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Assessing novel modalities for the therapy of inflammatory bowel disease

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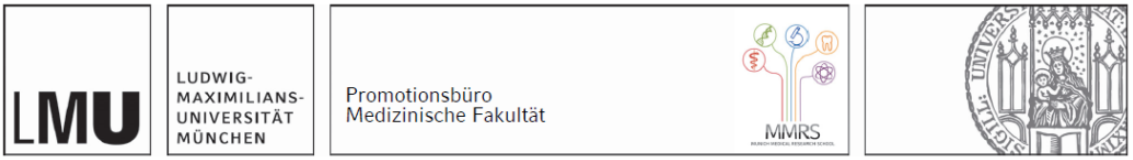

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List of abbreviations

ABBREVIATION	DEFINITION
ACD	allergic contact dermatitis
APC	antigen presenting cell
CD	Crohn's disease
CD3	cluster of differentiation - 3
CKD	chronic kidney disease
CRAC	calcium release-activated
CRP	C-reactive protein
CFSE	carboxyfluorescein succinimidyl ester
DAG	diacylglycerol
DC	dendritic cell
DSS	dextran sodium sulphate
ECM	extracellular matrix
ER	endoplasmic reticulum
FACS	fluorescence-activated single cell sorting
HGF	hepatocyte growth factor
IBD	inflammatory bowel diseases
IFN-γ	interferon-γ
IgG	immunoglobulin G
IHC	immunohistochemistry
IL	interleukin
IP3	inositol-1,4,5-triphosphate
MCP-3	monocyte chemotactic protein - 3
NFAT	nuclear factor of activated T-cells
NSG	NOD-scid IL2R γnull
PAP-1	5-(4-phenoxybutoxy)-psoralen
PBMCs	peripheral blood mononuclear cells
PLC	phospholipase C
SHK	Stichodactyla toxin
TCR	T-cell receptor
TEM	effector memory T-cells

TH	T helper cells
TNBS	2,4,6- trinitrobenzenesulfonic acid
TNF-α	tumor necrosis factor-α
TREG	regulatory T-cell
TSLPR	thymic stromal lymphopoietin receptor
UC	Ulcerative colitis

List of publications

I. “Suppressing Kv1.3 Ion Channel Activity with a Novel Small Molecule Inhibitor Ameliorates Inflammation in a Humanized Mouse Model of Ulcerative Colitis.“

A Unterweger, M Ø. Jensen, F Giordanetto, V Jogini, A Rüschher, M Seuß, P Winkelmann, L Koletzko, D E. Shaw, M Siebeck, R Gropp, F Beigel, A Aszodi, Journal of Crohn's and Colitis, April 2021, DOI:10.1093/ecco-jcc/jjab078

II. NOD/scid IL-2Rγnull mice reconstituted with peripheral blood mononuclear cells from patients with Crohn’s disease reflect the human pathological phenotype.”

A Unterweger, A Rüscher, M Seuß, P Winkelmann, F Beigel, L Koletzko, S Breiteneicher, M Siebeck, R Gropp, A Aszodi, Immunity, Inflammation and Disease, September 2021, DOI: 10.1002/iid3.516

Contribution to the publications

1.1 Contribution to paper I

"Suppressing Kv1.3 Ion Channel Activity with a Novel Small Molecule Inhibitor Ameliorates Inflammation in a Humanized Mouse Model of Ulcerative Colitis."

A-L.U. ex vivo experiments (cell culture, flow cytometric analysis, Luminex assay, immunohistochemistry of PBMCs), animal studies (conducting the study protocol, immunohistochemistry, histology) and data analysis (flowjo, R statistics).

M.Ø.J., F.G., V.J., D.E.S. inhibitor design, study design, writing of the manuscript; M.Ø.J. data analysis; A.R. IHC, animal study; M.S., P.W. animal study; L.K. patient recruitment, patient history; M.Sie. conception of the study; F.B. conception of the study, patient recruitment, patient history; R.G. conception of the study, data analysis, writing of the manuscript; A.A conception of the study.

1.2 Contribution to paper II

"NOD/scid IL-2Rγ^{null} mice reconstituted with peripheral blood mononuclear cells from patients with Crohn's disease reflect the human pathological phenotype."

A.-L.U. ex vivo analysis (flow cytometric analysis, Luminex assay), animal studies (conducting the study protocol, immunohistochemistry, histology).

