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Analysis of T cells as potential sources of interleukin-22 in colorectal cancer

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1. Table of Content

2. Summary

Interleukin-22 (IL-22) is a unique cytokine produced by immune cells and acting exclusively on IL-22 receptor-1 (IL-22-R1) positive epithelial cells. IL-22 plays a central role in epithelial regeneration and contributes to pathogen clearance. In contrast, the proliferation enhancing effects of IL-22 may be used by cancer cells to promote and sustain their growth. This has been shown in genetically modified mouse strains with spontaneous development of colorectal cancer.

The cellular sources of IL-22 in tumor tissue are not yet fully identified.

In a pilot study with tumor tissue of patients with colorectal cancer, we could identify $CD3^+CD4^+$ T cells that produce IL-22. This confirms findings of other groups published in recent years. Peripheral T cells from healthy donors only contained low frequencies of IL-22 producing $CD3^+CD4^+$ cells. Upon stimulation, IL-22 induced STAT3 phosphorylation and proliferation of human colon cancer cell lines. In one cell line, we could also show the induction of the cytokines IL-10 and VEGF. IL-22 suppressed IFN-γ-induced synthesis of the chemokine CCL22 in HT29 cells while enhancing TNF-α-induced expression of CCL22 in the same cell line. This indicates differential effects of IL-22 on IFN-γ- and TNF-α-signaling. IFN-γ as well as TNF-α led to IL-22-R1 upregulation in human colon cancer cell lines.

In summary, we find evidence that in tumor tissue $CD3^+$ $CD4^+$ T cells produce IL-22. IL-22 acts directly on cells of colorectal cancer cell lines. It enhances their proliferation. IL-22 presumable serves as a mediator in the genesis of colorectal cancer and a potential therapeutic target.

Analysis of T cells as potential sources of interleukin-22 in colorectal cancer

Zusammenfassung

Interleukin-22 (IL-22) ist ein einzigartiges Zytokin welches von Immunzellen produziert wird und exklusiv auf IL-22-Rezeptor (IL-22-R1) positive epitheliale Zellen wirkt. IL-22 spielt eine zentrale Rolle in der epithelialen Regeneration und trägt zur Pathogenbeseitigung bei. Im Gegensatz dazu könnten die Effekte von IL-22 von Krebszellen benutzt werden um ihr Wachstum zu fördern. Dies wurde in genetisch modifizieren Mauslinien mit spontaner Entwicklung von Kolonkarzinomen nachgewiesen.

Die zellulären Quellen von IL-22 im Tumorgewebe sind noch nicht vollständig identifiziert.

In einer Pilotstudie an Tumorgewebe von Patienten mit kolorektalem Karzinom konnten wir $CD3^+CD4^+$ T Zellen identifizieren, die IL-22 produzieren. Dies bestätigt in den letzten Jahren publizierte Befunde anderer Gruppen. Periphere T Zellen von gesunden Spendern enthielten nur geringe Frequenzen von IL-22 produzierenden $CD3^+CD4^+$ T Zellen. Nach Stimulation induziert IL-22 in Zellen von humanen kolorektalen Karzinomzellllinen die Phosphorylierung von STAT3 und Proliferation. In einer Zelllinie konnten wir auch die Induktion der Zytokine IL-10 und VEGF nachweisen. IL-22 unterdrückt IFN-γ-induzierte CCL22 Proteinexpression in HT29 Zellen wohingegen es die TNF-α-induzierte Expression von CCL22 in derselben Zelllinie verstärkt. Dies spricht für differenzielle Effekte von IL-22 auf die IFN-γ- und TNF-α-Signalwege. Sowohl IFN-γ als auch TNF-α führten zur Hochregulation von IL-22-R1 in Zellen von humanen kolorektalen Karzinomzellllinen.

Zusammenfassend finden wir Hinweise, dass im Tumorgewebe IL-22 von $CD3^+$ $CD4^+$ T Zellen produziert wird. IL-22 wirkt direkt auf kolorektale Karzinomzellen. Es steigert deren Proliferation. IL-22 bildet vermutlich einen Mediator in der Genese von kolorektalen Karzinomen und ein potenzielles therapeutisches Target.

3. Introduction

3.1. Physiology of interleukin-22

Interleukin-22 (IL-22) is a member of the interleukin-10 (IL-10) family of cytokines which also comprises IL-10, IL-19, IL-20, IL-24, IL-26, IL-28α, IL-28β, and IL-29 (Wolk et al, 2010). IL-22 was first described in 2000 as a secreted α-helical protein in IL-9-stimulated murine BW5147 T lymphoma cells (Dumoutier et al, 2000).

3.1.1. Cellular sources of IL-22

IL-22 is expressed by a number of immune cells of the innate and adaptive immune system. The cellular sources of IL-22 are immune cells. In general T cells and innate lymphoid cells are considered to be the major cellular sources of IL-22 (Sabat et al, 2014). $CD4^+$ T cells, including Th-1 cells, Th-17 cells and Th-22 cells produce IL-22 (Duhen et al, 2009; Witte et al, 2010; Wolk et al, 2002).

Th-17 cells produce IL-22 as an effector cytokine together with IL-17 (Chung et al, 2006; Liang et al, 2006; Ouyang et al, 2008). The retinoic-acid-related orphan nuclear receptor RORγt is the master regulator of Th-17 cells (Ivanov et al, 2006). Among the factors that drive Th-17 differentiation and IL-17 expression, IL-6 and IL-23 induce IL-22 expression while transforming growth factor-β (TGF-β) antagonizes IL-22 induction (Rutz et al, 2011).

Th-22 cells are defined as expressing the chemokine receptors CCR4, CCR6, CCR10 and producing IL-22 but not any other known T-helper-prototypical cytokine, such as IL-17 or IFN-γ. Th-22 cells have a high expression of the aryl hydrocarbon receptor (AHR) but a low expression of RORγt and T-bet (Duhen et al, 2009; Trifari et al, 2009). *In vitro*, Th-22 cells may develop in interaction with plasmacytoid dendritic cells (pDCs) with IL-6 und TNF-α as driving factors (Duhen et al, 2009). Activation of the aryl hydrocarbon receptor (AHR) is important for IL-22 production in $CD4^+$ memory T cells suggesting a role for AHR-ligands in Th-22 polarization (Stockinger et al, 2009; Trifari et al, 2009; Veldhoen et al, 2009). In addition, it has been reported that IL-23 stimulation is a prerequisite for the IL-22 production of $CD4^+$ T cells (Zheng et al, 2007). IL-1β has been described to further promote Th-22 differentiation (Ivanova & Orekhov, 2015).

In addition to $CD4^+$ T cells, $CD8^+$ T cells (Nograles et al, 2009) are also capable of producing IL-22 (Liu et al, 2011b; Ortega et al, 2009). Innate immune cells also belong to the IL-22 producers: IL-22 is expressed by certain natural killer (NK) cell subsets, natural killer T (NKT) cells (Goto et al, 2009), γδ T cells (Martin et al, 2009), LTi (lymphoid tissue inducer) cells (Sonnenberg et al, 2011) and LTi-like (lymphoid tissue-like inducer) cells (Takatori et al, 2009).

Although IL-22 is produced by the above described immune cells it does not act on those. IL-22 is a unique cytokine, produced by immune cells, which does not act on cells of hematopoietic origin but on epithelial cells via the Janus kinase (JAK) - signal transducer and activators of transcription (STAT) pathway (Wolk et al, 2010).

3.1.2. IL-22 receptor-1 and its signaling pathway

IL-22 acts via a heterodimeric transmembrane receptor, composed of the IL-22-receptor-1 (IL-22-R1) and the IL-10-receptor-2 (IL-10-R2) (Wu et al, 2008). The IL-22-R1 chain holds the docking site for STAT signal molecules. The IL-10-R2 component is ubiquitously expressed due to its function as part of several cytokine receptors. Cellular responsiveness to IL-22 is thus determined by the expression of IL-22-R1. IL-22-R1 is not expressed on cells of hematopoietic origin but on cells of epithelial and endothelial origin (Wolk et al, 2002; Wolk et al, 2004).

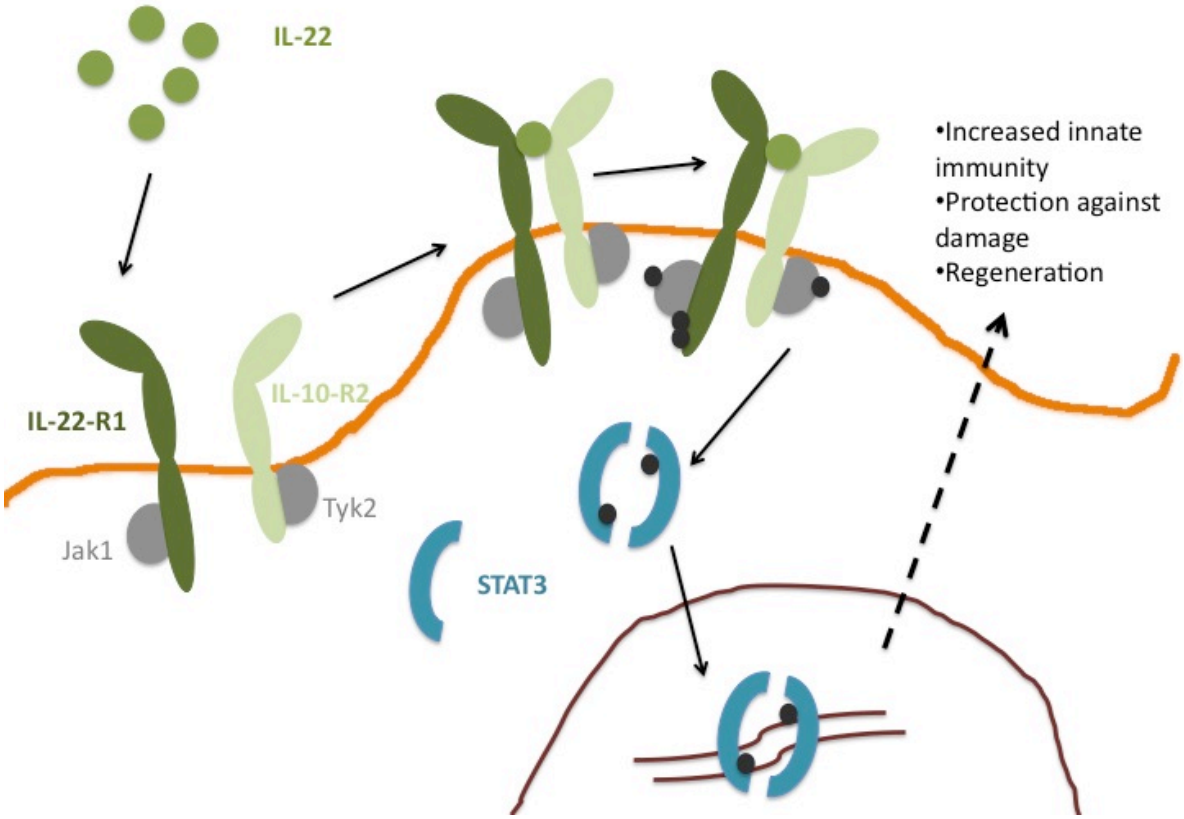


Figure 1: IL-22 signaling cascade. IL-22 signals via the activation of the Jak - STAT pathway. Receptor-associated tyrosine kinases Jak1 and Tyk2 are phosphorylated leading to phosphorylation of IL-22-R1 pre-associated STAT3. STAT3 dimerizes and translocates to the nucleus leading to transcription of target genes (modified from Wolk et al, 2010).

IL-22 activates the JAK-STAT pathway. First phosphorylation of the receptor associated tyrosine kinases JAK1 and TYK2 takes place which in turn phosphorylate specific receptor tyrosine residues serving as docking sites for sarcoma homology 2 (SH2) domains of STAT3. Subsequently, receptor-docked STAT3, is phosphorylated itself, dimerizes and translocates into the nucleus. There, pSTAT3 can bind responsive elements and regulate gene expression (Lejeune et al, 2002).

3.1.3. Physiological functions of IL-22

Unlike most interleukins, IL-22 does not directly act on immune cells (Wolk et al, 2004), but regulates functions of cells of the body barriers (Sabat et al, 2014) where IL-22-R1 is expressed. The expression pattern identifies the main target cells: the digestive tract

(pancreas, small intestine, liver, colon), the respiratory system (lung, trachea), kidney and skin (Wolk et al, 2004).

The physiological function of IL-22 is to protect tissues from damage and to enhance their regeneration. In many of the above-mentioned cells IL-22 induces anti-microbial proteins and chemokines. Induction of an epithelial innate immune response directed against extracellular pathogens is one example of the protective function of IL-22.

In the skin, IL-22 mainly targets keratinocytes (Wolk et al, 2009; Wolk et al, 2004). There, IL-22 signaling has been shown to control innate immunity by upregulating the expression of β-defensins 2 and 3 as well as the anti-microbial heterodimer S100A8/9 (Kolls et al, 2008; Wolk et al, 2004; Wolk et al, 2006).

In bronchial epithelial cells IL-22 upregulates G-CSF and antimicrobial proteins such as BD2, S100A7 and A100A12 and the chemokines CXCL1 and CXCL5. IL-22 promotes epithelial regeneration and reduction of *K. pneumoniae* growth (Aujla et al, 2008).

