Harnessing immune regulation for treatment of human diseases : CD4+CD25+ regulatory T cells & antibody glycosylation

Wang, J.

CHAPTER 8

Summary and General Discussion

I. SUMMARY

1. Treg cells

The immune system displays a delicate balance between eradication of harmful "non-self" intruders and tolerance to "self" or harmless antigens to avoid tissue damage. The shifted immune balance to either arm will result in immune-mediated disorders, for instance, outgrowth of tumors or chronic infections due to compromised immune responses to "non-self" while autoimmune diseases or collateral tissue damage might arise from unwanted or exuberant immune reactions. In addition to the immune regulatory mechanisms occurring in the thymus and bone marrow, various subsets of immune cells in the periphery, including T, B lymphocytes and DCs, play an essential role in maintaining this balance as well (1-3). Among these cell populations with immune regulation capacities, $CD4^+CD25^+$ Treg cells have been intensively studied in the last 2 decades, as they can not only add weight to one side by promoting tolerance to "self", but also reduce weight from the other side by preventing excessive immune reactions against "non-self". Accordingly, disturbed numbers and/or functions of Treg cells, defined by the expression of CD25 and/or FOXP3, in patients with various human diseases have been suggested by numerous reports, but not by others (4-6).

Human FOXP3 has been reported to be significantly up-regulated in activated conventional T cells. This is associated with the acquisition of regulatory function of the bulk activated T cell population (7, 8). However, considering our previous findings that human Th1 cell clones upregulate FOXP3 mRNA after activation as well (9), we reasoned that endogenous expression of FOXP3 as such may not be specific for Treg cells in humans. Therefore, in **Chapter 2** we investigated the dynamics of endogenous FOXP3 expression and its relation to the suppressive function in activated human T cells at the single-cell level by using recently developed FOXP3 specific antibodies. We found that FOXP3 is expressed in a high percentage of activated T cells following *in vitro* stimulation of human $CD4^+CD25^-$ cells. Although FOXP3 expression in activated T cells is associated with hyporesponsiveness and reduced cytokine production upon TCR-mediated re-stimulation in all donors, it is not directly correlated to their suppressive abilities as it is transiently expressed in activated non-suppressive T cells in 6 out of 9 individuals tested. In contrast, it is stably expressed in activated T cells that do display suppressive function as well as isolated $CD4^+CD25^{++}$ Treg cells. Our data indicate that expression of endogenous FOXP3, in humans is therefore, not sufficient to induce regulatory T cell activity or to identify Tregs.

Our findings on the "non-specificity" of FOXP3 for human Tregs indicate that the conflicting data regarding the function of isolated $CD4^+CD25^+$ or $CD4^+CD25^{++}$ T cells in patients with inflammatory diseases could be related to contaminating nonregulatory $FOXP3^+$ cells within the purified cell compartments (4-6). Therefore, in **Chapter 3** we aimed to define surface makers for identifying and enriching Tregs with maximal regulatory ability within the $CD4^+CD25^{++}$ T cell compartment. In light of previous reports showing that anti-TNFα therapy could enhance the suppressive capacity of $CD4^+CD25^{++}$ T cells in RA patients (10), and that mTNFα-expressing T cells could be depleted by antibodies against TNFα both *in vitro* and *in vivo* (11-15), we hypothesized that the expression of mTNFα distinguishes $CD4^+CD25^{++}$ cells without or with inferior suppressive function. We showed that a substantial number of $CD4^+CD25^{++}$ T cells express mTNFα. $mTNF\alpha^+CD4^+CD25^{++}$ T cells display a reduced anti-inflammatory cytokine production and a less potent suppressor capacity. Moreover, treatment of RA patients with TNFα-specific antibodies leads to a reduction of $mTNF\alpha^+CD4^+CD25^{++}$ T cells from peripheral blood. These data indicate that the absence of mTNFα on

$CD4^+CD25^{++}$ T cells can be used to characterize and enrich for Tregs with maximal suppressor potency. Moreover, they also indicate that enrichment of $mTNF\alpha^-CD4^+CD25^+$ cells in the $CD4^+CD25^{++}$ T cell compartment may contribute to the reversal of the compromised suppressive ability of $CD4^+CD25^{++}$ T cell populations in RA patients after anti-TNFα treatment (Figure 1).

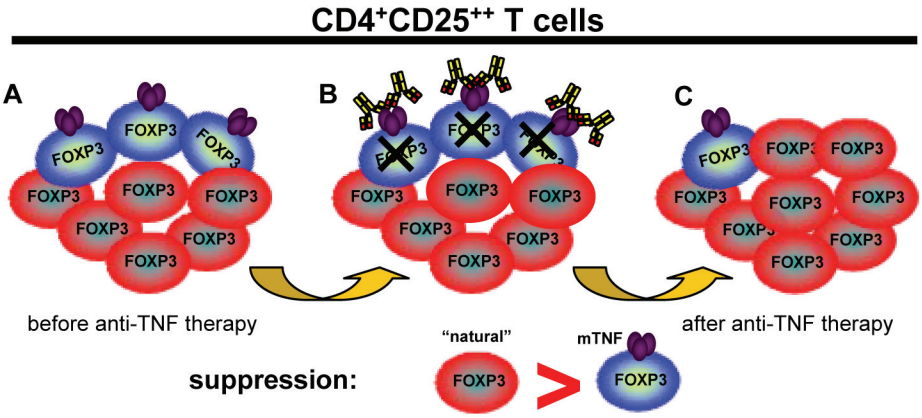

Figure 1. Anti-TNFα therapy removes less suppressive mTNFα-expressing cells from the $CD4^+CD25^{++}$ T cell compartment in RA peripheral blood.