M.S. and A.R. histological analysis; P.W. animal studies and cytokine expression analysis; S.B., F.B., L.K. recruitment of patients, patient history; M.S. study design and analysis of data, R.G. writing of the manuscript, data analysis, study design. A.A. writing of the manuscript.

2. Introduction

2.1 IBD

Ulcerative colitis (UC) and Crohn's disease (CD) are part of the chronic inflammatory bowel diseases (IBDs), which set up abdominalgia, diarrhea, and rectal bleeding (Conrad, Roggenbuck, & Laass, 2014). The symptoms of UC are dominated by the inflammation of the surface area of the colonic mucosa, whereas in CD the inflammatory process can impair the gastrointestinal system thoroughly and penetrates the mucosal wall leading to complications like strictures, abscesses, and fistulas. The reported incidence of IBD is a rate of 1.2 to 20.3 individuals in 100,000 in one year, and a prevalence of 7.6 to 245 developing diseases per 100,000 people in one year, which is increasing due to an adaption of a western lifestyle in many countries all over the world (Feuerstein & Cheifetz, 2014). The onset of the disease is primarily during adolescence and young adulthood and is not sex specific (Rosen, Dhawan, & Saeed, 2015). As the underlying cause of these pathologies is still not fully understood, current research investigates immunological, genetic, and environmental factors that can play an important role in these diseases and lead to the dysregulated mucosal immune response against commensal intestinal microbiota (Khor, Gardet, & Xavier, 2011).

One of the latest theory states that the overlapping factors of an increased T-cell immune response to a subgroup of nonpathogenic intestinal bacteria in a genetically predisposed host and the environment induce the onset or reactivation of IBD (Sartor, 2006).

Studies already showed that Western diet, left-handedness, and depression may increase the risk for UC. Furthermore, the patients show higher levels of specific inflammatory marker, increasing oxidative stress levels, a unbalanced intestinal milieu and a higher intestinal patency (Head & Jurenka, 2003).

At present, the main focus of research lies on the interactions of the intestinal flora and defense mechanisms of the local immune system (Khor et al., 2011). Mutations in functional genetic regions for autophagy, microbe recognition, lymphocyte and cytokine signaling and many more have been detected in IBD patients and are consequently special targets of research (Jostins et al., 2012).

In a recent study we could show that the inflammation in IBD patients is very dynamic and comprises two stages, a pro-inflammatory and a remodeling phase. The immunologic profiles revealed two subsets of patients that are characterized either by T- helper-cells (TH) 1, TH2 T-cells or by TH17 T-cells and monocytes (M1 and M2), respectively. Both processes can act solitarily and coexist at the same time with one or the other predominating (Jodeleit, et al., 2020).

Our research theory is based on the hypothesis that the inflammation in the intestine, to a certain degree, reflects to a certain degree a wound healing process (Fohlinger et al., 2016). This process involves cells from the immune system, from the epithelium, the endothelium, and from muscles, as well as fibroblasts (Medzhitov, 2010). Those ensure the intestinal defense from invasive pathogens, the restoration of wounds, while terminating inflammation (Werner & Grose, 2003). An injury of the intestine initiates an inflammatory response during the physiological wound healing process that repairs the damaged tissue and restores the normal structure and functionality (Speca, Giusti, Rieder, & Latella, 2012). If the mechanism of resolution collapse due to consistent injury, a repetitive cycle of continuous injury and repair is induced resulting in a chronic inflammatory condition that can be accompanied by the development of fibrosis (Lovisa, Genovese, & Danese, 2019).

A normal wound healing consists in general of restitution, proliferation, and differentiation of epithelial cells in the damaged tissue of the intestine (Iizuka & Konno, 2011). The first response after an injury is a pro-inflammatory type 1 immune reaction. Subsequently, an anti-inflammatory type 2 wound repair process induces tissue regeneration and homeostasis (Rankin & Artis, 2018). Hematopoietic immune cells (i.e., lymphocytes, dendritic cells (DCs), monocytes, macrophages, and granulocytes) communicate in these processes with progenitor and stromal cells via the secretion of cytokines and growth factors to promote wound repair in the injured tissue. The first cascade of a nonspecific and fast resistance against pathogens is the innate immunity, which consists of neutrophils, macrophages and DCs. The adaptive immunity (i.e., T- and B-cells) provides specific long-lasting memory after activation (Xue & Falcon, 2019). Neutrophiles are the first reacting leucocytes at the breached epithelial barrier and can have destructing and protecting effects in wound healing processes through controlling the infiltration of pathogens and the attraction of monocytes and/or macrophages (Nathan, 2006). Inflammatory macrophages (M1) fur-

ther eliminate pathogens. After closing of the wound, they convert into healing / remodeling macrophages (M2) through the efferocytosis of apoptotic neutrophils (Ferrante & Leibovich, 2012; Mosser & Edwards, 2008).