IL-22 also acts on the gastrointestinal tract. In the pancreas, the organ with the highest IL-22-R1 expression, it has been shown that IL-22 induced osteopontin and pancreatitis-associated protein (PAP) 1/RegIIIα (Aggarwal et al, 2001). In human intestinal epithelial cells IL-22 increased the expression of BD2 and IL-8 (Brand et al, 2006). The impact of IL-22 in the defense against infections with bacteria of the intestinal tract has been shown in a number of studies: IL-22 induces the expression of anti-bacterial peptides namely: β-defensins, RegIIIβ, and RegIIIγ, S100A7, S100A8 and S100A9 (Liang et al, 2006; Wolk et al, 2004; Wolk et al, 2006). The release of anti-bacterial peptides can be increased by the combination of IL-22 and IL-17 (Liang et al, 2006).

3.1.4. Pathophysiological functions of IL-22

IL-22 is upregulated in a number of chronic inflammatory and autoimmune diseases generating the hypothesis that IL-22 may be disease promoting in this context. The production of IL-22 by T cells may indicate that IL-22 is involved in the pathogenesis of T

cell-mediated diseases such as psoriasis, rheumatoid arthritis (RA) or inflammatory bowel disease (IBD).

The impact on keratinocytes is important in psoriasis and atopic skin pathogenesis where IL-22 has been found to be a key mediator. In atopic skin IL-22 has been found to be mainly produced by Th-22 cells (Nograles et al, 2009). In a psoriasis-like disease model it has been shown that upon treatment of mice with IL-22 neutralizing antibodies the expression of Th-17 cytokines was reduced as well as the recruitment of inflammatory infiltrates. In addition, this treatment prevented development of disease (Ma et al, 2008). In an imiquimod-induced mouse model of skin inflammation, skin lesions that are induced in wild-type mice were almost absent in IL-22-deficient mice or in mice treated with an IL-22 blocking antibody (Van Belle et al, 2012).

IL-22 is found in large quantities in the inflamed regions of the colonic mucosa in ulcerative colitis and Crohn's disease, which are predisposing diseases for colorectal cancer (Andoh et al, 2005). In IBD $CD4^+$ IL-22 expressing T cells have been shown to be present in the inflamed regions of the gut: In active Crohn's disease and ulcerative colitis IL-22 is abundantly found in the submucosa or in the lamina propria, respectively (Andoh et al, 2005; Brand et al, 2006). Furthermore, systemic IL-22 levels are increased in patients with Crohn's disease (Wolk et al, 2007). One study showed that intestinal tissue from patients with IBD expressed significantly less AHR compared to controls. Conversely, there are experimental data that IL-22 can protect mice from IBD. This protection was mediated by CD4 T cells and IL-22 expressing NK cells (Zenewicz et al, 2008). In a mouse model it has been shown that AHR induced IL-22 and inhibited inflammation in the gastrointestinal tract (Monteleone et al, 2011).

Th-22 cells are increased in the peripheral blood of patients suffering from rheumatoid arthritis (RA) and levels of IL-22 are increased in their plasma, which correlates with disease activity (da Rocha et al, 2012; Leipe et al, 2011; Zhang et al, 2011b; Zhang et al, 2012a). Fibroblasts from patients with RA that show high concentrations of IL-22 increased proliferation and the release of CCL2, which attracts monocytes (Ikeuchi et al, 2005). Further, IL-22 deficient mice showed a less severe form of arthritis as compared to wild-type mice, indicating the disease-promoting role of IL-22 (Geboes et al, 2009).

3.1.5. Influence of cyokines on IL-22 signaling

IL-22 is generally secreted with other cytokines. IL-22 effects as well as IL-22 expression are influenced by other cytokines like IFN-α, IFN-γ, TNF-α and IL-17 (Eyerich et al, 2009; Guilloteau et al, 2010; Liang et al, 2006; Pennino et al, 2013).

IL-22 can decrease the responsiveness of epithelial cells to T cell–mediated cytotoxicity by counteracting the IFN-γ –induced expression of MHC-I (Pennino et al, 2013). IFN-α, IFN-γ and TNF-α induce increased responsiveness of keratinocytes towards IL-22 by upregulation of IL-22-R1 and IL-10-R2 (Kunz et al, 2006; Tohyama et al, 2012).

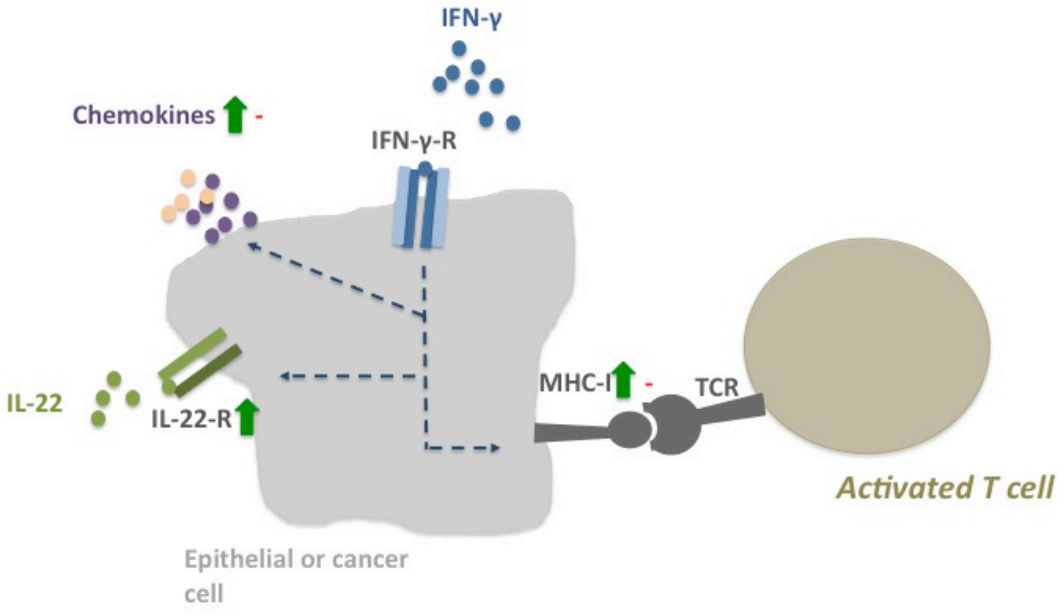


Figure 2: Crosstalk of IL-22 and IFN-γ in the context of effects on epithelial cells. IFN-γ induces chemokines in epithelial and cancer cells, upregulates MHC-I and IL-22-R1 (Kunz et al, 2006; Pennino et al, 2013). IL-22 antagonizes chemokine upregulation and upregulation of MHC-I by IFN-γ (Pennino et al, 2013). Green arrows indicate effects of IFN- γ, red - indicate antagonistic effects of IL-22.

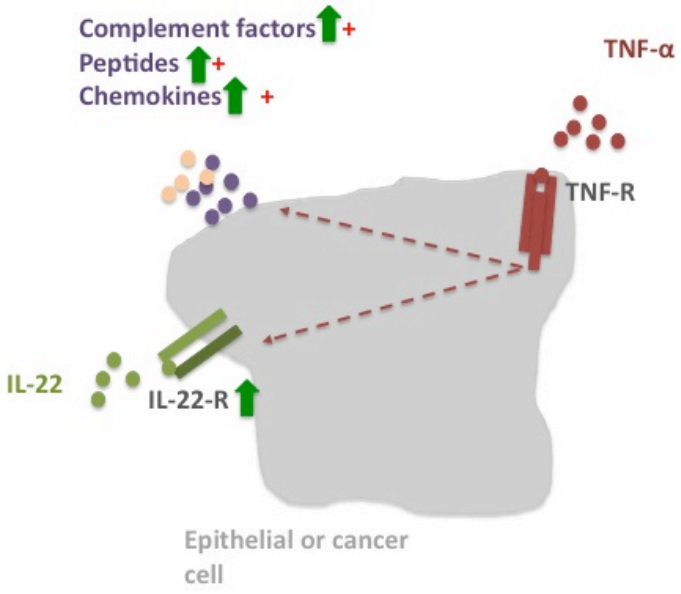


Figure 3: Crosstalk of IL-22 and TNF-α in the context of effects on epithelial cells. Stimulation leads to the upregulation of IL-22-R1(Wolk et al, 2009; Wolk et al, 2004). TNF-α upregulates complement factors C1r and C1s, peptides S100A7 and HBD-2 and chemokines CXCL-9, CXCL-10 and CXCL-11 (Eyerich et al, 2011). These effects are further promoted by IL-22. Green arrows indicate effects of TNF-a and red + indicate synergistic effects of IL-22.

IL-22 and TNF-α are both released by Th-22 cells (Eyerich et al, 2009). IL-22 enhances TNF-α-induced secretion of complement factors C1r and C1s, peptides S100A7 and HBD-2 and chemokines CXCL-9, CXCL-10 and CXCL-11 (Eyerich et al, 2011). Expression of IL-22-R1 in both keratinocytes and dermal fibroblasts is induced by TNF-α (Wolk et al, 2009; Wolk et al, 2004). In keratinocytes, IL-22 may also synergize with IL-17 and IFN-γ in inducing keratin 17 expression which indicates that IL-22 is involved in a positive feedback loop where keratin 17 induces the proliferation and activation of T cells (Zhang et al, 2012b).

One study showed that the function of IL-22 in a bleomycin-induced lung inflammation model differed in the presence or absence of IL-17. In the presence of IL-17, IL-22 led to more inflammation whereas in the absence of IL-17, IL-22 exerted tissue-protective functions (Sonnenberg et al, 2010).

3.1.6. Role of IL-22 in carcinogenesis

The proliferative, tissue regenerating and pro-inflammatory properties of IL-22 raised the question of a potential involvement in cancer of this particular cytokine where its effects may be tumor-promoting.

Table 1: Overview of literature connected to the role of IL-22 in cancer

Cancer type	Key messages of publication
Lymphoma	• Anaplastic large cell lymphoma shows aberrant expression of IL-22-R1 (Bard et al, 2008) as well as mantle cell lymphoma (Gelebart et al, 2011) • IL-22 stimulation of mantle cell lymphoma cell lines leads to increase of STAT3 activation and cell proliferation (Gelebart et al, 2011) • Patients suffering from cutaneous T cell lymphoma show increased levels of IL-22 in lesional skin, elevate IL-22 serum levels correlate with severity of disease (Miyagaki et al, 2011)
Gastric cancer	• Increased circulating Th-22 and Th17 cells of patients are associated with tumor progression (Liu et al, 2012) • Increased intratumoral $CD4^+IL\text{-}22^+$ T cells and Th-22 cells are linked to tumor progression and lower patient survival (Zhuang et al, 2012) • IL-22 produced by cancer-associated fibroblasts promotes cancer cell invasion (Fukui et al, 2014)
Pancreatic cancer	• IL-22 stimulated production of vascular endothelial growth factor (VEGF) and anti-apoptotic proteins (Curd et al, 2012). • Frequencies of $CD4^+IL\text{-}22^+$ cells and Th-22 cells were significantly increased in pancreatic cancer tissue compared to peripheral blood and healthy controls, intratumoral IL-22 levels and frequencies of Th-22 and $CD4^+$ IL-22-producing cells has been linked to progression of disease (Xu et al, 2014) • High expression of IL-22 and IL-22-R is linked to poor patient prognosis (Wen et al, 2014)

Hepatocellular carcinoma	• IL-22 in the tumor microenvironment, leads to increased growth, inhibition of apoptosis and promotion of metastases (Jiang et al, 2011) • Th-22 are associated with heptatocelluar carcinoma development (Qin et al, 2014) • IL-22 serum levels are a negative prognostic factor (Waidmann et al, 2014)
Renal cancer	• IL-22 dose-dependently suppresses renal cell carcinoma cells in vitro and induced growth inhibition of renal carcinoma cell-bearing mouse xenografts (Zhang et al, 2011a)
Kaposi sarcoma	• IL-22-R1 transcript levels are downregulated in Kaposi's Sarcoma (Su et al, 2011)
Lung cancer	• The number of IL-22 producing Th-22 cells is increased in malignant pleural effusion (Ye et al, 2012). • Autocrine production of IL-22 may contribute to human lung cancer cell survival and resistance to chemotherapy through the upregulation of anti-apoptotic proteins (Zhang et al, 2008) • IL-22 was frequently expressed in human lung cancer tissue (Kobold et al, 2013)
Skin cancer	• Proliferation and migration of human skin basal cell carcinoma and squamous-cell carcinoma cell were increased by IL-22, IL-22 induced STAT3 pathway and the antiapoptotic AKT protein, IL-22 enhanced tumor growth in nude mice injected with basal cell carcinoma cell lines (Nardinocchi et al, 2015)

3.1.7. Role of IL-22 in colorectal cancer development

Chronic mucosal inflammation predisposes patients to colorectal cancer development (Grivennikov et al, 2010). Patients suffering from ulcerative colitis and Crohn's disease have a higher risk of developing colorectal cancer (Bernstein et al, 2001; Schottenfeld & Beebe-Dimmer, 2006). However, it is still poorly understood what type of immune cells and cytokines trigger the development from chronic inflammation to colorectal cancer. IL-22 is considered to play a crucial role in this process (Kirchberger et al, 2013).

Analysis of T cells as potential sources of interleukin-22 in colorectal cancer

Experimental models of inflammation-associated colorectal cancer development suggest that inflammatory cell-derived cytokines (including IL-22) directly or indirectly stimulate the growth of cancer cells (Grivennikov et al, 2009; Huber et al, 2012; Hyun et al, 2012; Kirchberger et al, 2013). IL-22 seems to drive the development of colorectal cancer via the activation of STAT3 (Lim & Savan, 2014). Forced expression of IL-22 using retroviral transduction of Colon-26 carcinoma cell lines had no impact on tumor growth or metastasis but prolonged survival of inoculated mice (Nagakawa et al, 2004). Another study found an association of colorectal cancer and the human *IL22 rs1179251* G allele with a 52 % increase in risk of developing colorectal cancer by individuals with at least one of these alleles (Thompson et al, 2010). IL-22 enhances iNOS expression and contributes to tumorigenesis in colorectal cancer (Takahashi et al, 1997; Ziesche et al, 2007). Oxidative activity mediated by IL-22 is required for activation of STAT3. An oxidative inflammatory microenvironment containing IL-22 increases the risk of tumor development (Bansal et al, 2013).

