22 production by splenocytes when stimulated with cell-free tumor supernatant from murine 4T1 cells (Voigt, et al. 2017). Using MACS purification, it was demonstrated that IL-22 was conserved in the CD3$^+$ fraction while being significantly reduced in CD3-depleted fraction.

To further analyze the subgroup of IL-22-producing T cells, CD4$^+$ cells were purified and stimulated. Purity of the CD4$^+$ T cell fraction was between 82 and 99 %, while the CD4-depleted splenocytes contained less than 5 % CD4$^+$ cells (data not shown).

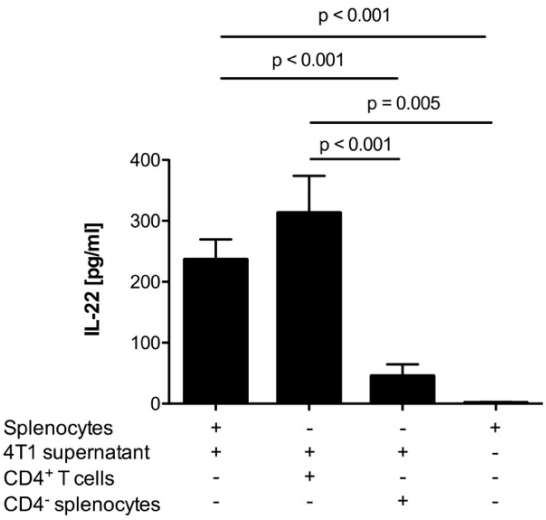

Figure 3: Murine IL-22 production in CD4$^+$ T cells and CD4$^-$ splenocytes after stimulation with tumor cell supernatant.
Stimulation of total splenocytes, MACS-enriched CD4$^+$ T cells and CD4$^-$ splenocytes (2 x 10^6 per ml) for six days with cell-free supernatant from 4T1 cell culture. IL-22 concentration was quantified by ELISA. Values of four independent experiments are shown, with n = 8 different donors. Error bars represent SEM and statistical significance was calculated using two-sided Student's t-tests.

MACS-enriched CD4$^+$ T cells produced IL-22 in similar amounts to unselected splenocytes upon stimulation with tumor-cell supernatant. In contrast, IL-22 secretion was significantly lower, though still detectable, in the CD4-depleted fraction. Consequently, CD4$^+$ T cells can be identified as the responsible CD3$^+$ T cell subgroup producing IL-22 after stimulation with 4T1 tumor supernatant.

Our group could also previously show that the IL-22 induction by 4T1 tumor cell supernatant was dependent upon IL-1α and IL-23 (Voigt, et al. 2017). To demonstrate that this effect was also relevant for and mediated through $CD4^+$ T cells, stimulation of $CD4^+$ and $CD4^-$ splenocytes was repeated with IL-1α and IL-23.

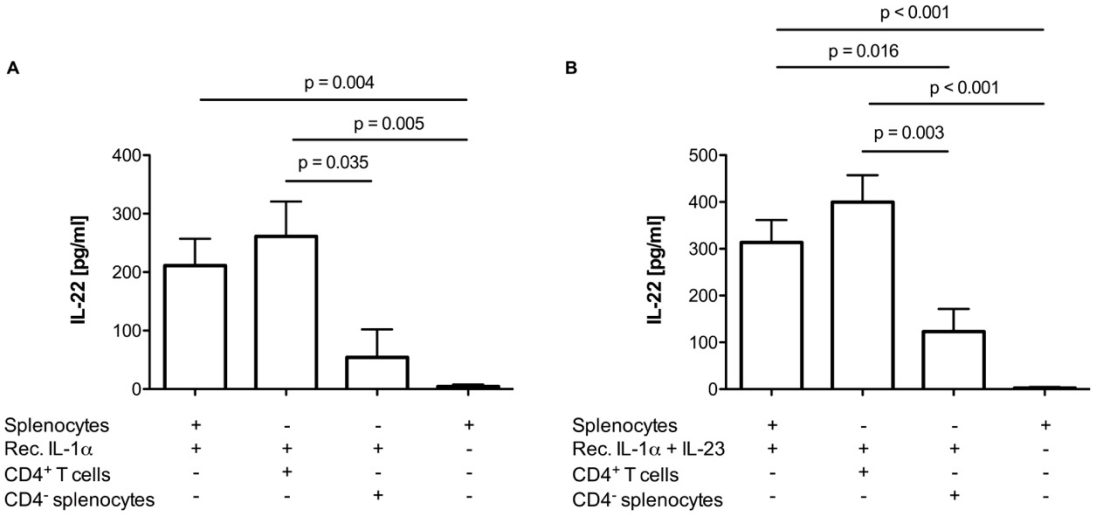

Figure 4: Murine IL-22 production in $CD4^+$ T cells and $CD4^-$ splenocytes after stimulation with recombinant proteins.
Stimulation of total splenocytes, MACS-enriched $CD4^+$ T cells and $CD4^-$ splenocytes (2 x 10^6 per ml) for six days with cell-free supernatant from (A) 100 ng/ml recombinant IL-1α or (B) 100 ng/ml recombinant IL-1α and IL-23. IL-22 concentration was quantified by ELISA. Values of a minimum of four independent experiments are shown, with n = 4 (A) and n = 7 (B) different donors. Error bars represent SEM and statistical significance was calculated using two-sided Student's t-tests.

IL-22 production was at comparable levels in $CD4^+$ T cells and mixed splenocytes after stimulation with recombinant IL-1α, but significantly reduced in the CD4-depleted fraction. The effect was slightly more pronounced when recombinant IL-23 was added. $CD4^+$ T cells are thus the main producers of IL-22 after stimulation with 4T1 tumor supernatant or recombinant IL-1α and IL-23.

4.1.2 Mixed $CD4^+$ T helper cell population produce IL-22

To further characterize the source of IL-22 within the population of $CD4^+$ T cells, MACS-enriched $CD4^+$ T cells were analyzed by flow cytometry after stimulation with 4T1 tumor cell supernatant or recombinant IL-1α.

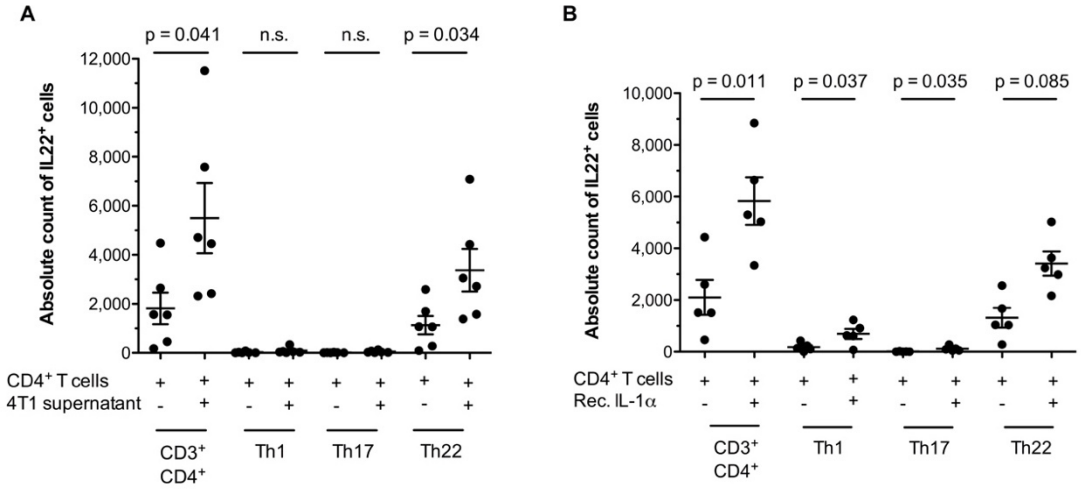

Figure 5: FACS analysis of murine T helper cell subgroup after stimulation with tumor cell supernatant or recombinant IL-1α.
MACS-enriched $CD4^+$ T cells (10^6 per well) were cultured in 96-well plates and stimulated with (A) 4T1 cell-free tumor supernatant or (B) 20 ng/ml recombinant IL-1α for 4 days. They were then restimulated using PMA and ionomycin in the presence of brefeldin A and stained intracellularly for IL-22. Subsequently, they were analyzed via flow cytometry for IL-22 expression in Th1 ($CD3^+CD4^+IFN\gamma^+$), Th17 ($CD3^+CD4^+IL\text{-}17^+$), and Th22 ($CD3^+CD4^+IL\text{-}17^-IFN\gamma^-$) cells. Values of a minimum of three independent experiments are shown, with n = 6 (A) and n = 5 (B) different donors. Error bars represent SEM and statistical significance was calculated using two-sided Student's t-tests.

