Analysis of the role of IL-10 signaling for T_R1 cell differentiation, stability and function

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Summary

Chronic inflammatory and autoimmune diseases, as well as allergies, are continuously increasing threats, especially in developed countries. The disruption of the immune homeostasis in response to self- or non-pathogenic foreign-antigens is likely to be caused by false regulation of $CD4^+$ T cells. This dysbalance can either be caused by an overreaction of effector T cells such as T_H1 and T_H17 cells, or by a dysfunction of regulatory T cells, such as $Foxp3^+$ Treg cells or type one regulatory T cells (T_R1 cells). Accordingly, adoptive transfer of regulatory T cells could potentially play a significant role in new therapies for these diseases. Indeed, initial clinical trials have already shown promising results. Regulatory T cells, both $Foxp3^+$ Treg cells and T_R1 cells, have the potential to re-introduce peripheral tolerance by releasing the anti-inflammatory cytokine IL-10. However, cell stability and therefore function of regulatory T cells is of great importance for the safety and success of a regulatory T cell-based therapy. Noteworthy, regulatory T cell therapies based on $Foxp3^+$ Treg cells have been recently challenged by studies in mouse models, which showed that some of these cells can indeed convert into pathogenic T cells and favor inflammatory diseases, rather than block them. Thus, signals and mechanisms that sustain the functional stability of regulatory T cells have to be intensively studied. T_R1 cell biology is still controversially discussed. IL-10 was (long) considered to be the driving cytokine for T_R1 cell differentiation, but recent studies showed that T_R1 cells can emerge in the complete absence of IL-10. Signals that maintain the stability of T_R1 cells still remain unknown. Nevertheless it has been shown that IL-10 signaling could sustain IL-10 production and in turn functional stability in $Foxp3^+$ Treg cells. Therefore, one aim of this thesis is to characterize the role of IL-10 for T_R1 cell differentiation, stability and function. To address this question we used murine models of intestinal inflammation and transgenic mice, which allowed us to analyze the role of IL-10 signaling in Tr1 cells. The use of a transgenic mouse model in which $CD4^+$ T cells display a specific blockade of IL-10 signaling revealed that IL-10 was not essential for T_R1 cell differentiation *in vivo*. But IL-10 signaling was crucial to maintain the regulatory function of T_R1 cells in a colitis model that resembles the use of T_R1 cells as T cell-based therapy for severe Crohn's disease in humans. Mechanistically, p38 MAP kinase was

identified to be activated downstream of IL-10 receptor signaling in T_R1 cells, thereby furthermore sustaining their IL-10 production. These findings were also confirmed using mature human T_R1 cells. Importantly, data obtained in a mouse model of GvHD also indicate that even if T_R1 cells lose their regulatory activity in the absence of IL-10 signaling, they still do not promote disease. This suggests that T_R1 cell–based therapies in humans would be safe.

Additionally, a second aim of this thesis is to identify highly suppressive T_R1 cells among the heterogeneous IL-10 producing $CD4^+$ T cell subset based on the use of two surface markers, CD49b and LAG-3. The use of surface markers to identify regulatory T cells, such as T_R1 cells, allows the identification and isolation of viable cells that could be used as T cell therapy to treat chronic inflammatory conditions and autoimmunity in human. Indeed, IL-10 producing $CD49b^+$ $LAG\text{-}3^+$ T cells could be identified to display the strongest suppressive capacity and regulatory phenotype compared to those that do not express CD49b and LAG-3. These findings support the efficiency of these two markers to identify T_R1 cells. Nevertheless, further experiments are required to analyze additional regulatory T cell markers and to confirm these findings in human T_R1 cells.

Zusammenfassung

Chronisch-entzündliche Krankheiten und Autoimmunerkrankungen, ebenso wie Allergien, stellen ein zunehmendes gesundheitliches Problem für die Bevölkerung in Industrieländern dar. Die Störung der Immunhomöostase durch eine Immunantwort gegen Autoantigene und Allergene wird sehr wahrscheinlich durch eine Fehlregulation von $CD4^+$ T-Zellen verursacht. Es kann dabei zu einer Überreaktion von Effektor-T-Zellen, wie T_H1 und T_H17 Zellen, oder zu einer Fehlfunktion von regulatorischen T-Zellen, wie $Foxp3^+$ Treg Zellen und Typ 1 regulatorischen T-Zellen (T_R1), kommen. Der adoptive Zell-Transfer von regulatorischen T-Zellen stellt hierbei einen neuen Ansatz dar, solche Krankheiten zu therapieren. Erste klinische Studien zeigen bereits positive Ergebnisse für den behandelten Patienten. Regulatorische T-Zellen, sowohl $Foxp3^+$ Treg Zellen als auch T_R1 Zellen, besitzen das Potential durch die Freisetzung des anti-inflammatorischen Zytokins IL-10 die periphere Toleranz wieder herzustellen. Allerdings ist für die Sicherheit und den Erfolg einer solchen Therapie, die Stabilität und Funktion der transferierten Zellen entscheidend. Erkenntnisse, nach welchen $Foxp3^+$ Treg Zellen in pathogene Effektor-T-Zellen konvertieren können und somit den Krankheitsverlauf begünstigen könnten, haben die Verwendung von diesen Zellen als T-Zell-basierte Therapie in Frage gestellt. Daher ist es von entscheidender Bedeutung Signalwege und Mechanismen zu identifizieren, welche die Stabilität und Funktionalität von regulatorischen T-Zellen erhalten. Vor allem das Wissen bezüglich T_R1 Zellen ist sehr kontrovers. IL-10 wurde als das entscheidende Zytokin für die T_R1 Differenzierung angesehen. Jedoch wurde in weiteren Studien gezeigt, dass T_R1 Zellen in der Abwesenheit von IL-10 *in vivo* entstehen können. Ferner sind Signalwege, welche die T_R1 Zellstabilität erhalten weitestgehend unbekannt. Doch konnte in $Foxp3^+$ Treg Zellen gezeigt werden, dass der IL-10 Signalweg die Produktion von IL-10 erhalten kann. Daher war ein Ziel dieser Arbeit die Rolle von IL-10 für T_R1 Differenzierung und Stabilität zu untersuchen. Hierzu wurde ein transgenes Mausmodell verwendet, in dem $CD4^+$ T-Zellen einen blockierten IL-10 Signalweg aufweisen. Es konnte gezeigt werden, dass IL-10 nicht notwendig für die T_R1 Differenzierung *in vivo*, jedoch essentiell zur Erhaltung der

regulatorischen Funktion der Zellen ist. Die Funktionalität der regulatorischen Zellen wurde in einem Kolitis-Mausmodell getestet, welches der Anwendung von T_R1 Zellen in Patienten mit einem schweren Verlauf von Morbus Crohn ähnlich ist. Auf mechanistischer Ebene konnte gezeigt werden, dass p38 MAP Kinase entscheidend ist, um als Antwort auf IL-10 die Produktion von IL-10 in T_R1 Zellen zu erhalten. Diese Erkenntnisse konnten ebenfalls in humanen T_R1 Zellen bestätigt werden. Auch wenn T_R1 Zellen in der Abwesenheit von IL-10 ihre regulatorische Kapazität verlieren, so weisen doch Ergebnisse aus einem Mausmodell für GvHD darauf hin, dass T_R1 Zellen dennoch nicht in pathogene Zellen konvertieren. Diese Ergebnisse bekräftigen die Hinweise, dass T_R1 Zellen sicher sind für eine T-Zell-basierte Therapie in Menschen, jedoch könnte der Erfolg dieser Therapie an die Anwesenheit von IL-10 gekoppelt sein.

Ein weiteres Ziel dieser Arbeit war die Identifizierung stark suppressiver T_R1 Zellen anhand von den Oberflächenmarkern CD49b und LAG-3 aus dem sehr heterogenen Pool an IL-10 produzierenden $CD4^+$ T-Zellen. Die Verwendung von Oberflächenmolekülen zur Identifikation von regulatorischen T-Zellen wie T_R1 Zellen ermöglicht die Isolation von vitalen Zellen, welche therapeutische Anwendungen finden können und stellt somit eine deutliche Verbesserung dar gegenüber der Notwendigkeit die Zytokinproduktion der Zellen zu bestimmen. Tatsächlich konnten $LAG\text{-}3^+$ $CD49b^+$ IL-10 produzierende T-Zellen als regulatorische T-Zellen mit einer starken suppressiven Kapazität und einem ausgeprägten regulatorischen Phänotyp identifiziert werden. Diese Ergebnisse bestätigen die Effektivität von LAG-3 und CD49b als T_R1 Zell-Marker, jedoch müssen diese Daten in zukünftigen Versuchen noch in humanen T_R1 Zellen bestätigt werden.

1. Introduction

To date around 80 to100 autoimmune and chronic inflammatory diseases are known. Among these are multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease (IBD), type 1 diabetes and psoriasis. These diseases are very heterogeneous and can affect different tissues (such as intestine in inflammatory bowel disease or skin in psoriasis). But most of them, such as rheumatoid arthritis and IBD also act systemically. However, all of these diseases share common hallmarks such as the involvement of strongly pathogenic T lymphocytes (T cells), they are mostly chronic and they require life-long monitoring and treatment. Exactly which pathogenic mechanisms cause the onset of an autoimmune disease is still under discussion and remains controversial. Genetic predispositions [1, 2] are known to play a major role in autoimmune diseases. But clearly, environmental factors also trigger the development of autoimmune diseases since identical twins do not necessarily both develop such diseases [3]. The prevalence of autoimmunity is higher in Western society than for example in Eastern society, further strengthening the hypothesis that environmental factors such as Western lifestyle (hygiene, food, stress etc.) influence autoimmunity [4, 5]. Mechanisms that lead to the onset of autoimmune diseases and possible treatments are ongoing research topics in the field of immunology. T cells notably are investigated as the cause of autoimmunity, but also as a possible therapeutic approach.

T cells are part of the adaptive immune system, with their origin in the thymus, and are distinct from other lymphocytes due to the expression of a T cell receptor (TCR). T cells are a heterogeneous cell population with different subsets that each fulfills a distinct function during the defense against pathogens, preventing cancer and maintaining immune homeostasis. Most T cells express a TCR consisting of α- and β-chains, but there is a small fraction of T cells that express γ- and δ-chains. Unlike conventional α/β T cells, these γ/δ T cells are considered as belonging to the innate immune system. Furthermore, T cells are divided into $CD4^+$ and $CD8^+$ T cells. $CD8^+$ T cells, which express CD8 glycoprotein on their surface, are also known as cytotoxic T cells and play an important role for the defense against virus infected cells and cancer cells. $CD8^+$ T cells recognize

antigens that are presented on the surface through MHC-I, which is expressed on all nucleated cells. $CD4^+$ T cells on the other hand, express CD4 glycoprotein and recognize antigens that are presented by antigen presenting cells (APC) through MHC-II. Naïve $CD4^+$ T cells, which circulate in the periphery, can differentiate into different $CD4^+$ T-helper cell subsets. Thus, this compartment of the adoptive immune system is able to respond to a broad range of environmental pathogens. Already more than 25 years ago, two major $CD4^+$ T-helper cell subsets were discovered: T_H1 cells and T_H2 cells [6]. T_H1 cells are characterized by their secretion of IFN-γ and expression of the master transcription factor T-bet. Whereas T_H2 cells mainly secrete IL-4 and express the transcription factor GATA3. More recently, in 2005 this T_H1/T_H2 paradigm was challenged by the identification of another T-helper cell subset distinct to T_H1 or T_H2 cells: T_H17 cells. T_H17 cells produce IL-17A as a signature cytokine and RORγt is known to be their master transcription factor. All these effector T-helper cell subsets are essential for the protection against pathogens. On the other hand, they also need to be regulated to prevent allergies and autoimmunity. Indeed, regulatory T cells, such as type one regulatory T cells (T_R1 cells) or $Foxp3^+$ regulatory T cells ($Foxp3^+$ Treg cells), can control effector T cells to maintain immune homeostasis and terminate an immune response.

1.1 Immune homeostasis

The first control mechanism to modulate immune responses already occurs in thymus. $CD4^+$ progenitor cells in thymus pass through a strict selecting process. Cells that recognize self-antigens, which might be potentially self-reactive and could cause autoimmunity, are either deleted or converted into regulatory T cells ($Foxp3^+$ Treg cells) with anti-inflammatory properties. This control process is called central tolerance and is an essential step to program T cells to only react against foreign antigens. However, this central tolerance is not sufficient to maintain the immune homeostasis. The immune system has developed several additional mechanisms to prevent an immune reaction against non-pathogenic foreign-antigens such as food antigens or antigens of commensal bacteria in the intestine. Some auto-reactive $CD4^+$ T cells might also escape the process of

central tolerance induction in thymus and these cells would also need to be controlled in the periphery. One of the best studied mechanisms to induce peripheral tolerance is that of regulatory T cells. Regulatory T cells can not only control effector T cells, but can also modulate APCs and B cells. An imbalance of effector and regulatory T cells can lead to a breakdown of the immune homeostasis with severe consequences: effector T cells can react against self-antigens and cause autoimmune or chronic inflammatory diseases such as IBD, type 1 diabetes or rheumatoid arthritis. The immune system can also overreact against harmless foreign-antigens causing an allergic reaction.

1.2 Differentiation of naïve T cells into T-helper cell subsets

Naïve T cells that are generated in thymus and circulate in the periphery are considered immature. Following TCR activation through an antigen and co-stimulatory signaling by APCs, the cytokine environment of naïve T cells is then critical to determine the fate of these cells. These cytokines activate varying “Signal Transducer and Activator of Transcription” (STAT) molecules in the T cells, which lead to activation of master transcription factors. The master transcription factors normally bind to the effector cytokine genes and modulate gene expression through activation, repression or epigenetic modification [7]. New evidence however shows that differentiated T-helper cells display a certain plasticity and can convert into a different T-helper cell subset under specific conditions [8].

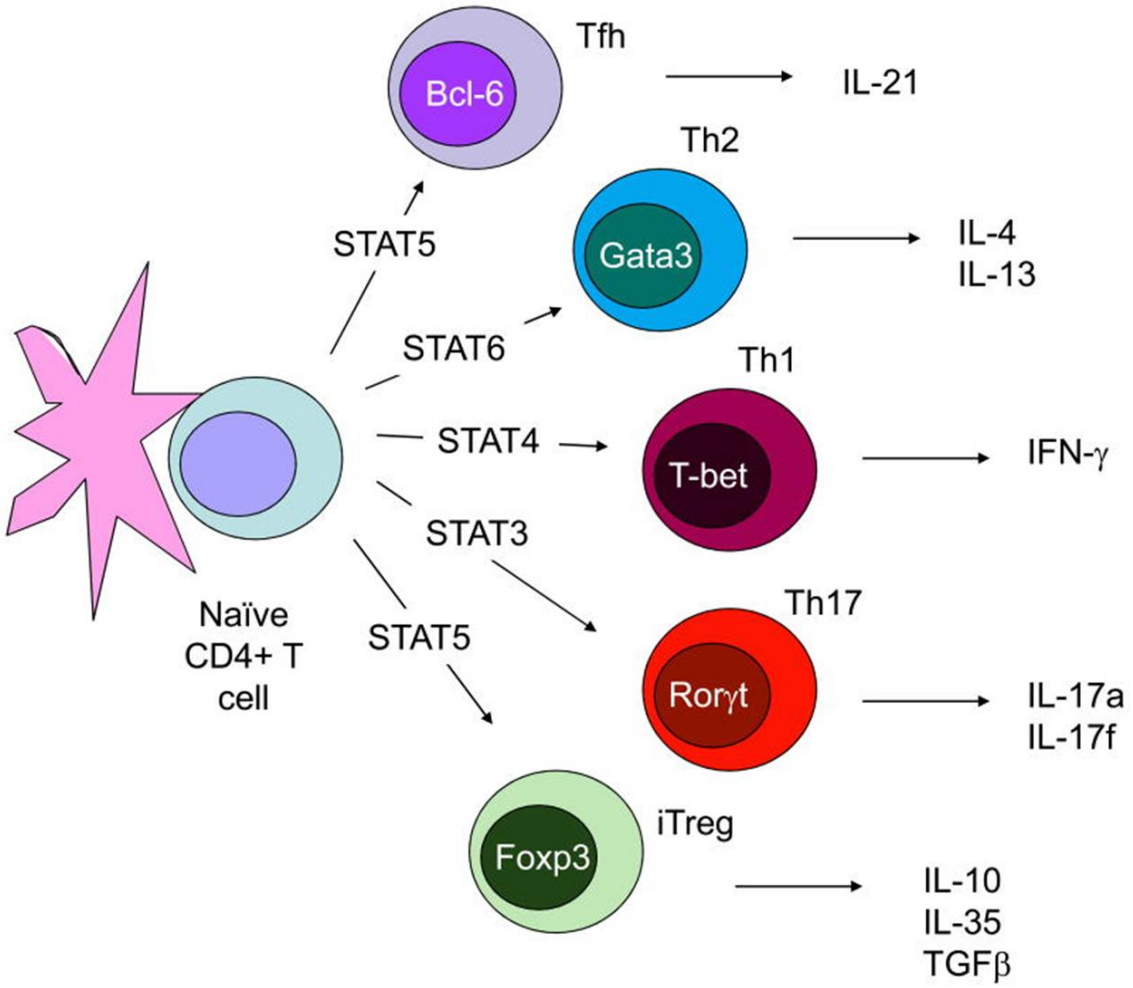

Figure 1: T-helper cell differentiation.

Classic view of T-helper cell linage commitment. Depending on the cytokine environment, an activated T cell differentiates into different T-helper cell subsets. This differentiation is driven by certain STAT molecules. Phosphorylated STAT molecules lead to the expression of linage master transcription factors that in turn regulate T-helper cell subset specific cytokine release. Modified from [9].

1.2.1 Differentiation and Function of T_H1 cells

The signature cytokine of T_H1 cells is IFN-γ. In addition to IFN-γ they also produce IL-2, TNF-α and lymphotoxin-α (LTα) [8]. The differentiation of naïve T cells into T_H1 cells is driven by IL-12, which is produced by activated monocytes/macrophages and dendritic cells. Its receptor is a heterodimer consisting of subunit IL-12Rβ1 and IL-12Rβ2. Accordingly, mice with deficiency in IL-12 receptor display defect in T_H1 based immune responses [8, 10]. Upon binding of IL-12 to the receptor complex, STAT4 is activated and STAT4 in turn

promotes expression of the T_H1 master transcription factor T-bet, which transcribes the *Ifng* gene [11, 12]. In addition to STAT4 activation, IL-12 signaling and later on IFN-γ signaling leads to activation of STAT1 and sustains the expression of T-bet and T_H1-specific cytokine production. Accordingly, mice with a deficiency in STAT1 also show an impaired T_H1 immune response [13-16].

T_H1 cells are especially important for defense against intra-cellular bacteria and viruses. In humans, T_H1 cells are particularly important for defense against mycobacteria infections, for example infections with *Mycobacterium tuberculosis* or *Mycobacterium lepromatosis*. Releasing the T_H1 cell signature cytokine IFN-γ results in activation of mononuclear phagocytes, including macrophages, and therefore increases the efficiency of phagocytosis of infected cells [17]. Consequently, deficiency of T_H1 cells in humans is associated with a higher susceptibility to infections with intracellular pathogens, particularly with *Mycobacterium tuberculosis* [18]. T_H1 cells are also associated with the development of autoimmune diseases. LTα has been especially implicated as a marker for disease progression in multiple sclerosis and it has been shown that blocking LTα inhibited disease development in a mouse model of this disease [19, 20].

1.2.2 Differentiation and Function of T_H2 cells

T_H2 cells can produce a broad range of cytokines such as IL-4, IL-5 and IL-13, which are considered T_H2 cell signature cytokines. Additionally, T_H2 cells are known to secrete IL-9, IL-10 and IL-25. The differentiation of T_H2 cells is strictly dependent on IL-4 and IL-2 signaling. The engagement of IL-4 with its receptor leads to activation of STAT6 and this promotes the expression of the T_H2 cell master transcription factor GATA3 [21-24]. Indeed, GATA3 is indispensable for a functional T_H2 immune response, as already shown that naïve T cells in mice with a deficiency of GATA3 show a strong T_H1 polarization [25]. GATA3 as the master transcription factor of T_H2 cells regulates *Il5* and *Il13* by directly binding to the promoter region of these genes and it can also bind to the enhancer of *Il4* [7]. However, recent studies showed that GATA3 needs to collaborate with STAT6 for the induction of several T_H2 related genes [26]. Besides STAT6, STAT5 also

plays a non-redundant role for the T_H2 lineage commitment. STAT5 is induced through IL-2 receptor signaling and binds to the *Il4* gene and together with GATA3 induces a sufficient *Il4* expression [27].

T_H2 cells are important for the defense against extracellular parasites such as helminths, which are often localized in the intestine, but are also strongly associated with atopic diseases such as asthma [6, 28, 29]. Accordingly, mice with a deficiency in either IL-4 receptor α-chain, STAT6 or GATA3 show a high susceptibility for helminth infections [30]. IL-4 secreted by T_H2 cells is not only the positive feedback cytokine of T_H2 differentiation, but also an important mediator for the IgE class switch in B cells [31]. Additionally, IL-4 also induces other pro-inflammatory cytokines and mediators such as IL-6 and GM-CSF [32]. A T_H2 cell related immune response also includes eosinophils. Especially IL-5, but also IL-13 released by T_H2 cells can activate eosinophils and prevent apoptosis of these cells [33]. IL-13 is essential for the expulsion of a helminth infection, but it is also thought to be a cytokine strongly linked to allergies, increased mucus secretion and airway hypersensitivity [30, 34]. Another allergy-related cytokine which is released by T_H2 cells is IL-9. IL-9 signaling leads to secretion of chemoattractant factors and activation of mast cells, B cells, eosinophils and neutrophils which can together result in allergic airway inflammation [35].

1.2.3 Differentiation and Function of T_H17 cells

The T_H17 cell signature cytokines are IL-17A and IL-17F. In addition, T_H17 cells also secrete IL-22 and TNF-α. T_H17 cell differentiation is independent of T_H1 or T_H2 related transcription factors such as T-bet, STAT1, STAT4 and STAT6. T_H17 cells are induced in the presence of IL-6 and TGF-β or IL-1β. Also IL-23 plays a crucial role for the T_H17 cell biology [36-38]. Naïve T cells express only a very low level of IL-23 receptor, but IL-6 signaling induces its expression, which is essential for the stability and expansion of T_H17 cells [39]. Indeed, IL-23 receptor deficient T_H17 cells fail to maintain their phenotype and are instable *in vivo* [40]. Both IL-6 and IL-23 signaling pathway activate STAT3. This activation is known to be essential for the IL-6-dependent differentiation of T_H17 cells [38]. Activated STAT3 dimers modulate gene expression in the cells and activate the expression

of the T_H17 cell master transcription factor RORyt as well as the expression of IL-17A and IL-17F. Another key transcription factor for the T_H17 cell biology is the Aryl hydrocarbon receptor (AhR). AhR has been reported to promote T_H17 cell differentiation and is already very highly expressed during the early polarization of T_H17 cells [41]. IL-6 also promotes the secretion of IL-21, which further stabilizes the T_H17 cell phenotype synergistically with IL-6, TGF-β and IL-1β in an autocrine self-amplifying loop [37]. Nevertheless, the role of TGF-β during the differentiation of T_H17 cells still remains controversial. In low concentrations it has been shown that TGF-β can inhibit IL-2 dependent STAT5 activation and expression of T-bet and GATA3 and therefore further promote T_H17 cell differentiation [42]. However, in high concentrations TGF-β also inhibits the expression of IL-23 receptor and consequently counteracts the differentiation and expansion of T_H17 cells [39]. Furthermore, T_H17 cells can develop in the absence of TGF-β signaling in gut mucosa, demonstrating that TGF-β is not essential for T_H17 cell differentiation *in vivo* [43].

During physiological conditions, T_H17 cells are mainly located in the small intestine, or more specifically, in the terminal ileum due to the presence of certain members of the microbiota in this organ [44]. T_H17 cells primarily contribute to the defense against extracellular bacteria and some fungal pathogens in the gastrointestinal tract as well as in the lung and skin [45, 46]. Consequently, T_H17 cells are rapidly induced at mucosal sites during infections. IL-17A and IL-17F both bind to the receptor IL-17RA and therefore a similar function of these two cytokines is assumed [47]. Both cytokines induce pro-inflammatory cytokines and chemokines, which are important for chemotaxis of inflammatory cells to the site of infection [48, 49]. IL-17 also induces production of β-defensin, which plays an important role in the defense against bacterial infections [50, 51]. IL-22 signaling promotes the secretion of antimicrobial peptides from epithelial cells. Thus IL-22 displays a crucial role for host defense against bacteria [45, 51]. Furthermore, IL-22 exhibits tissue protective properties. It can induce cell proliferation, survival and tissue repair in the mucosa [52-54]. Besides the important role of T_H17 cells for clearance of extracellular pathogens, this cell type is also strongly linked to the development of autoimmune and inflammatory diseases. In particular, T_H17 cells are involved in the pathogenesis of multiple sclerosis, rheumatoid arthritis

and inflammatory bowel disease, as well as in psoriasis and contact dermatitis [55, 56].

1.2.4 Differentiation and Function of Foxp3$^+$ regulatory T cells

One subset of regulatory T cells, which was discovered in 1995, was characterized by the expression of IL-2 receptor α-chain (CD25). This subset was named CD4$^+$CD25$^+$ Treg cells and was able to prevent autoimmunity in mice [57]. Intensive studies have then identified Foxp3 as the master transcription factor essential to differentiate and maintain Treg cell program and therefore this subset was entitled Foxp3$^+$ Treg cells to distinguish them from other regulatory T cell subsets [58, 59]. Among Foxp3$^+$ Treg cells two major subsets can be discriminated: On the one hand thymus derived Foxp3$^+$ Treg cells (tTreg) and on the other hand inducible Foxp3$^+$ Treg cells, which are induced in peripheral lymphoid organs (pTreg). tTreg cells are supposed to be the majority of Foxp3$^+$ Treg cells and can expand in the lymphoid organs of the periphery. Inducible Foxp3$^+$ pTreg cells can be differentiated *de novo* in the periphery from naïve T cells. The differentiation of pTreg cells is dependent on a combination of IL-2 and TGF-β [60-63]. TGF-β signaling leads to induction of Foxp3 [60, 64, 65], whereas IL-2 dependent activation of STAT5 further enhances and stabilizes expression of the master transcription factor [61, 66]. TGF-β signaling can induce both pTreg cells and T_H17 cells. Besides enhancing the expression of Foxp3, STAT5 has another important role during pTreg differentiation: STAT5 impairs binding of STAT3 to its binding sites and thus suppresses T_H17 cell differentiation [61, 67-69].

Foxp3$^+$ Treg cells have an indispensable role for maintenance of immune homeostasis because they are essential for controlling self-reactive T cells. Several studies in mouse models showed that Foxp3 deficiency, resulting in a lack of Foxp3$^+$ Treg cells, causes fatal autoimmune diseases [70, 71]. Accordingly, patients with a rare genetic defect in the Foxp3 gene, suffering from the immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), also develop severe autoimmune diseases [72]. Both mice and humans with a defect in Foxp3, show a very early onset and severe autoimmune colitis,

demonstrating the important effects of Foxp3$^+$ Treg cells for the immune homeostasis in the intestine [72]. Foxp3$^+$ Treg cells have several mechanisms to suppress and regulate an immune response. They secrete soluble factors such as IL-10 or TGF-β [73-75], but can also express negative T cell regulators, for example CTLA-4 and PD-1, which can suppress effector cells in a cell contact dependent manner [76].

1.2.5 Differentiation and Function of type one regulatory T cells

T_R1 cells were first described in 1994. These cells secrete a very high level of their signature cytokine IL-10 and are therefore known to play a crucial role in maintaining immune tolerance and preventing autoimmunity [77].

1.2.5.1 Differentiation of T_R1 cells

Some of the first protocols that were developed to differentiate T_R1 cells *in vitro* are based on repeated TCR-dependent activation of naïve T cells in the presence of IL-10 [78-80]. IL-10 is not only the signature cytokine, but was also thought to be the driving force during differentiation of T_R1 cells. In line with this, a tolerogenic DC subset (DC-10) has been identified in human peripheral blood. DC-10 can probably induce the differentiation of T_R1 cells *in vitro* through the release of IL-10 and the IL-10-dependent ILT4/HLA-G pathway [81]. IL-10 signaling leads to activation of STAT3. Also other kinases such as p38 MAP kinase can act downstream of the activated IL-10 receptor complex [82-84]. The differentiation of T_R1 cells is independent from Foxp3 since patients with IPEX syndrome, who show mutations in the Foxp3 gene, still display functional T_R1 cells [85].

However, conflicting studies showed that mouse T_R1 cells can develop *in vivo* in the complete absence of IL-10 [86]. Consequently, another cytokine has been described to promote the differentiation of mouse T_R1 cells *in vivo* and *in vitro*: IL-27. IL-27 is a cytokine from the family of IL-12/IL-23 cytokines. Its receptor is a heterodimer composed of IL-27Rα (WSX-1) and glycoprotein 130 (gp130) [87].

