Characterization of regulatory T cells in atopic dermatitis

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

Eva-Maria Garcia Caratozzolo (geb. Oesau)

aus

Mainz

Bonn 2018

Die vorliegende Arbeit wurde mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn angefertigt.

Erstgutachter: Prof. Dr. med. Natalija Novak

Zweitgutachter: Prof. Dr. rer. nat. Irmgard Förster

Tag der Promotion: 22.11.2018

Erscheinungsjahr: 2019

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1 INTRODUCTION

1.1 REGULATORY T CELLS

The aim of the immune system is to defend against harmful pathogens. For that reason it has to distinguish between harmless self-structures and environmental antigens and infectious nonself-structures (Medzhitov & Janeway, 2002; Povoleri et al., 2013). Hence, the immune system has to establish tolerance or immunity. Furthermore, it is vital that the immune reaction is controlled tightly. Once the defense has started and the pathogens are diminished, effector cells need to be stopped in their reaction. When this equilibrium is disturbed, severe autoimmune diseases, atopic diseases or immune deficiencies can occur.

The first concept of immune suppression was suggested by Gershon *et al* in the early 1970s (Gershon & Kondo, 1970; Gershon & Kondo, 1971). He and others found that thymectomy of 3 days old mice resulted in organ-specific autoimmune diseases and the appearance of tissue-specific auto-antibodies in the circulation. However, mice thymectomized on day 1 or 7 did not develop this pathology (Nishizuka & Sakakura, 1969; Taguchi & Nishizuka, 1981). Furthermore, day 3 thymectomized mice which got infusion of thymocytes, would not develop autoimmunity (Sakaguchi, Takahashi, & Nishizuka, 1982). These studies suggest that in the first 3 days auto-reactive T cells leave the thymus and are followed by suppressor cells which are able to control the former. The idea of suppressor T cells was born. It still took more than 20 years to discover that a subset of T cells with increased CD25 expression was responsible for the suppression. Sakaguchi *et al* were the first who identified $CD4^+$ $CD25^+$ T cells as suppressive T cells and they were later referred to as regulatory T cells (Tregs) (S. Sakaguchi, Sakaguchi, Asano, Itoh, & Toda, 1995). Since then Tregs have been the subject of active research in both basic and clinical immunology and substantial progress has been made in characterizing Tregs phenotypically and functionally. In the early years of Treg history it was assumed that this cell population was mainly important to suppress self-reactive T cells, which escaped the negative selection in the thymus. Therefore they were only associated with autoimmunity. Later it became clear, that Tregs are actually essential to suppress immune responses in general (Wing & Sakaguchi, 2010). Malfunction of Tregs can not only lead to severe auto-immune diseases, but is also associated e.g. with atopic diseases like allergy, atopic dermatitis, asthma or with tumors (Elkord, 2006; Sakaguchi et al., 2001; H. Y. Wang & Wang, 2007).

1.1.1 TREG MARKERS

Since the identification of $CD4^+$ $CD25^+$ Tregs in 1995 by Sakaguchi, intensive research was carried out to characterize those suppressive cells in more detail (Sakaguchi et al., 1995). Soon it became clear that CD25 is not only expressed by Tregs, but also by activated conventional T cells (Tconv) and is hence not a precise marker (Shimon Sakaguchi, Miyara, Costantino, & Hafler, 2010). Substantial progress was made in this field by studying the scurfy mouse, a natural occurring mouse mutant. Those mice suffer from multi-organ autoimmunity and uncontrolled $CD4^+$ T cell proliferation. Responsible for this phenotype is a two base-pair insertion in the Foxp3 transcription factor (Brunkow et al., 2001). Interestingly, $CD4^+$ $CD25^+$ Tregs are absent in these mice (Khattri, Cox, Yasayko, & Ramsdell, 2003). Furthermore, it was discovered that $CD4^+$ $CD25^+$ Tregs express Foxp3 and that ectopic Foxp3 expression provides murine naive T cells with regulatory capacity *in vivo* and *in vitro* (Hori, Nomura, & Sakaguchi, 2003; Ramsdell & Ziegler, 2014). Hence, it could be shown that Foxp3 plays a crucial role in Treg function and development in mice.

In humans, mutations in the *FOXP3* gene lead to the disorder immune dysregulation, polyendocrinopathy and enteropathy X-linked syndrome (IPEX). IPEX is associated with a deficiency in $CD25^+$ Tregs and results into severe immune dysregulation, including amongst others, eczematous dermatitis and characteristics of allergic disorder (reviewed in Ochs et al. 2005). This provides evidence for Foxp3 being a master regulator for Treg development and function in mice and human (Hori, Nomura, & Sakaguchi, 2003). The protein has been shown to act as a transcriptional repressor and inhibits the expression of cytokines such as IL-2 (Bettelli, Dastrange, & Oukka, 2005). Furthermore, Foxp3 activates genes like Cytotoxic T-lymphocyte-associated Protein 4 (CTLA4), Glucocorticoid-induced TNFR-related protein (GITR) and CD25, which are Treg-associated markers (see below) (Sakaguchi, Miyara, Costantino, & Hafler, 2010).

Foxp3 is regarded as a reliable marker for Tregs in mice. In humans, the case is more complicated. Foxp3 can, besides of Tregs, also be expressed in activated T cells (Ramsdell & Ziegler, 2014; Walker et al., 2003). However, Foxp3 is still widely used as a Treg marker. In the last decades, several markers have been proposed to be specific for Tregs. Cytotoxic T-lymphocyte-associated Protein 4 (CTLA4), Glucocorticoid-induced TNFR-related protein (GITR), Ox40, Helios, CD127low being some among them. However, none of them has been found to be exclusively expressed in Tregs (M. A. C. De Curotto de Lafaille & Lafaille, 2002;

Romagnani, 2006; Schmetterer, Neunkirchner, & Pickl, 2012; Rui Wang, Wan, Kozhaya, Fujii, & Unutmaz, 2008).

Lastly, a protein of high interest in Treg physiology has been brought into focus: the glycoprotein A repetitions predominant (GARP), a leucine-rich transmembrane protein. GARP is highly expressed on the surface of activated Tregs and has been shown to increase the suppressive function of Tregs (Stockis, Colau, Coulie, & Lucas, 2009; Rui Wang et al., 2008). GARP has been suggested to be a *bona fide* Treg marker (Schmetterer et al., 2012). GARP will be introduced in more detail in chapter 1.6.

1.1.2 CLASSIFICATION OF TREGS

Tregs constitute 5-10% of $CD4^+$ T cells in the peripheral blood of healthy individuals (Shevach, 2002). They can be grouped into two categories. Natural Tregs (nTregs), which arise in the thymus, and induced Tregs (iTregs), which arise in the periphery (Povoleri et al., 2013) (Figure 1). However, the nomenclature is not uniform. nTregs are also called thymus Tregs (tTregs), whereas iTregs are sometimes called peripheral Tregs (pTregs) or acquired Tregs (aTregs). In this work, they will be named "nTregs" or "iTregs", respectively. nTregs are generated in the thymus from thymocytes without specific antigen stimulation (H. Y. Wang & Wang, 2007). They express CD4, CD25 and Foxp3 and work on a cell-cell contact dependent mechanism (H. Y. Wang & Wang, 2007). The majority of Tregs in peripheral blood are expected to be nTregs (Shevach & Thornton, 2014). iTregs are generated from $CD4^+$ $CD25^-$ naïve T cells in the periphery and are further divided into $Foxp3^-$ cells and $Foxp3^+$ cells (Figure 1). Several $CD4^+$ $CD25^-$ $Foxp3^-$ Tregs have been described, Tr1 and Th3 being the most important/ frequent. Tr1 cells are induced *in vivo* and *in vitro* by MHC-peptide stimulation in the presence of IL-10. They suppress immune response by secreting IL-10 and or TGF-β in a cell contact-independent manner (Roncarolo, Bacchetta, & Levings, 2006). So far, no specific marker for Tr1 cells has been identified yet. Th3 cells have been described as Th2-like-TGF-β secreting cells, which are mainly induced by the oral administration of antigen, in the presence of TGF-β and IL-4 (Inobe et al., 1998; Weiner, 2001). It is a controversial cell subset, although they are essentially associated with TGF-β secretion, it has been found that Th3 cells might also secrete IL-4 and IL-10 (Povoleri et al., 2013).

Foxp3$^+$ iTregs are the most studied cell population within iTregs. They are generated from naïve CD4$^+$ CD25$^-$ T cells in the periphery and can induce their immunosuppressive function by cell-cell contact or through soluble factors. The conversion of CD4$^+$ CD25$^-$ T cells into CD4$^+$ CD25$^+$ Foxp3$^+$ iTregs will be discussed in more detail in 1.1.5.

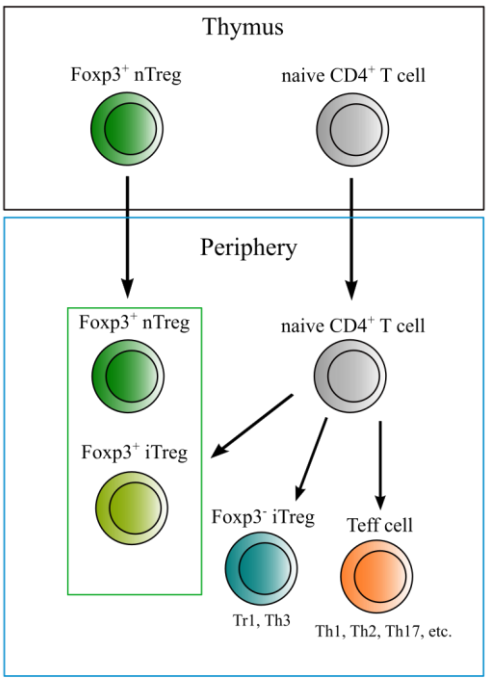

Figure 1: Classification of Tregs
Tregs are classified by their place of origin. Foxp3$^+$ nTregs (in dark green) arise in the thymus (upper panel) and migrate then into peripheral tissue (lower panel). iTregs originate from naïve Th cells (gray) in the periphery. They are further divided into Foxp3$^-$ Tr1 and Th3 Tregs (blue) or Foxp3$^+$ iTregs (light green). Naïve Th cells can also differentiate into Teff cells (orange).

It is speculated that nTregs are primarily responsible for preventing autoimmunity and setting the activation threshold for all immune responses. Whereas the task of iTregs is mainly to suppress immune response to environmental and food allergens and to diminish chronic inflammation (Maria A. Curotto de Lafaille & Lafaille, 2009).

1.1.3 THE TREG SPECIFIC DEMETHYLATED REGION

Due to the difficulty of finding a reliable marker, it is an ongoing challenge to identify *bona fide* Tregs. Lately, analysis of the methylation pattern of a distinct area in the *FOXP3* gene has found to be a promising approach to ensure a reliable identification of Tregs. Foxp3 gene expression is controlled by four elements, specifically the promoter region and three

evolutionary highly conserved non-coding sequences (CNS) (Povoleri et al., 2013). Each of the CNS has different regulatory functions and is regulated by epigenetic modifications that determine chromatin-structure and DNA methylation (Povoleri et al., 2013; Ramsdell & Ziegler, 2014). CNS2 is located upstream of exon-1 and is of particular interest. Demethylation of CpG motifs within CNS2 region which is called Treg specific demethylated region (TSDR), is related to the stability of Foxp3 expression. While complete demethylation was related with high stable Foxp3 expression in nTregs, iTregs only displayed incomplete demethylation, even though Foxp3 expression was high (Baron et al., 2007; Floess et al., 2007). These TGF-β induced $Foxp3^+$ Tregs lose Foxp3 expression and suppressive function. Therefore, demethylation of this region not only allows to identify *bona fide* Tregs, but also enables to distinguish between nTregs and iTregs (Shevach & Thornton, 2014).

1.1.4 SUPPRESSIVE MECHANISMS OF TREGS

Tregs exert their function in regulating homeostasis by inhibiting pro-inflammatory immune response in several ways. One main feature is the inhibition of differentiation, activation, proliferation, cytokine secretion and migration of T cells into inflamed tissue (Loser & Beissert, 2012). Furthermore, they have been shown to suppress the function of natural killer (NK) T cells, B cells, macrophages, osteoclasts and DCs (Miyara & Sakaguchi, 2007; Shevach, 2006; Q. Tang & Bluestone, 2008; von Boehmer, 2005).

Regarding the suppressive mechanism on a molecular basis, many open questions remain. So far, a set of different suppressive mechanisms has been identified. Generally, they can be grouped into four modes: suppression by inhibitory cytokines, suppression by cytolysis, suppression by metabolic disruption and suppression by modulation of DC maturation or function (Vignali, Collison, & Workman, 2008) (Figure 2).

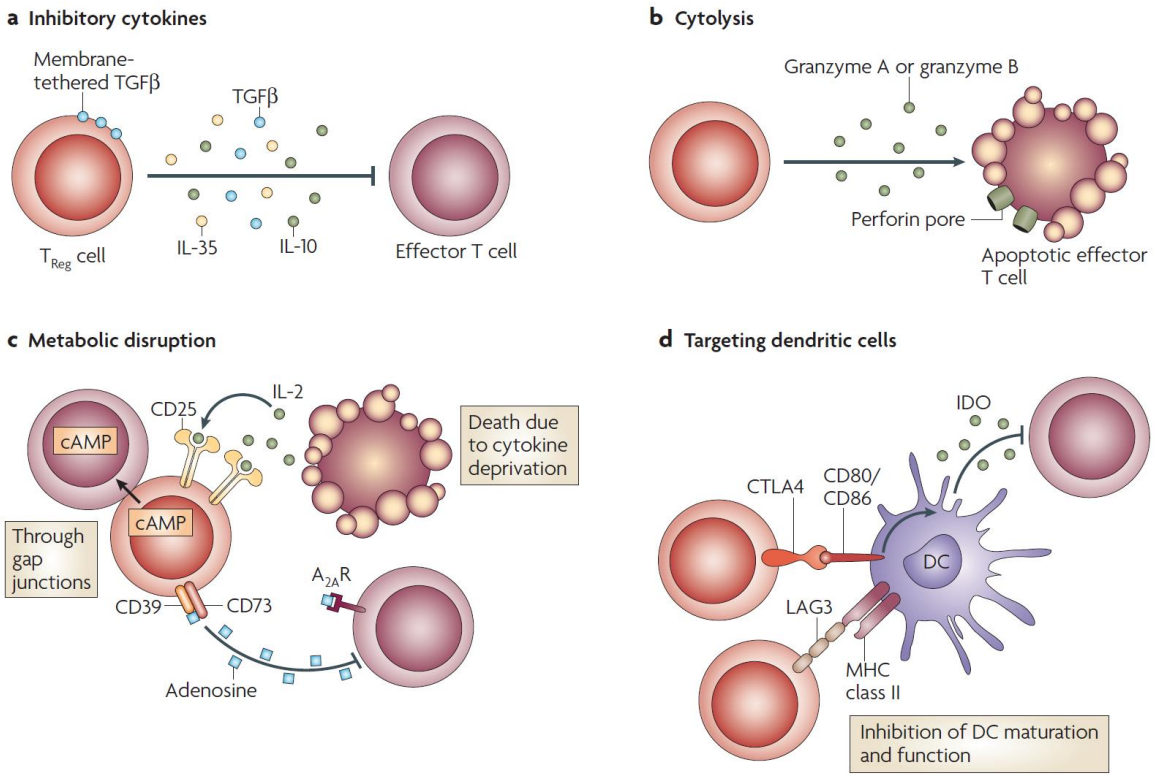

Figure 2: Mechanisms of Treg suppression
Tregs can mediate suppression by different modes.
a: Secretion of inhibitory cytokines (TGF-β, IL-10, IL-35).
b: Cytolysis of Teff by granzyme A or B and building a perforin pore.
c: Metabolic disruption by IL-2 deprivation, transfer of immune suppressive cAMP or generation of pericellular adenosine by CD39 and CD73.
d: Targeting of DC. Down regulation of CD80/86 and inducing DCs to produce IDO.
Adapted from (Vignali et al., 2008).

Suppression by inhibitory cytokines

It is generally undisputed that Tregs are able to suppress immune response by secretion of anti-inflammatory cytokines such as TGF-β, IL-10 or the relatively newly discovered IL-35, a member of the IL-12 heterodimeric cytokine family (Figure 2a). Besides direct mediation of suppression, TGF-β might affect responder T cells to be sensitive to suppression. Furthermore, this cytokine influences Tregs to maintain Foxp3 expression and suppressive activity (von Boehmer, 2005). This mechanism of suppression by inhibitory cytokines is more important for iTregs, since nTregs are thought to work mainly by cell contact (Shevach, 2006).

Suppression by cytolysis

There is evidence that activated human Tregs express the serine proteases granzyme A and granzyme B and that target-cell killing occurs by granzyme A/B and cytolytic perforin through the adhesion of CD18 (Grossman et al., 2004). Granzyme A/B is usually assocciated with cytotoxic T cells and NK cells, but a line of evidences indicates the participation of granzyme A/B and perforin in the suppression mechanism of Tregs (Figure 2b).

Suppression by metabolic disruption

Several ways of suppression by metabolic disruption have been described. One mechanism has been discussed for years, namely IL-2 deprivation by Tregs. It might be that the high expression of the IL-2 receptor CD25 on Tregs captures the free IL-2 and diminishes thereby the proliferation of dividing Teff cells, which need IL-2 to survive (de la Rosa, Rutz, Dorninger, & Scheffold, 2004; Thornton & Shevach, 1998). It has been shown that the concordant expression of the ectoenzymes CD39 and CD73 on Tregs generate pericellular adenosine, which suppresses effector T cell function by binding to the adenosine receptor 2A (A2AR) (Deaglio et al., 2007; Kobie et al., 2006). Furthermore, activation of the A2AR seems to increase the generation of Tregs by inhibiting IL-6 expression and promoting TGF-β secretion (Zarek et al., 2008). Moreover, Tregs inhibit Teff cell differentiation and proliferation by hydrolyzing tissue-destructive adenosine triphosphate (ATP) to adenosine monophosphate (AMP) by the ectoenzyme CD39 (Borsellino et al., 2007; Shevach, 2009). Also, adenosine has been shown to influence DC maturation and to favor a tolerogenic phenotype. Another mechanism to suppress pro-inflammatory immune response by metabolic disruption is to transfer the inhibitory second messenger cyclic AMP (cAMP) into effector T cells through gap junctions (Bopp et al., 2007) (Figure 2c).

Suppression by targeting DCs

Moreover, Tregs can also suppress immune response by targeting DCs (Figure 2d). They may down regulate the expression of the co-stimulatory molecules CD80 and CD86 on DCs and thereby down modulate the capacity to activate Teff cells (Misra, Bayry, Lacroix-Desmazes, Kazatchkine, & Kaveri, 2004).

CTLA4 plays an important role as its deficiency or treatment with blocking antibodies reduces Treg mediated suppression (Shevach, 2009; Wing et al., 2008). Tregs can also modulate DCs by inducing them to express indoleamine 2,3-dioxygenase (IDO), a regulatory molecule particularly toxic to T cells. IDO induction is mediated by binding of CTLA4 to CD80/CD86 and is therefore CTLA4-dependent (Gravano & Vignali, 2012; Grohmann et al., 2002).

1.1.5 CONVERSION INTO iTREGS

In 2003, *in vitro* conversion of $CD4^+$ $CD25^-$ T cells into suppressive $CD4^+$ $CD25^+$ $Foxp3^+$ iTregs was shown for the first time in mice (W. Chen et al., 2003). Since then, iTregs induction from $CD4^+$ $CD25^-$ T cells has been reported many times in *in vitro* and *in vivo* studies (see reviews Curotto de Lafaille and Lafaille 2009; Schmitt and Williams 2013; Dons et al. 2012). *In vivo,* iTregs have been studied intensively in oral tolerance in mice (Mucida et al., 2005). Indeed, it is suspected that one of the main functions of iTregs is to establish tolerance to harmless food antigens (Maria A. Curotto de Lafaille & Lafaille, 2009).