Stimulation with either 4T1 tumor supernatant or recombinant IL-1α resulted in an upregulation of IL-22. As expected, the majority of $IL\text{-}22^+$ cells were $CD3^+CD4^+$. They consisted of a mixed Th1, Th17 and Th22 population, with Th22 cells representing the most prominent subpopulation. Results were similar for stimulation with either 4T1 cell-free tumor supernatant or recombinant IL-1α.

4.1.3 $CD4^+CD44^+$ memory T cells as main source of IL-22

Mechanistically, IL-1α can either lead to differentiation of naïve T cells into IL-22-producing subsets, or directly induce IL-22 production from an existing pool of Th1, Th17, and Th22 cells. In order to differentiate whether naïve or memory T cells were the source of IL-22 in this model, splenocytes were sorted using MACS. Mouse naïve $CD4^+$ T cells were isolated by depletion of $CD44^+$ memory T cells and non-$CD4^+$ T cells. Purity of the $CD4^+$ T cell fraction was between 84 and 98 %. The naïve $CD4^-$ splenocyte fraction contained less than 22 % $CD4^+$ cells and less than 1 % $CD4^+CD44^+$ memory T cells (data not shown).

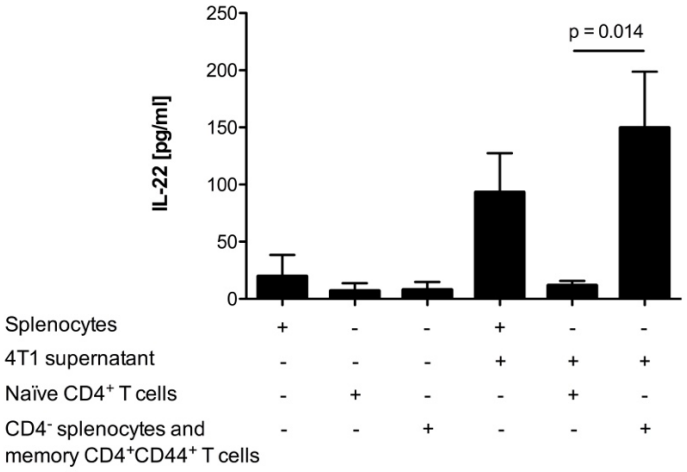

Figure 6: Murine IL-22 production in naïve CD4$^+$ T cells and CD4$^+$CD44$^+$ memory T cells. Stimulation of total splenocytes, MACS-enriched naïve CD4$^+$ T cells, and CD4$^+$CD44$^+$ memory T cells with CD4$^-$ splenocytes (2 x 10^6 per ml) for six days with cell-free supernatant from 4T1 cell culture. IL-22 concentration was quantified by ELISA. Values of three independent experiments are shown, with n = 8 different supernatants. Error bars represent SEM and statistical significance was calculated using two-sided Student's t-tests.

Naïve CD4$^+$ cells showed no relevant IL-22 production after stimulation with recombinant IL-1α. In contrast, CD4$^+$CD44$^+$ memory T cells with CD4-depleted splenocytes produced IL-22 levels similar to that of the mixed splenocyte population. Given that CD4$^-$ splenocytes on their own were shown to induce only low levels of IL-22 (see Figure 3), these findings suggest that IL-1 containing tumor supernatant does not lead to differentiation of naïve T cells into IL-22-producing subsets. Instead, pre-existing memory T cells are the target population for IL-1-induced IL-22 production in mice.

4.2 Effects of blocking IL-1 *in vivo*

4.2.1 Blocking of IL-1 reduces tumor progression and production of IL-22 cells

To confirm the *in vitro* findings of IL-1 induced IL-22 production by $CD4^+$ cells through cancer cells, the impact of IL-1 blockade on IL-22 production and tumor growth was tested *in vivo*. First, 4T1 tumor-bearing mice were treated every second day with a neutralizing IL-1R antibody.

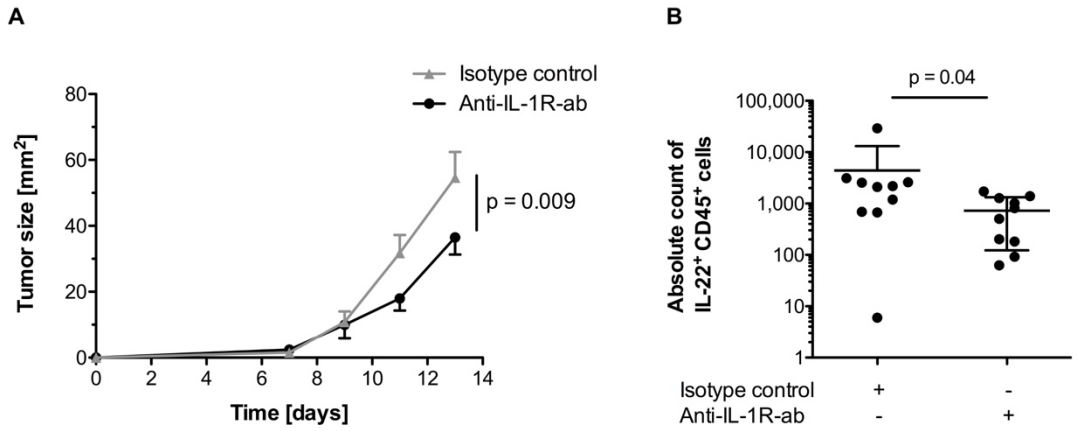

Figure 7: Neutralizing IL-1R antibody reduces tumor growth and IL-22 production in 4T1 breast cancer model.
1.25 x 10^5 4T1 tumor cells were injected in the right flank of BALB/c mice (n = 10 mice per group). Beginning on day 0, mice were treated with 300 µg InVivoMAb anti-mouse IL-1R antibody (clone: JAMA-147) or isotype control (InVivoMAb polyclonal Armenian hamster IgG) i. p. every second day. (A) shows tumor growth curves, error bars representing SEM and statistical significance was calculated by two-way ANOVA with correction for multiple testing. (B) shows absolute number of $IL\text{-}22^+CD45^+$ cells in the lung as determined by FACS analysis. Error bars represent SEM and statistical significance was calculated by Mann-Whitney U test.

Tumor growth was significantly reduced in the IL-1R antibody-treated group compared to the isotype control. As suggested by the *in vitro* findings, IL-22 production, as measured by the number of $IL\text{-}22^+$ cells in the lung, was also reduced.

Similarly, mice bearing the E0771 breast cancer model were treated with anakinra, a soluble IL-1R antagonist. Similar to the results in the 4T1 model, tumor growth was decreased, and IL-22 production was reduced through inhibition of IL-1 activity.

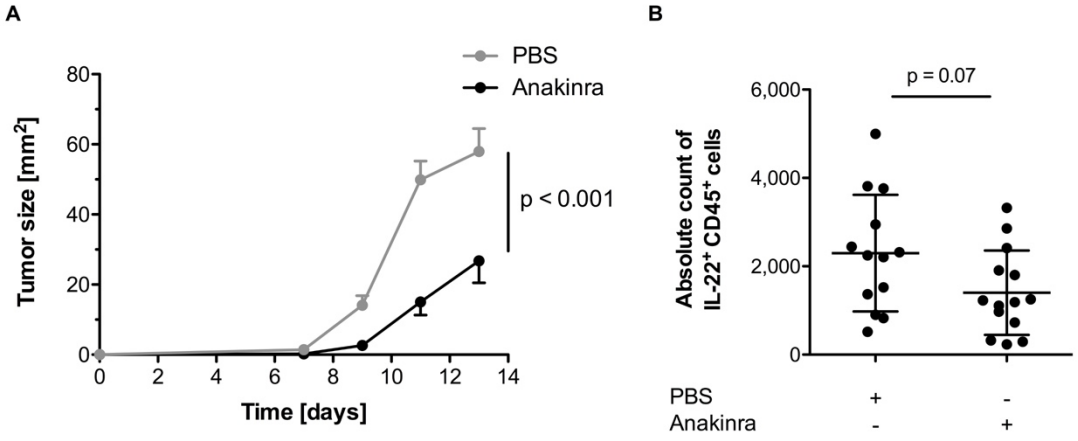

Figure 8: Soluble IL-R antagonist anakinra reduces tumor growth and IL-22 production in E0771 breast cancer model.
2.5 x 10^5 E0771 tumor cells were injected in the right flank of C57BL/6 mice (n = 15 mice per group). Beginning on day 0, mice were treated with 1 mg anakinra or PBS i. p. every day. (A) shows tumor growth curves, error bars representing SEM and statistical significance was calculated by two-way ANOVA with correction for multiple testing. (B) shows absolute number of IL-22^+CD45^+ cells in the lung as determined by FACS analysis. Error bars represent SEM and statistical significance was calculated by Mann-Whitney U test.