Adoptive transfer of $CD4^+CD25^+$ Tregs has been successfully used in several animal models of graft rejection and autoimmune diseases (16-19). However, Treg-based immunotherapy has been hampered in the clinic by the difficulties to obtain large numbers of homogenous Treg populations with stable and potent suppressive function due to the lack of specific markers for Treg isolation/characterization. Moreover, the possibly compromised function of *ex vivo* Tregs from patients, potentially decreased suppressive function and/or the re-differentiation into IL-17-producing cells of "genuine" Tregs from healthy individuals during *in vitro* expansion further complicates the clinical usage of the expanded "Treg" population (10, 20-23). Inspired by recent findings showing the role of all-*trans* retinoic acid (ATRA) on the induction of murine Tregs (24-28), we investigated the effect of ATRA on the *in vitro* conversion of Tregs from conventional effector cells as well as the expansion of isolated $CD4^+CD25^{++}$ T cells in humans. The results of these studies are described in **Chapter 4**. We found that together with TGF-β, ATRA efficiently converts adult human peripheral blood naive $CD4^+$ T cells into $FOXP3^+$ Tregs with stable and potent suppressive ability. Moreover, treatment of isolated $CD4^+CD25^{++}$ T cells with ATRA/TGF-β enhances their suppressive potential during expansion. However, we also found that memory $CD4^+$ T cells are not only resistant to FOXP3 induction, but also inhibit Treg-conversion of naive T cells partially through the production of IL-4. This negative effect of memory cells on Treg induction can not be reversed by ATRA, thereby leading to the inconsistent induction of Tregs from total $CD4^+CD25^-$ cells among different donors.

ATRA has been shown, mainly in the C57BL/6 (B6) mouse system, to enhance TGF-β-induced Treg generation through multiple mechanisms, either directly on naive cells by enhancing TGF-β-signaling pathway or indirectly *via* memory cells by decreasing their cytokine secretion (26, 29-31). Intrigued by our human results in **Chapter 4** showing the inconsistent induction of Tregs by TGF-β and ATRA from $CD4^+CD25^-$ cells partially because of IL-4 produced by memory cells, we sought to investigate whether the aforementioned findings obtained in B6 mouse system can be extrapolated into other commonly used mouse strains (26, 29-31). Moreover, we wished to determine, in case differences are present, the underlying mechanism(s) responsible and whether these differences could be overcome by manipulation of the responsible pathway(s). Our data in

Chapter 5 show that although purified naive cells are highly susceptible to Tregs generation, total $CD4^+$ T cell populations from different mouse strains display significantly different sensitivities to TGF-β/ATRA-induced Treg conversion. The resistance of "non-responder" strains is associated with an enhanced production of IL-4 by memory T cells as well as an increased sensitivity of naive T cells to the action of IL-4. Importantly, neutralization of IL-4 overcomes the differences, thereby enabling TGF-β/ATRA to generate large numbers of functional Treg cells from total $CD4^+$ T cells in a consistent manner across different strains. These results show that the inherent resistance of "non-responder" mouse strains to Treg conversion induced by TGF-β and ATRA can be reverted by neutralization of IL-4, and offer hope for the extrapolation of these results into the human settings.

2. Glycosylation of IgG

It has been established that effector functions of IgG are not only influenced by the amino acid sequence of the constant region, but also by the carbohydrate structures attached to the asparagine 297 (Asn297) residue in the Cγ2 domain of each IgG Fc portion (32). Moreover, patients with inflammatory disorders (i.e. RA) display a clearly different glycan profile of serum IgG as compared to those from healthy individuals, and the glycosylation patterns may also be associated with disease activity (32-36), indicating its relevance for the pathogenesis (37, 38). However, little is known about the glycosylation profile of pathogenically relevant antibodies, for example autoantibodies in autoimmune diseases, because most of the studies were performed by using total serum IgG. Thus, in **Chapter 6** we developed a method for the microscale purification and Fc-glycosylation analysis of anti-citrullinated peptide antibodies (ACPA). ACPA are the autoantibodies that occur in RA patients with unique specificity and are associated with rapid disease progress (39, 40).