IL-27 receptor is most abundant on activated T cells and NK cells, but also expressed on naïve T cells [88-90]. IL-27 signaling in T cells leads to activation of STAT1 and STAT3 [91-93]. The WSX-1 subunit of the receptor activates Janus kinas 1 (JAK1) and results in phosphorylation of STAT1. This in turn is essential for the IL-27-dependent T-bet activation [94]. p38 MAP kinase signaling downstream of WSX-1 further enhances T-bet expression. T-bet is important not only for the secretion of IFN-γ, but also for the inhibition of T_H17 polarization [95]. STAT1 signaling also plays a significant role for the induction of IL-10, but the underlying mechanism is still unknown. The IL-27 receptor subunit gp130 activates STAT3 and this subsequently results in expression of the transcription factor c-Maf (c-avian musculoaponeurotic fibrosarcoma) [96]. c-Maf is essential for the IL-10 production of T_R1 cells, since c-Maf deficient $CD4^+$ T cells fail to produce IL-10 in response to IL-27 [97]. Furthermore, it is known that c-Maf can transactivate both *il10* and *il21* promoters. The effect of c-Maf is amplified by the transcription factor Ahr. c-Maf and Ahr can form a complex leading to a more efficient transcription of *il10* and *il21*. IL-10 is fundamental for the function of T_R1 cells, whereas IL-21 seems to be important for the maintenance and expansion of T_R1 cells. It has been shown that IL-27 acts synergistically with TGF-β to induce T_R1 cells [98]. The TGF-β dependent induction of Foxp3 is thereby inhibited by IL-27 signaling. It is assumed that activated STAT3 can bind to a gene silencer region of the *Foxp3* gene and inhibits Smad3-dependent transcription [99, 100]. Therefore, IL-27 signaling favors the differentiation of T_R1 cells, but inhibits the induction of $Foxp3^+$ pTreg. This finding underlines that these two regulatory T cell subsets presumably have different functions *in vivo*.

However, several other factors such as Egr-2 and Blimp1 have also been proposed to play a key role during T_R1 cell differentiation. The transcription factor Blimp1 can also act in synergy with c-Maf to induce IL-10 production from naïve T cells or T_H1 cells in response to IL-27 [101, 102]. Egr-2 is induced upon IL-27 signaling and can activate the expression of IL-10 and LAG-3 [103]. But a T_R1 cell master transcription factor has not been discovered to date.

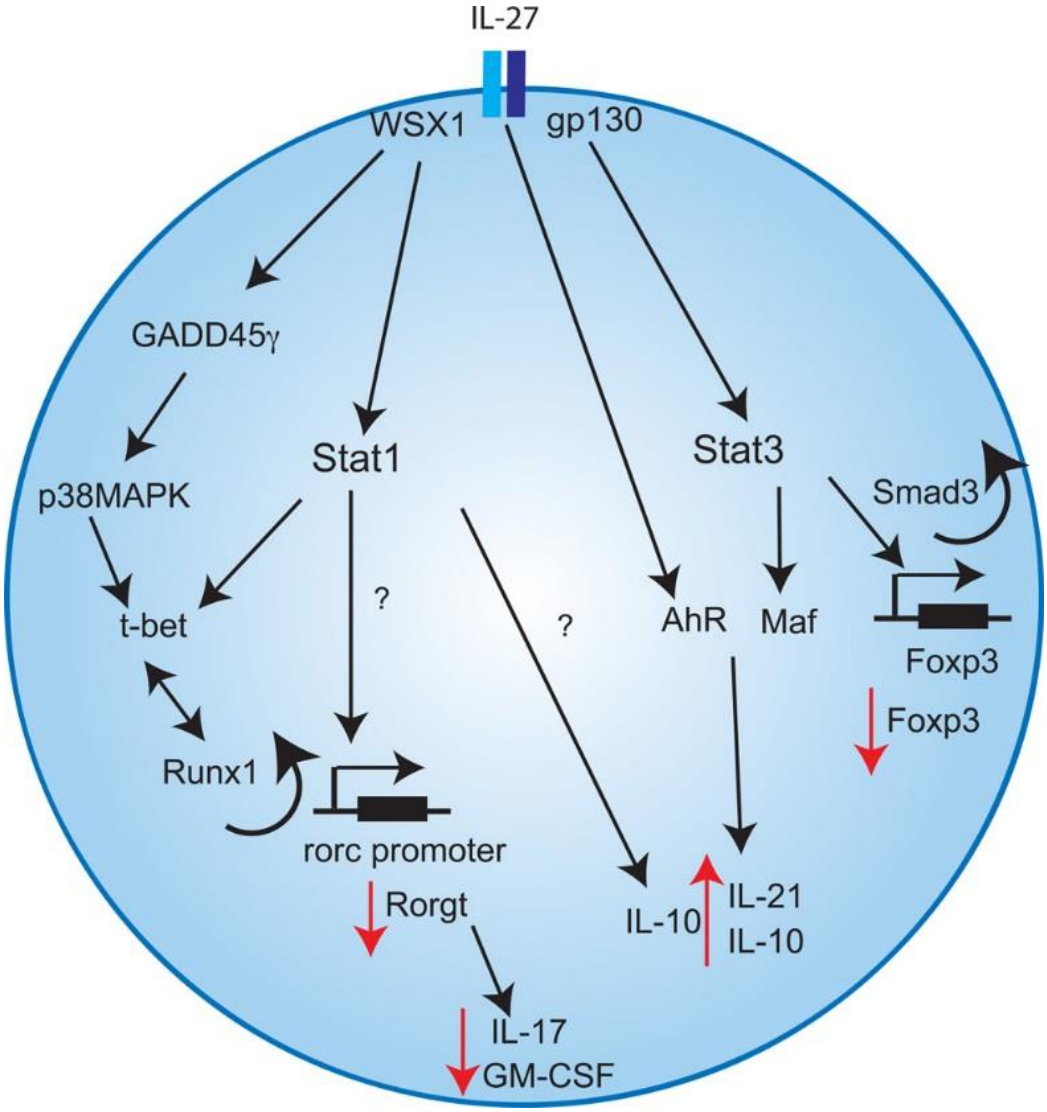

Figure 2: IL-27 dependent T_R1 cell differentiation.

IL-27 dependent molecular mechanisms that lead to the differentiation of T_R1 cells are shown. The WSX-1 subunit activates STAT1. This inhibits T_H17 cell polarization by suppressing Roryt expression. Additionally, signaling through gp130 leads to the phosphorylation of STAT3. STAT3 induces the expression of c-Maf and c-Maf acts in synergy with Ahr to activate *il10* and *il21* promoters. Furthermore, IL-27 inhibits Foxp3 transcription in a STAT3/Smad3 dependent manner. Modified from [93].

1.2.5.2 Biology and function of T_R1 cells

Lack of knowledge about their master transcription factor increases the difficulties to definitely identify T_R1 cells. It is yet already known that T_R1 cell cytokine profile discriminates them from T_H1, T_H2 or T_H17 cells: T_R1 cells secrete higher levels of

IL-10 compared to IL-4 or IL-17A, which are the signature cytokines of T_H2 and T_H17 cells respectively. T_R1 cells also secrete TGF-β [75, 104, 105]. T_R1 cells produce variable levels of IFN-γ depending on the surrounding setting. But their marked regulatory function clearly distinguishes them from effector T cells such as T_H1 cells. T_R1 cells are likewise distinct to $Foxp3^+$ Treg cells since T_R1 cells do not constantly express Foxp3 [106]. Importantly, two surface markers, CD49b (Integrin α2) and LAG-3 (Lymphocyte activation gene 3) have been recently discovered. These markers identify human and mouse T_R1 cells [107]. They allow the identification of T_R1 cells without testing their suppressive capacity and their unique cytokine profile. Neither CD49b nor LAG-3 was exclusively expressed on T_R1 cells, but their co-expression profile distinguishes T_R1 cells from other T-helper cell subsets during helminth infection or inflammatory bowel disease. CD49b is constantly expressed on these cells and LAG-3 expression is induced upon activation. This suggests that these two markers together classify strongly suppressive IL-10 producing T_R1 cells [107].

The production of IL-10 displays the strongest immune-regulatory mechanism of T_R1 cells. IL-10 is essential to control inflammation and terminate immune response. Thus, a dysregulation of IL-10 such as in patients with mutations either in genes encoding IL-10 or IL-10 receptor leads to severe autoimmune diseases, for example an early-onset colitis [108, 109]. Likewise, mice with an IL-10-deficiency develop spontaneous inflammatory diseases, demonstrating the fundamental role of IL-10 for the immune system [110]. IL-10 can directly inhibit T_H17 cells in the intestine. It down-regulates the expression of co-stimulatory molecules such as CD80, CD86 and MHC-II. IL-10 also down-regulates pro-inflammatory cytokine production from APCs and therefore dampens a pro-inflammatory immune response [111]. Additional suppressive mechanisms by T_R1 cells besides the secretion of IL-10 are production of Granzyme B and TGF-β. Like IL-10, TGF-β down-regulates the function of APCs and can inhibit proliferation and cytokine production of T cells [112, 113]. Granzyme B expressing human T_R1 cells, which were generated from naïve T cells with CD3/CD46 antibodies, have been shown to kill target cells in a perforin-dependent manner [114]. T_R1 cells can specifically lyse myeloid cells, but not other APCs, T or B cells [106]. Furthermore, T_R1 cells express CTLA-4, PD-1 and ICOS, which are receptors that serve as negative T cell regulators [115-117].

Thus, T_R1 cells can modulate an immune response also in a cell-contact-dependent manner. T_R1 cells are induced and expanded upon antigen specific TCR activation. But especially through the high secretion of IL-10, T_R1 cells can exert bystander suppressive activity against other antigens and cells.

1.3 T cell plasticity

Mosmann and Coffman divided $CD4^+$ T cell-dependent immune responses strictly in T_H1 or T_H2 related [6] and this view lasted until the identification and characterization of other, clearly distinct T-helper cell subsets such as T_H17 cells or newly described T_H9 or T_H22 cells. Nevertheless, even this conventional concept of distinct T-helper cell linages has been challenged in the recent years of immunological research. Once differentiated, the fate of a T-helper cell seemed to be settled, but now it is very clear that $CD4^+$ T-helper cells display remarkable flexibility. It is known that T_H17 cells can start producing IFN-γ, the signature cytokine of a T_H1 cell and even completely convert into a T_H1 cell [118]. Particularly, this conversion seems to display an important aspect of immune-pathogenesis in autoimmune diseases [119]. But also T_H2 cells can start to produce IFN-γ and thereby express both GATA3 and T-bet, the master transcription factors of T_H2 and T_H1 cells respectively [120]. Even $Foxp3^+$ Treg cells have been shown to inherit a certain plasticity with the potential to become effector $CD4^+$ T cells, although these results are discussed controversially [121-123]. The newly emerging questions regarding factors and mechanisms regulating $CD4^+$ T cell plasticity and stability are the topic of recent immunological research.

1.3.1 Plasticity in T_H1 and T_H2 subsets

Initial experiments with T_H1 and T_H2 cells, which were the first T-helper cell subsets ever described, supported the idea of distinct linage commitments. Differentiation of T_H1 cells inhibits the development of T_H2 cells and *vice versa*. On the one hand, IL-4 signaling hinders the production of IFN-γ and IL-12 and on

the other hand, IFN-γ prevents the production of T_H2 related cytokines [124]. Nevertheless, more recent findings demonstrated that IFN-γ and IL-4 can be produced parallel to each other early after naïve T cell activation, indicating a more complex view on the relation between the two subsets [125, 126]. Furthermore, *in vitro* experiments showed that T_H1 polarized cells cultured in the presence of IL-4 start to produce T_H2 related cytokines, promoting the idea that T_H1 and T_H2 cells have the potential to switch between the two phenotypes. However, these findings were obtained with *in vitro* differentiated cells. More importantly, also *in vivo* generated T_H1 cells maintained the capacity to switch to a T_H2 related phenotype: Mice develop a strong T_H1 dependent immune response when infected with *Leishmania major* and these T_H1 cells *ex vivo* exposed to IL-2 and IL-4 acquire a T_H2 like phenotype [126]. But substantial data regarding the relevance of a switch between T_H1 and T_H2 cells *in vivo* during an infection is still missing to date.

1.3.2 T_H17 cell plasticity

T_H17 cells seem to display an even greater plasticity than T_H1 and T_H2 cells. T_H17 cells have a bivalent expression of T-bet and GATA3, the master transcription factors of T_H1 and T_H2 cells respectively, and can be converted *in vitro* into either T_H1 or T_H2 like cells [127]. More importantly, the acquisition of IFN-γ production by T_H17 cells frequently occurs during inflammation in humans and T_H17+T_H1 cells, cells that simultaneously produce IL-17A and IFN-γ, are associated with disease progression [118, 128]. T_H17 cells can induce type 1 insulin-dependent diabetes mellitus in a mouse model only when they convert into T_H1 cells [119, 129]. Also, in humans T_H17+T_H1 cells are known to be present in autoimmune arthritis and in IBD [130-132]. T_H1 cells originated from T_H17 cells can be distinguished from classical T_H1 cells based on different markers: T_H17-derived T_H1 cells express the T_H17 marker CD161 and are positive for CCR6 whereas classical T_H1 cells do not express CD161 and express only very low levels of CCR6 [118, 133]. T_H17 cells also have the capacity to convert into cells co-producing IL-17A and IL-4, a signature cytokine of T_H2 cells [134]. These T_H17+T_H2 cells are more frequent in patients suffering from allergic asthma and

in a mouse model of induced asthma. T_H17+T_H2 cells displayed a greater potential to induce disease than conventional T_H2 cells [134, 135].

1.3.3 Regulatory T cell plasticity

Regulatory T cell plasticity is a current topic broadly discussed and investigated. Most researchers assume that Foxp3$^+$ tTreg are indeed very long lived and stable [136]. Nevertheless, some studies have shown that Foxp3$^+$ Treg cells can convert into pathogenic T_H17 cells in rheumatoid arthritis. In the mouse model used Foxp3$^+$ regulatory T cells lost the expression of Foxp3 and acquired a T_H17 like phenotype. The underlying process was dependent on IL-6 signaling and the cells became highly pathogenic [137]. However, which origin these instable Foxp3$^+$ Treg cells have is still unknown. One possibility is that Foxp3$^+$ tTregs are indeed stable whereas Foxp3$^+$ pTregs display a certain plasticity. Recent studies identified IL-10 as a crucial cytokine to maintain Foxp3$^+$ Treg stability and IL-10 production. Foxp3$^+$ Treg cells with an impaired IL-10 signaling were not able to suppress T_H17 cells. IL-10 signaling maintained the IL-10 production in Foxp3$^+$ Treg cells in a STAT3-dependent manner [138, 139].

Many unanswered questions remain regarding the T_R1 cell biology. The master transcription factor is still unknown and the differentiation is controversially discussed. In addition, whether T_R1 cells are plastic and which factors regulate their stability is currently being investigated.

1.4 Regulatory T cell-based therapies

The immune system has to be modulated very accurately not only to protect against infections or cancer, but also to prevent overreaction against commensal bacteria or food allergens and most importantly, to inhibit autoimmunity. Regarding this, the identification and characterization of regulatory T cells displayed a significant discovery in the field of immunology. In recent years, researchers have focused on new approaches to use regulatory T cells for the treatment of several human diseases such as autoimmune diseases or chronic

inflammatory diseases and for the prevention of graft rejection and graft-versus-host disease (GvHD). Graft-versus-host disease is a severe complication following a hematopoietic stem cell transplantation (HSCT) [140]. It occurs when the engrafted allogeneic immune cells recognize polymorphic major histocompatibility (MHC) and minor histocompatibility antigens as non-self, and start a strong immune reaction against the host. This donor anti-host allo-response results in a strong multi-organ inflammation that cannot be controlled even with the use of immunosuppressive drugs and is therefore fatal.

1.4.1 Treg-based clinical trial

Regulatory T cells have been successfully tested in human trials to prevent GvHD. In the first clinical trial freshly isolated Foxp3$^+$ Treg cells were adoptively transferred to 5 post-HSCT patients. An increase of infection or GvHD could not be observed [141]. Another trial by Di Ianni et al. [142] confirmed the safety of fresh isolated and unmanipulated Foxp3$^+$ Treg cells and demonstrated a beneficial effect for the patients: Out of 28 patients pre-treated with Foxp3$^+$ Treg cells, only 2 patients developed a low grade GvHD following HSCT, furthermore Foxp3$^+$ Treg promoted lymphoid reconstitution. One struggle for designing an efficient approach for a T cell-based therapy using Foxp3$^+$ Treg cells is the difficulty to purify a sufficient amount of pure and potent Foxp3$^+$ Treg cells. Thus, other studies tested the safety and efficiency of *ex vivo* expanded Foxp3$^+$ Treg cells. Brunstein et al. [143] demonstrated that umbilical cord blood derived Foxp3$^+$ Treg cells, which were expanded *ex vivo* and injected in patients undergoing allo-HSCT, prevented the development of grade II-IV GvHD. Furthermore, Foxp3$^+$ Treg cells were challenged in a clinical trial to treat autoimmunity in type 1 diabetes. Autologous CD4$^+$CD25^{high}CD127$^-$ cells (mainly Foxp3$^+$ Treg cells) were therefore expanded *ex vivo* and administered to children with a recent onset of type 1 diabetes. While the data obtained cannot yet provide a clear answer regarding the treatment's effectiveness, the use of Foxp3$^+$ Treg cells was safe [144].

Besides Foxp3$^+$ Treg cells T_R1 cells are also of great interest for future T cell-based therapies to treat inflammatory diseases due to their strong and varied

immune suppressive mechanisms. To date there have already been two successful human trials to test the safety and efficiency of T_R1 cell-based therapy. A proof-of-concept study using IL-10 anergized T cells from haplo-identical hematopoietic stem cell donors indicated a positive outcome for patients undergoing HSCT. Donor-derived T cells were *in vitro* activated with host-derived APCs in the presence of high amounts of IL-10, these T cells were specific for host allo-antigens and contained T_R1 cells [145]. Another study tested the safety and efficiency of T_R1 cells as a treatment of severe Crohn's disease (IBD). Autologous antigen-specific T_R1 cells were generated *in vitro* and adoptively transferred. T_R1 cells treatment showed a good tolerability and potential to benefit the patients [146].

1.5 Aims

Autoimmunity and chronic inflammatory diseases are widespread in Western countries. These diseases are linked to the breakdown of the immune homeostasis and dysregulation of the immune system. Regulatory T cells are an important player to control potential auto-reactive T cells and to induce peripheral tolerance. Therefore these cells are the main focus for new approaches in the field of T cell-based therapies to treat these diseases. T_R1 cells in particular are of great interest. Nevertheless, many unanswered questions regarding T_R1 cell biology remain, which cause difficulties for the assessment of the potential risks of using T_R1 cells for T cell-based therapy. IL-10 is the signature cytokine of T_R1 cells, but the role of IL-10 for T_R1 cell differentiation and function is controversial, therefore the following questions were addressed:

1. Which roles do IL-10 and IL-27 play in T_R1 cell differentiation *in vivo*?
2. Can mature T_R1 cells respond to IL-10?
3. Is IL-10 signaling in T_R1 cells important for their stability and function?

The identification of CD49b and LAG-3 as T_R1 cell markers leads to further questions. T_R1 cells are a very heterogeneous cell population and among the IL-10 producing Foxp3$^-$ T cells (T_R1 cells) there are T cells which express CD49b and LAG-3, and also cells that are negative for these two markers. In order to

further deepen the understanding of these different subsets, the following questions were addressed:

1. Do CD49b$^+$ LAG-3$^+$ T_R1 cells have a higher suppressive capacity?
2. Do the different subsets display a different genetic phenotype?

By addressing these questions regarding the functionality and stability of T_R1 cells and by identifying the most potent suppressor subset, this study ultimately aims to identify the best and safest T cell subset for T_R1 cell-based therapy to re-induce tolerance in autoimmune or inflammatory diseases and to prevent GvHD and graft rejection after a transplantation.

2. Material and Methods

2.1 Material

2.1.1 Reagents

Basic chemicals and reagents were purchased from the companies, Merck, Sigma-Aldrich and Roth. Specific chemicals are listed in the tables below.

Table 1: Reagents for animal experiments

Reagent	Company
5-Bromo-2′-deoxyuridine (BrdU)	BD Bioscience
Cotrim K (Cotrimoxazol, Sulfamethoxazol, Trimethoprim)	Ratiopharm
Forene (Isofluran)	abbvie

Table 2: Reagents for cell isolation, cell culture and *in vitro* assays

Reagent	Company
Click's Medium	Irvine Scientific
β-Mercaptoethanol	Gibco
Dulbecco's Phosphate Buffered Saline (PBS), 1 x and 10 x	PAA
Fetal calf serum (FCS)	PAA
Penicillin/Streptomycin, 10,000 units/ml	Invitrogen
Percoll	GE Healthcare
Trypan blue solution, 0.4%	Sigma-Aldrich
L-Glutamine	Invitrogen
Ethylenediaminetetraacetic acid (EDTA)	Fluka
Collagenase IV (100 U), from Clostridium histolyticum	Sigma-Aldrich
Biocoll separation solution	Biochrom
Streptavidin microbeads	Miltenyi Biotec
CD4 microbeads, mouse	Miltenyi Biotech

CellTrace Violet dye Proliferation kit	ThermoFisher Scientific
Dimethylsuloxide (DMSO)	Merck
SB203580 (p38 MAP kinase inhibitor)	Invivogen
PD98059 (ERK1/2 inhibitor)	Invivogen
JNK inhibitor II	Invivogen
STAT3 inhibitor VI	Invivogen
Dynabeads Human T-Activator CD3/CD28	ThermoFisher Scientific
mouse $T_H1/T_H2/T_H17$ Cytokine Kit	BD Bioscience
Human T helper Cytokine Panel	BioLegend

Table 3: Reagents for Flow Cytometry

Reagent	**Company**
FACS Clean Solution	BD Bioscience
FACS Flow, 20l	BD Bioscience
FACS Rinse Solution	BD Bioscience
7-AAD Viability Staining Solution	BioLegend
Phorbol 12-Myristate 13-Acetate (PMA)	Sigma-Aldrich
Ionomycin	Sigma-Aldrich
Monensin A	BioLegend
Formaldehyde solution	Sigma-Aldrich
Nonidet P40 (NP40)	Sigma-Aldrich
PhosFlow Lyse/Fix Buffer	BD Bioscience
Perm Buffer III	BD Bioscience
Cytofix/Cytoperm buffer	BD Bioscience
Perm/Wash buffer	BD Bioscience
Cytoperm Permebilization buffer Plus	BD Bioscience
DNase	BD Bioscience

Table 4: Reagents for DNA extraction and genotyping PCR

Reagent	**Company**
Agarose Ultra Pure	Life Technologies

dNTP mix	Fermentas
GeneRuler 1 kb Plus DNA Ladder	Fermentas
DreamTaq DNA Polymerase	ThermoFisher Scientific
10x DreamTag Green buffer	ThermoFisher Scientific
Proteinase K	Roche
Ethidiumbromid	Sigma-Aldrich

Table 5: Reagents for RNA extraction, cDNA synthesis and real-time PCR

Reagent	**Company**
Ethanol, absolute	Th. Geyer
2-Propanol (Isopropanol)	Th. Geyer
Chloroform	JT Baker
Trizol LS reagent	ThermoFisher Scientific
Glycogen	Merck
High Capacity cDNA Reverse Transcription kit	AB applied biosystems
TaqMan Fast Advanced Master Mix	ThermoFisher Scientific

Table 6: Reagents Western blot

Reagent	**Company**
BCA Protein Assay	ThermoFisher Scientific
BSA	Roche
Methanol	Roth
PageRuler Plus Prestained Protein Ladder	ThermoFisher Scientific

2.1.2 Cytokines

Table 7: Cytokines

Cytokine	**Company**
Interleukin-27	BioLegend
hTGF-β1	R&D Systems

Interleukin-6	BioLegend
Interleukin-23	BioLegend
Interleukin-1β	R&D Systems

2.1.3 Antibodies

Table 8: Antibodies for Flow Cytometry

Antigen	Clone	Staining	Dilution	Fluorochrome	Company
CD4	RM4-5	Surface	1:400	ParcificBlue	BioLegend
CD11b	M1/70	Surface	1:400	PE-Cy7	BioLegend
CD11c	N418	Surface	1:200	PE-Cy7	BioLegend
CD8α	53-6.7	Surface	1:400	PE-Cy7	BioLegend
NK1.1	PK136	Surface	1:200	PE-Cy7	BioLegend
TCRγ/δ	GL3	Surface	1:200	PE-Cy7	BioLegend
CD49b	HMa2	Surface	1:100	PE	BioLegend
LAG-3	C9B7W	Surface	1:100	APC	BioLegend
CD45.1	A20	Surface	1:400	APC	BioLegend
CD45.2	104	Surface	1:400	PE-Cy7	BioLegend
IL-10Rα	1B1.3a	Surface	1:400	PE	BioLegend
BrdU	Bu20a	Intracellular	1:100	FITC	BD Bioscience
pSTAT3	4/P-STAT3	Intracellular	1:5	ParcificBlue	BD Bioscience
pp38	36/p38	Intracellular	1:5	ParcificBlue	BD Bioscience
h CD4	OKT4	Surface	1:500	ParcificBlue	BioLegend
h CD45RA	HI100	Surface	1:400	AlexaFluor700	BioLegend
h LAG-3	3DS223H	Surface	1:20	PerCP-eFluor710	eBioscience
h CD49b	P1H5	Surface	1:20	APC	eBioscience

Table 9: Antibodies for animal experiments, cell culture and *in vitro* assays

Antigen	Clone	Company
CD3	2C11	BioLegend
CD28	37.51	BioLegend
Ultra-LEAF Purified anti-mouse IL-27 p28	MM27-7B1	BioLegend

Mouse IgG2a isotype	MOPC-173	BioLegend
Monoclonal Anti-Interleukin-10 Receptor α, human	37607	Sigma-Aldrich
mouse IgG1 isotype	MG1-45	Sigma-Aldrich

Table 10: Antibodies for Western blot

Antigen	Clone	Host	Label	Company
STAT3	EPR361	rabbit	None	Abcam
pSTAT3	Polyclonal	rabbit	None	Abcam
rabbit IgG	Polyclonal	goat	HRP	Abcam

2.1.4 Primers and real-time PCR assays

Table 11: Primers for genotyping PCR

Genotyping Primer	Sequence 5'-3'
FIR1	CAA AAC CAA GAA AAG GTG GGC
FIR2	GGA ATG CTC GTC AAG AAG ACA GG
FIR3	CAT CTT GGA GAG TCG GTG TG
IL10KOF	GTG TGT ATT GAG TCT GCT GGA C
IL10KOR1	GTG TGG CCA GCC TTA GAA TAG
IL10KOR2	GGT TGC CTT GAC CAT CGA TG
GFP-3	AAG TCG TGC TGC TTC ATG TG
GFP-5	ACG TAA ACG GCC ACA AGT TC
IL-17A KI sense	CAC CAG CGC TGT GTC AAT
IL-17A KI anti sense	ACA AAC ACG AAG CAG TTT GG
IL-17A IRES	ACC GGC CTT ATT CCA AGC

Table 12: Real-time PCR assays

Gene name	Taqman Assay ID	Company
Maf	Mm02581355_s1	ThermoFisher Scientific
Ahr	Mm00478932_m1	ThermoFisher Scientific
Prdm1	Mm00476128_m1	ThermoFisher Scientific

Gzmb	Mm00442837_m1	ThermoFisher Scientific
Tgfb1	Mm01178820_m1	ThermoFisher Scientific
Ctla4	Mm00486849_m1	ThermoFisher Scientific
Tbx21	Mm00450960_m1	ThermoFisher Scientific
Pdcd1	Mm01285676_m1	ThermoFisher Scientific
MAF	Hs04185012_s1	ThermoFisher Scientific
AHR	Hs00907314_m1	ThermoFisher Scientific
PRDM1	Hs00153357_m1	ThermoFisher Scientific
GZMB	Hs00188051_m1	ThermoFisher Scientific
TGFB1	Hs00998133_m1	ThermoFisher Scientific
CTLA4	Hs00175480_m1	ThermoFisher Scientific

2.1.5 Buffers and solutions

10x ACK buffer	20.05 g NH_4Cl, 2.5 g $KHCD_3$, 0.093 g EDTA, ad 250 ml distilled H_2O
FACS buffer	0.5% FCS, 0.03% Natriumazid in PBS
MACS buffer	2 mM EDTA, 1% FCS in PBS
complete medium	Click's medium supplemented with 10% FCS, 1% l-glutamine, 1% penicillin/streptomycin and 1:1000 β-Mercaptoethanol
Fix buffer	3.64% Formaldehyde in MACS buffer
Perm buffer	0.1% NP40 in MACS buffer
10x TBS	12.1 g Tris, 87.7 g NaCl, ad 1L distilled H_2O
1x 0,05% TBS-T	100 ml 10x TBS, 500 µl Tween20, ad 1L distilled H_2O
20 % SDS Stock	200 g SDS, ad 1L distilled H_2O
Proteinase K Buffer	12.1 g Tris, 10 ml 0.5 M EDTA, 11.7 g NaCl, 5 ml SDS (from 20% Stock), ad 1L distilled H_2O
Lysis buffer	5.9 g HEPES, 4.4 g NaCl, 5 ml TritonX-100, 1.05 g NaF, 2 ml 0.5M EDTA, 50 ml Glycerol, ad 400 ml distilled H_2O

1,5 M Tris-HCl	45.43 g Tris, ad 100 ml distilled H_20, adjusted to pH 6.8 with HCl
5x Laemmli buffer	60 mM Tris-HCl pH 6, 8.2% SDS, 10% glycerol, 5% β-Mercaptoethanol, 0,01% bromphenol blue
Running buffer	25 mM Tris, 200 mM Glycin (pH 8.3), 0.1% SDS
Transfer buffer	292.8 g Glycine, 121.1 g Tris, ad 1L distilled H_2O

2.1.6 Animals

Mice were kept under specific pathogen free conditions in the facility of the University Medical Center UKE. Food and water was provided *ad libitum*. BALB/c, C57BL/6 and C57BL/6 *Rag1*$^{-/-}$ CD45.1^{+} were obtained from the Jackson Laboratory. CD4-DNIL-10R transgenic mice, Foxp3^{RFP}, IL-17A^{eGFP}, IL-17A^{FP635} and IL-10^{eGFP} reporter mice are described elsewhere [75, 147-149]. Age and sex matched littermates between 8-16 weeks were used.