By now, generation of iTregs has been also shown in humans (Amarnath, Dong, Li, Wu, & Chen, 2007; Fantini et al., 2004; Tran, Ramsey, & Shevach, 2007; Yamagiwa, Gray, Hashimoto, & Horwitz, 2001). Intensive research has been carried out to address the key players in iTreg induction. TGF-β has been proven to be crucial for the generation of iTregs (W. Chen et al., 2003). The cytokine promotes the binding of NFAT and Smad 3 to the CNS1 enhancer and induces Foxp3 expression (Tone et al., 2008). Besides, polarization into a regulatory phenotype does not occur without T cell receptor stimulation, which can be polyclonal or antigen specific. Furthermore, IL-2 plays a key role in iTreg generation, since it is critical factor to TGF-β-mediated Foxp3 induction (Q. Chen, Kim, Laurence, Punkosdy, & Shevach, 2011; Fantini et al., 2004; Zheng & Rudensky, 2007). Likewise, IL-2 is not only central for iTreg generation and proliferation, but also for iTreg stability, at least in mice (Q. Chen, Kim, Laurence, Punkosdy, & Shevach, 2011). Another well descried factor is CD28. It has been demonstrated that co-stimulation of CD28 is required for the generation of nTregs, but not for iTregs (Horwitz, Zheng, & Gray, 2008; Tai, Cowan, Feigenbaum, & Singer, 2005). Strong CD28 stimulation is even assumed to be detrimental for Foxp3 induction (Dons, Raimondi, Cooper, & Thomson, 2012; Povoleri et al., 2013). Shevach *et al.* suggest

that the role of CD28 signaling in the conversion progress is only related to its capacity to increase endogenous production of IL-2 (Shevach & Thornton, 2014). In summary, the conversion of $CD4^+$ T cells into Foxp3 expressing iTregs is TGF-β-dependent and follows TCR activation in the presence of IL-2.

1.2 TGF-β SIGNALING

TGF-β plays a crucial role in Treg functions. As described above, TGF-β drives the conversion of $CD4^+$ $CD25^-$ T cells into Foxp3 expressing iTregs. Furthermore, Tregs utilize TGF-β to suppress immune response by secretion of this anti-inflammatory cytokine. Finally, TGF-β produced by Tregs protects and maintains Tregs against apoptosis and destabilization when surrounded by inflammation and constant stimulation (reviewed in Tran 2012).

TGF-β is a pleiotropic cytokine which controls numerous cellular processes and plays an important role in tissue homeostasis and development. In humans, TGF-β superfamily consists of three isotypes: TGF-β 1, TGF-β 2 and TGF-β 3. In the immune system, TGF-β 1 is the isotype most abundantly expressed (Wan & Flavell, 2007). TGF-β is synthesized in an inactive form (pro-TGF-β), which homodimerises and is cleaved and activated by a pro-protein convertase furin (Dubois, Laprise, Blanchette, Gentry, & Leduc, 1995). The C-terminal section corresponds to mature TGF-β, whereas the N-terminal part is known as latency-associated peptide (LAP). Both parts stay non-covalently bound to each other. This construct is called latent TGF-β (Stockis, Colau, Coulie, & Lucas, 2009). TGF-β 1, in its activated form, binds to a heterodimeric transmembrane receptor complex consisting of the serin/threonin kinase receptors TGF-β R I and TGF-β R II, which is further supported by TGF-β R III (Wrana & Attisano, 2000). After the TGF-β binding, TGF-β R II phosphorylates TGF-β R I on serine and threonine residues in a highly conserved juxta-membrane “GS” domain. TGF-β R I itself subsequently phosphorylates downstream molecules, so called Smads, on a conserved carboxy-terminal SSXS motif. Once the also called R-Smads (Receptor-mediated Smads) 2/3 are phosphorylated, they form a heteromeric complex with the second class of Smads, the common (Co)-Smads, namely Smad 4. The complex is then translocated into the nucleus, where it regulates gene expression (Figure 3) (Piek, Heldin, & Ten Dijke, 1999; Wrana & Attisano, 2000). TGF-β signaling can be suppressed by Smad 7, an inhibitory molecule (I-Smad) that prevents recruitment and phosphorylation of Smad 2/3

through the binding of MH2 domains to TGF-β RI (Derynck & Zhang, 2003; Nakao et al., 1997).

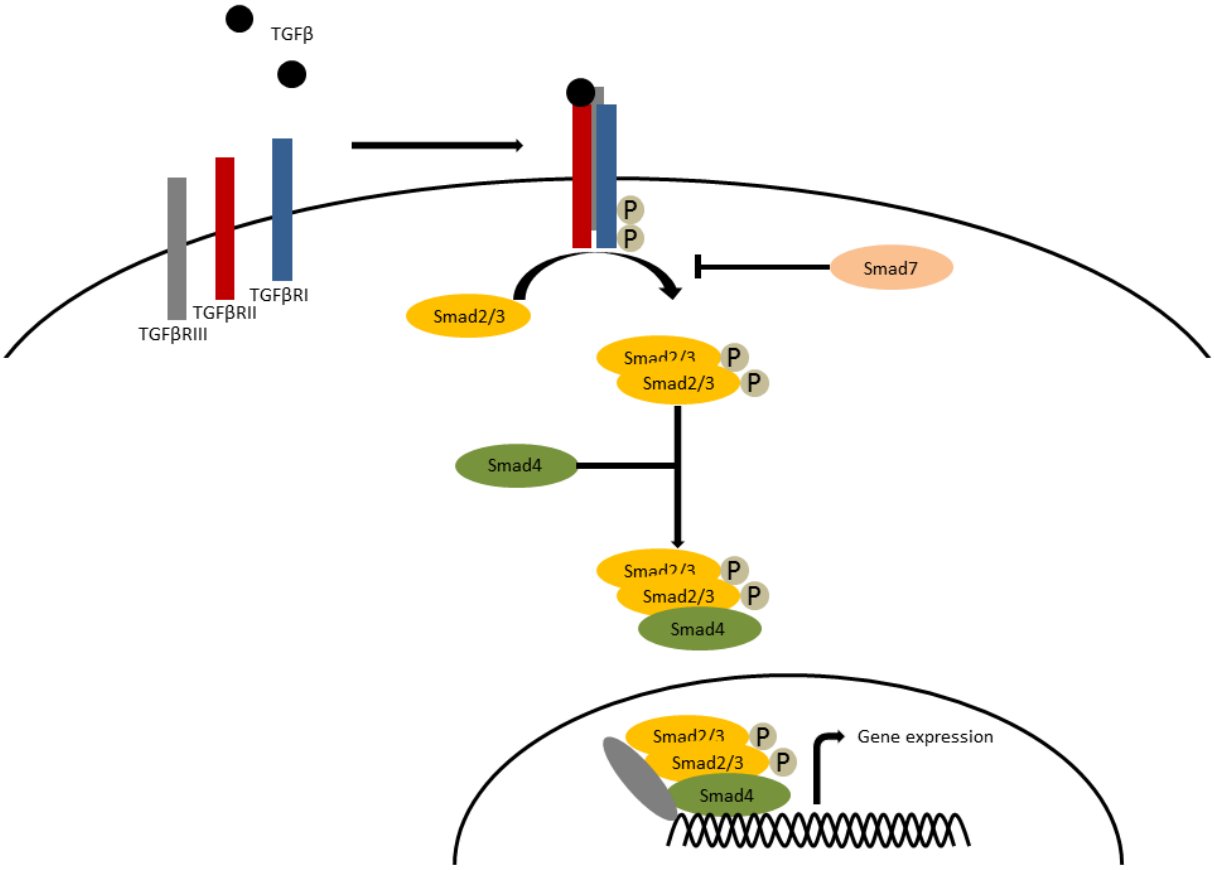

Figure 3: TGF-β Signaling pathway
Mature TGF-β binds to a heterodimeric transmembrane receptor complex, consisting of TGF-β RI and TGF-β RII. The binding of TGF-β to the complex is supported by TGF-β RIII. The binding of TGF-β leads to phosphorylation of TGF-β RI by TGF-β RII. TGF-β RI subsequently phosphorylates downstream molecules. After Smad 2/3 are phosphorylated they form a heteromeric complex with Smad4. The complex is then translocated into the nucleus, where it regulates gene expression. Smad 7 is inhibiting TGF-β signaling by preventing phosphorylation of Smad 2/3.

1.3 ATOPIC DERMATITIS

Atopic dermatitis (AD) belongs, together with allergic rhinitis and asthma, to the atopic diseases (Novak & Bieber, 2003). “Atopic”, derived from the Greek word “atopos”, means “out of place” and can be translated as “strange”, “unnatural” or “monstrous” (Liddell & Scott, 1996). Atopic diseases are defined as the familial propensity to become IgE-sensitized to environmental allergens (Weidinger & Novak, 2016). AD is one of the most common chronic inflammatory skin diseases. In developed countries, up to 30% of children and 20% of adults are affected. The prevalence of AD in industrialized countries has doubled during

the last 30 years and it is still rising in developing countries (Deckers, Mclean, Linssen, Mommers, & Schayck, 2012). Commonly, AD begins in early infancy (early-onset AD). 45% of all AD cases start within the first 6 months of life, 60% during the first 12 months of life and 85% before the 5 years of age (Bieber, 2008). In most cases, AD vanishes within the first 6 years. Often a remission is observed in adulthood or it is present the whole time. Late-onset AD frequently starts in puberty and often continues into adulthood, however it can start at any age (C. Akdis et al., 2006; Garmhausen et al., 2013). The disease varies from mild to moderate and severe forms (Ballardini et al., 2013). AD commonly starts with skin dryness and roughness. Thereafter eczematous lesions establish, going along with inflammation, pruritus and excoriations, scaling and furthermore, susceptibility for cutaneous bacterial and mycotic infections. Lesions can be acute, subacute or chronic. The course of the disease is of characteristic relapsing–remitting nature with repeated flare-ups, even though it can be persisting. Any part of the body can be affected, however, lesions show typically age-related morphology and distribution (Weidinger & Novak, 2016). In many cases the disease affects the life quality of the patients immensely, amongst others due to itch, insomnia and embarrassment as a result of visible lesions, inflicting a strong psychosocial burden on patients.

The pathogenesis of AD is highly complex and multi-factorial. It is commonly thought that genetic, environmental and immunological factors play together (Novak & Leung, 2011; W. Peng & Novak, 2015). There is a strong indication for genetic influence, as twin studies demonstrated a higher concordance rate (0.77) in monozygotic twins than in dizygotic twins (0.15). Besides, the incidence rate will be doubled if one parent is affected by AD and tripled if both parents have AD (Bieber, 2010). Interestingly, there is a strong association between loss-of-function mutation in the pro-filaggrin gene (*FLG*) and AD (O'Regan, Sandilands, McLean, & Irvine, 2008). Pro-filaggrin, the precursor of filaggrin, is significantly involved in building the epidermal structure and barrier function. Furthermore, other genes have been described to be associated with AD, e.g. the gene that encodes for the protein claudin-1 (*CLDN1*) (Benedetto et al., 2011). This protein is part of the tight junctions and is hence involved in building the epithelial barrier. Interestingly, also other factors besides the genetic influence can impair the skin barrier. It has been shown that an increased pH and Th2 cytokines lead to a susceptibility to elevated amounts of *S. aureus* (Rippke, Schreiner, Doering, & Maibach, 2004). On the other hand, *S. aureus* secrets virulence factors, such as α-toxin, which induce cell death of keratinocytes and promotes Th2-type inflammation leading

to an impairment of skin barrier function (Brauweiler, Goleva, Leung, & Health, 2015; Vu, Baba, Chen, Le, & Kinoshita, 2010).

It is commonly agreed that genetic factors play a crucial role, but they are not the only cause for developing AD. A hyper-reactive immune reaction is a hallmark of AD. Presumably, antigens are able to enter through a disrupted epithelial barrier, where they encounter Langerhans cells and inflammatory dendritic epidermal cells bearing IgE. The antigen is presented by these APCs leading to the initiation of T cell driven immune response. Infiltration of T cells is another indicator for AD (Agrawal R, Wisniewski, MD and Woodfolk, 2011). In the acute state Th2 response dominates. IL-4 and IL-13 are suspected to be responsible for isotype-switch to IgE (Hamid, Boguniewicz, & Leung, 1994). Interestingly, even non-lesional skin already reveals subclinical signs of inflammation as Th2, Th22 and Th17 cells are detected, along with pro-inflammatory cytokines (Suárez-Fariñas et al., 2012). In the chronic phase Th1 response is the strongest (IFN-γ, IL-2), while Th2 still contributes (Cabanillas, Brehler, & Novak, 2017). In addition to T cell infiltration, other immune cells contribute to the inflammatory reaction, including mast cells, eosinophils, DCs and macrophages (Ilkovitch, 2011; W. Peng & Novak, 2015). There is an ongoing discussion, whether the impaired skin barrier on one side or the immunologic disturbance on the other side is the primary or secondary cause of the disease (Novak & Leung, 2011). In other words, is the impaired skin barrier dysfunction leading and the immunologic phenotype a collateral phenomenon? Or is the primary effect located in a defect immune reaction resulting in disrupted epithelial-barrier due to local inflammation (Bieber, 2008). The complexity of AD pathogenesis is still under intense debate and further investigations are needed to understand the mechanisms behind the disease better.

1.4 THE ROLE OF TREGS IN AD

It is likely that Tregs play a pivotal role in the pathogenesis of AD, as Tregs regulate immune homeostasis by modulating the immune system response (Shimon Sakaguchi et al., 2010; Shevach & Thornton, 2014). In fact, it has been shown that they are involved in many inflammatory disorders (Agrawal R, Wisniewski, MD and Woodfolk 2011; Ilkovitch 2011). Interestingly, Tregs express a number of skin-homing receptors like CCR4, CCR6 and cutaneous lymphocyte-associated antigen (CLA) and are therefore expected to be recruited to the skin to take part in cutaneous immune surveillance (Hirahara et al., 2006). Indeed, it has

been proposed that most human Tregs reside in the skin, where they might be involved in peripheral tolerance (Kupper & Fuhlbrigge, 2004).

Major evidence that Tregs limit cutaneous inflammation is derived from patients with Foxp3 mutation, leading to IPEX (see 1.1.1). Patients suffer, amongst other systemic abnormalities, from eczematous dermatitis with pruritus and hypereosinophilia as seen in AD (Halabi-Tawil et al., 2009; Ochs et al., 2005). Another indication that Tregs play a role in AD comes from a contact hypersensitivity mouse model, where adoptive transfer of Tregs can prevent contact dermatitis (Ring, Schäfer, Mahnke, Lehr, & Enk, 2006). In ovalbumin-sensitized mice, depletion of Tregs increases the severity of skin inflammation (Fyhrquist, Lehtima, Lahl, Savinko, & Lappetela, 2012). Some groups have shown that $CD25^+$ $Foxp3^+$ Tregs are present in lesional skin of AD patients (Caproni et al., 2007; Fujimura, Okuyama, Ito, & Aiba, 2008; Szegedi et al., 2009). In contrast, a work from Verhagen *et al.*, demonstrates that Tr1 cells are present, but $CD25^+$ $Foxp3^+$ Tregs could not be found (Verhagen et al., 2006). Regarding the amount of circulating Tregs in the blood, conflictive results have been reported in literature. Some studies indicate a higher number of Tregs in AD or allergy (Ou *et al.*, 2004; Vukmanovic-Stejic *et al.*, 2005; Agrawal R, Wisniewski, MD and Woodfolk, 2011; Lesiak *et al.*, 2012; Roesner *et al.*, 2015). Others have observed less Tregs in AD (Stelmaszczyk-Emmel, Zawadzka-Krajewska, Szypowska, Kulus, & Demkow, 2013) or allergy (J. Lee, Yu, & Wang, 2007; Xu et al., 2007). Even more, others did not find a change in Treg numbers (Bellinghausen, Klostermann, Knop, & Saloga, 2003). Reasons for such a contradiction are probably lying in the diversity of age, disease stage and cell subsets analyzed in the particular study.

Taken together, there are indications that Tregs play an important role in AD, but due to several factors it is still difficult to get a clear picture. The complicacy of a suitable marker and furthermore, the complexity of the disease are a big challenge and make further investigations necessary.

1.5 TGF-β SIGNALING IN AD

Growing evidence has demonstrated that TGF-β signaling is compromised in AD (Anthoni et al., 2007; Katagiri, Arakawa, & Hatano, 2007; W. M. Peng, Maintz, Allam, & Novak, 2013). It has been shown that TGF-β mRNA is reduced in freshly isolated PBMC in AD patients in

comparison to healthy controls (Katagiri et al., 2007; H. Lee, Lee, Ha, Byun, & Kim, 2000). Gambichler *et al.* reported a reduced mRNA expression of Smad 3/4 in lesional skin of AD patients as compared to healthy subjects (Gambichler, Tomi, Skrygan, Altmeyer, & Kreuter, 2006). Anthoni *et al*. showed that in a murine AD model using Smad 3-deficient mice, Smad 3-pathway regulates allergen-induced skin inflammation and systemic IgE antibody production (Anthoni et al., 2007). These studies highlight, that TGF-β might play an important role in AD pathology. Little is known about TGF-β R expression on $CD4^+$ T cells in AD. In allergic diseases it has been shown that TGF-β R mutations lead to a strong predisposition (Frischmeyer-guerrerio et al., 2013). Revealing how TGF-β Rs are expressed and regulated in $CD4^+$ T cells in AD would gain important inside in TGF-β signaling in the context of the disease.

1.6 GARP

As mentioned above, GARP is of high interest in Treg physiology. This protein is focus of intensive study and promises valuable inside regarding Treg function, generation and its role in diseases such as AD. GARP is a leucine-rich transmembrane protein. It anchors latent TGF-β at the cell surface of Tregs and might be further involved in the production and activation of TGF-β by activated Tregs (Edwards, Thornton, & Shevach, 2014; Hahn et al., 2013; Probst-Kepper & Buer, 2010). Interestingly, GARP has been shown to be a specific marker for activated human $Foxp3^+$ Tregs (Stockis et al., 2009; Rui Wang et al., 2009) and furthermore, it seems to play a role in the inhibitory function of Tregs. It has been demonstrated that down regulation of GARP in human Tregs by gene knockdown approaches diminished their suppressive qualities (Probst-Kepper & Buer, 2010; Rui Wang et al., 2009). Another study shows a decrease in Treg-suppressive function after addition of GARP blocking antibodies (Miller, Fogle, Ross, & Tompkins, 2013). By contrast, GARP over-expression can phenotypically and functionally re-program Teff cells into Tregs (Probst-kepper et al., 2009; Rui Wang et al., 2008). There is evidence that GARP regulates Foxp3 gene expression (Probst-kepper et al., 2009; Rui Wang et al., 2008). On the other hand, GARP induction seems to be dependent on Foxp3 expression (Rui Wang et al., 2008). This is indicating a close relation between GARP and Foxp3 expression. Even though there is contradiction (Tran et al., 2009; Rui Wang et al., 2009), most people agree that GARP and Foxp3 are tightly interacting with each other.

Interestingly, GARP might function not only when bound to the membrane, but also in its soluble form and therefore, might be acting as an immune modulator (Fridrich et al., 2016). Hahn et al demonstrated that soluble GARP (sGARP) induces the expression of TGF-β1 and Foxp3, resulting in differentiation of naive T cells into induced Tregs. These effects were abolished after TGF-β R blockade, indicating the participation of TGF-β in sGARP mechanism. Specially, GARP is of high interest in AD pathology, because it has been shown that low-frequency missense variants in the *LRRC32* gene (coding for the protein GARP) are significant contributors to AD risk (Manz et al., 2016). Therefore, understanding the role of GARP in Tregs would not only gain valuable insides in Treg function, but also in their role AD.

1.7 AIM OF THE THESIS

AD is one of the most common chronic inflammatory skin diseases with increasing incidence. Although the role of Tregs in AD has been investigated for the last 10-15 years, their contribution to the pathogenesis of this disease is still not clear. The aim of this study was therefore to characterize the phenotype, the induction and function of Tregs in patients with AD compared with healthy controls.

More specifically, there is evidence that the frequency of circulating Tregs in the peripheral blood of AD patients might be altered. Therefore, the first objective of this work was to quantify and compare circulating Tregs and T cell subsets of AD patients with that of healthy individuals.

Furthermore, it was indicated that TGF-β signaling might be malfunctioning in AD. Therefore, TGF-β-dependent *in vitro* conversion of $CD4^+$ $CD25^-$ $Foxp3^-$ T cells into $CD4^+$ $CD25^+$ $Foxp3^+$ iTregs in AD patients and healthy donors was investigated within this work. This part of the study was based on the hypothesis that iTreg induction might be diminished, and therefore contribute to AD pathogenesis.

Since mutations in the gene encoding for GARP has been associated with the risk to develop AD, the final goal of this thesis was to determine the role of this transmembrane protein in the context of AD. In particular, the objective was to analyze cells from AD patients with a missense mutation in the gene encoding for GARP phenotypically and functionally.