T-cell activation is induced by antigen presenting DCs, which are representing a juncture between the innate and adaptive immunity (Rossi & Young, 2005). T-cells have a variety of functions such as for naïve T cells the response to novel antigens, memory T-cells maintain a lasting immunity and regulatory T-cells (Tregs) regulate immune reactions (Kumar, Connors, & Farber, 2018). The innate immune responses trigger naïve T helper cells to develop into effector T-cells. TH1 cells release interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) for the activation of macrophages and cytotoxic T-cells and induce the release of immunoglobulin G (IgG) antibodies from B-cells (Zhang, Liu, & Shao, 2020). TH2 cells produce interleukin 4, 5, 10 and 13 (IL-4, -5, -10- and -13) to avert pathogens and to stimulate B-cells to secrete most classes of antibodies (Zhu, 2018). TH17 cells produce IL-17A and IL-17F to attract neutrophiles and stimulate different cell types to release cytokines and IL-22 to provoke the release of antimicrobial peptides and pro-inflammatory cytokines and chemokines of cells at mucosal barriers (Liang et al., 2006).

In the course of an inflammation, the infiltrating immune cells like T-cells, neutrophils and macrophages generate tissue damage by the distribution of reactive oxygen species and tissue harming enzymes. Furthermore, immunomodulating cytokines and cell-activating and chemotactic peptides are secreted. This results in the damage of epithelial cells and the extracellular matrix (ECM) in the lamina propria, clinically resulting in ulcerations. In severe cases of injuries, myofibroblasts can migrate to the wound and produce ECM. This process plays an important part in the physiological wound healing and remodeling process and is altered during chronic inflammation. Insufficient wound healing induces abscesses or fistulas, while excessive wound healing and remodeling can lead to fibrosis. These complications are the main causes for surgeries in IBD patients. The trigger of increased fibrosis in some patients is still unclear yet it is described as a pathologically increased healing reaction to an inflammation-induced impairment of the mucosa (Rieder, Brenmoehl, Leeb, Scholmerich, & Rogler, 2007).

2.2 The KV1.3 potassium channel

The inhibition of T-cell/lymphocyte activation and proliferation has for a long time been a therapeutic target, as IBD (UC/CD) is characterized by T-cell/lymphocyte infiltration and excessive cytokine expression (Lin, 1993). Immunomodulating components like azathioprine, 6-mercaptopurine and cyclosporine are administered to patients to suppress the T-cell functions, but despite their efficacy, they are not favored due to many severe side effects accompanying the highly immune suppressive therapy (Jani & Regueiro, 2002).

An alternative target to inhibit T-cell activation is KV1.3, a voltage gated potassium channel. This channel is especially found in human inflammatory immune cells like activated T-cells, as well as B-cells and monocytes (Land et al., 2017; Winslow, Neilson, & Crabtree, 2003).

The KV1.3 potassium channel is crucial for the control of the membrane potential, as the efflux of potassium balances the influx of calcium during the T-cell activation. The first model that describes the impact of KV1.3 of activated T-cells is the “membrane potential model” (Teisseyre, Palko-Labuz, Sroda-Pomianek, & Michalak, 2019). The channel is located in the “immunological synapse” in-between the T-cell receptor (TCR) and an antigen presenting cell (APC). Upon initiation of the TCR and cluster of differentiation (CD) -3 complex by antigen APCs like B-cells, macrophages, or dendritic cells (DCs), the enzyme phospholipase C (PLC) is activated. This leads to an increasing release of inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). After the binding of DAG to its receptor on the endoplasmic reticulum (ER), calcium ions can efflux from the ER, as the receptor is a calcium-selective receptor-gated channel. Calcium influx is subsequently ensured by the activation of a voltage-independent calcium release-activated (CRAC) channel located in the plasma membrane (Perez-Garcia et al., 2017). In response to the increased cytosolic calcium levels, the transcription factor nuclear factor of activated T-cells (NFAT) gets dephosphorylated by the phosphatase calcineurin. As a result, NFAT relocates to the nucleus and activates the production IL-2. This cytokine is a T-cell growth factor that promotes T-cell proliferation (Chandy et al., 2004). To preserve a fluctuating calcium influx, the alternate activation of the potassium channel Kv1.3 activates the efflux of potassium ions resulting in a hyperpolarization of the plasma membrane. Inhibition of these channels depolarizes the cells, as the influx