Using the method described in **Chapter 6**, we recently showed that the glycan pattern of ACPA varies considerably from total serum IgG in RA (41). Moreover, autoreactive, but not total IgG, present in local inflammatory sites display a further decreased galactosylation and sialylation levels as compared to those in sera (41). Interestingly, active immunization preferentially reduces sialylation of antigen-specific IgG in mice (42). Together, these findings indicate that glycosylation of IgG is regulated in an antigen-specific manner during the course of immune responses. Inspired by these results, we sought to identify environmental factors present during B cell activation and differentiation that could influence the gylcan pattern of secreted IgG by using an *in vitro* B-cell culture system. The results of these endeavors are described in **Chapter 7**. Our data show that treatment of B cells with IL-21 and CpG significantly increases IgG1 Fc-linked galactosylation and sialylation levels, while an opposite effect was observed with the addition of ATRA. Moreover, IL-21 and CpG reduce the incidence of IgG1 with a bisecting GlcNAc. Furthermore, these effects appeared to be stable and specific for immunoglobulins as no significant changes in the overall glycoforms of cellular proteins were observed. Intriguingly, no significant changes in the overall glycosylation profiles of cellular proteins among different culture conditions were observed. Together, these data indicate that soluble "microenvironmental" factors present during the activation and differentiation of B cells into antibody-secreting cells (ASCs) modulate the carbohydrate structures of secreted IgG without affecting the general cellular glycosylation machinery. Our study, could, therefore, further our understandings of the regulation of IgG glycosylations at the cellular level under different conditions.

II. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

1. Expression and role of FOXP3 in human effector T cells

In mice, Foxp3 was originally thought to be the master switch gene that is both necessary and sufficient for the development and regulatory function of $CD4^+CD25^+$ Tregs as 1) its mutation results in the absence of Tregs, thereby causing the systemic and fatal autoimmune disorders in Scurfy mice (43-45); 2) its expression is highly restricted in Tregs (44-47); and 3) Foxp3 deficiency does not affect the function of effector cells, while forced expression of Foxp3 converts conventional $CD4^+CD25^-$ T cells towards a regulatory phenotype (44-47). However, recent findings have shown that expression of Foxp3 is not required for the lineage commitment of Tregs as cells with disrupted Foxp3 protein expression maintain the characteristic Tregs "genetic signature" (48-50). Nonetheless, Foxp3 stabilizes the pre-established Treg lineage and controls its regulatory function through modification of cell surface and signaling molecules by directly binding to DNA and/or indirectly by interacting with other transcription factors including NFAT, AML1 and Runx (48-59). Accordingly, attenuated Foxp3 expression results in reduced or even the absence of suppressive abilities (50).

As in mice, a defect in naturally occurring Tregs was observed in IPEX (immune dysregulation, polyendocrinopathy, enteropthy, X-linked syndrome) patients with mutations in the FOXP3 gene, and this was thought to be responsible for the systemic autoimmune symptoms (60-64). However, recent studies suggest that apart from dysfunctional Tregs, altered effector T cell functions may also contribute to the immunological abnormalities in these patients (60, 63). Compared to those from healthy individuals, effector T cells from IPEX patients display reduced sensitivity to suppression and cytokine producing capabilities (60, 63). Together, these results not only demonstrate the importance of FOXP3 in the development/function of Tregs, but also point to a possible role of FOXP3 in the function of human effector cells.

Indeed, it has been shown that, at the bulk T cell level, FOXP3 is up-regulated in human $CD4^+$ effector T cells after activation (7-9, 65). Moreover, FOXP3 expression is associated with the acquisition of regulatory function in some studies, indicating that as in mice, FOXP3 controls the suppressive function of human T cells as well (7, 8). In this thesis, we have shown that FOXP3 is rapidly induced and highly expressed in virtually all human $CD4^+$ T cells after activation. However, expression of FOXP3 in activated effector cells is transient and not sufficient to confer a regulatory function, albeit that it may lead to some "Treg-like" features such as hyporesponsiveness and reduced cytokine production (**Chapter 2 & 4**). These results are consistent with the findings describing that the FOXP3 promoter region is in an open conformation and accessible to the transcription machinery in human effector T cells (66). Because the FOXP3 promoter contains binding sites for NF-AT and AP-1 (66) and it has been reported that FOXP3 could interact directly with NF-AT or NF-κB (67), which are well known mediators of T cell activation, it is therefore conceivable that FOXP3 gene expression is regulated in a similar manner as other immune-related genes controlled by NF-AT/AP-1 (66). Subsequently, FOXP3 is capable of attenuating TCR signal and repressing cytokine production by direct binding to their promoter regions or indirectly through interacting with NF-AT/NF-κB (53, 67-70). Thus, the transient upregulation of FOXP3 during activation provides a negative feedback loop for the activation and function of effector cells (65, 70-72). This putative role of FOXP3 could also contribute to the altered function of effector cells in IPEX patients. In this way, the increased resistance of effector cells from IPEX patients to suppression might be attributed to the reduced "infection tolerance" induction conferred by cocultured Tregs *via*

TGF-β, whereas decreased cytokine production upon *in vitro* activation might result from the exhaustion of these *in vivo* activated cells (60, 63, 73, 74).

