Charles University in Prague

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The role of cytokines in development and differentiation of regulatory T cells

Role cytokinů ve vývoji a diferenciaci regulačních T buněk

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis

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List of abbreviations

APCs	Antigen presenting cells
CD40L	CD40 ligand
CTLA-4	Cytotoxic T lymphocyte antigen 4
DCs	Dendritic cells
EAE	Experimental autoimmune encephalomyelitis
Foxp3	Forkhead box p3
GITR	Glucocorticoid-induced TNFR-related protein
IFNγ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
IL-2R	IL-2 receptor
iTregs	Induced Tregs
KO	Knock out
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MSCs	Mesenchymal stem cells
NFAT	Nuclear factor of activated T cells
NK	Natural killer
nTregs	Naturally occuring Tregs
ROR	Retinoic acid receptor-related orphan receptor
RUNX	Runt-related transcription factor
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TNFR	Tumor necrosis factor receptor
Tregs	Regulatory T cells

Abstract

The development and function of T helper (Th) cells and regulatory T cells (Tregs) are plastic processes that are regulated by cytokines. In our project we first analyzed the effect of different cytokines on the development of induced (i) Tregs. It has been demonstrated that iTregs arise from $CD4^+CD25^-$ T cells upon stimulation with alloantigen in the presence of transforming growth factor β (TGF-β). The development of these Tregs and their proliferation were inhibited by interleukin (IL)-4 and IL-12. The aquired results also demonstrated distinct responses of naturally occuring (n) Tregs and iTregs to the regulatory action of IL-4 and an opposite role of IL-4 in maintenance of nTregs and iTregs phenotype.

An important role in the induction of T cell subsets may play also mesenchymal stem cells (MSCs) which can, under specific conditions, produce TGF-β and IL-6. Depending on the current production of TGF-β or IL-6, MSCs can qualitatively regulate the ration between Tregs and Th17 cells. Anti-inflammatory Tregs and pro-inflammatory Th17 cells are induced upon stimulation in the presence of TGF-β and TGF-β and IL-6, respectively. In addition to our previous work we studied the role of IL-12 in the development of Tregs and Th17 cells. It was shown that Treg and also Th17 cell differentiation was prevented by IL-12 as was the induction of Foxp3 transcription factor expression by TGF-β or RORγt transcription factor expression by TGF-β and IL-6. Moreover, IL-12 was able to alter the development of iTregs and Th17 cells even when added to the differentiating cells after 48 h of the culture. The cells activated in the presence of TGF-β and IL-12 had an increased expression of the Th1 transcription factor T-bet, produced Th1 cytokines interferon γ and IL-2 and expressed the phenotypic markers IL-18 receptor and C-C chemokine receptor type 5 which are characteristic for Th1 cells.

In conclusion, our findings contributed to the field of developmental plasticity of Tregs and Th cells and demonstrated the significant role of cytokines in this process. The results can also contribute to the improvement of therapeutic procedures where Tregs are used to treat severe autoimmune diseases or transplantation.

Abstrakt

Vývoj a funkce pomocných T (Th) lymfocytů a regulačních T buněk (Treg) je plastický proces regulovaný pomocí cytokinů. Proto jsme se v tomto projektu nejprve zaměřili na sledování vlivu různých cytokinů na vývoj indukovaných (i) Tregs. Bylo prokázáno, ze iTregs se vyvýjejí z $CD4^{+}CD25^{-}$ T buněk po stimulaci alloantigenem a v přítomnosti transformujícího růstového faktoru β (TGF-β). Vývoj a proliferaci takto indukovaných Tregs je možno potlačit pomocí interleukinu (IL)-4 and IL-12. Výsledky také ukázaly, že IL-4 má zcela rozdílný vliv na udržení fenotypu přirozeně se vyskytujících (n)Treg a iTreg.

Další populací s imunoregulačními vlastnostmi jsou mezenchymální kmenové buňky (MSC), které mohou za určitých podmínek produkovat TGF-β a IL-6 a tak se výrazně podílet na indukci Treg a Th subpopulací. Podle aktuální produkce TGF-β či IL-6 tak MSC mohou kvalitativně regulavat poměr mezi Treg a Th17 buňkami. Protizánětlivé Treg a prozánětlivé Th17 buňky se vyvýjejí po stimulaci za přítomnosti TGF-β, resp. v přítomnosti TGF-β a IL-6. V další části této studie jsme se zaměřili na studování role IL-12 při vývoji Treg a Th17 buněk. Bylo ukázáno, že diferenciace Treg a Th17 buněk byla v přítomnosti IL-12 potlačena, stejně jako TGF-β-indukovaná exprese transkripčního faktoru Foxp3 a TGF-β/IL-6-indukovaná exprese transkripčního faktoru RORγt. IL-12 potlačil vývoj Treg a Th17 buněk i po přidání do kultur, ve kterých byla indukována diferenciace, po 48 hodinách. Buňky indukované pomocí TGF-β a IL-12 vykazovaly zvýšenou expresi transkripčního faktoru Th1 buněk T-bet, produkovaly Th1-specifické cytokiny IL-2 a interferon γ a na svém povrchu exprimovaly další fenotypové znaky Th1 buněk IL-18 receptor a C-C chemokinový receptor typu 5.

Získané výsledky přinesly nové poznatky o vývojové plasticitě Treg a Th buněk a ukázaly na důležitou roli některých cytokinů v tomto procesu. Tyto výsledky mohou také přispět ke zdokonalení terapeutického využití Treg při léčbě závažných imunopatologických onemocnění a při transplantacích.

1. Introduction

Regulatory T cells (Tregs) are one of the key components of the immune system. They maintain homeostasis of an organism and regulate many processes of the immune system securing, in ideal case, its proper function. Therefore, Tregs have a vast clinical potential. Although they are already used in treatment of certain diseases, mechanisms of their development and function have not been fully recognized yet which disables their wider use in clinics.

1.1. Types of regulatory T cells

To date, several types of regulatory cells have been described, each one of them with different characteristics and also different developmental criteria. Cells with regulatory properties were found within $CD4^+$ and $CD8^+$ T cells, however, even $CD4^-CD8^-$ cells and NKT cells were described.

$CD4^+$ regulatory cells can be further divided into the following subpopulations: Tr1 cells, T helper (Th) 3 cells and Tregs (Figure 1). Tregs as a main topic of this thesis will be described in detailed in next chapters. The phenotypical and mainly functional differences between particular types of $CD4^+$ regulatory cells are not precisely defined and might overlap. Therefore, a short overview of these cells and their role in the immune system is provided here.

1.1.1. Tr1 cells

Tr1 cells are generated in the periphery by antigen stimulation in the presence of interleukin (IL)-10 which is also a vital growth factor for these cells (Groux et al., 1997). These cells can be either produced in vitro or in vivo in appropriate conditions. This tolerogenic environment is in vivo provided mainly by IL-10-producing dendritic cells (DCs)

which are immature or made tolerogenic (Jonuleit et al., 2000; Levings et al., 2005). One of the in vitro protocols for Tr1 induction employs immunosuppressive drugs, vitamin D3 and dexamethasone (Barrat et al., 2002). Cells stimulated in such conditions produce high amounts of IL-10 and exert potent suppressive activity. In addition, cytokines determining the development of Th1 and Th2 cells impair the development of these cells (Barrat et al., 2002). Tr1 cells are characterized by a high production of IL-10 and transforming growth factor β (TGF-β), a low production of IL-2, no secretion of IL-4 and by the ability to suppress effector T cells by a cytokine-dependent mechanism (Groux et al., 1997; Papiernik et al., 1998). Tr1 cells, although capable of a similar range of suppression as Tregs, do not express forkhead box p3 (Foxp3), a marker of Tregs (Vieira et al., 2004). IL-10 was found crucial as an effector mechanism used mainly by Tr1 cells in the gut where it mediated suppression in an antigen-specific, cytokine-mediated fashion. Besides inhibiting cell-mediated immune reactions, Tr1 cells were also shown to specifically downregulate immunoglobulin (Ig)E production in an antigen-dependent manner, thus suggesting a role of these regulatory cells in preventing allergy (Cottrez et al., 2000). IL-10 is capable of abrogating experimentally induced colitis and other forms of inflammatory responses to intestinal antigens (Groux et al., 1997; Asseman et al., 1999).

1.1.2. Th3 cells

Th3 cells are present in the gut and on other mucosal surfaces. They are generated in the presence of TGF-β which is also later produced by these cells and regulates local immune reactions. These cells were shown to express an autoreactive T cell receptor (TCR) and produce both, TGF-β and IL-10. The in vitro suppression assays revealed that only the neutralization of TGF-β abolished the inhibitory effects of these cells (Kitani et al., 2000).

Th3 are connected with the phenomenon of oral tolerance, an antigen specific induction of peripheral immune tolerance, which was demonstrated to contribute to the tolerance of ingested antigen but can be also used to suppress autoimmune diseases. In a model of exprerimental autoimmune encephalomyelitis (EAE) it was shown that oral administration of myelin basic protein, one of the major antigens in EAE, suppressed this disease by induction of tolerance to this antigen. The tolerance was mediated by T cells producing TGF-β, IL-4 and IL-10 which were later confirmed as regulatory cells (Chen et al., 1994). The mechanism of oral tolerance is, however, not only induction of Th3 cells. Chen et

al. (1995) concluded that the dose of the administered antigen is of high importance. This group showed that lower doses of antigen indeed support the induction of regulatory cells which act mainly by suppressive cytokines such as TGF-β and IL-10. High antigen doses, on the other hand, lead to the clonal deletion of antigen-specific Th1 and Th2 cells by apoptosis in Peyers patches. The TGF-β producing Th3 cells were described in different models where they had the ability to suppress unwanted inflammatory or autoimmune diseases (Bridoux et al., 1997, Maloy et al., 2003).

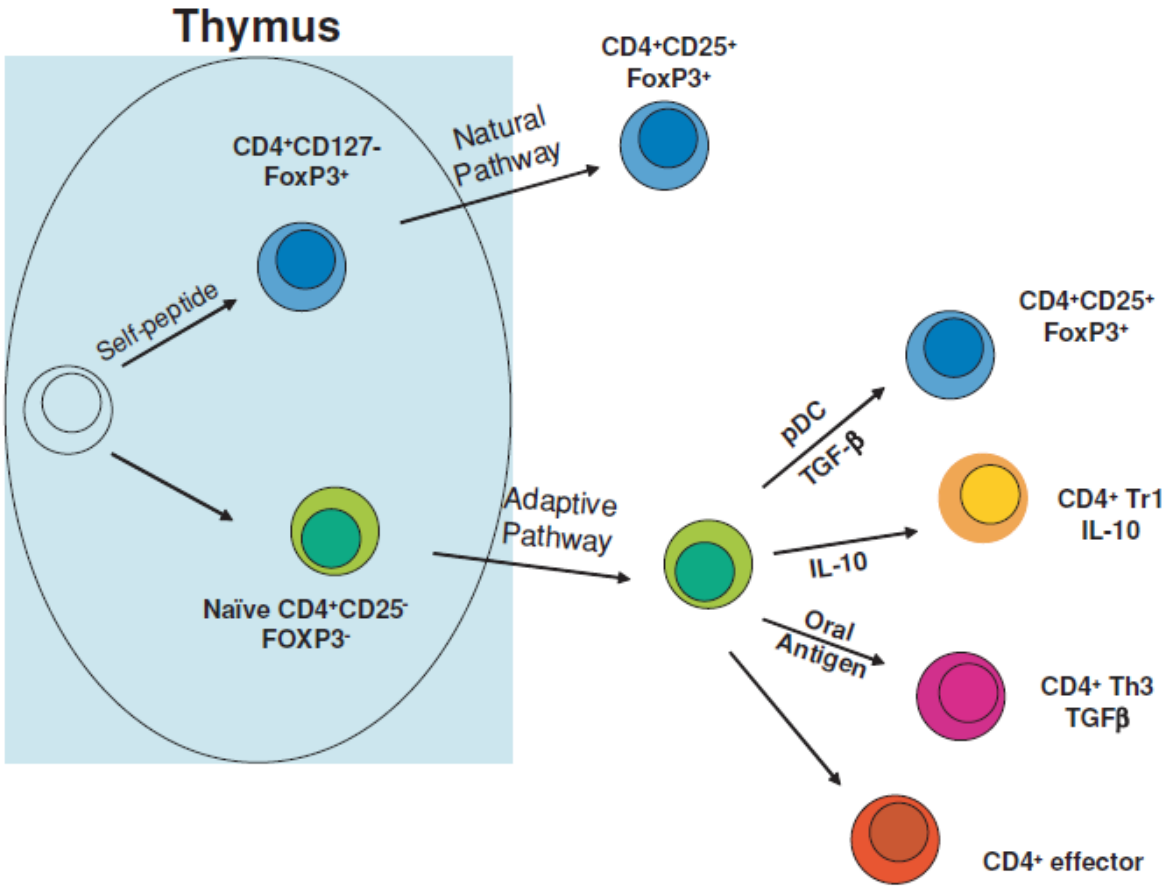

Figure 1: Developmental pathways of natural and induced Tregs. (pDC - plasmacytoid dendritic cell) Adapted from Kang et al., 2007.