2.2 Methods

2.2.1 Genotyping

Tail biopsies

To determine the genotype of the genetically modified mice, the mice were genotyped by polymerase chain reaction (PCR). To this end, tail biopsies were digested at 55°C overnight using Proteinase K (in Proteinase K buffer) to extract the genomic DNA from the sample.

The presence of Foxp3^{mRFP} reporter was confirmed using primers FIR1, FIR2 and FIR3. The PCR resulted in a 692 bp wild type amplicon and a 470 bp knock in amplicon. For the PCR reaction 2 µl of tail biopsy sample was added to a PCR master mix: 3 µl 10 x master mix buffer, 0.6 µl dNTP (10 mM), 0.25 µl Dream Tag polymerase, 0.9 µl primer each (10 µM) and 19.5 µl water. The amplification was run in a PCR cycler with the following program: 3 min 94°C, 35 cycles of 94°C, 65°C -0.3°C/cycle and 72°C for 40 sec each, 5 min of 72°C.

The presence of IL-10^{eGFP} reporter was confirmed using two different PCR reactions. One using primers IL10KOF, IL10KOR1 and IL10KOR2, this PCR reaction resulted in an *Il10* wild type amplicon of 340 bp, but the knock in amplicon of 550 bp could not be amplified efficiently enough. Therefore, a second PCR reaction was run using GFP-3 and GFP-5 primer to amplify the inserted *Gfp.* Both PCR reactions were run with the same master mix and PCR program as described above.

The presence of IL-17A^{eGFP} and IL-17A^{FP635} reporter was confirmed using the primers, IL-17A KI sense, IL-17A KI anti sense and IL-17A KI IRES. The PCR reaction amplified a wild type product of 370 bp and a knock in amplicon of 300 bp. The PCR reaction was performed in accordance with the Foxp3^{mRFP} PCR reaction mix and program (described above).

All PCR products were resolved by electrophoresis on a 1.5% agarose gel. DNA was stained with Ethidiumbromid and bands were visualized with a UV transilluminator.

Blood genotyping

The presence of the CD4-DN-IL10R transgene was assessed by Flow Cytometry of lymphocytes from the peripheral blood. To this end 800 µl ACK buffer was added to the blood samples to lyse the erythrocytes. After 5 min of incubation 500 µl PBS was added to stop the lysis and the sample was centrifuged (350 x g, 5 min, 4°C). The supernatant was removed and the cell pellet was stained with fluorescents labelled CD4 and IL-10 receptor α-chain antibodies for 15 min at 4°C (FACS surface staining protocol described in detail below). The sample was washed, pelleted and re-suspended in FACS buffer for analysis. Mice expressing CD4-DN-IL10R transgene showed a much stronger expression of IL-10 receptor α-chain on CD4$^+$ T cells than wild type mice due to the overexpression of the extracellular domain.

2.2.2 Mouse experiments

Anti-CD3 antibody model

One group of mice were injected with anti-CD3 (clone 2C11, 15 µg) intraperitoneally two times every other day (day 0, day 2), and sacrificed 4 hours or 48 hours after the second injection. As controls, another group of mice were injected with isotype-matched antibody or PBS.

To analyze the proliferative potential of T_R1 cells *in vivo* mice were injected with BrdU (1 mg in 100 µl PBS) intraperitoneally 8 hours prior to the second anti-CD3 injection or 4 hours after the second anti-CD3 injection mice were sacrificed.

To test the role of IL-27 for T_R1 cell induction in the anti-CD3 antibody model, neutralizing IL-27 antibodies or isotype control antibodies (BioLegend, 5 mg/kg in PBS) were injected intraperitoneally 12 hours prior to the first anti-CD3 injection or 4 hours after the second anti-CD3 injection. Mice were sacrificed either 4 hours, 48 hours or 96 hours after the second injection of anti-CD3 antibodies.

CD45RB^{high} colitis model

Splenocytes were collected from 8 to 12 week old Foxp3^{mRFP} IL-17A^{eGFP} double reporter mice (CD45.1/2). CD4$^+$ T cells were enriched using the MACS system (Miltenyi Biotec). CD4$^+$ T cells were further purified by FACS-sorting to collect CD45RB^{high} Foxp3^{RFP-} cells using FACS Aria II. 4 x 10^5 CD45RB^{high} cells were injected intraperitoneally into *Rag1$^{-/-}$* mice (CD45.1). Mice were weighed once a week to monitor colitis development. When the mice started to lose weight the colitis was further monitored by endoscopy. After the establishment of a colitis confirmed by endoscopy the mice were sacrificed. Lymphocytes were isolated from inflamed colon. The cells were further FACS-sorted to purify IL-17A^{eGFP+} Foxp3^{mRFP-} T cells (eT_H17 cells).

Adoptive T cell transfer model

(e)T_H17 cells (3 x 10^4) generated in the CD45RB^{high} colitis model were transferred intraperitoneally into Rag*1$^{-/-}$* (CD45.1) mice. In parallel, WT or CD4-DNIL-10R transgenic T_R1 cells were isolated and FACS-sorted from the small intestine of anti-CD3 treated Foxp3^{mRFP} IL-10^{eGFP} double reporter mice. T_R1 cells (3 x 10^4)

were transferred either alone or together with (e)T_H17 cells into CD45.1 *Rag1*$^{-/-}$ mice. Mice were weighed once per week to monitor colitis development. When mice started to lose weight the colitis was further monitored by endoscopy. After the establishment of colitis as confirmed by endoscopy the mice were sacrificed (about 5 weeks after the transfer).

Endoscopic procedure

Colitis scoring using endoscopy was performed in a blinded fashion using the Coloview system (Karl Storz, Germany) [150]. Mice were anesthetized with Isofluran and colitis scoring was based on the following parameters: granularity of the mucosal surface, stool consistency, vascular pattern, translucency of the colon and number of fibrin visible (0–3 points for each). If mice reached a score of 12 they had to be sacrificed by cervical dislocation and counted as colitis lethality.

Graft-versus-Host disease (GvHD)

Wild type BALB/c mice were lethally irradiated with a single-dose of 8 Gy at the age of 9 to 10 weeks. To protect the mice against infections they were given Cotrimoxazol (600 mg/l), Sulfamethoxazol (480 mg/l) and Trimethoprim (100 mg/l) in drinking water, starting 3 days prior to the irradiation until the end of the experiment. Approximately 24 hours after irradiation the mice received $5x10^6$ T cell-depleted BM cells from C57BL/6 mice and either $1x10^5$ T_H17 cells, $1x10^5$ wild type T_R1 or $3x10^4$, $1x10^5$, $3x10^5$ CD4-DNIL-10R transgenic T_R1 cells intravenously. To this end mice were anesthetized with Isofluran and the cells were injected intravenously via the retro-bulbar plexus. To obtain T cell-depleted BM cells, erythrocytes were lysed from total BM using ACK buffer, T cells were depleted using MACS anti-CD90.2 beads (BioLegend) and MACS columns in accordance with the manufacturer's instructions. Mice were monitored for survival daily. Clinical signs of acute GvHD, such as ruffled fur, weight loss (mild >10% of initial body weight; severe >25% of initial body weight), hunched back, inactivity, were monitored daily. Severity of each clinical sign was scored (no = 0; mild = 1; severe = 2), and animals with a total score >6 were sacrificed by cervical dislocation and counted as GvHD lethality.

2.2.3 Cell isolation

Immune cell isolation from spleen

Mice were first anesthetized with a mixture of 80% CO_2 and 20% O_2 and subsequently sacrificed by inhaling 100% CO_2 alone. Spleens were harvested with sterile instruments and collected in complete medium on ice. Spleens were homogenized using 40 µm cell strainers and pelleted by centrifugation (350 x g, 5 min, 4°C). Erythrocytes were lysed by treating the cells with ACK buffer for 5 min at 4°C. The lysis was stopped by adding PBS in excess and cells were pelleted by centrifugation (350 x g, 5 min, 4°C). Then cells were re-suspended in either FACS buffer, MACS buffer or medium depending on the following step.

Immune cell isolation from small intestine

Mice were sacrificed as described above and the small intestine was harvested with sterile instruments. The peyer's patches were removed, the small intestine was opened longitudinally and washed in PBS supplemented with 1% FCS. Then the small intestine was cut into small pieces of approximately 0.5 cm and incubated in the presence of 5 mM EDTA in complete medium at 37°C for 30 min while shaking. Intraepithelial lymphocytes (IEL) could be collected in the media after this step using centrifugation (350 x g, 5 min, 4°C). The tissue was collected to further isolate lamina propria lymphocytes (LPL). To this end, the small intestine was digested by collagenase IV (100 U, Sigma) in complete medium at 37°C for 45 min while shaking. The digested gut tissue was further homogenized through a metal strainer and lymphocytes (IEL fraction and LPL fraction were pooled prior to this step) were further separated with a Percoll gradient.

Percoll gradient: The osmolality of Percoll was first adjusted by adding 1 part (v/v) 10x PBS to 9 parts (v/v) of Percoll (90% Percoll). This isotonic Percoll was further diluted with 6 parts (v/v) 1x PBS supplemented with 1% FCS and 4 parts (v/v) isotonic Percoll to create a 40% Percoll solution. In a 15 ml tube 4 ml of isotonic 90% Percoll was added. Cells were re-suspended in 40% Percoll (4 ml) and overlaid. The gradient was centrifuged to separate the cells (400 x g, 20 min, RT). Lymphocytes could be collected from the interphase.

Immune cell isolation from colon

Mice were sacrificed as described above. The colon was harvested, opened longitudinally and washed in PBS supplemented with 1% FCS. Then the colon was cut into small pieces of approximately 0.5 cm and incubated in the presence of 5 mM EDTA in complete medium at 37°C for 30 min while shaking. Intraepithelial lymphocytes (IEL) could be collected in the media after this step by centrifugation (350 x g, 5 min, 4°C). The tissue was collected to further isolate lamina propria lymphocytes (LPL). To this end the colon was digested with collagenase IV (100 U, Sigma) in complete medium at 37°C for 45 min while shaking. The digested gut tissue was further homogenized through a metal strainer and lymphocytes (IEL fraction and LPL fraction were pooled prior to this step) were further separated with a Percoll gradient in analogy to the Percoll gradient for small intestinal lymphocytes.

Immune cell isolation from lung

Mice were sacrificed as described above and lungs were perfused with 10 ml PBS through the right ventricle until the lungs were clear of blood (a slit was cut in left ventricle to allow blood to leave). Lung tissue was collected in complete medium on ice. Lung tissue was cut into small pieces of approximately 0.5 cm and digested with collagenase IV (100 U) in complete medium at 37°C for 45 min while shaking. The digested lung tissue was further homogenized through a metal strainer and lymphocytes were further separated with a Percoll gradient in analogy to the Percoll gradient for small intestine lymphocytes.

Immune cell isolation from liver

Mice were sacrificed as described above and livers were perfused with 10 ml PBS through the portal vein until the livers were clear of blood. Livers were harvested with sterile instruments into complete medium. Liver tissue was homogenized through a metal strainer and lymphocytes were further separated with a Percoll gradient in analogy to the Percoll gradient used to isolate small intestinal lymphocytes.

Isolation of bone marrow cells (BM)

Mice were sacrificed as described above. The muscles from the lower extremities were cut off with sterile scissors and the acetabulum was carefully dislocated from the hip joint without breaking the femur head. The remaining muscles were removed from femur and tibia and the bones were collected in sterile complete medium. The epiphyses of bones was cut off under sterile conditions. The bones were flushed with sterile PBS supplemented with 1% FCS using a needle. Bone marrow cells were filtered through a 100 µm strainer. Cells were centrifuged (350 x g, 5 min, 4°C) and erythrocytes were lysed using ACK buffer for 3 min at 4°C. After ACK lysis cells were washed with sterile PBS, centrifuged (350 x g, 5 min, 4°C) and re-suspended in PBS for further use.

Human PBMC isolation from buffy coat

To isolate PBMCs from buffy coat the buffy coat (50 ml on average) was diluted with sterile PBS up to a final volume of 350 ml. 15 ml of Biocoll separation solution was added in a 50 ml tube and 35 ml of the diluted buffy coat was carefully overlaid. The Biocoll gradient was centrifuged at 400 x g for 20 min at RT. Human lymphocytes could be collected from the interphase.

2.2.4 Magnetic-activated cell sorting (MACS)

Naïve $CD4^+$ T cells were isolated from splenocytes using magnetic-activated cell sorting (MACS) in accordance with the manufacturer's instructions (Miltenyi Biotech). In brief, cells were re-suspended in MACS buffer with biotinylated antibodies against CD25 and CD44 for 15 min at 4 °C. The antibody-bound $CD25^+$ (tTreg) and $CD44^+$ (Memory T cells) cells were targeted by streptavidin-beads that bind to biotin and depleted from the suspension via a MACS LS column. Non-labelled cells were recovered from the flow through and centrifuged (350 x g, 5 min, 4°C). Cells were re-suspended in MACS buffer containing CD4-microbeads and incubated for 45 min at 4°C. Labelled $CD4^+$ T cells were separated from the suspension via a MACS LS column. $CD4^+$ T cells were recovered from the column by flushing with MACS buffer after the column was

removed from the magnetic field. The flow through containing non-$CD4^+$ T cells was centrifuged (350 x g, 5 min, 4°C) and cells were re-suspended in MACS buffer with biotinylated antibodies against CD3 for 15 min at 4°C. The antibody-bound $CD3^+$ cells were targeted by streptavidin-beads that bind to the biotin and depleted from the suspension via a MACS LS column. The flowthrough contained APCs. APCs were irradiated with 30 Gy to inhibit proliferation, but the appearance of co-stimulatory molecules was preserved.

2.2.5 Flow Cytometry (FACS)

Identification of dead cells

To identify dead cells a 7-AAD staining (BioLegend) was performed. To this end cells were incubated in 0.5 ml FACS buffer including 5 µl of 7-AAD (per 1 x 10^6 cells) for 10 min in the dark after the surface staining. The cell suspension was analyzed without additional washing.

Surface staining

Lymphocytes (1 x 10^6 cells) were transferred to a 5 ml tube, centrifuged and re-suspended in 100 µl FACS buffer containing Fc-block (1:100) antibody and directly fluorochrome labelled antibodies against surface markers. Cells were stained in the dark for 20 min at 4°C, washed with FACS buffer and pelleted. Then cells were either re-suspended in 300 µl FACS buffer for direct acquisition or further proceeded to intracellular staining. The staining for mouse and human CD49b and LAG-3 was performed for 30 min at 37°C.

Intracellular staining

For intracellular cytokine staining (ICS), cells were re-stimulated with PMA (50 ng/mL) and ionomycin (1 mM) for 4 hours at 37°C prior to the staining. Monensin A was added during the last 3 hours of re-stimulation. Cells were washed and pelleted and surface markers were stained as described above. Cells were fixed in 100 µl 4% Formaldehyde (Fix buffer) for 20 min at RT, washed, pelleted and re-suspended in 100 µl 0.1% NP40 (Perm buffer) for 4 min at RT in the dark. After the permeabilization, cells were washed with FACS buffer,

centrifuged (350 x g, 5 min, 4°C) and re-suspended in 100 µl FACS buffer containing fluorochrome labelled antibodies against intracellular cytokines. Cells were incubated for 1 hours at RT in the dark. Cells were washed, pelleted and re-suspended in 300 µl FACS buffer for acquisition.

For intracellular pSTAT3 and pp38 MAPK staining cell were not re-stimulated with PMA/Iono or pre-stained with surface markers. Cells were fixed with PhosFlow Lyse/Fix Buffer for 10 min at 37°C and permeabilized with Perm Buffer III for 30 min on ice. Cells were incubated for 1 hour at RT in the dark in 100 µl FACS buffer containing fluorochrome labelled antibodies against pSTAT3 and pp38 MAP kinase as well as surface markers.

Cells were transferred into a 5 ml tube and stained for surface markers as described above in order to stain intracellular DNA-integrated BrdU. In brief, after washing with FACS buffer and centrifugation (350 x g, 5 min, 4°C), cells were re-suspended in 100 µl Cytofix/Cytoperm buffer for 15 min at RT. Cells were washed with 1 ml Perm/Wash buffer and pelleted. For nucleus permeabilization cells were re-suspended in Cytoperm Permebilization buffer Plus for 5 min on ice. After washing and pelleting, cells were treated with 100 µl DNAse buffer to expose incorporated BrdU (300 µg/mL DNAse; 1 hour at 37°C). Then, washed and pelleted cells were re-suspended in 50 µl Perm/Wash buffer containing fluorescent anti-BrdU antibodies and incubated for 20 min at RT. After washing, the cells were re-suspended in 300 µl Perm/Wash buffer for acquisition.

Data acquisition and analysis

The samples were analyzed on a LSR II flow cytometer running FACD Diva software. Data analysis was performed using FlowJo vX for Windows analysis software.

2.2.6 Fluorescence-activated cell sorting (FACS-sort)

Cells were stained for surface markers as described above and filtered through a 40 µm cell strainer to remove debris that could occlude the cell sorter nozzle. Cell sorting was performed on a BD FACS Aria. Cells were sorted in a 5 ml tube

containing cold complete medium and purity of sorted cells was routinely above 95%.

2.2.7 *In vitro* T_R1 cell and T_H17 cell differentiation

$CD4^+$ naïve T cells were enriched from splenocytes of either wild type or CD4-DN-IL10R transgenic $Foxp3^{RFP}$ $IL\text{-}10^{eGFP}$ double reporter mice or $Foxp3^{RFP}$ $IL\text{-}17A^{eGFP}$ double reporter mice using MACS. In brief: Erythrocytes were lysed prior to the $CD4^+$ T cell enrichment using ACK buffer. $CD44^+$ and $CD25^+$ T cells were depleted using biotinylated antibodies and Streptavidin beads. $CD4^+$ T cells were enriched using CD4-micobeads.

For T_R1 cell differentiation naïve T cells were cultured for 5 days at a density of 10^6 cells/ml with plate-bound anti-CD3 (2 µg/ml) and soluble anti-CD28 (2 µg/ml) in complete medium under T_R1 polarizing conditions (0.5 ng/ml hTGF-β1, 30 ng/ml IL-27). IL-10 (eGFP) and Foxp3 (mRFP) expression was determined by Flow Cytometry.

For T_H17 cell differentiation naïve T cells were cultured for 5 days at a density of 10^6 cells/ml with soluble anti-CD3 (3 µg/ml) and soluble anti-CD28 (1 µg/ml) in the presence of irradiated APCs (ratio 1:4) in complete medium under T_H17 polarizing conditions (0.5 ng/ml hTGF-β1, 10 ng/ml IL-6, 20 ng/ml IL-23, 10 ng/ml IL-1β). IL-17A (eGFP) and Foxp3 (mRFP) expression was determined by Flow Cytometry.

2.2.8 *In vitro* assays

In vitro suppression assay

T_R1 cells or T_R1 cell subsets and $Foxp3^+$ Treg cells (all CD45.2) were isolated from small intestine or spleens of anti-CD3 treated animals (protocol described above) via FACS-sorting. Responder T cells and APCs were isolated from spleens of untreated wild type mice via MACS. APCs (feeder cells) were further irradiated with 30 Gy to prevent proliferation and washed twice (350 x g, 5 min,

4°C) using complete medium. Responder T cells (CD45.1/2) were labelled with violet dye in accordance with the manufacturer's instructions. In brief, violet dye was solved in DMSO to generate a stock solution of 5 mM. 1 µl of the stock solution was added to every 1 ml of cell suspension (working concentration 5 µM, cells were re-suspended in pre-warmed PBS without FCS) and incubated for 8 min at 37°C in the dark. 5 times the original staining volume of FCS was added to the cell suspension to stop the staining process. Cells were centrifuged (350 x g, 5 min, 4°C) and washed twice using complete medium. 5 x 10^4 responder cells were plated in a 96 well flat-bottom plate together with 2 x 10^5 APCs. Regulatory T cells were added to achieve the following final Treg : Responder ratios 1:1, 1:2, 1:4 and 0:1 as control. T cells were further stimulated with 1.5 µg/ml soluble anti-CD3 antibodies. The final volume in each well was adjusted to 200 µl and all conditions were plated in duplicates. Cells were cultured for 72 hours and the proliferation of responder T cells was measured via FACS depending on the dilution of violet dye. For better distribution of responder T cells and regulatory T cells, cells were also stained for CD45.1 and CD45.2.

STAT3 responsiveness in vitro

T_R1 cells (wild type and CD4-DNIL-10R transgenic) were isolated from small intestine of anti-CD3 treated mice and FACS-sorted. Foxp3$^+$ Treg cells and naïve T cells were isolated from spleens of untreated wild type animals and also FACS-sorted. Cells were rested overnight in complete medium at 4°C. The next day, cells were plated in 96 well plates and either stimulated with indicated concentrations of IL-10 or IL-6 at 37°C (time as indicated) or remained unstimulated (Figure 8). Phosphorylated STAT3 was stained intracellularly (detailed protocol described above) and analyzed using Flow Cytometry. IL-10 or IL-6 stimulated samples were compared to unstimulated control cells.

Kinase inhibitor assay

Wild type Foxp3^{RFP} IL-10^{eGFP} CD4$^+$ T cells were isolated from spleen and cultured for 5 days under T_R1-polarizing conditions. Cells were washed and centrifuged (350 x g, 5 min, 4°C) and re-plated in 96 well flat-button plates (2 x 10^5 cells/well) in the presence of 10 µg/ml plate-bound anti-CD3 antibodies and soluble 10 µg/ml anti-CD28 antibodies at 37°C. SB203580 (p38 MAP kinase inhibitor), PD98059

(ERK1/2 inhibitor), JNK inhibitor II or STAT3 inhibitor VI were solved in DMSO and added to the culture medium in the indicated concentrations (Figure 14) every 24 hours. DMSO was added to control cultures at equivalent concentrations. Frequency of IL-10^{eGFP+} cells was assessed after 48 hours via Flow Cytometry.

Re-stimulation of human T_R1 cells

Human T_R1 cells were isolated from PBMCs via FACS-sorting (CD4$^+$ CD45RA^{low} LAG-3$^+$ CD49b$^+$). T_R1 cells were plated in 96 well round-button plates (1 x 10^4 cells/well) and the volume was adjusted to 150 µl medium/well. The cells were re-stimulated using CD3/CD28 Dynabeads (Dynabeads Human T-Activator CD3/CD28). In brief, beads were re-suspended in the vial and the desired volume of beads was transferred to a new tube (0.25 µl beads per 1 x 10^4 T cells for a bead-to-cell ratio of 1:1). Beads were washed by adding medium in excess. The tube was then placed in a magnet and the liquid was removed and the beads re-suspended in complete medium and added to the cell culture. Either 50 µg/ml human IL-10Rα or isotype control antibodies were added to the cell culture. Cells were cultivated for 96 hours at 37°C. Cell culture supernatants were stored at –80°C.

2.2.9 Cytometric Bead array (CBA)

Mouse CD4$^+$ T cells (4 x 10^6 cells/ml) were stimulated with plate-bound CD3 antibodies (10 µg/ml) and soluble CD28 antibodies (10 µg/ml) in complete medium for 60 hours at 37°C. Cytokines in the cell culture supernatants were quantified by Cytometric Bead Array (mouse T_H1/T_H2/T_H17 Cytokine Kit) in accordance with the manufacturer's instructions. In brief, the supernatant was incubated together with cytokine-specific beads and detection antibodies for 2 hours at RT while mildly shaking. The cytokines in the supernatant bind to the specific beads and are labelled with the detection antibody. The samples were then washed and analyzed by Flow Cytometry. The different beads (specific for different cytokines) could be distinguished dependent on different fluorescence intensities, whereas the cytokine concentration in the supernatant was quantified based on the MFI of the detection antibody in relation to a standard dilution series.

Human T_R1 cells (3 x 10^4 cells/200 µl) were stimulated with CD3/CD28 beads (Dynabeads Human T-Activator CD3/CD28) in a bead:T cell ratio of 1:1 in full medium for 96 hours at 37°C. Cytokines in the supernatants were quantified by Legendplex Assay (Human T helper Cytokine Panel) in accordance with the manufacturer's instructions. The principle of the Legenplex assay is comparable to the Cytometric Bead Array that is described above.

2.2.10 RNA isolation, cDNA synthesis and real-time PCR (RT PCR)

RNA isolation

For RNA isolation Trizol LS Reagent was used in accordance with the manufacturer's instructions. In brief, cells were pelleted and re-suspended in 1 ml of Trizol LS Reagent for 5 min at RT. 10 µl Glycogen was added to the sample for better recovery of RNA. After carefully pipetting to mix the sample with the glycogen 200 µl of Chloroform was added to the tube. The closed tube was vortexed for approximately 30 seconds and incubated for 10 min at RT. Samples were centrifuged at 12,000 x g for 15 minutes at 4°C. The mixture separates into a lower phenol-chloroform phase and an upper aqueous phase containing RNA. The upper phase was carefully removed and transferred in a new tube by pipetting. 500 µl of Isopropanol was added to the aqueous phase and the sample was incubated for 4 hours or overnight at -20°C followed by centrifugation at 12,000 x g for 15 minutes at 4°C. The RNA was pelleted and the supernatant was carefully removed. The RNA pellet was washed with 1 ml 70% Ethanol following centrifugation at 12,000 x g for 10 minutes at 4°C. The supernatant was removed from the RNA pellet and the pellet was air-dried. After drying, the RNA was re-suspended in RNAse free water (28 µl). RNA isolation was directly followed by cDNA synthesis or RNA samples were stored at -80°C.

cDNA synthesis

For cDNA synthesis, RNA concentration was adjusted to 500 ng/µl. 2 µl of 10 x reaction buffer, 0.8 µl of dNTP mix (100 mM), 2 µl of 10 x random primers, 1 µl of reverse transcriptase (50 U/µl), 0.5 µl RNAse inhibitor (20 U/µl) and 12.7 µl RNAse free water was mixed. 1 µl of RNA (500 ng) was added and carefully

mixed and incubated for 10 min at 25°C. Reverse transcription was performed at 37°C for 2 hours and terminated by heat inactivation of the enzymes at 85°C for 5 min. cDNA was stored at -20°C.

Real-time PCR

RT PCR was performed using TaqMan assays containing a set of primers and reporter probes. All TaqMan assays are listed in table 12. cDNA was diluted 1:5 with RNAse free water and run in the RT PCR in accordance with the manufacturer's instructions. In brief, 5 µl TaqMan Fast Advanced Master Mix were mixed with 0.5 µl TagMan primer/probes. 4.5 µl of diluted cDNA was added and run in a 96-well plate. Reaction was initialized by heating to 50°C for 2 min following 95°C for 10 min. In total 40 cycles with 95°C for 15 sec and 60°C for 1 min were run. All results were normalized to *Hprt* quantified in parallel amplification reactions during each PCR quantification. To analyze the data the ΔCt (change in cycle threshold) method was used.

2.2.11 Western blot

Cell lysis

T_R1 cells were generated *in vitro* and FACS-sorted. After resting overnight cells were re-stimulated with 100 ng/ml IL-10 for 20 min at 37°C. Cells were washed and centrifuged (350 x g, 5 min, 4°C) and the supernatant was removed from the pellet. Cells were re-suspended in 50 µl Lysis buffer (including proteinase inhibitors) and samples were sonificated with ultrasound (10 sec, 4 cycles). Samples were centrifuged for 30 min with 1000 x g at 4°C. The supernatant which contained protein was transferred to a new tube and mixed with Laemmli buffer in a protein:Laemmli ratio of 1:5.

Determination of protein concentration

Protein concentration was assessed using BCA Protein Assay in accordance with the manufacturer's instructions. In brief, 10 µl of protein sample and standard was added to a 96 well plate and carefully mixed with 200 µl/well freshly prepared BCA solution. Plate was incubated for 30 min at 37°C and absorbance at 562 nm

was assessed on a spectrophotometer. Protein concentration of the samples was calculated based on the protein standard curve.