Higher IL-22 serum levels are found in chemotherapy-resistant patients in contrast to chemotherapy-sensitive patients and IL-22 treatment of colorectal cancer cell lines enhances chemotherapy-resistance of these cancer cells (Wu et al, 2013).

As a further line of evidence for the importance of IL-22 in colorectal cancer development, the lack of the natural antagonist to IL-22: IL-22-binding protein (IL-22BP) leads to enhanced colitis-associated colon carcinogenesis (Huber et al, 2012). In this setting, IL-22 secretion is driven by infiltrating leukocytes, as peripheral leukocytes are weaker producers of IL-22. These IL-22 producing tumor-infiltrating lymphocytes enhance tumor growth and metastasis in a xenograft mouse model via STAT3 activation (Jiang et al, 2013). In colorectal cancer, $CD4^+$ T cells seem to be the main source of IL-22. IL-22 promoted activation of STAT3 induces the methyltransferase DOT1L. The DOT1l complex in turn induces the core stem cell genes Sox2, Nanog and Pou5F1, leading to the conclusion that IL-22 promotes colorectal cancer stemness (Kryczek et al, 2014).

Th-22 accumulation is linked to colorectal cancer development. IL-22 expression is significantly higher in tumor tissue than in peritumoral tissue (Huang et al, 2015).

3.2. Objectives

Our group could previously show increased proliferation and STAT3 phosphorylation in human lung cancer cell lines upon IL-22 stimulation (Kobold et al, 2013). This study seeks to gain a better understanding of the effects of IL-22 and of IL-22 producing T cells on colorectal cancer cells.

Tumor-infiltrating lymphocytes are important in colorectal cancer biology. Thereby, T cells producing tumor promoting IL-22 are significant players in this population. In addition, the exact impact of IL-22 at the cellular and sub cellular level in colorectal cancer is incompletely understood.

To gain further understanding of IL-22 in colorectal cancer, IL-22-producing T cells will be analyzed:

- Proportions of IL-22^{+} among Th-1, Th-17 and Th-22 cells in healthy human PBMC
- Proportions of IL-22^{+} among Th-1, Th-17, Th-22 and CD8^{+}IL-22^{+} T cells in tissue taken from patients with cancer of the colon or of the rectum

Additionally, the effect of IL-22 will be studied on human colorectal cancer cell lines:

- Ability to phosphorylate STAT3 in colorectal cancer cells proliferation upon IL-22 stimulation
- Impact of IL-22 on cytokine release from colorectal cancer cells
- Effect of IL-22 on IFN-γ as well as on TNF-α

4. Material and Methods

4.1. Materials

4.1.1. Chemicals and reagents

Bovine serum albumine (BSA)	Sigma Aldrich, Steinheim, Germany
Brefeldin A	Sigma Aldrich, Steinheim, Germany
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, Steinheim, Germany
Dulbecco's PBS (1x)	PAA, Pasching, Austria
Ethylenediamine tetraacetic acid (EDTA)	Sigma Aldrich, Steinheim, Germany
FACSFlow, FACSSafe	Becton Dickinson, San Jose, USA
Fixable Viability Dye eFluor® 780	eBioscience
Ionomycin	Sigma Aldrich, Steinheim, Germany
Lysis buffer	Cell Signaling, USA
Isopropanol (70 Vol %)	Apotheke Innenstadt, LMU Munich
Phorbol 12-myristate 13-acetate (PMA)	Sigma Aldrich, Steinheim, Germany
Propidium iodide	Sigma Aldrich, Steinheim, Germany
Protease inhibitor cocktail	Sigma-Aldrich, Steinheim, Germany
Rotiphorese® NF-Acrylamid/Bis-Lösung 30 % (29:1)	Roth, Karlsruhe, Germany
Trypan blue	Sigma Aldrich, Steinheim, Germany
Tween® 20	Roth, Karlsruhe, Germany

4.1.2. Cell culture reagents and media

Dulbecco's modified Eagle´s medium (DMEM)	PAA, Pasching, Austria
Fetal calf serum (FCS)	GibcoBRL (invitrogen), Karlsruhe, Germany
L-glutamine 200 mM	PAA, Pasching, Austria
McCoy's 5A (modified)	Invitrogen, Carlsbad, USA
NEM-NEAA (non-essential amino acids)	GibcoBRL (invitrogen), Karlsruhe, Germany

Opti-MEM® I Reduced Serum Medium	GibcoBRL (invitrogen), Karlsruhe, Germany
1 x PBS	PAA, Pasching, Austria
Phosphate-buffered saline (PBS)	PAA, Pasching, Austria
Penicillin plus streptomycin (100 x)	PAA, Pasching, Austria
Roswell Park Memorial Institute (RPMI) 1640 medium	PAA, Pasching, Austria
Sodium pyruvate	PAA, Pasching, Austria

Disposable plastic equipment for cell culture experiments were purchased from Becton Dickinson (Heidelberg, Germany), Eppendorf (Hamburg, Germany), Falcon (Heidelberg, Germany), Greiner (Frickenhausen, Germany) or Sarstedt (Numbrecht, Germany).

4.1.3. Western blot buffers

Table 2: Western blot buffers

Separating gel buffer (4-fold) pH 8.5 600 ml H_2O 182 g tris hydrochloride HCl to pH 8.8 4 g SDS	Stacking gel buffer (4-fold) pH 6.8 300 ml H_2O 30.25 g tris hydrochloride HCl to pH 6.8 H_2O to 500 ml 2 g SDS
SDS-PAGE running buffer (10-fold) 0.25 M Tris 2 M Glycin 1 % SDS pH 8.3	Wet transfer buffer 25 mM Tris 190 mM Glycin 20 % (v/v) Methanol pH 8.0
Wash buffer (10-fold) pH 7.8 261.4 g tris HCl 53.99 tris 876.6 g NaCl 100 ml tween-20 H_2O to 10000 ml	Blocking solution 5 % milk powder in wash buffer
Laemmli buffer (6-fold) 1.2 g SDS 0.06 g bromphenol blue 4.7 ml glycerol 1.2 ml tris pH 6.8 (0.5 M) 4.1 ml H_2O 0.939 g DTT	

4.1.4. Kits

InviTrap® Spin Cell RNA Mini Kit	Stratec

4.1.5. Cytokine ELISA sets

Human IL-22	R & D Systems, Minneapolis, USA
Human IL-10	R & D Systems, Minneapolis, USA
Human VEGF	R & D Systems, Minneapolis, USA

4.1.6. Enzymes

Collagenase D	Roche, Mannheim, German
DNase II	Roche, Mannheim, Germany
Trypsin (10 x)	PAA, Pasching, Germany

4.1.7. Cytokines

Recombinant human IL-22	Peprotech
Recombinant human IFN-γ	Peprotech
Recombinant human TNF-α	Peprotech

4.1.8. Antibodies

4.1.8.1. Western blot antibodies

Table 3: Primary antibodies

Description	Isotype	Clone	Company
Mouse IL-22 Rα1 antibody	Rat IgG2A	496504	R&D Systems
β-actin (C4) sc-47778 HRP	Mouse IgG1	C4	Santa Cruz
Phospho-Stat3 (Thyr705)	Mouse IgG2b	B-7	Santa Cruz
Stat3	Mouse IgG1	F-2	Santa Cruz

Table 4 Secondary antibodies

Description	Isotype	Clone	Company
Goat anti-rabbit IgG HRPsc-2301	Goat IgG	Polyclonal	Santa Cruz
Goat anti-rat IgG HRP sc-2006	Goat IgG	Polyclonal	Santa Cruz
Anti-Mouse IgG, HRP-linked antibody	Horse IgG	Polyclonal	Cell signaling

4.1.8.2. FACS antibodies

Table 5: Directly conjugated FACS antibodies

Description	Isotype	Clone	Company
Annexin V	Chicken recombinant	n.a.	Immunotools
Anti-Human IL-17A eFluor® 450	Mouse IgG1, κ	eBio64DEC17	eBioscience
Anti-human PE/Cy7 IL-17A antibody	Mouse IgG1, κ	BL168	Biolegend
Anti-human APC CD3 antibody	Mouse IgG2a, κ	OKT3	Biolegend
Anti-human IL-22 PE	Mouse IgG1, κ	22URTI	eBioscience
Anti-human APC IFN-γ antibody	Mouse IgG1, κ	4S.B3	Biolegend
Anti-human PerCP/Cy5.5 IFN-γ antibody	Mouse IgG1, κ	4S.B3	Biolegend
Anti-human FITC CD4 antibody	Mouse IgG2b, κ	OKT4	Biolegend
Anti-Human CD8a eFluor® 450	Mouse IgG2a	OKT8	eBioscience
Mouse IgG1, κ Isotype Control PE/Cy7	Mouse (BALB/c) IgG1, κ	MOPC-21	Biolegend
Mouse IgG1, κ Isotype Control PerCP/Cy5.5	Mouse (BALB/c) IgG1, κ	MOPC-21	Biolegend
Mouse IgG1 K Isotype Control PE	Mouse IgG1, kappa	P3.6.2.8.1	eBioscience
Mouse IgG1 K Isotype Control eFluor® 450	Mouse IgG1, kappa	P3.6.2.8.1	eBioscience

4.1.9. Primers for qRT-PCR

Table 6: qRT-PCR primers

Description	Sequence 5´- 3´	Primer efficiency	Molecular Probe #
CCL20 forward	5´- GCT GCT TTG ATG TCA GTG CT-3´	1,809	39
CCL20 backward	5´- GCA GTC AAA GTT GCT TGC TG-3´		
CCL22 forward	5´- CCC CCT GAC CCC TCT AAC -3´	1,926	13
CCL22 backward	5´- GGA ACA GGA CCC TCT GAC TG-3´		
HPRT forward	5´-CGA GCA AGA CGT TCA GTC CT-3´	1,952	73
HPRT backward	5´-TGA CCT TGA TTT ATT TTG CAT ACC-3´		
IL-22 forward	5´- CAA CAG GCT AAG CAC ATG TCA -3´	2,042	6
IL-22 backward	5´- ACT GTG TCC AGC TTT TGC -3´		
IL-22-R1 forward	5´-CAC CTC CCA ACT CCC TGA-3´	2,130	28
IL-22-R1 backward	5´-CGT GCT CCT GGA TGA AGC-3´		
IP10 forward	5´-GAA AGC AGT TAG CAA GGA AAG GT-3´	2,104	34
IP10 backward	5´-GAC ATA TAC TCC ATG TAG GGA AGT GA-3´		

4.1.10. Human cell lines

Human colorectal cancer cell lines

Caco-2 kindly provided by Dr. Andreas Herbst, Medizinische Klinik II, Klinikum der Universität München

Colo205 kindly provided by Universitätsklinikum Hamburg Eppendorf, UCCH Hubertus Wald Tumorzentrum

DLD-1 kindly provided by Dr. Andreas Herbst, Med. Klinik II Klinikum der Universität München

HCT116 kindly provided by Dr. Andreas Herbst, Med. Klinik II Klinikum der Universität München

HT29 kindly provided by Dr. Andreas Herbst, Med. Klinik II Klinikum der Universität München

SW480 kindly provided by Dr. Andreas Herbst, Med. Klinik II Klinikum der Universität München

SW620 kindly provided by Prof. Dr. Klaus-Peter Janssen, Department of Surgery, Technische Universität München

Other human cell lines

Karpas 299	kindly provided by Deutsches Krebsforschungszentrum Heidelberg (DKFZ)

4.1.11. Equipment

Balance (LP 6209)	Sartorius, Göttingen, Germany
Cell culture CO2 incubator (BD 6220)	Heraeus, Hanau, Germany
Cell culture laminar flow	Heraeus, Hanau, Germany
Centrifuge 5417 R	Eppendorf, Hamburg, Germany
Centrifuge 5424	Eppendorf, Hamburg, Germany
FACSCanto II	Becton Dickinson, San Jose, USA
Gel electrophoresis systems	Bio-rad, Munich, Germany
LightCycler 2.0 System	Roche, Mannheim, Germany
Mithras LB940 multilabel plate reader	Berthold, Bad Wildbad, Germany
Multifuge 3L-R	Heraeus, Hanau, Germany
Nanodrop ND-1000	NanoDrop, Wilmington, USA
Neubauer hemocytometer Optik Labor	FrischknechtBalgach, Germany
Omnifuge 2 ORS	Heraeus, Hanau, Germany
pH meter	WTW, Weilheim, Germany
Power Supply 200/2.0	Biorad, Munich, Germany
Refrigerators (4°C, -20°C, -80°C)	Thermo Scientific, Waltham, USA
Shaker	NeoLab, Heidelberg, Germany
Thermocycler T3	Biometra, Göttingen, Germany
Thermomixer	Eppendorf, Hamburg, Germany
Vortex VF2	Janke & Kunkel, Staufen, Germany

4.1.12. Patient samples

Irreversibly anonymized consecutive tumor samples from patients suffering from cancer of the colon or the rectum were collected by Dr. Jens Neumann, Pathologisches Institut, LMU, Munich. Analysis of anonymised patient tissue samples was performed as approved by the local ethics committee.