2 MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 EQUIPMENT

Devices	Manufacturer / type
Analytic scales	Sartorius BP110, BP221D, BP6100, Secura 6102-1S
Autoclave	H+P Labortechnik Varioklav 500
Centrifuges	Heraeus Biofuge Pico, Heraeus Biofuge Fresco, Multifuge 3 S-R, Eppendorf Centrifuge 5415R
Electrophoretic transfer cell	Trans-Blot SD Semi Dry Transfer Cell, Bio-Rad
Flow cytometer	BD FACSCanto
Gel electrophoresis chamber	PerfectBlue™ Gelsystem Mini L
Gel electrophoresis system	Mini-PROTEAN Tetra Cell, Bio-Rad
Incubators	Heraeus HeraCell, Memmert Drying Cabinet U 40
Microplate reader	BioTek Synergy HAT
Microscopes	Leitz Laborlux K light microscope
pH-Meter	WTW pH 526
Pipettes	Abimed Labmate, Discovery Comfort DV, Eppendorf Reference 2
Pipette controller	Hirschmann Laborgeräte Pipetus
Real-time PCR system	Applied Biosystems 7300 System
Shakers	Biosan Mini Rocker MR-1
Spectrophotometer	Peqlab NanoDrop ND-1000
Sterile work benches	Heraeus HeraSafe
Thermo cycler	Eppendorf Mastercycler Gradient
Thermo mixer	Eppendorf Thermomixer compact
Ultrapur water unit	Millipore Milli-Q Integral Water Purification System
Vortex device	IKA MS 1 Minishaker
Water bath	GFL1012

2.1.2 CONSUMABLES

Consumables were purchased from Axygen, BD, Braun, Corning, Eppendorf, Greiner bio-one, Millipore, Nunc, Sarstedt, Thermo Fisher Scientific and Whatman.

2.1.3 CHEMICALS

Chemicals	Company
Antibiotic/Antimycotic (AB/AM)	Invitrogen
Agarose	Sigma
Aprotinin	Sigma
APS (Ammoniumpersulfate)	Applichem
Acrylamide (30%)	Bio-Rad
Bromphenol blue	Plusone
Bovine serum albumin (BSA)	Sigma
Carboxyfluorescein succinimidyl ester (CFSE)	Thermo Fisher Scientific
Dimethylsulfoxide (DMSO)	Applichem
Ethanol	Merck
Foetal calf serum (FCS)	Sigma
Glycerol	Merck
Glycine	Merck
Halt Phosphatase Inhibitor Cocktail	Thermo Fisher Scientific
Leupeptin	Sigma
L-Glutamine	Gibco
Lymphoprep	Progen
Methanol	Merck
Mercaptoethanol	Sigma
Milk powder	Applichem
Mouse serum	Jackson Immuno Research
Sodium chloride (NaCl)	VWR
Sodium azide (NaN_3)	Merck
Sodium hydroxide (NaOH)	Merck
PagerRuler Plus Prestained Protein Ladder	Thermo Fisher Scientific
Phenylmethylsulfonyl fluoride (PMSF)	Sigma

Rat serum	Sigma
RIPA buffer	Sigma
Sodium dodecyl sulfate (SDS)	Sigma
Tetramethylethylendiamine (TEMED)	Fluka
Tris-Base	Sigma
Tris hydrochloride (Tris-HCl)	Sigma
Tween 20	Sigma

2.1.4 SOLUTIONS AND BUFFERS

Solution / buffer	Composition
Blocking solution	5% milk powder (w/v) in TBST and 5% BSA (w/v) in TBST
FACS buffer (10 x), pH 7.4	85 g/l NaCl, 10.7 g/l Na_2HPO_4, 4.5 g/l NaH_2PO_4 ($\cdot H_2O$), 10% FBS (v/v), 10 g/l NaN_3
MACS buffer	8 g/l NaCl, 0.2 g/l KCl, 1.2 g/l NaH_2PO_4 ($\cdot H_2O$), 0.2 g/l KH_2PO_4, 5 g/l BSA, 10 ml of 0.5 M EDTA
NaN3 solution	0.01% and 10% NaN3 (w/v) in PBS
PBS	8.0 g/l NaCl, 0.2 g/l KCl, 1.5 g/l Na_2HPO_4 ($\cdot 2H_2O$), 0.2 g/l KH_2PO_4
10 x SDS Page Running buffer	30.3 g/l Tris-Base, 144 g/l Glycine, 1% SDS (w/v), pH 8.3
50 x TAE Puffer	242 g/l Tris base, 57,1 ml/l glacial acetic acid, 100 ml/l 0.5 M EDTA (pH 8.0)
20 x TBST	24.22 g/l Tris-Base, 175.32 g/l NaCl, 2% Tween 20 (v/v), pH 8
1 x Transfer buffer	15% CH_3OH, 20% 5x Transfer buffer, 65% H_2O
5 x Transfer buffer	15.15 g/l Tris-Base, 72 g/l Glycine, 10, 1% SDS (w/v), pH 8.3
Tris buffer for separation gel	181.8 g/l Tris-Base, pH 8.8
Tris buffer for stacking gel	121.1 g/l Tris-Base, pH 6.8

2.1.5 KITS

Kit	Company
CD4+CD25+ Regulatory T cell Isolation Kit, human	Milteniy biotec
DNeasy Blood & Tissue Kit	Qiagen
EpiTect Bisulfite Kit	Qiagen
Foxp3 / Transcription Factor Staining Buffer Set	Thermo Fisher (eBioscience)
NucleoSpin Gel and PCR Clean-up	Macherey-Nagel
NucleoSpin RNA	Macherey-Nagel
peqGOLD Plasmid Miniprep Kit I	Peqlab
Pierce BCA Protein Assay Kit	Thermo Fisher (eBioscience)
RNase-free DNase Set	Qiagen
TaqMan Gene Expression Master Mix	Applied Biosystems
TaqMan Reverse Transcription Kit	Applied Biosystems
Treg suppressor kit human	Milteniy biotec
WesternBright Sirius	Advansta

2.1.6 STIMULI, CYTOKINES, REAGENTS

Stimuli / cytokine / reagents	Company
recombinant human IL-2	PeproTech
recombinant human TGF-β	R&D Systems
anti-CD3 antibody	R&D Systems
anti-CD28 antibody	R&D Systems
anti-IL-4 antibody	R&D Systems
sGARP	R&D Systems

2.1.7 GENE EXPRESSION ASSAYS

Gene name	Assay ID	Label
FOXP3	Hs01085835_m1	FAM
Smad 3	Hs00969210_m1	FAM
Smad 7	Hs00998193_m1	FAM
18S ribosomal RNA	4310893	VIC-TAMRA

2.1.8 ANTIBODIES AND SERUM

Isotype control	Clone	Concentration	Company
non conjugated			
Mouse IgG1, k	MOPC21	2.5 µg/ml	Sigma-Aldrich
Mouse IgG2a,k	UPC-10	10 µg/ml	Sigma-Aldrich
conjugated			
Mouse IgG1-APC	MOPC-21	1:20	BD Biosciences
Mouse IgG1-FITC	MOPC-21	1:20	BD Biosciences
Mouse IgG1, k, PE	P3.6.2.8.1	1:20	eBioscience
Mouse IgG2a, k PE	eBM2a	1:20	eBioscience
Mouse IgG2b, k APC	27-35	1:20	BD Biosciences
Rat IgG2a, k -PE	eBR2a	1.25 µg/ml	eBioscience
Primary antibody	**Clone**	**Concentration**	**Company**
non conjugated			
CD3	UCHT1	5 µg/ml	R&D Systems
CD28	37407	2 µg/ml	R&D Systems
GARP	7B11	2.5 µg/ml	Biolegend
GAPDH	411	1:5000	Santa Cruz Biotechnology
IL-4	34019	10 µg/ml	R&D Systems
Smad2/3 (rabbit)	polyclonal	1:1000	Cell Signaling Technology
Phospho-Smad2/Smad3 (rabbit)	polyclonal	1:1000	Cell Signaling Technology
TGF-β Receptor I	MM0015-8G33	10 µg/ml	Abcam
TGF-β Receptor II	MM0056-4F14	2.5 µg/ml	Abcam
TGF-β Receptor III	31349	2.5 µg/ml	R&D Systems
conjugated			
CD3-PE Cy5	UCHT1	1:10	BD Biosciences
CD4-APC	RPA-T4	1:20	BD Biosciences
CD14-PE	M5E2	1:10	BD Biosciences
CD19-PE	LT19	1:10	Miltenyi Biotec
CD25-FITC	M-A251	1:20	BD Biosciences
CD25-PE	4E3	1:20	Miltenyi Biotec
CD56-PE	B159	1:10	BD Biosciences
GARP-APC	7B11	1:20	BD Biosciences
GITR-APC	110416	1:20	R&D Systems
Foxp3-FITC	236A/E7	1:20	eBioscience
Foxp3-PE	PCH101	1.25 µg/ml	eBioscience
Secundary antibody	**Clone**	**Concentration**	**Company**
conjugated			
Goat anti mouse-APC	polyclonal	2.5 µg/ml	Jackson ImmunoResearch
Donkey anti-rabbit-HRP	polyclonal	1:2000	Santa Cruz Biotechnology

Goat anti mouse IgG-HRP	polyclonal	1:5000	Santa Cruz Biotechnology
Serum		**Concentration**	**Company**
Mouse serum		1:20	Jackson ImmunoResearch
Rat serum		1:50	Sigma

2.1.9 PRIMARY CELLS

Human $CD4^+$ $CD25^-$ T cells were isolated from peripheral blood of patients with AD and healthy donors and cultured *in vitro.*

2.2 METHODS

2.2.1 CELL CULTURE

2.2.1.1 PATIENTS AND HEALTHY CONTROLS

Peripheral blood was obtained from 72 AD patients (aged 18-76, mean age 38) and 63 healthy donors (aged 19-61, mean age 33 years). The patients did not receive any systemic therapy. Each donor gave written informed consent and all studies were approved by the local Ethics Committee.

$CD4^+$ $CD25^-$ T cells were cultured at 1 million cells per ml in RPMI 1640 supplemented with 10% inactive FCS, 1% AB/AM, 1% L-glutamine and 0.2% (v/v) β-mercaptoethanol. Cells were kept untreated or treated with cytokines (2.2.1.4.1 Conversion of $CD4^+$ $CD25^-$ T cells into iTregs). iTregs were harvested after 1, 3 or 4 days (D1, D3 or D4) of culture.

2.2.1.2 PURIFICATION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

Human peripheral blood mononuclear cells (PBMC) were isolated from patients with AD or healthy controls. The peripheral blood was collected in 6 S-Monovettes 9 ml LH (Sarstedt) containing heparin as anticoagulant. 25 ml of blood was mixed with the same volume of MACS Buffer. 25 ml were layered over 15 ml Lymphoprep™ and centrifuged for 28 min at 2150 rpm at room temperature. This step leads to division of cell types depending on their density. The cloudy interphase between plasma and Lymphoprep, containing the lymphocytes, was aspirated and washed with MACS buffer 3 times. Before the last centrifugation step cells were passed through a cell strainer to remove cell clumps. Finally, the cell number was determined.

2.2.1.3 ISOLATION OF $CD4^+$ $CD25^-$ AND $CD4^+$ $CD25^+$ T CELLS

Human $CD4^+$ $CD25^-$ T cells were isolated from peripheral blood from patients with AD or CTR by density gradient centrifugation of PBMC and subsequent magnetic separation of cells (MACS isolation).

Magnetic separation was performed by using magnetic beads (Miltenyi Biotech) according to the manufacturer´s instructions. The separation was carried out in a two-step procedure. First, $CD4^+$ T cells were collected by negative selection. Therefore, cells were re-suspended in 90 µl MACS buffer per 10 x 10^6 cells and incubated with a biotin-antibody (Ab) cocktail of monoclonal (mc) Ab against CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and CD235a for 5 min. Afterwards the suspension was incubated with Microbeads conjugated to mc anti-biotin Ab for 10 min. Cells were then washed with MACS buffer and re-suspended in 500 µl MACS buffer. Subsequently, the labeled non-$CD4^+$ cells were depleted by separation over a MACS column, which was placed in the magnetic field of an autoMACS® Separator. In the second step, $CD25^+$ T cells were directly labeled within the pre-enriched $CD4^+$ T cell fraction with CD25 Microbeads. Therefore, cells were incubated with the beads for 15 min and washed with MACS buffer. After centrifugation at 300 x g the cell pellet was re-suspended in 500 µl MACS buffer and magnetic separation with autoMACS® Separator was carried out. Both, the positive fraction ($CD4^+$ $CD25^+$) and the negative fraction $CD4^+$ $CD25^-$) were used for subsequent applications. All steps were carried out at 4 °C.

2.2.1.4 STIMULATION OF CELLS

2.2.1.4.1 CONVERSION OF $CD4^+$ $CD25^-$ T CELLS INTO iTREGS

First, $CD4^+$ $CD25^-$ T cells were magnetically isolated from PBMC. The cells were then incubated for up to 4 days in T cell media in the presence of 100 U/ml IL-2 and 5 ng/ml TGF-β on anti-CD3 (5 µg/ml) coated plates. In some experiments (2.2.1.4.3 Treatment of $CD4^+$ $CD25^-$ T cells with sGARP) 2 µg/ml anti-CD28 Ab was added. Cells were cultured at 10^6 cells / ml in 96-well plates (flat bottom). After 3 or 4 days of culture cells were used for subsequent experiments. The culture consisted of a mixture of $CD4^+$ $CD25^-$, $CD4^+$ $CD25^+$ $Foxp3^-$ and $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (Figure 4).

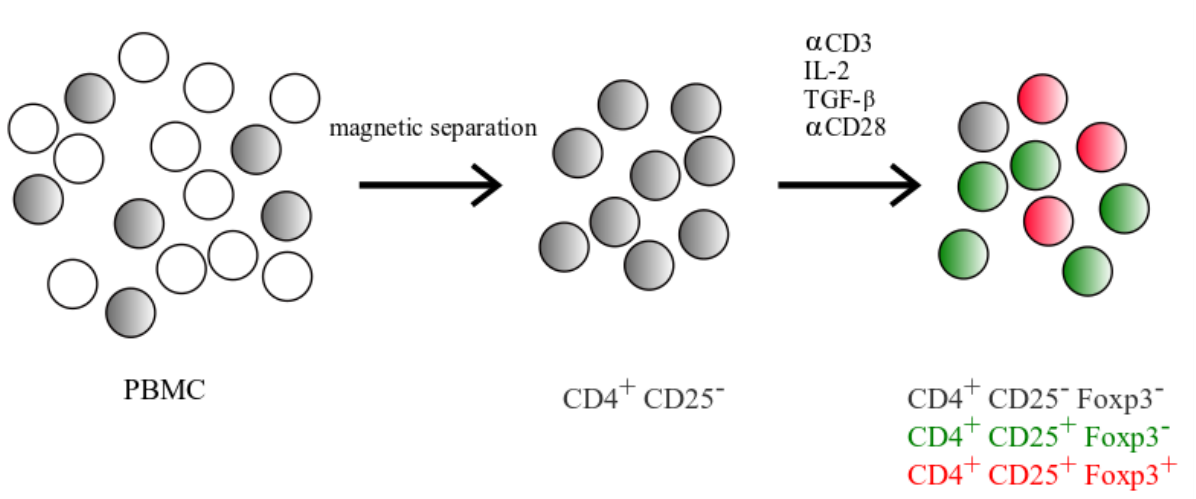

Figure 4: Schematic workflow of *in vitro* generation of iTregs.
First, PBMC were isolated from human peripheral blood. $CD4^+$ $CD25^-$ T cells (green) were then magnetically isolated from PBMC by MACS isolation. Finally, $CD4^+$ $CD25^-$ T cells were cultured for up to 4 days on anti-CD3 coated 96 well plates in the presence of IL-2 and TGF-β (and in some cases anti-CD28 Ab). Cells differentiated into $CD4^+$ $CD25^-$, $CD4^+$ $CD25^+$ $Foxp3^-$ and $CD4^+$ $CD25^+$ $Foxp3^+$ T cells.

2.2.1.4.2 TREATMENT OF $CD4^+$ $CD25^-$ T CELLS WITH ANTI-IL4

Magnetically isolated $CD4^+$ $CD25^-$ T cells were treated as described in 2.2.1.4.1. Additionally 10 µg/ml of neutralizing anti-IL-4 antibody was added (or not added in the control condition) on D0. CD25 and Foxp3 expression was measured by flow cytometry on D1 and D3 of culture.

2.2.1.4.3 TREATMENT OF $CD4^+$ $CD25^-$ T CELLS WITH SGARP

Magnetically isolated $CD4^+$ $CD25^-$ T cells were stimulated with 100 U/ml recombinant human IL-2, 2 µg/ml anti-CD28, 5 ng/ml recombinant human TGF-β or 10 µg/ml sGARP at 1 million cell on 5 µg/ml anti-CD3 (clone UCHT1) coated plates for 3 days (all from R&D systems, Wiesbaden, Germany). Expression was measured on day 3 of culture by flow cytometry. Condition without sGARP or stimulation was used as control.

2.2.1.5 ENRICHMENT OF ITREGS WITH CD25 MICROBEADS

After 3 days of culture cells were collected in a 15 ml tube and washed with MACS buffer twice (centrifugation at 300 x g for 10 min). The cell pellet was then re-suspended in 270 µl of MACS buffer and incubated with 30 µl CD25 Microbeads for 15 min at 4 °C. The cells were washed again in MACS buffer and separation with the autoMACS® Separator was

carried out. The positive fraction contained $CD4^+$ $CD25^+$ iTregs. The cells were subsequently used for co-culture experiments.

2.2.1.6 CO-CULTURE OF ITREGS WITH $CD4^+$ T RESPONDER CELLS

To evaluate the inhibitory capacity of iTregs on proliferating $CD4^+$ $CD25^-$ T responder cells (Tres), both cell types coming from the same donor, were co-cultured in different ratios and proliferation of Tres was measured after 3 days by flow cytometry. $CD4^+$ $CD25^-$ T cells were frozen on the day of isolation and thawed 3 days later. For staining Tres with CFSE, cells were washed with pre-warmed T cell media and the cell number was determined. After centrifugation at 800 rpm for 10 min 10^6 cells / ml were re-suspended in PBS and incubated with 0.05 µM CFSE for 20 min at room temperature whereat cells were carefully shaken after 10 min of incubation. To stop the reaction 5 x volumes of T cell media containing 10% FCS was added, mixed carefully with the cells and incubated for 5 min at 37 °C in a water bath. Finally, cells were centrifuged (800 rpm for 10 min), re-suspended in pre-warmed T cell media and counted.

2.2.2 PROTEIN BIOCHEMISTRY

2.2.2.1 *IN VITRO* STIMULATION OF $CD4^+$ $CD25^-$ T CELLS WITH TGF-β AND CELL LYSIS

$CD4^+$ $CD25^-$ T cells were cultured overnight (16 hours) in RPMI 1640 + 0.25% FCS. Cells were then stimulated with 5 ng/ml TGF-β for 1 hour or left untreated. Subsequently, cells were washed with ice cold PBS and were then lysed in RIPA buffer with 1 mM PMSF, 5 µg/ml aprotinin, 5 µg/ml leupeptin and 1% phosphatase inhibitor cocktail. After 30 min of extraction on ice, cell lysates were collected by centrifugation at 15 000 x g for 15 min.

2.2.2.2 PROTEIN QUANTIFICATION

The amount of total protein was measured using the bicinchoninic acid (BCA)-assay according to the manufacturer´s instructions with little modifications. In short, solution A and B were mixed 1 to 50 and 200 µl of the mixture per reaction was transferred to 96 well plates

(flat bottom). 5 µl of the BSA standards (125 µg/ml to 1500 µg/ml), the blank (RIPA buffer with proteinase inhibitors) and the samples were added, mixed with the reaction solution and incubated for 30 min at 37 °C. All probes were carried out in doublets. The absorbance was measured at 562 nm with the microplate reader BioTek Synergy HAT and analyzed with the Gen5 software.

2.2.2.3 DETECTION OF PHOSPHORYLATED SMAD 2/3 AFTER *IN VITRO* STIMULATION BY WESTERN BLOT

Protein content was measured by BCA-assay (2.2.2.2 Protein quantification) and 10 µg of total protein was loaded onto a 12% polyacrylamide gel. Proteins were separated by electrophoresis using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Proteins were then blotted onto a PVDF membrane (100 mA for 80 min with the semi dry system) and blocked with 5% BSA in Tris-buffered saline (20 mM Tris, pH 7.5, and 0.15% M NaCl) containing 0.1% Tween-20 (TBST) for 1 h at room temperature. Rabbit anti-Smad 2/3 and rabbit anti-phosphorylated Smad 2/3 were incubated overnight at 1:1000 in 5% BSA/TBST. Mouse anti-GAPDH (1:5000) in 5% milk/TBST was used as loading control. Donkey anti-rabbit IgG-HRP (1:2000 in 5% BSA/TBST) and goat anti-mouse IgG-HRP (1:5000 in 5% milk/TBST) respectively was used for detection of the primary Ab. Detection was performed using WesternBright Sirius (Advansta, Menlo Park, USA) on Fujifilm LAS 3000 analysis.