4.3 *In vitro* characterization of IL-22-producing cells in humans

4.3.1 Purified CD4$^+$ T cells but not CD4-depleted PBMCs produce IL-22

The next step was to evaluate whether the findings seen in mice can be translated into the human setting. Our group could previously show that human cancer cells induce IL-22 production from human PBMCs via IL-1β. Mirroring the approach with murine splenocytes, PBMCs from human donors were isolated and CD4$^+$ cells were purified. In total, supernatants from three human lung cancer cell lines (H1339, A549, and HCC827) and three human breast cancer cell lines (MD-AMB-231, CAMA1, and MCF-7) were tested. Cells were stimulated first with supernatants from human lung cancer cell lines. In the following three figures, purity of the CD4$^+$ T cell fraction was between 92 and 98 %, while the CD4$^-$ PBMC fraction contained less than 31 % CD4$^+$ cells (data not shown).

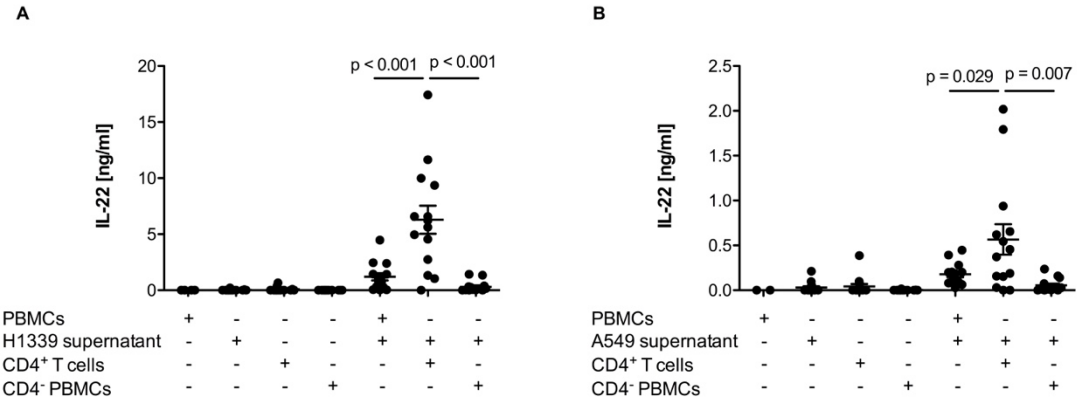

Figure 9: Human IL-22 production in CD4$^+$ T cells and CD4$^-$ PBMCs after stimulation with human lung cancer cell lines.
Stimulation of total PBMCs, MACS-enriched CD4$^+$ T cells or CD4$^-$ PBMCs (2 x 10^6 per ml) for six days with cell-free supernatant from (A) H1339 or (B) A549 cell culture. IL-22 concentration was quantified by ELISA. Values of a minimum of five independent experiments are shown, with n = 14 (A) and n = 14 (B) different donors. Error bars represent SEM and statistical significance was calculated using two-sided Student's t-tests.

When stimulated with supernatants from H1339 or A549 lung cancer cell lines, production of IL-22 by CD4$^+$ T cells was higher compared to that from whole PBMCs, while being almost absent in the CD4-depleted fraction.

The same experiments were repeated with supernatants from human breast cancer cell lines.

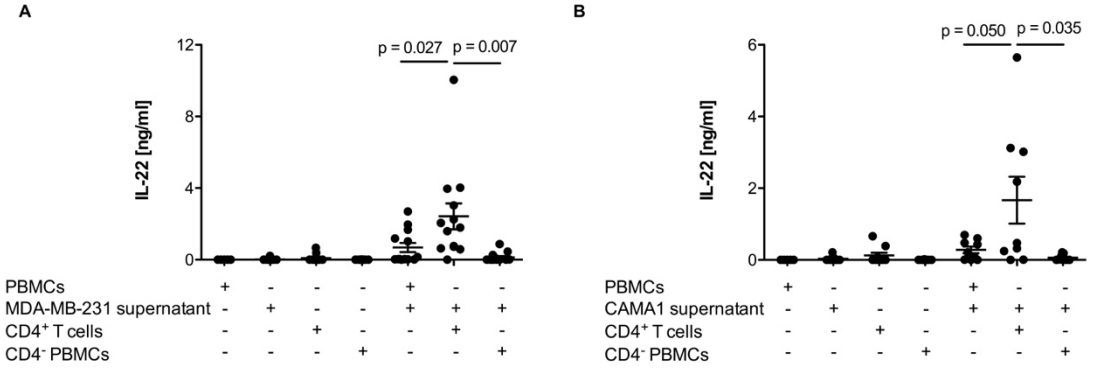

Figure 10: Human IL-22 production in CD4$^+$ T cells and CD4$^-$ PBMCs after stimulation with human breast cancer cell lines.
Stimulation of total PBMCs, MACS-enriched CD4$^+$ T cells or CD4$^-$ PBMCs (2 x 10^6 per ml) for six days with cell-free supernatant from (A) MDA-MB-231 or (B) CAMA1 cell culture. IL-22 concentration was quantified by ELISA. Values of a minimum of five independent experiments are shown, with n = 13 (A) and n = 9 (B) different donors. Error bars represent SEM and statistical significance was calculated using two-sided Student's t-tests.

Previous data from our group indicated that IL-1 family members induce IL-22 from PBMCs. To transfer these findings to the identified subpopulation of CD4$^+$ T cells, MACS enriched, MACS-enriched CD4$^+$ T cells were subsequently stimulated by recombinant proteins.

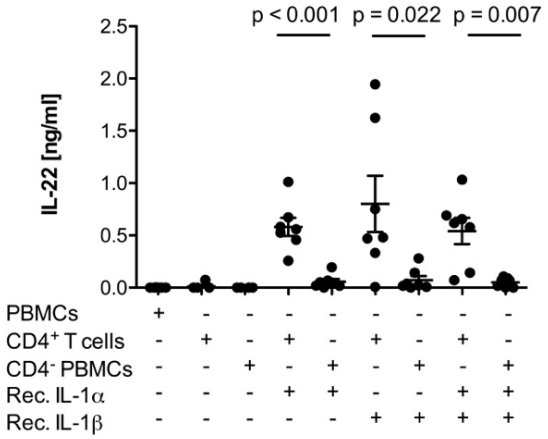

Figure 11: Human IL-22 production in CD4$^+$ T cells and CD4$^-$ PBMCs after stimulation with recombinant IL-1α and/or IL-1β.
Stimulation of total PBMCs, MACS-enriched CD4$^+$ T cells or CD4$^-$ PBMCs (2 x 10^6 per ml) for six days with 10 ng/ml recombinant IL-1α and/or IL-1β. IL-22 concentration was quantified by ELISA. Values of four independent experiments are shown, with n = 8 different donors. Error bars represent SEM and statistical significance was calculated using two-sided Student's t-tests.

Stimulation of human CD4$^+$ T cells with recombinant IL-1α or IL-1β led to significant IL-22 production, but IL-1β was shown to be a stronger inductor in the human setting. Combining both IL-1α and IL-1β also caused IL-22 induction in CD4$^+$ PBMCs, though this was not

increased compared to either cytokine alone. As seen in the stimulation with tumor supernatants, CD4-depleted PBMCs did not respond to either cytokines. This indicates that the $CD4^+$ T cell fraction is the target of IL-1 and the source of IL-22 production within the proposed mechanism.

