Chapter 2
FoxP3 and Regulatory T Cells

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Abstract Some regulatory T cells express the Foxp3 transcription factor and such Tregs have an essential function of preventing autoimmune disease in man and mouse. Foxp3 binds to Forkhead motifs of about 1100 genes and the strength of binding increases when Foxp3-expressing T cells are stimulated by PMA and ionomycin. In Foxp3-expressing T cell hybridomas, Foxp3 binding to DNA does not lead to the activation or suppression of genes which becomes only visible after T cell activation. These findings are in line with observations by others that Foxp3 exerts important functions through association with T cell receptor-dependent transcription factors in a DNA-binding complex.

Tregs can be generated when developing T cells encounter TCR agonist ligands in the thymus. This process does not require TGF-β signaling in the T cells but requires costimulatory signals. In contrast, the conversion of naïve T cells into Tregs in peripheral lymphoid tissue essentially depends on TGF-β and is inhibited by costimulation. In fact retinoic acid, produced by some dendritic cells, helps the conversion process by counteracting the negative impact of costimulation on the conversion process. Since AP-1 is produced after costimulation and appears to interfere with a Foxp3-NFAT transcription complex, it is of interest to note that retinoic acid interferes with AP-1-dependent transcription. Thus, retinoic acid may interfere with the negative impact of costimulation on Treg conversion by interfering with the generation and/or function of AP-1.

Peripherally converted Tregs have a stable Foxp3$^+$ phenotype and in mice can survive for several months in the absence of the antigen that induced their formation. In fact the prospective induction of Tregs can be used to generate antigen-specific tolerance that relies on immunosuppression of neighboring CD4 and CD8 T cells by Foxp3$^+$ Tregs in antigen-draining lymph nodes. The mechanisms of suppression may involve cytokines such as TGF-β and IL-10 but also

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S. Jiang (ed.), *Regulatory T Cells and Clinical Application*,
DOI: 10.1007/978-0-387-77909-6_2,

other mechanisms that involve suppressive purine-metabolites such as adenosine or adenosine-monophosphate.

Introduction

Cellular therapy employing Foxp3-expressing regulatory T cells (Tregs) holds the promise to replace and/or supplement indiscriminatory immunosuppression by drugs. In order to achieve this goal in the clinic we need to learn more about the generation, lifestyle and function of Tregs. One way to generate Tregs of any desired antigen specificity is the retroviral introduction of the Foxp3 gene into activated CD4 T cells. Foxp3 is a transcriptional repressor and activator that interferes with T cell receptor (TCR) –dependent activation of genes and may exert its effect, at least in part, by compromising NFAT-dependent gene activation. Another way of generating Tregs extrathymically in vivo is the introduction of low amounts of peptides under subimmunogenic conditions. Such artificially induced Tregs have a long lifespan in the absence of the inducing antigen and can thus mediate antigen-specific tolerance. Antigen specificity of Treg-mediated immunosuppression is due to effective co-recruitment and expression of Tregs and T effector cells to antigen-draining lymph nodes and sites of inflammation such that Tregs effectively suppress neighboring effector T cells at early or late stages of their differentiation. The latter allows for interference with already established unwanted immunity and may thus be employed to treat rather than prevent unwanted immune reactions.

The notion that the immune system employs different mechanisms to prevent autoimmune disease or maintain self-tolerance has been around for decades but definitive evidence emphasizing the essential role of negative selection as well as that of suppressor or regulatory T cells is of more recent origin. Today we distinguish negative selection in the form of deletion [1] of certain antigen-specific cells as well as in the form of "anergy" [2] by cell-autonomous mechanisms, also referred to as "recessive" tolerance, from tolerance that relies on the silencing of immune cells by regulatory or suppressor T cells by non-cell-autonomous mechanisms [3], also referred to as "dominant" tolerance. Both forms of tolerance can achieve antigen-specific non-responsiveness of the immune system in contrast to pharmacological interventions that usually result in undesirable general immunosuppression with potentially deadly side effects. In many clinical situations antigen-specific non-responsiveness represents the desired goal but present day treatment does generally not achieve that goal. For that reason it remains a great challenge for immunologists to design strategies and protocols that achieve antigen-specific non-responsiveness since there is little hope that the pharmaceutical industry will come up with suitable procedures to effectively and specifically interfere with unwanted immunity in the near future. Given this goal, it appears a reasonable strategy to exploit evolutionarily selected mechanisms effective in self-tolerance for clinical purposes. This requires a thorough understanding of how the immune system manages to avoid self-aggression. It is now appreciated that so-called negative selection