2.2.3 FLOW CYTOMETRIC ANALYSIS

Flow cytometry was utilized to determine expression of CD4, CD25, Foxp3, GITR, GARP, TGF-β R I, TGF-β R II and TGF-β R III. Cells were acquired in 100 µl of FACS buffer on a FACS-Canto and analyzed with FACSDiva software (both Becton Dickinson GmbH, Heidelberg, Germany).

Immunofluorescence staining of surface markers

Monoclonal (mc) mouse anti-CD4, anti-CD25 and anti-GITR were incubated for 15 min at room temperature. Mc mouse anti-CD3, anti-CD14, anti-CD19 and anti-CD56 were applied for 15 min at 4 °C. Conjugated mc mouse anti-GARP was incubated for 20 min at 4 °C, whereas unconjugated mc mouse anti-GARP was applied for 30 min at 4 °C, followed by 20 min of goat anti-m-APC at rt. Unconjugated mc mouse anti-TGF-β R I, TGF-β R II and TGF-β R III were applied for 20 min followed by 20 min of goat anti-m-APC. Cells were then incubated with 5% mouse serum for 15 min followed by extracellular staining of anti-CD25. Consecutive intracellular anti-Foxp3 staining was carried out as described below.

Immunoflourescence staining of intracellular markers

Intracellular mc rat anti-FOXP3 staining was carried out according to the manufacturer's instructions. Briefly, cells were first stained with extracellular markers and then fixed with Transcription Factor Staining Buffer Set for 30 min. Consecutive permeabilisation was carried out for 10 min with permeabilisation buffer + 2% rat serum. Anti-Foxp3 was then added and incubated at room temperature for 30 min.

2.2.4 RNA PREPARATION AND QUANTITATIVE REAL-TIME PCR

2.2.4.1 RNA PREPARATION

Total RNA was isolated from $CD4^+$ $CD25^-$ T cells after 3 days of culture with NucleoSpin RNA kit. First, cells were washed once with 1 ml ice cold PBS. After centrifugation PBS was removed carefully and the reaction tube with the cell pellet ("dry pellet") was shocked frozen in liquid nitrogen and then stored at -80°C. Total RNA was then isolated from the "dry pellet" according to the manufacturer´s instructions.

2.2.4.2 cDNA PREPARATION AND QUANTITATIVE REAL-TIME PCR

Complementary DNA (cDNA) synthesis was carried out with TaqMan reverse transcription reagents with random hexamers (Applied Biosystems, Darmstadt, Germany). 0.1 µg of total RNA was diluted with RNase-free water to 7.7 µl or 25 µl (depending of the concentration of the RNA). The reaction was set up as following and carried out in a thermocycler using the indicated program:

Temperature	Time
25 °C	10 min
48 °C	45 min
95 °C	5 sec
4 °C	hold

cDNA was amplified using TaqMan Gene Expression Master Mix and predesigned TaqMan Gene Expression Assays, according to the recommendations of the manufacturer´s instructions. Per reaction 9 µl of diluted cDNA, 1 µl of TaqMan Gene Expression Assay and 10 µl of TagMan Gene Expression Master Mix were set up. The PCR reaction was carried out in an Applied Biosystems 7300 System using the following protocol.

Temperature	Time
50 °C	2 min
95 °C	10 min
95 °C	15 sec
60 °C	1 min
Number of cycles	50

Expression levels of *FOXP3*, *SMAD3* and *SMAD7* were quantified with the given primers (see 2.1.7 Gene expression assays) and 18S was used as an endogenous reference. Each reaction was performed in duplicate. All assays were performed according to the manufacturer´s instructions. Relative quantification and calculation of the range of confidence was performed using the comparative CT method (Schmittgen & Livak, 2008).

2.2.5 QUANTIFICATION OF DEMETHYLATION AT THE TSDR

Methylation Specific-Real Time PCR (MS-RT PCR) was used to quantify demethylation at the TSDR on intron 1 of the *FOXP3* gene. Therefore, genomic DNA (gDNA) was isolated from $CD25^+$ $Foxp3^+$ T cells, bisulfite treated and then amplified with methylation (M) and demethylation (D) specific primers by RT-PCR. Plasmids, carrying the methylated- or demethylated-specific DNA sequence were used as controls for specificity and to create a standard curve. In a final step the amount of demethylation was calculated by making ratio of the copy number taking in account the amount of X chromosomes (2.2.5.5 Analysis).

2.2.5.1 GDNA ISOLATION

$CD4^+$ $CD25^+$ T cells magnetically isolated from AD patients and healthy controls were washed once with 1 ml ice cold PBS. After centrifugation PBS was removed carefully and the cell pellet was shock frozen in liquid nitrogen and then stored at – 80°C. Subsequently, gDNA was isolated using the DNeasy Blood & Tissue Kit according to the manufacturer´s instructions. Thereafter the amount of DNA was measured with the NanoDrop.

2.2.5.2 SODIUM BISULFITE TREATMENT

To measure the methylation status of the TSDR, isolated gDNA was treated with sodium bisulfite. Sodium bisulfite leads to conversion of unmethylated cytosine residues into uracil, whereas methylated cytosines are kept unchanged. Hence, treatment with bisulfite gives rise to different DNA sequences for methylated and unmethylated DNA. Treatment was done using the EpiTect® Bisulfite kit according to the manufacturer´s instructions. In short, the first step is the conversion of unmethylated cytosines by bisulfite. Next, the single-stranded DNA binds to the membrane of an EpiTect spin column. Thereafter, the membrane-bound DNA is desulfonated and eluted. For the bisulfite reaction the following components were pipetted into a 200 µl PCR tube:

Component	Volume per reaction (µl)
DNA solution (1 ng- 2 µg)	Variable (maximum 40µl)
RNase-free water	Variable
Bisulfite Mix	85
DNA protection Buffer	15
Total volume	140

After mixing the conversion was performed using a thermal cycler with the following program (taking approximately 5 hours).

Step	Time	Temperature
Denaturation	5 min	95 °C
Incubation	25 min	60 °C
Denaturation	5 min	95 °C
Incubation	85 min	60 °C
Denaturation	5 min	95 °C
Incubation	175 min	60 °C
Hold	Indefinite	20 °C

The clean-up of bisulfite converted DNA was done according to the protocol in small amounts of fragmented DNA.

2.2.5.3 PLASMIDS

Plasmids were purchased from Prof. Sophie Lucas (Institut de Duve, Brussels). One plasmid (p53.1A) carried the unmethylated sequence (human FOXP31l OPC1931-1932) in a pCR4-TOPP vector. The other plasmid carried the methylated sequence (human FOXP31l OPC1920-1921) in a pcDNA3.1 / V5-His-TOPO vector. After amplification and purification of the plasmids, they were digested with EcoR1 and purified with the NucleoSpin Gel and PCR Clean-up kit. The product was loaded on a 2% agarose gel to control the specificity regarding to its size.

To digest the plasmids, H_2O, NEB buffer 2, 10x BSA, the plasmid (DNA) and the digestion enzyme (EcoR1) was pipetted in this order (see table for the amounts) in a reaction tube and incubated for 2 hours at 37 °C at 400rpm.

Plasmid (2 µg)	Volume (µl)
Enzyme	1
BSA (10x)	5
NEB Buffer 2	5
H_2O	Fill up to get total volume
Total volume	40 or 50

2.2.5.4 METHYLATION SPECIFIC-REAL TIME PCR

Methylation specific-real time PCR (MS-RT-PCR) was carried out using methylated and unmethylated specific primers and probes for the TSDR. The sequence is given below. The underlined nucleotides correspond to LNA® modified bases (Eurogentec).

Form detected	Primer type	Sequence (5´to 3´)
Methylated TSDR	sense	CTCTTCTCTTCCTCCGTAATATCG
	antisense	GTTATTGACGTTATGGCGGTC
	FAM-TAMRA probe	AAACCCGACGCATCCGAC
Demethylated TSDR	sense	TCTACCCTCTTCTCTTCCTCCA
	antisense	GATTTTTTTGTTATTGATGTTATGGT
	FAM-TAMRA probe	AAACCCAACACATCCAACCA

20 ng of bisulfite treated gDNA was diluted with water to a total volume of 10 µl per reaction. The PCR reaction was carried out using methylated specific primers and probe and unmethylated specific primers and probe, respectively as indicated below.

Reagent	Volume for 1 reaction [µl]
300 nM sense primer	0.75
300 nM antisense primer	0.75
200 nM probe	0.5
TaqMan Gene Expression Master Mix	12
Total volume	14

Plasmids carrying the methylated or demethylated specific sequence were used for quantification and as a control for specificity. 10 µl of the plasmids with the following concentration were pipetted in 96 well plates.

Concentration	Copy number
$4.5 * 10^{-8}$ (M) / $8.5 * 10^{-8}$ (D)	$1 * 10^{1}$
$4.5 * 10^{-7}$ (M) / $8.5 * 10^{-7}$ (D)	$1 * 10^{2}$
$4.5 * 10^{-6}$ (M) / $8.5 * 10^{-6}$ (D)	$1 * 10^{3}$
$4.5 * 10^{-5}$ (M) / $8.5 * 10^{-5}$ (D)	$1 * 10^{4}$
$4.5 * 10^{-4}$ (M) / $8.5 * 10^{-4}$ (D)	$1 * 10^{5}$

The following PCR program was used:

Temperature	Time
50 °C	2 min
50 °C	10 min
50 °C	15 sec
50 °C	1 min
Number of cycles	60

2.2.5.5 ANALYSIS

The proportion of cells with demethylated TSDR was calculated as follows:

[number of demethylated TSDR sequences / (number of demethylated TSDR sequences + number of methylated TSDR sequences)].

To compensate x-chromosomal inactivation, the value of male donors was divided by 2.

2.2.6 STATISTICS

Statistical analysis was performed using Graph Pad Prism software 5.01. Student´s t-test was used to compare the data of patients with AD to those from healthy donors. Data are presented as mean ± SEM and $p = 0.05$ was considered statistically significant. The Welch test was used to compare the data of AD patients with the missense mutations A407T within the LRRC32 gene to those with the WT form. Those data are presented as mean ± confidence interval and $p = 0.05$ was considered statistically significant.

3 RESULTS

3.1 QUANTIFICATION OF $CD4^+$ T CELL SUBSETS IN PATIENTS WITH AD AND HEALTHY DONORS

There is evidence that Tregs play a role in AD pathogenesis and that their cell properties are altered. Therefore, the frequency of $CD4^+$ T cell subsets in the peripheral blood of AD patients and healthy controls was determined. To address this, the amount of $CD4^+$ $CD25^-$ $Foxp3^-$, $CD4^+$ $CD25^+$ $Foxp3^-$ and $CD4^+$ $CD25^+$ $Foxp3^+$ T cells in the peripheral blood of AD patients and healthy controls was measured by flow cytometry.

3.1.1 $CD4^+$ $CD25^+$ $FOXP3^+$ TREGS WERE INCREASED IN AD PATIENTS

The frequency of resting $CD4^+$ $CD25^-$ $Foxp3^-$ T cell (Figure 5 B) and $CD4^+$ $CD25^+$ $Foxp3^-$ Teff cells (Figure 5 C) were found to be almost the same in peripheral blood of AD patients and healthy donors. In contrast, a significant higher percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (Tregs) was observed in AD patients compared to healthy controls (Figure 5 A+D). The amount in peripheral blood of AD patients was more than double as high (2.1% in AD vs. 0.8% in healthy donors).

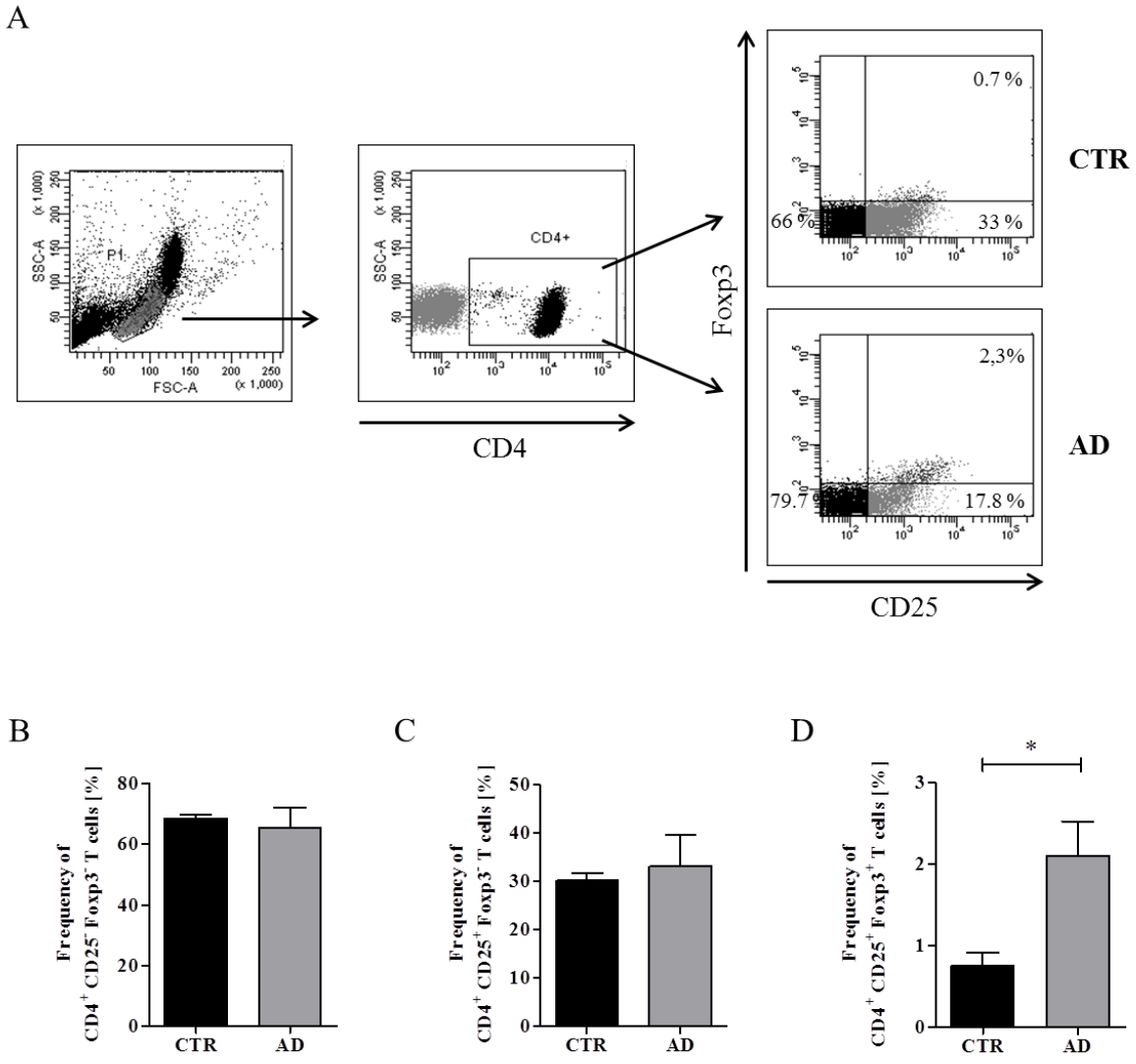

Figure 5: Quantification of different CD4$^+$ T cell subsets in AD and healthy donors.
The frequency of CD4$^+$ CD25$^-$ Foxp3$^-$, CD4$^+$ CD25$^+$ Foxp3$^-$ and CD4$^+$ CD25$^+$ Foxp3$^+$ T cells in the peripheral blood of AD patients and healthy donors were measured by flow cytometry after isolation of PBMC (n =6).
A: Representative Dot plot is shown.
B: Frequency of CD4$^+$ CD25$^-$ Foxp3$^-$ T cells in AD and healthy CTR [%].
C: Frequency of CD4$^+$ CD25$^+$ Foxp3$^-$ T cells in AD and healthy CTR [%].
D: Frequency of CD4$^+$ CD25$^+$ Foxp3$^+$ T cells in AD and healthy CTR [%] (p = 0.014).
Data are shown as mean ± SEM. Statistical significance was determined by t-test.

3.1.2 RNA EXPRESSION OF FOXP3 AND GARP IN PERIPHERAL BLOOD CD4$^+$ CD25$^+$ FOXP3$^+$ T CELLS

Flow cytometric measurements revealed that the frequency of CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs was increased in peripheral blood of AD patients in comparison to healthy donors. Next, expression level of Foxp3 and GARP RNA of CD4$^+$ CD25$^+$ isolated cells from peripheral blood was determined. No difference in the RNA level of Foxp3 and GARP was detected in

AD patients versus healthy donors, despite increased amounts of $CD4^+$ $CD25^+$ Tregs (Figure 6).

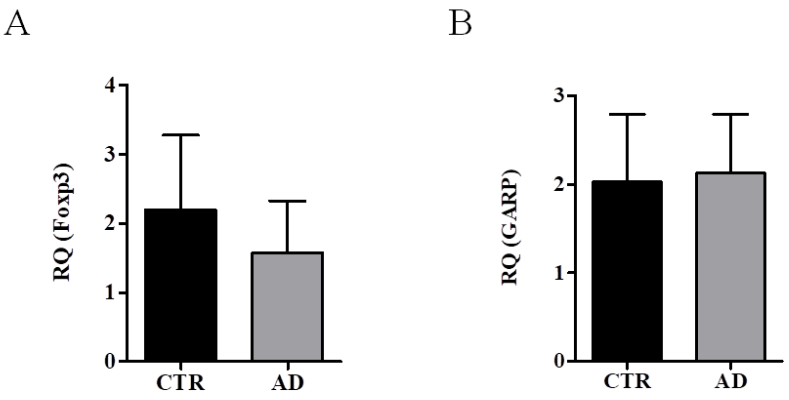

Figure 6: RNA expression of Foxp3 and GARP of $CD4^+$ $CD25^+$ T cells isolated from peripheral blood of AD patients and healthy donors.
RNA expression of Foxp3 and GARP of AD patients and healthy controls was measured by RT-PCR after isolation of $CD4^+$ $CD25^+$ T cells from PBMC.
A: RNA expression of Foxp3 (AD: n= 10; CTR: n= 9).
B: RNA expression of GARP (AD: n= 12; CTR: n= 9).
Data are shown as mean ± SEM. Statistical significance was determined by t-test.

3.1.3 QUANTIFICATION OF *BONA FIDE* TREGS BY MS-RT-PCR

To investigate the increase of $CD4^+$ $CD25^+$ cells in the peripheral blood of AD patients, the demethylation of TSDR in total circulating Tregs cells were quantified. M and D specific plasmids were used for generating a quantification curve and as a control for specificity (Figure 7 A). In AD patients close to 80% of peripheral $CD4^+$ $CD25^+$ T cells revealed a demethylated TSDR, whereas in healthy donors about 40% exhibited demethylated TSDR (Figure 7 B). Thus, within $CD4^+$ $CD25^+$ T cells more cells were *bona fide* nTregs in patients with AD in comparison to control donors. In order to determinate whether conversion of circulating $CD4^+$ $CD25^-$ cells to iTregs cells was altered, *in vitro* generation of iTregs was analyzed next.