Table 7: Tumor and normal tissue samples details

Patient	Sample number	Histology	Age at diagnosis	Gender	Localization
1	**C1**	Adenocarcinoma	70	Male	Coecum
	C2	Adenocarcinoma	70	Male	Colon ascendens
	N1/2	Normal tissue	70	Male	Colon ascendens
2	**C3**	Adenocarcinoma	67	Female	Colon ascendens
	N3	Normal tissue	67	Female	Colon ascendens
3	**C4**	Adenocarcinoma	66	Female	Rektum
	N4	Normal tissue	66	Female	Rectum
4	**C5**	Adenocarcinoma	66	Male	Colon ascendens
	N5	Normal tissue	66	Male	Colon ascendens
5	**C6**	Adenocarcinoma	85	Female	Rectum
	N6	Normal tissue	85	Female	Rectum
6	**C7**	Adenocarcinoma	82	Male	Rectum
	N7	Normal tissue	82	Male	Rectum
7	**C8**	Adenocarcinoma	59	Male	Sigma
	N8	Normal tissue	59	Male	Sigma

Table 8: Tumor and normal tissue sample staging of tumor. Abbreviations are: T = tumor, N = nodes, Ln = lymph nodes, M = metastasis, L = lymphatic vessel invasion, V = vein invasion, Pn = perineural invasion, UICC = Union internationale contre le cancer

Patient	Sample number	Grading	T	N	Number Ln	M	L	V	Pn	UICC-Stadium	R-Status
1	C1	2	3	1b	3/47	1b (PER)	0	0	0	IVB	Rx
	C2	2	3	1b	3/47	1b (PER)	1	0	0	IVB	Rx
	N1/2										
2	C3	3	4b	2b	18/23	1b (PER)	1	1	0	IVB	Rx
	N3										
3	C4	2	3	2b	12/42	x	1	0	0	IIIC	R0
	N4										
4	C5	2	3	1b	2/24	x	1	2	3	III	R0
	N5										
5	C6	3	3	2a	4/13	1a (HEP)	1	0	1	IVA	R0
	N6										
6	C7	3	4b	0	0/17	x	0	0	0	IIC	R0
	N7										
7	C8	3	4b	0	0/32	x	0	0	0	IIC	R0
	N8										

4.1.13. Computer programs

Adobe Creative Suite	Adobe Systems, San Jose, USA
BD FACSDiva	BD Biosciences, San Diego, USA
Endnote X4	Thompson Reuter, Carlsbad, USA
Flowjo887	Tree Star, Ashland, USA
GraphPad Prism, Version 5.0b	GraphPad Software, La Jolla, USA
Microsoft Office	Microsoft, Redmond, USA
Light Cycler 480 software release 1.5.0 SP4	

4.2. Methods

4.2.1. Molecular biology

4.2.1.1. Isolation of cytoplasmatic RNA

To isolate RNA from human colon cancer cell lines the InviTrap® Spin Cell RNA Mini Kit (Stratec) was used. Purification was done according to the protocol provided by the manufacturer.

4.2.1.2. Reverse transcription

Complete RNA was reversely transcribed in first strand copy-DNA (cDNA) using the RevertAid First Strand cDNA Synthesis Kit. 1 - 2 µg of total RNA was used. For low concentrated samples a maximum of 11 µl of RNA was used. The RNA was mixed with Oligo-$(dT)_{18}$ primer, 5 x Reaction buffer, RiboLock RNase Inhibitor, dNTP Mix and RevertAid M-MuI reverse transcriptase. cDNA was either directly used or stored at -20°C.

4.2.1.3. Real time quantitative reverse transcription PCR (qRT-PCR)

To quantify mRNA levels qRT-PCR was used. 2 µl of cDNA was mixed with 5 µl of molecular probe master mix, 0.4 µl forward primer, 0.4 µl backward primer, 3 µl H_2O and 0.2 µl molecular probe.

Analysis was performed using a LightCycler 2.0 System (Roche) and quantification using Light Cycler 480 software release 1.5.0 SP4. HPRT was used as reference gene. For stimulations of colorectal cancer cells with cytokines, expression was normalized to unstimulated controls (set to 1).

4.2.2. Cell culture methods

4.2.2.1. General conditions

Cell culture was carried out under a laminar flow hood to ensure sterile conditions. The cells were grown in commercially available disposable tissue culture flasks and dishes, and incubated at 37ºC, 5% CO2 - air mixture and 95% atmospheric humidity.
Cell numbers and viability were tested by trypan blue staining. Cells were counted using a Neubauer hemocytometer.

4.2.2.2. Culturing medium for human cell lines

Table 9: Culturing medium for human cell lines and primary cells

Cell line(s)	Medium
Caco-2 HCT116 DLD-1 SW480 SW620	DMEM supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 U/l), streptomycin (0.1 mg/ml)
Colo205 Karpas299	RPMI medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, penicillin (100 U/l), streptomycin (0.1 mg/ml)
HT29	McCoy's 5A medium supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, penicillin (100 U/l), streptomycin (0.1 mg/ml)

4.2.2.3. Cell stimulations using recombinant cytokines

Colorectal cancer cell lines were stimulated with IL-22 (50 ng/ml), IFN-γ (10 ng/ml) and TNF-α (20 ng/ml) alone or in combination. For stimulation, cells were plated onto 6- or 24-well plates and allowed to adhere overnight. Medium was removed and cells were washed once with PBS. For STAT3 and pSTAT3 stimulation cells were starved 4 h in Opti-MEM prior to stimulation with recombinant IL-22. For proliferation assays, a Neubauer chamber was used for cell quantification.

4.2.2.4. Preparation of whole protein lysates from colorectal cancer cell lines

Cells grown in suspension were collected and adherent cells were detached by trypsinization. Cells were washed once with PBS and pellets were resuspended in 1 x lysis buffer supplemented with protease inhibitor cocktail. Cells were kept 30 - 45 min on ice and vortexed shortly every 10 min. Cell debris was removed by centrifugation for 15 min at maximum rpm at 4 °C. Cell lysates were stored at -20 °C.

4.2.3. Immunological methods

4.2.3.1. Bradford assay

Total protein concentration of lysates from colorectal cancer cells was assessed using the Bradford method (Bradford, 1976).

4.2.3.2. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

To separate proteins according to their molecular mass, SDS-PAGE was used. Protein samples were boiled for 5 min at 95 ºC in Laemmli buffer (Laemmli, 1970) under reducing conditions (including β-mercaptoethanol). Proteins were separated at a constant voltage of 100 V.

4.2.3.3. Western blot

After SDS Page, proteins were transferred onto a PVDF membrane at 360 Ampere for 60 min. Unspecific binding was blocked with 5% milk powder in TBST for 60 min at room temperature (RT). RT is defined as the temperature that is currently in the laboratory. Membranes were incubated with primary antibody overnight at 4 °C. Secondary antibody was incubated 60 - 120 min at RT. Afterwards the membrane was washed 3 x and Western blotting substrate was applied to the membrane. Chemo luminescence was detected using a CCD camera system (ImageQuant LAS 4000 mini).

4.2.3.4. Enzyme-linked immunosorbent assay (ELISA)

Cytokine concentrations of culture supernatant were determined by using sandwich ELISA, according to the protocol of the manufacturer.

4.2.3.5. Flow cytometry

All measurements were performed on an analytical FACS Canto II three laser system.

4.2.3.5.1. Analysis of surface molecule expression

Expression of surface molecules was assessed by adding fluorochrome-conjugated monoclonal antibodies.

4.2.3.5.2. Intracellular cytokine staining

Cells were stimulated with PMA (50 ng/ml) and ionomycin (1µg/ml) in the presence of Golgistop (brefeldin A, 4 µg/ml) for 4 - 5 h at 37 °C of intracellular cytokine expression analysis. Afterwards cells were washed 2 x with PBS and surface antigens were stained. Cells were then fixed using IC Fixation (eBioscience) for 20 min at RT. Cells were washed 1 x with PBS and left over night in PBS at 4 °C.
After washing cells twice with 1 x permeabilisation buffer (eBioscience), cells were stained in 100 µl 1 x permeabilisation buffer supplemented with fluorochcrome-conjugated monoclonal antibodies against the cytokines of interest for 30 min at 4 °C. Afterwards cells were washed 1 x with 1 x permeabilisation buffer, 1 x with PBS.

4.2.3.5.3. Apoptosis assay: Annexin V and PI staining

Apoptosis assay was performed using Annexin V and propidium iodide (PI) staining. Cells were harvested and transferred to FACS tubes and washed 1 x with PBS. Afterwards 200 µl 1 x Annexin V buffer was added per tube together with Annexin V APC antibody. Shortly before FACS analysis, PI was added.

4.2.3.5.4. Preparation and staining of colorectal cancer tissue

Samples from colorectal cancer patients were collected and stored at 4 °C in complete RPMI until digestion of the sample with 1 mg/ml collagenase and 0.05 mg/ml DNAse I at 37 °C for 30 min under mild shaking. Tissues were then sequentially dissociated by mashing through 100 µm and 40 µm cell strainers to generate single cell suspension. Cells were stimulated as described above. Cells were washed 2 x with PBS and fixable live dead staining was performed in 1 ml PBS for 30 min at 4°C. Cells were fixed and stored overnight at 4 °C after surface staining.

Intracellular staining was performed using IL-22, IL-17A and IFN-γ specific antibodies as well as appropriate isotype controls.

4.3. Statistical analysis

Columns and error bars indicate means and standard errors of the mean (SEM). Differences in experimental data sets were analyzed using unpaired (patient samples) or paired (cell culture experiments) two-sided Student's t-test. $p < 0.050$ are considered as statistically significant. Number of independent experiments is stated for each figure.

5. Results

5.1. Cellular sources of IL-22 in human tissue

5.1.1. Frequencies of IL-22 producing T cells in human PBMC

To evaluate Th-22 cell function in experiments, the frequency of Th-22 cells was evaluated in PBMC of 8 healthy individuals. Th-22 cells are defined as $CD4^+$ T cells that produce IL-22 in the absence of IL-17 and IFN-γ. The gating strategy is shown in figure 4. We defined Th-22 cells as $CD3^+$ $CD4^+$ $CD8^-$ $IL\text{-}17^-$ $IL\text{-}22^+$ and $IFN\text{-}\gamma^-$. Th-17 cells were defined as $CD3^+$ $CD4^+$ $CD8^-$ $IL\text{-}17^+$ and $IFN\text{-}\gamma^-$, and Th1 cells as $CD3^+$ $CD4^+$ $CD8^-$ and $IFN\text{-}\gamma^+$. Th-22 and Th-17 cells were found at low frequencies, in contrast to Th-1 cells which were the most abundant Th-subpopulation. The frequency range of Th-22 cells was 0.3 – 0.7 %, the range of Th-17 was 0.3 – 0.8 % and the range of Th-1 cells was 3.7 – 10.1 % (see figure 5).

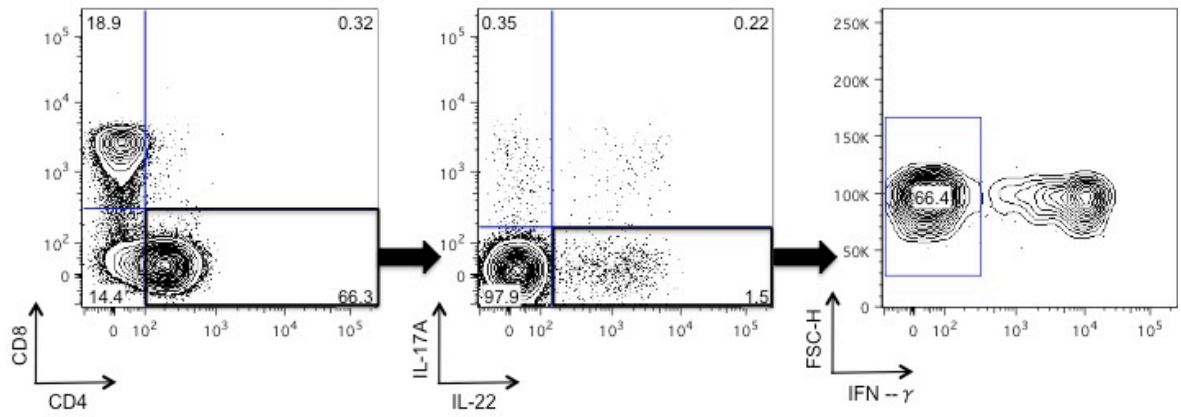


Figure 4: Gating strategy example of one healthy donor. Samples from a total of 8 healthy donors were purified by Biocoll density gradient centrifugation and restimulated with PMA and ionomycin in the presence of brefeldin A. PBMC were stained for surface CD3, CD4 and CD8 as well as for intracellular IL-22, IL-17A and IFN-γ. Results are shown for one representative donor. $CD3^+$ $CD4^+$ $CD8^+$ T cells were analyzed for their IL-22 and IL-17A production. Numbers indicate relative percentages per quadrant.