Although the expression of FOXP3 *per se* is not sufficient to induce a regulatory phenotype, our data in **Chapter 2 & 4** indicate that the stability and levels of FOXP3 determine whether $CD4^+$ cells acquire Treg function, which is consistent with the reports that only ectopic expression of stable and high levels of FOXP3 confers a regulatory phenotype to human $CD4^+CD25^-$ cells (75, 76). Because it has been suggested that the balance between the NFAT-binding partners, FOXP3 and AP-1, determines the function of cells, and because AP-1 is upregulated during cell activation and promotes the expression of activation-associated genes by cooperation with NFAT (53, 77), it is conceivable that higher levels of FOXP3 are needed to convert activated $CD4^+$ effector cells into Tregs. In this scenario, the ratio of FOXP3/AP-1, rather than the levels of FOXP3 alone, controls the function of $CD4^+$ cells. This may also explain the variable suppressive capabilities of cells with similar levels of FOXP3 described in this thesis (**Chapter 2-4**) as well as in other studies (65, 78). Intriguingly, expression of FOXP3 in human activated $CD8^+$ T cells has been reported to be associated with a regulatory phenotype (79-84).

In summary, FOXP3 is not a Treg-specific molecule but an activation marker that serves as a negative regulator for cell activation and function in humans. Thus, conclusions on the phenotype/function of Tregs presented by numerous studies that were predominantly based on FOXP3 and/or CD25 expression should be interpreted with caution (4-6). The latter is especially the case in the context of patients with autoimmune diseases where chronic T cell activation occurs. The heterogeneity of the isolated $CD4^+CD25^{++}$ T cell population may explain the discrepancy reported by different groups claiming either a hampered Treg cell function, respectively, or an unaffected Treg cell function in patients.

2. Strategies to enhance/generate Tregs

Given the essential role of Tregs in immune regulation, Tregs-based immunotherapies may offer promises in many immune-mediated disorders. There is evidence indicating that the therapeutic effect of many agents/regimens in the treatment of immune-mediated disorders may relate to the modulation of Tregs. For example, induction of Tregs contributes to antigen inhalation-induced allergen desensitization and amelioration of autoimmune diseases as well as enhanced graft survival induced by donor blood transfusion (85-87). Moreover, many immunosuppressive reagents, such as glucocorticoids, vitamin D, intravenous immunoglobulin (IVIG) and rabbit anti-thymocyte globulin (rATG), can induce, expand and/or enhance the suppressive function of Tregs (88-91).

In vivo expansion & induction. In rats and mice, it has been shown that injection of CD28 superagonist or IL-2/IL-2 mAb preferentially expands $CD25^+$ Tregs *in vivo*, thereby inhibiting the induction/progression of experimental autoimmune encephalomyelitis (EAE) and prolonging the survival of allograft (92-96). Moreover, combined with rapamycin, this IL-2/IL-2 mAb complex is also effective in the treatment of ongoing EAE (96). However, translation of these animal studies to human cases should be performed with extreme cautions considering the lessons from TGN1412 trial, where administration of TGN1412 resulted in life-threatening systemic organ failure in all 6 healthy volunteers despite the promising preclinical *in vivo* and *in vitro* data with this humanized CD28 superagonist (97, 98).

Apart from expansion of pre-existing Tregs, recent studies have showed that *de novo* generation of Tregs *in vivo* is also feasible. It has been shown that administration of a humanized, Fc-region modified CD3ε specific monoclonal Ab can promote the tolerance

to allograft and ameliorate the symptoms in patients with T1D and psoriatic arthritis (99-102). Further studies in mice suggested that the short-term beneficial effect of this regimen was mediated by depletion of pathogenic effector cells, while the long-term restoration of tolerance was achieved through the induction of $CD4^+CD25^+$ Tregs in a TGF-β dependent manner (100, 103). Nonetheless, whether the same mechanisms are responsible for its effect in humans needs to be further investigated.

In vitro expansion & induction. In light of the great therapeutic potentials of *in vitro* activated/expanded Tregs for various inflammatory diseases in animals (16-19, 104), protocols have been developed for efficient isolation of cells with regulatory function (i.e., high CD25 but low CD127 expression etc) in humans as well as for the subsequent *in vitro* expansion (105-109). However, it is difficult to obtain, in a reproducible manner, a population with potent and stable suppressive capacity after expansion due to 1) the lack of specific markers for human Tregs (70, 110); 2) a potentially declined suppressive capacity of "genuine" Tregs during expansion (109, 111-113); and 3) the possibly compromised Treg function in patients (10, 21-23, 114). Therefore, an efficient approach to preserve/enhance the suppressive capacity during expansion is still highly desired.

An alternative approach is to convert conventional $CD4^+CD25^-$ effector cells into Tregs by forced over-expression of FOXP3 (65, 75, 76, 115) or *in vitro* reprogramming *via* TCR-mediated stimulation with or without exogenous TGF-β (7, 8, 81, 116-119). However, neither of these two methods can result in consistent acquisition of functional Tregs, possibly because of the variable stability and levels of FOXP3 induced among different studies (75, 76).