4.3.2 NLRP3 inflammasome plays an important role in IL-1-induced IL-22 production

Production of IL-1β is predominantly regulated by cytosolic molecular complexes, called inflammasomes. Nod-like receptor protein 3 (NLRP3) inflammasome activity has been described as responsible for IL-1 activation in tumor cells (Dostert, et al. 2008). To identify whether this mechanism also applies to the IL-1 induced IL-22 secretion described above, the specific NLRP3 inhibitor CRID3 was used to antagonize IL-22 induction in PBMCs through cell-free supernatant from human breast cancer cell lines.

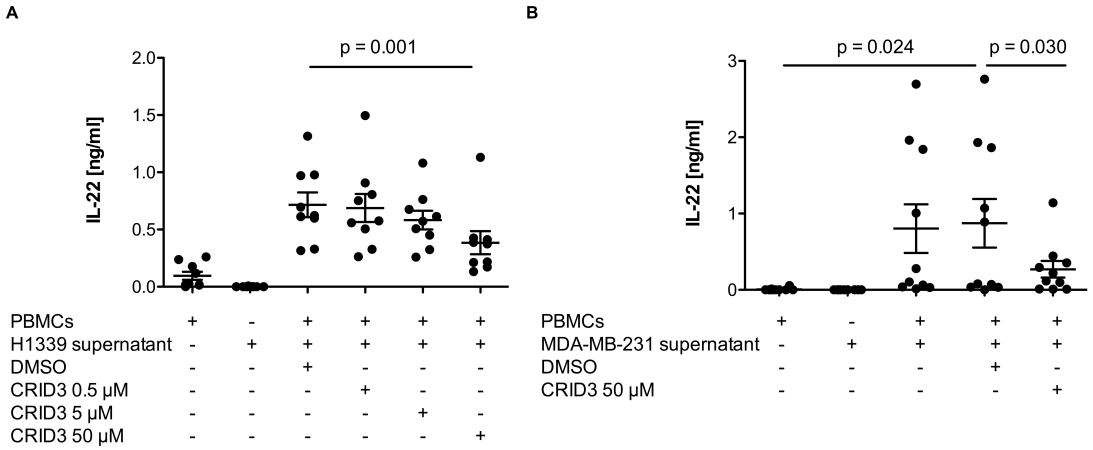

Figure 12: IL-22 induction by human breast cancer cell lines can be dose-dependently antagonized through NLRP3 inhibition.
Stimulation of PBMCs for six days with 50 % cell-free supernatant from (A) H1339 or (B) MDA-MB-231 in the presence of 0.5, 5 and 50 µM (A) or 50 µM (B) CRID3. DMSO served as a negative control. IL-22 concentration was quantified by ELISA. Values of a minimum of five independent experiments are shown, with n = 9 (A) and n = 10 (B) different donors. Error bars represent SEM and statistical significance was calculated using two-sided Student's t-tests.

Addition of CRID3 reduced IL-22 secretion in a dose-dependent manner and consistently across different human breast cancer entities. Similar results could be attained by using the pan-caspase inhibitor Z-VAD (data not shown). This points towards the contribution of inflammasome activation in the stimulation of the IL-1-IL-22 axis by tumor cells.

To see whether the identified $CD4^+$ T cell fraction was also the target of this inflammasome activation, the experiments were replicated in MACS-enriched $CD4^+$ T cells.

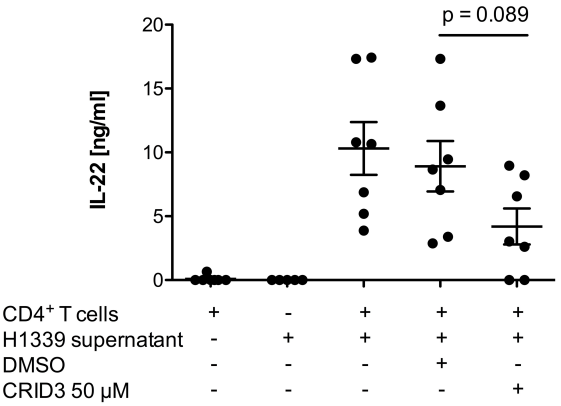

Figure 13: IL-22 induction by human breast cancer cell lines in CD4$^+$ T cells can be antagonized through NLRP3 inhibition.
Stimulation of MACS-enriched CD4$^+$ T cells for six days with 50 % cell-free supernatant from H1339 in the presence of 50 µM CRID3. DMSO served as a negative control. IL-22 concentration was quantified by ELISA. Values of four independent experiments are shown, with n = 7 different donors. Error bars represent SEM and statistical significance was calculated using two-sided Student's t-tests.

While not significant with the current number of repetitions, the data suggests that CD4$^+$ T cells are indeed the main target of NLRP3 inflammasome activation. NLRP3 inhibition was not able to fully suppress IL-22 induction, pointing towards the existence of additional factors responsible for cytokine production in the IL-1-IL-22 axis.

4.3.3 CD4$^+$CD44$^+$ memory T cells as main source of IL-22

To further characterize the involved T cell population, naïve and memory CD4+ T cells were isolated. Purity of the CD4$^+$ T cell fraction was between 84 and 98 %. The naïve CD4$^-$ splenocyte fraction contained less than 9 % CD4$^+$CD44$^+$ memory T cells, while the mixed CD4$^-$ contained at least 23 % CD4$^+$CD44$^+$ memory T cells (data not shown).

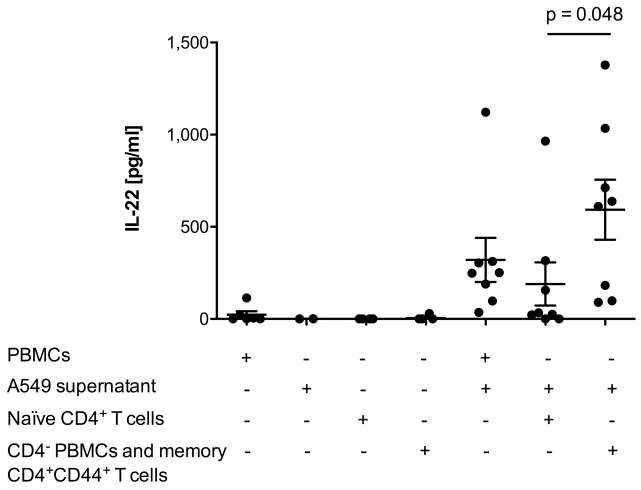

Figure 14: Human IL-22 production in naïve CD4⁺ T cells and CD4⁺CD44⁺ memory T cells after stimulation with human lung cancer cell line.
Stimulation of total splenocytes, MACS-enriched naïve CD4⁺ T cells, and non-naïve T cells (CD4⁺CD44⁺ memory T cells with CD4⁻ splenocytes) (2 x 10^6 per ml) for six days with cell-free supernatant from A549 cell culture. IL-22 concentration was quantified by ELISA. Values of four independent experiments are shown, with n = 8 different donors. Error bars represent SEM and statistical significance was calculated using two-sided Student's t-tests.

It was found that isolated memory CD4⁺CD44⁺ T cells were proficient IL-22 producers in this model. The naïve CD4⁺ T cell-depleted fraction had lower levels of IL-22 secretion, but in contrast to the murine model partially retained their ability to produce IL-22.

To investigate whether these memory T cells are also the target cells of the proposed mechanism via IL-1α and IL-1β, MACS-enriched cells were subsequently stimulated with recombinant proteins.

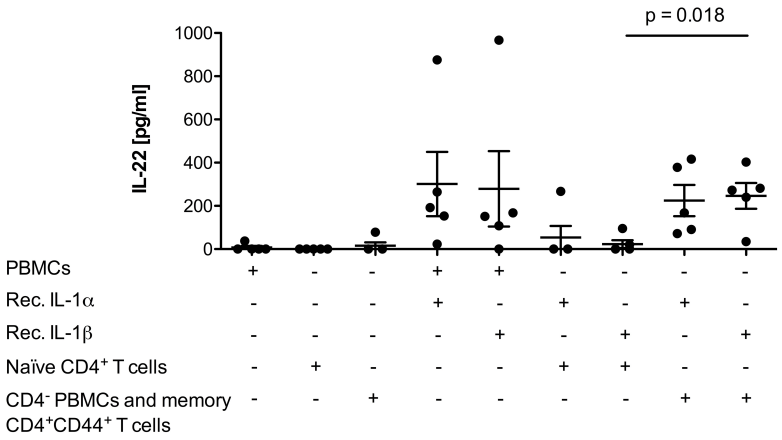

Figure 15: Human IL-22 production in naïve CD4⁺ T cells and CD4⁺CD44⁺ memory T cells after stimulation with recombinant proteins.
Stimulation of total splenocytes, MACS-enriched naïve CD4⁺ T cells, and non-naïve T cells (CD4⁺CD44⁺ memory T cells with CD4⁻ splenocytes) (2 x 10^6 per ml) for six days with 10 ng/ml recombinant IL-1α and/or IL-1β. Values of three independent experiments are shown, with n = 5 different donors. Error bars represent SEM and statistical significance was calculated using two-sided Student's t-tests.