of calcium required for a calcium-dependent cell activation and proliferation is impaired without affecting other lymphoid subsets (Shah, Tom Blake, Huang, Fischer, & Koo, 2003).

As KV1.3 mRNA expression is increased in active UC compared to controls, targeting potassium channels could therefore be a promising therapeutic approach in IBD because the channel is especially expressed in activated immune cells (Koch Hansen et al., 2014). Furthermore, Hansen et al., 2014 could reveal the role of upregulated Kv1.3 in regulating effector memory T-cells (TEM), which mediate autoimmune diseases. Increased expression has also been observed in indications like asthma (Koshy et al., 2014), arterio-sclerosis (Wu et al., 2015), allergic contact dermatitis (ACD) (Azam, Sankaranarayanan, Homerick, Griffey, & Wulff, 2007), age-dependent hypertension (L. P. Wang et al., 2015) and chronic kidney disease (CKD) (Kazama, 2015). Moreover, it is linked to insulin sensitivity, cell proliferation and apoptosis, and plays also a role in obesity, non-insulin-dependent type II diabetes mellitus and cancer (Perez-Garcia, Cidad, & Lopez-Lopez, 2018).

It has been shown that the suppression of the activity of T-cells via Kv1.3 inhibitors is a complicated process depending on a variety of factors like the qualities of the stimulus, the T-cell identity and response (Fung-Leung et al., 2017). Thus, also a partial inhibition can be possible. Furthermore, as the channel inhibition targets predominantly activated lymphocytes, overall a milder immune suppression is induced (Chiang et al., 2017). Therefore, this form of therapy form could be safer and more effective for patients with autoimmune diseases than existing immune suppressants drugs like cyclosporine A (Fung-Leung et al., 2017).

Furthermore, there is already promising data from clinical research about molecules that inhibit KV1.3, like the Stichodactyla peptide toxin (ShK) (Castaneda et al., 1995) and 5-(4-phenoxybutoxy)-psoralen (PAP-1), a small molecule (Schmitz et al., 2005). The data of preclinical experiments showed promising results in models of diseases like asthma and rheumatoid arthritis (Koshy et al., 2014); (Beeton et al., 2006). The efficacy of the variant ShK-186 (dalazatide) was also proved during early clinical trials. Phase 1a and 1b trials were completed in 2016, where it was well tolerated and ameliorated skin lesions in mild-to moderate plaque psoriasis (Tarcha et al., 2017). Since then, dalazatide shows a good efficacy in the treatment of numerous autoim-

mune diseases, like arthritis, lupus, ANCA vasculitis, psoriatic arthritis, rheumatoid Type 1 diabetes, inclusion body myositis, multiple sclerosis, psoriasis and inflammatory bowel diseases (Chandy & Norton, 2017; Liao, Feng, Yang, & Lee, 2019; X. Wang et al., 2019).

2.3 NSG-UC mouse model

Mouse models reflecting the human disease are important tools in clinical development. Conventional animal models for UC use toxins like, dextran sodium sulphate (DSS), oxazolone or 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Kiesler, Fuss, & Strober, 2015) to induce symptoms of colitis. However, these models poorly reflect the human disease characterized by highly variable manifestations regarding the onset of the disease, severity, length of phases of relapse and remission, and response to therapies. In addition, therapeutics developed against human targets cannot be tested if the interaction of ligand and receptor requires high structure homology, as it is descripted for the KV1.3 potassium channel.

Mouse T-cells are regulated by more than one KV1.x channel (Ishida & Chused, 1993). Therefore, studies so far have been conducted in rats which show more consistency with the human mode of action and revealed the coexistence of the channels KCa3.1 and KV1.3 (Koo et al., 1997).