of potentially self-reactive T cells by antigens in- and probably also out-side the thymus essentially contributes to self-tolerance [4]. Likewise it has become clear that the generation of Foxp3-expressing regulatory T cells is mandatory to achieve self-tolerance [5]. The progress in understanding the contribution of such reasonably well-defined mechanisms to tolerance has thus established the somewhat limited usefulness of models that solely consider the absence of "danger" signals as an essential feature of self-tolerance.

While we have some basic ideas about mechanisms that can be exploited to induce antigen-specific non-responsiveness much needs to be learned in detail before this will become clinically applicable. Experiments have shown that over-expression of certain crucial self-antigens (such as insulin) that results in more profound tolerance by negative selection [6], can be helpful in preventing autoimmune disease, perhaps because certain autoimmune diseases, such as in type 1 diabetes, begin with a rather limited autoimmune response to antigens such as insulin [6,7], while later on a variety of other antigens in pancreatic β cells are recognized. However, clinically, such maneuvers would be limited to introducing such antigens prior to disease outbreak or when the immune system is "reset" after elimination of mature lymphocytes by x-irradiation and/or cytotoxic drugs.

In contrast, the manipulation of regulatory T cells appears to represent a more widely applicable approach to not only prevent but potentially also interfere with already ongoing unwanted immunity. With such a clinical goal in mind it is clear that we need to have a much better understanding of how antigen-specific regulatory T cells are and can be generated and/or amplified and how they can achieve antigen-specific non-responsiveness. It is the purpose of this little chapter to review recent progress in the understanding of several aspects of regulatory T cells with the hope that some of this information may find its way into the clinic with the challenge that ensuing procedures will eventually replace or at least supplement the present day practice of indiscriminatory immunosuppression.

Characteristics of Regulatory T Cells

Recent years have seen rapid progress in the characterization of regulatory T cells (Tregs). There is not one particular cell surface marker that defines Tregs but the CD25 surface molecule is at least expressed on the vast majority of cells that express the Foxp3 transcription factor, which has become a signature gene expressed in Tregs. The recognition that $CD25^+$ cells are enriched in Tregs has thus contributed considerably to establishing their role in suppressing activation and function of other lymphocytes [8]. In the meantime other molecules such as neuropilin 1[9], CD103 [10], GPR83 [11], GITR [12] and CTL-A4 [13] have been shown to have a characteristic expression profile in Tregs and thus can be helpful in achieving optimal purification in combination with the CD25 marker. Recent evidence shows that $CD4^+25^+$ Tregs are IL-7R-negative in contrast to $CD4^+25^+$ cells that just represent activated T cells without obvious regulatory function [14]. Intracellular staining by Foxp3 antibodies represents a useful means to identify Tregs in various tissues [15]

and in the meantime various Foxp3 reporter mice [16,17] have become available which allow functional purification of Foxp3-expressing cells. While Foxp3 expression represents a good signature for Tregs it can have its drawbacks because Foxp3 can be transiently expressed in activated T cells that, however, do not qualify as stable Tregs [15].

A variety of studies indicate that stable Foxp3 expression is sufficient to confer a regulatory T cell phenotype to CD4 T cells [18–20]. Thus retroviral Foxp3 transduction is a valuable means to endow antigen-specific T cells with a regulatory phenotype. This represents an important tool because unlike the in vitro expansion [21,22] of Tregs preformed in vivo it allows to produce Tregs of any desired specificity.