Regarding the generation of Tregs, this thesis mainly focused on the *in vitro* conversion of effector cells into Tregs (**Chapter 4 & 5**). Our data in **Chapter 4** reveal that functional Tregs could only be obtained in a consistent fashion across donors by stimulating purified naive $CD4^+CD25^-CD45RA^+$ T cells in the presence of both TGF-β and ATRA. Although ATRA alone has little effect on FOXP3 expression, together with TGF-β, it induces high levels of FOXP3 that is sufficient to confer a regulatory phenotype. This effect of ATRA could be attributable to its role in enhancing TGF-β signaling and/or epigenetically modifying FOXP3 promoter region (26, 30, 31, 120). Nonetheless, in contrast to the reports showing that TGF-β and ATRA together induce high Foxp3 expression from unfractioned $CD4^+CD25^-$ cells in B6 mice (24-31), our results show that in the presence of memory T cells, even the combination of TGF-β and ATRA is not sufficient to consistently induce Tregs from naive T cells in humans as well as in DBA and BALB/c mouse strains. However, neutralization of IL-4 greatly enhances such conversions, indicating that IL-4 plays a dominant role in hampering the generation of adaptive Tregs across different species (**Chapter 4 & 5**). Given that not only differences in IL-4 production by memory T cells, but also differences in the sensitivity to IL-4 by naive T cells were observed between different mouse strains, it is conceivable that these two aspects (IL-4-producing ability of memory T cells and IL-4-sensitivity of naive T cells) contribute to the considerable donor-to-donor variations in TGF-β/ATRA-induced Treg conversions in humans as well. Thus, starting with purified naive T cells and neutralization of IL-4 may be necessary for inducible Treg generation in Th2-proned/dominated diseases and/or IL-4 sensitive hosts.

The inhibitory role of IL-4 in TGF-β-induced Foxp3 expression has been attributed to its role in inducing the expression of Stat6 and GATA-3, as they both can prevent the transcription of Foxp3 by binding to its promoter region (121, 122). Contrary to this notion, our data in **Chapter 5** on the delayed effect of IL-4 on Foxp3 expression suggest that IL-4 mainly affects the maintenance of TGF-β/ATRA-induced Foxp3 levels at later

time points rather than preventing its induction shortly after T-cell activation. These data are compatible with recent findings showing the unstable expression of Foxp3 in induced and natural Tregs, especially under pro-inflammatory conditions (112, 113, 123-125). The accelerated downregulation of Foxp3 induced by IL-4 may relate to the interaction between GATA-3 and Foxp3 and/or the epigenetic modification of Foxp3 promoter, for example, increased methylation levels, after occupying by GATA-3/Stat6, as methylated promoter regions are associated with instable Foxp3 expression (123, 124, 126).

Collectively, the data presented in **Chapter 4 & 5** of this thesis are relevant for the development of Treg-based immunotherapies, as they show that ATRA/TGF-β can be employed to generate large numbers of highly suppressive $CD4^+FOXP3^+$ Tregs from adult human peripheral blood through the conversion of conventional naive T cells and/or preservation/augmentation of the regulatory function of isolated $CD4^+CD25^{++}$ T cells during expansion. Moreover, our data also indicate that neutralization of IL-4 could facilitate, and even be necessary for inducible Treg generation in Th2-dominant diseases and/or IL-4 hypersensitive individuals. However, the *in vivo* function of these converted and/or expanded Treg populations still needs to be intensively investigated.

3. Hurdles & perspectives for Tregs-based therapy

As discussed above, despite many advances, the main obstacle for the clinical application of Tregs-based immunotherapy, the lack of specific markers for human Tregs, remains unsolved. This precludes the specific administration/manipulation of Tregs *in vitro* and *in vivo*. Moreover, little is known about the *in vivo* function of human Tregs so far, which is extremely important as studies have shown that the ability of Tregs to control unwanted immune reactions *in vivo* can be influenced by many extrinsic as well as intrinsic factors.

Hampered suppression by pro-inflammatory cytokines. Both in mice and humans, there is evidence showing that many pro-inflammatory cytokines, derived from activated effector T cells or APCs, can either directly (IL-6, TNF-α, IFN-α) or indirectly (IL-4, IL-15 and IL-21) hamper the function of Tregs (22, 127-134). The indirect effect is mediated by rendering effector cells resistant to Treg-mediated suppression (132-134). These findings may explain the better therapeutic efficacy of adoptively transferred Tregs in mice with newly developed rather than established diseases (17, 135). Therefore, *in vivo* neutralization of these inflammatory cytokines may help and even be necessary for Tregs to exert their regulatory function in patients with ongoing or chronic inflammatory reactions (10, 22, 130, 131, 135, 136).