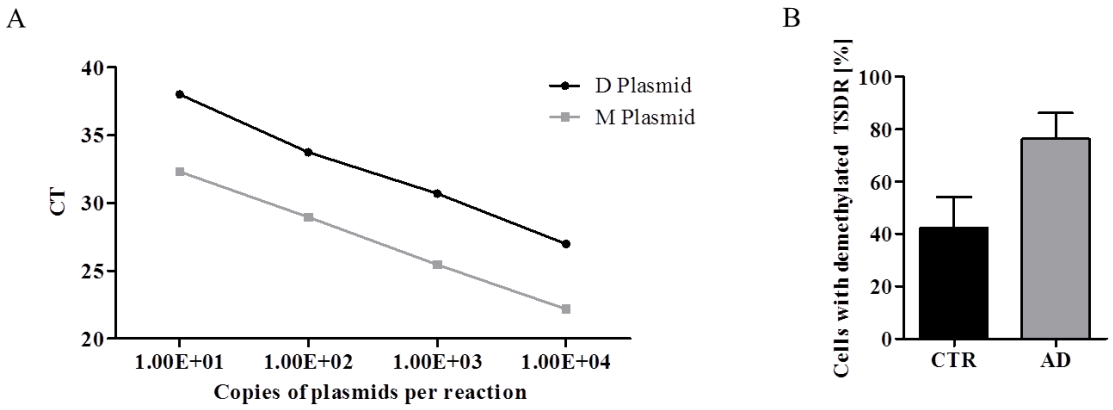

Figure 7: Quantification of demethylated TSDR in CD4$^+$ CD25$^+$ T cells of AD patients and healthy donors.
Demethylated TSDR was quantified using MS-specific RT-PCR.
A: Specificity of M and D primers on cloned DNA fragments
B: Frequency of demethylated TSDR in AD and CTR [%] (AD: n = 4; WT: n = 5; p = 0.0699).
Data are shown as mean ± SEM. Statistical significance was determined by t-test.

3.2 CONVERSION OF CD4$^+$ CD25$^-$ T CELLS INTO iTREGS

After it was revealed that the numbers of circulating naïve CD4$^+$ CD25$^-$ T cells versus CD4$^+$ CD25$^+$ Teff cells were not varied in the blood of AD patients, the ability of CD4$^+$ CD25$^-$ T cells to polarize towards a regulatory phenotype (CD4$^+$ CD25$^+$ Foxp3$^+$) was analyzed.

3.2.1 CONVERSION OF CD4$^+$ CD25$^-$ T CELLS INTO CD4$^+$ CD25$^+$ FOXP3$^+$ iTREGS WAS DIMINISHED IN AD

The *in vitro* conversion of peripheral blood CD4$^+$ CD25$^-$ T cells into CD4$^+$ CD25$^+$ Foxp3$^+$ iTregs without antigen-specific stimulation was determined after 3 days of culture. It was observed that more cells maintained the CD25$^-$ Foxp3$^-$ phenotype in AD patients (Figure 8). According to these data, the percentage of iTregs (CD25$^+$ Foxp3$^+$ T cells) was found to be lower in AD-derived T cells in comparison to T cells from healthy donors (Figure 8). Besides, the data revealed that the frequency of CD4$^+$ CD25$^+$ Foxp3$^-$ T cells was equal in AD and healthy subjects (Figure 8).

Cell population	Healthy control [%]	Atopic dermatitis [%]	p-value
CD25$^-$ FOXP3$^-$	61.3 ± 5.0	70.7 ± 4.1	0.16
CD25$^+$ FOXP3$^-$	17.7 ± 3.6	17.7 ± 3.4	0.91
CD25$^+$ FOXP3$^+$	14.6 ± 2.0	8.5 ± 1.7	**0.03**

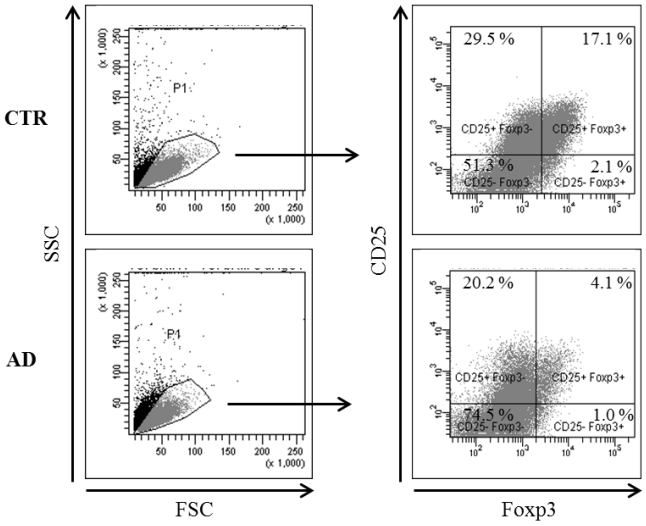

Figure 8: Conversion of CD4$^+$ CD25$^-$ T cells into iTregs was reduced in AD.
CD4$^+$ CD25$^-$ T cells were cultured for up to 3 days on CD3-coated plates in the presence of IL-2 and TGF-β and analyzed by flow cytometry. Frequency of CD25$^-$ Foxp3$^-$, CD25$^+$ Foxp3$^-$ and CD25$^+$ Foxp3$^+$ cell subsets after 3 days of culture is shown (n = 12 / 10 (AD / CTR)). One representative dot plot from CTR and AD is shown on the right sight.
Data are shown as mean ± SEM. Statistical significance was determined by t-test.

3.2.2 THE EXPRESSIONS OF GITR WAS REDUCED IN CD4$^+$ T CELLS OF AD PATIENTS

To further confirm the observation that less CD25$^+$ Foxp3$^+$ iTregs were induced in cells from AD patients, surface GITR was measured on CD4$^+$ T cells after *in vitro* stimulation. This marker is constitutively expressed on Tregs. It was found that extracellular expression of GITR was dramatically reduced in AD patients. Whereas about 30% of CD4$^+$ T cells revealed GITR on the cell surface, the frequency was reduced to a third in AD patients (Figure 9).

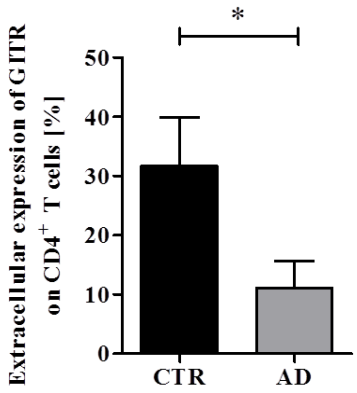

Figure 9: Extracellular expression of GITR was reduced in cells from AD patients.
$CD4^+$ $CD25^-$ T cells were cultured for 4 days on CD3-coated plates in the presence of 100 U/ml IL-2 and 2ng/ml TGF-β and analyzed by flow cytometry (AD: 11.1%; CTR: 31.7%; n = 9; p = 0.044).
Data are shown as mean ± SEM. Statistical significance was determined by t-test.

3.2.3 GARP EXPRESSION WAS REDUCED IN AD-DERIVED iTREGS

In order to detect the activation level of iTregs, the extracellular expression of GARP was measured on $CD4^+$ T cells of AD and healthy control subjects after stimulation. The results showed that T cells from AD patients displayed significantly lower GARP surface protein levels after 16 h of stimulation with anti-CD3, TGF-β and IL-2 (Figure 10). Only half as many cells expressed the marker on the cell surface in AD-$CD4^+$ T cells in comparison to $CD4^+$ T cells of healthy donors. Therefore, AD-T cells were less able to be reprogrammed towards an activated Treg-$GARP^+$ phenotype comparing to healthy-T cells.

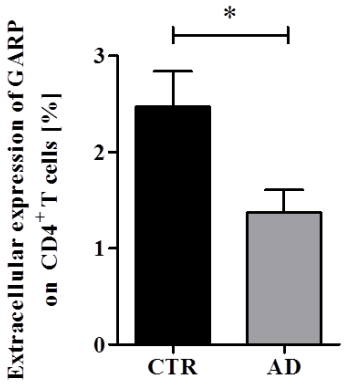

Figure 10: Extracellular expression of GARP after stimulation of $CD4^+$ $CD25^-$ T cells.
Expression of GARP on $CD4^+$ T cells measured by flow cytometry after 16 h of culture is shown (n = 13 / 10 (AD / CTR); p = 0.02).
Data are shown as mean ± SEM. Statistical significance was determined by t-test.

3.2.4 THE INFLUENCE OF IL-4 ON *IN VITRO* CONVERSION OF $CD4^+$ $CD25^-$ T CELLS INTO iTREGS

In order to exclude that the reduction of *in vitro* iTreg conversion in AD might occur due to the presence of IL-4 secreting Th2 cells within the $CD4^+$ $CD25^-$ population, $CD4^+$ $CD25^-$ T cells from AD patients and healthy donors were treated with or without neutralizing anti-IL-4 antibody in the presence of anti-CD3 antibody, IL-2 and TGF-β.

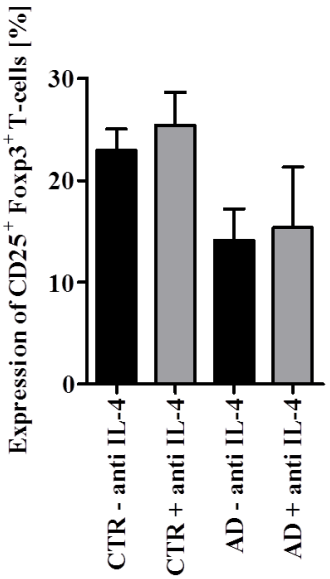

Figure 11: Treatment with anti-IL-4 increased the conversion of $CD4^+$ $CD25^-$ T cells into $CD25^+$ $Foxp3^+$ iTregs slightly.
$CD4^+$ $CD25^-$ T cells were cultured for 3 days on anti-CD3-coated plates in the presence of IL-2, TGF-β and with (+) or without (-) anti-IL-4 (10 µg/ml). Cells were analyzed by flow cytometry on day 3 of culture (CTR: n = 4; AD: n = 3).
Data are shown as mean ± SEM. Statistical significance was determined by t-test.

The conversion into $CD25^+$ $Foxp3^+$ iTregs was increased slightly in the presence of anti-IL-4 in AD patients and healthy donors (Figure 11). However, the polarization in cells from AD patients towards iTregs was not increased up to the level of healthy donors. This indicated that the amount of IL-4-secreting Th2 cells was not significantly higher in the T cell population of AD patients. Furthermore, this data set illustrated the antagonistic effect of IL-4 and TGF-β. In the presence of neutralizing anti-IL-4 TGF-β signaling was not longer diminished by the Th2 cytokine and hence, iTreg conversion increased.

3.3 ANALYSIS OF TGF-β SIGNALING IN $CD4^+$ T CELL SUBSETS OF AD PATIENTS AND HEALTHY DONORS

After observing that $CD4^+$ $CD25^-$ $Foxp3^-$ T cells conversion into $CD4^+$ $CD25^+$ $Foxp3^+$ iTregs was reduced in cells obtained from AD patients, and considering that induction of Tregs depends on TGF-β stimulation, the question arose whether TGF-β signaling pathway was altered in AD patients.

3.3.1 TGF-β RS WERE DIFFERENTLY EXPRESSED IN AD ON $CD4^+$ T CELLS BEFORE AND AFTER STIMULATION

First, the surface expression of TGF-β R I-III on freshly isolated $CD4^+$ $CD25^-$ T cells was examined by flow cytometry. Protein expression of TGF-β R II was found to be the highest, whereas expression of TGF-β R I and TGF-β R III was faint (Figure 12A). No differences in the protein expression of TGF-β R I-III between $CD4^+$ $CD25^-$ T cells obtained from blood of AD patients in comparison to cells from healthy controls were observe (Figure 12A).

Next, the surface expression of TGF-β R I-III after 3 days of culture was measured. It was distinguished between two cell populations: $CD25^+$ $Foxp3^-$ T cells, representing activated T cells and $CD25^+$ $Foxp3^+$ T cells, representing iTregs. Interestingly, surface TGF-β R II expression was significantly up-regulated in cells derived from AD patients in comparison to healthy controls in both cell populations (Figure 12 B and C). Besides, TGF-β R III was found to be up-regulated in AD-$CD25^+$ $Foxp3^-$ T cells (Figure 12 B). However, the expression of TGF-β R I was similar between AD and healthy donors. Elevated expression of TGF-β R II and TGF-β R III in AD-$CD25^+$ $Foxp3^-$ was revealed only after 3 days of culture. Representative dot blots (from data of B and C) of TGF-β R I-III expression on $CD25^+$ T cells after 3 days of culture are shown in Figure 12 D.

Thus, it was revealed that surface TGF-β R expression is partially altered in activated T cells ($CD25^+$ $Foxp3^-$) and iTregs ($CD25^+$ $Foxp3^+$).

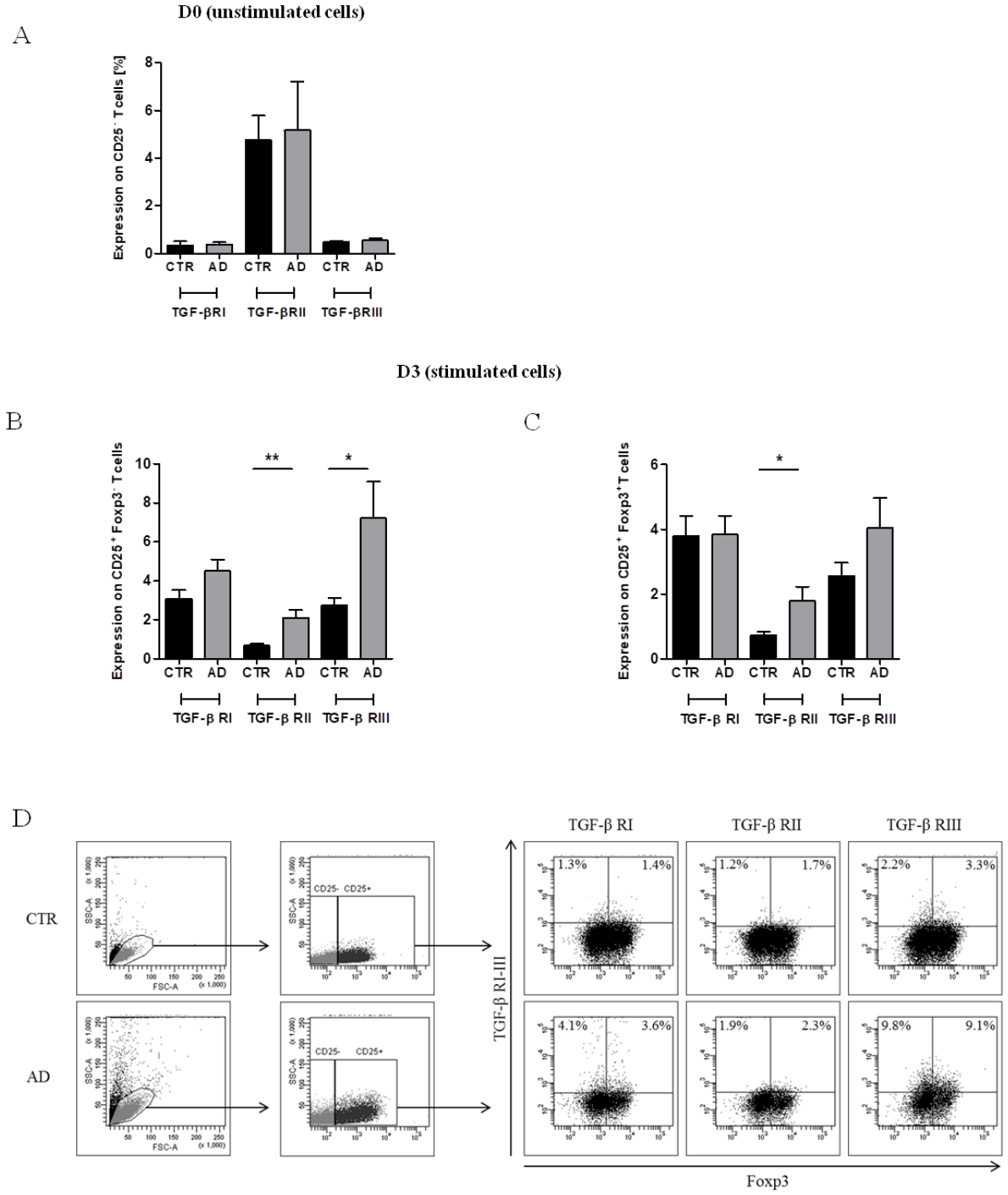

Figure 12: TGF-β Rs were differently expressed in AD on CD4⁺ T cells before and after stimulation.
A: Protein surface expression of TGF-β R I-III measured on CD25⁻ cells directly after isolation.
B: CD4⁺ CD25⁻ T cells were cultured on CD3-coated plates in the presence of IL-2 and TGF-β for 3 days. Expression of TGF-β R I-III on CD25⁺ Foxp3⁻ cells measured with flow cytometry is shown (TGF-β R II: $p = 0.0013$; $n = 17$; TGF-β R III: $p = 0.021$; $n = 16/17$).
C: CD4⁺ CD25⁻ T cells were cultured on CD3-coated plates in the presence of IL-2 and TGF-β for 3 days. Expression of TGF-β R I-III on CD25⁺ Foxp3⁺ cells measured with flow cytometry is shown. (TGF-β R II: $p = 0.023$; $n = 16/17$).
D: Representative dot blots (from data of C and D) of TGF-β R I-III expression on CD25⁺ T cells after 3 days of culture are shown.
Data are shown as mean ± SEM. Statistical significance was determined by t-test.

3.3.2 COMPARISON OF TGF-β R I-III EXPRESSION ON $CD25^+$ $FOXP3^-$ AND $CD25^+$ $FOXP3^+$ T CELLS

Next, TGF-β R I-III surface expression on activated T cells ($CD25^+$ $Foxp3^-$) was compared with that on iTregs ($CD25^+$ $Foxp3^+$). It was revealed that there was no difference in the expression level between activated T cells and iTregs within the CTR group. In AD patients less extracellular expression of TGF-β R I-III in $CD25^+$ $Foxp3^+$ iTregs in comparison to $CD25^+$ $Foxp3^-$ Teff cells was observed (Figure 13). However, the difference in the expression level was not statistically significant according to Students t-test.

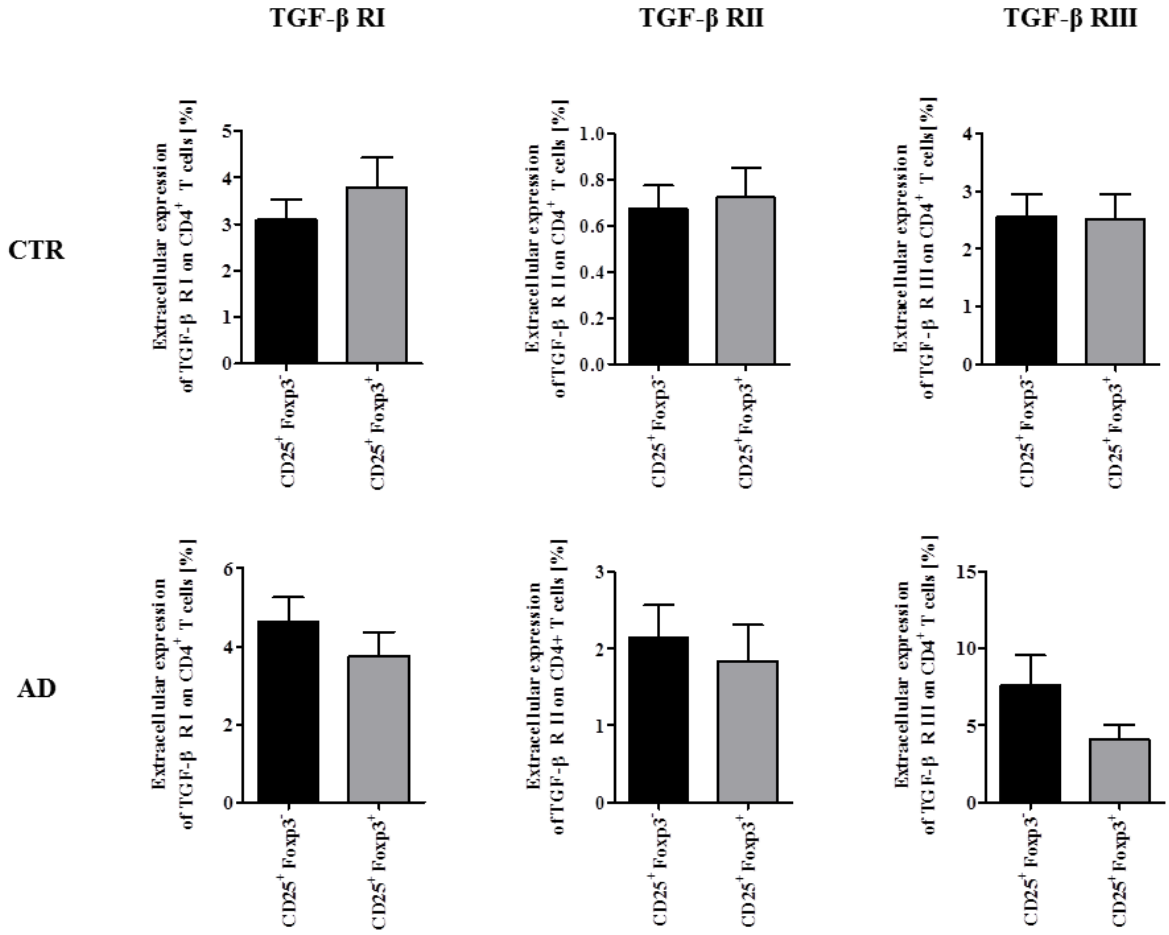

Figure 13: TGF-β R I-III expression on $CD25^+$ $Foxp3^-$ and $CD25^+$ $Foxp3^+$ T cells within AD patients or healthy donors.
TGF-β RI-III expressions on $CD25^+$ $Foxp3^-$ and $CD25^+$ $Foxp3^+$ T cells within AD patients or healthy donors were measured on D3 of culture after stimulation of $CD4^+$ $CD25^-$ T cells with anti-CD3, IL-2 and TGF-β (AD: n = 16; WT: n = 17).
Data are shown as mean ± SEM. Statistical significance was determined by t-test.