In 2013, a humanized UC mouse model was developed which comprises immunocompromised NOD-scid IL2R γnull (NSG) mice reconstituted with peripheral blood mononuclear cells (PBMCs) derived from UC patients (NSG-UC). In this mouse model, disease-like symptoms were triggered with a rectal challenge with ethanol or oxazolone (Nolte et al., 2013). Since then, the model has been further characterized and used to validate a variety of therapeutics (Jodeleit et al., 2018; Jodeleit, et al., 2020; Unterweger et al., 2021; Winkelmann et al., 2021). One important feature of the NSG mice is the partial conservation of the immunological profile of the respective patient. Therefore, the model not only enables the validation of agents for human target molecules but also the stratification of donors in later clinical trials.

Rectal application of ethanol provokes the infiltration of human immune cells into the lamina propria containing CD8+, CD4+ T-cells, CD11b+ macrophages, B-cells, neutrophils and CD14+ mon-

ocytes. CD14+ monocyte subtypes included CD64-(M1), CD163-(M2), thymic stromal lymphopoietin receptor (TSLPR)- and CD1a-expressing monocytes. C-reactive protein (CRP), monocyte chemotactic protein-3 (MCP-3), hepatocyte growth factor (HGF) and IL-6 were identified as inflammatory biological markers. The inflammation caused a modified colon architecture characterized by oedema, fibrosis, hemorrhage and crypt abscesses (Jodeleit, et al., 2020; Palamides et al., 2016; Winkelmann et al., 2021).

In summary, this chimeric mouse model reflects a clinically relevant phenotype to validate novel therapeutics for IBD such as KV1.3 channel antagonists.

Therefore, we chose this NSG-UC mouse model for the preclinical testing of DES1, a novel small molecule Kv1.3 inhibitor obtained from the research company D.E. Shaw Research (New York, USA). To further assess, whether DES1 is also efficacious in CD, we developed a mouse model for CD based on the same principal as the NSG-UC model.

2.4 Aims of the thesis

The aims of this thesis consisted first of the preclinical validation of the KV1.3 potassium channel as a potential therapeutic target in IBD, secondly of the preclinical validation of the novel small molecule KV1.3 inhibitor DES1 and thirdly, of the establishment of a novel Crohn's disease mouse model.

For the preclinical validation of KV1.3 two approaches were planned. Firstly, the expression pattern of KV1.3 was examined by fluorescence-activated single cell sorting (FACS) analysis in colon biopsies of UC patients, in PBMC samples of IBD patients and non-IBD donors. Secondly, the distribution of the channel was visualized by immunohistochemistry (IHC) staining of PBMCs and colon biopsies. An increased expression of the channel in patient samples corroborated the KV1.3 channel as a possible therapeutic target in IBD.

The next goal of this thesis was the preclinical validation of a novel small molecule KV1.3 inhibitor DES1. In vitro, the suppressing effect of the KV1.3 inhibitor DES1 was examined in cell cultures of PBMCs derived from healthy donors in which T- cells were activated by anti-CD3 monoclonal antibodies and treated with DES1. The readout was the frequencies of activated CD4+ T-cells (CD4+ CD25+, CD4+ CD69+) determined by FACS analysis and quantity of cytokines in the cell culture supernatant determined by Luminex analysis. In addition, carboxyfluorescein succinimidyl ester (CFSE) flow cytometry revealed the proliferation of CD4+ T-cells. The data showed that levels of activated T-cells and cytokines decreased upon exposure to DES1 in a dose dependent manner and the inhibitor impaired the proliferation of T-cells. These results corroborated DES1 as a promising inhibitor and allowed for taking the validation to the next level.

In vivo, DES1 was validated using the NSG-UC model in a head-to-head study with standard of care therapeutics infliximab and tofacitinib. To assess the therapeutic efficacy of all three therapeutics the following parameters were determined: the clinical-, histological- and colon scores of the mouse model and inflammatory markers detected in colon and serum. A significant amelioration of the scores, as well as a decline of the inflammatory markers in the samples of the treated mice was proof of the efficacy of DES1. Orthogonal partial least square discrimination analysis

showed a slightly higher efficacy of DES1 as compared to the effect of infliximab and tofacitinib. Moreover, immune profiles of the donors selected for reconstitution were generated and the response to the treatment with DES1 was analyzed in the respective mice. This analysis suggested a stronger response to DES1 in patients exhibiting higher frequencies of activated T-cells. This observation might lead to a stratification of patients for future clinical trials.