1.2. Tregs

1.2.1. Discovery and function

Tregs play a crucial role in the regulation of the entire immune system. They provide immune surveillance and control harmful autoimmune cell clones. Moreover, Tregs efficiently suppress inflammatory immune responses and actively regulate various cells and components of the immune system.

Tregs possess classical αβTCR and CD4 antigen. They constitutively express on the cell surface high quantities of CD25 molecule which is an α subunit of IL-2 receptor (IL-2R). Tregs are vitally dependent on IL-2, they require this cytokine for their development and proper function (Malek, 2003). Indeed, in 1995 Sakaguchi et al. (1995) proved that self-tolerance is maintained by a population of $CD4^+$ T cells that express high levels of IL-2R α subunit CD25. This group found that when $CD4^+CD25^-$ T cells are transferred into immunized athymic nu/nu mice, the mice spontaneously develop sever autoimmune diseases. However, when $CD4^+CD25^+$ T cell are transferred together or shortly after the inoculation of $CD25^-$ cells, the development of autoimmune diseases was prevented. Similarly, $CD25^+$ T cells were also able to suppress rejection of allogeneic skin grafts transplanted together with $CD25^-$ cells onto nu/nu mice. Together, these results showed that $CD4^+CD25^+$ T cells contribute to maintaining self-tolerance by inhibiting immune responses to self-antigens and also suppress reactions to non-self antigens in an antigen-nonspecific manner (Sakaguchi et al., 1995). Moreover, removal of this suppressive population or performing of neonatal thymectomy at day three after birth caused the onset of various organ-specific autoimmune diseases. This suggested that $CD4^+CD25^+$ T cells population occurs in mouse thymus as soon as three days after birth and depleting this population causes uncontrolled action of previously produced autoimmune T cell clones (Asano et al., 1996). This thymectomy-induced autoimmunity can be prevented by reconstitution of the animals with lymphocytes from normal adult animals. The cells responsible for the suppression are $CD4^+CD25^+$ T cells (Suri-Payer et al., 1998). Similar results were later achieved also in other models of autoimmune diseases where Tregs were shown to protect from these pathological states such as EAE (Olivares-Villagomez et al., 1998; Van der Keere and Tonegawa, 1998).

It is clear that Tregs play an essential role in the regulation of autoimmune responses. Moreover, it was described that they also regulate immune responses to pathological conditions such as cancer. It has been demonstrated that Tregs may be responsible for the supression of immunity in cancer (Wang and Wang, 2007). It was shown that removal of $CD4^+CD25^+$ T cell population elicited potent immune responses to syngeneic tumors in vivo. The depletion of these cells resulted in eradication of the tumor by $CD8^+$ T cells and NK cells which were activated by secretion of large amounts of IL-2 by $CD4^+CD25^-$ T cells and thus called lymphokine activated killers (Shimizu et al., 1999). It was confirmed that an accumulation of Tregs at the tumor site is often connected with bad prognosis, however, it has been proven only for certain types of tumors (Curiel et al., 2004, Dannull et al., 2005).

1.2.2. Tregs in transplantation tolerance

Another important field of immunology where Tregs have indispensable function is transplantation tolerance. It was demonstrated long ago that the anti-CD4 monoclonal antibody (mAb) could induce tolerance and even permanent acceptance of allografts (Qin et al., 1993). It was shown that this tolerance is mediated by $CD4^+CD45RC^+$ T cells, is transferable or so called infectious, is partly mediated by soluble TGF-β and is not permanent because it can be abrogated by high doses of IL-2 (Josien et al., 1998; Zhai et al., 2001; Jonuleit et al., 2002). Later it was shown that anti-CD4-induced tolerance is dependent on $Foxp3^+$ Tregs which accumulate in the graft (Cobbold et al., 2004). Moreover, a successful protocol for a long-term engraftment implicated the use of anti-CD4 antibody together with donor-specific transfusion before transplantation. This procedure generated $CD4^+CD25^+$ Tregs which mediated suppression of allograft rejection by a cytokine-dependent mechanism since the administration of anti-IL-10 or anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) antibody abrogated the suppressive effect (Kingsley et al., 2002). In addition, it was demonstrated that the mere donor-blood transfusion before transplantation induced tolerance and it was suggested that alloantigens alone are sufficient to induce Tregs (Bushell et al., 2003). A similar approach was adopted in generating tolerance to Y chromosome-encoded transplantation antigens (Verginis et al., 2008). The persistence of donor alloantigens is essential for induction and maintenance of tolerance in anti-CD4-treated allografted mice (Hamano et al., 1996). Similarly, the blockade of costimmulatory molecules CD28 and CD40 effectively prevented T cell expansion in a model of heart allotransplantation, lead to long-

term graft survival and inhibited chronic rejection (Larsen et al., 1996). Anti-CD40 ligand (CD40L) antibody was used to prevent acute rejection and to induce prolonged graft survival which was strengthened by anti-CD8 antibody (Honey et al., 1999; Kirk et al., 1999). Transplantation tolerance induced by blockade of CD40 – CD40L interaction was partly mediated by enhanced apoptosis of antigen-specific aggressive T cells (Graca et al., 2000). Blockade of costimulatory molecules such as CD40/CD40L or inducible costimulator (ICOS)/ICOSL has been used in different settings to prevent or inhibit rejection of allografts or graft versus host disease (GVHD) (Taylor et al., 2005). Moreover, this blockade was shown to induce regulatory $CD4^+CD25^+$ T cells, the depletion of which abrogated the tolerance (Taylor et al., 2002; van Maurik et al., 2002). In addition, it was shown that anti-CD3/blood transfusion-induced Tregs arise predominantly in the lymph nodes and that the homing T cell receptor CTLA-4 played a non-redundant role in this process (Ochando et al., 2005). Tregs were, however, found also directly at the site of a tolerated allograft. These cells were alloantigen-specific and able to prevent host T cells from rejecting the transplant (Graca et al., 2002). Direct and indirect alloantigen presentation also play a role in tolerance induction. It was demonstrated that when the antigen is presented by host antigen presenting cells (APCs), e. g. indirectly, the $CD4^+CD25^+$ mediated immunoregulation is significantly more pronounced (Sanchez-Fueyo et al., 2007).

1.2.3. Molecular markers of Tregs

1.2.3.1. CD25 and IL-2

CD25, IL-2R α subunit, has long been known as an activation marker of T cells (Ortega et al., 1984). However, it was proven that its expression on a population of suppressive cells is much higher and persistent in time. $CD4^+CD25^-$ T cells gain CD25 expression after activation but this expression is only transient and lower than in case of suppressive $CD4^+CD25^+$ T cell population (Kuniyasu et al., 2000). It has been demonstrated that IL-2 and CD25 are critically required for Treg dvelopment and function (Fontenot et al., 2005a). Tregs express also CD122 which is a β subunit of IL-2R and its deficiency results in lethal autoimmunity soon after birth (Suzuki et al., 1995; Stephens et al., 2001; Malek et al., 2002). Another activation-induced marker was suggested by the use of global gene expression studies. A molecule called neuropilin-1 was identified as a potential surface marker of Tregs

(Bruder et al., 2004). It is a receptor which is involved in angiogenesis and T cell activation but its role in Tregs is still unclear.

IL-2 is an important growth factor for T cells. Surprisingly, the experiments with IL-2 knock out (KO) mice showed that this cytokine is not vital for all T cells but mainly for Tregs. (Krämer et al., 1995; Malek and Bayer, 2004) Mice lacking IL-2, CD25 or CD122 develop fatal lymfoproliferative diseases soon after birth (Papiernik et al., 1998; Malek and Bayer, 2004). However, it was demonstrated that IL-2 deficient mice bear $CD4^+$ T cells capable of protection against EAE whereas CD25 deficient mice do not display this ability (Furtado et al., 2002). This finding suggests that it is IL-2 signaling that is crucial for Tregs function. It was also demonstrated that IL-2R antagonist KO mice with lymphoproliferative syndrome can be rescued by administration of $CD4^+CD25^+$ T cells and mice lacking IL-2 with a similar disease can be cured by IL-2 producing cells (Almeida et al., 2002). This finding suggests that whereas CD25 is required for the development of Tregs, IL-2 is essential for their peripheral maintenance. Apart from its role in Treg development, IL-2 is a key factor for Tregs homeostasis in the periphery. Neutralization of IL-2 triggers development of various autoimmune diseases in different mouse strains. This treatment directly reduces the number of Tregs and inhibits their proliferation (D'Cruz and Klein, 2005; Setoguchi et al., 2005). Isolated Tregs cultured without IL-2 are susceptible to apoptosis and the administration of IL-2 averts this state and restores their suppressive capacity (Levings et al., 2001; Taams et al., 2001). IL-2 also mediates the ability of Tregs to suppress memory CD8 T cell proliferation as shown by Murakami et al. (2002). On the contrary, other experiments with IL-2 and CD25 deficient mice showed that the development of functional Tregs is possible in these mice (Fontenot et al., 2005a). Instead, IL-2 seemed to be important for maintaining homeostasis and fitness of Tregs in the periphery. These studies also revealed that the γ subunit of IL-2R is absolutely essential for Treg development.

$CD4^+CD25^+$ T cells do not respond to TCR stimulation in vitro and remain in an anergic state. For activation they require much lower concentration of antigen than $CD4^+CD25^-$ require for stimulation of proliferation (Ronchese et al., 1994; Takahashi et al., 1998). In vitro stimulation of $CD4^+CD25^+$ T cells with high doses of IL-2 or anti-CD28 antibody breaks the anergic state of these cells and also abrogates their suppressive activity (Takahashi et al., 1998; Stephens et al., 2001).

1.2.3.2. Foxp3

Mouse Tregs were so far characterized by one reliable cell marker, Foxp3 which is a key regulatory gene for the development and function of Tregs (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). In comparison, human Tregs also bear Foxp3 but its expression is not exclusive for this cell type. Non-regulatory human $CD4^+$ and $CD8^+$ T cells also upregulate Foxp3 expression after stimulation in vitro. These $Foxp3^+$ cells, however, do not alter their surface markers nor do they suppress the cytokine production of activated Th1 cells (Gavin et al., 2006). The absence of Foxp3 was shown to cause a severe lymphoproliferative disease (Khattri et al., 2003). This phenotype has long been known in both, in mice as scurfy and in humans as the immune dysregulation, polyendokrinopathy, enteropathy, X-linked syndrom (IPEX) and it has been proven that both syndromes are caused by deficiency in or mutation of Foxp3 gene (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001). The Foxp3 deficient mice can be rescued by timely administration of $CD4^+CD25^+$ Tregs (Fontenot et al., 2003).

Foxp3 is a zinc-finger transcription factor which is localized in cell nucleus (Brunkow et al., 2001). Tregs develop primarily in the thymus and the expression of Foxp3 is first apparent in the CD4 single positive phase. Foxp3 is required for Tregs development and function (Fontenot et al., 2003). As a transcription factor, Foxp3 can bind to the promotor and regulatory regions of various genes and thus regulate their expression. Its target genes are e.g. genes for proinflammatory cytokines IL-2 and interferon-γ (IFN-γ) or genes for CD25 or a regulatory molecule CTLA-4. By binding to the promotors of these genes, Foxp3 can effectively inhibit or enhance the expression of these molecules. Overexpression of Foxp3 in CD4 T cells attenuates activation-induced cytokine production and proliferation (Schubert et al., 2001). Using genom-wide chromatin immunoprecipitation assay it has been shown that Foxp3 regulates the expression of plenty different genes, many of them the key components of T cell activation and function (Marson et al., 2007). Moreover, regulatory components of gene expression such as microRNA and genes for chromatin remodeling machinery were also identified as Foxp3 targets (Zheng et al., 2007a).

Foxp3 can also directly interact with other transcription factors such as nuclear factor of activated T cells (NFAT) and by blocking their function regulate different cells processes and responses. NFAT is a key regulator of T cell activation and anergy. Together with another transcription factor activator protei 1 (AP-1), NFAT activates the transcription of IL-2 gene and other T cell activation-associated genes (Jain et al., 1993). By binding to NFAT, Foxp3

represses the IL-2 transcription and drives the expression of Treg-specific molecules such as CTLA-4 and CD25. Thus, Foxp3 converts the T cell activation program into the Treg suppressive program (Wu et al., 2006). Similarly Foxp3 interacts with another transcription factor AML1 (acute myeloid leukemia 1) and prevents gene expression of IL-2 and IFN-γ (Ono et al., 2007).