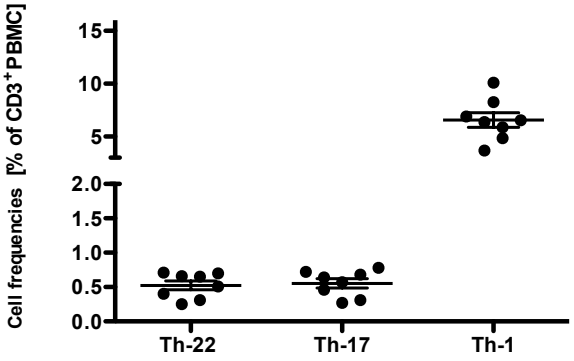
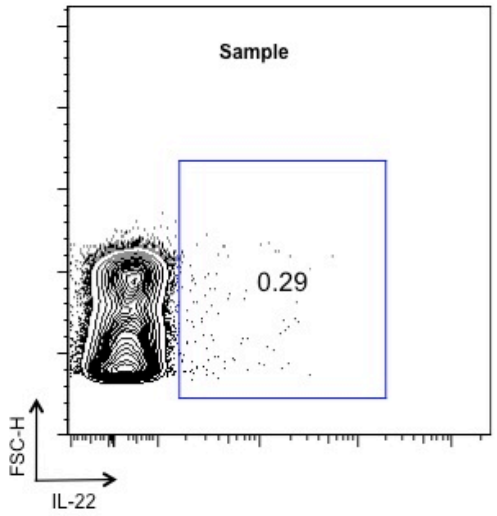


Figure 5: Frequencies of IL-22-producing T cells in PBMC of healthy donors. Samples from a total of 8 healthy donors were purified by Biocoll density gradient centrifugation and restimulated with PMA and Ionomycin in the presence of brefeldin A. PBMC were stained for CD3, CD4 and CD8 surface staining as well as intracellular staining for IL-22, IL-17A and IFN-γ. Frequencies of Th-22 (CD3+ CD4+ CD8- IL-22$^+$ IL-17A$^-$ IFN-γ$^-$), Th-17 (CD3+ CD4+ CD8- IL-17$^+$IFN-γ$^-$), and Th-1 (CD3+ CD4+ CD8- IFN-γ$^+$) cells within the CD3$^+$ cell population. Mean with SEM is indicated.

While CD4$^+$ T cells are the main producers of IL-22, low frequencies of IL-22$^+$ CD8$^+$ T cells were also detected (see figure 6).

A



B

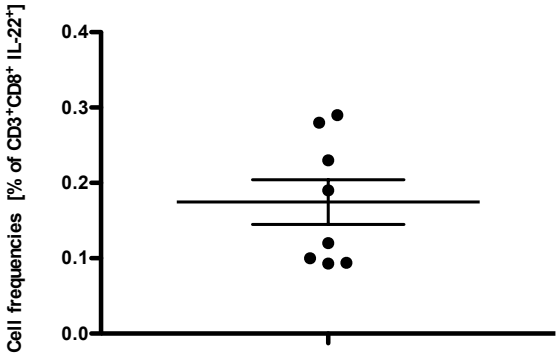


Figure 6: CD8$^+$ IL-22 producing T cells in PBMC from healthy donors. PBMC of a total of 8 healthy donors were purified by Biocoll density gradient centrifugation and restimulated with PMA and Ionomycin in the presence of brefeldin A. CD3, CD4 and CD8 surface staining as well as intracellular staining for IL-22, IL-17A and IFN-γ was performed. (A) One representative donor is shown as FACS blot gated from CD3$^+$ CD8$^+$ T cells. (B) Frequencies of IL-22$^+$ cells within CD3$^+$ CD8$^+$ T cells of 8 donors. Mean with SEM is indicated.

5.1.2. T-helper cell subpopulations producing IL-22 are found in the tumor tissue of colorectal cancer patients

To further dissect the role of IL-22 in tumor biology, we applied the above described technique to analyze IL-22-producing cells in primary colorectal cancer tissue. For each patient a tissue sample from the tumor and a sample from neighboring tissue macroscopically not affected by the tumor (herein referred to as 'normal tissue') were collected.

Samples from patients with colorectal cancer were analyzed for CD3, CD4 and CD8 surface expression and IL-22, IL-17 and IFN-γ intracellular cytokine staining.

Figure 7 shows the numbers of Th-1, Th-17 and Th-22 as proportions of $CD3^+$ T cells Numbers of Th-17 and Th-22 were low compared to Th-1 cells. For Th-17 and Th-1 cells there is a trend towards higher numbers in tumor tissue. However, the frequencies of Th-1, Th-17 and Th-22 cells are not significantly different in tumor compared to normal tissue.

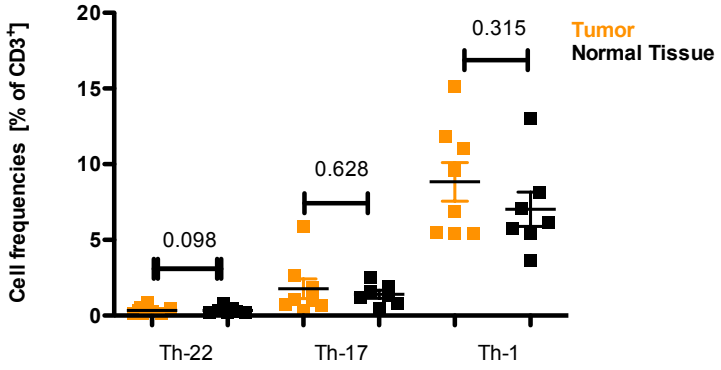


Figure 7: Frequencies of Th-22, Th-17 and Th-1 in samples from colorectal cancer patients. CD3, CD4 and CD8 surface staining as well as intracellular staining for IL-22, IL-17A and IFN-γ were performed. Frequencies of Th-22 (CD3+ CD4+ CD8- $IL\text{-}22^+$ $IL\text{-}17A^-$ $IFN\text{-}\gamma^-$), Th-17 (CD3+ CD4+ CD8- $IL\text{-}17^+IFN\text{-}\gamma^-$), and Th-1 (CD3+ CD4+ CD8- $IFN\text{-}\gamma^+$) cells within the $CD3^+$ $CD4^+$ $CD8^-$ cell population of tumor and normal tissue from 7 patients are shown, for one patient with dual carcinoma two specimens were taken. Mean with SEM is indicated.

A population of $IL\text{-}22^+$ T cells that was $IFN\text{-}\gamma^+$ ($CD3^+CD8^-CD4^+IL\text{-}17^-IL\text{-}22^+$) T cells was found in higher numbers in normal tissues compared to tumor tissue (p=0.046) (figure 8A).

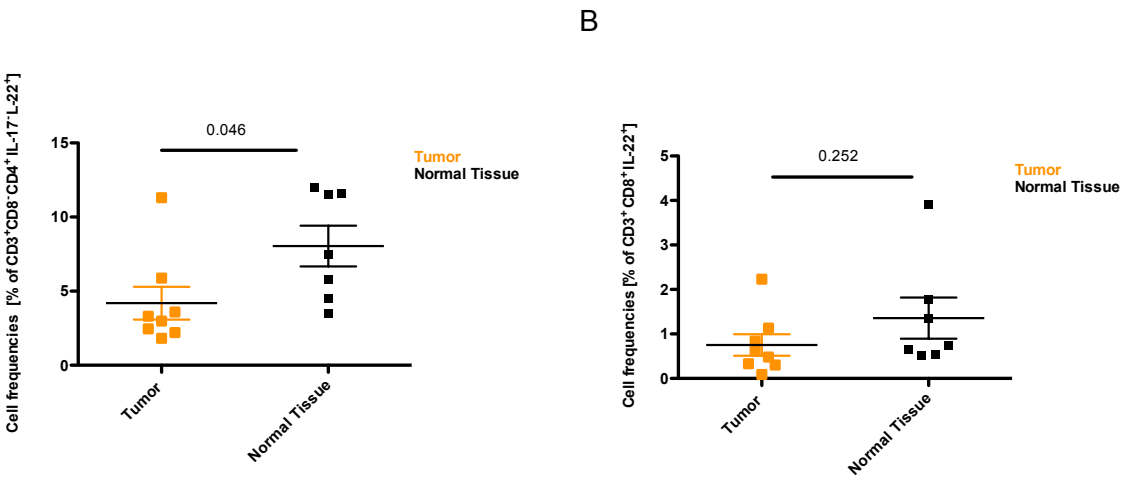


Figure 8: Frequencies of tumor infiltrating lymphocytes. Lymphocytes were isolated from colorectal cancer biopsies and restimulated with PMA and Ionomycin in the presence of brefeldin A. Afterwards CD3, CD4 and CD8 surface staining as well as intracellular staining for IL-22, IL-17A and IFN-γ was performed. (A) Frequencies of $CD3^+CD8^-CD4^+IL\text{-}17^-IL\text{-}22^+$ within CD4+ T cells. (B) $CD8^+$ IL-22 producing T cells are found in normal and colorectal cancer tissue. Results of normal and cancer tissue from 7 patients are shown, for one patient with dual carcinoma two specimens were taken. Mean with SEM is indicated.

Using flow cytometry, we found a proportion of the $CD8^+$ T cells to stain positive for IL-22. Equal amounts of IL-22 producing $CD8^+$ T cells were found in normal and tumor tissue ($p=0.252$) (figure 8B).

5.2. Effect of IL-22 on human colorectal cancer cell lines

To understand the effect of IL-22 production at the tumor site, we next performed *in vitro* assays using a panel of human colorectal cancer cell lines (n = 6).

5.2.1. Colorectal cancer cell lines do not produce IL-22

To test if colorectal cancer cell lines have the ability to produce IL-22, we tested Colo205, CaCo2, DLD-1, HCT116, HT29 and SW480 for IL-22 secretion by ELISA and IL-22 expression by qRT-PCR. The tested cell lines do not secrete IL-22 and no significant mRNA levels were found for the IL-22 transcript, indicating that these colorectal cancer cell lines do not produce IL-22 (figure 9)

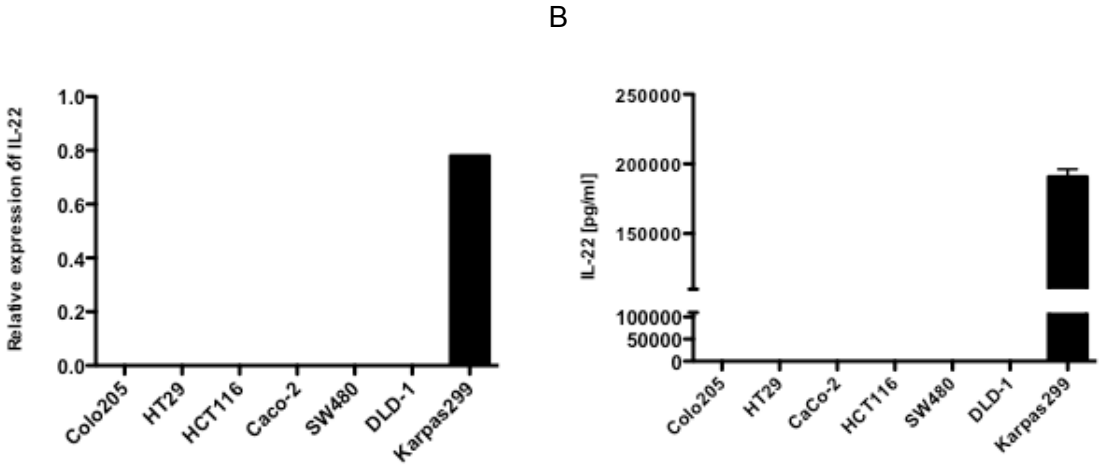


Figure 9: Colorectal cancer cell lines do not produce IL-22. (A) Analysis of IL-22 mRNA in colorectal cancer cell lines by qRT-PCR. For qRT-PCR data relative expression of IL-22 to hypoxanthin-phosphorybosyltransferase (HPRT) for each cell line was calculated by the Light Cycler relative quantification software. The human lymphoma cell line Karpas 299 served as positive control. (B) Supernatants of colorectal cancer cell lines were tested for IL-22 content by ELISA. The cell line Karpas 299 served as positive control. The error bars represent standard error of the mean of duplicates. Results are representative for at least two independent experiments.

5.2.2. Colorectal cancer cells express IL-22-R1

To further dissect the potential role of IL-22 in colorectal cancer, we next studied expression of IL-22-R1 on colorectal cancer cell lines. IL-22-R1 expression was assessed by Western blot analysis and qRT-PCR. IL-22-R1 was found in all cell lines analyzed on mRNA and on protein level (figure 10).

A B

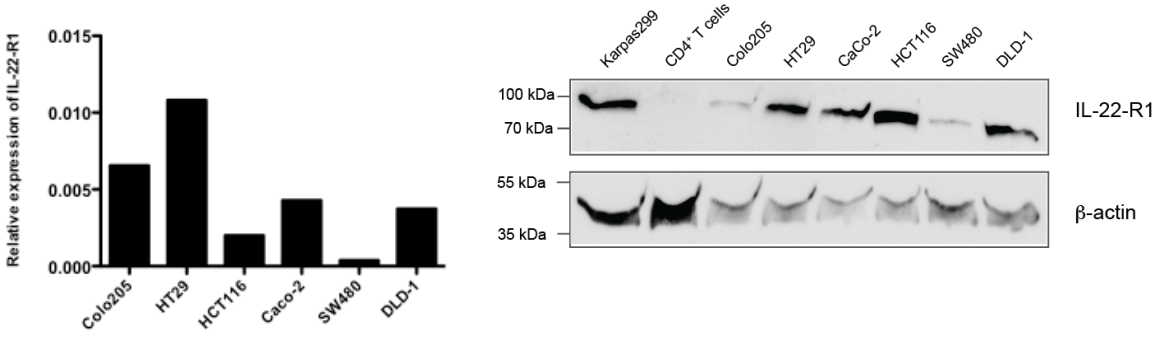


Figure 10: Colorectal cancer cell lines express IL-22-R1 on mRNA and protein level. (A) Analysis of IL-22-R1 mRNA in colorectal cancer cell lines by qRT-PCR. For qRT-PCR data relative expression of IL-22-R1 to HPRT for each cell line was calculated by the Light Cycler relative quantification software. (B) 40 µg protein of whole cell lysate was loaded per lane. IL-22-R1 expression was detected by Western blot. β-actin served as loading control. One representative of at least two is shown.