3.3.3 EXPRESSION OF ACTIVATING AND INHIBITORY SMAD MOLECULES IN $CD4^+$ $CD25^-$ T CELLS

After analyzing the surface expression of TGF-β R in Tregs cells derived from AD patients and healthy controls individuals, the next question was focused on TGF-β-downstream signaling molecules. The constitutive and induced TGF-β expression of activating Smad 3 and inhibitory Smad 7 was determined at transcriptional and protein level.

The *SMAD3* mRNA expression level was determined in directly isolated $CD4^+$ $CD25^-$ T cells. A significant reduction in cells from AD patients in comparison to healthy controls was revealed (Figure 14 A). Likewise, the protein amount of Smad 3 was detected by Western Blot, but no differences between AD and healthy $CD4^+$ $CD25^-$ T cells were found. Interestingly, also mRNA expression of the inhibitory *SMAD7* was reduced in $CD4^+$ $CD25^-$ T cells in AD patients compared to healthy donors (Figure 14 B).

Thereafter, phosphorylation of Smad 2/3 was investigated in $CD4^+$ $CD25^-$ T cells of AD patients and healthy control after starving conditions (0.25% FCS medium, 16 h), followed by 1 h of TGF-β stimulation. The data showed that AD-$CD4^+$ $CD25^-$ T cells displayed lower Smad 2/3 phosphorylation levels compared to healthy donors cells, although the difference was not significant ($p = 0.13$) (Figure 14 C+D). Therefore, these results suggest that TGF-β signaling is altered in $CD4^+$ $CD25^-$ T cells from AD patients in comparison to healthy controls.

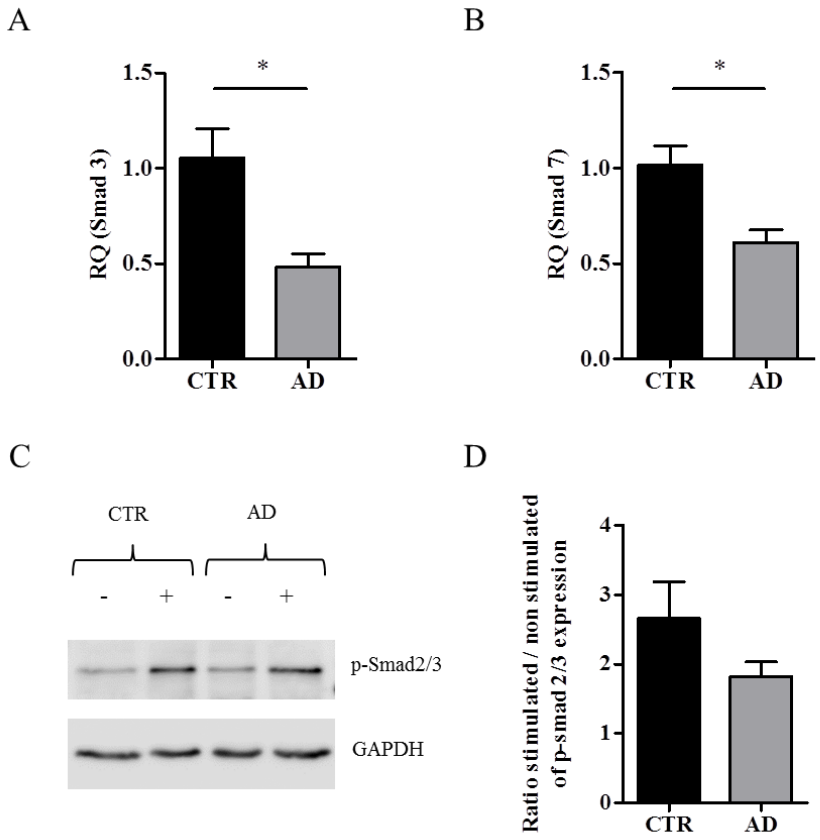

Figure 14: TGF-β signaling was impaired in AD.
A: RNA expression of *SMAD 3* in $CD4^+$ $CD25^-$ T cells of AD patients in comparison to healthy controls after isolation is shown (AD: 0.48; CTR: 1.05; n = 6 / 5; p = 0.0059).
B: RNA expression of *SMAD 7* in $CD4^+$ $CD25^-$ T cells of AD patients in comparison to healthy controls after isolation is shown (AD: 0.61; CTR: 1.02; n = 6 / 5; p = 0.0065).
C: Phosphorylated (p-) Smad 2/3 was detected with Western Blot. $CD4^+$ $CD25^-$ T cells of AD patients and healthy controls were isolated from PBMC and cultured overnight (16 h) in RPMI media containing 0.25% FCS to starve the cells. On the next day cells were stimulated with 5 ng/ml TGF-β for 1 h, lysed in RIPA buffer and immunoblotted. One representative experiment out of 10 is shown.
D: The ratio of p-Smad 2/3 expression of stimulated and non-stimulated cells evaluated by Western Blot (see C) is shown (AD: 1.8-fold increase; CTR: 2.4-fold increase; n = 10; p = 0.3).
Data are shown as mean ± SEM. Statistical significance was determined by t-test.

3.4 iTREGS FROM AD PATIENTS WERE FUNCTIONALLY NOT IMPAIRED

Tregs are considered anti-inflammatory cells that, amongst other, suppress proliferation of Teff cells. It was observed that conversion into iTregs was diminished in cells derived from AD, that TGF-β R expression on $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^+$ T cells was altered and TGF-β signaling was modified. Subsequently it was tested whether iTregs ($CD4^+$ $CD25^+$) arising from $CD4^+$ $CD25^-$ T cells had the same inhibiting qualities in AD patients and in healthy donors. For this purpose $CD4^+$ $CD25^+$ T cells were purified after 3 days of culture and co-cultured in different ratios with autologous $CD4^+$ $CD25^-$ T responder (Tresp) cells in the

presence of unspecific polyclonal stimulation for 3 days. Both, iTregs from AD patients and healthy controls were able to inhibit proliferation of Tresp, increasing with the ratio of Tresp to Tregs. No difference in the function of iTregs derived from AD patients was observed in comparison to healthy individuals (Figure 15).

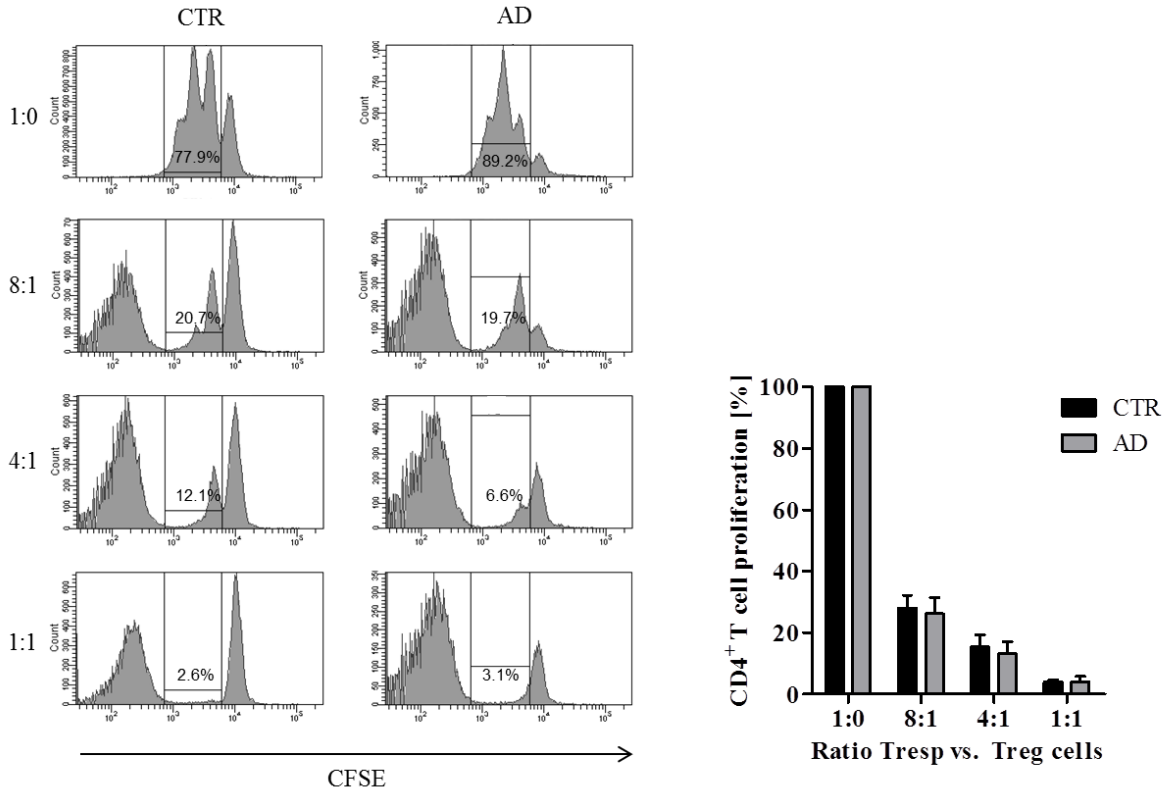

Figure 15: Functionality of iTregs derived from AD was not reduced.
Peripheral $CD4^+$ $CD25^-$ T cells were cultured on CD3-coated plates in the presence of IL-2 and TGF-β. Induced $CD25^+$ cells were purified with anti-CD25-coated beads on day 3 and co-cultured for 3 days with autologous CFSE stained $CD25^-$ T responder cells at ratios. Representative histograms are shown on the left. T cell proliferation of $CD4^+$ T cells is shown on the right. Data are shown as mean ± SEM (n = 6/7 (AD/CTR)). Statistical significance was determined by t-test.

3.5 CHARACTERIZATION OF T CELLS FROM AD PATIENTS WITH A MUTATION IN THE *LRRC32* GENE

In a former study the low-frequency and rare missense mutations A407T within the *LRRC32* gene encoding the protein GARP has been identified as a susceptibility locus for AD and other inflammatory diseases (Manz et al., 2016). To enlighten the impact of GARP in Treg function in the context of AD in more detail, cells derived from AD patients with either the mutant A407T or the WT variant were characterized and functional assays were carried out. Parts of this section has been published in (Manz et al., 2016).

3.5.1 SURFACE GARP EXPRESSION WAS ALTERED ON DIFFERENT IMMUNE CELLS

First of all, PBMC from AD patients with the A407T and the WT variant were isolated and the surface expression of GARP was measured on different immune cells. It was discovered that GARP expression was significantly down regulated on B cells ($CD19^+$), natural killer (NK) cells ($CD56^+$) and pan-T cells ($CD3^+$) from carriers with the mutant A407T form. No difference was observed in PBMC and monocytes. Furthermore, $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^+$ T cells revealed significant less surface GARP expression in those donors (Figure 16). The reduction was most profound in $CD4^+$ $CD25^+$ T cells. Whereas in wild type carriers 2.6% of $CD4^+$ $CD25^+$ T cells expressed GARP on the cell surface, donors with the A407T mutation only revealed 0.5% of GARP positive $CD4^+$ $CD25^+$ T cells.

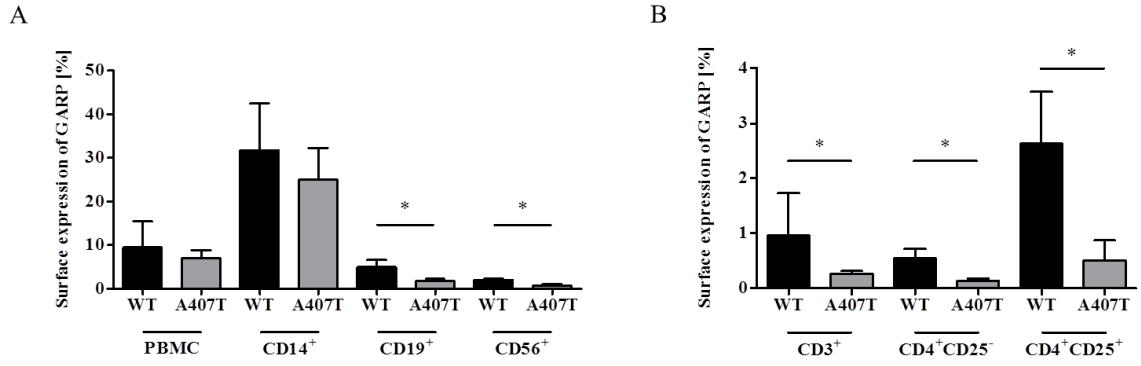

Figure 16: GARP cell surface expression on different immune cells from AD patients carrying either the WT or the A407T form of *LRRC32*.
GARP cell surface expression on different immune cells and T cell subtypes from AD patients with either the WT form of *LRRC32* (n=7) or the A407T variant (n=6). Bars indicate the percentage (%) of GARP positive cells on
A: PBMC, monocytes ($CD14^+$), B cells ($CD19^+$), NK cells ($CD56^+$) and
B: Pan-T cells ($CD3^+$), $CD4^+$ $CD25^-$ T cells, $CD4^+$ $CD25^+$ T cells.
Mean values + 95% confidence intervals are presented. Statistical significance was determined by Welch-test.

3.5.2 GARP EXPRESSION WAS REDUCED AFTER ACTIVATION OF $CD4^+$ $CD25^-$ T CELLS

Next, the surface GARP expression on activated iTregs was determined. Therefore, $CD4^+$ $CD25^-$ T cells were stimulated with anti-CD3, anti-CD28, IL-2 and TGF-β for up to 3 days and the surface expression on cells from AD patients with the A407 variant and the WT variant was measured after one (Figure 17 A) and three days (Figure 17 B) of culture. It was observed that surface GARP expression was significantly reduced on $CD25^+$ $FOXP3^+$ T cells (iTregs) from donors with the mutant variant at both time points (Figure 17). As expected,

surface GARP expression was the highest after 16 h of stimulation and was subsequently down regulated to half of the amount on D3 of culture.

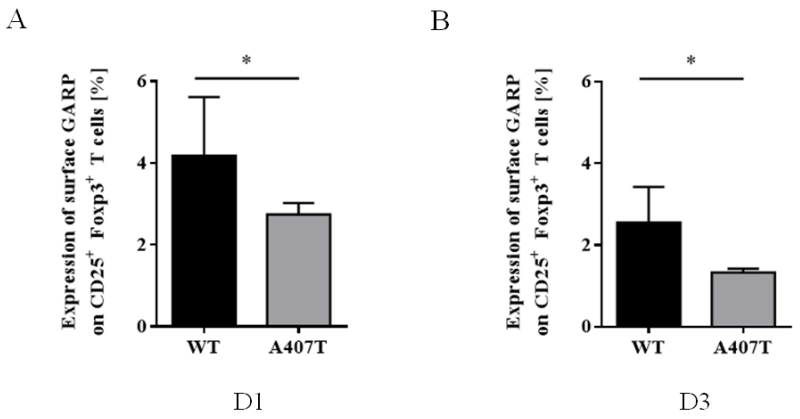

Figure 17: GARP cell surface expression on CD25$^+$ FOXP3$^+$ T cells from AD patients after stimulation of the TCR and TGF-β.
GARP cell surface expression on CD25$^+$ FOXP3$^+$ T cells after stimulation of CD4$^+$ CD25$^-$ T-cells from AD patients with anti-CD3, anti-CD28, IL-2 and TGF-β.
A: after 1 day of culture (16 h) (WT: n= 5; A407T: n=5; p = 0.024)
B: after 3 days of culture. (WT: n=6; A407T: n=4; p = 0.017)
Mean values + 95% confidence intervals are presented. Statistical significance was determined by Welch-test.

3.5.3 STIMULATION OF CD4$^+$ CD25$^-$ T CELLS FROM A407T- AND WT-CARRIERS WITH TGF-β

To identify if the mutant A407T variant in the *LLRC32* gene has an effect of *in vitro* conversion into iTregs, the ability of CD4$^+$ CD25$^-$ T cells derived from donors with the A407T or the WT variant to polarize towards CD25$^+$ Foxp3$^+$ T cells was analyzed. Already one day (16 h) after the stimulation a diminished induction into CD25$^+$ Foxp3$^+$ T cells (iTregs) was observed (p = 0.09) (Figure 18 A). On D3 of culture the amount of CD25$^+$ Foxp3$^+$ T cells was increased, but lower numbers were observed in the group with the mutant form of GARP (Figure 18 B) (p = 0.2). Hence, there is strong indication that the heterozygote mutation of *LLRC32* leads to an impaired conversion into iTregs.

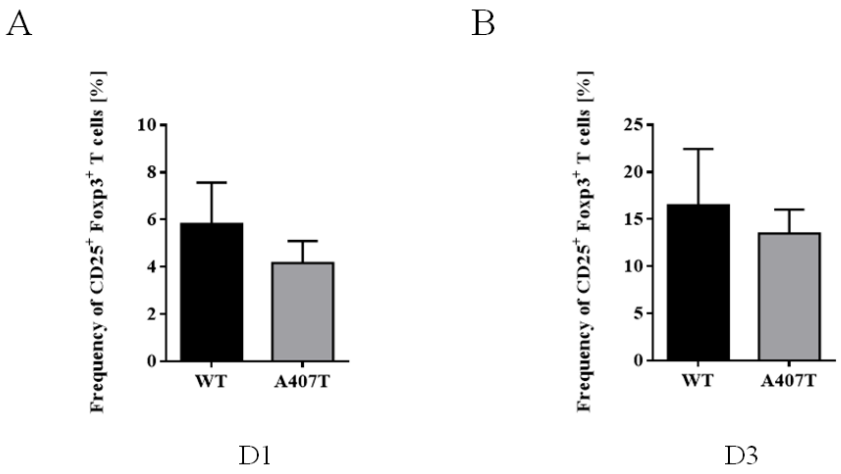

Figure 18: Conversion of CD4$^+$ CD25$^-$ T cells into iTregs in cells derived from patients with the A407T mutant variant of *LLRC32* in comparison to patients with the WT form after stimulation with TGF-β.
Frequency of CD25$^+$ FOXP3$^+$ T cells was determined after stimulation of CD4$^+$ CD25$^-$ T-cells from AD patients with anti-CD3, anti-CD28, IL-2 and TGF-β for up to 3 days.
A: after 1 day of culture (16 h) (WT: n=6; A407T: n=5; p =0.09).
B: after 3 days of culture (WT: n=6; A407T: n=5; p = 0.2).
Mean values + 95% confidence intervals are presented. Statistical significance was determined by Welch-test.

3.5.4 STIMULATION OF CD4$^+$ CD25$^-$ T CELLS FROM A407T- AND WT-CARRIERS WITH SGARP

CD4$^+$ CD25$^-$ T cells stimulated with sGARP are able to polarize towards an iTreg phenotype (Hahn et al., 2013). To understand more of the regulatory function of GARP in the conversion into iTregs we stimulated CD4$^+$ CD25$^-$ T cells with anti-CD3, anti-CD28, IL-2 and sGARP respectively, for up to 3 days and measured the frequency of CD25$^+$ Foxp3$^+$ T cells after one and three days of culture. Fewer cells of patients with the A407T variant converted into CD25$^+$ Foxp3$^+$ iTregs, when stimulated with sGARP (along with anti-CD3, anti-CD28, IL-2) in comparison to donors with the WT form (Figure 19). This effect was already seen after 1 day of culture (Figure 19 A), but was more profound after 3 days of culture (Figure 19 B).