In addition, reducing the sensitivity of effector T cells and/or Tregs to the action of pro-inflammatory cytokines would also lead to increased suppression of Tregs *in vivo*. Recent studies from Dustin's group showed that blockade of PKC-θ not only enhances the function of human Tregs, but also protects them from inactivation by TNF-α and restores the *in vitro* activity of Tregs from RA patients. *In vivo*, Tregs pre-treated with PKC-θ inhibitor provide a significantly better protection in a murine colitis model induced by adoptively transferred effector cells (137). Likewise, ATRA sustains the stability and function of murine natural Tregs in an inflammatory milieu (138). Although the (long term) effect of these reagents on the *in vivo* function of human Tregs is not clear yet, further dissecting the different signaling pathways involved in the activation, survival and/or function of effector *vs.* Treg cells may provide valuable components to modulate the immune system to achieve desired effect.

Intrinsic defect of effector T cells to suppression. It has been suggested that effector T cells from patients with active autoimmune diseases may bear intrinsic defects to regulation

conferred by Tregs (139, 140). Schneider et al found that effector T cells from T1D patients are resistant to suppression. This escape from regulation is not transferred to co-incubated control effector cells, indicating that the resistance to regulation of T1D effector cells is not attributable to high levels of cytokines in the surroundings but is a characteristic intrinsic to these cells (139). For instance, effector cells in these T1D patients may display increased sensitivity to the action of cytokines, as we described in some mice strains (**Chapter 5**). Thus, elucidating the mechanisms underlying this phenomenon and thereby manipulating the responsible pathways, rather than simply increasing the number of Tregs, may be a better approach to restore immune balance in these patients.

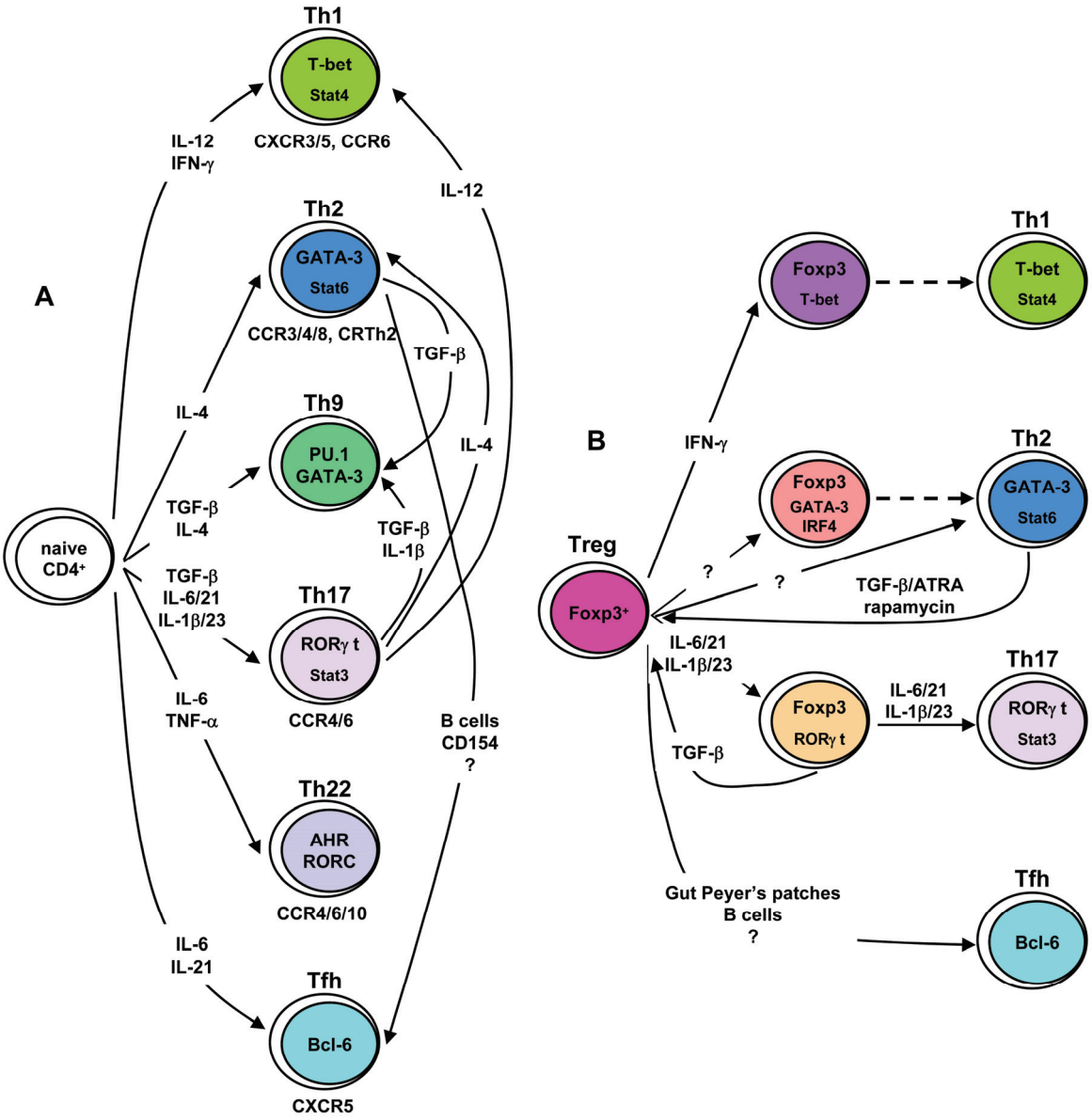

Figure 2. Plasticity of CD4$^+$ T cell lineage differentiation. A, conversion among different effector T cell subsets. **B,** conversion between Tregs and effector T cells. Solid lines indicate the reported differentiation directions, while directions represented by dashed lines have not been investigated yet.