5.2.3. IL-22 signaling pathway is functional in colorectal cancer cells

In cancer cells, STAT3 is the major signal transducing molecule upon IL-22 stimulation. To test ability of the six colorectal cancer cells lines to respond to IL-22 stimulation, cells were treated with 100 ng/ml IL-22 for 10, 30 and 60 min and analyzed for STAT3 phosphorylation by Western blot.

All tested colorectal cancer cell lines (Colo205, CaCo2, DLD-1, HCT116, HT29 and SW480) responded to IL-22 stimulation by STAT3 phosphorylation. Phosphorylation of STAT3 is shown exemplarily in figure 11 for the cell lines HT29 and Colo205.

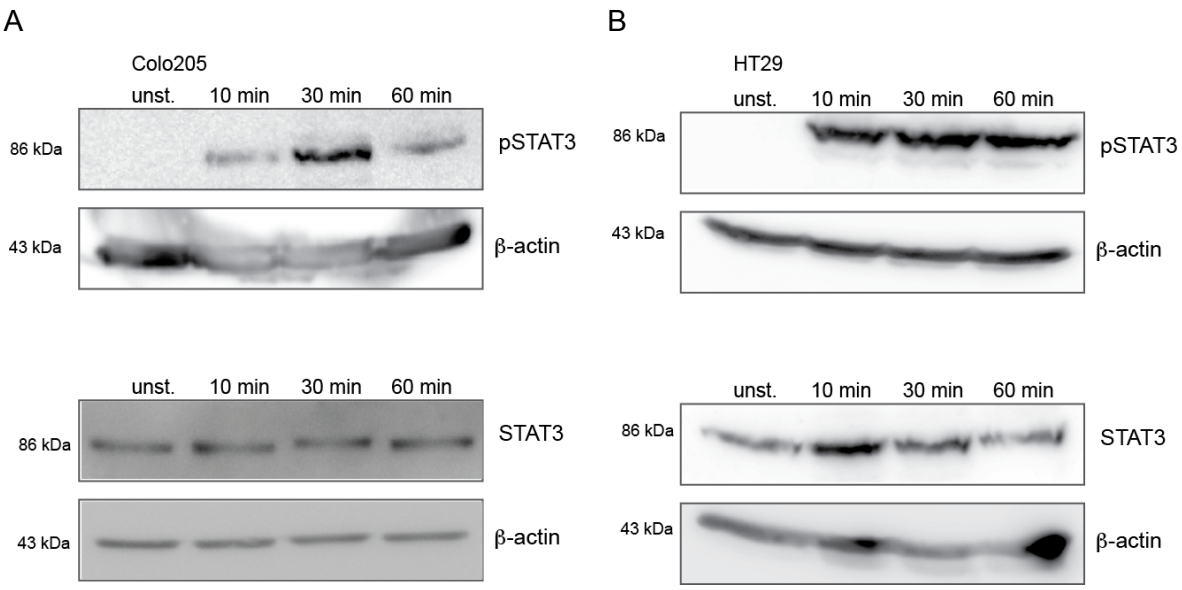


Figure 11: STAT3 is phosphorylated in colorectal cancer cell lines upon IL-22 stimulation. Colorectal cancer cell lines were either left unstimulated (unst.) or stimulated with IL-22 (100 ng/ml) for the indicated time periods. Phospho-STAT3 (pSTAT3) was evaluated by Western blot analysis. STAT3 and β-actin served as loading controls. Colo205 (A) and HT29 (B) showed STAT3 phoshorylation upon IL-22 stimulation. One representative of at least two independently performed experiments is shown.

5.2.4. IL-22 stimulation induces release of IL-10 and VEGF from Colo205 cells

To further dissect the downstream effects of IL-22 stimulation, we analyzed the secretion of IL-10 and VEGF, which are both induced by pSTAT3 activation. Upon IL-22 stimulation, IL-10 secretion was induced (figure 12A) and VEGF secretion was enhanced in a time dependent manner (figure 12B).

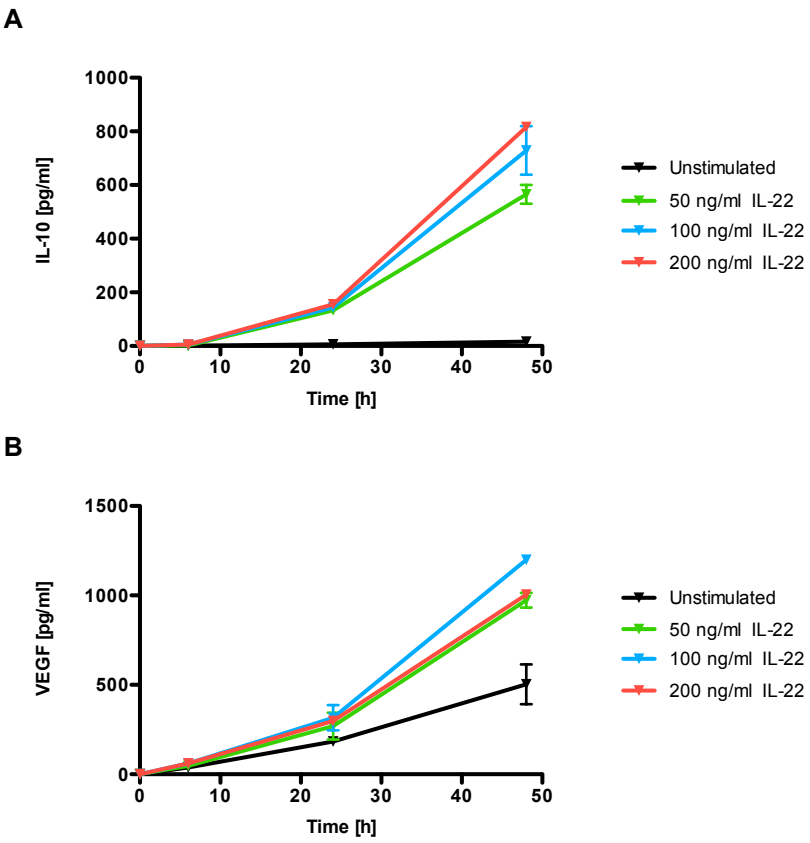


Figure 12: IL-22 induces IL-10 secretion from Colo205 cells. Colo205 cells were seeded in 96 well plates and stimulated with 50, 100 or 200 ng/ml IL-22. Supernatants were collected at 6, 24 and 48 h and the (A) IL-10 content and (B) VEGF concentration was measured by ELISA. One representative experiment of three independent experiments is shown. Error bars represent SEM of duplicates.

5.2.5. IL-22 induces proliferation of Colo205 cells

Another consequence of STAT3 phosphorylation in cancer cells is cell proliferation. To analyze if IL-22 stimulation would induce proliferation of colorectal cancer cells, we stimulated Colo205 cells with different concentrations of IL-22 (see figure 13). Shown are fold change normalized to unstimulated controls after 48 hours. IL-22 stimulation dose-dependently induced proliferation of Colo205 cells (figure 15, p = 0.023) for the 100 ng/ml condition versus unstimulated).

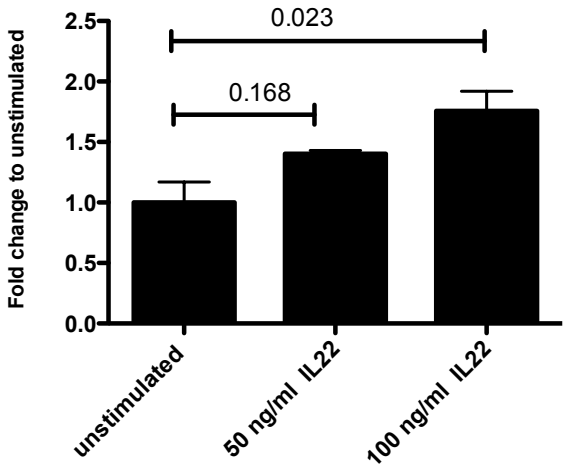


Figure 13: Colo205 cells proliferate upon IL-22 stimulation in a concentration dependent manner. Proliferation is measured as cell number by Neubauer chamber counting in the presence or absence of IL-22 (50 ng/ml or 100 ng/ml) for the cell line Colo205. Number of cells was normalized to unstimulated controls. The results of one representative experiment of three independent experiments are shown. Error bars represent Mean with SEM of triplicates. Paired t-test, $p<0.05$ represents significant difference.

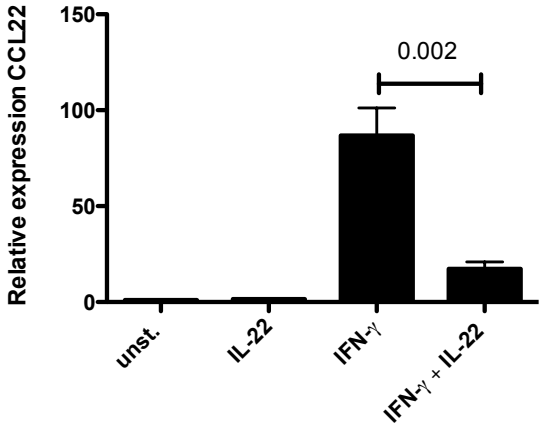
5.3. Effect of IL-22 on IFN-γ-induced CCL22 production of tumor cells

5.3.1. IL-22 inhibits IFN-γ-induced CCL22 secretion in HT29 cells

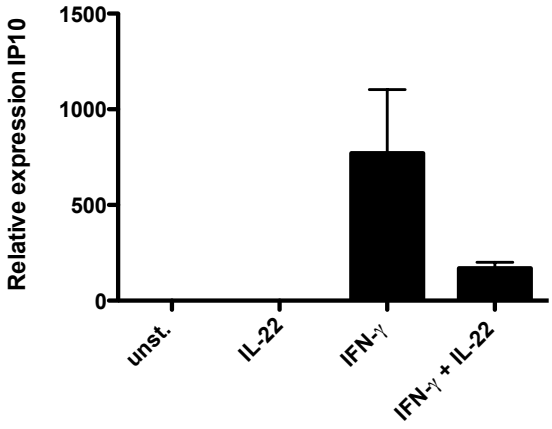
Because IL-22 may interact with the downstream effects of IFN-γ, we sought to further investigate the effect on other IFN-γ-mediated effects such as chemokine secretion (Berin et al, 2001; Kaplan et al, 1987; Pennino et al, 2013). The colorectal cancer cell line HT29 produces CCL22 after IFN-γ stimulation (Berin et al, 2001).

HT29 cells were either stimulated with IFN-γ, IL-22 or the combination of both. CCL22 induction was analyzed by qRT-PCR and ELISA. IFN-γ stimulation induced CCL22 (see figure 14A). Addition of IL-22 significantly reduced IFN-γ-induced CCL22 secretion (p = 0.002). However, for IP10 and CCL20 no significances were calculated since the sample number was too low (figure 14B und 14C).

A



B



C

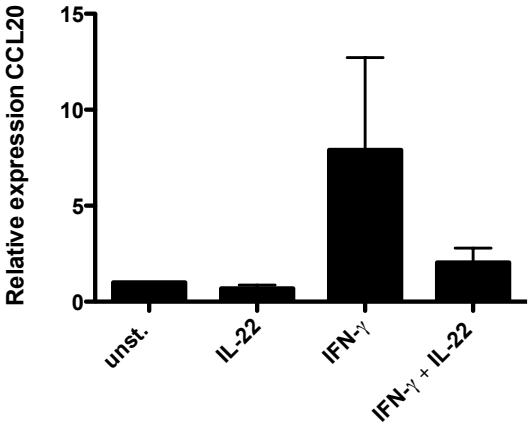


Figure 14: IL-22 downregulates IFN-γ-induced CCL22 in HT29 cells. HT29 cells were treated with IFN-γ (10 ng/ml) or IL-22 (50 ng/ml) for two days in Opti-MEM. Cells were harvested and RNA was extracted. mRNA expression was determined by qRT-PCR for CCL22, IP10 and CCL20. For qRT-PCR data relative expression of IL-22-R1 to HPRT for each cell line was calculated by the Light Cycler relative quantification software and the mean of untreated control HT29 cells was set to 1. (A) Relative expression of CCL22 in HT29 cells is shown. Error bars represent mean with SEM of seven independent experiments. (B) Relative expression of IP10 in HT29 cells is shown. Error bars represent mean with SEM of two independent experiments. (C) Relative expression of CCL20 expression in HT29 cells. Error bars represent mean with SEM of two independent experiments.

To exclude that the IL-22 effect on IFN-γ –induced CCL22 production was due to apoptosis induction, Annexin V and PI staining on HT29 cells after stimulation was performed. The combination of IL-22 and IFN-γ did not significantly increase the number of Annexin V and PI positive cells (p=0.265) (figure 15).

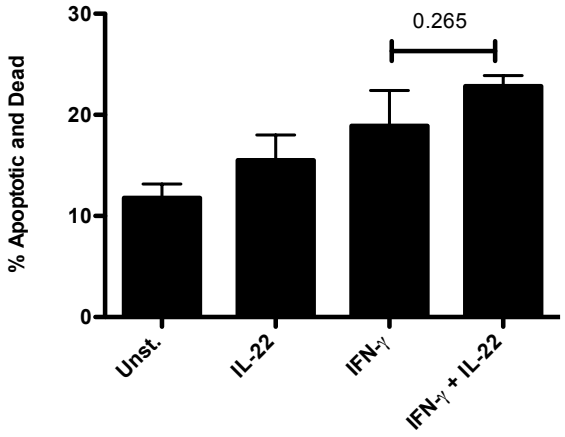


Figure 15: Apoptosis and Death of HT29 cells stimulated 2 days in Opti-MEM supplemented with IFN-γ, IL-22 or both. Data are representative for at least two independent experiments. Error bars represent mean with SEM of triplicates. Paired t-test, $p<0.05$ represents significant difference.