As seen in the murine model, the stimulatory effect of tumor supernatant on primarily CD4⁺CD44⁺ memory T cells can be replicated with recombinant proteins. In the human setting, both recombinant IL-1α and IL-1β seem to be similarly potent inducers of IL-22 production from these memory T cells. Naïve CD4⁺ T cells are induced to produce some IL-22, though significantly less than their memory counterparts.

4.4 *Ex vivo* characterization of IL-22-producing cells in humans

4.4.1 T helper cells are the main producers of IL-22 in primary breast cancer tissue

To validate the existence of IL-22-producing T helper cell populations and to illustrate the relevance of the findings in human breast cancer, tumor samples of patients with breast cancer were analyzed for the presence of IL-22-producing cells. Single cell suspensions were stimulated for intracellular staining and IL-22-producing subpopulations were identified via flow cytometry. T helper cell subpopulations were defined as Th1 ($CD3^+CD4^+IL\text{-}17^-IFN\gamma^+$), Th17 ($CD3^+CD4^+IL\text{-}17^+IFN\gamma^-$), and Th22 ($CD3^+CD4^+IL\text{-}17^-IFN\gamma^-$); additional cell populations included $CD3^+CD8^+$, $CD3^+CD4^-CD8^-$, and $CD11b^+$ IL-22-producing cells.

On average, 0.23 % of the mononuclear cell fraction expressed IL-22 in breast cancer samples. Among these $IL\text{-}22^+$ cells, the main fraction, accounting for 41 %, was of Th1 phenotype, followed by Th22 and Th17 phenotypes (22 % and 9 %, respectively), making up almost ¾ of all $IL\text{-}22^+$ cells. The remaining IL-22-producing cell populations included $CD3^+CD8^+$ (12 %), $CD11b^+$ (12 %), and $CD3^+CD4^-CD8^-$ (2 %). Expression of IL-22 in breast cancer tissue was confirmed in protein lysates of the same tumor samples. On average, a concentration of 63.9 pg/mg protein IL-22 was measured.

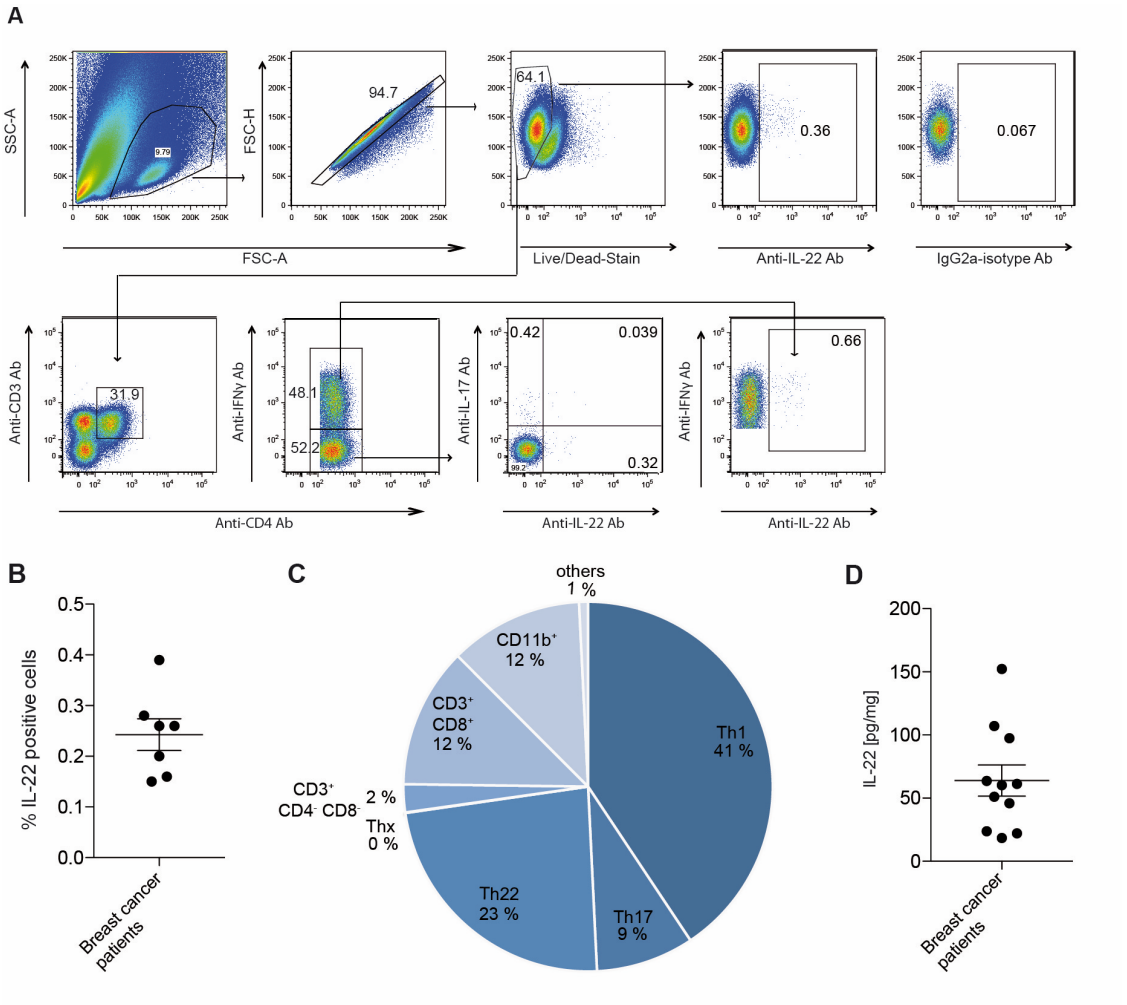

Figure 16: Identification of IL-22-producing cells in primary breast cancer tissue via FACS.
Single cell suspensions of breast cancer tissue were stimulated with PMA and ionomycin in the presence of brefeldin A and stained intracellularly for IL-22. Subsequently, they were analyzed via flow cytometry for IL-22 expression in Th1 ($CD3^+CD4^+IFN\gamma^+$), Th17 ($CD3^+CD4^+IL\text{-}17^+$), Th22 ($CD3^+CD4^+IL\text{-}17^-IFN\gamma^-$), $CD3^+CD8^+$, $CD3^+CD4^-CD8^-$, and $CD11b^+$. Dead cells were excluded per live-dead staining (A to C). (D) Quantification of IL-22 production in tumor lysates with ELISA. B to C represent n = 7 and D n = 11 patient samples. Error bars represent SEM and statistical significance was calculated using two-sided Student's t-tests.

5 Discussion

5.1 Summary of the mechanism of IL-22 induction in cancer

IL-22 has been characterized as a tumor-promoting cytokine in previous literature. IL-22-producing T cells have been found in lung and breast cancers, but also colorectal, gastric and pancreatic tumors (reviewed in: Lim and Savan 2014). In the majority of cases, IL-22 expression was correlated with cancer development and progression and poor survival outcome. So far, few studies have addressed the exact mechanism by which IL-22 expression is influenced by cancer cells. In addition, the classification of the target cells of this axis has been missing.

This set of experiments characterizes a subset of IL-22-producing $CD4^+$ T cells in mice and in humans which are induced by tumor cells in an IL-1-dependent manner. In the murine setting, $CD4^+$ but not CD4-depleted T cells are capable of producing IL-22 when stimulated by tumor cell supernatants. With naïve $CD4^+$ T cells shown to be irresponsive to stimulation, this project identified $CD4^+CD44^+$ memory T cells as the major IL-22-producing subset. The mechanism was shown to be dependent upon and replicable by IL-1 activation and conserved across different tumor entities. In murine cancer models, IL-1α was shown to be responsible for IL-22 production, whereas human cancer cell lines seem to induce IL-22 through IL-1β via the activation of the NLRP3 inflammasome. In line, IL-22 content and tumor growth were reduced when IL-1 was neutralized in two different murine breast cancer models, pointing toward the potential use of IL-1- and IL-22-antagonists for cancer therapy.