Plasticity of Tregs. It has become increasingly clear that subsets of T cells, defined by the function and expression of transcription factors, are not stable (**Figure 2**), although it is unlikely that a given subset can transform into any other subsets (141, 142). For example, Th17 cells tend to convert to Th1 or Th2 cells in the presence of IL-12 or IL-4, respectively (143, 144), and Th2 cells may easily switch to Th9 but not Th1 cells (145).

Moreover, evidence also suggests that human Th17 cells can switch to IL-9-producing cells by stimulation in the presence of TGF-β and IL-1β (146), and murine Th2 cells acquire canonical Tfh cell markers in response to helminth antigens in a B cell or CD154 dependent manner (147, 148). Furthermore, murine Th2 cells can become Tregs upon activation in the presence of TGF-β, ATRA, rapamycin and neutralizing Abs to IL-4 and IFN-γ (149).

Similar to effector cells, Tregs are also subject to differentiation and functional specialization (**Figure 2B**). It is well established that both murine and human $Foxp3^+$ Tregs can be converted into IL-17-producing cells, possibly through a $Foxp3^+ROR\gamma t^+$ intermediate stage (150, 151), in a pro-inflammatory cytokine milieu such as IL-6/21/23 (20, 152-155). Moreover, the double-positive $Foxp3^+ROR\gamma t^+$ T helper intermediates may bear regulatory function *in vivo* and switch to Foxp3 single positive Tregs under Treg-favorable conditions, for instance, in the presence of TGF-β (150, 151). Likewise, some Tregs start expressing T-bet or GATA-3/IRF-4 under a respective type 1 or type 2 inflammatory conditions. Studies have indicated that expression of these transcription factors endow Tregs with a unique homeostatic and migratory property, thereby conferring them a superior ability to control corresponding type of immune reactions (149, 156, 157). Nonetheless, so far it still remains unknown whether these double positive "Th1/Th2" like Tregs can switch back to Foxp3 single-positive "classical" Tregs or further proceed to conventional Th1/Th2 cells after the resolution of inflammations. In addition, recent studies have demonstrated that murine Tregs could lose Foxp3 and become auto-aggressive effector T cells even under physiological conditions (50, 125, 155, 158-160). Accordingly, IL-17-producing $Foxp3^+ROR\gamma t^+$ memory Tregs were present in PBMCs from healthy human individuals, indicating their pro-inflammatory potentials (154, 161, 162).

Given the above-mentioned obstacles for Tregs to properly exert their function *in vivo*, it is unlikely that Tregs-based immunotherapy will be widely employed to treat patients in the near future, especially in those with actively ongoing immune responses. In these patients, the overwhelming effector immune reactions are very likely beyond reach of adequate Treg-mediated suppression, and moreover, Tregs may lose their regulatory function and even be converted into harmful effector cells by inflammatory mediators. Moreover, antigen-specific, but not polyclonal, Tregs should be used to selectively target the immune components causing diseases without a general inhibition of the recipient's immune system. However, apart from lacking an efficient protocol to obtain sufficient numbers of antigen-specific Tregs, our knowledge on the antigen(s) responsible for the pathology, such as auto-antigen(s) in patients like RA, is still very limited. Furthermore, since Tregs-based immunotherapy is a patient-tailored therapy that requires the generation of clinical-grade Tregs for each patient under GMP conditions, the high economic- and labor-intensive investments required will also prevent its applications in life-unthreatening diseases, such as RA. On the other hand, Treg-mediated adoptive immunotherapy can be used to maintain/promote remission in inflammatory diseases when the active immune responses have been controlled by other immunosuppressive treatments. Likewise, Tregs could be effective to promote engraftment or prevent graft-versus-host-disease (GVHD) not only because of the presence of a short period with "quiet" immune reactions in these patients after transplantation, but also because of the feasibility to generate Tregs specific for allo-antigens (163-166). In these situations, combined treatment of Treg cell-transfer with other immunosuppressive reagents, such as rapamycin, may result in superior therapeutic efficacy if the latter selectively targets effector T cells without compromising the function and survival of Tregs *in vivo* (167).

In summary, despite recent advances in our understandings of Treg biology and their roles in maintaining immune tolerance, many technical and theoretical challenges remain and Tregs-based therapy in humans are still in early stages. In addition to the clinical trials initiated to test the safety and efficacy of Tregs (167, 168), more basic research on the generation, functionality and stability of Tregs as well as assays to predict their *in vivo* functions are required for the effective translation of this strategy from bench to bedside.