Retroviral transfection of peripheral $CD4^+$ T cells with Foxp3 construct converted these into Tregs which were unable to produce IL-2 upon stimulation, upregulated CD25 and CTLA-4 and suppressed in vitro proliferation of effector cells (Fontenot et al., 2003; Hori et al., 2003; Yagi et al., 2004). The ectopic expression of Foxp3 in T cells leads to dramatically decreased proliferative responses of T cells and to a halt in IL-2 expression. Moreover, it was shown that $CD4^+$ T cells with ectopic expression of Foxp3 were able to suppress proliferation of $CD4^+Foxp3^-$ T cells in vitro and in vivo. Thus, Foxp3 expression causes generalized inability in cellular activation and renders conventional T cells suppressive and is sufficient to induce Treg phenotype in conventional $CD4^+CD25^-$ T cells (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2001; Khattri et al., 2003). In contrast, lack of functional Foxp3 gene or its mutation results in lymphoproliferative diseases cause by complete lack of Treg function (Fontenot et al., 2003; Khattri et al., 2003; Fontenot et al., 2005b). In conclusion, Foxp3 is necessary for the development and proper function of Tregs.

1.2.3.3. CTLA-4

Tregs express other molecules that are characteristic for these cells or are important for their function. One of those molecules is CTLA-4 which belongs to the CD28 family of surface molecules and is considered as negative regulator of costimulation which is normally expressed on T cells 2-3 days after activation (Walunas et al., 1994). It was stated that CTLA-4 binds to CD80/86 with higher affinity than CD28 and blocks T cell activation in vitro. As shown by Manzotti et al. (2002), CD80 is a preferential ligand for CTLA-4 compared to CD86 and the results of such interactions might be qualitatively different.

It has been demonstrated that CTLA-4 is constitutively expressed on suppressive $CD4^+CD25^+$ T cells and that the blockade of CTLA-4 abrogates their suppressive function. Moreover, in vivo blockade of CTLA-4 in normal mice leads to spontaneous development of autoimmune diseases which indicates that CTLA-4^+ CD4 T cells control self-reactive T cell clones (Takahashi et al., 2000). Similarly, selective engagement of CTLA-4 on T cells by

antigen-presenting DCs renders the T cells suppressive and capable to control and suppress autoimmune thyroiditis (Li et al., 2007a). Moreover, it was demonstrated that anti-CTLA-4 antibody prevented allogeneic rejection in mice by blocking the interaction of T cells with APCs. In addition, this antibody induced a donor-specific, long-term tolerance of the graft (Lenschow et al., 1992; Hwang et al., 2002). CTLA-4 might also be responsible for induction of peripheral T cell tolerance in vivo (Perez et al., 1997). Further studies revealed that suppressive function of Tregs in the gut is dependent on signaling through CTLA-4 (Read et al., 2000). CTLA-4 thus seems to play an important role in effector mechanisms of Tregs. CD28, another costimulatory T cell molecule, however, is not required for the suppressive function of Tregs which was shown in experiments with CD28 deficient mice. The $CD4^+CD25^+$ T cells from these animals were still able to mediate suppression and only the blockade of CTLA-4 disabled the suppression (Takahashi et al., 2000). How the suppressive action of CTLA-4 is mediated still remains a question. One possible mechanism is that CTLA-4 restricts the clonal expansion of proliferating $CD4^+$ T cells. It was shown that after approximately 3 cell divisions the Th1 and Th2 precursors start to express CTLA-4 which leads to proliferative arrest or even death of these cells. The precursors of Th1 and Th2 effector cells may be rescued from proliferative arrest by IL-12 and IL-4 signaling which is a stronger stimulus in case of infection (Doyle et al., 2001; Wells et al., 2001). In experiments with mutated extra- and intra-cellular part of CTLA-4 it was demonstrated that the cytoplazmic domain of this costimulatory molecule is required for regulation of Th cell differentiation in vivo whereas the extracellular part of the molecule can compete for ligands and thus mediate the inhibitory effect of CTLA-4 (Masteller et al., 2000).

1.2.3.4. GITR

Another molecule which is present on Tregs, belongs to the tumor necrosis factor receptor (TNFR) superfamily and is called glucocorticoid-induced TNFR-related protein (GITR). Since it can be expressed by other cell types it is not an exclusive marker of Tregs, however, it was found to be constitutively expressed on the surface of $CD4^+CD25^+$ T cells (McHugh et al., 2002, Shimizu et al., 2002). It was shown that stimulation of cells through GITR abrogated the suppression mediated by Tregs which indicates that it might have a negative role in the regulation of Tregs activity (McHugh et al., 2002). On the other hand, removal of GITR-expressing cells or administration of anti-GITR antibody led to the

induction of autoimmune diseases in mice which is consistent with the fact that T cells from GITR-deficient mice proliferate more and produce more IL-2 after activation (Ronchetti et al., 2002; Shimizu et al., 2002).

1.2.4. Mechanisms of Treg function

Although many mechanisms of Treg function have been suggested, it is not clear which can be considered as general. To date, the data acquired from functional studies of suppressive mechanisms of Tregs remain controversial with major differences between in vivo and in vitro studies. It was demonstrated that the prevailing mechanism of action of Tregs in vitro is contact dependent (Thorton and Shevach, 2000; Taams et al., 2001). On the other hand, in vivo mechanisms range from suppressive cytokines to cytokine starvation and even cytotoxicity.

In general, there are four major types of suppression which will be discussed in the next chapters and those are: production of soluble factors such as cytokines, targeting DCs and modulating their function, direct cytolysis of target cells or their metabolic disruption (Figure 2).

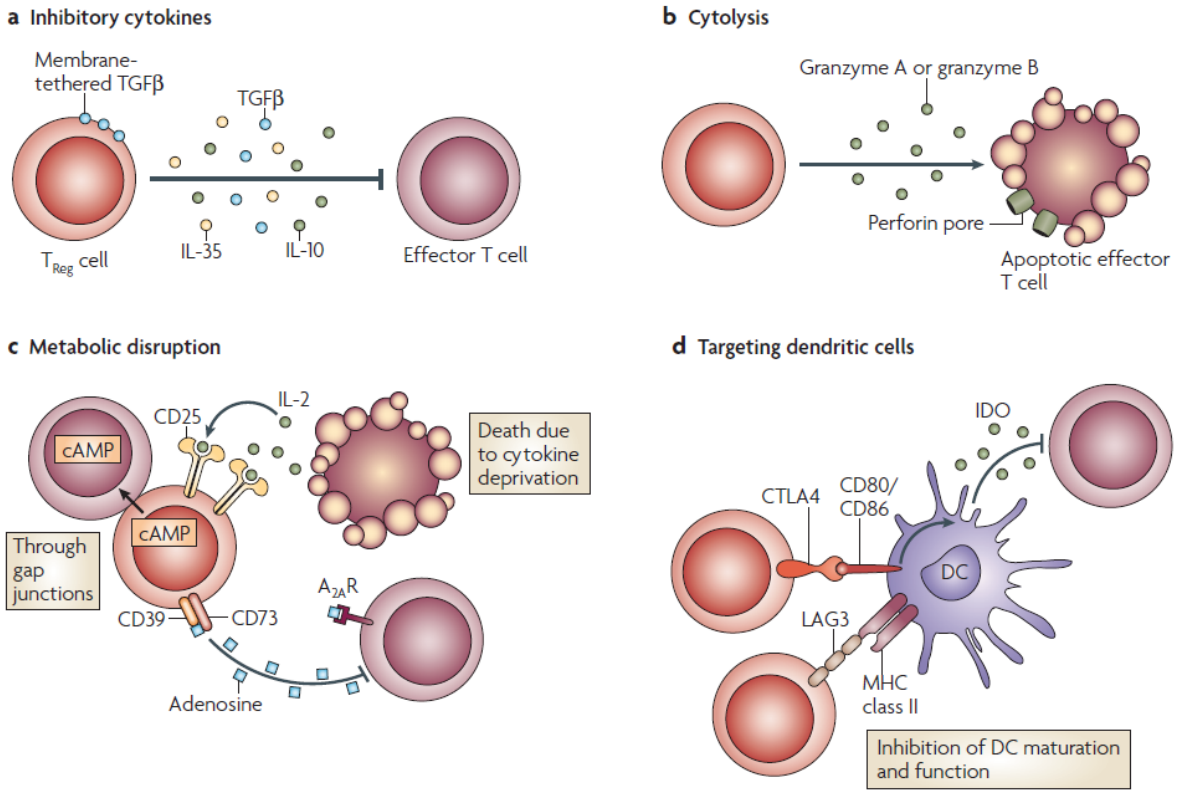

Figure 2: Basic mechanisms of Treg function. (a) Inhibitory cytokines including IL-10, TGF-β and IL-35. (b) Cytolysis dependent on granzyme A, B and perforin. (c) Metabolic disruption of target cells. (d) Modulation od DC function. Adapted from Vignali et al., 2008.

1.2.4.1. Production of inhibitory cytokines

Tregs can secrete several cytokines that have immunosuppressive functions. It has been demonstrated that IL-10 plays an important role in Tregs suppression, especially in vivo where it inhibits the expansion of effector T cells (Annacker et al., 2001). TGF-β is another cytokine that is produced by Tregs and that regulates the immune system. Its immunosuppressive function has been demonstrated on many models. Induction of autoimmune disease or inflammation such as colitis in adult mice can be prevented by administration of Tregs. This protective effect, however, can be abrogated by injection of anti-TGF-β mAb (Seddon and Mason, 1999; Nakamura et al., 2004). Powrie and Mason (1990) also suggested a role for CD45RBlow $CD4^+$ T cells that are able to suppress autoagressive T cell clones. It was shown that a Th1-mediated colitis was successfully treated by a transfer of these CD45RBlow T cells. Later it was demonstrated that this protecting effect was reversed by an anti-TGF-β antibody which suggested a key role of TGF-β in the mechanism of suppression of inflammation in the gut (Powrie et al., 1996; Fuss et al., 2002). Some groups also pointed out that TGF-β which is bound to the surface of Tregs might play an important role in suppression. TGF-β neutralization by mAb abolished the Treg-mediated suppression of $CD4^+CD25^-$ T cells stimulated by plate-bound antibody (Nakamura et al., 2001). Others showed, however, using TGF-βR or TGF-β deficient mice, that Treg-mediated suppression in these animals was not abrogated (Piccirillo et al., 2002).

In addition to IL-10 and TGF-β there has been another cytokine proposed to play an important role in Tregs function (Collison et al., 2007). IL-35 is secreted exclusively by Tregs and consists of two different subunits p35 and Epstein-Barr virus induced gene 3 (Ebi3). p35 is an alpha subunit of IL-12 and thus IL-35 is another member of heterodimeric IL-12 family. These cytokines are, however, mostly considered as proinflammatory. IL-35, in contrast, was shown to have inhibitory properties. Deficiency of its subunits leads to autoimmune diseases in vivo and reduced suppressive activity in vitro. On the other hand, its ectopic expression renders naïve T cells suppressive and recombinant IL-35 suppresses T cell proliferation (Collison et al., 2007). This novel inhibitory cytokine might play an important role in Treg-mediated suppression.

1.2.4.2. Cell contact with DCs and modulation of DC function

There is multiple evidence of direct interaction of Tregs with DCs. Tang et al. (2006) used a two photon microscopy technique to demonstrate that Tregs form stable contacts with DCs in lymph nodes. In a model of non-obese diabetic mice, the auto-reactive $CD4^+CD25^-$ T cells could not be activated by DCs bearing the autoantigen because of stable associations of Tregs with DCs. Thus, Tregs prevent the priming of diabetogenic effector T cells and it was also shown that they decrease the duration of the contacts between T cells and antigen-loaded DCs (Tadokoro et al., 2006). In vitro studies demonstrated that nTregs downregulate the expression of costimulatory molecules CD80 and CD86 on APCs. Their expression was inhibited even when APCs were stimulated to express higher levels of costimulatory molecules (Cederbom et al., 2000). Another group showed that also in vivo antigen-induced iTregs use downregulation of costimulation as an effector mechanism of suppression (Grundström et al., 2003). Later it was demonstrated that this ability of Tregs was dependent on their expression of CTLA-4 because a specific blockade of this molecule disabled Tregs to downmodulate the CD80/86 molecules (Oderup et al., 2006). Thus, CTLA-4 seems to play a non-redundant role in this mechanism of Tregs suppression since anti-CTLA-4 antibody completely abrogated their suppressive function (Tang et al., 2004). Moreover, another effect of Tregs on APCs might occur. Downregulation of costimulatory molecules occurs when immature APCs are stimulated, however, the stimulation of mature APCs leads to their apoptotic cell death mediated by Tregs (Frasca et al., 2002).