Recent data suggest that Foxp3 can interact with NFAT to regulate gene expression such as downregulation of the IL-2 gene and upregulation of CTL-A4 and CD25 molecules [23]. It is presently not clear whether all Foxp3-dependent gene regulation involves NFAT and whether NFAT plays a crucial role in the generation of Tregs. It has become clear from the combined analysis of Foxp3 binding and genome-wide gene expression, however, that Foxp3 is predominantly but not exclusively a repressor that silences genes that are normally activated after T cell stimulation, especially genes associated with T cell receptor (TCR) signaling [24]. This fact may contribute to the relatively poor response of Tregs in response to antigenic stimulation in vitro while exogenous growth factors may permit effective clonal expansion in vivo. The latter feature that is likely essential for effective in vivo suppression.

Among the genes that fail to be upregulated in Foxp3-expressing cells is the PTPN22 phosphatase that has a role in dephosphorylating $p56^{lck}$ and Zap-70 [24]. Interestingly a gain of function mutation of this gene has been postulated to affect several autoimmune diseases and it is presently not clear whether this mutant affects Tregs that control autoimmune disease or effector T cells that cause autoimmune disease [25].

Another important characteristic of Tregs is that they do express an αβTCR that confers antigen specificity. This is worthwhile pointing out since many studies on Tregs ignore this fact. It is our belief that antigen specificity of Tregs is absolutely crucial for antigen-specific suppression of immune responses and hence considerable attention has to be paid to the role of TCR specificity in the generation, homing and effector function of Tregs [26]. As all T cells with αβTCRs, Tregs also undergo stringent TCR-dependent selection in primary and secondary lymphoid organs [27] which eventually may be exploited to generate Tregs of any desired specificity and to interfere specifically with unwanted immune responses in the clinic.

Intra- and Extra-Thymic Generation of Tregs

Experiments in TCR transgenic mice in which the transgenic TCR was the only TCR expressed by developing T cells have clearly shown that ligation of the αβTCR by strong agonist ligands plays an essential role in the intrathymic generation of

Tregs [28,29]. These results are compatible with analysis of the Treg TCR repertoire in normal mice suggesting a focus on self-antigens [30]. It became especially obvious that expression of TCR ligands by thymic epithelial cells represented a powerful means to commit developing $CD4^+$ T cells to the Treg lineage [29]. In this context it is of considerable interest to note that thymic epithelial cells and especially thymic medullary epithelial cells can express "ectopically" a variety of proteins that otherwise would be considered "organ-specific" such as preproinsulin 2 that is expressed in pancreatic β cells but also in thymic medullary epithelial cells [31,32]. Such ectopic expression can be regulated, at least in part, by the AIRE (for *a*utoimmune *i*mmune *r*egulation) transcription factor [33] and it is thus conceivable that the ectopic expression of "organ-specific" antigen by thymic epithelium plays a decisive role in the generation of Tregs specific for such antigens, even though experiments addressing that question have so far yielded negative results [34,35]. However, negative results by no means rule out that AIRE-regulated antigens contribute to the generation of Tregs under more favorable experimental conditions.

The intrathymic generation of Tregs by strong agonist ligands appears to require costimulation of developing cells by B7-1 (CD80) [36] ligands that are expressed on thymic epithelial cells as well as on antigen-presenting cells of hemopoietic origin at least under certain experimental conditions. This is a somewhat astonishing observation in the light of findings that Treg generation in peripheral lymphoid tissue is most effective under conditions that avoid costimulation (see below). Conceivably this could be due to the different stages of development of thymic and extrathymic T cells which may require different signaling inputs for Treg commitment. From thymus transplantation experiments it is clear that Treg-generated by ligands expressed on thymic epithelium only, can migrate into peripheral lymphoid tissue and patrol the body for long periods of time without being confronted with the same ligand that was involved in their generation [29,37]. This does not exclude that lower affinity ligands in peripheral lymphoid tissue may contribute to survival much like they can contribute to survival of CD4 and CD8 conventional T cells [38].