5.2 IL-1α secreted by tumor cells may stimulate IL-22 production from murine $CD4^+CD44^+$ memory T cells

In the first set of experiments, murine $CD4^-$ cells did not secrete IL-22 upon stimulation, but non-naïve splenocytes, that is $CD4^-$ and $CD4^+CD44^+$ cells, produced significant cytokine levels. From this it can be concluded that the $CD4^+CD44^+$ subpopulation is most likely the major source of IL-22 and that IL-22 production is driven by reactivation of memory T cells rather than differentiation of naïve T cells.

In the murine setting, it was shown that IL-1α is mainly responsible for this induction. IL-1α is expressed predominantly intracellularly or membrane-bound (Gross, et al. 2012, Orjalo, et al. 2009). Its precursor (pro-IL-1α) is activated through the protease Calpain into IL-1α N-terminal peptide (IL-1αNTP) (Voronov, et al. 2013). Its expression is upregulated through cell stress such as hypoxia and necrosis, which is common in fast-growing tumors both *in vitro* and *in vivo* (Cooper and Beasley 1999, Di Mitri, et al. 2014). Accordingly, IL-1α concentrations in tumor supernatants were strongly correlated with length of incubation

time (data not shown). The effects on IL-1R$^+$ cells can be further enhanced by effect that IL-1α can induce its own release from immune cells, causing positive feedback loops (Ghezzi and Dinarello 1988). So far, it has only been shown that murine NCR$^-$ ILC3 secrete IL-22 upon stimulation with IL-1α (Hernandez, et al. 2015). While murine NCR$^-$ ILC3 also secrete IL-17 and low levels of IFNγ, they are CD3$^-$CD4$^-$ and localized primarily in the mucosa (Walker, et al. 2013). Given the identification of CD4$^+$CD44$^+$ memory T cells as the main target of IL-1α induced IL-22 production, these experiments seem to describe a new pathway.

Naïve CD4$^+$ T cells were unable to produce significant amounts of IL-22 upon stimulation with tumor cell supernatants containing IL-1α. This suggests that IL-1α stimulation does not share the same pathway with IL-6, a cytokine that is sufficient to induce IL-22 from naïve T cells *in vitro* in mouse through differentiation (Zheng, et al. 2007). Instead, the findings promote the hypothesis that IL-1α activates an existing pool of T helper cells by upregulating their IL-22 production. Nonetheless, low levels of IL-22 were secreted by naïve cells in most experiments. This was most likely due to either non-identified components released by tumor cells or direct stimulation by activated immune cells. The above experiments also cannot rule out that there exists some form of soluble or cell-to-cell interaction between these cell subpopulations. Naïve CD4$^+$ T cells could still be a source of IL-22 after contact with stimulated CD4$^-$ or CD4$^+$CD44$^+$ cells. Moreover, suboptimal MACS purities in this experimental setup could have contributed to confounding factors.

FACS analysis of stimulated splenocytes confirmed the presence of primarily Th22 cells, and smaller counts of Th1 and Th17 cells. In comparison to recombinant IL-1α, stimulation with tumor cell supernatants shifted the phenotype more towards Th22 cells, which is most likely due to the existence of additional factors such as TNFα (Duhen, et al. 2009). But although murine Th22 cells have been identified in literature, they are still not defined as an individual subset *in vivo*; according to literature, the main producers of murine IL-22 are Th17 cells. This mismatch might be explained by the fact that the presence of IL-17 can shape the role of IL-22 in cancer *in vivo* (Eyerich, et al. 2017). Moreover, IL-17 and IL-22 are components of a highly redundant and regulated system involved in epithelial homeostasis, making it very difficult to predict the role of an individual cytokine in any given environment. Studies have also demonstrated plasticity of Th cell subsets, which appears to be dependent upon regulatory transcription factors that act as master regulators (Plank, et al. 2017).

5.3 $CD4^+CD44^+$ memory T cells are also responsible for IL-22 production in humans, but primarily upon IL-1β stimulation

Similar to the murine setting, $CD4^+CD44^+$ memory T cells were identified as the primary source of IL-22 after stimulation with human tumor cell supernatant. In humans, IL-1β, more than IL-1α, was shown to be responsible for this induction. Naïve T cells were shown to be relatively irresponsive to IL-1 stimulation in terms of IL-22 production. This is in contrast with recent findings of significant polarization of the naïve T cell compartment in patients with psoriatic arthritis (Ezeonyeji, et al. 2017). One explanation why differentiation of naïve T cells was not observed in the experiments might be missing co-stimulation. In addition to IL-1β, Th17 cells, for example, are known to require additional stimulation through IL-6 and TGFβ and interaction between the MHC class II and the cognate TCR for polarization (Liang, et al. 2006, Zheng, et al. 2007). Th22 cells, on the other hand, require IL-6 co-stimulation (Duhen, et al. 2009). Hence, it cannot be fully determined whether IL-1β activates preexisting Th cells or drives their differentiation from naïve T cells.

It was also shown that $CD4^+$ cells co-secrete IL-17 and IFNγ with IL-22, which is reversible through inhibition of the IL-1R-axis with anakinra (Voigt, et al. 2017). This may lead to the conclusion that a mixed Th1, Th17, and Th22 population produces IL-22 upon stimulation in an IL-1-dependent manner. Mechanistically, IL-1β could drive the differentiation of naïve T cells to Th1, Th17, and Th22 lineages, or IL-1β might be critical for activating or maintaining IL-22 from existing T helper cell subpopulations. In this set of experiments, IL-22 production was only seen after reactivation of memory T cells. So far, this has only been shown in the context of C. albicans infections, where $IL\text{-}22^+CD4^+$ cells displayed a phenotype of both central and effector memory T cells (Liu, et al. 2009). As memory T cells can be heterogeneous in terms of effector functions and tissue-homing capacities, further analysis of chemokine receptor co-expression would be necessary to determine whether central or effector memory T cells are activated after IL-1 induction.

Because cells were not sorted for CD3, human $CD4^+$ ILCs cannot be excluded as a potential source of IL-22. $CD4^+CD3^-$ ILCs have been described in literature to produce IL-22 upon stimulation (Bekiaris, et al. 2013). However, in this experimental model it was shown that $CD3^-$ cells did not produce significant amounts of IL-22 using the proposed pathway (Voigt, et al. 2017).

5.4 Mixed T helper cell populations are the main producers of IL-22 in primary human breast cancer tissue

In literature, Th1 and Th17 cells have been identified in breast cancer and linked to dismal clinical outcomes (Gu-Trantien, et al. 2013). In this FACS analysis of primary human breast cancer tissue, a significant amount of IL-22 was detected via flow cytometry and protein

lysates. Th1 cells were identified as the main producers of IL-22 (41 %), followed by Th22 and Th17 phenotypes (22 % and 9 %, respectively). In total, T helper cells made up almost ¾ of all IL-22$^+$ cells. The remaining IL-22-producing cell populations included CD3$^+$CD8$^+$ (12 %) and CD11b$^+$ (12 %). This corresponds well with the *in vitro* analysis, where IFNγ and IL-17 co-secretion with IL-22 was observed after stimulation with supernatants from cancer cell lines, most likely in an IL-1-dependent manner. It is also compatible with the findings generated in mice. The analysis would have further benefitted from comparison with healthy breast tissue. However, it was not possible to isolate sufficient number of cells from such tissue.

5.5 Targeting the IL-1-IL-22 axis for cancer treatment

The murine *in vivo* experiments link endogenous IL-1 activity to IL-22 production in two breast cancer models by showing downregulation of IL-22 positive cells in murine tissue after inhibition of endogenous IL-1 activity. This confirms the mechanism that was identified *in vitro*. Antagonizing IL-1 also led to significant retardation of tumor growth in both breast cancer models. While these findings are in line with prior literature, it has not yet been linked to the role of IL-22. IL-1 is detectable in human lung and breast cancer, as well as melanoma, head and neck, and colon cancers and its presence is usually associated with worse prognosis (Ye, et al. 2012). The experiments done here suggest a possible pathway: blockade of IL-1 reduces production of IL-22 in a specific T helper cell subset, thereby lessening its pro-tumorigenic effects leading to better tumor control. This indicates a potential role in cancer treatment in humans, either by blocking IL-1 or IL-22 directly.