Protein electrophoresis

Protein samples were run on a 12% tris-gylicine SDS-page. Prior to the separation protein samples were heated to 95°C for 5 min. Equal concentrations for each sample and a protein standard ladder were loaded to the gel. The gel chamber was filled with 1 x running buffer and electrophoresis was performed at 80 volt for 10 min followed by 30 min at 120 volt (until the prestained protein ladder bands were clearly separated).

Protein transfer and detection

Proteins were transferred from the gel to a nitrocellulose membrane using wet-blot electrophoresis. Membrane and gel were carefully assembled in the blotting chamber filled with 1 x transfer buffer and electrophoresis was performed for 1 hour at 300 mA. The blotted membrane was blocked for 1 hour in 5% BSA (in TBS-T) solution at RT while mildly shaking. After blocking, the membrane was incubated overnight at 4°C with the first antibody (STAT3 or pSTAT3) in 5% BSA/TBS-T solution. The next day membrane was washed three times with TBS-T for 10 min each and incubated for 1 hour with the second antibody in 5% BSA/TBS-T solution at RT. After washing 3 times the blot was developed (5 min) with chemiluminescent HRP substrate (Millipore) before placing a film on the membrane (in the dark) for 10 min. The photo film was run through a developer and the ladder was carefully marked in the film.

2.2.12 Histology

Tissue samples were kept in 4% PFA solution for 24 hours. For dehydration samples were transferred every 2 hours in solutions containing different proportions of isopropanol (30%, 50%, 70%, 85%, 95% and 100% Isopropanol in H_2O) at RT. Then samples were transferred to a 1:1 (v/v) chloroform/paraffin solution at 60°C. Finally, samples were embedded in paraffin and stored at room temperature or 4°C until myotome sectioning. For histological analysis, sample slides were stained with haematoxylin and eosin (HE staining). Slides were kept

for 30 min at 68°C to melt the paraffin. After 2 times 5 min bath in xylene, slides were transferred to a glass coplin jar containing 100%, 90%, 70% EtOH in H_2O for 4 min each, respectively. Slides were stained in haematoxylin for 2 to 5 min and bathed in warm water for 10 min. After that, slides were stained with eosin for 3 to 5 min and dehydrated with a fast rinse in H_2O, 30%, 50%, 70%, 85%, 95% and 100% of EtOH in H_2O, respectively. Glass slides were mounted with RotiR Histokit II.

2.2.13 Statistical analysis

The Mann–Whitney U test, paired t test or one-way ANOVA (post-test Tukey) were used to calculate statistical significance. A p-value <0.05 was considered significant. Statistical calculations were performed using Prism program 5.0 (GraphPad Software, Inc.)

3. Results

3.1 IL-10 signaling in T_R1 cells

3.1.1 IL-10 is dispensable for T_R1 cell differentiation, whereas IL-27 promotes T_R1 cell induction *in vivo*

It has previously been shown that both IL-10 and IL-27 are sufficient to induce the differentiation of T_R1 cells *in vitro* [80, 104, 148], whereas the role of IL-10 during the differentiation of T_R1 cells *in vivo* remained controversial [86]. CD3-specific antibody treatment that leads to a strong induction of T_R1 cells in the small intestine of mice served as a model to study the impact of these two cytokines on T_R1 cell differentiation *in vivo [75]*.

Wild type Foxp3^{RFP} IL-10^{eGFP} double reporter mice (WT) and CD4-DN-IL10R transgenic Foxp3^{RFP} IL10^{eGFP} double reporter mice (Tg) [147, 149, 151] were treated with anti-CD3 antibodies. CD4-DN-IL10R transgenic mice overexpress a dominant-negative IL-10 receptor α-chain in CD4$^+$ T cells and consequently display a strongly impaired IL-10 signaling [147]. Cells were isolated from the small intestine 4 hours after the last anti-CD3 injection, because it had previously been shown that CD4$^+$ T cells in the small intestine show the highest IL-10 expression at this time point [75]. Neither the frequency of T_R1 cells (CD4$^+$IL-10$^+$Foxp3$^-$) nor the frequency of Foxp3$^+$ Treg cells (CD4$^+$Foxp3$^+$) was altered in CD4-DN-IL10R transgenic mice compared to wild type mice (Figure 3).

Beside the expression of IL-10 and the lack of Foxp3 expression, mature and functional T_R1 cells are characterized by additional criteria such as expression of the T_R1 cell markers CD49b and LAG-3, expression of T_R1 cell signature genes and suppressive capacity [104, 106, 107]. Further analyses revealed that CD4-DN-IL10R transgenic T_R1 cells showed similar expression levels of CD49b and LAG-3 compared to wild type T_R1 cells (Figure 3).

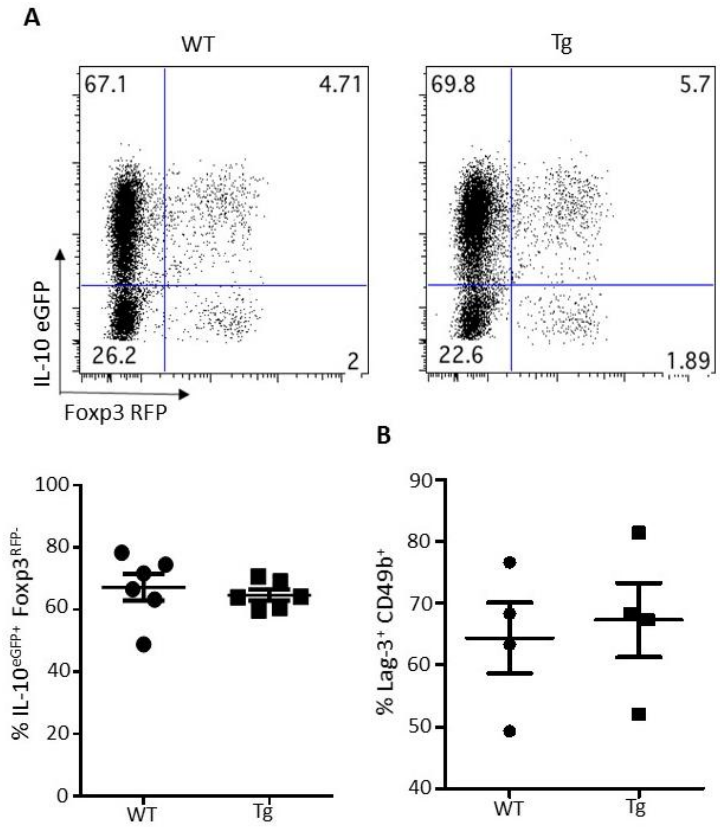

Figure 3: IL-10 signaling in T cells is not essential for the differentiation of T_R1 cells.

Wild type (WT; n=6) or CD4-DNIL-10R transgenic (Tg; n=6) $Foxp3^{RFP}$ $IL\text{-}10^{eGFP}$ double reporter mice were treated with 15 µg anti-CD3 antibodies twice (day0, day2). Cells were isolated from the small intestine 4 hours after the second injection and analyzed by Flow Cytometry. Representative dot plots and scatter plots (lines indicate mean ± SEM) are shown. Data are cumulative of three independent experiments.

Furthermore, wild type T_R1 cells and CD4-DN-IL10R transgenic T_R1 cells were FACS-sorted from small intestines of anti-CD3 treated animals and the mRNA levels of T_R1 cell signature genes (*Maf, Ahr, Prdm1, Gzmb, Tgfb1, Ctla4*) were analyzed. *Maf*, *Ahr* and *Prdm1* (encoding Blimp1), as well as *Gzmb*, *Tgfb1* and *Ctla4* were not differentially expressed between wild type and CD4-DN-IL10R transgenic T_R1 cells (Figure 4).

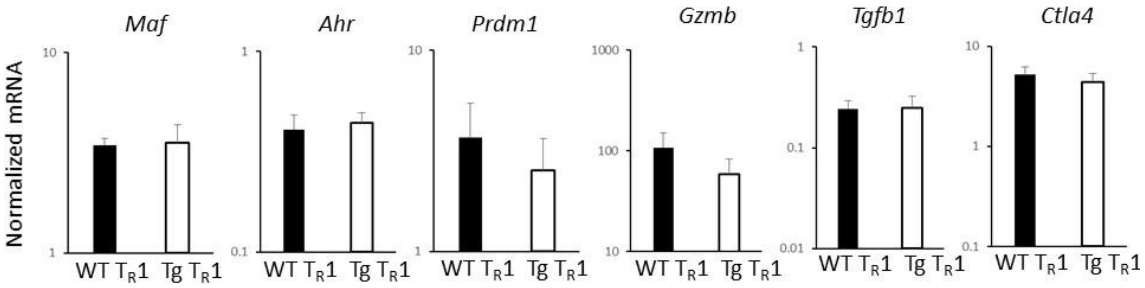

Figure 4: Mature T_R1 cells can rise in the absence of IL-10 signaling.

Maf, *Ahr*, *Prdm1*, *Tgfb1*, *Ctla4* and *Gzmb* mRNA expression (normalized to *Hprt)* of wild type (WT) and CD4-DNIL-10R transgenic (Tg) T_R1 cells were isolated from small intestine cells of anti-CD3 treated mice and FACS-sorted. Data are cumulative of three independent experiments.

To ensure that the CD4-DN-IL10R transgenic T_R1 cells also exhibit suppressive potential, the functionality of the cells was tested in an *in vitro* suppression assay. Wild type or CD4-DN-IL10R transgenic T_R1 cells were isolated and FACS-sorted from the small intestines of anti-CD3 treated wild type and transgenic mice and cultured *in vitro* for 4 days together with violet dye labelled $CD4^+$ T cells (Responder cells).

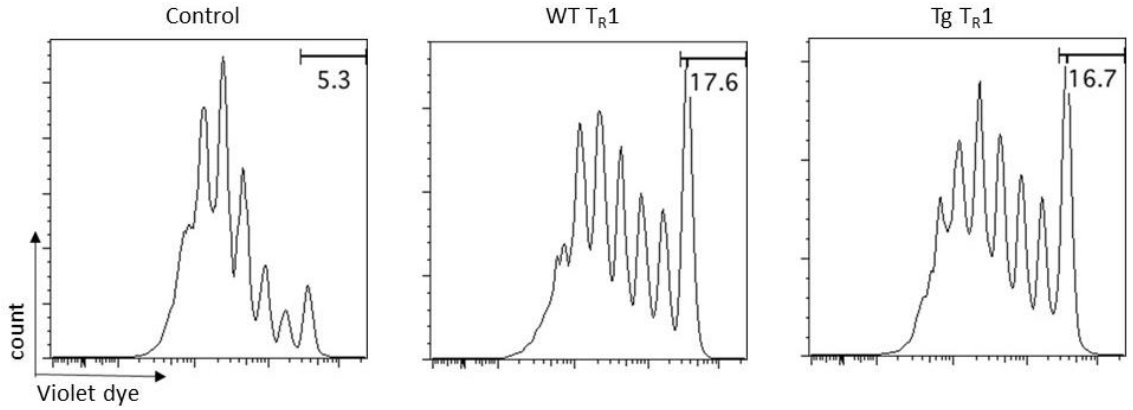

Figure 5: Wild type (WT) and CD4-DN-IL10R transgenic (Tg) T_R1 cells have the same *in vitro* suppressive capacity.

Wild type (WT) or CD4-DNIL-10R transgenic (Tg) $Foxp3^{RFP}$ $IL\text{-}10^{eGFP}$ double reporter mice were treated with 15 µg anti-CD3 antibodies twice (day0, day2). Cells were isolated from the small intestine 4 hours after the second injection and FACS-sorted. T_R1-mediated suppression was measured by violet dye dilution. Responder T cells were isolated from C57Bl/6 mice and labelled with 5 µM violet dye. The cells were activated in the presence of irradiated APCs and 1.5 µg/ml anti-CD3 antibody and cultured either alone (Responder+Responder, control) or in the presence of WT or Tg T_R1 cells at a 1:2 (T_R1: Responder) ratio. After 72 hours the proliferation of the responder T cells was measured via Flow Cytometry. Data are representative of five independent experiments.

This short term *in vitro* suppression assay demonstrated the functionality of CD4-DN-IL10R transgenic T_R1 cells: T_R1 cells with an impaired IL-10 signaling exhibited equal suppressive potential *in vitro* as wild type T_R1 cells (Figure 5).

Taken together, these data indicate that mature and functional T_R1 cells can emerge in mice with T cell specific impairment of IL-10 signaling *in vivo*.

To test the role of IL-27 in T_R1 cell differentiation *in vivo,* $Foxp3^{RFP}$ $IL\text{-}10^{eGFP}$ $IL\text{-}17A^{FP635}$ triple reporter mice were treated with anti-CD3 antibodies to induce T_R1 cells. 12 hours prior to the first anti-CD3 injection the mice were treated with IL-27 neutralizing antibodies (IL-27 mAb) or isotype control antibodies (control). Flow Cytometric analysis of cells from the small intestine revealed that the blocking of IL-27 during the induction of T_R1 cells *in vivo* caused a significant reduction in the T_R1 cell pool ($CD4^+IL\text{-}10^+Foxp3^-$) 4 hours after the last anti-CD3 injection compared to isotype-control-treated animals (Figure 6A). In contrast, the frequency of IL-10 producing T_H17 cells or IL-10 producing $Foxp3^+$ Treg cells in the small intestine was not altered between anti-IL-27 treated animals and animals treated with isotype-control antibodies, indicating a specific effect of IL-27 on the differentiation of T_R1 cells *in vivo*.

To test the effect of IL-27 on already existing T_R1 cells $Foxp3^{RFP}$ $IL\text{-}10^{eGFP}$ $IL\text{-}17A^{FP635}$ triple reporter mice were treated with neutralizing IL-27 antibodies or isotype-control antibodies after the induction of T_R1 cells with anti-CD3 antibodies. 48 hours or 96 hours after the last anti-CD3 antibody injection respectively, cells were isolated from the small intestine. No reduction in the frequency of T_R1 cells could be observed between anti-IL-27 treated animals and animals treated with isotype-control antibodies at any time (Figure 6B). These findings suggest that IL-27 does not affect already existing T_R1 cells, but rather contributes to their differentiation.

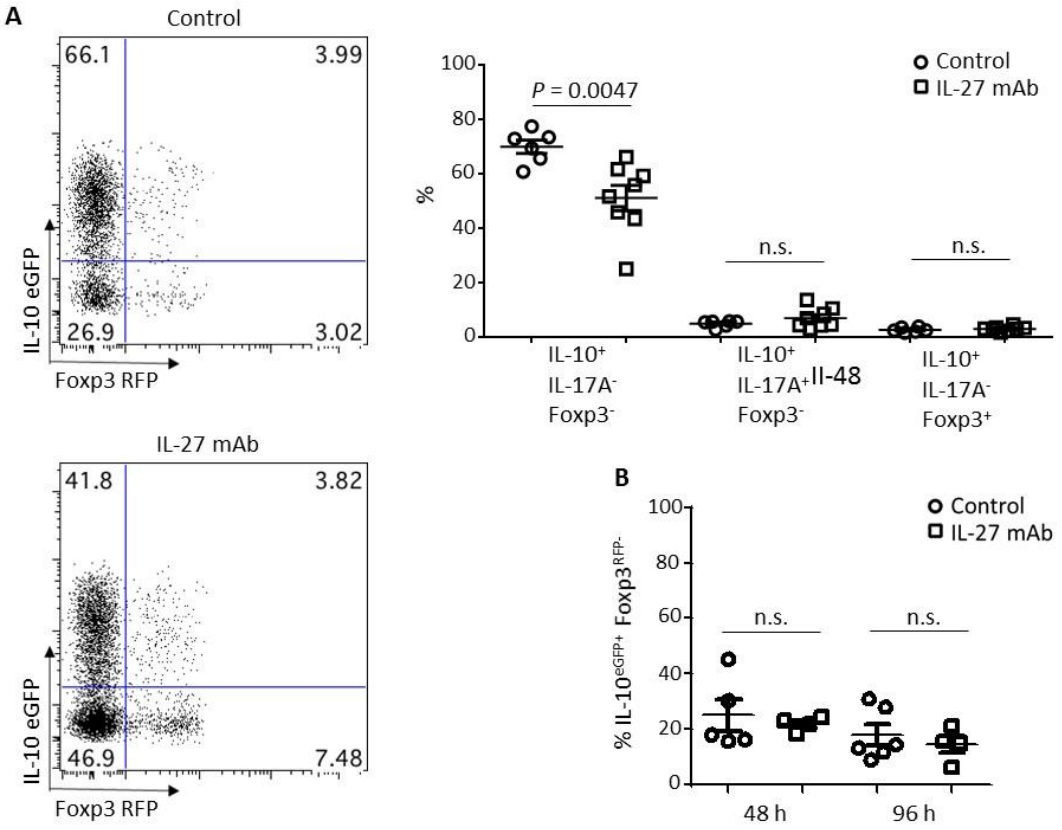

Figure 6: IL-27 promotes T_R1 cell differentiation.

(**A**) Foxp3^{RFP} IL-10^{eGFP} IL-17A^{FP635} triple reporter mice were treated with 15 µg anti-CD3 antibodies twice (day0, day2) and either received neutralizing IL-27 antibodies (5 mg/kg; n=8) or isotype control antibodies (5 mg/kg; n=6) 12 hours before the first injection of anti-CD3 mAb. Cells were isolated from the small intestine 4 hours after the second anti-CD3 injection. Representative dot plots (left) and scatter plots (right; lines indicated mean ± SEM) are shown. Data are cumulative of two independent experiments. (**B**) Foxp3^{RFP} IL-10^{eGFP} IL-17A^{FP635} triple reporter mice were treated with 15 µg anti-CD3 antibodies twice (day0, day2) and either received neutralizing IL-27 antibodies (5 mg/kg) or isotype control antibodies (5 mg/kg) 4 hours after the second injection of anti-CD3 mAb. Cells were isolated from the small intestine at the indicated time points (48h: control n=5; IL-27 mAB n=4; 96h: control n=6; IL-27 mAb n=4; lines indicated mean ± SEM). Results are cumulative of two independent experiments.

3.1.2 Mature T_R1 cells respond to IL-10

IL-10 signaling seems to be dispensable for the differentiation of T_R1 cells *in vivo*, but the role of IL-10 for the biology of mature T_R1 cells remained unknown. To address whether mature T_R1 cells can in principal respond to IL-10, the expression of the IL-10 receptor α-chain (IL-10Rα) was assessed by Flow Cytometry. Foxp3^{RFP} IL-10^{eGFP} IL-17A^{FP635} triple reporter mice were treated with

CD3-specific antibodies to induce T_R1 cells and cells were isolated from the small intestine 4 hours after the last anti-CD3 injection.

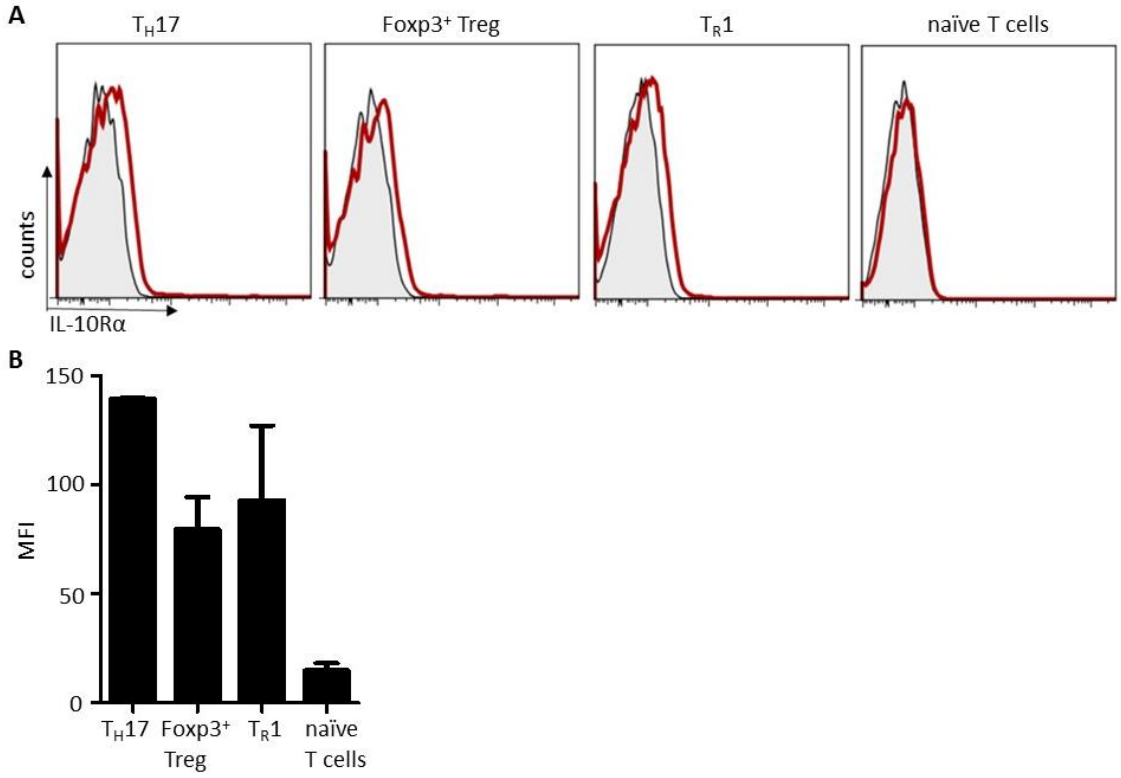

Figure 7: T_R1 cells express IL-10Rα.

$Foxp3^{RFP}$ $IL\text{-}10^{eGFP}$ $IL\text{-}17A^{FP635}$ triple reporter mice were treated with 15 µg anti-CD3 antibodies twice (day0, day2). Cells were isolated from the small intestine 4 hours after the second injection. IL-10Rα expression (**A**) and MFI (**B**) of $Foxp3^+$ Treg cells ($CD4^+Foxp3^+$), T_R1 cells ($CD4^+IL\text{-}10^+Foxp3^-$) and T_H17 cells ($CD4^+Foxp3^-IL\text{-}10^-IL\text{-}17A^+$) were measured by Flow Cytometry. Splenocytes were isolated from untreated wild type mice and IL-10Rα expression (**A**) and MFI (**B**) of naïve T cells ($CD4^+CD44^{low}CD62^{high}$) were measured by Flow Cytometry. Grey area represents the isotype control. Data are representative of two independent experiments.

As control the expression of IL-10 receptor was also assessed on T_H17 cells and $Foxp3^+$ Treg cells. These cells are also induced in the anti-CD3 antibody model in the small intestine [147] and both cell subsets are known to express IL-10 receptor [75, 138]. Naïve T cells, isolated from the spleen of untreated mice, served as negative control since it is known that these cells express no or only very low amounts of IL-10 receptor [138]. The expression levels of IL-10 receptor

were comparable between T_R1 cells and Foxp3$^+$ Treg cells in the small intestine, whereas T_H17 cells showed a slightly higher expression. IL-10 receptor could not be detected on naïve T cells as published before [148] and the expression of IL-10 receptor on T_R1 cells was 6 times higher compared to the expression on naïve T cells (Figure 7A and B).

Next, the functionality of the expressed IL-10Rα was analyzed. IL-10 signaling is known to lead to phosphorylation of STAT3 [152]. Therefore, T_R1 cells were stimulated with IL-10 and STAT3 phosphorylation was measured by Flow Cytometry. T_R1 cells were isolated and FACS-sorted from the small intestine of anti-CD3 treated animals and stimulated *ex vivo*. Foxp3$^+$ Treg cells and naïve T cells were isolated and FACS-sorted from the spleens of untreated wild type mice and served as positive and negative control respectively. To also test the functionality of the CD4-DN-IL10R transgenic mouse model, pSTAT3 levels were assessed in CD4-DN-IL10R transgenic (Tg) T_R1 cells upon stimulation with IL-10. Wild type (WT) T_R1 cells showed a time and dose dependent increase in pSTAT3 level upon stimulation with IL-10, whereas CD4-DN-IL10R transgenic (Tg) T_R1 cells showed only an incremental increase in pSTAT3 levels (Figure 8A and B). The remaining responsiveness to IL-10 by CD4-DN-IL10R transgenic T_R1 cells was most likely caused by the residual IL-10 signaling in these cells as published before [148]. These findings were confirmed with *in vitro* generated and FACS-sorted T_R1 cells using Immunoblotting (Figure 8D).

Stimulation of wild type (WT) and CD4-DN-IL10R transgenic (Tg) T_R1 cells with IL-6 led to a comparable increase in pSTAT3 levels in both cell types, demonstrating that the STAT3 activation was not altered in CD4-DN-IL10R transgenic T_R1 cells *per se* (Figure 8C). Foxp3$^+$ Treg cells showed a strong increase in pSTAT3 level in a time dependent manner, whereas STAT3 was not activated in naïve T cells upon stimulation with IL-10 (Figure 8A).

In conclusion, mature T_R1 cells express IL-10 receptor α-chain and can respond to IL-10. Nevertheless, the question remained as to which role IL-10 signaling played in mature T_R1 cells.

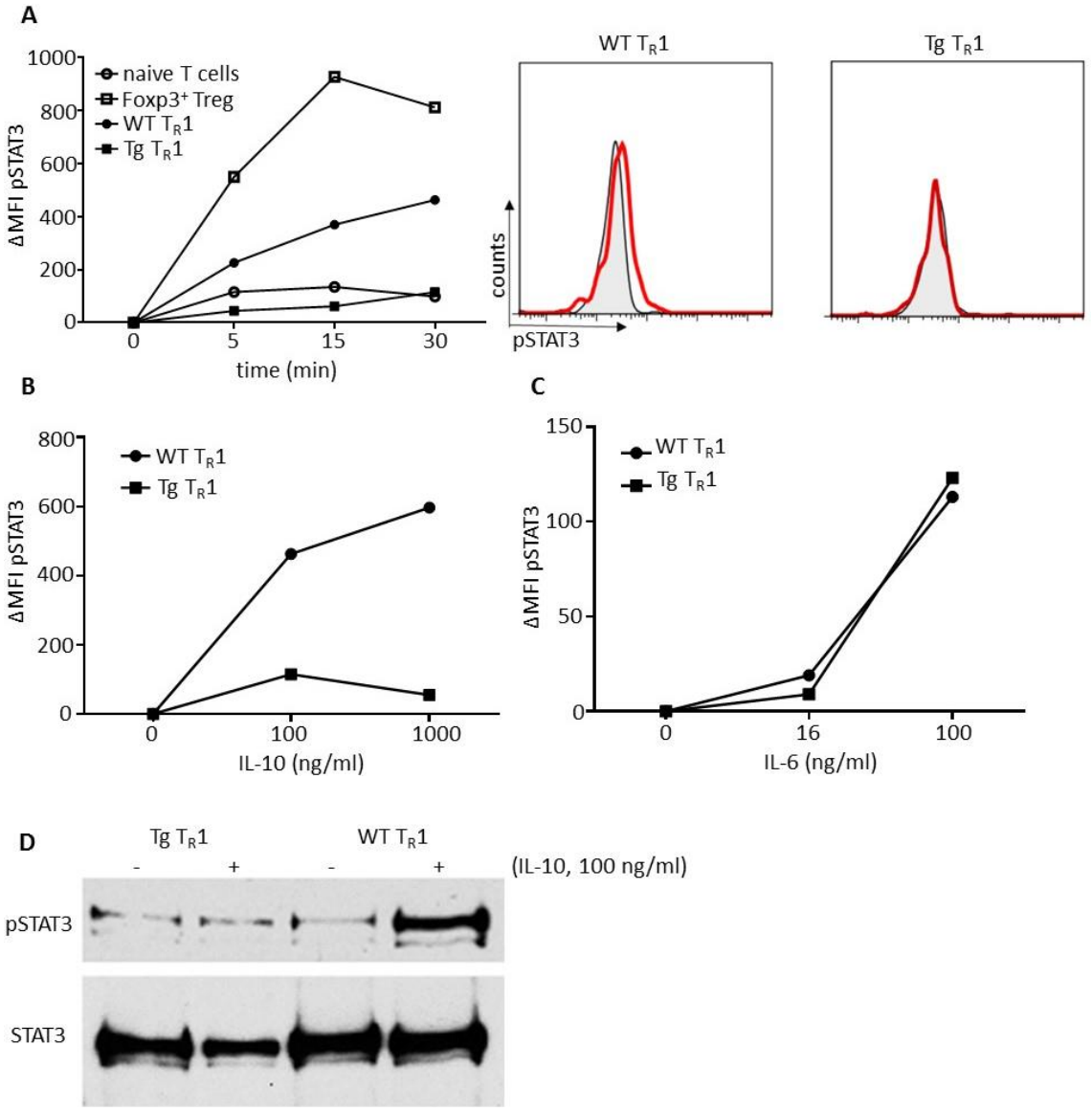

Figure 8: IL-10 signaling is functional in T_R cells.