5.3.1.1. IFN-γ upregulates IL-22-R1 on colorectal cancer cells

Because IFN-γ has been described to upregulate IL-22-R1 on keratinocytes (Kunz et al, 2006), we next asked whether this effect could also be observed in colorectal cancer cell lines. HT29 and Colo205 cells were stimulated with IFN-γ or IL-22 and IL-22-R1 expression was analyzed by RT-PCR. IL-22 did not significantly enhance IL-22-R1 expression in HT29 (p=0.067) (figure 16A) and in Colo205 (0.139) cells (figure 16B). IFN-γ enhanced IL-22-R1 expression significantly in HT29 cells (p=0.001). However the induction of IL-22-R1 was not significant in Colo205 cells (p=0.091).

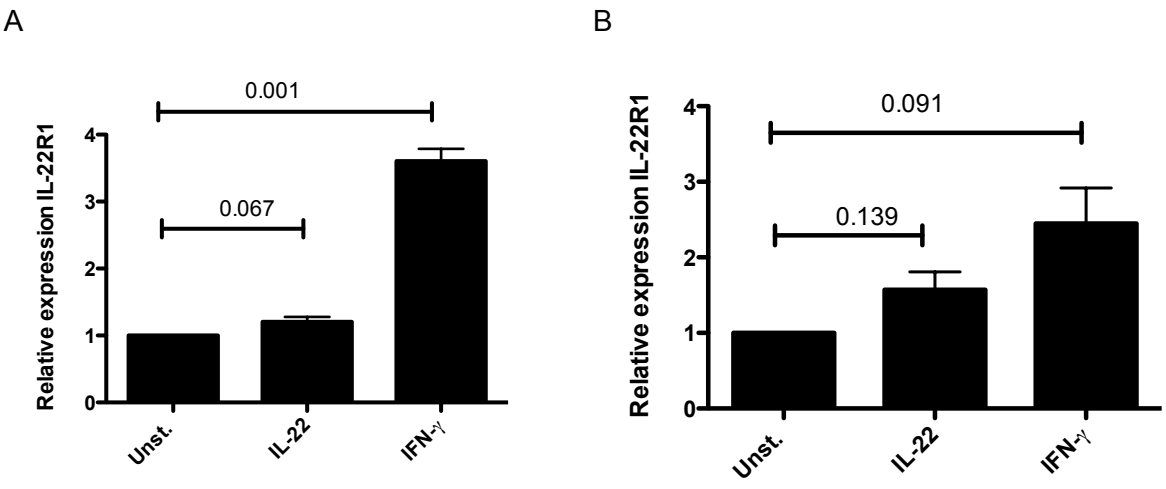


Figure 16: IFN-γ upregulates IL-22-R1 expression in HT29 and Colo205 cells. HT29 (A) and Colo205 (B) cells were treated with IFN-γ (10 ng/ml) or IL-22 (50 ng/ml) for two days. Cells were harvested and cDNA was prepared. mRNA expression was determined by qRT-PCR for IL-22-R1. The mRNA expression of each condition was normalized to expression of HPRT mRNA and the mean of untreated control HT29 and Colo205 cells was set to 1.0, respectively. Error bars represent mean with SEM of four (HT29 cells) and three (Colo205) independent experiments. Paired t-test, $p<0.05$ represents significant difference.

5.3.1.2. IL-22 and TNF-α synergistically induce CCL22 in HT29 cells

HT29 and Colo205 cells were stimulated with IL-22, TNF-α or the combination of both. TNF-α induced CCL22 expression in HT29 cells. Addition of IL-22 to TNF-α significantly increased CCL22 expression in HT29 cells (p = 0.021) (figure 17).

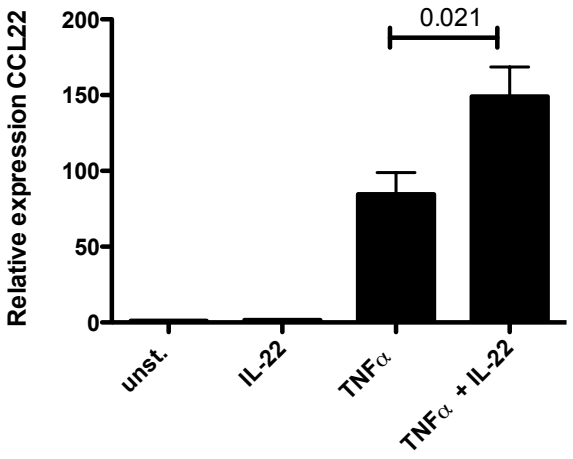


Figure 17: IL-22 synergizes with TNF-α- to induce CCL22 expression in HT29 cells. HT29 cells were treated with TNF-α (20 ng/ml) or IL-22 (50ng/ml) for two days in Opti-MEM. Cells were harvested and mRNA and subsequent cDNA was prepared. mRNA expression was determined by real-time PCR for CCL22. The mRNA expression of each condition was normalized to expression of HPRT mRNA and the mean of untreated control HT29 cells was set to one. CCL22 expression in HT29 cells is shown. Error bars represent mean with SEM of five independent experiments.

5.3.1.3. TNF-α induced upregulation of IL-22-R1 expression in HT29 cells

To further understand the interaction of IL-22 and TNF-α on colorectal cancer cells, we next investigated the impact of TNF-α on IL-22-R1 expression. IL-22 did not significantly upregulate IL-22-R1. TNF-α and the combination with IL-22 both upregulated IL-22-R1 expression in HT29 cells significantly, p=0.018 and p=0.036, respectively (see figure 18). The combination of TNF-α with IL-22 showed a trend towards further enhancement of IL-22-R1 expression.

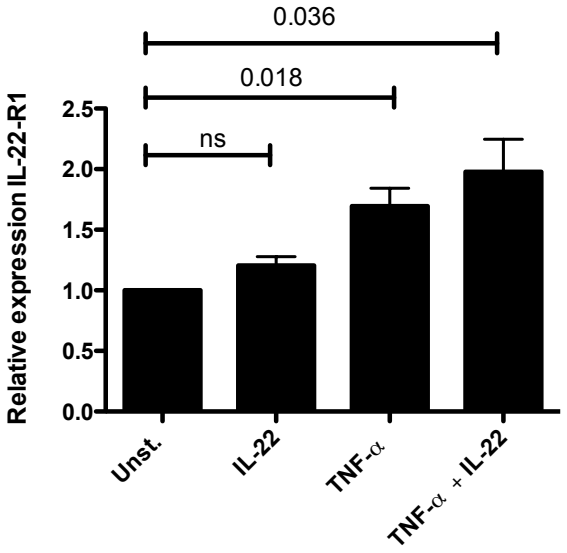


Figure 18: TNF-α upregulates IL-22-R1 in HT29 cells. HT29 cells were treated with IL-22 (50 ng/ml) or TNF-α (20ng/ml) alone or in combination for two days in Opti-MEM. Cells were harvested and cDNA was prepared. mRNA expression was determined by real-time PCR for IL-22-R1. The mRNA expression of each condition was normalized to expression of HPRT mRNA and the mean of untreated control HT29 and Colo205 cells was set to one. Error bars represent Mean ± SEM of four independent experiments.

6. Discussion

The present work shows that human PBMC from healthy donors as well as colorectal cancer tissue contain subclasses of IL-22 producing T cells, including Th-1, Th-17 and Th-22 cells as detected by flow cytometry. Low frequencies of $CD8^+$ IL-22 secreting T cells were also detected.

Colorectal cancer cell lines did express IL-22-R1 but not IL-22. IL-22 stimulation induced STAT3 phosphorylation in all tested cell lines. In Colo205 cells IL-22 led to VEGF and IL-10 release as well as cell proliferation. We also showed that IL-22 suppresses IFN-γ-induced CCL22 protein expression in HT29 cells. In addition, IL-22 enhanced TNF-α-induced expression of CCL22 in the same cell line. Both IFN-γ and TNF-α upregulated expression of IL-22-R1.

6.1. IL-22 expression in human PBMC from healthy donors

Our study shows that IL-22 producing T cells can be detected in PBMC. Different Th cell subtypes are present within circulating healthy PBMC (Lu et al, 2015; Luan et al, 2015; Oliveira et al, 2015).

In this thesis, it was shown that Th-1, Th-17 and Th-22 are present in the peripheral blood of healthy donors, consistent with the literature. The focus of the thesis was Th-22 cells that represent a novel T cell subpopulation. The frequency of Th-22 cells was low. Studies by other groups have identified Th-22 cells as a very rare population within PBMC from healthy human donors (Liu et al, 2012; Zhang et al, 2012a). One study detected a mean of 0.8 % circulating Th-22 cells with a range of 0.3 % to 2.1% for Th-17 cells a mean of 1.9 % was found (range, 0.4 % to 4.3 %) (Lu et al, 2015). Another study described low frequencies with up to approximately 0.1 % $CD3^+$ $CD4^+$ IL-22^+ cells (Oliveira et al, 2015). An additional study showed for Th-22 cells a median of 0.2 % and a range of 0.5 %, for Th-17 cells a median of 0.4 % and a range of 1.5 % and for Th-1 cells a median of 1.4 % and a range of 15.5 % (Luan et al, 2015). The levels we found for Th-22 cells were 0.3 to 0.7 % and 0.3 to 0.8 % for Th-17 cells. For Th-1 cells we found levels between 3.7 % and 10.1 %.

Th frequencies of Th-22 cells detected in the present thesis are compatible with the low frequencies described in literature. Minor differences may result from different cohorts of donors as well as from methodological differences. Th-22 cells are found in low frequencies in peripheral blood, however they are abundant in tissue. One explanation could be the expression of their characteristic chemokine receptors CCR4 and CCR10. Chemokines binding these receptors are for instance strongly expressed in the skin (Eyerich & Eyerich, 2015).

IL-22 does not act on immune cells but on cells of epithelial origin and therefore represents novel kind of immune modulator within the IL-10 family of cytokines (Wolk et al, 2010). The main functions of IL-22, in healthy individuals, are increase of innate immunity, protection of tissue from damage and enhancement of tissue regeneration.

Particular Th-22 cells have been described to protect epithelial barriers in organs and also modulate injured and inflamed tissue (Eyerich & Eyerich, 2015). IL-22 also plays a role in the defense against bacterial infections (Siegemund et al, 2009) inducing, for instance, antimicrobial molecules (Siegemund et al, 2009; Wolk et al, 2004).

6.2. Expression of IL-22 in colorectal cancer tissue

A Th-22 staining protocol was established and applied to tumor samples from colorectal cancer patients. The presence of IL-22 and/or of Th-22 cells has previously been demonstrated within tumor tissues from patients with colorectal, gastric, hepatocellular, small-and large-cell lung carcinoma (Jiang et al, 2013; Kobold et al, 2013; Liu et al, 2012; Qin et al, 2014).

We detected IL-22 producing Th-1, Th-17 and Th-22 within tumor samples of colorectal cancer patients. These findings are in line with published data as all three T cell types have been described in solid tumors.

$CD4^+$ T cells were shown to exert a role in both the induction and the effector phases of the antitumor response (Protti et al, 2014). It has been described that activated $CD4^+$ T cells modulate antitumor immune response. Th-1 cells support cytotoxic T cells and

secrete cytokines such as IL-2 or IFN-γ and that promote an antitumor response and tissue destruction (Disis, 2010; Pernot et al, 2014).

Th-17 cells are known to be present in the tumor environment of colorectal cancer patients. Tumor-infiltrating IL-17 producing $CD4^+$ T cells and macrophages have been associated with poor prognosis for colorectal cancer patients (Fridman et al, 2011; Fridman et al, 2012; Liu et al, 2011a).

Th-22 cells were the focus of this thesis; their involvement in tumor development and role in sustaining established tumors was investigated. We detected low frequencies of Th-22 in both tumor tissue and non-affected tissue. However, it has been shown that Th-22 cell accumulation may be connected to colorectal cancer development, according to published evidence. The authors found increased levels of Th-22 in tumor tissue compared to peritumoral tissue (Huang et al, 2015). In this thesis up to 0.9 % of Th-22 cells as subset of $CD4^+$ T cells were found whereas Huang et al., found up to 1.5 % Th-22 cells. The cited publication analyzed a cohort of 18 patients for their Th-22 content via FACS as opposed to the seven patients analyzed here. Thus, discrepancies may be explained by the low power of either study. A larger cohort might be more suitable for comparison of Th-22 in tumor tissue and peritumoral tissue. Peritumoral tissue was used in this thesis as control. However, while the cancer cells do not directly affect this tissue, it is extracted from the same patient suffering from cancer. The T cell repertoire might also be affected by changes resulting either from neighboring cancer cells or by the factors leading to cancer development in the first place. Therefore, the peritumoral tissue may not be strictly considered a healthy tissue and may not have identical characteristics to tissue from a true healthy individual.

Circulating Th-22 are increased in patients with gastric cancer compared to healthy donors and are associated with tumor progression (Liu et al, 2012). Another study demonstrated increased intratumoral $CD4^+IL\text{-}22^+$ T cells. The authors correlated Th-22 to tumor progression and lower patient survival (Zhuang et al, 2012). Circulating Th-22 cells are also associated with hepatocellular carcinoma development and disease progression (Qin et al, 2014). For pancreatic cancer patients, intratumoral IL-22 levels and frequencies of $CD4^+$ IL-22 producing cells are as well linked to disease progression (Xu et al, 2014).

In summary, available data suggests a disease-promoting role of Th-22 in several cancer entities.