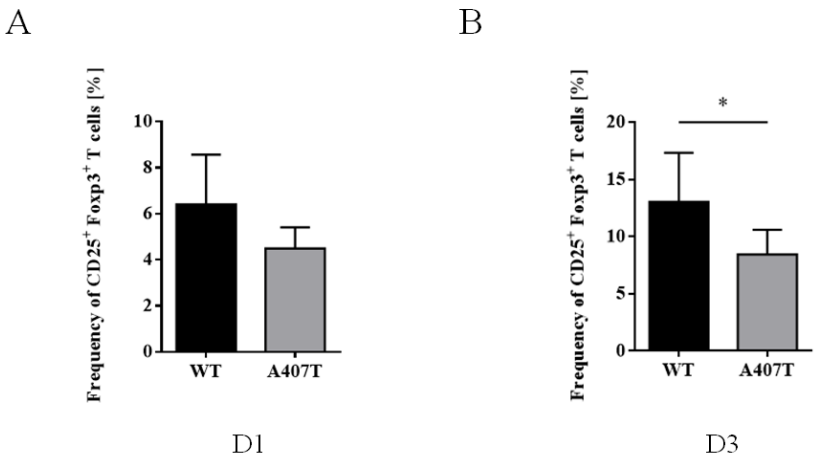

Figure 19: Conversion of CD4$^+$ CD25$^-$ T cells into iTregs in cells derived from patients with the A407T mutant variant of *LLRC32* in comparison to patients with the WT form after stimulation with sGARP.
CD4$^+$ CD25$^-$ T cells were stimulated with anti-CD3, anti-CD28, IL-2 and sGARP for 3 days.
A: Frequency of CD25$^+$ FOXP3$^+$ T cells after 1 day of culture (16 h) (WT: n=6; A407T: n=5; p =0.081).
B: Frequency of CD25$^+$ FOXP3$^+$ T cells after 3 days of culture (WT: n=6; A407T: n=5; p = 0.024).
Mean values + 95% confidence intervals are presented. Statistical significance was determined by Welch-test.

No profound difference was seen in the level of conversion when comparing stimulation with TGF-β and sGARP after 1 day of culture (Figure 19 A and Figure 18 A). However, the frequency of CD25$^+$ FOXP3$^+$ T cells was lower when stimulated with sGARP after 3 days of culture (Figure 19 B and Figure 18 B). Stimulating cells derived from donors with the A407T mutant form with sGARP did not restore the amount of conversion to the level of WT-cells.

3.6 TREGS OBTAINED FROM THE BLOOD OF A407T CARRIERS WERE FUNCTIONALLY NOT IMPAIRED

In order to evaluate whether CD4$^+$ CD25$^+$ Tregs isolated from peripheral blood from AD patients with the A407T variant were functionally impaired in comparison to cells from AD patients with the WT form, the inhibiting quality was tested. Therefore, CD4$^+$ CD25$^+$ Tregs from AD patients with the A407T variant and the WT form were isolated and subsequently co-cultured in different ratios with autologous CD4$^+$ CD25$^-$ T responder cells in the presence of unspecific polyclonal stimulation for 3 days. No significant change in the inhibiting qualities of CD4$^+$ CD25$^+$ T cells was observed (Figure 20).

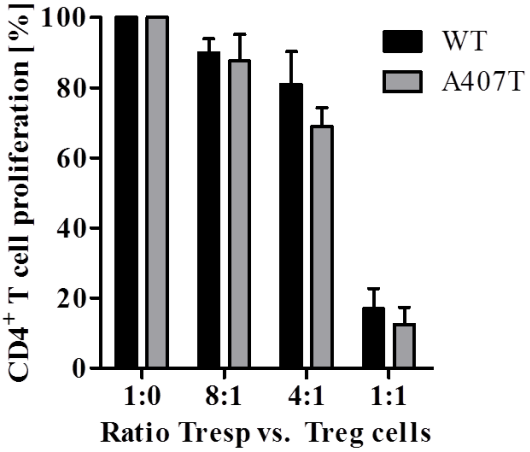

Figure 20: Functionality of CD4$^+$ CD25$^+$ Tregs derived from AD with A407T variant was not reduced. Peripheral CD4$^+$ CD25$^+$ T cells were isolated and co-cultured for 3 days with autologous CFSE stained CD25$^-$ T responder cells at ratios. T cell proliferation of CD4$^+$ T cells is shown in %. Data are shown as mean ± CI (n = 3/5 (A407/WT)). Statistical significance was determined by Welch-test.

4 DISCUSSION

4.1 TREGS IN AD

AD is one of the most common chronic inflammatory skin diseases in clinical practice. It has been suggested that Tregs play an important role in the pathogenesis of this disease. Even though Tregs in AD have been investigated intensively the contribution of these anti-inflammatory cells in the pathogenesis is still not resolved and many questions remain open, especially in the chronic phase of the disease. The aim of this thesis was to characterize Tregs in the context of AD and thereby to enlighten their role. Several alterations in Treg number, development and function were revealed and strengthened the assumption of Tregs playing an important role in AD pathogenesis.

4.1.1 THE NUMBER OF TREGS IN PERIPHERAL BLOOD IS INCREASED IN AD PATIENTS

Interestingly, the number of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs was found to be increased in peripheral blood of AD patients compared to healthy donors. The frequency of Tregs in peripheral blood of AD patients has been addressed for years with conflictive results. Some studies indicate a higher number of Tregs in AD or allergic rhinitis in accordance to the presented data (Ou *et al.*, 2004; Vukmanovic-Stejic *et al.*, 2005; Reefer *et al.*, 2008; Lesiak *et al.*, 2012; Roesner *et al.*, 2015). Others observed less Tregs in AD (Stelmaszczyk-Emmel et al., 2013) or allergic rhinitis (Lee, Yu, & Wang, 2007; Xu et al., 2007). Even more, one group did not find a change in Treg numbers (Bellinghausen et al., 2003). Hence, the data presented here are in line with the majority of preceding studies. The reason for the discrepancy in the investigations might be due to variances in the clinical form of the disease, the age of patients or the way Tregs were characterized (Stelmaszczyk-Emmel et al., 2013). Furthermore, differences in the disease stage, the genetic background and treatments the patients underwent might be convincing explanations for the discrepancy.

However, the fact that the amount of Tregs is increased in the blood of AD patients is surprising as diminished numbers of Tregs would explain the overreacting immune system more easily. One assumption might be that Tregs are less functional. This presumption is

supported by the finding that Foxp3 and GARP RNA expression level was equal in purified $CD4^+$ $CD25^+$of AD patients and healthy donors despite a higher number of Tregs in the peripheral blood of AD patients. Both Treg markers play a pivotal role in their inhibiting function and a decrease might reflect a functional deficit (Probst-Kepper & Buer, 2010). The functional properties of Tregs in AD will be discussed in more detail below.

Another explanation for the increased number of Tregs in the peripheral blood may be offered by the theory of "parallel amplification" whereby expansion of Teff cells is counter-regulated by an expansion of Tregs (Agrawal, Wisniewski and Woodfolk, 2011). In fact, due to the inflammatory background, the amount of activated T cells is expected to be higher in AD patients than in healthy controls (Akdis, Akdis, Weigl, Disch, & Blaser, 1997). It is possible that Teff cells and Tregs are specific for the same antigen. However, the amount of $CD4^+$ $CD25^+$ $Foxp3^-$ Teff cells was found to be equal in the blood of AD patients and healthy donors and does not support this theory.

It is a well-known challenge to distinguish between activated T cells and Tregs, or even more between nTregs and iTregs. One reliable method to identify *bona fide* nTregs is based on the methylation pattern of the TSDR. Only nTregs reveal a fully demethylated TSDR. Here, an increased number of nTregs was found in the peripheral blood of AD patients. This is supporting the FACS data, showing expanded numbers of $CD25^+$ $Foxp3^+$ T cells in the blood. Roesner *et al*. showed recently, that the number of Tregs was increased in peripheral blood of adult AD patients, utilizing quantification of demethylated TSDR (Roesner et al., 2015). So far, this was the only study found in the literature, using this method in AD. Thus, the data provided here are within the first data sets achieved by a reliable method and are therefore a valuable addition to the current field. However, investigating demethylation of the TSDR does not allow a conclusion about iTreg frequency. Hence, it could be that iTreg frequency is diminished in the peripheral blood of AD patients, despite increased amounts of nTregs. In fact, malfunctioning *in vitro* conversion into iTregs was revealed in AD patients and will be discussed below.

It can be suggested that increased amounts of Tregs are not an AD-specific feature, but a bystander effect without major influence on the development of the disease. Interestingly, a higher frequency of $CD25^+$ T cells was observed in patients with the chronic inflammatory skin disease psoriasis in the context of this thesis. Increased amounts of Tregs have been reported for other chronic diseases, including mouse models of asthma (M A Curotto de Lafaille et al., 2008; Weiss et al., 2012) and systemic lupus erythematosus (SLE) (Alexander

et al., 2013; Roesner et al., 2015). The paradox of large numbers of Tregs at the sites of chronic inflammation is still obscure (M A Curotto de Lafaille et al., 2008). However, similar to the theory of parallel amplification, it has been proposed that under strong inflammatory conditions Tregs might compensate diminished suppression of the pro-inflammatory response by increased expansion (Alexander et al., 2013).

It should be noted that an increased amount of Tregs in the blood does not refer to the amount of Tregs in the skin. A possible future experiment would be to analyze skin homing markers on Tregs such as CCR4, CCR6 or CLA or the number of Tregs in skin lesions of AD patients. However, due to low numbers of Tregs in the blood and even less in the skin such investigations are highly challenging. They are even more complicated by the problematic of suitable markers and, not least, by the difficulty to obtain skin samples. Rare data exist about Tregs in the skin of AD patients, but with conflictive results. Whereas Verhagen *et al.* did not detect $CD25^+$ $Foxp3^+$ Tregs in lesional AD skin, others demonstrated the presence of $CD25^+$ $Foxp3^+$ Tregs at lesional sites (Caproni et al., 2007; Fujimura et al., 2008; Szegedi et al., 2009). Thus, analyzing skin homing markers on Tregs would gain important insights and could be a next step to characterize Tregs in AD in more depth.

To sum up, it was shown that Tregs in peripheral blood are expanded in AD. This is in line with the majority of previous investigations. However, increased Treg numbers are probably only a side effect and supposable not the main factor in the pathogenesis of the disease.

4.1.2 *IN VITRO* CONVERSION OF $CD4^+$ $CD25^-$ INTO $CD25^+$ $FOXP3^+$ iTREGS IS DIMINISHED IN AD PATIENTS

A major aim in this thesis was to investigate *in vitro* iTreg generation, which depends on the presence of the anti-inflammatory cytokine TGF-β. It has been demonstrated that TGF-β signaling is compromised in AD (Anthoni et al., 2007; Katagiri et al., 2007; W. M. Peng et al., 2013). Here it is shown for the first time that *in vitro* conversion of $CD4^+$ $CD25^-$ T cells into $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs was significantly reduced in cells derived from peripheral blood of patients with AD in comparison to healthy donors. This finding is highly interesting, since it might imply that the inflammatory immune response in the skin cannot be down regulated properly due to reduced amount of iTregs. Hence, this is supporting the assumption that Tregs play a role in AD pathogenesis.

Reduced amount of Foxp3 expressing T cells was accompanied by a diminished number of $GITR^+$ T cells. The latter protein is typically expressed on Tregs and is supporting the data of a diminished iTreg generation. In addition, less $GARP^+$ cells were detected in the *in vitro* cultures. GARP is a *bona fide* marker for activated Tregs. This data provides not only evidence of less iTreg generation, but might also indicate impairment in iTreg function. It has been shown previously that down regulation of GARP in human Tregs by gene knockdown diminished their suppressive qualities (Probst-Kepper & Buer, 2010; Rui Wang et al., 2009). Due to the fact that Foxp3 and GARP expression seem to tightly regulate each other, it remains obscure which factor is the dominating one and is influencing the other. However, another yet unknown factor might influence both Foxp3 and GARP expression. The role of GARP in Treg function in the context of AD was investigated in more detail by analyzing cells derived from AD patients with a mutation in the *LRRC* gene, encoding for GARP. This will be discussed in more detail below.

However, the observation of decreased amounts of iTregs seems to be conflictive with the *ex vivo* data, where an increase in the number of Tregs was detected in the blood of AD patients in comparison to healthy donors. Yet, there are possible explanations enlightening the contradictory result. Even though the peripheral conversion into $CD25^+$ $Foxp3^+$ iTregs might be diminished in AD patients, generation of nTregs might not be affected. iTreg development depends on TGF-β signaling and evidence that the latter is affected was revealed in this work. It is presumed that the generation of nTregs in the thymus does not require TGF-β and hence could be normal (Dons, Raimondi, Cooper, & Thomson, 2012). In fact, even an expansion of nTregs was detected. nTregs are generated by negative selection to prevent autoimmunity and to raise the activation threshold for immune reaction in general (Maria A. Curotto de Lafaille & Lafaille, 2009). In contrast, iTregs have been described to suppress immune responses to environmental and food allergens and to decrease chronic inflammation (Maria A. Curotto de Lafaille & Lafaille, 2009). Hence, iTregs might be more crucial in the context of the disease than nTregs as the latter are not specific against the antigens entering the skin. A possible future experiment could be to induce iTregs in an antigen-specific way instead of polyclonal CD3-activation to reveal whether impaired conversion occurs.

It might be that diminished conversion of naive T cells into $CD25^+$ $Foxp3^+$ iTregs enables the Th2 driven immune response to dominateas it is seen in the acute phase of AD (Hamid, Boguniewicz, & Leung, 1994). The Th2 cytokine IL-4 has been described to inhibit TGF-β and thereby Foxp3 expression (Dardalhon, Awasthi, Kwon, Galileos, & Gao, 2008; Macey,

Sturgill, Morales, & Falanga, 2012). Hence, Th2 dominance might further impair the polarization towards the iTreg phenotype. It was shown that treatment of the cultures with anti-IL4 antibody leads to an increase in the conversion of $CD4^+$ $CD25^-$ T cells into $CD25^+$ $Foxp3^+$ iTregs in cells derived from AD patients as well as in healthy donors. However, even treatment with anti-IL4 antibody did not increase the conversion up to a level of the healthy donors. This data reveals on the one hand that IL-4 and TGF-β are indeed counter players and blocking IL-4 leads to an increase in the conversion into iTregs. On the other hand, this data shows that some of the $CD4^+$ $CD25^-$ T cells were actually IL-4 secreting (and presumably Th2) T cells. However, the amount of those cells did not differ significantly in AD and healthy donors. This signifies that the patients enrolled in this study may not have a dominant Th2 response. This is probably due to the fact that they were rather in the chronic stage of the disease. On the other hand this data exclude the possibility that diminished conversion into iTregs derives from the presence of IL-4 secreting Th2 cells in the cell culture.

Interestingly, a decreased amount of Tregs has been reported in childhood AD (Stelmaszczyk-Emmel, Zawadzka-Krajewska, Szypowska, Kulus, & Demkow, 2013). Probably, lower numbers of Tregs in the blood could be a feature of early or acute AD. This goes along with Th2 dominance and further suppresses Treg generation. In chronic AD Th1 response dominates and the number of Tregs could increase due to lower amount of IL-4 and other factors. The exact mechanism remains obscure and requires further investigation. This means, that the *in vitro* condition might reflect the situation of acute or early AD (when it appears for the first time). In contrast, the *ex vivo* data (increased amounts of Tregs in the blood) could reflect a feature of chronic AD. The patients in this study were older than 18 years and the disease pattern was rather heterogeneous. A more homogeneous selection of patients would ease the interpretation of the data.

To conclude, here it was shown for the first time that *in vitro* iTreg induction was impaired in AD patients. This new insight is giving strong indication that Tregs play a role in AD pathogenesis.

4.2 TGF-β SIGNALING IN T CELLS OF AD PATIENTS AND HEALTHY DONORS

Possibly, diminished conversion in AD-derived $CD4^+$ $CD25^-$ T cells into iTregs is due to malfunctioning of TGF-β signaling. Indeed, several alterations in TGF-β signaling were

revealed in $CD4^+$ T cells. Lower expression of Smad 3 mRNA was detected in $CD4^+$ $CD25^-$ T cells. This is line with the data from Gambichler *et al*., showing reduced amounts of Smad 3/4 mRNA in lesional skin of AD patients compared to healthy subjects (Gambichler, Tomi, Skrygan, Altmeyer, & Kreuter, 2006). Furthermore, Anthoni *et al*. reported that in a murine AD model using Smad 3-deficient mice, Smad 3-pathway regulates allergen-induced skin inflammation and systemic IgE antibody production (Anthoni et al., 2007). Thus, diminished expression of Smad 3 RNA fits into previous studies and confirms the relevance of TGF-β signaling in AD pathology. Less RNA of this signaling molecule was associated with decreased phosphorylation of the Smad 2/3 protein. Thus, these alterations in TGF-β signaling pathway is supporting the assumption that malfunction in TGF-β signaling might result in diminished iTreg conversion. However, Smad 3 protein was not found to be reduced. This might be due to different expression time points and stability of protein and mRNA. Kinetic studies might enlighten Smad regulation in AD and healthy CTR.

Interestingly, the surface expression of TGF-β R II and TGF-β R III was higher in $CD25^+$ $Foxp3^-$ Teff cells and $CD25^+$ $Foxp3^+$ iTregs derived from AD patients in comparison to healthy control. Regulation of TGF-β R in iTregs in the context of AD has not been explored yet. In general, data on TGF-β R expression on $CD4^+$ T cells in AD is rare. In allergic diseases it has been shown that TGF-β R mutations lead to a strong predisposition (Frischmeyer-Guerrerio et al., 2013). This is in contrast with the observation of equal amounts of TGF-β Rs in $CD4^+$ $CD25^-$ $Foxp3^-$ and increased expression on $CD4^+$ $CD25^+$ $Foxp3^-$ T cells $CD4^+$ $CD25^+$ $Foxp3^+$. Investigation on TGF-β R are challenging for their low expression. The alterations detected in this project are, even though statistically significant, within a low range and might not be relevant on a functional level.

In summary, several alterations in the TGF-β pathway were observed. Changes in the R-Smads (Smad 3 RNA, p-Smad 2/3) could have a direct effect on TGF-β signaling driven gene expression such as Foxp3. In contrast, up regulation or less down regulation respectively, of TGF-β Rs and decrease in Smad7 RNA seems to be rather a secondary effect. This is in line with the observation that surface expression of TGF-β Rs in $CD4^+$ $CD25^-$ $Foxp3^-$ T cells was equal in AD and healthy CTR. Hence, TGF-β R expression per se does not seem to be affected, but their regulation seems to be altered. This might imply that in stimulated T cells from AD patients the negative feedback loop has not been initiated yet or not as much as in cells from healthy donors. That is probably due to the fact that gene expression driven by the signaling has not been carried out sufficiently.

Malfunction in TGF-β signaling might be, as mentioned above, derive from abnormal expression of Smad 3 or reduced phosphorylation of Smad 2/3. However, it cannot be excluded that another member of the downstream TGF-β signaling cascade might be affected, such as Smad anchor for receptor activation (Sara). This protein interacts directly with Smad 2/3 and recruits Smad 2/3 to the TGF-β R. It further might be involved in the dephosphorylation of TGF-β R I mediated by Smad 7 (Tang, Ling, Sun, & Liu, 2010). Hence, Sara might play a crucial role in TGF-β signaling and therefore in iTreg conversion.

Another hot candidate, which might be responsible for a dampened signaling is GARP, as it is directly associated with TGF-β. Because TGF-β release from Tregs is mediated by GARP, it plays a pivotal role in Tregs function through TGF-β. Presumably, reduced surface expression of GARP leads to diminished TGF-β release / signaling and this might result in a dampened conversion (Sun, Jin, & Li, 2016). Interestingly, reduced GARP expression has been associated with impaired Treg function and reduced Foxp3 expression (Probst-Kepper & Buer, 2010; Rui Wang et al., 2009). Also, it was shown that a fewer number of $GARP^+$ Tregs was expressed after TCR stimulation. Furthermore, a direct link between GARP and AD was found when cells derived from AD patients with a mutation in the *LLRC* gene were analyzed. This will be discussed below. In conclusion, alteration in the TGF-β signaling might be responsible for diminished iTreg conversion. Indeed, evidences for abnormal signaling such as reduced Smad 3 RNA expression were observed. However, more investigations need to be carried out to reveal the mechanism and further involved proteins respectively, behind it.

4.3 FUNCTIONAL CHARACTERIZATION OF ITREGS

Another aim of the thesis was to characterize iTregs derived from AD patients and healthy donors functionally. iTregs enriched with $CD25^+$ magnetic beads were able to suppress proliferation of Teff cells and the suppression quality was not impaired in AD patients in comparison to healthy donors. This implies that cells, which were able to convert might not possess a reduction in suppression quality. This result is rather unexpected since several indications for impaired iTregs function in AD were provided. First, expanded numbers of Tregs in peripheral blood were detected. Since a higher frequency of immune suppressing cells was not able to dampen inflammation in the skin, it was hypothesize that Treg function might be impaired. Furthermore, alterations in the TGF-β signaling and in GARP and GITR expression were detected, which might indicate impaired suppressive activity.