(**A**-**C**) Cells were isolated from the small intestine of anti-CD3 treated mice and T_R1 cells were isolated by FACS-sorting. ΔMFIs (compared to unstimulated cells) of pSTAT3 levels as assessed by Flow Cytometry are shown. (**A**) Naïve T cells and Foxp3$^+$ Treg cells were isolated and FACS-sorted from the spleen of untreated mice. Naïve T cells (CD4$^+$CD44^{low}CD62^{high}), Foxp3$^+$ T cells (CD4$^+$Foxp3$^+$) and wild type (WT) or CD4-DNIL-10R transgenic (Tg) T_R1 cells (CD4$^+$IL-10$^+$Foxp3$^-$) were stimulated with IL-10 (100 ng/ml) for indicated time points. (Right) Representative histogram plots are shown. Grey area represents the unstimulated control. (**B** and **C**) WT or Tg T_R1 cells were stimulated for 20 min with the indicated concentrations of IL-10 or IL-6. Data are representative of two independent experiments. (**D**) Naïve CD4$^+$ T cells were isolated from WT or Tg Foxp3^{RFP} IL-10^{eGFP} double reporter mice and cultured under T_R1 polarizing conditions. FACS-sorted T_R1 cells (CD4$^+$IL-10$^+$Foxp3$^-$) were re-stimulated in the presence or absence of 100 ng/ml IL-10 for 20 min. Immunoblotting of pSTAT3 and STAT3 is shown.

3.1.3 IL-10 signaling is crucial for the suppressive function of T_R1 cells *in vivo*

Even though IL-10 seems to be dispensable for T_R1 cell differentiation *in vivo,* mature T_R1 cells can respond to IL-10 signaling. However, the role of IL-10 for mature T_R1 cells remained elusive. The question how T_R1 cells sustain their suppressive function is of great importance since T_R1 cells are already being tested as T cell therapy to treat human inflammatory diseases such as Cohn's disease (IBD) and GvHD [145, 146, 153]. It is known that $Foxp3^+$ Treg cells depend on IL-10 signaling to maintain their function [138]. Therefore, the function of CD4-DN-IL10R transgenic T_R1 cells was tested in a challenging *in vivo* colitis model. For this purpose $CD4^+$ IL-$17A^{eGFP+}$ effector (e)T_H17 cells were generated using the $CD45RB^{hi}$ transfer colitis model [75] and isolated from diseased mice. The adoptive transfer of these (e)T_H17 cells into lymphopenic *Rag1*$^{-/-}$ mice caused severe colitis in the recipients, determined based on weight loss, endoscopy and histology (Figure 9A and B). The mice were observed weekly for colitis development. Wild type T_R1 cells (WT) and CD4-DN-IL10R transgenic T_R1 cells (Tg) were generated *in vivo* using the anti-CD3 antibody model. Cells were isolated via FACS-sorting from the small intestine of diseased mice and co-transferred with (e)T_H17 cells in a ratio of 1:1. The co-transfer of wild type T_R1 cells completely prevented the development of the disease. Mice that received (e)T_H17 cells together with wild type T_R1 cells showed a significant lower endoscopic (Figure 9B) and histological (Figure 9B) colitis score compared to animals that only received (e)T_H17 cells. Strikingly, CD4-DN-IL10R transgenic T_R1 cells could not control (e)T_H17 cells *in vivo*. The weight loss, endoscopic and histological colitis score of mice that received (e)T_H17 cells together with CD4-DN-IL10R transgenic T_R1 cells was comparable with that of mice that only received a single transfer of (e)T_H17 cells (Figure 9A and B). Notably, neither the single transfer of CD4-DN-IL10R transgenic T_R1 cells (Tg) into lymphopenic *Rag1*$^{-/-}$ mice nor the transfer of wild type T_R1 cells caused colitis (Figure 9C).

These findings demonstrate that IL-10 signaling in T_R1 cells is crucial to maintain their suppressive function. However, T_R1 cells with an impaired IL-10 signaling did not aggravate disease caused by (e)T_H17 cells or caused disease on their own.

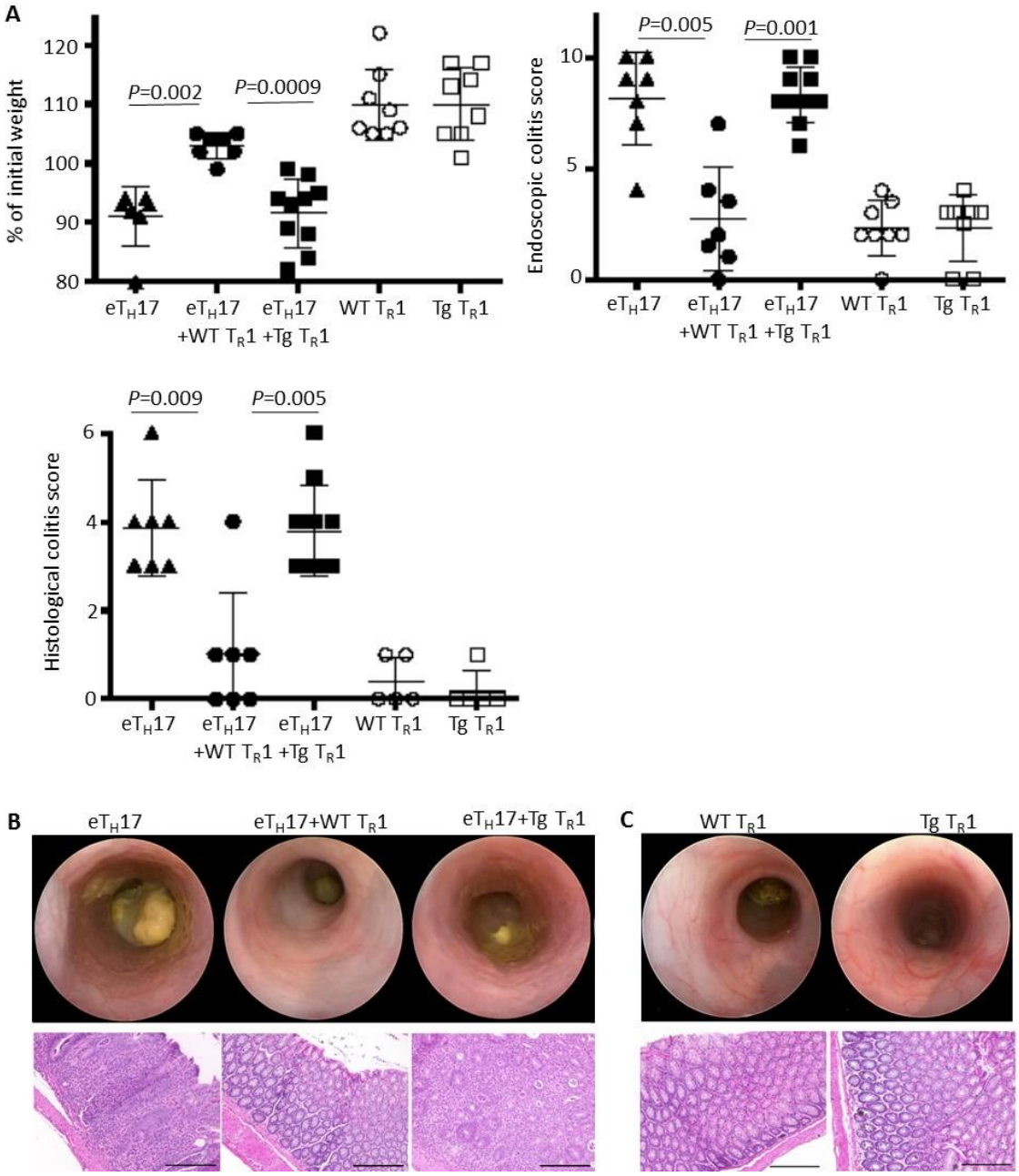

Figure 9: IL-10 signaling in T_R1 cells is essential to maintain their suppressive function.

Wild type (WT) or CD4-DNIL-10R transgenic (Tg) T_R1 cells were isolated from the small intestine of anti-CD3 treated mice and injected alone or together with *in vivo* differentiated effector (e)T_H17 cells. (**A**) Mass loss, endoscopic and histological colitis score 5 weeks upon transfer (eTh17 n=7; eTh17+WT T_R1 n=7; eTh17+Tg T_R1 n=10; WT T_R1 n=8; Tg T_R1 n=8; lines indicate mean ± SEM). Representative endoscopic and histological (**B** and **C**; scale bars, 200 µm) findings are shown. Results are cumulative of two independent experiments. Mann-Whitney U test was used to calculate significance.

3.1.4 T_R1 cells do not cause disease in the absence of IL-10 signaling

As mentioned above, T_R1 cells are currently tested in human trials to treat inflammatory diseases [145, 146, 154], therefore it is of great significance to determine if T_R1 cell–based therapy is safe in a pro-inflammatory environment even in the absence of IL-10. Especially since there is new evidence that shows that Foxp3$^+$ Treg cells can convert into pro-inflammatory T cells and thus challenge the use of this cell type as T cell therapy [137]. The data obtained in the transfer colitis model backs up the argument of the safety of T_R1 cells since T_R1 cells with impaired IL-10 signaling did not mediate disease progression even though they failed to suppress colitis caused by (e)T_H17 cells. To reinforce these findings, CD4-DN-IL10R transgenic T_R1 cells were tested in an alternative mouse model using various dosages. The mouse model of Graft-versus-Host disease (GvHD) is based on the adoptive transfer of MHC-mismatched cells into a lethally irradiated recipient. This model was chosen because it resembles the current application of T_R1 cells in humans upon bone marrow transplantation. To this end, naïve T cells were isolated from the spleens of Foxp3^{RFP} IL-17A^{eGFP} double reporter mice as well as from wild type or CD4-DN-IL10R transgenic Foxp3^{RFP} IL-10^{eGFP} double reporter mice. (e)T_H17 cells, wild type T_R1 cells and CD4-DN-IL10R transgenic T_R1 cells were generated *in vitro* and FACS-sorted. (e)T_H17 cells, wild type and CD4-DN-IL10R transgenic T_R1 cells were all generated from mice on C57/Bl6 background. These T cells were adoptively transferred into irradiated BALB/c wild type mice together with T cell depleted bone marrow cells (BM) from C57/Bl6 wild type mice. The transfer of T cell depleted bone marrow cells alone served as negative control since the recipients did not develop GvHD (Figure 10A and B). Mice were monitored daily for development of GvHD, which is characterized by weight loss, reduced activity and reduced skin integrity, ruffled fur and a hunched back. In this model the adoptive transfer of (e)T_H17 cells alone caused a rapid disease development (Figure 10A). After only 2 weeks, over 80% of the mice died or had to be euthanized in accordance with our animal protocol as a consequence of the (e)T_H17 cell transfer (Figure 10B).

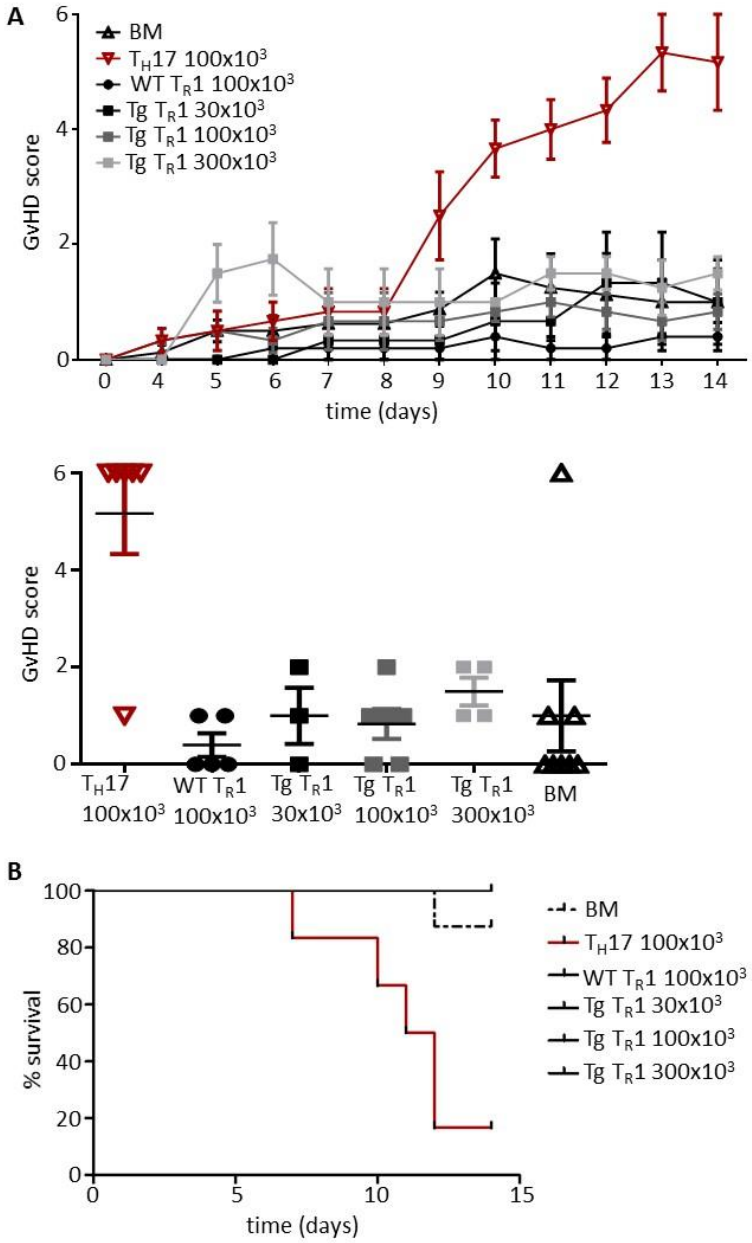

Figure 10: Wildtype (WT) and transgenic (Tg) T_R1 cells do not cause acute GvHD.

$CD4^+$ T cells were isolated from wild type (WT) or CD4-DN-IL10R (Tg) $Foxp3^{RFP}$ $IL\text{-}10^{eGFP}$ double reporter mice and cultured under T_R1 polarizing conditions. Naïve $CD4^+$ T cells from $Foxp3^{RFP}$ $IL\text{-}17A^{eGFP}$ double reporter mice were cultured under T_H17 polarizing conditions. T_R1 (WT or Tg) or T_H17 cells were FACS-sorted based on GFP and mRFP expression and injected intravenously together with $5x10^6$ T cell depleted BM cells into irradiated BALB/c mice. (**A**) The degree of clinical GvHD was assessed daily (upper panel) and on day 14 (lower panel) based on five parameters: weight loss, activity, posture, fur texture and skin integrity. (**B**) Kaplan-Meier curve of survival. Results are cumulative of two independent experiments (BM n=8; T_H17 n=6; WT T_R1 n=5; Tg T_R1 $3x10^4$ n=4, Tg T_R1 $1x10^5$ n=6, Tg T_R1 $3x10^5$ n=4; lines indicate mean ± SEM). Mann-Whitney U test was used to calculate significance.

T_R1 cells did not induce GvHD and more importantly also CD4-DN-IL10R transgenic T_R1 cells did not mediate disease. Even recipient BALB/c mice that received a three times higher dosage of CD4-DN-IL10R transgenic T_R1 cells compared to (e)T_H17 cells did not show signs of GvHD two weeks post transfer (Figure 10A). These findings indicate that T_R1 cells with an impaired IL-10 signaling, even though they are unable to suppress colitis as shown in Figure 9, do not acquire pathogenic properties.

3.1.5 IL-10 signaling in T_R1 cells sustains their IL-10 production

Since T_R1 cells with an impaired IL-10 signaling failed to suppress (e)T_H17 cells in a transfer colitis model, the cytokine profile of wild type and CD4-DN-IL10R transgenic T_R1 cells was analyzed in more detail to identify the underlying mechanisms. For this purpose wild type Foxp3^{RFP} IL-10^{eGFP} double reporter mice and CD4-DN-IL10R transgenic Foxp3^{RFP} IL10^{eGFP} double reporter mice were treated with anti-CD3 antibodies and cells were isolated from the small intestine. Wild type or CD4-DN-IL10R transgenic T_R1 cells were FACS-sorted and re-stimulated *in vitro* with anti-CD3 and anti-CD28 antibodies for 60 hours. The cytokines in the supernatant of the cell culture were assessed by Cytometric Bead Array (CBA) analysis. Wild type T_R1 cells showed a typical T_R1 cytokine profile [104] characterized by high production of IFN-γ and IL-10 and low secretion of IL-2, IL-4, IL-17A, TNF-α and IL-6 (Figure 11A). Remarkably, CD4-DN-IL10R transgenic T_R1 cells produced significantly lower amounts of IL-10 and higher amounts of IL-17A upon re-stimulation than wild type T_R1 cells (Figure 11A). The production of the other tested cytokines, IFN-γ, IL-2, IL-4, IL-6 and TNF-α was not altered in CD4-DN-IL10R transgenic T_R1 cells compared to wild type T_R1 cells (Figure 11A). To further test if CD4-DN-IL10R transgenic T_R1 cells lose the expression of T_R1 cell signature genes (*Maf*, *Ahr, Prdm1*, *Gzmb*, *Tgfb1* and *Ctla4*), mRNA was extracted from re-stimulated wild type and CD4-DN-IL10R transgenic T_R1 cells. The expression of transcription factors related to the differentiation of T_R1 cells and IL-10 expression, *Maf*, *Ahr* and *Prdm1*, was not significantly changed between CD4-DN-IL10R transgenic and wild type T_R1 cells (Figure 11B).

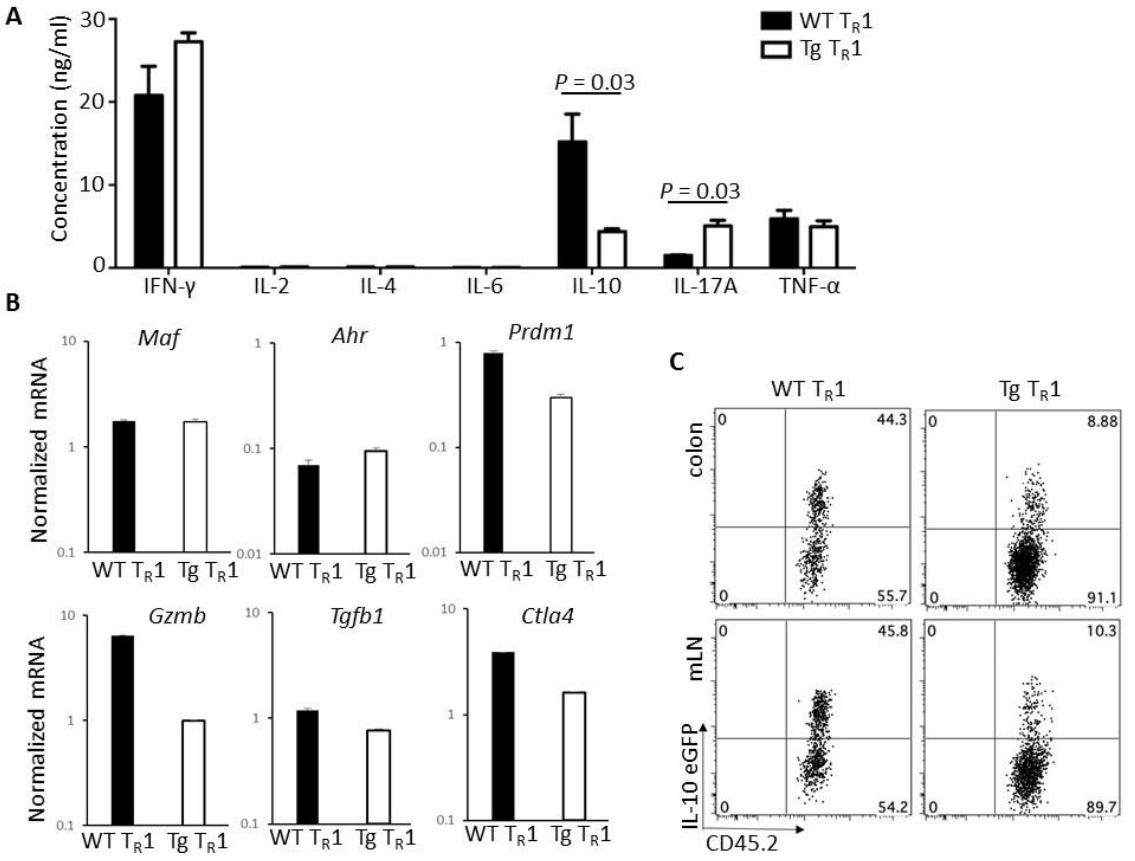

Figure 11: IL-10 signaling in T_R1 cells sustains IL-10 expression.

T_R1 cells were isolated and FACS-sorted from the small intestine of anti-CD3 treated wild type (WT) or CD4-DNIL-10R transgenic (Tg) Foxp3^{RFP} IL-10^{eGFP} double reporter mice. (**A**) T_R1 cells were re-stimulated with anti-CD3 and anti-CD28 for 60 hours, and cytokine production was quantified from the cell culture supernatants using Cytometric Bead Array. Mann-Whitney U test was used to calculate significance. (**B**) *Maf*, *Ahr*, *Prdm1*, *Tgfb1*, *Ctla4* and *Gzmb* mRNA expression (normalized to *Hprt*) of re-stimulated WT and Tg T_R1 cells. Data are representative of three independent experiments. (**C**) 5 x 10^4 WT or Tg T_R1 cells were injected into *Rag1*$^{-/-}$ mice. Cells were isolated 5 weeks after transfer and IL-10^{eGFP} expression of the transferred cells was analyzed by Flow Cytometry. Representative dot plots of 4 pooled mice per group gated on CD4$^+$CD45.2$^+$ events are shown. Data are representative of three independent experiments.

Furthermore, the expression of genes encoding Granzym B, TGF-β and CTLA-4, which have been linked to the suppressive function of T_R1 cells [104, 106], was likewise not significantly altered in CD4-DN-IL10R transgenic T_R1 cells. Only the expression of *Gzmb* was by trend lower in CD4-DN-IL10R transgenic cells compared to wild type T_R1 cells (Figure 11B). Collectively, these data indicate that while IL-10 signaling is essential to maintain the IL-10 production by T_R1 cells *in vitro*, the T_R1 cell signature genes are not affected by impaired IL-10 signaling.

In order to additionally test T_R1 cell stability in a more challenging way, *in vivo* generated wild type and CD4-DN-IL10R transgenic T_R1 cells, which had been isolated from the small intestine of anti-CD3 treated Foxp3^{RFP} IL-10^{eGFP} double reporter mice, were adoptively transferred into lymphopenic *Rag1*$^{-/-}$ mice. Five weeks after the transfer, cells were isolated from the colon and mesenteric lymph nodes. IL-10 expression of the isolated cells was analyzed by Flow Cytometry. Interestingly, only around 10% of the transferred CD4-DN-IL10R transgenic T_R1 cells remained IL-10 producing, whereas still approximately 45% of the wild type T_R1 cells secreted IL-10 (Figure 11C). This result further substantiate the findings obtained *in vitro*: Mature T_R1 cells depend on IL-10 signaling to sustain their IL-10 production and thereby their suppressive function.

The anti-CD3 antibody model of transient intestinal inflammation is a well-established model to study T_R1 cell induction. As shown above, differentiation of T_R1 cells was not dependent on an intact IL-10 signaling in this model (Figure 3). On the basis of these data, the question of whether the maintenance of IL-10 production would be altered in CD4-DN-IL10R transgenic T_R1 cells in this model was analyzed. Thus, wild type and CD4-DN-IL10R transgenic Foxp3^{RFP} IL10^{eGFP} double reporter mice were treated with anti-CD3 antibodies and cells were isolated from the small intestine 48 hours after the last anti-CD3 injection. As shown above, the frequency of wild type and CD4-DN-IL10R transgenic T_R1 cells was equal 4 hours after the last anti-CD3 injection during the peak of IL-10 expression (Figure 3). However, 48 hours after induction, the frequency of T_R1 cells in CD4-DN-IL10R transgenic mice was significantly reduced compared to wild type animals (Figure 12A). Finally, to exclude that the observed effect was due to an altered, more specifically a reduced proliferation of CD4-DN-IL10R transgenic T_R1 cells, wild type (WT) and CD4-DN-IL10R transgenic (Tg) mice were treated with anti-CD3 antibodies and injected with BrdU 8 hours prior to the last anti-CD3 treatment. T_R1 cells from the small intestine of diseased mice were FACS-sorted and their DNA was stained for implemented BrdU and analyzed by Flow Cytometry. BrdU can only be integrated in the genome of dividing cells, thus the frequency of BrdU positive cells reflects the proliferative potential of the cell subset *in vivo*. CD4-DN-IL10R transgenic T_R1 cells showed not only the same, but even a higher proliferative activity compared to wild type T_R1 cells (Figure

12B), indicating that IL-10 signaling in T_R1 cells is not essential for an efficient proliferation of these cells.

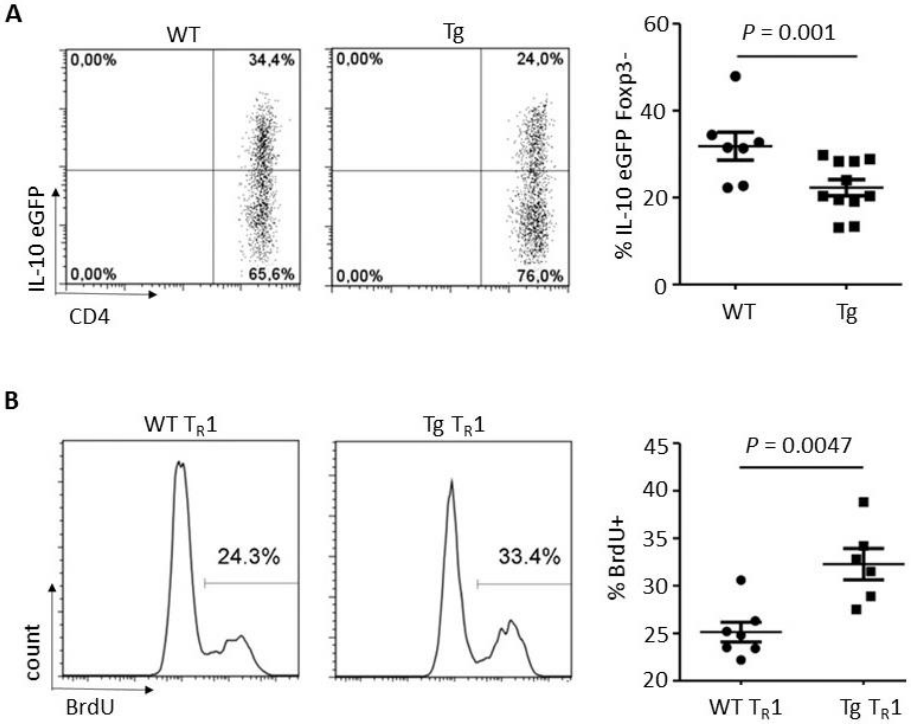

Figure 12: CD4-DN-IL-10R transgenic (Tg) T_R1 cells lose IL-10 expression *in vivo*, but proliferate more than wild type T_R1 cells.

Wild type (WT) or CD4-DNIL-10R transgenic (Tg) $Foxp3^{RFP}$ $IL\text{-}10^{eGFP}$ reporter mice were treated with 15 µg anti-CD3 antibodies twice (day0, day2). (**A**) Cells were isolated from the small intestine 48 hours after the second injection. Representative dot plots (left) and scatter plots (right; WT n=7; Tg n=11; lines indicate mean ± SEM) are shown. Data are cumulative of three independent experiments. (**B**) Mice were injected with BrdU 8 hours before the second anti-CD3 injection. T_R1 cells were FACS-sorted 4 hours after the second anti-CD3 injection and stained for BrdU. Representative histograms (left) and scatter plots (right; WT n=7; Tg n=6; lines indicate mean ± SEM) are shown. Results are cumulative of two independent experiments. Mann-Whitney U test was used to calculate significance.

In summary, these findings demonstrate that IL-10 signaling in T_R1 cells sustains their IL-10 production *in vitro* and *in vivo*.

3.1.6 IL-10 promotes IL-10 production via activation of p38 MAP kinase in T_R1 cells

Stimulation of the IL-10 receptor leads to the activation of several kinases, STAT3 notably being the best studied during this process. It is known that IL-10 production of Foxp3$^+$ Treg cells is dependent on STAT3 activation and that the differentiation of T_R1 cells with IL-27 requires the phosphorylation of STAT3 [92, 139]. Nonetheless, p38 MAP kinase is also linked to the regulatory function of pTreg cells and IL-10 production of macrophages and monocytes [155-158]. Therefore, the role of STAT3 and p38 MAP kinase for IL-10 production of T_R1 cells was addressed. To this end, naïve T cells were isolated from wild type or CD4-DN-IL10R transgenic Foxp3^{RFP} IL-10^{eGFP} double reporter mice and cultured under T_R1 polarizing conditions *in vitro*. T_R1 cells were FACS-sorted and re-stimulated *in vitro*. Read-out was the expression level of IL-10^{eGFP}, phosphorylated p38 MAP kinase and phosphorylated STAT3 in CD4-DN-IL10R transgenic T_R1 cells compared to wild type T_R1 cells over time. Further strengthening the validity of the data presented above (Figure 11), CD4-DN-IL10R transgenic T_R1 cells showed a faster decrease of IL-10^{eGFP} over time than wild type T_R1 cells (Figure 13A).