In contrast, the function of IL-22 may vary according to the stage of the disease where IL-22 is expressed. For example, transgenic mice that are characterized by constant overproduction of IL-22 in the liver do not spontaneously develop liver tumors (Park et al, 2011). However, IL-22 may support the progression of already developed tumors, as IL-22 induces proliferation in a number of tumor cells from various origins (Kobold et al, 2013).

6.3. Expression of IL-22-R1 and activation of STAT3

To address the question of the effect of IL-22 on colorectal cancer, colorectal cancer cell lines were used as an in vitro model.

To verify that colorectal cancer cells themselves do not produce IL-22, six cell lines were tested for their IL-22 expression. It is widely accepted that IL-22 is not produced by epithelial cells (Wolk et al, 2010). Consistent with this statement, we found no IL-22 expression on protein level in Colo205, HT29, SW480, DLD-1, HCT-116 and Caco-2 cells. The findings of this thesis strengthen the hypothesis that IL-22 found in tumor tissues is expressed by infiltrating immune cells and not by the tumor cells.

To test the responsiveness of these cell lines, IL-22-R1 receptor expression was analyzed. The IL-10-R2 chain of the functional IL-22 receptor complex is ubiquitously expressed and is not limiting in defining IL-22-responsive cells (Wolk et al, 2004). Thus, cellular responsiveness to IL-22 is determined by the expression of IL-22-R1. All tested colorectal cancer cell lines did express IL-22-R1 on protein level. The lymphoma cell lines Karpas299 served as positive control as this cell lines has been described to express IL-22-R1 (Bard et al, 2008).

The findings of this thesis corroborate previously published findings on colorectal cancer cell lines and IL-22-R1 expression (Brand et al, 2006; Di Lullo et al, 2015; Nagalakshmi et al, 2004; Ziesche et al, 2007). However, it cannot be concluded that any cancer cell

from any origin necessarily expresses IL-22-R1: one study showed that not all tested multiple myeloma cell lines did express IL-22-R1. They found IL-22-R1 mRNA in approximately one third of the tested cell lines (Di Lullo et al, 2015).

All tested colorectal cancer cell lines showed induction of STAT3 phosphorylation upon IL-22 stimulation, as previously published (Brand et al, 2006; Cella et al, 2009; De Simone et al, 2014; Nagalakshmi et al, 2004). However, IL-22 can also activate other STAT members namely 1 and 5. For all three STATs the major MAPK pathway is activated, leading to phosphorylation of ERK1/2, JNK, and p38 kinase after interaction with the IL-22-R1/IL-10-R2 receptor complex (Lejeune et al, 2002). In cancer cells, most of the downstream effects of IL-22 are thought to be mediated by activation of STAT3 (Boniface et al, 2005; Nagalakshmi et al, 2004; Xie et al, 2000). STAT3 activation has been shown in multiple carcinoma cell lines other than colorectal cancer, such as: in human lung carcinoma cell lines (Kobold et al, 2013), gastric carcinoma cell lines (Fukui et al, 2014), skin basal-cell carcinoma and squamous-cell carcinoma cell lines (Nardinocchi et al, 2015). Since we focused on STAT3 activation for these reasons, we cannot rule out a contribution to IL-22 signaling by other downstream effector molecules.

6.3.1. IL-22 induces VEGF, IL-10 and proliferation in Colo205 cells

We could show that Colo205 secrete IL-10 upon IL-22 stimulation as previously published (Nagalakshmi et al, 2004). Production of IL-10 by Colo205 cells could be a mechanism by which tumor cells suppress inflammatory immune responses, since IL-10 is a crucial anti-inflammatory cytokine (Fiorentino et al, 1991).

On the other hand, we showed that Colo205 cells increased VEGF production upon IL-22 stimulation, as previously demonstrated in HPAFII human pancreatic cancer cells (Curd et al, 2012). A finding which is further corroborated by IL-22^{+} tumor-infiltrating lymphocytes of colorectal cancer patients increasing VEGF expression in mice with colorectal cancer xenografts (Jiang et al, 2013).

VEGF directly acts on endothelial cells and is the major angiogenic growth factor for cancers and may thus represent a mechanism by which IL-22 could help colorectal

cancer progression (Carmeliet & Jain, 2000; Pepper et al, 1992).
However, none of the other tested colorectal cancer cell lines did produce IL-10 or VEGF after IL-22 stimulation, which may indicate a cell line specific over a disease specific effect of IL-22.

A number of studies demonstrated proliferation of tumor cells in response to IL-22 (Brand et al, 2006; Di Lullo et al, 2015; Kobold et al, 2013; Nardinocchi et al, 2015) which is further supported by our results of increased proliferation of Colo205 cells upon IL-22 stimulation. Supernatants derived from NK-22, which produce IL-22, stimulate Colo205 cells to proliferate, which is in line with our data (Cella et al, 2009).

In summary, these findings support the idea that IL-22 may contribute to enhanced tumor growth, progression and angiogenesis by cytokine, growth factor and proliferation induction.

6.4. Crosstalk of IL-22 pathway with signaling of IFN-γ and TNF-α

Multiple cytokines are involved in tumor formation and growth. However, their pro- or antitumor role depend on the balance of the different cytokines as well as on the stage of the cancer (Landskron et al, 2014). IL-22 can act pro- and anti-inflammatory on tissue homeostasis. On the one hand, IL-22 promotes epithelial cell regeneration and protects tissues from damage while playing a crucial inflammatory role in a pathological context (Nikoopour et al, 2015). The reasons for the dual role of IL-22 in intestinal homeostasis are not elucidated but might be related to microbial stimulation and that maintained production in response to chronic bacterial stimulation eventually promotes disease (Kirchberger et al, 2013). These paradox functions may be explained by the presence or absence of other cytokines.

6.4.1. IL-22 inhibits IFN-γ-induced CCL22 secretion in HT29 cells

IFN-γ is one of the Th1 prototypical cytokines and often co-secreted with IL-22. However the possible interplay of IL-22 and IFN-γ has not been systematically investigated.

IFN-γ can lead to the upregulation of MHC-I, MHC-II and ICAM-1 in epithelial cells thus enhancing adhesion of $CD4^+$ and $CD8^+$ T cells and apoptosis induction in epithelial cells affected (Miyahara et al, 2004; Pennino et al, 2010; Schwarze et al, 1999; Traidl et al, 2000). IL-22 can antagonize IFN-γ-induced MHCI, MHCII and CD54-intercellular adhesion molecule upregulation in bronchial epithelial cells indicating a regulatory function of IL-22 in the context of IFN-γ-induced inflammation (Pennino et al, 2013). These findings led us to hypothesize that IL-22 may also affect the regulation of other proteins such as chemokines.

IFN-γ induces chemokines in epithelial cells such as colorectal cancer cells. In the intestinal mucosa, intestinal epithelial cells are an important source of chemokines. The observed CCL22 upregulation in HT29 upon IFN-γ stimulation is consistent with published data (Berin et al, 2001).

In the intestinal mucosa, multiple immune cells are capable of producing IFN-γ: B and T lymphocytes, mononuclear phagocytes, and dendritic cells, as well as smaller numbers of eosinophils and mast cells (Berin et al, 2001). We hypothesized that IL-22 might play a role in the tumor microenvironment by regulating chemokine expression in tumor cells. In our experiments, IL-22 did impair IFN-γ-induced chemokine release for CCL22 in HT29 cells.

Our findings suggest that IL-22 can inhibit some effects of IFN-γ. Induction of CCL22 in the tumor microenvironment can lead to the attraction of CCR4-positive immune cells such as Th-17 cells, Th-22 and Tregs. Further, Th-17 cells are attracted by CCL22 and their signature cytokine IL-17 exerts an inflammatory and protumorigenic role (He et al, 2012; Hyun et al, 2012). Th-22 cells also express CCR4 and produce mainly IL-22 (Duhen et al, 2009) and IL-22 can exert a protumorigenic role itself. The inhibition of CCL22 expression by IL-22 in this case would lead to less attraction of these cells and thus to an antitumor response.

However, Tregs play a crucial role during the control of antitumor immune responses. Tregs can produce immunosuppressive cytokines such as IL-10, TGF-ß and IL-35 (Nishikawa & Sakaguchi, 2010). Thus by inhibiting CCL22 secretion, IL-22 may reduce the attraction of immunosuppressive Tregs and thus exert a protumorigenic function.

IFN-γ upregulated IL-22-R1 in HT29 cells. This finding is supported by a study where keratinocytes upregulated IL-22-R1 upon IFN-γ (Kunz et al, 2006).

6.4.1.1. IL-22 and TNF-α synergistically induce CCL22 and in HT29 cells

We next investigated whether IL-22 can interfere with the TNF-α pathway in colorectal cancer cells. This is of particular importance, as these two cytokines are co-secreted by Th-22 cells (Eyerich et al, 2009).

TNF-α is a cytokine with well-described anti-cancer effects and has been used in anti-cancer therapy for patients suffering from solid tumors (Bertazza & Mocellin, 2010). However, in the recent years a dual role of TNF-α has been described, as this cytokine is also capable of driving tumor elimination (Lebrec et al, 2015). In colorectal cancer it has been reported that TNF-α might play a tumor-promoting role (Grivennikov & Karin, 2011).

For HT29 cells, TNF-α highly upregulates CCL22 (Berin et al, 2001) which is consistent with our data, showing upregulation of CCL22 by TNF-α on mRNA level. Addition of IL-22 to TNF-α on tumors cells led to a functional synergism of TNF-α and IL-22: the combination lead to upregulation of CCL22 in HT29 cells. As mentioned above, CCL22 is a chemokine that is a chemoattractant for Tregs and may thus anti-cancer immune responses.

So far, few studies investigated the functional interplay of IL-22 and TNF-α. Eyerich et al., could demonstrate that TNF-α and IL-22 synergistically induce an innate immune profile in human keratinocytes including IP10, CXCL12 and HBD-2 (Eyerich et al, 2011). This synergism is mediated by the MAP kinase p38.

IL-22-R1 in both keratinocytes and dermal fibroblasts is induced by TNF-α (Wolk et al, 2009; Wolk et al, 2004). These data strengthen our finding that TNF-α upregulates IL-22-R1 in HT29 cells. By upregulating IL-22-R1 on tumor cells, these cells become more sensitive to IL-22 and thus the synergistic effect of TNF-α and IL-22 might be enhanced.

6.5. Conclusion and perspectives

Since IL-22 plays a crucial role in inflammation and proliferation, it might have promise as a potential therapeutic for different cancer entities including colorectal cancer. Our data that IL-22 has an impact on colorectal cancer cells promotes the idea that IL-22-targeting may be a reasonable strategy for cancer therapy. As IL-22 does not act on immune cells itself, modulation of the IL-22/IL-22-R1 axis may have a different safety profile than the targeting of other cytokine pathways which than have profound immune suppressive effects.

Neutralizing antibodies could target IL-22. IL-22 neutralizing antibodies are being tested in autoimmune disorders such as psoriasis and RA (https://clinicaltrials.gov/ct2/show/NCT01941537). On the other hand, blockade of chemokines that attract IL-22 producing T cells such as CCL20 may diminish the impact of IL-22 on the tumor milieu.

Since the data presented here only touches on the biology of IL-22 further studies will be needed to better understand the role of IL-22 in cancer genesis and progression.

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8. Appendices

8.1. Abbreviations

AHR	Aryl hydrocarbon receptor
APC	Allophycocyanin
BSA	Bovine serum albumin
CCR	Chemokine receptor
CCL	Chemokine ligand
CD	Custer of differentiation
cDNA	Complementary deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle´s medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
HPRT	Hypoxanthine phosphoribosyltransferase
HRP	Horseradish peroxidase
h	Hours
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-22BP	IL-22 binding protein
IL-10-R2	IL-10 receptor-2
IL-22-R1	IL-22 receptor-1
IBD	Inflammatory bowel disease
JAK	Janus kinase
LTi	Lymphoid tissue inducer
LTi-like	Lymphoid tissue-like inducer
mRNA	Messenger ribonucleic acid

NK cells	Natural killer cells
NKT cell	Natural killer T cell
O	
PBS	Phosphate-buffered saline
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PI	Propidium iodide
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RORγt	Retinoic-acid-related orphan nuclear receptor
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH2	Sarcoma homology 2
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor-β
Th	T helper
TNF	Tumor necrosis factor
TYK2	Tyrosine kinase 2

8.2. Publications

Work performed during my doctoral thesis

1. Kobold S, Volk S, Clauditz T, **Küpper NJ**, Minner S, Tufman A, Duwell P, Lindner M, Koch I, Heidegger S, Rothenfuer S, Schnurr M, Huber RM, Wilczak W, Endres S (2013). Interleukin-22 is frequently expressed in small- and large-cell lung cancer and promotes growth in chemotherapy-resistant cancer cells.
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Work performed prior to doctoral thesis:

1. Muller AM, Poyser J, **Küpper NJ**, Burnett C, Ko RM, Kohrt HE, Florek M, Zhang P, Negrin RS, Shizuru JA (2014). Donor hematopoiesis in mice following total lymphoid irradiation requires host T-regulatory cells for durable engraftment.
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3. Muller AM, Shashidhar S, **Küpper NJ**, Kohrt HE, Florek M, Negrin RS, Brown JM, Shizuru JA (2012). Co-transplantation of pure blood stem cells with antigen-specific but not bulk T cells augments functional immunity.
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Abtracts

1. **Küpper NJ**, Völk S, Ochs C, Clauditz T, Minner S, Tufman A, Düwell P, Lindner M, Koch I, Heidegger S, Rothenfußer S, Schnurr M, Huber RM, Wilczak W, Endres S, Kobold S (2013).
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Analysis of T cells as potential sources of interleukin-22 in colorectal cancer

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Eidesstattliche Versicherung

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