The third aim of this thesis was the development of a Crohn's disease mouse model. This model will be useful in the future to assess whether DES1 could be also efficacious in CD. First, immune profiles of CD patients were compared to those of UC patients. Data revealed that most of CD patients exhibited a profile characterized by TH1/TH2 cells. Furthermore, this analysis revealed a role of CD14+ CD163+ monocytes in CD.

As opposed to the NSG-UC mouse model, the NSG-CD mice were reconstituted with PBMCs from CD patients. The clinical-, histological- and colon scores, frequencies of leukocytes in the spleen and colon as well as inflammatory markers in the colon were determined and compared to those of the NSG-UC model. These analyses revealed significant differences in both models. In contrast to NSG-UC mice that were characterized by a highly inflammatory phenotype, NSG-CD mice exhibited a remodeling phenotype which could reflect the ongoing fibrosis in CD patients. To further support this observation, immunohistochemistry of fibrotic areas of the mouse colon sections were analyzed for the presence of fibrocytes using the markers CD45+ and COL1A1. This analysis demonstrated the presence of fibrocytes in areas of fibrosis.

In summary, these results show that the mouse model partially reflects the human disease and can be used for preclinical in vivo studies in the future.

3. Zusammenfassung

Im ersten Schritt der präklinischen Validierung des KV1.3 Kaliumkanal als therapeutisches Ziel in der Behandlung von IBD stand die Untersuchung des Expressionsvorkommen des KV1.3 Kanals in Dickdarmbiopsien von UC Patienten.

Die Analyse mittels Durchflusszytometer konnte den Nachweis von KV1.3 exprimierenden CD4+ T-Zellen in Dickdarmbiopsien aufzeigen. Es wurde aber kein Unterschied zwischen den entzündlichen und nicht-entzündlichen Stellen des Dickdarms festgestellt.

In den mononukleäre Zellen des peripheren Blutes (PBMCs, Akronym für englisch: Peripheral Blood Mononuclear Cells) zeigten die Daten eine höhere Expression von KV1.3 auf den CD4+ T-Zellen in UC Patienten als in PBMCs von nicht-IBD Spendern. Diese Ergebnisse spiegeln sich auch in den erhöhten Vorkommnissen von TH1 und TH2 Zellen wider.

Zusätzlich zeigte die immunohistochemische Färbung (IHC) der PBMCs und der Dickdarmbiopsien eine Ko-expression von CD4+ und KV1.3 an den fokalen Bereichen der äußeren Membran der T-Zellen.

Zweitens wurde der hemmende Effekt des KV1.3 Kaliumkanal Inhibitor DES1 wurde auf die Aktivierung von anti-CD-3 aktivierten T-Zellen in einem in vitro Versuch demonstriert.

Die Daten des Durchflusszytometers zeigten, dass DES1 den Anstieg von aktivierten CD4+ T-Zellen (CD4+ CD25+, CD4+ CD69+) nach der Stimulierung mit anti-CD-3 Antikörpern dosisabhängig hemmen konnte. Diese Ergebnisse konnten durch die Luminex Analyse der Zellkulturüberstände bestätigt werden, da hier ein Rückgang der IFNy-, IL-4-, IL-10- Zytokin Ausschüttung erkennbar war. Des Weiteren, zeigte die Carboxyfluorescein-succinimidyl-ester (CFSE) durchflusszytometische Analyse eine Inhibition der CD4+ T-Zell Proliferation.

Als drittens wurde die Wirksamkeit von DES1 in vivo in dem NSG-UC Mausmodell im Vergleich zu den standardisierten Therapeutika Infliximab und Tofacitinib validiert.

Die Daten der Experimente zeigten eine Verbesserung des Zustandes der erkrankten Mäuse durch die Behandlung und eine erhöhte Wirksamkeit von DES1 gegenüber den anderen Medikamenten. Eine Verbesserung hinsichtlich der klinischen-, Dickdarm-, und histologischen Bewer-

tung war deutlich nach der Gabe von DES1 zu sehen. Genauso die Ausschüttung der Entzündungsmarker msMCP-3 im Dickdarm und Tryptophan im Serum der Mäuse nahm ab. Außerdem konnte durch die Analyse der immunologischen Profile der Spender eine bessere Wirksamkeit bei rekonstituierten Mäusen mit PBMCs mit aktivierten CD4+ T-Zellen nachgewiesen werden. Folglich könnte diese Methode der Stratifizierung von Patienten für zukünftige klinische Versuche verwendet werden.