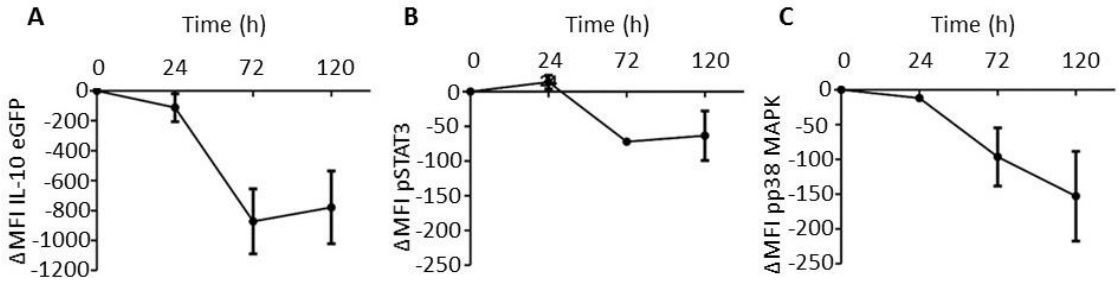

Figure 13: CD4-DN-IL-10R transgenic (Tg) T_R1 cells lose IL-10 expression and p38 MAP kinase phosphorylation *in vitro*.

(**A-C**) CD4$^+$ T cells were isolated from wild type (WT) or CD4-DN-IL10R (Tg) Foxp3^{RFP} IL-10^{eGFP} double reporter mice and cultured under T_R1 polarizing conditions. FACS-sorted T_R1 cells were re-stimulated and IL-10^{eGFP} (**A**), pSTAT3 (**B**) and pp38 MAP kinase (**C**) levels were measured using Flow Cytometry. ΔMFI of Tg T_R1 cells compared to WT T_R1 cells are shown. Results are cumulative of three independent experiments.

Surprisingly, only a mild change could be observed in the phosphorylation status of STAT3 in CD4-DN-IL10R transgenic T_R1 cells compared to wild type T_R1 cells, while the phosphorylation of p38 MAP kinase was distinctively reduced in CD4-

DN-IL10R transgenic T_R1 cells upon re-stimulation (Figure 13B and C). This finding suggest a possible correlation between IL-10 production in T_R1 cells and p38 MAP kinase and imply a minor role of STAT3 during this process.

To further validate these data, the functional role of STAT3 and p38 MAP kinase was tested using specific kinase inhibitors. Naïve T cells were isolated from Foxp3^{RFP} IL-10^{eGFP} double reporter mice and cultured under T_R1 cell polarizing conditions *in vitro*. After 5 days of culture cells were re-activated in the presence of anti-CD3 and anti-CD28 antibodies for 48 hours. During the re-activation a specific p38 MAP kinase inhibitor (SB203580) or an equal amount of DMSO was added to the culture, as well as an inhibitor for STAT3 (STAT3 inhibitor).

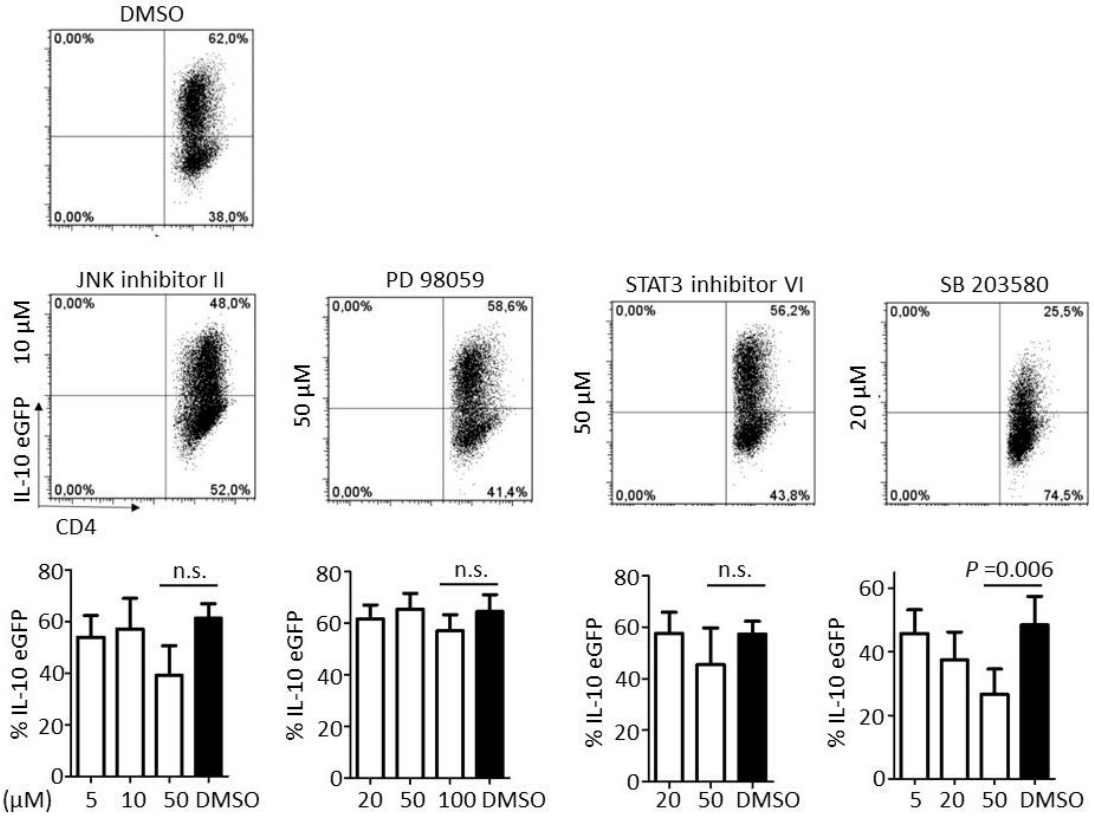

Figure 14: p38 MAP kinase maintains IL-10 production in T_R1 cells.

CD4$^+$ T cells were isolated from Foxp3^{RFP} IL-10^{eGFP} double reporter mice, cultured under T_R1 polarizing conditions and re-activated for 48 hours with or without JNK inhibitor II, PD 98059 (ERK1/2 inhibitor), STAT3 inhibitor VI or SB 203580 (p38 inhibitor). Representative dot plots and frequency of IL-10^{eGFP+} cells are shown (mean ± SEM of three independent experiments). One-way ANOVA (post-test Tukey) was used to calculate significance.

As control, other major kinase pathways were blocked using an ERK1/2 inhibitor (PD98059) and JNK inhibitor (JNK inhibitor II) since these kinases are linked to IL-10 production in other immune cells such as T_H1 and T_H2 cells or monocytes and macrophages [159, 160]. The frequency of T_R1 cells (IL-10^{eGFP+} $Foxp3^{RFP-}$) in the presence or absence of the specific inhibitors was assessed by Flow Cytometry. Inhibition of STAT3 and JNK, led to a mild reduction of T_R1 cells in the culture compared to DMSO treated cells, but this difference was not significant (Figure 14). The ERK1/2 inhibitor did not show an effect on IL-10 production of T_R1 cells in the culture (Figure 14). Remarkably, the inhibition of p38 MAP kinase led to a reduction of IL-10^{eGFP} positive cells in the culture in a dose dependent manner (Figure 14).

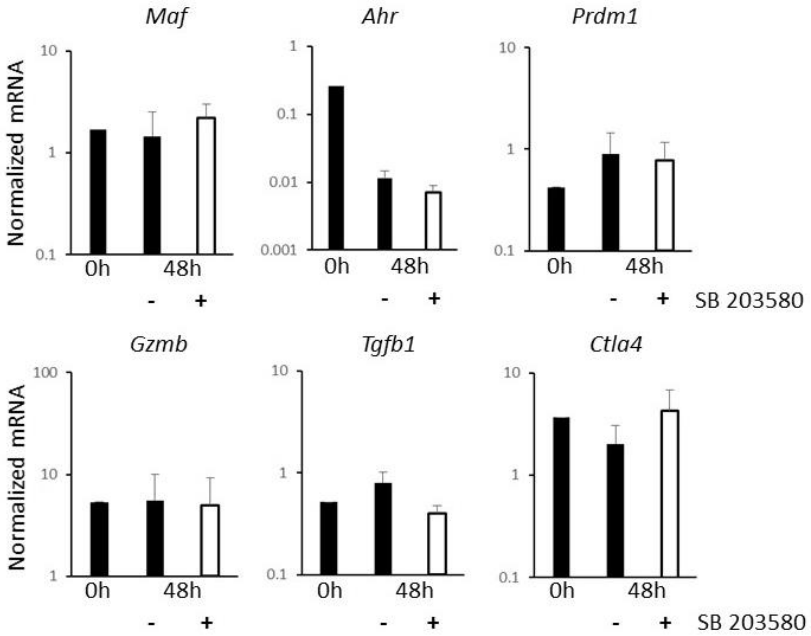

Figure 15: p38 MAP kinase does not regulate T_R1 cell signature genes.

$CD4^+$ T cells from $Foxp3^{RFP}$ IL-10^{eGFP} double reporter mice were cultured under T_R1 cell polarizing conditions and T_R1 cells were FACS-sorted. *Maf*, *Ahr*, *Prdm1*, *Tgfb1*, *Ctla4* and *Gzmb* mRNA expression (normalized to *Hprt*) of fresh or re-activated T_R1 cells with SB 203580 or DMSO are shown. Results are cumulative of three independent experiments.

To test if the blockade of p38 MAP kinase influenced the expression of T_R1 cell signature genes, T_R1 cells were generated *in vitro*, FACS-sorted and re-activated in the presence or absence of p38 MAP kinase inhibitor. The mRNA levels of *Maf*, *Ahr* and *Prdm1* as well as the expression of *Gzmb*, *Tgfb1* and *Ctla4* was not altered between treated and untreated T_R1 cells (Figure 15)

In conclusion, IL-10 signaling appears to activate p38 MAP kinase and this kinase in turn sustains the IL-10 production in a positive-feedback loop. However, STAT3 seems to play only a minor role in the maintenance of IL-10 production in T_R1 cells.

3.1.7 p38 MAP kinase and STAT3 are important during the differentiation of T_R1 cells

STAT3 plays a non-redundant role during the differentiation of T_R1 cells with IL-27 [92]. To confirm the functionality of the STAT3 inhibitory compound and to also test the role of p38 MAP kinase, JNK and ERK during the differentiation of T_R1 cells with IL-27, naïve T cells were cultured *in vitro* in T_R1 polarizing conditions in the presence or absence of different kinase inhibitors. As expected, blockade of STAT3 (STAT3 inhibitor VI) during the differentiation led to a strongly decreased frequency of T_R1 cells (IL-10^+ Foxp3^-) in comparison to DMSO treated control culture (Figure 16). Inhibition of JNK (JNK inhibitor II) or ERK1/2 (PD 98059) did not alter the differentiation of T_R1 cells. Strikingly, in the presence of p38 MAP kinase inhibitor (SB 203580) the differentiation of naïve T cells into T_R1 cells was strongly diminished compared to DMSO treated control cells, similar to the results obtained with the STAT3 inhibitor (Figure 16).

In conclusion, these results imply that IL-27 initiates the differentiation of T_R1 cells through activation of STAT3 and p38 MAP kinase. However, T_R1 cells are required to respond to IL-10 in order to maintain their IL-10 production and in turn their *in vivo* functionality. The maintenance of IL-10 production is mainly sustained via p38 MAP kinase signaling whereas STAT3 seems to play a redundant role during this process.

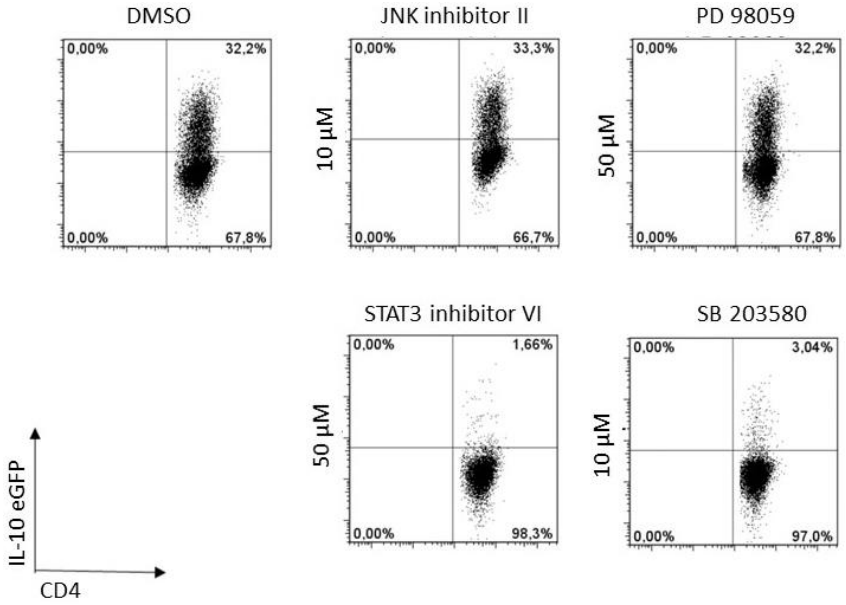

Figure 16: p38 MAPK and STAT3 inhibition block the differentiation of T_R1 cells *in vitro.*

$CD4^+$ T cells were isolated from wild type $Foxp3^{RFP}$ $IL\text{-}10^{eGFP}$ reporter mice and cultured under T_R1 polarizing conditions in the presence or absence of JNK inhibitor II (50 µM), PD 98059 (ERK1/2 inhibitor; 50 µM), STAT3 inhibitor VI (50 µM) or SB 203580 (p38 MAP kinase inhibitor; 20 µM). Representative dot plots of two independent experiments are shown.

3.1.8 IL-10 signaling in human T_R1 cells sustains IL-10 production

Human T_R1 cells are known to have a strong potential to induce immune tolerance [106, 161, 162] and therefore are of major interest as possible T cell-based therapy in humans. Therefore, it is of great importance to understand the mechanisms that maintain the functionality of these cells. Thus, the role of IL-10 signaling in human T_R1 cells was then studied. LAG-3 and CD49b are two markers that identify human and mouse T_R1 cells [107]. Using these markers, it was possible to isolate circulating human T_R1 cells. To this end, PBMCs were enriched from buffy coats of healthy donors and T_R1 cells ($CD4^+$ $CD45RA^{low}$ $CD49b^+$ $LAG\text{-}3^+$) were purified from the PBMCs using FACS (Figure 17A). The isolated T_R1 cells were re-activated with anti-CD3 and anti-CD28 antibodies for 4 days *in vitro* in the presence of either human IL-10Rα blocking antibodies or isotype control antibodies. The concentration of IL-10 and IFN-γ in the cell culture supernatants was measured using a Cytometric Bead Assay. Remarkably, IL-10

production of human T_R1 cells was significantly decreased by more than 30% in the presence of blocking IL-10Ra antibodies compared to T_R1 cells stimulated in the presence of isotype control antibody (Figure 17B), demonstrating the importance of IL-10 signaling for human T_R1 cells. However, no difference could be observed in the concentration of IFN-γ in the cell culture supernatants (Figure 17B), indicating IL-10 signaling is indeed needed to maintain the IL-10 production in human T_R1 cells but not cytokine release *per se*.

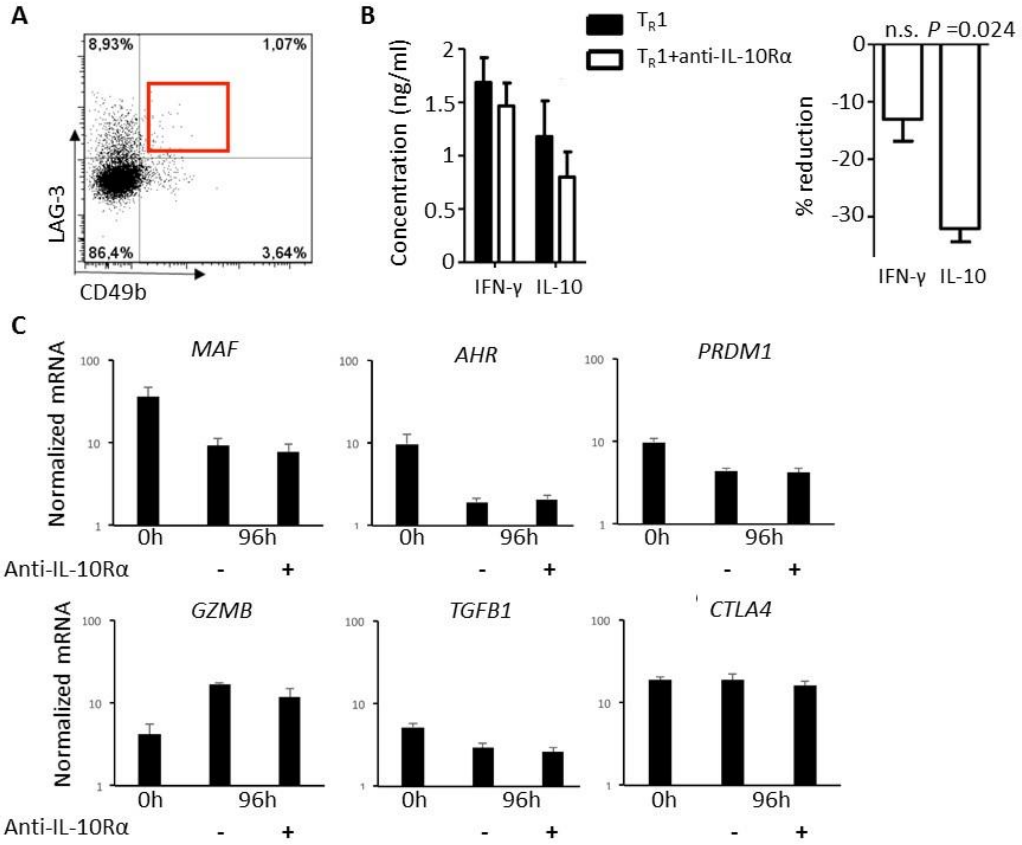

Figure 17: IL-10 receptor signaling is essential to maintain IL-10 production in human T_R1 cell.

(**A-C**) Circulating human T_R1 cells ($CD4^+CD45RA^{low}CD49b^+LAG\text{-}3^+$) were FACS-sorted from PBMCs of healthy donors (n=5). T_R1 cells were re-stimulated with anti-CD3 and anti-CD28 for 96 hours with either 50 µg/ml human IL-10Rα antibodies or isotype control antibodies and the indicated cytokines were quantified. A paired t test was used to calculate significance. (**B**). *MAF*, *AHR*, *PRDM1*, *TGFB1*, *CTLA4* and *GZMB* mRNA expression (normalized to *HPRT*) of freshly isolated or re-stimulated T_R1 cells in the presence or absence of IL-10Rα antibody are reported (**C**). Data (**A-C**) are cumulative of five independent experiments.

Next the T_R1 cell gene signature was analyzed in human T_R1 cells which were re-activated in the presence of either human IL-10Rα blocking antibodies or

isotype control antibodies. In line with the murine data no altered mRNA expression of *MAF*, *AHR* or *PRDM1* could be found in T_R1 cells re-activated together with IL-10Rα blocking antibodies compared to control. Likewise, *GZMB*, *TGFB1* and *CTLA4* were not differently expressed between the two groups (Figure 17C).

Collectively, the data obtained analyzing human T_R1 cells confirmed the findings from the murine models. IL-10 signaling in human T_R1 cells is likewise critical to maintain their IL-10 production.

3.2 Analysis of IL-10 producing T cell subsets based on LAG-3 and CD49b expression

3.2.1 LAG-3$^+$ CD49b$^+$ double positive cells are enriched in the IL-10$^+$ cell subset

Co-expression of CD49b and LAG-3 has been proposed as surface markers identifying human and murine T_R1 cells [107]. Previously T_R1 cells were defined on the basis of their cytokine expression profile, namely high expression of IL-10, low IL-4 and IL-17A production and IFN-γ production dependent on the environment of the cell, a lack of Foxp3 expression and strong immune-suppressive potential [104]. However, CD49b and LAG-3 are not co-expressed by all T cells fulfilling these criteria. Also some CD49b and LAG-3 double positive cells are indeed IL-10 negative. These data raised the question as to which parameters would best define a T_R1 cell. In order to address this question the expression of CD49b and LAG-3 among the CD4$^+$ T cells that produce IL-10 and lack Foxp3 expression (CD4$^+$IL-10$^+$Foxp3$^-$) was studied using the above described model of anti-CD3 specific antibody induced tolerance. On the peak of T_R1 cell induction cells were isolated from the small intestine, spleen, lung and liver and the expression of IL-10, CD49b and LAG-3 was analyzed via Flow Cytometry.

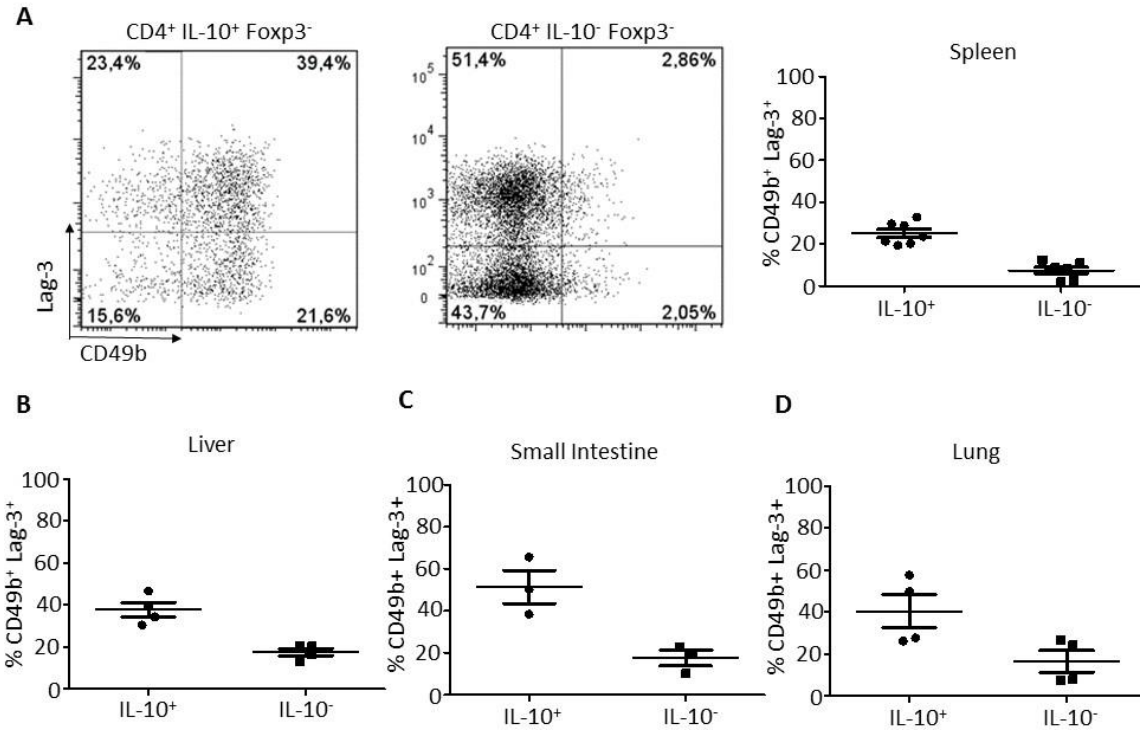

Figure 18: CD49b$^+$ LAG-3$^+$ cells are enrich among the CD4$^+$ IL-10$^+$ Foxp3$^-$ cells.

Foxp3^{RFP} IL-10^{eGFP} reporter mice were treated with 15 µg anti-CD3 antibodies twice (day0, day2). Cells were isolated 4 hours after the second anti-CD3 injection from the small intestine, liver, lung and spleen. Representative dot plots of LAG-3 and CD49b staining from splenocytes are shown. Scatter plots (lines indicate mean ± SEM; Spleen n=7; Liver n=4; Small Intestine n=3; Lung n=4) are cumulative from two independent experiments.

Among the cell subset expressing CD4$^+$ IL-10$^+$ Foxp3$^-$ cells, formally defined as T_R1 cells, cells also co-expressing CD49b and LAG-3 were enriched compared to the cell subset of CD4$^+$ IL-10$^-$ Foxp3$^-$ cells (Figure 18). The strongest enrichment could be found in the small intestine where around 60% of the IL-10$^+$ Foxp3$^-$ cells also expressed CD49b and LAG-3 (Figure 18C). In spleen, liver and lung these cells were still enriched in the IL-10$^+$ compartment, but not as strong as it was in the small intestine (Figure 18A, B and D). Applying a different gating strategy, namely gating on CD4$^+$ CD49b$^+$ LAG-3$^+$ cells, revealed that IL-10 producing cells were also strongly enriched in this subset in the small intestine supporting the efficiency of the two markers (Figure 19C). Nevertheless, in spleen, liver and lung this effect was not as prominent, only around 20-30% of the LAG-3$^+$ CD49b$^+$ cells also produced IL-10 which is however still an enrichment compared to the CD49b$^-$ LAG-3$^-$ cell subset (Figure 19A, B and D).

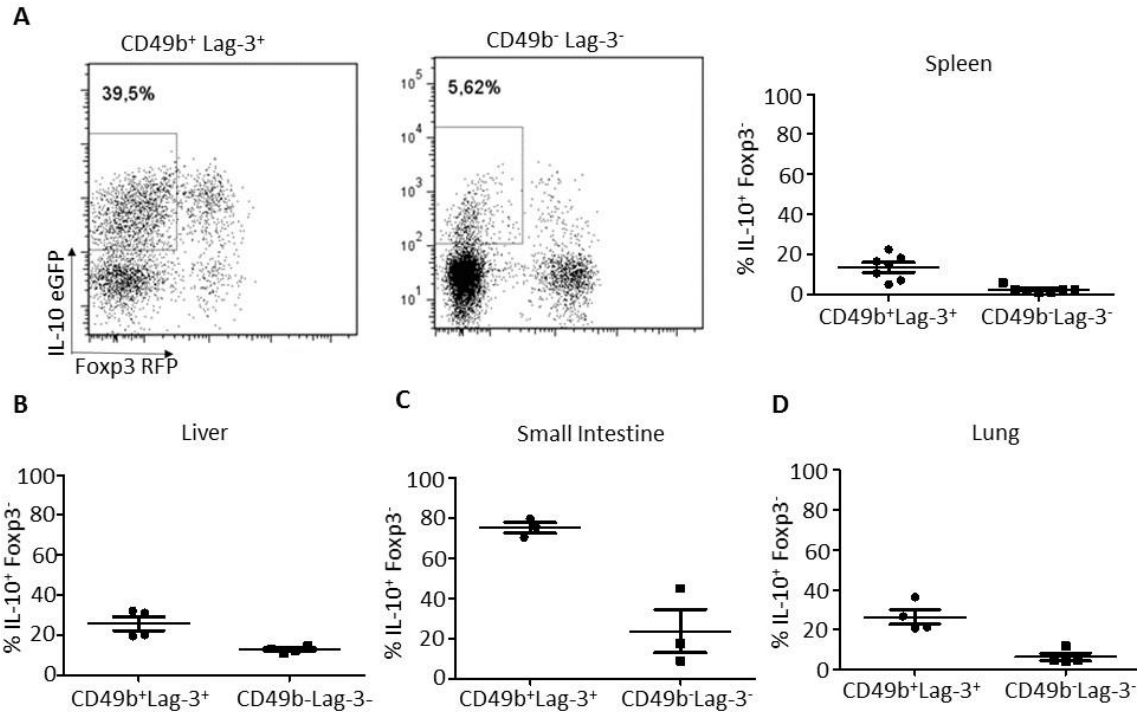

Figure 19: IL-10$^+$ cells are enrich among the CD49b$^+$ LAG-3$^+$ cells.

Foxp3^{RFP} IL-10^{eGFP} reporter mice were treated with 15 µg anti-CD3 antibodies twice (day0, day2). Cells were isolated 4 hours after the second anti-CD3 injection from the small intestine, liver, lung and spleen. Representative dot plots of LAG-3 and CD49b staining from splenocytes are shown. Scatter plots (lines indicated mean ± SEM; Spleen n=7; Liver n=4; Small Intestine n=3; Lung n=4) are cumulative from two independent experiments.

Collectively, these data show that IL-10 producing T cells are a very heterogeneous population. CD49b and LAG-3 are expressed by some of these cells, but not by all. Which subset can be considered a T_R1 cell and which cells display the highest suppressive capacity was still unknown.

3.2.2 IL-10$^+$ CD49b$^+$ LAG-3$^+$ cells display the strongest suppressive potential

The data shown above (Figure 18 and 19) indicate that T_R1 cells are a heterogeneous cell population. The IL-10$^+$ Foxp3$^-$ T cell subset contained LAG-3 and CD49 co-expressing cells, but also a significant number of cells, which did not co-express these markers. Likewise, IL-10$^-$ Foxp3$^-$ T cells contained a subset that co-expressed CD49b and LAG-3. Thus it was unclear which subset could be

considered a T_R1 cell. The key characteristic of T_R1 cells is IL-10 production and suppressive capacity. Thus, it was next aimed to analyze the different subsets based on the expression of IL-10, CD49b and LAG-3. To this end Foxp3^{RFP} IL10^{eGFP} double reporter mice were treated with anti-CD3 antibodies to generate T_R1 cells *in vivo*. The different subsets, namely IL-10$^+$ LAG-3$^+$ CD49b$^+$, IL-10$^+$ LAG-3$^-$ CD49b$^-$ and IL-10$^-$ LAG-3$^+$ CD49b$^+$, were FACS-sorted from splenocytes. Foxp3$^+$ Treg cells were also isolated to serve as positive control for the suppression assay.