IL-1 antagonisation is already commonly used in a variety of diseases and has been classified as safe with a small spectrum of adverse effects (Dinarello 2010). Anakinra is currently tested in clinical trials in patients with pancreatic or metastatic breast cancer as an addition to chemotherapy regimens because it reduces peritumoral inflammation and cachexia. However, administration of anakinra has shortcomings such as a short half-life that necessitates high-dose daily injections as well as the simultaneous inhibition of both IL-1α and IL-1β, two cytokines with different biology and distinct effects on immune responses in inflammation and tumorigenicity (Rider, et al. 2011). The experiments above suggest that in the human setting, IL-1β – activated through the NLRP3 inflammasome – plays a more prominent role in activation of the IL-1-IL-22 pathway. This finding is in line with results from the recent CANTOS trial, which evaluated the efficacy of the anti-IL-1β antibody canakinumab on reducing atherosclerosis. While not its primary endpoint, the study noted a significant reduction lung cancer incidence and mortality (Ridker, et al. 2017). The experiments of this thesis imply that blockade of IL-1β might have reduced IL-22 production in these patients, which could have slowed down cancer development and metastasis

formation. Further evaluations, preferably in respect to IL-22 secretion and T helper cell subsets in canakinumab treated patients, are necessary to expose the pathway underlying this interesting finding.

The experiments also show that the NLRP3 inflammasome is necessary for the activation of the IL-1-IL-22 axis. Thus, another therapeutic approach could be the inhibition of IL-1β processing through specific inflammasome inhibitors. Recently, IL-1β released via NLRP3 has been demonstrated to be pro-tumorigenic by promoting metastasis and lymphangiogenesis (Weichand, et al. 2017). NLRP3 inhibitors are currently tested for treatment of inflammatory diseases and could turn out to be promising cancer therapies once their efficacy in decreasing IL-1β production *in vivo* has been demonstrated (Coll, et al. 2015).

Finally, direct inhibition of IL-22-IL-22-R signaling without IL-1 targeting should be assessed in terms of anti-tumoral effects in humans. Anti-IL-22 antibodies have already demonstrated positive effects in inflammatory diseases such as psoriasis and atopic dermatitis, again showing limited side effects. Larger trials, preferably with a focus on cancer development and spreading, are necessary to see if data from the CANTOS trial can be replicated by direct IL-22 inhibition. IL-22-BP, the endogenous antagonist of IL-22, also appears to be an attractive choice for targeting IL-22. However, further verification of its biological properties is necessary, as some studies have shown that IL-22BP may lead to stabilization of IL-22 (Jiang, et al. 2011). Finally, direct inhibition of IL-22-Rα1 should be evaluated. Direct blockade of IL-22-Rα1 is interesting because its expression is primarily limited to epithelial cells and potentially also reduces the pro-tumoral effects of IL-20 and IL-24. IL-22 antagonists can therefore potentially spare patients from adverse effects, which often lead to dangerous states of immunodeficiency or severe autoimmune disorders (reviewed in: Lim and Savan 2014).

When comparing different treatment approaches, heterogeneity of and responses by tumor cells need to be carefully evaluated. For instance, it was recently found that IL-1 is an important inflammatory driver in pancreatic cancer, but IL-1 inhibition with anakinra was unable to inhibit tumor growth of cells with EMT phenotype due to continuous activation of IL-6 by EMT transcription factors (Siddiqui, et al. 2018).

5.6 Conclusion and perspectives

In summary, this thesis describes a previously unknown mechanism through which cancer cells induce IL-22 and characterizes the target cell as memory T cells of primarily Th22 subtype (see Figure 17). Through the release of IL-1α in mice or the induction of IL-1β via the NLRP3 inflammasome in humans, IL-22 production is upregulated in the tumor

microenvironment. In the murine system, IL-1α and to a lesser extent IL-23 release from (hypoxic) tumor cells activates memory T cells, which leads to IL-22 secretion from T cells that are primarily of Th22, but also Th1 and Th17 subtype. In the human setting, the NLRP3 inflammasome is activated in memory T cells, either directly through cancer cells or indirectly via myeloid cells. Subsequent IL-1β release acts on memory T cells to produce IL-22 by either reactivating pre-existing or inducing differentiation into a mixed Th cell population.

In this context, IL-22 can be seen as a double-edged sword: by increasing cell proliferation, migration and angiogenesis, it is an important cytokine for tissue regeneration and host defense, but its functions can be hijacked by cancer cells to enhance progression. Together with the results from *in vivo* experiments, the findings support the idea of developing therapeutic approaches that intervene in the IL-1-IL-22 axis in cancer and provide a rationale for targeting various points along this axis. What remains elusive, however, is the exact mechanism which links IL-22 to worse prognosis in cancer. Given its role as a complex homeostatic cytokine, it is likely that pathways differ across cancer entities and stages of disease, including induction of stemness, EMT, angiogenesis and proliferation. Uncovering these mechanisms would help to understand the links between cancer and the immune system and offer further treatment options.

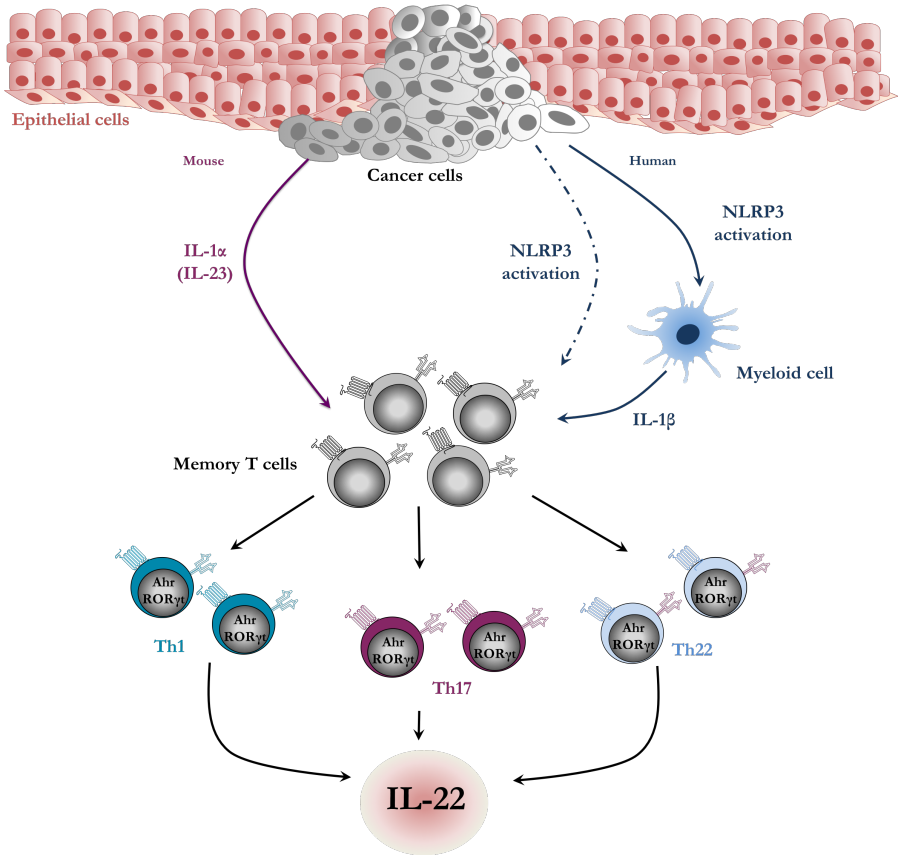

Figure 17: Schematic model of the IL-1-IL-22 axis (modified from: Voigt, et al. 2017).

6 Summary

Interleukin-22 (IL-22) is a unique cytokine which is secreted exclusively by cells of hematopoietic origin, but its direct effects are restricted to interleukin-22-receptor (IL-22-R) expressing epithelial cells. By facilitating cross-talk between hematopoietic and non-hematopoietic cells, IL-22 plays an important role in tissue regeneration, repair and epithelial defense. Dysfunction of the IL-22-IL-22-R axis has been linked to several autoimmune diseases such as psoriasis or inflammatory bowel diseases. In cancer, IL-22 has been suggested to promote tumorigenesis, induction of stemness and metastasis formation. However, neither the exact mechanism of induction nor the exact cellular sources have been characterized for lung and breast cancers.