4. IgG glycosylation, another checkpoint in immune regulation

It has been long recognized that changes in sera total IgG glycosylation profiles are associated with various physiological (i.e., the age and/or pregnancy) and pathological conditions (i.e. autoimmune diseases and/or tumors) (32-36, 169-175). Recently, we and others found that, compared to total or irrelevant sera IgG in patients with autoimmune diseases, auto-antigen specific IgGs exhibit a further different glycosylation pattern, indicating that structural features of glycan moieties in the Fc portion of IgG are regulated in an antigen specific fashion (41, 176, 177). Moreover, considering the signals required for the production of antibodies against T-cell dependent antigens from B cells (signals through B-cell receptor, CD40L-CD40 interaction and cytokines derived from Th or other cells), these data also indicate that the composition of IgG Fc glycoforms is regulated by the microenvironmental factors present during the activation and differentiation of B cells into ASCs. Indeed, our data in **Chapter 7** demonstrate that some (i.e., CpG, ATRA, IL-21 & IFN-γ), but not all factors known to modulate the outcome of B-cell responses have a direct impact on the Fc-coupled glycosylation of IgGs produced by B cells. It appears that these effects mediated by IL-21 and ATRA are long-lasting and therefore they are not required to maintain the glycan profiles of secreted IgG1 once ASCs are generated. Because glycan profiles of IgG1 are not associated with the cell proliferation or levels of IgG, our data also indicate that the posttranslational modification of IgG sugar chains is a process independent of cell growth and antibody titers. Given the relevance of Fc-glycan moieties for the effector function of IgGs (32), modulation of the carbohydrate structures of IgG may represent an additional and independent checkpoint, next to the amount and type of antibodies, to control the strength of humoral immune responses by some factors known to influence the outcome of B-cell responses.

Our findings showing the effect of B-cell stimuli on IgG glycosylation profiles are relevant for the design of new treatment for patients, especially those with antibody-mediated diseases. For instance, because of the role of ATRA in tipping the balance between effector and regulatory T cells (178) and its effect in inhibiting collagenase production by rheumatoid synovial cells (179), it has been proposed to use retinoids for the treatment of autoimmune diseases such as RA (180, 181). However, our data in **Chapter 7** indicate that the presence of retinoids during the activation & differentiation of B cells not only increases the amount of IgGs produced, but also possibly increases their pro-inflammatory activity due to decreased glycosylation and sialylation levels (37, 42, 170, 182, 183). Given the continuously occurring activation and differentiation of B cells into ASCs in patients like RA (184), the above-mentioned effects of ATRA may result in the acceleration, rather than the amelioration of antibody-mediated tissue/organ destructions in patients. Thus, the effect on antibody glycoforms should be taken into account when introducing a new therapy into patients.

Apart from the soluble factors (signal 3, Figure 1 in **Chapter 7**) present during the activation and differentiation of B cells, the effect of the duration/strength of signals delivered through B-cell receptor (BCR) *via* crosslinking of antigens (signal 1) and through CD40 *via* interaction with CD40L expressed on activated Th cells (signal 2) on IgG Fc

glycosylations remains unknown. Moreover, the mechanisms behind the findings presented in **Chapter 7** of this thesis as well as their *in vivo* relevance await further investigations. In addition of the direct effect, many other factors could also have an indirect impact on IgG Fc glycan structures. For example, cytokines, such as TGF-β and IL-4/6, may indirectly influence the glycan pattern of IgG through regulating the amount of IL-21-producing Th17 and Tfh cells *in vivo* (185-187), albeit no direct effect was detected in this thesis (**Chapter 7**). Likewise, given the capacity of certain antigens to elicit certain types of immune responses (i.e., Th1 *vs.* Th2 *vs.* Th17) (188), it would be interesting to compare the glycosylation profiles of IgG directed against Th1-, Th2- or Th17- associated antigens.

III. CONCLUDING REMARKS

This thesis focuses on two aspects that are involved in immune regulations 1) $CD4^+CD25^+$ Tregs and 2) Fc-glycosylations of IgG. In the first part (**Chapters 2-5**), we tried to define markers to identify and isolate cells with maximal regulatory ability. We also developed new protocols to generate large numbers of functional Treg cells by expansion of isolated "Tregs" or by conversion of effector T cells. Our findings in this part contribute to the identification and generation of Tregs in humans and thereby may help the translation of Tregs-based therapy from animal models to clinics. In the second part (**Chapters 6-7**), we attempted to obtain insights into how IgG glycosylation is regulated at the cellular level during the activation and differentiation of B cells. We identified some factors that could directly influence the Fc-glycoforms of secreted IgGs *via* B cells. Studies like the one described could open up possibilities for the identification of pharmaceutical mediators to "introduce" a desired function of antibodies by modulating their Fc-glycan composition. However, many caveats and difficulties still remain for the application of Tregs-based therapy in humans. Moreover, further studies are needed to show how IgG glycosylation is modulated *in vivo* and which underlying mechanisms are responsible for its regulation.

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