Tregs can suppress APCs by modulation of the capacity of APCs to activate other T cells. Coculture of APCs with Tregs rendered APCs unable to activate naïve T cells and the APCs retained these suppressive features permanently also after removal of Tregs from the culture (Taams et al., 2000). This study thus indicated a possible mechanism by which Tregs maintain the pool of suppressor T cells. APCs stimulated by lipopolysaccharide (LPS) after such culture inhibited the proinflammatory cytokine production and T cell activation (Taams et al., 2005).

Another type of APCs are monocytes and their tissue form macrophages. These cells can be also affected by coculture with Tregs to become alternatively activated macrophages. Such cells lose the ability to become good APCs but gain rather anti-inflammatory and regulatory functions (Tiemessen et al., 2007).

The suppressive function of Tregs can be attenuated in certain conditions. A strong microbial stimulation which triggers toll-like receptors (TLRs) on DCs leads to the production

of IL-6 which acts in favour of proinflammatory response rather then responses dependent on Tregs (Pasare and Medzhitov, 2003). In contrast, it was shown that LPS-stimulated Tregs have higher suppressor activity (Caramalho et al., 2003).

1.2.4.3. Metabolic disruption of effector cells

It has been shown that Tregs might interact with their target cells and modulate their metabolic activity in order to suppress their function. Several studies revealed that Tregs might stimulate the ability of DCs to catabolize tryptophan. The expression of CD80/86 molecules on DCs and CTLA-4 on Tregs was required for this type of interaction (Fallarino et al., 2003). Tryptophan can be degraded by the enzyme indoleamine 2,3-dioxygenase (IDO) and subsequent tryptophan starvation can inhibit proliferation of T cells in vivo (Munn et al., 2004).

It is known that IL-2 is an activator of Tregs suppressive function and expansion in vitro and in vivo (Thorton et al., 2004; Brandenburg et al., 2008). Tregs are anergic in vitro even after TCR stimulation or the addition of IL-2 alone. Coculture of these cells with activated $CD4^{+}CD25^{-}$ cells inhibits the production of IL-2 by the effector cells and thus leads to suppression of proliferation of the responder cells. In this system the Treg-mediated suppression is cytokine independent, cell-contact dependent and requires activation of Tregs through TCR. Moreover, this inhibition could be overcome by addition of IL-2 or anti-CD28 to the cocultures (Thorton and Shevach, 1998). Experiments with mice transgenic for a certain antigen showed that when Tregs are activated through their TCR they are able to inhibit immune responses to a different antigen and thus their effector mechanism is antigen-nonspecific (Thorton and Shevach, 2000). Competition for IL-2 was shown to be one of the effector mechanisms of Tregs (Pandiyan et al., 2007). It was shown that Tregs are able to bind and intake great amounts of IL-2 due to the high expression of CD25 on their surface. Thus, Tregs cause IL-2 starvation of effector T cells which can lead even to apoptotic death. It was also suggested that the uptake of IL-2 might be a flexible mechanism by which Tregs adapt their activity to the strength of the responder T cell reaction (de la Rosa et al., 2004).

1.2.4.4. Direct cytolysis of target cells

Cytotoxic killing of various cell types is one of the effector mechanisms of Tregs. It was demonstrated that activated Tregs express granzyme A and are able to kill effector T cells and DCs (Grossman et al., 2004). Similarly, the expression of granzyme B by Tregs which caused the lysis of activated lymphocytes was shown (Gondek et al., 2005). Granzyme B deficient mice were able to reject tumor cells more efficiently than wild type mice (Cao et al., 2007).

Another cell population which can be lysed by Tregs is B cells. Tregs were shown to be potent suppressors of B cell proliferation induced by B cell mitogens. The inhibition was in this case cell contact-dependent and caused by an increased death rate of B cells. Surprisingly, the cell-mediated apoptosis was caused by production of perforin and granzyme by Tregs rather than dependent on a more common T cell killing mechanism that is FasL (Zhao et al., 2006).

1.3. Development of Tregs

According to the developmental criteria we can divide Tregs into two distinct subpopulations, naturally occuring (n) and induced (i) Tregs. nTregs are a constitutive part of a healthy immune system. iTregs, on the other hand, develop under specific conditions and might be a useful tool for manipulating the immune system. The possibility of iTregs induction is thus an important goal of clinical immunology.

1.3.1. nTregs development

nTregs develop in the thymus like conventional T cells. Their development is dependent on major histocompatibility antigen (MHC) class II^+ thymic cortical epithelium (Bensinger et al., 2001). It is also likely to be directed by medullary thymic epithelial cells which express a transcription factor autoimmune regulator (AIRE) (Zuklys et al., 2000). These thymic cells have a wide range of self-antigens on their surface and together with MHC class II they control the development of Tregs (Aschenbrenner et al., 2007). It was indicated that AIRE regulates the processing and presentation of self-proteins so that the thymocytes recognize the self-antigen and are able to develop into autoreactive Tregs (Kuroda et al., 2005). Indeed it was previously shown that $CD25^+$ thymocytes have self-antigen specificity and the affinity of their TCR to the antigen must be relatively high (Jordan et al., 2001). In experiments with transgenic mice that expressed ovalbumin (OVA) antigen it was shown that the T cells with TCR specific for OVA developed as $CD25^+$ Tregs (Kawahata et al., 2002). TCR on Tregs is therefore self-specific but they are not eliminated by negative selection because they probably posses mechanisms by which they avoid this process (Jordan et al., 2001). One of the possible mechanisms is the presence of the molecule GITR on their surface. GITR can mediate anti-apoptotic signals to the cell which render the cell resistant to apoptosis (McHugh et al., 2002). Tregs are released from thymus into circulation and can later settle in lymphoid tissue.

The induction of Foxp3 in thymus and the conditions in which it occurs are still unclear. Recently it has been shown that myeloid DCs present in thymic stroma express

receptors for thymic stromal lymphopoietin (Hanabuchi et al., 2010). In response to this factor myeloid DCs produce chemokines that attract T cells but they don't produce Th1 or Th2 polarizing cytokines. It was shown that such myeloid DCs induce the differentiation of thymocytes into Tregs and probably also induce the expression of Foxp3 in these cells (Hanabuchi et al., 2010). Moreover, Tregs development is dependent on the presence of recombination activating gene (RAG)-2 rekombinase and Tregs exhibit a classical αβTCR on their surface (Itoh et al., 1999). It has also become apparent that the costimulatory molecule CD28 is indispensable for the thymic development of T cells. By the use of molecular mapping of CD28 costimulation it was demonstrated that CD28 signaling induces expression of Foxp3 and thus directly initiates Tregs differentiation program (Tai et al., 2005). Moreover, CD28 maintains peripheral homeostasis of Tregs probably by regulating IL-2 production by other cells and thus supporting Tregs survival and self-renewal (Salomon et al., 2000; Tang et al., 2003). Recently, the crucial role for TGF-β in thymic development was clarified. The conditional deletion of TGF-β gene expression in T cells at postnatal days 3-5 lead to a complete blockade of appearance of nTregs. Interestingly, when the TGF-β expression was abrogated one week after birth, nTregs developed normally but the presence of IL-2 in this situation was essential for nTregs development. Thus, TGF-β signaling was shown to be of utmost importance even in the development of nTregs (Liu et al., 2008). Similar results were achieved by Marie et al. (2005) who showed that TGF-β deficiency at days 8-10 after birth does not abrogate thymic development of Tregs but rather decrease the numbers of Tregs in the periphery. They concluded that TGF-β signaling is important for maintenance of Foxp3 expression and suppressive function of peripheral Tregs.

Although it was hypothesized that the antigen specificity of nTreg is mostly restricted to self-antigens, their repertoire seems to be wider. nTregs have been shown to recognize foreign antigens too. It was demonstrated that the nTregs that accumulate at the site of chronic infection with Leishmania major in mice are able to respond specifically to the Leishmania antigen. Such Tregs strongly proliferate in response to Leishmania-infected DCs, they maintain Foxp3 expression, and Leishmania-specific Treg cell lines can be generated from infected mice (Suffia et al., 2006). Moreover, the persistence of Leishmania in the skin is controlled by Tregs that suppress the ability of $CD4^{+}CD25^{-}$ effector T cells to eliminate the parasite (Belkaid et al., 2002).

Until recently, no specific marker which would distinguish nTreg from other Tregs was known. However, a transcription factor Helios, a member of the Ikaros transcription factor family, has recently been assigned a distinct role in nTreg development. It was shown

that Helios is expressed by 100% of $CD4^+CD8^-Foxp3^+$ thymocytes and 70% of peripheral $Foxp3^+$ T cells. On the other hand, this transcription factor was neither found in any kind of iTregs that were prepared in vitro by stimulation with TGF-β nor was it found in iTregs induced in vivo by antigen feeding under specific conditions (Thorton et al., 2010). Thus, Helios seems to be a unique marker of nTreg that develop in thymus. This observation also indicates that thymus might not be the only site of nTregs generation. There is some evidence that $CD4^+CD25^+CD45RB^{low}$ cells that possess suppressive activity are generated in the periphery. These cells were described to have a broad TCR repertoire and memory phenotype with short telomeres which indicates that they were repeatedly stimulated by a specific antigen to proliferate in vivo. These Tregs might thus induce tolerance to antigens that are not encountered in thymus (Taams et al., 2002).

1.3.2. iTregs development

iTregs, compared to nTregs, can develop at any time during the whole life but only under specific conditions in the periphery. There have been reported many ways of iTreg induction in vivo in the periphery or in vitro. The conditions, in which iTregs arise, however, seem to be very distinctive. It was demonstrated that the conversion can be achieved in vivo by small antigen doses with suboptimal DC activation. In such conditions naïve $CD4^+$ T cells are converted into $Foxp3^+$ Tregs which possess regulatory activity. Their induction can be further enhanced by the addition of TGF-β (Kretschmer et al., 2005). Another important player in the generation of iTregs, especially in the gut, is DCs-derived retinoic acid which probably acts together with TGF-β and helps the conversion process by counteracting the negative impact of costimulation (Apostolou et al., 2008).

In conclusion, iTregs develop from naive $CD4^+Foxp3^-$ T cells upon stimulation through the TCR and in the appropriate cytokine environment. The essential cytokine for their development is TGF-β (Chen et al., 2003a). It is a pleiotropic cytokine which has an impact on many different cell types, not only of immune character. TGF-β has long been known as negative regulator of immune cells, regulator of cell phenotype and cell adhesion. It regulates proliferation and differentiation of a wide spectrum of cell types and thus has an important role in homeostasis (Massague et al., 1992). TGF-β signaling is also essential for the maintenance of peripheral tolerance. With the use of TGF-RII deficient mice, it was

demonstrated that these mice develop fatal autoimmunity mainly due to the activation and expansion of Th1 cells, cytotoxic T cells and NK cells (Marie et al., 2006).

Development of iTregs thus encompasses many different mechanisms which depend on the chosen model and conditions. Detailed description of in vitro mechanisms of iTregs generation will follow in the next chapters.

1.4. The role of cytokines in development of Tregs and Th cells

The developmental pathways of Tregs and Th cells are diverse and their determination depends on many factors. The quality and quantity of TCR stimulation and cytokine environment at the site of T cell priming are the most important aspects. In this chapter the role of cytokines in the development of Tregs will be described.

1.4.1. Generation of iTregs

The development of iTregs has always been an intriguing question. The conditions and the original T cell populations from which they arise seem to be diverse. However, the possibility of in vitro manipulating Tregs population is tempting and provides many opportunities for clinical usage.

The expansion of a certain Treg population might be a difficult task. Instead, a great effort was put into finding ways of Treg induction from non-Treg precursors. Several groups demonstrated that TGF-β can trigger Foxp3 expression in $CD4^+CD25^-$ T cells (Fu et al., 2004; Park et al., 2004). The conditions for this induction have, however, not been well described. It was suggested that stimulation of precursor cell is required for the induction of Foxp3 but the costimulation of CD28 did, in contrast, prevent this induction (Fu et al., 2004). Similarly, the necessity to stimulate TCR in the presence of TGF-β was shown in order to induce suppressive cells which produce TGF-β and inhibit cell proliferation (Chen et al., 2003a). TCR engagement can be performed with the use of antigen presented by APCs but also by general stimulation of TCR through anti-CD3 mAb (Rao et al., 2005). Fantini et al. (2004) also showed that TGF-β induces Foxp3 expression in $CD4^+CD25^-$ T cells. Moreover, they pointed out that Smad7 which plays a negative regulatory role in TGF-β signaling is downregulated by Foxp3 and thus revealed a positive regulatory loop in TGF-β-triggered induction of iTregs. TGF-β signaling is probably not sufficient to induce Foxp3 expression in naïve T cells. Studies with IL-2 conditional KO mice revealed that IL-2 is necessary to convert $CD25^-$ cells into $CD25^+Foxp3^+$ Tregs and also to sustain Foxp3 expression and suppressive function of these cells (Davidson et al., 2007; Zheng et al., 2007b).