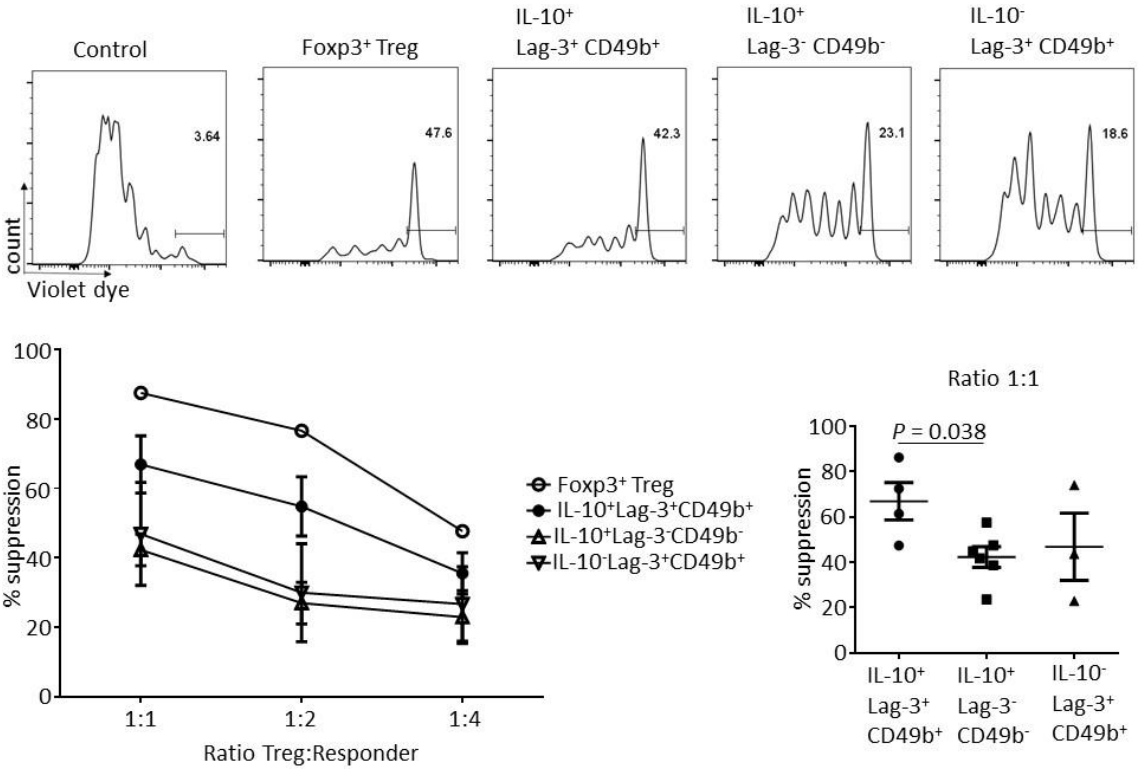

Figure 20: IL-10$^+$LAG-3$^+$CD49b$^+$ cells show the highest suppressive capacity *in vitro*.

Foxp3^{RFP} IL-10^{eGFP} double reporter mice were treated with 15 µg anti-CD3 antibodies twice (day0, day2). Cells were isolated from the spleen 4 hours after the second injection and indicated subsets were FACS-sorted. T_R1- and Treg-mediated suppression was measured by violet dye dilution. Responder T cells were isolated from C57Bl/6 mice and labelled with 5 µM violet dye. The cells were activated in the presence of irradiated APCs and 1.5 µg/ml anti-CD3 antibody and cultured either alone (Responder+Responder, control) or in the presence of Treg cells or T_R1 cell subsets. After 72 hours the proliferation of the responder T cells was measured via Flow Cytometry. Data are representative of six independent experiments.

In parallel CD4$^+$ T cells were isolated from untreated mice and labelled with violet dye to serve as responder T cells. These responder T cells were co-cultured with

Foxp3$^+$ Treg cells or one of the above mentioned T_R1 cell subsets. The proliferation of the responder cells was measured via the dilution of violet dye. As expected, co-culture of responder T cells with Foxp3$^+$ Treg cells resulted in a strong impairment of the proliferation of responder T cells (Figure 20).

IL-10$^+$ CD49b$^+$ LAG-3$^+$ cells also showed a very high suppressive potential, with around 70% of suppression at a ratio of 1:1. Interestingly, both IL-10$^+$ LAG-3$^-$ CD49b$^-$ cells and IL-10$^-$ LAG-3$^+$ CD49b$^+$ cells, only exhibited an intermediate suppressive capacity that was however significantly lower than the capacity of IL-10$^+$ CD49b$^+$ LAG-3$^+$ cells (Figure 20).

In summary, only CD49b and LAG-3 co-expressing cells that also produced IL-10 had a considerable suppressive potential.

3.2.3 IL-10$^+$ CD49b$^+$ LAG-3$^+$ cells produce more IL-10 then IL-10$^+$ CD49b$^-$ LAG-3$^-$ cells

IL-10 production is one of the major suppressive mechanisms of T_R1 cells [97, 104, 163]. Therefore, it was not surprising, that LAG-3 and CD49b co-expressing cells that did not produce IL-10 (IL-10$^-$ CD49b$^+$ LAG-3$^+$) did not show a strong suppressive potential *in vitro*. Nevertheless, IL-10$^+$ CD49b$^-$ LAG-3$^-$ cells, which formally would also have been defined as T_R1 cells, could also not sufficiently suppress the proliferation of responder T cells *in vitro*. To further understand the difference between the subsets, the cytokine production was analyzed in more detail. Foxp3^{RFP} IL10^{eGFP} double reporter mice were treated with anti-CD3 antibodies and T_R1 cells were FACS-sorted from splenocytes. The cell subsets were re-stimulated *in vitro* for 60 hours and IL-10^{eGFP} expression was analyzed by Flow Cytometry. Additionally, the concentration of IL-10 and IFN-γ was assessed in the supernatants of the cell culture. Flow Cytometric analysis revealed that IL-10 producing cells that co-expressed LAG-3 and CD49b had a higher stability regarding IL-10 production than cells that did not co-express the two markers (IL-10$^+$ LAG-3$^-$ CD49b$^-$) (Figure 21). IL-10$^-$ CD49b$^+$ LAG-3$^+$ cells did not acquire IL-10 production upon re-stimulation *in vitro* (Figure 21). Also, supernatant of IL-10$^+$ CD49b$^+$ LAG-3$^+$ cells contained a significantly elevated concentration of IL-10 compared to the other two tested subsets analyzed

confirming the results obtained by Flow Cytometry (Figure 21). IFN-γ is another cytokine known to be produced by T_R1 cells [117]. The concentration of IFN-γ was also highest in the IL-10$^+$ CD49b$^+$ LAG-3$^+$ cell subset, whereas IL-10$^+$ CD49b$^-$ LAG-3$^-$ cells and IL-10$^-$ CD49b$^+$ LAG-3$^+$ cells produced low and similar amounts of IFN-γ (Figure 21).

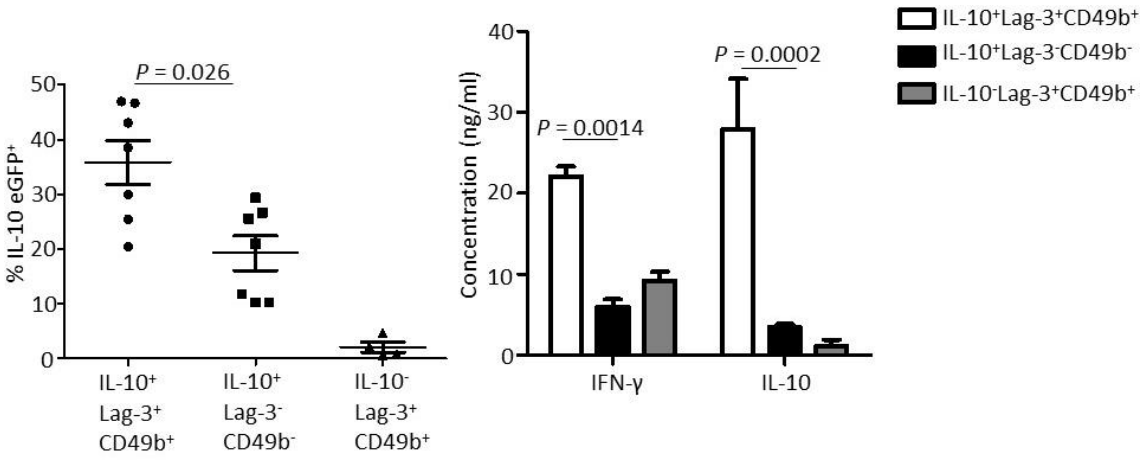

Figure 21: IL-10$^+$LAG-3$^+$CD49b$^+$ cells produce more IL-10 and IFN-γ.

Foxp3^{RFP} IL-10^{eGFP} double reporter mice were treated with 15 µg anti-CD3 antibodies twice (day0, day2). Cells were isolated from the spleen 4 hours after the second injection and indicated subsets were FACS-sorted. Cells were re-stimulated with anti-CD3 and anti-CD28 for 60 hours and IL-10^{eGFP} was assessed by Flow Cytometry (left). Cytokine production was quantified in the cell culture supernatants using Cytometric Bead array (right). Mann-Whitney U test was used to calculate significance.

To get a better insight into the molecular differences between the cell subsets IL-10$^+$ LAG-3$^+$ CD49b$^+$ cells, IL-10$^+$ LAG-3$^-$ CD49b$^-$ cells and IL-10$^-$ LAG-3$^+$ CD49b$^+$ cells were isolated from spleens of anti-CD3 treated mice. The mRNA expression of T_R1 cell signature genes (*Maf, Ahr, Prdm1, Gzmb, Tgfb1, Ctla4*) and additionally *Tbx21*, encoding T-bet the master transcription factor of T_H1 cells [11], and *Pdcd1*, encoding the negative T cell regulator PD-1, were examined. IL-10$^-$ cells that co-express CD49b and LAG-3 did not show a high expression of T_R1 cell associated transcription factors such as *Maf*, *Ahr* or *Prdm1* (Figure 22). The mRNA levels of these factors were elevated in IL-10 producing cells. But no significant difference could be found regarding the mRNA levels of *Maf* and *Ahr* between IL-10$^+$ LAG-3$^+$ CD49b$^+$ cells and IL-10$^+$ LAG-3$^-$ CD49b$^-$ cells. However,

Prdm1 was higher expressed in cells that co-expressed the T_R1 markers, LAG-3 and CD49b (Figure 22). In these cells the level of *Tbx21* was also elevated, positively correlating with the increased IFN-γ concentration in the supernatant of these cells (Figure 22 and Figure 21). The expression of *Tgfb1* was similar between the three subsets (Figure 22). *Pdcd1*, *Ctla4* and *Gzmb* were low in IL-10^- LAG-3^+ $CD49b^+$ cells. *Pdcd1* was similarly expressed between IL-10^+ LAG-3^+ $CD49b^+$ cells and IL-10^+ LAG-3^- $CD49b^-$ cells. But interestingly, *Ctla4* and *Gzmb* were highly expressed in the subset that co-expressed CD49b and LAG-3 (Figure 22).

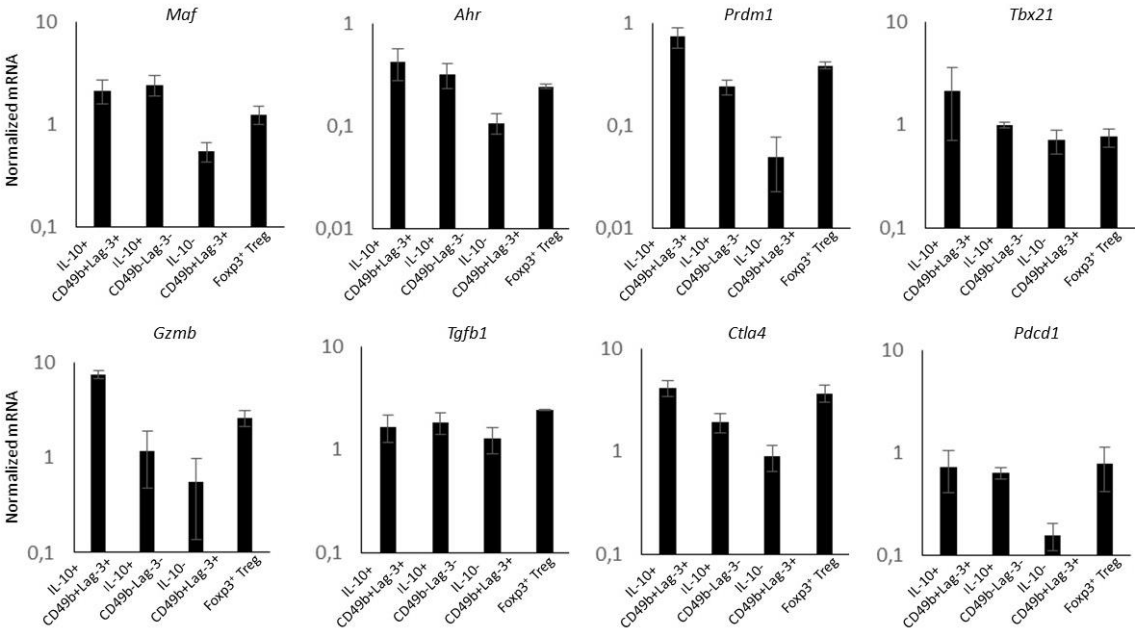

Figure 22: IL-10^+LAG-$3^+$$CD49b^+$ show a high expression of T_R1 cell signature cytokines.

Foxp3^{RFP} IL-10^{eGFP} double reporter mice were treated with 15 µg anti-CD3 antibodies twice (day0, day3). Cells were isolated from the 4 pooled spleens 4 hours after the second injection and indicated subsets were FACS-sorted. *Maf*, *Ahr*, *Prdm1*, *Tbx21*, *Tgfb1*, *Pdcd1*, *Ctla4* and *Gzmb* mRNA expression (normalized to *Hprt*) of the indicated cell subsets are shown. Data are cumulative of four independent experiments.

In summary, only IL-10 producing cells co-expressing LAG-3 and CD49b display a distinctive T_R1 cell phenotype, namely high production of IL-10 and IFN-γ and a gene signature typical for T_R1 cells.

4. Discussion

Chronic inflammatory diseases, autoimmunity and allergies are a major health threat. Of note, the frequency of these diseases is steadily increasing especially in Western countries. These diseases are characterized by a disruption of the immune homeostasis. The causes for this disruption are multifarious. The current hypothesis is that genetic predispositions in combination with environmental factors promote the development of autoimmunity, for example a SNP in the gene encoding IL-12B, a subunit of IL-12 and IL-23 receptor, is linked with the development of IBD [164, 165]. However, as shown by twin studies the presence of most mutations *per se* is insufficient to induce disease, which highlights the key role of the environment. In 1998 one out of five children already suffered from diseases such as asthma, allergic rhinitis or atopic dermatitis with increasing tendencies in the last decades [166, 167]. During the same period the incidences of infectious diseases declined [168]. This indicated again the role of the environment and led to the development of the hygiene hypothesis, assuming that early childhood exposure to pathogens can modulate the immune system, and therefore the lack of this can increase the risk of an imbalance in the immune response to self-antigens and harmless foreign antigens. Nevertheless, the mechanisms that are dysregulated are still poorly understood. $CD4^+$ T cells are thought to be a major player contributing to disease progression and chronic inflammation, either by an overreaction of effector T cells or by an impaired function of regulatory T cells. Thus a more comprehensive understanding of $CD4^+$ T-helper cells is essential to understand these diseases and to develop new therapies. One approach for new therapies is the adoptive transfer of regulatory T cells as T cell therapy. Animal models of chronic inflammatory diseases or GvHD showed that the transfer of regulatory T cells such as $Foxp3^+$ Treg cells or T_R1 cells can prevent disease development through the release of IL-10 providing first indications for the functionality of regulatory T cell transfers as therapy during autoimmune and chronic inflammatory diseases [79, 163, 169, 170]. Also in humans, first clinical trials demonstrated the safety and functionality of a regulatory T cell-based therapy [143-146]. Nevertheless, functional stability of the regulatory T cells is key for a successful treatment of humans. The use of

regulatory T cells as T cell-based therapy has been challenged by new evidence indicating that $CD4^+$ T cells display certain plasticity [127, 132-134]. Especially findings showing that $Foxp3^+$ Treg cells can convert into highly pathogenic T_H17 cells [137] demonstrated the necessity to further investigate which factors and mechanisms favor plasticity and stability of T-helper cell subsets.

IL-10 is known to be one of the strongest anti-inflammatory cytokines of the immune system with a broad range of effects on both adaptive immune cells and innate immune cells. IL-10 is essential to maintain the immune homeostasis and terminate immune response and inflammation. Especially in the intestine these properties play a key role in preserving the balance of pro-inflammatory and anti-inflammatory cells. Mice deficient in IL-10 develop severe spontaneous colitis [110]. Similarly, humans with a rare genetic defect in either IL-10 or IL-10 receptor develop early onset IBD [108, 109]. Due to its prominent role for peripheral tolerance and immune homeostasis, it is not surprising that IL-10 can be produced by a broad range of immune cells, including T cells, but also macrophages and B cells [171, 172]. Several immune cells can also directly respond to IL-10, memory $CD4^+$ T cells and particularly potential pathogenic T_H17 cells can be directly controlled by IL-10 signaling [75]. But IL-10 can also act on cells in a self-amplifying way: $Foxp3^+$ Treg cells, but also regulatory macrophages depend on IL-10 signaling to maintain their regulatory function and IL-10 production [138, 139, 173]. The signature cytokine of T_R1 cells is IL-10, which also displays the major suppressive mechanism of these cells. The role of IL-10 signaling for T_R1 cell biology is still controversial, especially the effect of IL-10 on T_R1 cell differentiation is debatable [86, 104, 116]. Whether T_R1 cells could respond to IL-10 signaling, and which effect IL-10 had on mature T_R1 cells had never been investigated prior to this study.

The aim of this thesis was thus to study the stability and therefore safety of T_R1 cells. More particularly, the aim was to investigate the role of IL-10 for T_R1 cell biology. To this end, different mouse models resembling human diseases were analyzed. One other key problem in studies using T_R1 cells as T cell therapy is the heterogeneity of IL-10 producing T-helper cells. Accordingly, another aim was to further characterize IL-10 producing T cells and to identify markers, which

characterize highly suppressive regulatory T cells among the heterogeneous population of IL-10 producing $CD4^+$ T cells.

4.1 IL-10 is dispensable for T_R1 cell differentiation, whereas IL-27 promotes the induction of T_R1 cells

IL-10 was the first cytokine, which was found to induce T_R1 cells *in vitro* [78-80]. Nevertheless, over the past decades several other factors such as IFN-α, vitamin D3, IL-27 and IL-21 have also been found to induce T_R1 cells in synergy or independent of IL-10 [80, 112, 174]. Strongly contradicting the first findings regarding IL-10 driven differentiation of T_R1 cells, was the finding by Maynard *et al.* (2007) demonstrating that T_R1 cells can develop in the complete absence of IL-10 *in vivo*. In this study T_R1 cells were still present in the intestine in a total IL-10 deficient mouse [86]. Nevertheless, in this study the direct effect of IL-10 on T_R1 cell differentiation was not investigated. To study this effect, a transgenic mouse that overexpressed a dominant negative IL-10 receptor α-chain [148, 149] was used. In this mouse model only $CD4^+$ T cells display strongly impaired IL-10 signaling, therefore excluding possible extrinsic effects and side effects related to the use of a total IL-10 deficient mouse model. The anti-CD3 model is very efficient to generate and study T_R1 cells *in vivo* [75, 149]. In this model T_R1 cells are induced during the course of inflammation in the small intestine with a peak of induction around 4 hours after the second injection of anti-CD3 antibodies. Therefore, this model was chosen to investigate the role of IL-10 and IL-27 on T_R1 cell differentiation *in vivo*.

The frequency of T_R1 cells was not altered in the absence of IL-10 signaling in $CD4^+$ T cells *in vivo*, supporting the theory that IL-10 can be substituted by other factors to induce T_R1 cells. However, whether or not T_R1 cells, which were differentiated in the absence of IL-10 signaling, could be considered mature and functional remained unresolved. One possibility was that in the absence of IL-10 signaling, $CD4^+$ T cells can acquire transient IL-10 expression instead of differentiating into suppressive regulatory T cells. Hence, deeper analyses were required and further T_R1 defining criteria were analyzed. T_R1 cells are defined as CD49b and LAG-3 expressing cells [107] and indeed the frequency of the marker

expression was the same on T_R1 cells whether they had been differentiated in the presence or in the absence of IL-10 signaling. This result adds to the findings that T_R1 cells can develop in the absence of IL-10. A master transcription factor of T_R1 cells has not been found to date, therefore the identification of T_R1 cells is more complex than for example the identification of $Foxp3^+$ Treg cells. c-Maf and Ahr are strongly linked to the differentiation of T_R1 cells [93, 96, 97]. But both transcription factors are not exclusively expressed by T_R1 cells, for example also the differentiation of T_H17 cells is strongly dependent on Ahr expression [41]. More recent studies also linked Blimp-1 (encoded by *Prdm1*) to the emergence of T_R1-like cells either originating from naïve T cells or T_H1 cells [101, 102]. However, all three transcription factors were independently regulated from IL-10 signaling. Besides IL-10 production, T_R1 cells display several additional regulatory functions. Among these are the production of TGF-β and Granzyme B and the expression of the negative T cell regulator CTLA-4 [106, 114, 117]. Neither of these factors were altered in expression in the absence of IL-10 signaling. Still the most important confirmation that mature T_R1 cells can be induced without IL-10 was the functional analysis. T_R1 cells showed equal suppressive capacity *in vitro* regardless of a functional IL-10 signaling during the differentiation. The early differentiation protocols to generate T_R1 cells using IL-10 are based on multiple rounds of TCR-dependent activation of naïve $CD4^+$ T cells in the presence of IL-10 [78, 79]. IL-10 signaling is therefore sufficient to induce the differentiation of T_R1 cells via activation of STAT3 after TCR stimulation. But the data obtained with the dominant negative IL-10 receptor α-chain mouse model revealed that IL-10 is not essential for the differentiation of mature and functional T_R1 cells *in vivo*.

Moreover, IL-27 had previously been shown to play an important role during T_R1 cell differentiation [91-93, 175] and furthermore the induction of T_R1 cells in a model of induced oral tolerance by CD3 antibodies was previously linked to IL-10 and IL-27 producing DC subsets, although it was still not known which of the two cytokines plays the bigger role [176]. Therefore, also IL-27 should be investigated in the process of T_R1 cell induction in the anti-CD3 mouse model. Neutralization of IL-27 *in vivo* caused a significant decrease in the T_R1 cell pool in the small intestine in this model. No effect could be observed on other IL-10 producing T-helper cell subsets such as T_H17 cells or $Foxp3^+$ Treg cells. This suggests that

IL-27 is only important for T_R1 cell differentiation and not for IL-10 production of $CD4^+$ T cells *per se*. Interestingly, neutralizing IL-27 after the induction of T_R1 cells did not alter the frequency of IL-10 producing $CD4^+$ T cells in the small intestine.

IL-27 has been reported to induce T_R1 cell differentiation through induction of STAT3 and STAT1 [91, 92, 94, 97] followed by the induction of c-Maf, Ahr and Blimp-1. The data obtained indicate that while IL-27 in conjunction with STAT molecules and transcription factors can be the driving force for T_R1 differentiation, IL-10 signaling is not strictly required for differentiation of T_R1 cells. However, IL-27 was not important for the maintenance of the T_R1 cell phenotype, therefore factors supporting T_R1 cell stability were still elusive.

4.2 Mature T_R1 cells express functional IL-10 receptor

IL-10 plays a highly important role for the suppressive function of T_R1 cells and IL-10 has always been linked to T_R1 cell biology. Therefore, the role of IL-10 signaling ought to be further investigated, even though it seems to be dispensable for differentiation. Whether or not mature T_R1 cells express IL-10 receptor and can respond to IL-10 was unknown. Thus, IL-10 receptor expression was assessed on T_R1 cells and compared to IL-10 receptor expression on $Foxp3^+$ Treg cells and T_H17 cells, which had previously been shown to express IL10 receptor [75, 139]. Naïve T cells served as negative control, since it was reported that they do not express IL-10 receptor or if so, then only at a low level [138]. T_R1 cells expressed IL-10 receptor compared to the positive controls and further analysis also revealed that this IL-10 receptor was functional. T_R1 cells with an overexpressed dominant negative IL-10 receptor α-chain showed a strongly impaired responsiveness to IL-10, demonstrating the functionality of the transgene, but also revealing the limitations of the model: IL-10 signaling in $CD4^+$ T cells in this mouse is impaired, but not completely blocked [148]. The use of a conditional IL-10 receptor knock-out mouse model would be more superior to the transgene model, however the observed phenotype, especially in the *in vivo* transfer models discussed in detail below, is sufficient to draw a valid conclusion for the role of IL-10 for T_R1 cell biology. Some of the experiments on the

differentiation of T_R1 cells however should be confirmed using a complete knock out to preclude that the remaining IL-10 signaling is still sufficient to influence the T_R1 cell differentiation. Nevertheless, naïve T cells did not respond to IL-10 stimulation, which is in line with the finding that they do not express IL-10 receptor. These data further support the finding that IL-10 signaling plays a minor role in the differentiation of T_R1 cells from naïve T cells. Still, T_R1 cells acquire the expression of IL-10 receptor during the course of differentiation. The function of IL-10 signaling for mature T_R1 cells had never been studied before. But studies showing that IL-10 signaling in other cells such as $Foxp3^+$ Treg cells and macrophages sustain their functional stability [138, 173] led to the hypothesis that IL-10 signaling could be important for the stability of T_R1 cells.

4.3 IL-10 signaling maintains T_R1 cell stability and function

T_R1 cells are currently being tested in clinical trials to treat severe Crohn's disease and GvHD [145, 146]. Consequently, the importance of IL-10 signaling for the function and stability of T_R1 cells was tested in a challenging mouse model of colitis resembling the T_R1 cell transfer applied in humans with Crohn's disease. Strikingly, the data obtained showed that IL-10 signaling in T_R1 cells was essential to maintain their suppressive function *in vivo*.

Mature T_R1 cells display a broad variety of immune suppressive mechanisms. They secrete TGF-β and Granzyme B and also express PD-1 and CTLA-4 [78, 177]. However, the most striking regulatory mechanism is the characteristic and defining high IL-10 production [104, 178]. Previous studies have shown that T_R1 cells can suppress (e)T_H17 cells directly via IL-10 [75]. However, IFN-γ released by T_R1 cells has also been shown to be critical for the immune regulatory activity of these cells and for contributing to suppress the potentially pathogenic (e)T_H17 cells [179].

The mechanism underlying the dysfunction of CD4-DNIL-10R transgenic T_R1 cells overexpressing a dominant negative IL-10 receptor α-chain ought to be further investigated. Therefore, the cytokine profile of T_R1 cells with an impaired IL-10 signaling compared to wild type T_R1 cells was analyzed upon re-stimulation

in vitro or adoptive transfer *in vivo*. Of note, IL-10 expression of T_R1 cells right after *in vivo* generation did not differ regardless of a functional IL-10 signaling. However, the production of IL-10 by T_R1 cells with an impaired IL-10 signaling was significantly reduced upon *in vitro* cultivation and re-stimulation, suggesting that IL-10 signaling is important to sustain IL-10 production by T_R1 cells. Other tested cytokines, including IFN-γ, were unchanged. IL17-A expression is inhibited by IL-10 signaling [75], therefore it was not surprising that T_R1 cells produce significantly higher amounts of IL-17A in the absence of IL-10 signaling. The finding regarding the dependence of IL-10 production by T_R1 cells on IL-10 signaling could be further verified *in vivo*: upon adoptive transfer into lymphopenic hosts, in the absence of IL-10 signaling in T_R1 cells the stability of IL-10 expression was drastically reduced. These findings therefore revealed not only that IL-10 does not directly act on T_H17 cells but also that its signaling is mandatory for T_R1 cells to maintain IL-10 expression and thus their regulatory function. Interestingly, the mRNA expression of neither *Ahr*, *Maf, Prdm1* nor *Tgfb1*, *Gzmb* and *Ctla4* was altered in the absence of IL-10 signaling upon re-stimulation. These data support the notion that IL-10 signaling might not be essential to maintain the T_R1 cell phenotype *per se*, but it is strictly required to sustain the production of IL-10 and consequently T_R1 cell regulatory activity. Furthermore, these results suggest a dominant role of IL-10 over the other suppressive mechanisms such as TGF-β and Granzyme B secretion or expression of CTLA-4. However, other suppressive functions of T_R1 cells could be more important in other tissues and different inflammatory settings that include diverse types of pro-inflammatory cells as recently described for human T_R1 cells in which Granzym B is essential to control APCs [106].