In the literature, the suppressive quality of Tregs in atopic diseases has been discussed intensively, but the outcome of the studies is highly diverse. In allergic rhinitis there is evidence of a possible impairment in the function of nTregs (Bellinghausen et al., 2003; Ling et al., 2004). Another study in AD reports that the suppressive function of (autologous) Teffs was attenuated (Zhang et al., 2016). On the contrary, Vukmanovic-Stejicet *et al*. demonstrated that $CD4^+$ $CD25^+$ T cells derived from peripheral blood of patients with AD have a similar suppressive capacity *in vitro* to that of Tregs from healthy donors (Vukmanovic-Stejic et al., 2005). One study is of particular interest as it reports that Tregs in peripheral blood possessed normal immune-suppressive activity when stimulated with anti-CD3, but they lost it after stimulation with superantigen (SAg), which is produced by *S. aureus*, generally colonizing the skin of AD patients (Ou et al., 2004). This work is highly interesting as it is in line with the present results (no functional impairment after anti-CD3 stimulation) and furthermore, provides a possible mechanism of Tregs losing their inhibiting feature upon SAg contact in the skin. The inhibitory GITR/GITR-L pathway has been implicated as a mechanism for the abrogation of immune-suppressive activity (Cardona, Goleva, Ou, & Leung, 2006). As a next step it would be highly interesting to stimulate Tregs in an antigen-specific manner. Preferably with the allergen the patients are sensitized. This would enlighten whether *in vitro* induced iTregs behave differently when stimulated polyclonal or antigen-specific respectively.

Another possible future experiment could be to investigate suppressive function of circulating Tregs. As a reduced expression level of Foxp3 and GARP RNA was detected in AD-Tregs isolated from blood in comparison to healthy donors, this might indicate impairment in functionality. However, since Ou *et al*. have already demonstrated, that Tregs isolated from blood of AD patients did not show impaired function, a next step could be to stimulate those Tregs antigen-specifically (Ou, Goleva, Hall, & Leung, 2004).

4.4 ANALYSIS OF T CELLS OF AD PATIENTS WITH A MUTATION IN THE *LRRC32* GENE OR THE WT FORM

The present results are supporting the assumption that GARP is involved in the pathogenesis of AD. Indeed, GARP has been associated with this inflammatory chronic skin disease. Recently, Manz *et al* revealed that there is an excess of rare missense mutations within the *LRRC32* gene encoding the protein GARP in individuals with AD and mutant carriers have an

increasing risk of developing this disease (Manz et al., 2016). Therefore, to get more insight into the role of GARP in Tregs in the context of AD cells derived from patients carrying either a missense mutation (here called A407T) in the *LRRC32* gene or the WT form respectively were analyzed. Several alterations were detected, indicating the significance of GARP in Treg function, and furthermore, in AD.

The expression of surface GARP on several cell types of heterozygous A407T carriers and AD patients with the WT form was found to be lower. Significant changes were observed in $CD19^+$ B cells, $CD56^+$ NK cells and $CD3^+$ pan-T cells derived from peripheral blood. Within T cells, $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^+$ T cells revealed a strong reduction in surface expression of GARP. AD patients with an A407T mutation expressed less protein, giving indication for functional impairment in the mutant carriers. This has not been reported yet. Presumably, the function of GARP in $CD19^+$ B cells and $CD56^+$ NK cells is tightly connected with TGF-β signaling related functions.

To further illuminate the functional role of GARP in Tregs in the context of AD, TGF-β dependant conversion of $CD4^+$ $CD25^-$ T cells into iTregs was investigated. A strong tendency of less $CD4^+$ $CD25^-$ T cells derived from mutant carriers converted into iTregs was found in comparison to AD patients with the WT form. Surface GARP expression was significantly reduced in cells derived from carriers with the A407T mutation in comparison to cells with the WT form. This result reveals a direct link of a diminished GARP expression to an impaired conversion into iTregs. As described above, an impairment of iTreg conversion in cells derived from AD patients in comparison to healthy donors was detected. However, the genetic background of the donors regarding the *LRRC32* gene was not known. The present results suggest that GARP plays a role in the generation of iTregs since low expression of the protein resulted in a reduced conversion rate in the context of AD.

The known function of GARP is to bind and accommodate latent TGF-β before the activation and release of the mature cytokine (Fridrich et al., 2016; Stockis, Colau, Coulie, & Lucas, 2009; Tran et al., 2009; Rui Wang et al., 2009). It is possible that defective or down-regulated GARP impairs the release of mature TGF-β, which might affect the autocrine positive feedback loop. Less GARP would lead to less TGF-β release / signaling and this would lead to less conversion (Sun et al., 2016). Because the TGF-β release process from Tregs is mediated by GARP, it plays a role in Tregs function through TGF-β. Furthermore, it has been shown previously that GARP not only exist in its membrane bound form, but also as a soluble TGF-β1-GARP complex due to proteolytic shedding (Fridrich et al., 2016). Fridrich *et al*

hypothesize that sGARP can bind free latent TGF-β non-covalently and thereby enhance its activation. sGARP could probably capture even small amounts of free latent TGF-β and activate it. This would lead to a positive feedback by up regulating TGF-β expression, which again can bind to sGARP. The soluble protein would therefore work as immune modulator (Fridrich et al., 2016). It has been shown that treatment of naive T cells with sGARP induces expression of TGF-β and Foxp3 which converts them into iTregs (Hahn et al., 2013). This effect was extinguished by application of TGF-β R blocking ab. To elucidate the role of GARP in the process of conversion in the context of AD in more detail, a functional assay was performed in which the generation into iTregs with sGARP instead of TGF-β was induced. Treatment with sGARP enabled the generation of $CD25^+$ $Foxp3^+$ iTregs in both patient groups. However, fewer iTregs were generated in the group with the A407T mutation. Hence, sGARP was not able to overcome the diminished conversion rate. There are several possible reasons for this observation. It might be that GARP is not the only factor responsible for diminished conversion into iTregs and for that reason, treatment with sGARP is not sufficient to rescue the conversion rate. Indeed, in this work indications were found for a malfunction in TGF-β signaling such as reduced p-Smad2/3 when comparing iTreg generation of cells derived from AD patients with that of healthy donors. This implies that GARP plays a pivotal role in Treg generation in the context of AD, but in interplay with other factors resulting in the observed phenotype. Secondly, it is possible that sGARP is not working in the same way as the membrane bound form, even though this has been reported (Hahn et al., 2013). However, others have suggested that GARP has to be membrane bound and TGF-β coupled by disulfide bridges in order to be able to activate TGF-β and to function properly (R. Wang et al., 2012). The GARP field is still under intensive investigation and data has to be confirmed and further illuminated. Another reason might lay in the experimental set up. It cannot be excluded, that adjustments in titration of protein and cytokines might lead to an improvement of conversion. More importantly, using naive T cells from cord blood might result in a different outcome as the state of differentiation might be important. Hence, $CD4^+$ $CD25^-$ T cells might be not as potent for sGARP treatment. Unfortunately, it is a big challenge to obtain such patient samples and in general it is difficult to receive sufficient amounts of cells for such an experimental set up.

GARP plays an important role in the suppression mechanism of Tregs as knock down of GARP by blocking antibodies or siRNA impairs the immune suppressive capacity of Tregs (Miller, Fogle, Ross, & Tompkins, 2013; Rui Wang et al., 2009). Therefore, the inhibition quality of Tregs derived from peripheral blood of AD patients with the A407T mutation or the

WT form respectively, was tested. It is suggestive, that Tregs bearing fewer GARP (A407T carriers) might be less potent. However, a co-culture assay with autologous Tresp cells did not reveal a difference between both groups. As mentioned above, when functionality of iTregs was tested, it is possible that the *in vitro* situation differs from that *in vivo* as the microenvironment might be crucial and change the outcome. However, surprisingly, the reduction of surface GARP did not change the inhibition quality in this experimental set up. It should be noted that only 3 donors with A407T mutation could be tested. More repetitions are necessary to confirm the data.

In conclusion, the present results demonstrate for the first time that GARP is tightly associated with AD since the missense mutation A407T in the *LRRC32* gene led to reduced surface GARP expression and malfunction in conversion into iTregs. In addition, these results are supporting the assumption that GARP might be jointly responsible for malfunction of TGF-β signaling, along with reduced Smad 3 RNA expression and phosphorylation of Smad 2/3. Thus, it can be stated that Tregs play an important role in AD.

4.5 FUTURE PROSPECTS

AD often lays a heavy burden unto patients, influencing their daily life significantly and impairing their life quality dramatically. Although, there are different therapy strategies available to control the symptoms, there is still a strong need for superior approaches in terms of efficiency and less side effects, especially for the severe form of AD. At present the main principles are application of emollients to improve epidermal barrier repair and anti-inflammatory therapy with topical corticosteroids or calcineurin inhibitors (Weidinger & Novak, 2016). As the disease gets more severe, phototherapy or systemic immunosuppressants (e.g. cyclosporine) are applied (Weidinger & Novak, 2016). However, side effects are a noteworthy by-product and there is still a need for well adapted treatment.

Tregs play an important role in the immune system as they are suppressing immune response. Furthermore, they have been shown to play a role in diseases such as autoimmunity, atopic diseases or cancer (Elkord, 2006; Shimon Sakaguchi et al., 2001). Hence, Tregs are under intense investigation regarding their potency for immune therapy. One approach of using Tregs in diseases with abnormal amount or functionally impairment of Tregs such as autoimmune diseases (e.g. type 1 diabetis or SLE), atopic diseases or graft-versus-host disease

(GVHD) after transplantation), is the expansion of autologous nTregs (Miyara, Wing, & Sakaguchi, 2009; Trzonkowski et al., 2009). This cellular therapy is based on *ex vivo* expansion of nTregs and thereafter, transfer of the cells to the patient. This approach has been effective in mice, especially in prevention of experimental autoimmune encephalomyelitis (Coombes, Robinson, Maloy, Uhlig, & Powrie, 2005; Hoffmann & Edinger, 2006; La Cava, 2008; O'Connor & Anderton, 2008; Salomon et al., 2000; Xia et al., 2006). There is at least indication that adoptive transfer of Tregs is also beneficial in ongoing inflammation (Shimon Sakaguchi, Yamaguchi, Nomura, & Ono, 2008).

However, several factors hamper / complicate investigations and need to be addressed before their clinical use. First, $Foxp3^+$ T cells are heterogeneous and the Treg population used for isolation and expansion must be determined carefully (Tran, Ramsey, & Shevach, 2007). Moreover, to gain a 100% pure population is rather complex and probably the biggest challenge (Tran et al., 2007). Second, the cytokine cocktail for *in vitro* expansion must be chosen and established thoroughly, due to the risk of outgrowth of non-Treg cells, which are producing pro-inflammatory cytokines (Tran, Ramsey and Shevach, 2007; Sakaguchi *et al.*, 2010). Rapamycin might be a beneficial supplement as it eliminates non-Treg cells (Shimon Sakaguchi et al., 2010). However, it has been suggested that generation of iTregs may be more effective due to the fact that they can be generated in an antigen-specific fashion, resulting in a disease /organ-specific treatment, providing higher safety and efficiency (Dons, Raimondi, Cooper, & Thomson, 2012). Moreover, it would be easier to gain a pure population and to generate an adequate number of cells.

Even though, adoptive Treg transfer seems to be a promising therapeutically attempt, there are more pitfalls waiting, concerning both nTregs and iTregs. Treg stability is a problem, which cannot be ignored. Especially iTregs exhibiting a demethylated TSDR are suspected to be not long lasting (Shevach & Thornton, 2014). However, there is a study indicating sustained Foxp3 expression in effective antigen-specific iTregs *in vivo* in mice after *in vitro* generation (DiPaolo et al., 2007). Still, little is known about the stability *in vivo* in human and more investigations are necessary for clarification (Dons et al., 2012). Furthermore, Tregs have been shown to play a negative role by inhibiting anti-tumor immunity and maintaining chronic infections (Shimon Sakaguchi et al., 2010). Despite all the pitfalls and challenges, Tregs remain to be highly interesting in terms of therapeutically usefulness. Especially iTregs might be proven to be valuable due to antigen-specificity, safety and easily obtainable numbers of cells as mentioned above.

In this project, it was shown that *in vitro* iTreg generation of cells derived from AD patients was impaired in comparison to healthy donors. In contrast, nTreg generation did not seem to be affected as frequency of nTregs were even higher in peripheral blood in comparison to healthy donors. There is indication, that iTregs and nTregs possess different regulatory functions *in vivo* e.g. due to their different developmental origin and their TCR repertoires (Maria A. Curotto de Lafaille & Lafaille, 2009). Indeed, iTregs have been associated mainly with establishing tolerance to harmless foreign antigens (Maria A. Curotto de Lafaille & Lafaille, 2009; Schmitt & Williams, 2013). Even though it might be possible that due to malfunctioning iTregs, adoptive transfer of (organ-specific) iTregs is beneficial in AD patients, it is a rather unlikely that this approach would find its way into praxis. Adoptive transfer of iTregs will stay a hot research field, but in the context of cancer and autoimmunity. However, revealing the importance of Tregs in AD helps to understand the pathogenesis of the disease and probably to find an appropriate therapy.

5 SUMMARY

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases with increasing incidence. It has been suggested that Tregs play a role in the pathogenesis of AD, as Tregs regulate immune homeostasis by modulating the immune system response. Even though the role of Tregs in this disease has been studied for several years, their part in it is still not clear. The objective of this study was to characterize the phenotype, the induction and function of Tregs in patients with AD compare with healthy controls.

Therefore, circulating Tregs and T cell subsets of AD patients and healthy individuals were quantified by flow cytometry. Interestingly, only $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs were found to be expanded in the blood of AD patients. Quantification of the TSDR, a specific region in the *Foxp3* locus, affirmed that the frequency of nTregs was increased in AD. This was in line with several preceding studies. In contrast, the TGF-β-dependent conversion of $CD4^+$ $CD25^-$ $Foxp3^-$ into $CD4^+$ $CD25^+$ $Foxp3^+$ iTregs was impaired in AD patients, which supported the evidence that TGF-β signaling might be malfunctioning in AD. Indeed, reduced expression of Smad 3 mRNA was revealed in $CD4^+$ $CD25^-$ T cells, which was accompanied by a tendency of less p-Smad 2/3. This might have a direct effect on TGF-β signaling driven gene expression, such as Foxp3. Interestingly, Smad 3 has already been associated with AD as Smad 3/4 mRNA was down-regulated in human skin lesions of AD patients. Additionally, Smad 3 deficiency in an AD mouse model led to allergen-induced skin inflammation. Alterations observed in TGF-β R and Smad 7 expression might display a secondary effect as a result of impaired signaling. Besides the expressional characterization of Tregs, their suppressive function was tested in an *in vitro* co-cultured with assay autologous T responder cells. iTregs derived from AD patients inhibited proliferation similar to those from healthy donors. Hence, their suppressive function was not impaired *in vitro*. Furthermore, the role of the transmembrane protein GARP was investigated in Tregs in the context of AD. It was shown that the missense mutation A407T in the *LRRC32* gene led to a reduced surface expression of GARP together with a malfunction of AD-T cells conversion into iTregs.

In conclusion, this study supplied strong indication for an important involvement of Tregs in AD pathology. Evidence was provided for malfunction in TGF-β signaling leading to

impaired iTreg generation which could explain why inflammatory immune response is not down regulated properly in the skin of AD patients.

6 ABBREVIATIONS

A2AR	Adenosine Receptor 2A
ab	Antibody
AB/AM	Antibiotic/Antimycotic
AD	Atopic dermatitis
APC	Allophycocyanin
APC	Antigen presenting cell
BCA	Bicinchoninic acid
BCR	B-cell receptor
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CTLA4	Cytotoxic T-lymphocyte-associated Protein 4
CTR	Control
D	Day
D	Demethylated
DC	Dentritic cell
DNA	Desoxyribonucleic acid
DNase	Desoxyribonuclease
FACS	Fluorescence-activated cell sorting
FAM	6-Carboxyfluorescein
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead-Box-Protein P3
GITR	Glucocorticoid-induced TNFR-related protein
GARP	Glycoprotein A repetitions predominant
gDNA	Genomic DNA
GVHD	Graft-versus-host disease
IDO	Indoleamine 2,3-dioxygenase
IgE	Immunoglobulin-E

IgG	Immunoglobulin-G
IL	Interleukin
iTreg	Induced T regulatory cell
LAP	Latency Associated Peptide
M	methylated
MACS	Magnetic activated cell sorting
mAb	Monoclonal antibody
mc	Monoclonal
MHC	Major histocompatibility complex
mRNA	messenger RNA
MS-RTPCR	Methylation Specific-Real Time PCR
NK cell	Natural killer cell
nTreg	Natural T regulatory cell
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
rpm	Rotations per minute
rt	Room temperature
RT-PCR	Real Time-Polymerase chain reaction
SAg	Superantigen
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
SNP	Single-nucleotide polymorphism
TAMRA	Tetramethylrhodamine
TBST	Tris-buffered saline with Tween20
TCR	T cell receptor
Teff	T effector cell
TGF-β	Transforming growth factor-β
TGF-β R	Transforming growth factor-β receptor
Th	T helper

Tr1	T regulatory cells 1
Treg	T regulatory cell
Tres	T responder cells
TSDR	T cell Specific Demethylated Region
tTreg	Thymus-derived Treg
U	Unit
WT	Wildtype

7 LIST OF FIGURES

8 REFERENCES

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9 DANKSAGUNGEN

Mein besonderer Dank gilt Frau Prof. Natalija Novak für die Möglichkeit diese Arbeit bei ihr zu verfassen und ihre Betreuung. Ihre Hilfestellung, Richtungsweisung und Inspiration waren stets sehr hilfreich für mich. Desweiteren danke ich Frau Prof. Irmgard Förster vom LIMES Institut für die Zweitbetreuung. Auch danke ich Herrn Prof. Koch für die Übernahme des 3. Gutachters und Herrn Prof. Wiegandt für die Übernahme des 4. Gutachters.

Ich danke Frau Prof. Dr. Sophie Lucas (Institut de Duve, Brüssel, Belgien) für das zur Verfügung stellen der demethyliert- und methyliert-spezifischen Plasmide.

Den Mitgliedern und Ehemaligen der AG Novak gilt ein herzliches Dank für Unterstützung jeglicher Art! Alica, Mareike, Elena, Jorge, Wenming, Chunfeng, Nils, Ivan, Beatriz, Yvonne, Markus, Africa und Ann-Christin, ihr habt mir die vielen Stunden im Labor und Büro versüßt! Auf ganz verschiedenen Ebenen habt ihr mir unheimlich geholfen! Mit mir gelacht, in den schweren Stunden beigestanden oder ganz praktisch im Labor, bei der Korrektur von Texten, wissenschaftlichen Diskussionen oder organisatorischen Dingen!

Desweiteren danke ich Mitarbeitern und Ärzten der Allergologie: Kirsten, die unermüdlich für mich Probenmaterial besorgt hat! Serhat, der mir bei den organisatorischen Dingen geholfen hat und mir immer hilfreich zur Seite stand! William und all den anderen Ärzten aus der Ambulanz, für die Beschaffung von Probenmaterial. Nicht vergessen möchte ich die Patienten und all die anderen Freiwilligen, die mir so bereitwillig ihr Blut zur Verfügung gestellt haben!

Besonderen Dank geht an meinen Verlobten Alex, meine Familie und meine Freunde! Ohne euch hätte ich wohl nicht durchgehalten! Ihr habt immer an mich geglaubt und mich in so vieler Hinsicht unterstützt und ermutigt! Ihr macht mein Leben schön und auf euch ist Verlass!!

10 PUBLICATIONS

JOURNAL ARTICLES

Manz, J., Rodríguez, E., ElSharawy, A., **Oesau, E.-M.**, Petersen, B.-S., Baurecht, H., Mayr, G., Weber, S., Harder, J., Reischl, E., Schwarz, A., Novak, N., Franke, A., Weidinger, S. (2016). 'Targeted Resequencing and Functional Testing Identifies Low-Frequency Missense Variants in the Gene Encoding GARP as Significant Contributors to Atopic Dermatitis Risk', Journal of Investigative Dermatology **136**(12):1–7.

POSTER PRESENTATIONS

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