In vitro TGF-β-induced iTregs significantly suppressed colitis when administered to diseased animals (Fantini et al., 2006). Moreover, IL-2 that is secreted by effector T cells at the site of inflammation was confirmed to be crucial for survival and expansion of iTregs. TGF-β was convincingly proven to induce Foxp3 expression in naïve cells in certain conditions, however, the exact mechanism of this induction remains elusive. Recently it was shown that TGF-β signaling triggers expression of two members of the Runt-related transcription factor family, RUNX1 and RUNX3. These factors bind to three distinct regions on Foxp3 promotor and probably initiate the expression of this Tregs master gene (Klunker et al., 2009).

It was observed in vivo that antigen-specific Th3 regulatory cells which produce TGF-β can induce the differentiation of Tregs and thus maintain peripheral tolerance to various antigens (Oida et al., 2006; Carrier et al., 2007a,b). Another possible source of TGF-β in vivo for the induction of iTregs might be Tregs that are already present in the system and become activated. It was shown that such cell express on their surface TGF-β that is bound to latency-associated peptide. These cells can induce the TGF-β-dependent differentiation of iTregs in a contact-dependent manner and thus spread and strengthen suppression (Andersson et al., 2008). TGF-β can be secreted also by a distinct type of stem cells with immunoregulatory properties which are described in the next chapter.

1.4.2. The role of mesenchymal stem cells in the generation of Tregs

Another type of immunomodulatory cells which can profoundly affect immune reactions are mesenchymal stem cells (MSCs). These cells are a subtype of adult stemm cells and can be found and isolated from bone marrow, adipose tissue and other organs and tissues in the body. After appropriate stimulation these cell are capable of differentiation into several cell types and importantly, they can produce soluble immunomodulatory factors. MSC affect functions of a spectrum of immune cells of both, innate (DCs, NK cells, neutrophils) and adaptive (T and B cells) immune system. Thus, MSCs were shown to inhibit maturation of DCs and their antigen presenting propertis which hinders inflammatory potential of DCs. In addition, it was demonstrated that MSCs inhibit mitogen- and alloantigen-induced T cell proliferation and cytokine production (Uccelli et al., 2008).

MSC behaviour and effects on other cells vary in different conditions. Cytokine environment is the decisive factor which influences the outcome of MSC responses. It has been demonstrated that MSCs can upregulate their surface expression of MHC class II and

thus can interact with CD4+ T cells. MHC class II expression can be elevated by IFN-γ. However, only low levels of IFN-γ favour MHC class II expression which decreases again with higher IFN-γ levels in the culture (Chan et al., 2006). As indicated, the cytokine environment is crucial for MSC features and function but the data in this field are scarce and controversial.

Immunomodulation mediated by MSCs is dependent on cell-to-cell contact and soluble factors such as cytokines. In a quiescent state, MSCs produce TGF-β and it was demonstrated that such production can induce differentiation of Tregs (Di Nicola et al., 2002). Moreover, in a pro-inflammatory environment MSCs can produce simultaneously TGF-β and IL-6. This cytokine environment creates ideal conditions for the preferential induction of inflammatory Th17 cells (Di Nicola et al., 2002).

1.4.3. Other important factors in iTreg development

Another candidate molecule necessary for the induction of iTregs is CTLA-4. Experiments of CTLA-4 deficient mice revealed that although the development of nTregs in these mice is normal, the differentiation of iTregs is impaired. CTLA-4 signaling might therefore be crucial for the induction of Foxp3 and suppressive activity in $CD4^+CD25^-$ T cells (Zheng et al., 2006a).

An experimental model of allogeneic transplantation showed that ex vivo TGF-β-induced Tregs injected together with alloantigen evoke transplantation tolerance (Zheng et al., 2006b). The mechanism by which the suppressor ability is spread through generation of suppressor cells was indicated previously by the same group and encompasses cell contact as well as the effect of TGF-β and IL-10 (Zheng et al., 2004). Similarly, it was shown that the addition of IL-10 and TGF-β to an allogeneic mixed lymphocyte culture resulted in antigen-specific hyporesponsiveness to alloantigens (Chen et al., 2003a). $CD4^+$ T cells recovered from such cultures were capable of protecting from GVHD. The role of CD28 in the induction of iTregs is still controversial. It was shown, in CD28 deficient mouse, that it is possible to convert $CD25^-$ T cells into $Foxp3^+$ iTregs using TGF-β and antigen stimulation. The resulting populations displayed comparable qualities to nTregs, however, the survival of these cells was decreased. CD28 signaling might thus contribute to maintenance of survival of iTregs (Liu et al., 2006).

It is well known that the mucose-associated immune system in the gut exerts specific features and has evolved multiple strategies to maintain homeostasis and to favor induction of tolerance. The mechanisms of such tolerance are probably diverse but it was shown that cells with regulatory characteristics can be induced in the gut from non-regulatory precursors. A specialized population of DCs which are $CD103^+$ and present in lamina propria selectively drive the development of $Foxp3^+$ Tregs via a production of vitamin A metabolite, retinoic acid in a process dependent on TGF-β which is abundant at this site (Coombes et al., 2007; Sun et al., 2007). A protocol employing TGF-β and retinoic acid promises an efficient way for ex vivo production of large numbers of Tregs with good suppressive function (Wang et al., 2009). In vivo it was demonstrated that Tregs can be induced also by contact with a special type of DCs that matured in the presence of IL-10 and that were suggested to induce oral tolerance and to prevent allergy (Huang et al., 2010). In vitro it was shown that such induction can be performed with allogeneic DCs and thus alloantigen-specific Tregs can be produced (Moore et al., 2010). DCs in the periphery play an important role in clearance of apoptotic cells. It was shown that the uptake of apoptotic thymocytes renders the DCs tolerogenic. In addition, DCs pulsed with allogeneic apoptotic cells have an increased capacity to induce the development of fully functional iTregs in a cell contact-dependent manner (da Costa et al., 2011). Another agent affecting the induction of iTregs is the costimulatory molecule OX40. It was shown that OX40 signaling inhibits the TGF-β mediated development of iTregs and rather supports the development of effector T cells (So and Croft, 2007).

A distinct type of suppressive iTregs was shown to develop from $CD4^+CD25^-$ T cells in coculture of these cells with nTregs. The newly generated iTregs were anergic to stimulation with anti-CD3, responded to IL-2 and were capable of suppressing the proliferation of $CD4^+CD25^-$ T cells (Qiao et al., 2007). It was thus shown that converting $CD25^-$ T cells into suppressive iTregs might also be another mechanism of suppression employed by nTregs.

The regulation of iTregs induction is a very complex process and many new players affecting it still emerge. Transcription factor interferon regulatory factor 1 was indicated as just another transcription factor which binds to the Foxp3 promotor and represses transcription of this gene. Mice deficient for this transcription factor exhibited a strong tendency of $CD4^+CD25^-$ T cells to convert into Tregs and all Tregs had enhanced suppressive activity (Fragale et al., 2008). Another important transcription factor that is essential for the induction of Foxp3 expression is signal transducer and activator of transcription (STAT)3. Ablation of this molecule leads to abrogation of suppressive activity of Tregs and restores IL-

2 and IFN-γ production by these cells. STAT3 inhibits Foxp3 expression in nTregs and induction of Foxp3 in the absence of STAT3 is also prevented (Pallandre et al., 2007). Transcription factors acting upstream of Foxp3 may thus be a potential target for manipulation of the immune system.

It is well known that during chronic inflammatory diseases Tregs fail to maintain homeostasis probably because activated effector cells prevent the induction or proper function of Tregs. It was demonstrated that IL-6 signaling into T cells completely abrogates induction of iTregs by increasing the expression of TGF-β signaling inhibitor SMAD-7 which renders the cells resistant to Foxp3 induction. Moreover, IL-6 signaling inhibited Tregs-mediated suppression of autoimmune colitis in vivo (Dominitzki et al., 2007). In vivo it was demonstrated that IL-6 produced after allogeneic renal transplantation gradually decreases the number of Tregs in the graft and its blockade prolongs graft survival (Wang et al., 2008).

The origin of iTregs is still not clearly defined also due to their diversity and number of experimental settings. It was speculated that nTregs are the parent population from which iTregs develop (Yamagiva et al., 2001). Induction of transplantation tolerance which is triggered by anti-CD4 generates iTregs. It was shown recently that these iTregs arise in vivo from both, $CD4^{+}Foxp3^{-}$ T cells and also from nTregs (Francis et al., 2011).

Epigenetic control of gene expression is one of key aspects of cell differentiation. Foxp3 as a master switch of Tregs and its regulatory sequences were analyzed by bisulphite sequencing and a Treg-specific demethylation region was discovered within Foxp3 locus (Floess et al., 2007). It was shown that DNA was completely demethylated in this region in nTregs but not in conventional T cells. Moreover, DNA in TGF-β-induced iTregs was only incompletely demethylated despite high Foxp3 expression (Floess et al., 2007). This finding suggests that epigenetic modification of Foxp3 plays an important role in Treg differentiation and maintenance. Later, it was showed that mere inhibition of DNA methylation by demethylating agent azacytidine promoted de novo induction of Foxp3 expression in T cells and stabilized expression of this transcription factor after restimulation (Polansky et al., 2008). Both these findings demonstrate that epigenetic imprinting in Foxp3 locus is critical for establishment of a stable Treg lineage. Similarly, it has been shown that a histone-deacetylase inhibitor increased expression of Foxp3 and production of Tregs in vivo. Moreover, this inhibitor, together with rapamycin, induced permanent and Treg-dependent allograft survival and donor-specific allograft tolerance (Tao et al., 2007).

Th2 cells were originally regarded as a potential regulatory population mainly owing to the production of IL-10 (Mosmann and Coffman, 1989). These cells also produce

substantial amounts of IL-4 and IL-13 and these cytokines were then suggested as inducers of iTregs from conventional peripheral $CD4^+CD25^-$ T cells. This Treg generation was dependent on antigen stimulation and costimulation of T cells but independent of IL-10 or TGF-β. The importance of this process was demonstrated in a model of oral tolerance where the blockade of IL-4 and IL-13 prevented the induction of iTregs (Skapenko et al., 2005). Another cytokine that has been regarded as proinflammatory was shown to induce functional Tregs. Ex vivo T cells stimulated by allogeneic APCs and in the presence of IFN-γ differentiated into suppressive $Foxp3^+$ Tregs. The authors speculate that in this case IFN-γ inhibits production of IL-4 which is detrimental for allograft survival which also indicates some controversy with the results of Skapenko's group (Feng et al., 2008). In contrast it was shown that IL-4 supports proliferation and suppressive competence of nTregs (Pace et al., 2006). It was demonstrated in vitro that isolated nTregs gradually lose Foxp3 expression and often undergo apoptosis. However, Tregs exposed to IL-4 maintain the level of CD25 and had better suppressive functions (Maerten et al., 2005). In addition to this controversial role of IL-4 in iTreg generation, it might also have distinct effect on suppressive function of Tregs which are able to inhibit proliferation of effector $CD4^+CD25^-$ T cells. It was demonstrated that engagement of IL-4 receptor on target effector cells renders these cells resistant to suppression (Pace et al., 2006). Indeed, it was shown that Tregs are able to completely suppress Th1 cell proliferation but only partly proliferation of Th2 clones (Cosmi et al., 2004). This might be caused be a higher expression of IL-4 receptor and IL-9 receptor on Th2 cells. Moreover, the addition of IL-4 to the culture significantly reduced the Treg-mediated suppression (Cosmi et al., 2004).

The development of iTregs is currently an intensively studied field. However, the findings are frequently different in various models and often even contradictory. Further research is needed to clarify all the aspects of this important issue in Treg biology.

1.5. Developmental plasticity of Th cells

In this chapter the main Th cell subsets will be introduced together with cytokines that drive their differentiation. In addition, the complex relationships between the Th lineages will be discussed. Th cell types are represented by proinflammatory Th1 and Th17 cells and by Th2 cells. Their regulatory counterparts are different types of Tregs.