Um abschätzen zu können, ob DES1 auch in Morbus Crohn (CD, Akronym für englisch: Crohn's Disease) wirksam sein kann, haben wir ein Mausmodell für CD entwickelt, welches auf den gleichen Prinzipien wie das NSG-UC Mausmodell basiert. In diesem NSG-CD Mausmodell, wurden Mäuse mit CD-Spender PBMCs rekonstituiert.

Wie bei dem NSG-UC Mausmodell wurde vor der Rekonstitution der Mäuse ein Immunprofil der Spender erstellt. Die Immunprofile der CD-Patienten gruppieren vorwiegend in dem entzündungstreibenden Arm, welcher durch erhöhte Zahlen an TH1/TH2 und isotyp-gewechselte B-Zellen charakterisiert ist. Ebenso wurden weniger M2 (CD14+ CD163+) Monozyten in CD-Patienten PBMCs nachgewiesen.

Im nächsten Schritt wurde der Phänotyp der NSG-Mäuse mit den der NSG-UC und NSG-nicht-IBD Mäusen in histologischen Untersuchungen verglichen. Der Phänotyp der NSG-CD Mäuse wies deutliche Unterschiede zu den NSG-UC Mäusen auf. Im Gegensatz zu dem NSG-UC Model, war der Einstrom der Entzündungszellen und das Entstehen der Ödeme weniger ausgeprägt. Stattdessen konnten signifikante Veränderungen in der Struktur des Dickdarmes gesehen werden, angedeutet durch Becherzellverlust, Krypelongation und Fibrose.

Zusätzlich zeigte die Duchflusszytometrie, dass die Anzahl der M2 Monozyten und die der aktivierten T-Zellen (CD4+ CD69+) in den NSG-CD Maus Milzen und Dickdärmen höher waren als die in denen der NSG-UC Mäusen. Maus Neutrophile waren nur in NSG-UC Mäusen erhöht. Diese Daten unterstützten die These einer umformenden/exzessiv wundheilenden Entzündungsreaktion in NSG-CD Mäusen.

Die Entzündungsmarker der beiden Mausmodelle spiegeln die verschiedenen pathologischen Phänotypen wider. Die Luminex Analyse wies einen erhöhten CRP Spiegel in NSG-UC Mäusen

nach, welcher auf ein proinflammatorisches Profil hinweist. Wohingegen die erhöhten HGF- und TGFbeta- Spiegel in den NSG-CD Mäusen wiederum auf ein umformendes Profil deutet und die ablaufende Fibrose und Kyptelongation im Dickdarm erklären kann.

Zuletzt konnten mittels IHC humane Fibrozyten (CD45+ COL1+) und erhöhte Kollagenablagerungen (COL1A1) in den fibrotischen Bereichen der Dickdarmschnitte identifiziert werden.

Diese Ergebnisse zeigten den umformenden Prozess der Entzündung des Dickdarms, welcher exzessive Fibrose beinhaltet und somit zusammenfassend die klinische Manifestation von CD aufzeigt.

Obwohl dieses Modell einige Limitationen aufweist, konnten wir in dieser Studie zeigen, dass unser neues Mausmodell die humane Krankheit Morbus Crohn teilweise widerspiegelt und für zukünftige präklinische in vivo Versuche von großem Nutzen sein kann.

4. Abstract

For the preclinical validation of the Kv1.3 potassium channel as a possible therapeutic target in IBD, we first revealed the expression pattern of the KV1.3 channel in colon biopsies of UC patients.

The flow cytometric analysis detected KV1.3 expressing T-cells in colon biopsies. No difference was discovered between the inflamed and the un-inflamed regions.

The expression pattern of the KV1.3 channel in PBMCs of IBD patients in the comparison to non-IBD donors showed higher expression levels of the KV1.3 channel on CD4+ T-cells in UC donors. This result correlates also with elevated incidences of TH1 and TH2 T-cells. Additionally, the IHC staining of PBMCs and the colon biopsies revealed the co-expression of KV1.3 with CD4+ in focal regions of the surface membrane of the T-cells.