The important finding that IL-10 signaling sustains IL-10 production in T_R1 cells was confirmed in the anti-CD3 antibody model. Initially the frequency of T_R1 cells was independent of functional IL-10 signaling, but IL-10 secretion by T_R1 cells could only be maintained in the presence of IL-10 signaling and this effect was not caused by a defective proliferation of T_R1 cells in the absence of IL-10 signaling. The results obtained in the anti-CD3 model substantiate the conclusions drawn from the transfer model in a more physiological situation: Also T_R1 cells generated *in vivo* need to respond to IL-10 to maintain IL-10 expression, even though cytokines that originally led to their differentiation such as IL-27

which is possibly absent in the artificial transfer situation are still present in the cell environment.

One significant observation in the course of the transfer colitis model was the finding that T_R1 cells regardless of functional IL-10 signaling and therefore regardless of the maintenance of IL-10 production did not cause disease on their own. The biggest threat that slows down the design of regulatory T cell-based therapies is the caveat that regulatory T cells, both Foxp3$^+$ Treg cells as well as T_R1 cells, could convert into pro-inflammatory effector cells and in turn worsen instead of curing the inflammatory condition [137]. In the mouse model of GvHD T_R1 cells with impaired IL-10 signaling did not cause disease, supporting the safety of a T_R1 cell-based therapy. Accordingly, the findings from two different mouse transfer models, colitis and GvHD, suggest that T_R1 cells remain functional as long as they can respond and are exposed to IL-10. Nonetheless, T_R1 cells could migrate into an environment poor in IL-10 and rich in pro-inflammatory cytokines and eventually enter a resting phase in which they stop producing IL-10. Even though T_R1 cells might lose their regulatory activity, the data obtained especially in the GvHD model, indicate that T_R1 cells do not convert into pathogenic cells. This finding is also substantiated by the result achieved upon re-stimulation of T_R1 cells *in vitro*: mRNA levels of typical T_R1 cell transcription factors were not influenced by IL-10 signaling.

In summary, IL-10 signaling is mandatory for the regulatory activity but not essential to maintain the T_R1 cells phenotype *per se*. These data are in line with two recent clinical trials that also support the safety of T_R1 cells as therapy in humans [145, 146].

Further experiments are needed to investigate the fate of T_R1 cells that lose IL-10 expression to establish if they are going through a resting phase or could potentially convert into a different T-helper cell subset. The best established mouse model to study this is the fate mapping of specific cytokines *in vivo*. First designed for T_H17 cells and fate mapping of IL-17A expression, this mouse model is a new tool used to follow the fate of a T-helper cell subset based on the expression of signature cytokines [180]. With the help of this fate mapping mouse model, recent research reported that T_H17 cells could not only convert into T_H1 cells [118, 119], but could also transdifferentiate into T_R1 cells [181]. Fate

mapping of IL-10 *in vivo* could provide a powerful tool to better understand the dynamics of the T_R1 cell biology.

4.3. IL-10 signaling sustains IL-10 expression through p38 MAP kinase in T_R1 cells

In the next step the molecular mechanism maintaining the IL-10 production in T_R1 cells in response to IL-10 signaling was investigated. Several signaling molecules are known to act downstream of the IL-10 receptor. Likewise, a variety of molecules have been linked to the induction of IL-10 in different cell types. Accordingly, STAT3 is the best-studied kinase activated upon IL-10 receptor signaling. After dimerization of the IL-10 receptor, JAK1 is recruited to the intracellular domain of the receptor and activated, JAK1 in turn can phosphorylate STAT3 and STAT3 dimers can act as transcriptional regulators [82]. More importantly, it was previously shown in $Foxp3^+$ Treg cells that IL-10 signaling sustains the production of IL-10 via activation of STAT3 [138]. Nevertheless, in human macrophages and monocytes, IL-10 production requires the activation of p38 MAP kinase and ERK1 and ERK2 (ERK1/2) which are, like STAT3, direct downstream targets of IL-10 receptor signaling [83, 84]. Noteworthy, also the suppressive capacity of inducible pTreg cells has been associated to p38 MAP kinase signaling [155-158]. However, in contrast to STAT3, p38 MAP kinase does not act as a transcription factor but rather as a mediator in a signaling cascade. Furthermore, T_H1 cells also require signaling through ERK1/2 and STAT4 to induce IL-10 expression, whereas it has been reported that T_H2 or T_H17 cells require STAT6 and STAT3, respectively, together with ERK1/2 to induce IL-10 production [25, 92, 159, 160, 182].

Since STAT3 is essential for the differentiation of highly IL-10 producing T_R1 cells [93], this kinase was analyzed regarding its role during the maintenance of IL-10 production in response to IL-10 signaling. Surprisingly, STAT3 phosphorylation was not affected by IL-10 signaling upon re-stimulation of T_R1 cells *in vitro* and also the inhibition of STAT3 in mature T_R1 cells did not affect the IL-10 production. Unlike $Foxp3^+$ Treg cells in which STAT3 is essential to maintain IL-10 production [138, 139], STAT3 seems to be dispensable in mature T_R1 cells for the secretion

of IL-10. One explanation for this finding could lie in the expression of Foxp3. It has been shown that STAT3 dimers can directly bind to the *Il10* promoter region, but this interaction also requires the formation of a complex of STAT3 with Foxp3 as co-transcriptional regulator and histone acetyl transterase-1. This complex can epigenetically modify the *Il10* promoter region to enable transcriptional regulation through STAT3 in Foxp3^{+} Treg cells [183]. T_R1 cells lack Foxp3 expression and therefore STAT3 may not be able to directly modulate the *Il10* promoter region.

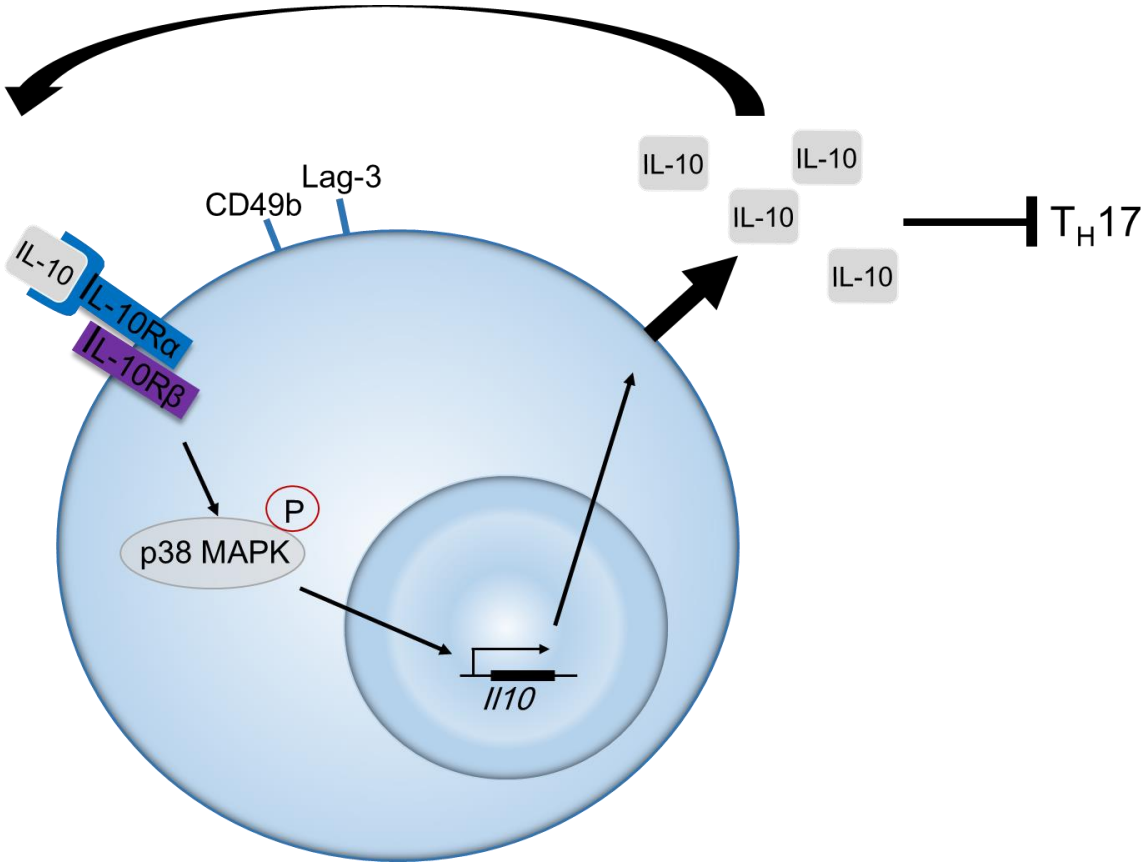

Figure 23: Maintenance of IL-10 production in T_R1 cells.

IL-10 receptor activation on T_R1 cells leads to the phosphorylation of p38 MAP kinase and this in turn modulates the expression of IL-10. Thus, IL-10 signaling maintains the regulatory activity of T_R1 cells, which is mainly linked to high production of IL-10. IL-10 can directly inhibit potential pathogenic T_H17 cells in the intestine, but it can also provide bystander suppression to other cell subsets that lack IL-10 receptor expression.

In addition to STAT3, the phosphorylation status of p38 MAP kinase during the course of *in vitro* re-stimulation was tested. Remarkably, the phosphorylation and

therefore activation of p38 MAP kinase decreased in the absence of IL-10 signaling in T_R1 cells and more importantly, the functional inhibition of p38 MAP kinase revealed that T_R1 cells are dependent on p38 MAP kinase signaling to maintain their IL-10 production. Inhibition of ERK1/2 and JNK did not affect the production of IL-10 in T_R1 cells, suggesting a minor role of these kinases for T_R1 cell biology. Even though p38 MAP kinase regulates IL-10 expression, no influence on the T_R1 cell signature genes (*Maf*, *Ahr*, *Prdm1* or *Ctla4*, *Tgfb1* and *Gzmb*) was observed. These results are in line with the finding that IL-10 signaling in T_R1 cells is crucial for IL-10 production, but not essential for the maintenance of the general T_R1 cell phenotype.

Overall, these findings indicate that IL-10 signaling in mature T_R1 cells sustains the activation of p38 MAP kinase and this in turn maintains IL-10 production via a positive feedback loop. Further analysis is however required to identify factors that act downstream of p38 MAP kinase and directly regulate the IL-10 expression via transcriptional regulation in T_R1 cells.

4.3.1 Both, STAT3 and p38 MAP kinase, are necessary for T_R1 cell differentiation

The differentiation of T_R1 cells with IL-27 is dependent on the activation of STAT3 and STAT1, hereby STAT3 is mainly required to induce the expression of c-Maf and Ahr, two transcription factors strongly linked to a T_R1 cell phenotype and IL-10 production [96-98]. Nonetheless, STAT3 seems to play a redundant part for maintaining IL-10 expression in mature T_R1 cells. However, blocking STAT3 during the differentiation phase of T_R1 cells with IL-27 confirmed the studies cited above: Blockage of STAT3 almost completely blocked the differentiation of T_R1 cells *in vitro* and demonstrated the functionality of the inhibitory compound. Interestingly, also the blockage of p38 MAP kinase inhibited T_R1 cell differentiation, while ERK1/2 and JNK did not influence T_R1 cell induction *in vitro*.

Together, these data suggest that IL-27 and not IL-10 in conjunction with STAT3 and p38 MAP kinase can be the driving force for T_R1 cell differentiation. However, during the maintenance phase, IL-10 signaling is essential to sustain IL-10 production and in turn functional stability via a positive feedback mechanism,

which is dependent on p38 MAP kinase signaling and independent of STAT3. Considering the fundamental role of IL-10 and T_R1 cells for peripheral tolerance and immune homeostasis, one hypothesis is that IL-10 production is sustained by a dynamic network of signaling molecules and transcription factors rather than by only one master regulator such as T-bet for IFN-γ production in T_H1 cells [11, 12].

4.4 IL-10 signaling in human T_R1 cells sustains IL-10 expression

One major focus in immunological research is finding new approaches to design T cell-based therapies to treat autoimmune and chronic inflammatory diseases or to prevent GvHD [161, 162]. Thereby T_R1 cells have been of great interest, because of their strong potential to maintain and re-establish immune homeostasis. The positive effect of T_R1 cell-based therapies has been mainly linked to their high IL-10 production in human trials [184]. Hence, understanding the mechanism maintaining the functional stability of human T_R1 cells and in this regard mainly the IL-10 production, is critical for the conduction of a T_R1 cell-based clinical trial. The functional stability of human T_R1 cells is by this means important for both the success and the safety of the therapy. The experiments performed using human T_R1 cells, identified on the basis of two T_R1 cell markers, CD49b and LAG-3 [107], indicate that IL-10 signaling is also critical for human T_R1 cells to maintain their IL-10 production, whereas the expression of factors which confer the T_R1 phenotype including *c-MAF*, *AHR* and *PRDM1* or *CTLA-4*, *TGFb1* and *Granzyme B* were not affected by blocking IL-10 signaling. These findings are in line with the data obtained from various mouse models described above. Nonetheless, further experiments regarding the involvement of STAT3 and p38 MAP kinase for maintaining IL-10 expression in human T_R1 cells are needed to gain deeper insight into the molecular mechanism.

Collectively, these results add to the notion that T_R1 cell-based therapy is safe, but more importantly, the success of the treatment might be linked to the presence of IL-10 in the environment in which T_R1 cells are present. Therefore, the capacity of T_R1 cells to sustain their IL-10 production in an autocrine manner might be key for re-establishing physiological levels of IL-10 in tissue in order to

cure inflammatory mediated diseases. Hence, there is a need to perform further experiments addressing these newly arising questions.

4.5. IL-10 producing cells are a heterogeneous population

One major unresolved problem in the field of T_R1 cell research is the identification of a master transcription factor. Therefore other criteria have been defined to describe a T_R1 cell: IL-10 production, lack of Foxp3 expression and suppressive capacity [104-106]. Recently, CD49b and LAG-3 have been described to be co-expressed by T_R1 cells, these makers can identify both human and murine T_R1 cells [107]. However, other markers have also been proposed to be expressed by T_R1 cells, for example TIM-3, TIGIT or PD-1 and CD49b and LAG-3 seem to be efficient in some models, but may not be the best marker combination in all settings [185]. Nonetheless, LAG-3 and CD49b have been successfully used to identify human T_R1 cells in a recent study by Clemente-Casares et. al. [186]. Taken together these studies certainly demonstrate that there are different subsets among the IL-10 producing Foxp3- (T_R1 cells) T cell fraction.

It is of great importance to analyze which subset displays the highest suppressive capacity and therefore possesses a high potential to be as efficient as a pharmaceutical for a T_R1 cell-based therapy. The anti-CD3 antibody model was used to induce and study different T_R1 cell subsets. Among the IL-10 producing T cells, cells co-expressing CD49b and LAG-3 were enriched and similarly, the $CD49b^+$ $LAG\text{-}3^+$ subset showed the highest frequency of IL-10 producing cells compared to the marker negative subset. However, a high frequency of IL-10 producing and Foxp3 negative T cells did not co-express CD49b or LAG-3. The subset which could be considered highly suppressive T_R1 cells was unknown, therefore the regulatory capacity of the different subsets was further analyzed. Co-expression of CD49b and LAG-3 on IL-10 producing T cells was strongly correlated with a higher suppressive capacity *in vitro*. Also the IL-10 production was highest and more stable in the cells co-expressing CD49b and LAG-3 which was reflected in the higher expression of *Prdm1* in these cells. However, no difference could be observed in the expression levels of *Maf* and *Ahr*, suggesting

a dominant role of Blimp-1 in the highly suppressive IL-10 expressing T_R1 cells. CD49b and LAG-3 also identified IL-10 producing cells with the highest expression level of CTLA-4 and Granzyme B further explaining the higher suppressive capacity of these cells. PD-1 and TGF-β seemed to play a minor role in the suppressive capacity of the CD49b and LAG-3 co-expressing T_R1 cells since the expression levels were not altered between the different T_R1 cell subsets.

Additionally, IFN-γ production was elevated in the T_R1 cell subset co-expressing CD49b and LAG-3 which was in line with the higher expression level of *Tbx21* in these cells. The study cited above by Clemente-Casares et. al. (2016) also indicates an important function of IFN-γ signaling and T-bet expression for the development and function of T_R1 cells [186]. In the future, performing *in vitro* and *in vivo* suppression assays comparing IL-10^+ and IL-10^+ IFN-γ^+ T_R1 cells could provide further insight into the different regulatory properties and functions of these subsets.

Further experiments will be required to further characterize different IL-10 producing T cell subsets regarding the expression of other regulatory T cell markers such as TIM-3, TGIT and PD-1. Additionally, the analysis of human IL-10 producing cells will be of great interest. The ability to identify highly suppressive T_R1 cells is depended on surface markers. Cytokine and transcription factor expression will not enable the isolation and enrichment of vital cells that can be used in therapeutic applications. LAG-3 and CD49b identify human T_R1 cells, but also in humans the IL-10 expressing T cells, distinct of Foxp3^+ Treg cells, are very heterogeneous. Other markers besides CD49b and LAG-3 have been described to classify human T_R1 cells. This described subset of T_R1 cells belongs to the memory T cell compartment and displays a low expression of IL-7 receptor. Human T_R1 cells can be further enriched in this subset based on the expression of CCR5 and PD-1 [117, 187]. However, not all of these cells express LAG-3 and CD49b and preliminary data have already shown that some CD49b and LAG-3 co-expressing cells can be found in the IL-7 receptor positive subset. Thus, further research is needed to clarify which of these cells are T_R1 cells and from which cell subset they originate from and

especially which of the subsets display the highest suppressive capacity and safety for a T_R1 cell-based therapy.

Furthermore, these results evoke doubts about the efficiency of using signature cytokines to describe T cell subsets. IL-10 can be produced by several subsets of T cells including T_H1, T_H2 and T_H17 cells and regulatory T cells. Nonetheless, IL-10 has been considered to be the signature cytokine of T_R1 cells, but the identification of T_R1 cells based on cytokine expression seems to be insufficient. If IL-10 producing T cells that lack the expression of CD49b and LAG-3 cannot be considered a T_R1 cell, then the question on which other T-helper cell subset they belong to needs to be further investigated. To this end a next generation sequencing approach (NGS) could reveal further insight into different mouse and human T_R1 cell subsets. Comparing gene expression profiles of different T_R1 cell subsets (for example IL-10^+CD49b$^+$LAG-3^+ and IL-10^+CD49b$^-$LAG-3^-) and other T-helper cell subsets such as T_H17 cells could highlight specific differences and similarity. This could potentially lead to the identification of important transcription factors which are differentially expressed between the subsets.

T_R1 cells are a unique cell population with a remarkable feature that they can origin from different precursor cells. Recent studies revealed that T_R1 cells can not only differentiate from naïve T cells, but can also origin from T_H1 or T_H17 cells [101, 181]. In line with these results the old monolithic view of T-helper cell linage commitment is increasingly being replaced by a complex system including plasticity of T cells and conversion of one subset into another. Taking this into account, new ways to describe T cells have to be developed and the mechanisms that control T cell stability and plasticity have to be identified.

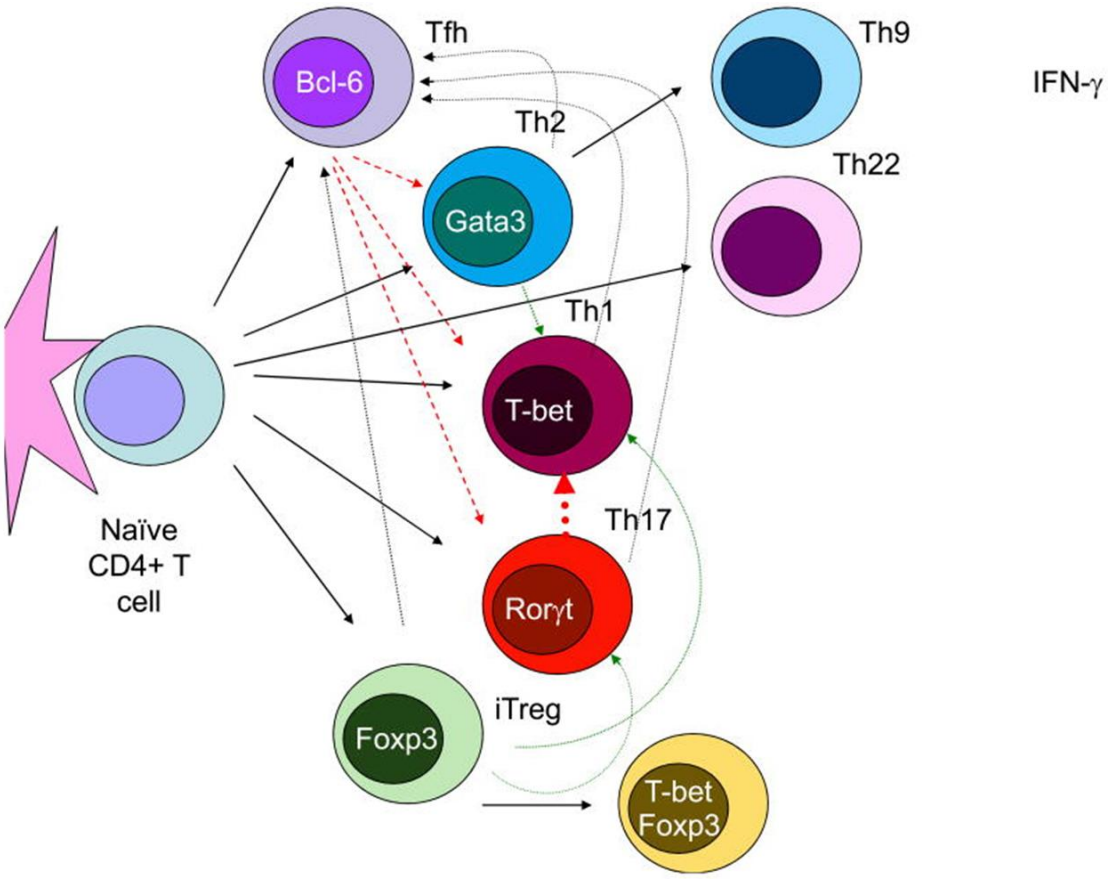

Figure 24: T-helper cell plasticity and flexibility.

Recent studies revealed that T-helper cell subsets display greater plasticity and flexibility regarding their cytokine production than predicted by earlier work. Not only can the cytokine secretion change (dotted lines), but the expression of master transcription factors also seems to be flexible. New T-helper cell subsets such as T_H22 and T_H9 (secreting IL-22 and IL-9 respectively) have been identified, ongoing research tries to clarify if these are distinct subsets or part of a complex network in which one T-helper cell subset can display a variety of phenotypic stages. Modified from [9].

4.6. Conclusions and outlook

Autoimmune and chronic inflammatory diseases are a constant health threat, but a sufficient cure for such diseases has not been found to date. Regulatory T cell-based therapy is a new approach to treat inflammatory diseases by re-establishing immune homeostasis. First clinical trials are already ongoing, even though many questions regarding the efficacy and safety of these therapies remain unresolved.

Collectively, the findings presented in this study demonstrate that mouse and human T_R1 cells require IL-10 receptor signaling to maintain their functional stability and IL-10 production. These findings are of significant importance since they indicate that T_R1 cells could potentially self-sustain their functional stability and would therefore be independent of the environment they could migrate to. Furthermore, this study substantiates the hypothesis that T_R1 cell-based therapy is safe, as T_R1 cells even, if the lost their regulatory function in the absence of IL-10 signaling, did not promote disease. Additionally, the markers CD49b and LAG-3 were identified to select the most effective T_R1 cells among the heterogeneous IL-10 producing T cell subset. Thus using CD49b and LAG-3 as markers to isolate T_R1 cells seems to be effective. Additional enrichment of those T_R1 cells with the highest expression of IL-10 receptor could potential be used for further T_R1 cell-based clinical trials. T_R1 cells enriched in this way could potentially display the highest efficiency to re-introduce immune homeostasis and also the highest potential to self-sustain their IL-10 production via IL-10 receptor signaling.

Many aspects of T_R1 cells biology still remain elusive. Most importantly the identification of a master transcription factor will be essential. One hypothesis is that T_R1 cells are sustained by a network of transcription factors rather than one single master transcription factor. Next generation sequencing analysis of different T_R1 cell subsets followed by functional analysis of candidate genes using conditional knock-out mice has potential to give further insight into this matter. Finally, one possibility is that different T_R1 cell subsets, such as IL-10^+IFN-γ^+ cells, have specialized functions to suppress one specific pro-inflammatory cell type. Thus one future aim is to analyze the suppressive potential of different T_R1 cell subsets in a variety of diseases models. Together these studies, if successful, could pave the way for more specific therapeutic approaches to treat and finally cure human inflammatory diseases in the future.

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6. Appendix

6.1 Abbreviation

ACK	Ammonium-Chloride-Potassium
Ahr	Aryl hydrocarbon receptor
APC	antigen presenting cell
Blimp1	PR domain zinc finger protein 1
BM	bone marrow cells
BrdU	5-Bromo-2′-deoxyuridine
BSA	bovine serum albumin
c-Maf	c-avian musculoaponeurotic fibrosarcoma
°C	degree Celsius
CBA	Cytometric Bead Array
CCR6	Chemokine receptor 6
CD	cluster of differentiation
CD49b	Integrin α2
cDNA	Complementary DNA
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DC	dendritic cell
DMSO	Dimethylsuloxide
DNA	Deoxyribonucleic acid
dNTP	nucleoside triphosphate
Fc	fragment crystallisable
EDTA	Ethylenediaminetetraacetic acid
Erg-2	ETS-related gene 2
ERK	Extracellular signal-regulated kinases
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FIR	FOXP3-IRES-mRFP
Foxp3	forkhead box P3
g	gram
GATA3	Trans-acting T-cell-specific transcription factor GATA-3
GFP	green fluorescent protein
GM-CSF	ranulocyte macrophage colony-stimulating factor
GvHD	Graft-versus-Host-Disease
Gy	gray
HLA-G	human leukocyte antigen G
HRP	Horseradish peroxidase
HSCT	Hematopoietic stem cell transplantation
IBD	inflammatory bowel disease
ICS	intracellular cytokine staining
ICOS	Inducible T-cell COStimulator
IEL	Intraepithelial lymphocytes
IFN	Interferon
IgE	Immunglobulin E
IL	Interleukin

ILT4	Ig-Like Transcripts 4
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
Jak1	janus kinase 1
JNK	c-Jun N-terminal kinases
Kg	kilogram
L	litre
LAG-3	Lymphocyte activation gene 3
LPL	lamina propria lymphocytes
LT-alpha	lymphotoxin-alpha
M	molar
mA	milliAmpere
mAb	Monoclonal antibody
MACS	magnetic-activated cell sorting
MFI	mean fluorescent intensity
MHC-I	polymorphic major histocompatibility-I
MHC-II	polymorphic major histocompatibility-II
Min	minute
ml	millilitre
mM	milli molar
mRNA	Messenger RNA
ng	nano gram
Nm	nano meter
NK cells	natural killer cells
PBMC	A peripheral blood mononuclear cell
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
PD-1	Programmed cell death protein 1
PFA	paraformaldehyde
pTreg	perhipheral induced Foxp3+ Treg cell
p38 MAPK	p38 mitogen-activated protein kinases
RFP	red fluorescent protein
RNA	Ribonucleic acid
RORyt	Orphan Nuclear Receptor RORgt
RT	room temperature
SEM	standard error of the mean
SFB	segmented filamentous bacteria
Smad3	SMAD Family Member 3
SNP	single nucleotide polymorphism
STAT	signal transducer and Activator of Transcription
T-bet	T-box transcription factor TBX21
T cells	Thymocytes cells
TCR	T cell receptor
Tg	transgene
TGF	Transforming Growth Factor
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TNF	Tumor necrosis factors
T_R1	Type 1 regulatory T cell
Treg	regulatory T cell
tTreg	thymus derived Foxp3+ Treg cells

UV	Ultravolet
WT	wild type
µl	microliter
µm	micrometre
µM	micro molar

6.2 List of tables

6.3 List of figures

6.4 Curriculum vitae

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Sprachkenntnisse

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Originalarbeiten

1. Gagliani N, Vesely MC, Iseppon A, **Brockmann L**, Xu H, Palm NW, de Zoete MR, Licona-Limón P, Paiva RS, Ching T, Weaver C, Zi X^7, Pan X, Fan R, Garmire LX, Cotton MJ, Drier Y, Bernstein B, Geginat J, Stockinger B, Esplugues E, Huber S, Flavell RA. 2015. Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. ***Nature*** doi: 10.1038/nature14452.
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Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hamburg, Juni 2016

Confirmation of linguistic correctness

I hereby declare, that I have read the doctoral thesis from Leonie Brockmann titled “Analysis of the role of IL-10 signaling for T_R1 cell differentiation, stability and function” and I confirm its linguistic correctness in English.

Hamburg, June 2016