1.5.1. Th1 and Th2 cells

Th1 cells which play an essential role in defence against intracellular pathogens, are characterized by production of IL-2 and IFN-γ upon activation. Their development is driven by IL-12, a proinflammatory cytokine produced mainly by cells of the innate immune system such as DCs (Mannetti et al., 1993; Macatonia et al., 1995). Th2 cells are crucial in defence against extracellular pathogens and their main effector cytokines are IL-4, IL-5, IL-10 and IL-13. Development of Th2 cells from uncommitted precursors is dependent on IL-4 (Swain et al., 1990).

Cytokines produced by Th1 and Th2 cells reciprocally regulate the development of these cell populations (Figure 3). IL-4 inhibits IL-12Rβ2 expression and thus represses the IL-12-mediated signaling and development of Th1 cells. Furthermore, IL-10 affects DCs and inhibits their ability to activate proliferation of Th1 cells and also reduces the production of Th1 cytokines in vitro (Kadowaki, 2007). Moreover, in vivo studies revealed that IL-10 synergizes with IL-4 to inhibit the proinflammatory activity of Th1 cells in delayed type of hypersensitivity (DTH) setting (Powrie et al., 1993). In addition, IL-4 was also able to inhibit IFN-γ production after antigen challenge comparably to IL-10 (Powrie et al., 1993). In contrast, IFN-γ promotes IL-12Rβ2 expression and enhances IL-12 responsiveness (Murphy et al., 1999). IL-12 induces IFN-γ production in naïve T cells and inhibits the development of IL-4 producing cells (Manetti et al., 1993). Moreover, IL-12 was also able to induce a transient IFN-γ production in Th2 cell clones (Manetti et al., 1994).

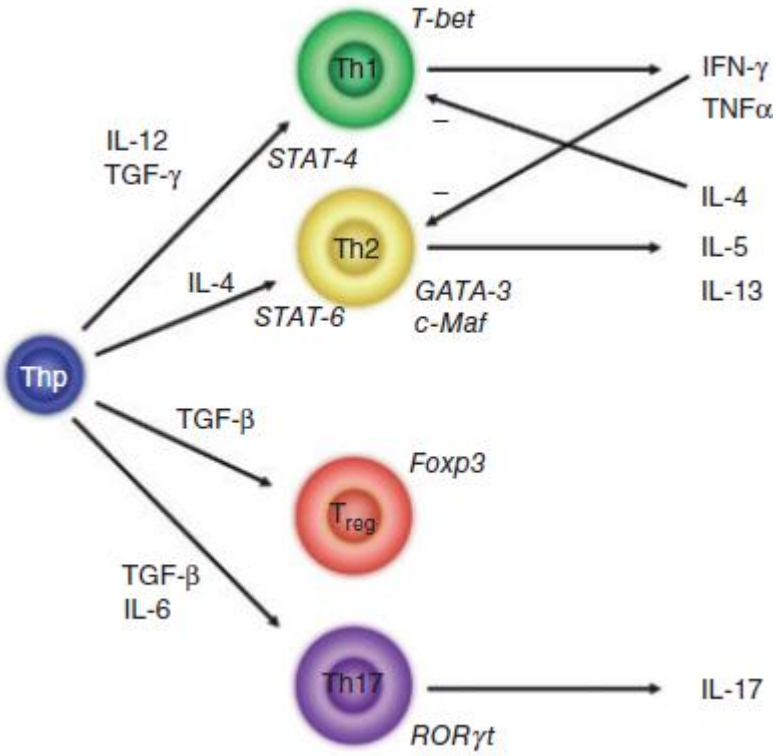

Figure 3: Th cell commitment towards specific lineages in mice. Th cell precursor (Thp) can be skewed towards mutually exclusive Th1, Th2, Th17 and Treg phenotypes on the basis of the cytokine environment. Adapted from Afzali et al., 2007.

1.5.2. Transcription factors in the development of Th1 and Th2 cells

Cell development is also regulated on the level of transcription factors. Each cell lineage expresses its unique transcription factor which is a master regulator of cell-specific transcription. Th1 cells are characterized by the presence of a specific transcription factor T-bet. This factor transactivates expression of IFN-γ, the main cytokine produced by Th1 cells (Szabo et al., 2000). Similarly to reciprocal regulation of Th cell subsets by cytokines, transcription factors regulate cell fates in a complex way. Forced expression of T-bet in highly differentiated Th2 cells decreases expression of IL-4 and induces production of IFN-γ providing a possibility to change cell fate and treat Th2-mediated diseases (Lametschwandtner et al., 2004). Another transcription factor RUNX3 is expressed in Th1 cells in a T-bet-dependent manner. It was shown that RUNX3 is, together with T-bet required for IFN-γ expression and for silencing the IL-4 gene. Both transcription factors bind to IFN-γ

and IL-4 promotor. It was demonstrated in RUNX3 KO mice that RUNX3 is essential for transcriptional repression of IL-4 and thus Th2 development (Djuretic et al., 2007). A conserved silencer of the IL-4 gene which is critical for the development of Th1 cells was identified. Its deletion causes IL-4 production in IFN-γ-differentiated Th1 cells and thus impaires function of these cells (Ansel et al., 2004).

Th2 cells selectively express transcription factor GATA3. It was shown that antisense GATA3 inhibited the expression of Th2 cytokine genes and that GATA3 directly activated IL-4 gene promoter (Zheng and Flavell, 1997). GATA3 was confirmed as a master switch for Th2 cells in an experiment where it was able to reconstitute Th2 cytokine expression and other markers of Th2 cells which were deficient for STAT6, a transcription factor activated by IL-4 signaling (Ouyang et al., 2000). GATA3 conditional KO mice were unable to differentiate T cells in Th2 direction. Instead, Th1 differentiation occurred even in the absence of IL-12 and IFN-γ. Moreover, GATA3 represses IL-12 signaling and production of Th1 cytokines (Ouyang et al., 1998). Therefore, GATA3 is not only a master transcription factor of Th2 cells but it also seems to be a principal switch determining Th1/Th2 responses (Zhu et al., 2004). Recent developments also revealed that specifically phosphorylated T-bet can interact with GATA3 and prevent its binding to DNA and thus repress Th2 lineage commitment (Hwang et al., 2005). Moreover, the ectopic expression of GATA3 in fully differentiated Th1 cells induced expression of Th2 cytokines IL-4 and IL-5 in these cells and at the same time downregulates their IFN-γ production (Ferber et al., 1999; Lee et al., 2000). The phenotype of committed Th1 cells was previously considered as irreversible, however, these experiments proved that GATA3 expression can change the phenotype of differentiated cells. Similarly, the ectopic expression of STAT6 which is also critical for Th2 differentiation, induced GATA3 and Th2 phenotype in developing Th1 cells (Kurata et al., 1999). It was shown that T-bet- and GATA3-dependent differentiation programs are cell cycle-dependent and thus proliferation is necessary for terminal differentiation of Th1 and Th2 cells (Mullen et al., 2001).

1.5.3. Th17 cells

Th17 cells are another type of proinflammatory Th cells. They are characterized by the production of IL-17 which drives inflammatory responses. Th17 cells develop from naïve CD4 T cells in the presence of TGF-β and IL-6 and this process is further amplified by IL-1β

and tumor necrosis factor-α (TNF-α) and other inflammatory stimuli such as TLR stimulation (Veldhoen et al., 2006). TGF-β-dependent Tregs development is in this case subverted by IL-6 which therefore decides about the pro- or anti-inflammatory cell fate (Bettelli et al., 2006). Another important cytokine for Th17 cells is IL-23 which is not a differentiation factor for Th17 cells but is required for their proliferation (Betelli et al., 2006). TGF-β and IL-6 were shown to induce the expression of IL-23 receptor rendering the developing Th17 cells sensitive to its effects – induction of retinoic acid receptor-related orphan receptor (ROR)-γt and IL-17 expression (Mangan et al., 2006; Zhou et al., 2007). Recent findings suggested, however, that Th17 might develop even in the absence of TGF-β. The combination of IL-6, IL-23 and IL-1β effectively induced the differentiation of Th17 cells (Ghoreschi et al., 2010).

A unique transcription factor of Th17 cells is ROR-γt. It was described that ROR-γt deficient mice lack tissue-infiltrating Th17 cells and that ROR-γt induces the expression of the gene for IL-17 (Ivanov et al., 2006). Furthermore, another transcription factor ROR-α was shown to induce IL-17 expression and development of Th17 cells. Only double KO of ROR-γt and Ror-α completely prevents generation of Th17 cells (Yang et al., 2008a).

Many factors can affect the balance between the TGF-β-dependent development of Tregs or Th17 cells. It was indicated that the development of IL-17-producing cells from naïve T cells is inhibited by IFN-γ and IL-4 although mature Th17 cells were no longer sensitive to inhibition by these cytokines (Harrington et al., 2005). The IL-6-driven induction of proinflammatory Th17 development can be inhibited by retinoic acid, preferentially in the gut (Mucida et al., 2007). It was shown that retinoic acid and other agonists of retinoic acid receptor α promote Foxp3 expression in the cells and shift the balance in Tregs favor. Conversely, inhibition of retinoic acid signaling prevents TGF-β-mediated Foxp3 induction (Elias et al., 2008).

As mentioned before, IL-6 is an important regulator of Th17 cell development which also negatively regulates the development of Tregs. On the other hand, IL-2 is a vital cytokine for Tregs survival and function (Malek et al., 2002). Interestingly, IL-2 blocade or disruption of its signaling resulted in a promoted Th17 cell development (Laurence et al., 2007). Therefore, IL-2 was identified to play a negative role in Th17 differentiation which further confirmed the opposing roles of Tregs and Th17 development.

1.5.4. TGF-β in the development of Th cell subsets

TGF-β has many pleiotropic functions within and also outside of the immune system. In chronic inflammatory diseases TGF-β mediates the repair of damaged tissues and triggers the induction of Tregs which suppress the effector T cells responsible for the original damage of the tissue. TGF-β directly inhibits T cell proliferation by abrogating the production of IL-2 and prevents apoptosis of T cells (Tiemessen et al., 2003). Furthermore, TGF-β enhances antigen presentation by B cells and induces the switch from IgM to IgA (Arai et al., 2003). The suppressive function of TGF-β on the immune system is one of the mechanisms of maintaining peripheral tolerance. It has been shown that TGF-β KO and TGF-β receptor KO mice develop severe autoimmune disease (Diebold et al., 1995; Gorelik and Flavell, 2000a). The role of TGF-β in Treg and Th17 development was decribed in previous chapters. Here I will focus on the effect of TGF-β on diffrentiation of Th1 and Th2 cells.

It is well know that TGF-β controls differentiation of Th1 and Th2 cells (Figure 4). It has been described that TGF-β interferes with GATA-3 at transcriptional level and thus it blocks differentiation of Th2 cells (Gorelik et al., 2000b; Heath et al., 2000a). TGF-β seems not to directly interact with IL-4 signaling pathway which is responsible for the development of Th2 cells. However, it has been shown that TGF-β can promote the production of IL-10 which is one of the effector cytokines of Th2 cells (Kitani et al., 2003). In addition, TGF-β was shown to inhibit the differentiation of Th1 and Th2 cells by interfering with the common pathway of T cell activation and thus it abrogates the possibility of differentiation of both, Th1 and Th2 cells (Chen et al., 2003b). Moreover, it was demonstrated that TGF-β in general inhibits T cell proliferation by reducing the cell cycle rate and by decreasing the expression of CD25, thus changing the activation status of T cells (Tiemessen et al., 2003). It was demonstrated that the differentiation of Th1 cells is abrogated in the presence of TGF-β (Gorelik et al., 2002). Here, TGF-β inhibits the expression of T-bet, the master regulator of Th1-specific transcription. Furthermore, TGF-β inhibits IL-12-induced IFN-γ secretion in Th1 cells (van Weyenbergh et al., 2001). Moreover, it has been suggested that the effect of TGF-β on Th1 development might be cause by TGF-β-mediated suppression of CD122 upregulation (Li et al., 2006). CD122 expression is enhanced in Th1 cells and promotes their development. On the other hand, it was shown that blockade of TGF-β signaling in NK cells caused accumulation of these cells and production of large amounts of IFN-γ which drives the differentiation of Th1 cells (Laouar et al., 2005). Moreover, in another experiment T cells primed with TGF-β secreted IFN-γ and expressed T-bet and that this Th1 induction was

independent of IL-12 and STAT4 and was mediated by the inhibition of IL-4 production (Smeltz et al., 2005).