Secondly, for the preclinical validation of a small molecule KV1.3 inhibitor (DES1), we demonstrated the suppressing effect of the inhibitor on the anti-CD3-driven T-cell activation in vitro. The flow cytometric analysis showed that DES1 reduced the increase of activated CD4+ T-cells (CD4+ CD25+, CD4+ CD69+) upon anti-CD3 antibody stimulation in a concentration dependent manner. These findings were further corroborated by the Luminex data of the cell culture supernatants, which also showed declining IFNy-, IL-4- and IL10- cytokine secretion. Furthermore, the CFSE flow cytometric assay revealed the inhibition of CD4+ T-cell proliferation.

Thirdly, the efficacy of the novel small molecule DES1 was validated in vivo in the NSG-UC mouse model in a head-to-head study with standard of care therapeutics infliximab and tofacitinib. The data revealed that the diseased mice benefited from the efficacy of the inhibitor DES1 and that it was slightly superior to that of infliximab or tofacitinib. The clinical-, histological- and colon scores ameliorated upon the administration of DES1. Likewise, the level of the inflammatory marker msMCP-3 in the colon and tryptophan in the serum declined. Moreover, immune profiles of the donors appointed for reconstitution suggested that the effectiveness was higher in the mouse model reconstituted with PBMCs from donors with activated CD4+ T-cells. Thus, this approach might be used for the stratification of donors for future clinical trials.

Furthermore, to assess whether DES1 could be also efficacious in CD, we developed the NSG-CD model, based on the same principal as the NSG-UC model, but the mice were reconstituted with PBMCs derived from CD patients.

Like in the NSG-UC mouse model, the analysis comprised the immune profiling prior to reconstitution. The immune profiles of CD patients clustered predominantly in the inflammatory arm characterized by increased frequencies of TH1/TH2 cells and switched B-cells. Additionally, a proportion of CD patients showed decreased frequencies of M2 (CD14+ CD163+) monocytes.

The phenotype of the NSG-CD mouse was compared to those of NSG-UC and NSG-non-IBD mice by histological analysis. The phenotype of NSG-CD differed significantly from that of the NSG-UC model. In contrast to the NSG-UC mice, the influx of inflammatory cells and the development of oedema were lesser pronounced. Instead, significant changes in colon architecture were observed, signified by goblet cell loss, crypt elongation and fibrosis.

In addition, flow cytometric analysis showed that the frequencies of activated T-cells (CD4+ CD69+) and M2 monocytes were higher in the NSG-CD spleen and colon as in those of the NSG-UC mice. Mouse neutrophiles were only increased in NSG-UC colons. These data corroborated a suggested remodeling inflammation in NSG-CD mice.

In the next step, the levels of biomarkers reflected the different pathological phenotype in both models. In the Luminex assay the NSG-UC mice showed higher levels of CRP, which represents a more pro-inflammatory profile. Furthermore, the levels of HGF and TGFbeta in NSG-CD mice are increased, which revealed a remodeling profile and could explain the ongoing fibrosis and crypt elongation in the colon.

Finally, immunohistochemistry could identify human fibrocytes (CD45+ COL1+) and increased collagen (COL1A1) deposition in the fibrotic areas of the mouse colon sections. These results showed a remodeling process of inflammation including extensive fibrosis, that also reflects the clinical manifestations of CD.

Regardless of certain limitations of this model, this study showed, that our novel mouse model can partially reflect the human disease and can be used in the future for preclinical in vivo studies.

5. Paper I

“Suppressing Kv1.3 Ion Channel Activity with a Novel Small Molecule Inhibitor Ameliorates Inflammation in a Humanized Mouse Model of Ulcerative Colitis.“

A Unterweger, M Ø. Jensen, F Giordanetto, V Jogini, A Rüschher, M Seuß, P Winkelmann, L Koletzko, D E. Shaw, M Siebeck, R Gropp, F Beigel, A Aszodi, Journal of Crohn's and Colitis, April 2021, DOI:10.1093/ecco-jcc/jjab078

6. Paper II

“NOD/scid IL-2Rγnull mice reconstituted with peripheral blood mononuclear cells from patients with Crohn’s disease reflect the human pathological phenotype.”

A Unterweger, A Rüscher, M Seuß, P Winkelmann, F Beigel, L Koletzko, S Breiteneicher, M Siebeck, R Gropp, A Aszodi, Immunity, Inflammation and Disease, September 2021, DOI: 10.1002/iid3.516

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