In previous analyses our group has shown that cancer cells can directly induce IL-22 production from murine and human immune cells. We could demonstrate that this process is driven by IL-1α in murine and IL-1β in human immune cells. We could also show that these cytokines primarily acted on $CD3^+$ T cells and mediated IL-22 production via the transcription factors AhR and RORγt. To further elucidate this pathway, we wanted to fully characterize the T cell subpopulation and confirm the mechanism by which cancer cells induce IL-22 secretion from these target cells. We also aimed to demonstrate the potential therapeutic benefits from intervening in the IL-1-IL-22 axis in cancer therapy.

This thesis identified $CD4^+CD44^+$ memory T cells as the major IL-22-producing subset. The mechanism was shown to be dependent upon and replicable by IL-1 activation and conserved across different tumor entities. In murine cancer models, IL-1α was shown to be responsible for IL-22 production from memory T cells, whereas human cancer cell lines appear to induce IL-22 through IL-1β via the activation of the NLRP3 inflammasome. It was concluded that IL-1 secreted by tumor cells promotes IL-22 production by either activating a pre-existing Th cell population or inducing differentiation of a mixed Th cell population, with a majority of the cells exhibiting a Th22 phenotype. Th22 cells were also shown to be the main source of IL-22 in primary breast cancer tissue using FACS analysis.

In line with these findings, IL-22 content and tumor growth were reduced when IL-1α and IL-1β were neutralized in two different murine breast cancer models. This points towards the potential use of IL-1- and IL-22-antagonists for cancer therapy.

7 Zusammenfassung

Interleukin-22 (IL-22) ist ein besonderes Zytokin, dessen Effekte auf Interleukin-22-Rezeptor (IL-22-R)-tragende Zellen epithelialen Ursprungs begrenzt ist. Es ermöglicht die Kommunikation zwischen hämatopoietischen und nicht-hämatopoietischen Zellen und spielt eine wichtige Rolle bei der Regeneration und Heilung von Gewebe sowie der epithelialen Abwehr. Störungen in der IL-22-IL-22-R-Achse sind bei einigen Autoimmunerkrankungen wie Psoriasis oder chronisch-entzündlichen Darmerkrankungen nachweisbar. Im Zusammenhang mit Krebserkrankungen wurde die tumorfördernde Rolle durch verstärkte Tumorgenese, Aktivierung von Stammzellen und Förderung der Metastasierung beschrieben. Bisher wurde jedoch weder der genaue Induktionsmechanismus von IL-22 noch die produzierende Zellpopulation bei Brust- und Lungenkrebs genau charakterisiert.

In bisherigen Untersuchungen unserer Arbeitsgruppe konnte gezeigt werden, dass Tumorzellen die IL-22-Produktion in murinen und humanen Immunzellen induzieren können. Wir konnten zudem zeigen, dass dies durch IL-1α in murinen und IL-1β in humanen Immunzellen geschieht. Des Weiteren konnte in Versuchen gezeigt werden, dass diese Zytokine vor allem auf $CD3^+$ T Zellen wirken und dass die IL-22-Produktion durch die Transkriptionsfaktoren AhR und RORγt reguliert wird. Auf dieser Basis sollte im Folgenden die IL-22 produzierende T-Zell-Subpopulation genau charakterisiert und der Mechanismus im humanen Modell näher untersucht werden. Darüber hinaus sollte durch Tierversuche der therapeutische Nutzen eines Eingriffs in die IL-1-IL-22-Achse aufgezeigt werden.

Die vorliegende Arbeit identifizierte $CD4^+CD44^+$ T-Gedächtniszellen als Hauptproduzenten von IL-22. Der Induktionsmechanismus war in allen getesteten Brust- und Lungenkrebsmodellen abhängig von IL-1. Es konnte gezeigt werden, dass im murinen Modell IL-1α die IL-22-Produktion durch Gedächtnis T-Zellen induziert, wohingegen im humanen Modell IL-1β über die Aktivierung des NLRP3-Inflammasoms verantwortlich ist. Daraus konnte geschlossen werden, dass durch Tumorzellen sezerniertes IL-1 die IL-22-Produktion durch Reaktivierung bereits vorhandener oder Induktion der Differenzierung einer gemischten T-Helferzell-Population mit einem überwiegend Th22-Phänotyp fördert. Th22-Zellen konnten zudem als Hauptproduzenten im primären Brustkrebsgewebe mittels FACS Analyse identifiziert werden.

Darauf aufbauend konnte gezeigt werden, dass nicht nur die Menge von IL-22, sondern auch das Tumorwachstum durch Hemmung von IL-1 im Tiermodell reduziert werden konnte. Dies deutet darauf hin, dass die Inhibition von IL-1 oder IL-22 eine Strategie in der Tumortherapie darstellen könnte.

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9 Appendices

9.1 Abbreviations

Ab	antibody
AhR	aryl hydrocarbon receptor
anti-IL-1-R	interleukin-1-receptor neutralizing antibody
APC	allophycocyanin
ATCC	American Type Culture Collection
BP	binding protein
BSA	bovine serum albumin
CCL	chemokine ligand
CD	cluster of differentiation
COPD	chronic obstructive pulmonary disease
Cy	cyanin
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
IBD	inflammatory bowel disease
IFN	interferon
IGF-1	insulin-like growth factor 1
IL	interleukin
IL-22-Rα1	interleukin-22-receptor-1 α chain
ILC	innate lymphoid cell

iNK	immature NK cell
iNKT	invariant NKT cell
i. p.	intraperitoneally
JAK	Janus kinase
LANA	latency-associated nuclear antigen
LTi cell	lymphoid tissue inducer cell
MAb	monoclonal Antibody
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
N. d.	not detectable
n. s.	not significant
NEAA	non-essential amino acids
NK cell	natural killer cell
NKT cell	natural killer T cell
NLRP3	Nod-like receptor protein 3
NSCLC	non-small cell lung cancer
NTP	N-terminal peptide
P3K/Akt	phosphoinositide-3 kinase/Akt
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll protein complex
PMA	phorbol-12 myristate-13 acetate
Rec.	recombinant
RNA	ribonucleic acid
ROR	retinoic acid-related orphan receptor
RPMI	Roswell Park Memorial Institute

SCLC	small cell lung cancer
SEM	standard error of mean
SNP	single nucleotide polymorphism
STAT	signal transducer and activator of transcription
STR	short tandem repeats
Tc	cytotoxic T cell
TCR	T cell receptor
TEMED	tetramethylethylenediamine
TGF	tumor growth factor
Th	T helper cell
TIL	tumor infiltrated leukocyte
TLR	Toll-like receptor
TNF	tumor necrosis factor
TYK	tyrosine kinase
VEGF	vascular endothelial growth factor
VLE	very low endotoxin
WT	wild type

9.2 Publications

9.2.1 Original publications

Voigt C*, **May P***, Gottschlich A*, Markota A, Wenk D, Gerlach I, Voigt S, Stathopoulos GT, Arendt K, Heise C, Rataj F, Janssen KP, Königshoff M, Winter H, Himsl I, Thasler WE, Schnurr M, Rothenfußer S, Endres S, Kobold S (*contributed equally).
Cancer cells induce interleukin-22 production form memory CD4+ T cells via interleukin-1 to promote tumor growth.
Proc Natl Acad Sci U S A 2017; 114(49):12994-12999.

9.2.2 Abstracts and Posters

Voigt C, **May P**, Gottschlich A, Markota A, … Endres S, Kobold S
Cancer cells regulate Interleukin-22 production to promote tumor growth
Immunotherapy of Cancer (ITOC)-5 Meeting 2018, Berlin, Germany

Ochs C, Gottschlich A, **May P**, Wenk D, Endres S, Kobold S
Impact of Interleukin-22 on two murine models of lung and breast cancer
Association for Cancer Immunotherapy (CIMT) Meeting 2016, Mainz, Germany

Ochs C, **May P,** Wenk D, Endres S, Kobold S
Impact of Interleukin-22 on two murine models of lung and breast cancer
4th European Congress of Immunology 2015, Vienna, Austria

Ochs C, **May P**, Wenk D, Endres S, Kobold S
Interleukin-22 does not impact methylcholantren-A mediated tumorigenesis
Association for Cancer Immunotherapy (CIMT) Meeting 2015, Mainz, Germany

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Eidesstattliche Versicherung

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Ich erkläre hiermit an Eides statt,
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