Reciprocal regulation between Th1 and Th2 cells has been well described. However, recent results indicate that even terminally differentiated cells might flexibly adapt their phenotype. Hegazy et al. (2010) demonstrated that the infection of Th2 cells with a lymphocytic choriomeningitis virus that promotes Th1 cell development induced the expression of T-bet and IFN-γ in these cells while the expression of GATA3 and IL-4 was preserved. This Th1/2 phenotype persisted in vivo for several months. Moreover, similar reciprocal inhibition of Tregs and Th17 cells was recently implicated. TGF-β is necessary for induction of both these cell types and drives the expression of Foxp3 and ROR-γt. At low concentrations TGF-β synergizes with IL-6 and IL-21 to promote IL-23 receptor expression which further supports the development of Th17 cells. On the other hand, high TGF-β concentrations suppress IL-23 receptor expression and promote Tregs development (Zhou et al., 2008). Foxp3 is able to inhibit Th17 differentiation by antagonizing the function of ROR-γt. This suppressive effect of Foxp3 is overcome by IL-6 signaling which steers the development in Th17 direction (Yang et al., 2008b).

Interestingly, it was also shown that Tregs with decreased Foxp3 expression are prone to become Th2 cells rather than Th1 cells upon stimulation in vivo even in Th1-polarizing conditions (Wan and Flavell, 2007). Later it was demonstrated that this conversion is dependent on induction of STAT6-independent IL-4 production by Tregs which suppressed the competitive IFN-γ and thus favored Th2 conversion (Wang et al., 2010). In vivo it was found that highly Th1 polarized environment during infection might induce the expression of T-bet and production of IFN-γ in Tregs (Oldenhove et al., 2009). Moreover, $Foxp3^+$ Tregs that simultaneously produce IFN-γ were identified in peripheral blood of diabetes patients (McClymont et al., 2011).

Moreover, it was shown that purified Th17 cell were able to induce diabetes in non-obese diabetic (NOD) immunodeficient mice (Bending et al., 2009). These cells begun to produce IFN-γ and express T-bet and their activity was not hindered by anti-IL-17 antibody. They also responded to IL-12 by increasing the production of IFN-γ. These results indicated that Th17 cells are prone to functional conversion in Th1 direction (Bending et al., 2009)

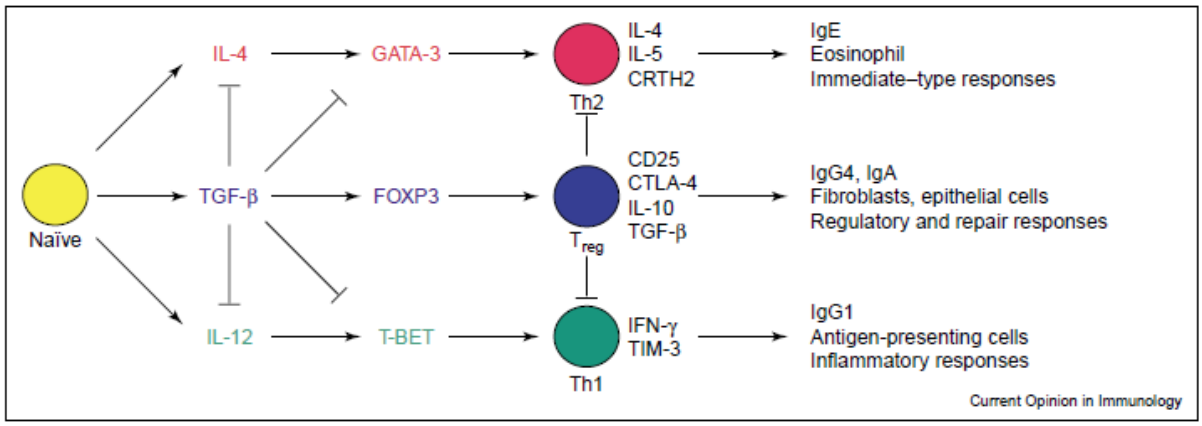

Figure 4: Influence of TGF-β on T cell differentiation. TGF-β inhibits the production of IL-4 and IL-12 and the expression of transcription factors GATA-3 and T-bet and thus inhibits Th1 and Th2 differentiation. TGF-β also induces Foxp3. Adapted from Schmidt-Weber and Blaser, 2004.

1.5.5. Flexible Treg phenotype

The subsets of Th cells seem no longer to be lineages but rather networks of different cell types. IL-17 has always been considered as proinflammatory cytokine produced mainly by Th17 cell. However, recent study introduced a novel cell subset of $Foxp3^+$ Tregs which can under certain conditions produce IL-17 and retain their suppressive potential at the same time (Voo et al., 2009). Recent studies suggested that some Treg populations might loose their suppressive phenotype and convert into inflammatory cell types which was shown in different settings. Upon activation, Tregs differentiate into IL-17-producing cells in the presence of IL-6 and in the absence of TGF-β (Xu et al., 2007; Figure 5). However, not all types of $Foxp3^+$ Tregs are sensitive to such conversion. iTregs that are induced in an appropriate environment using IL-2 and TGF-β seem to be refractory to this conversion and retain their suppressive phenotype in such conditions. It was found that IL-2 and TGF-β reduce the expression of IL-6 receptor and IL-6 signaling in the cells (Zheng et al., 2008). Thus, this group pointed out a major difference between nTregs and iTregs that may be important not only for immune homeostasis but also in clinical protocols.

TGF-β dependent differentiation of Tregs can be prevented by many factors. Besides IL-6 which, together with TGF-β, promotes the development of Th17 cells there is another cytokine that abolishes Tregs development. IL-4 was shown to block generation of Tregs and instead to induce a population of T cells which produced IL-9 and IL-10 (Dardalhon et al., 2008). Although IL-10 is considered rather anti-inflammatory and it mediates suppression of e.g. Tr1 cells, in this case the novel subtype of T cells seems to promote inflammation. This was shown in a model of RAG1 deficient mice which, after adoptive transfer of IL-9/IL-10 producing cells, developed colitis and neuritis and thus promoting induction of autoimmune diseases in mice (Dardalhon et al., 2008).

Another cytokine involved in the regulation of Th cell differentiation is an IL-12 family member, IL-27 which is often present at sites of inflammation. This cytokine was shown to prevent the development of anti-inflammatory iTregs and even more surprisingly prevent the development of pro-inflammatory Th17 cells, the latter by modulating expression of transcription factor STAT1 (Neufert et al., 2007).

Recently, it was shown that nTregs might convert into pro-inflammatory T cells under certain conditions. It has beed demonstrated that under inflammatory conditions Tregs can be

reprogramed into IL-17- or IFN-γ-producing cells (Duarte et al., 2009; Komatsu et al., 2009). Wang et al. (2010) demonstrated that Tregs expressing reduced levels of Foxp3 produced IL-4. In naïve $CD4^+$ T cells the IL-4 production is dependent on transcription factor STAT6, however, in the case of nTregs STAT6 was not necessary for IL-4 production. Instead, Foxp3 seemed to elevate the levels of another transcription factor GATA3 (Wang et al., 2010). Previous studies using transgenic mice with attenuated Foxp3 expression showed that Tregs from such mice have decreased suppressive function and can become Th2 effector cells (Wan and Flavell, 2007). One of the molecules expressed by Tregs, CTLA-4, inhibits expression of GATA3 in stimulated naïve mouse CD4+ T cells and thus prevents Th2 cells differentiation (Nasta et al., 2006). CTLA-4 engagement appears to inhibit STAT6 signaling pathway which prevent induction of Th2 differentiation program. Moreover, CTLA-4 also inhibits the expression of IL-4 receptor which further disables Th2 development. On the other hand, the expression of T-bet was not affected in these cells (Nasta et al., 2006). The development of Tregs might be prevented also by a Th1 cytokine, IFN-γ. It was documented that TGF-β-induced differentiation of Tregs was abrogated in the presence of IFN-γ (Wei et al., 2007).

In conclusion, Tregs as well as Th cells are phenotypically stable and fully functional in certain conditions but it was shown that they retain some degree of plasticity (Figure 6). This fact allows them to flexibly react to changing conditions in the body but it may as well constitute a major obstacle in their clinical usage.

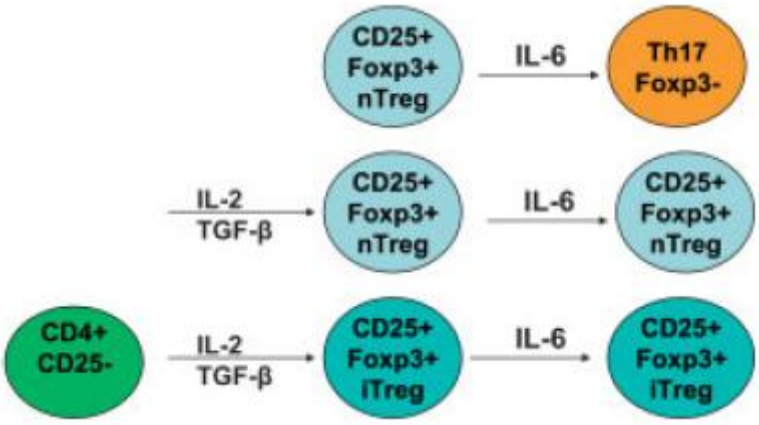

Figure 5: nTregs but not iTregs can be converted into Th17 cells by IL-6. iTregs induced by IL-2 and TGF-β are resistant to the effect of IL-6. nTregs can be protected from the effects of IL-6 by IL-2 and TGF-β. Adapted from Horwitz et al., 2008.

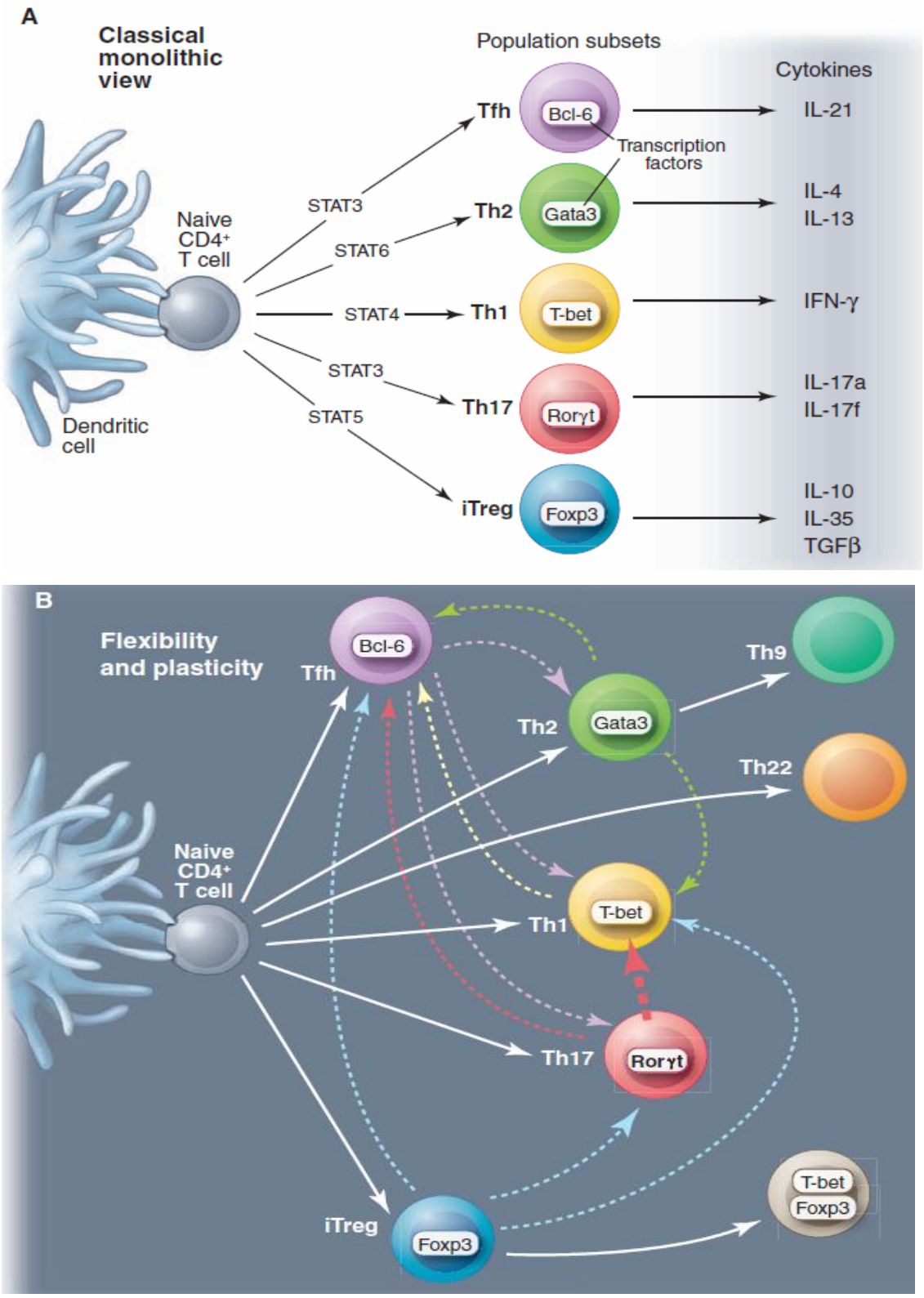

Figure 6: Th and Treg differentiation. (A) The classical view implied that T cells behave like lineages with inflexible phenotype. (B) Flexibility and plasticity of T cells. Recent data indicate that $CD4^+$ T cells can change their profile of cytokine production and that the expression of master regulators is also flexible. Adapted from O'shea and Paul, 2010.

2. Aims of the thesis

Regulatory T cells are one of the key components of the immune system. Their potential in clinical applications is vast, merging from therapy of autoimmune disease and alergy to treatment of transplantation reactions. These cells are, however, scarce in an organism and their routine usage is largely dependent on in vitro preparation. The induction of Tregs from conventional T cells is still a poorly understood process which can be affected by many different factors.

The aim of this thesis was to characterize the in vitro conditions necessary for induction of Tregs and mainly to describe the role of cytokines in this process. It has been known that TGF-β is a key cytokine responsible for the induction of Tregs but the role of other cytokines was not well specified. Therefore, we intended to test a panel of cytokines and to define their role in the de novo induction of Tregs. Both, iTregs and nTregs require certain specific conditions to maintain their viability and function. Thus we intended to describe the effect of tested cytokines not only on the induction of iTregs but also on the maintenance of nTregs and iTregs. Moreover, the suppression capacity of Tregs induced in various cytokine conditions was an important indicator in evaluation of iTreg and nTreg stability and function.

The developmnetal and functional plasticity of Th subtypes has been intensively studied in the recent years. The conventional helepr cells Th1 and Th2 were considered as terminaly differentiated. However, it has been lately hypothesized that a certain degree of plasticity prevails in these cells, both, in vitro and in vivo. It has been also demonstrated that Tregs are a highly plastic population which can be converted into other, proinflammatory cells types. The conditions responsible for the conversion are mostly activation of Th and Treg cells in a specific, local cytokine environment.

Another type of Th cells, Th17, was described several years ago. These cells are characterized by production of proinflammatory cytokine IL-17 and they develop from uncommited T cells in the presence of TGF-β and IL-6. Since Tregs develop also in the presence of TGF-β, there is a certain developmental link between these two cell types.

The regulation of Treg induction is another important issue. Another cell type with regulatory properties which has a vast immunomodulatory potential are MSCs. These cell are known for their secretion of TGF-β and, under certain conditions, also IL-6. Therefore we

wanted to evaluate the role of this cell population in the regulation of Treg and Th17 induction.

In addition, in course of our research we described another cytokine, IL-12, that plays an important role in the regulation of iTreg development where it inhibits the induction of these cells. Thus, the specification of the effect of IL-12 on the development of Tregs was another task of this thesis. Since Th17 cell development is also dependent on TGF-β, our goal was to characterize the role of IL-12 also in the developmnet of Th17 cells. Moreover, we intended to characterize the Th cell subpopulations which develop in the studied cytokine environment. We believed that redirecting Treg development in e.g. inflammatory conditions, has an important impact in possible clinical application of Treg.

3. Publications

The thesis was elaborated on the basis of the following publications:

Prochazkova J, Fric J, Pokorna K, Neuwirth A, Krulova M, Zajicova A, Holan V. Distinct regulatory roles of TGF-β and IL-4 in development and maintenance of natural and induced CD4+CD25+Foxp3+ regulatory T cells. Immunology. 2009 128:e670-8.

Holan V, Pokorna K, Prochazkova J, Krulova M, Zajicova A. Immunoregulatory properties of mouse limbal stem cells. J Immunol. 2010 184:2124-9.

Svobodova E, Krulova M, Zajicova A, Pokorna K, Prochazkova J, Trosan P, Holan V. The Role of Mouse Mesenchymal Stem Cells in Differentiation of Naive T-Cells into Anti-Inflammatory Regulatory T-Cell or Proinflammatory Helper T-Cell 17 Population. Stem Cells Dev. 2011 [Epub ahead of print].

Prochazkova J, Pokorna K, Holan V. IL-12 inhibits the TGF-β-dependent T cell developmental programs and skews the TGF-β-induced differentiation into a Th1-like direction. Immunobiology. 2011 *in press.*

4. Discussion

The development and differentiation of Th cell subsets is a highly plastic process which is regulated by cytokines. Local cytokine environment at the time of naïve T cell stimulation is determining for lineage-specific decisions. TGF-β has long been considered only as a differentiation factor for iTregs. Its high concentration in the absence of other cytokines is indeed decisive for induction of various cell types with regulatory properties. However, in the recent years it was shown that its effects are hindered in the presence of other cytokines. Simultaneous presence of TGF-β and IL-6 was demonstrated to induce development of highly inflammatory Th17 cells which play a physiological role in defense against extracellular parasites and are also important players in pathological autoimmune diseases.

Our study was based on the fact that TGF-β induces Tregs and with regard of the previous experience we wanted to determine the role of other cytokines in this process. It was also established that Tregs play an important role in pathogenesis of different diseases like autoimmunity, allergy, cancer and transplantation. All these immunologically relevant conditions are characterized by an increased cytokine secretion and typical cytokine profile at the site of question. It was therefore possible that different cytokines form specific conditions for the development of iTregs and other Th cells. To examine this hypothesis we used an in vitro model of allogeneic transplantation reaction and stimulated BALB/c splenocytes with irradiated B6 cells in the presence of various cytokines and we aimed to show their distinctive effects on the development of Tregs. The differentiation of iTregs was tracked by the expression of Foxp3 and their proliferation by carboxyfluorescein succinimidyl ester (CFSE). The results have surprisingly shown that except IL-6 two other cytokines affect iTregs development. Both, IL-4 and IL-12 significantly reduced the proportion and absolute numbers of Tregs when they were in the culture together with TGF-β. This was an interesting finding because IL-4 and IL-12 are the principal differentiation factors which drive the development of Th2 and Th1 cells, respectively. Similar results were reached by Mantel et al. (2007) who concluded that Th2 differentiation program which is induced in T cells by IL-4 or by GATA3 knock in prevented the TGF-β-driven differentiation of iTregs. Moreover, it was shown that GATA3 binds to the Foxp3 promotor and thus represses transcription of Tregs master gene (Mantel et al., 2007). Another group has demonstrated that Th2 and also Th1 differentiation

cytokine-mediated induction of transcription factors inhibits the development of iTregs (Wei et al., 2007). However, IFN-γ was used as a Th1-differentiation cytokine and its effects were responsible for blockade of iTregs development in this case (Wei et al., 2007). Moreover, we have demonstrated that anti-IL-4 mAb further supported the differentiation of iTregs.

We also intended to determine the cellular origin of iTregs. The primary studies were performed with bulk spleen cells and it was not possible to distinguish between the nTregs and newly arising iTregs. Therefore, we separated the population of naïve $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ nTregs and performed the experiments with the separate populations. We found that TGF-β-induced iTregs arise exclusively from naïve T cells where the de novo expression of Foxp3 occurs. Thus the iTregs which develop in allogeneic setting in the presence of TGF-β originate from conventional T cells. Other groups also showed that TGF-β generates Tregs from $CD25^-$ precursors (Chen et al., 2003c). On the other hand, other authors pointed out that iTregs might be derived from nTregs (Huber et al., 2004). Apart from the effect of IL-4 on the development of iTregs we have observed its distinctive effects on nTregs and already existing iTregs. Culture of nTregs with IL-4 alone or IL-4 together with TGF-β had a beneficial impact on this population. nTreg viability and maintenance was increased as was the expression of Foxp3 in these cells. In contrast, the effect of IL-4 on iTregs was basically opposite since the Foxp3 expression was dramatically decreased after the addition of IL-4. This was an interesting finding because we have also evaluated the functional properties of iTregs and found that iTregs were comparably suppressive as nTregs. The stability of iTregs has been discussed in a study by Floess et al. (2007) and it was speculated that the TGF-β-induced Foxp3 expression is only transient and unstable without further TGF-β treatment. The suppressive characteristics of iTregs were of great importance. Since we induced iTregs upon stimulation with alloantigen we evaluated their suppressive capacity in a mixed lymphocyte culture. TGF-β-induced iTregs suppressed proliferation of effector T cells in mixed lymphocyte reaction (MLR) in a dose-dependent manner.

In the recent years the state of terminal differentiation of Th cells was often questioned. Not only Tregs but also "classical" Th subsets were subjected to detailed analysis of their phenotypes. It has been shown that TGF-β inhibits Th1 and Th2 differentiation (Li et al., 2007b). Based on our previous results, we have studied the role of IL-12 in the TGF-β-dependent developmental programs, i.e. Tregs and Th17. We have demonstrated that IL-12 not only inhibits differentiation of Tregs but also of Th17 cells. It was recently shown that naïve T cells cultured with TGF-β and IL-4 differentiate into proinflammatory cells which produce IL-9 and were thus named Th9 cells (Dardalhon et al., 2008). Therefore, we intended

to characterize the cells that arise in the cultures with IL-12 instead of Tregs and Th17 cells. It was shown that Tregs are able to convert into non-regulatory cell types. Zheng et al. (2008) have shown that nTregs begin to express Th17 cytokines when cultured with IL-6. Interestingly, they demonstarated that this conversion was possible only within a population of nTregs which were considered as phenotypically more stable. This group demonstrated, however, that IL-2 and TGF-β which are used to induce iTregs decrease the expression of IL-6 receptor on iTregs which makes these cells refractory to IL-6-mediated conversion (Zheng et al., 2008). We have demonstrated that TGF-β-dependent Treg development was skewed in the presence of IL-12, the resulting cells lost Foxp3 expression and other Treg markers and acquired markers of a proinflammatory cells type. We have detected increased expression of T-bet in the newly arising cells which pointed at Th1 cell differentiation. Transcription factors typical for other T cell lineages were not expressed in these cells. The cytokine profile proved elevated production of IL-2 and IFN-γ and expression analysis revealed mRNA for IL-18 receptor and C-C chemokine receptor type 5 (CCR5) which are cell surface molecules characteristic for Th1 cells (Sebastiani et al., 2001).

It was previously demonstrated that Tregs might convert into a different cell type when cultured in specific cytokine conditions (Xu et al., 2007; Zheng et al., 2008; Zhou et al., 2009). It was reported for example that iTregs can secrete proinflammatory cytokines such as IL-17 and IFN-γ under certain conditions (Xu et al., 2007; Yang et al., 2008b; Wei et al., 2009). However, the role of IL-12 in this process was not studied yet. It was only shown that an ectopic expression of IL-12 receptor and stimulation with this cytokine does not change the phenotype of either developing or committed Th2 cells (Heath et al., 2000b).

The development of Th17 cells in the presence of IL-12 was converted similarly to development of iTregs. We have detected an enhanced expression of T-bet in these cells which is in concordance with the results of Bending et al. (2011). This group used a different model and stimulated already committed Th17 cells with IFN-γ which promoted T-bet expression. It was also shown that Th17 cells stimulated in the presence of TGF-β induced a subset of Th cells which produced IL-17 and IFN-γ (Lee et al., 2009). In our study, we have tested other markers of TGF-β/IL-12-activated T cells and detected also IL-2 and IFN-γ production by these cells but very low production of IL-17. Moreover, like in the case of converted iTregs, these cells expressed elevated levels of IL-18 receptor and CCR5. It was shown that the differentiation of Th17 cells is prevented by IFN-γ and IL-4, the product of Th1 and Th2 cells (Lexberg et al., 2008). We have shown that another Th1 cytokine, IL-12, prevents the development of Th17 cells. Similarly, it was shown that developing Th17 cells

expressed IL-12 receptor which enabled them to respond to IL-12 and thus retain some level of plasticity (Lee et al., 2009).

In our study it was possible to skew the phenotype of developing Treg and Th17 cells by IL-12. Importantly, IL-12 was able to do so even when it was added with 48-hour delay, at the time when the cells expressed markers of Tregs and Th17 cells. We have not studied a conversion of fully differentiated effector cells but the impact of cytokines in the process of development. The in vivo induction of Tregs is considerable as a tool for inducing tolerance in several pathological processes such as allergy or in transplantation. Therefore, it is important to make sure that cytokines already present in the system will not prevent Treg development and function. Besides our study there are other results that are often contradictory. Thus, the research in this field remains very important.

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