Considering the intrathymic generation of Tregs it is of interest to note that generation of Tregs from cells with one particular αβTCR is not mutually exclusive to deletion of some of these cells [29]. Thus both processes depend on recognition of agonist ligands by developing $CD4^+$ T cells but under some conditions such recognition results in deletion and under other conditions in Treg generation even within the same thymus, perhaps because some of these cells encounter their TCR ligands on different cells i.e. either on cross-presenting dendritic cells or directly on thymic epithelial cells [39].

Whereas the intrathymic generation of Tregs would mostly depend on instruction of lineage commitment by self-antigens, the peripheral generation of Tregs may also include instruction by foreign antigens. It is therefore of considerable interest to define conditions permissible for extrathymic Treg generation. To this end we have exploited protocols of subimmunogenic antigen presentation because circumstantial and historic evidence suggested that one might be able to induce "dominant" tolerance in this way. Indeed it was found that either constant delivery of peptides by osmotic mini-pumps [40] or by targeting dendritic cells with peptide-containing

fusion antibodies directed against the DEC205 endocytic receptor on dendritic cells [15] allowed the conversion of naïve T cells into Foxp3 regulatory T cells. The conversion process depended on an intact TGF-βRII receptor on naïve T cells and conditions that avoided activation of dendritic cells as well as IL-2 production by naïve T cells. It was clear that Tregs were generated by conversion rather than expansion of already committed Tregs since the experiments were performed in mice expressing only one particular transgenic TCR in the absence of coexpression of a TCR agonist ligand resulting in the unique constellation that none of the generated $CD4^+$ T cells exhibited initially a Treg phenotype and only a certain percentage (∼30%) assumed it after the artificial introduction of the respective TCR agonist ligand [15].

The generation of Treg by subimmunogenic antigen delivery and the negative impact of strong costimulation on conversion of naïve T cells into Treg [15] correlates with the fact that costimulation results in the accumulation of Fos and Jun containing AP-1 that interacts with NFAT and thereby blocks the formation of a Foxp3-NFAT complex that is required for the generation of functional Treg [23]. The latter data are well in line with the observation that Foxp3 overexpression inc T cell hybridomas, that are not activated through their TCR results in binding of Foxp3 to target genes but very little regulation of target genes, while in contrast activation of such hybridomas results in activation as well as Foxp3 dependent downregulation and upregulation of genes [24]. Thus TCR signals and Foxp3 need to synergize in the generation of functional Tregs [23,24].

Importantly, the peripherally generated Tregs exhibited the same global gene expression pattern as intrathymically generated Tregs [39] and much like intrathymically generated Tregs exhibited a long lifespan that was independent on further supply of the TCR agonist ligand. Thus by these maneuvers a Treg "memory" to external TCR ligands could be induced, resulting in the subsequent suppression of immune responses elicited by the same agonist ligand i.e. this protocol succeeded in generating specific immunological tolerance to one particular antigen. Hopefully this protocol can be extended to many other antigens and thus help the prevention of unwanted immune responses. Recently some of us (I.A., P.V., H. v. B.) succeeded to induce transplantation tolerance in wt female mice by infusing them with male peptide, resulting in the generation of male-specific $Foxp3^+$ regulatory T cells. Of note this particular protocol only works with naïve T cells and not with T cells that have been already activated in vivo and thus can presumably not be used to suppress already established autoimmunity in which most antigen-specific T cells are already activated. In such cases the in vitro generation of Tregs by Foxp3 transduction would likely be more appropriate (see below) [39].

Recently it has been reported that retinoic acid generated in CD103 positive DC in the gut helps the conversion of naïve T cells into $Foxp3^+$ Tregs thereby giving credibility to the disputed concept of oral tolerance [41–43]. Interestingly, retinoic acid appears to interfere with the negative effect of costimulation on TGF-β dependent conversion of naïve T cells into Tregs [43] providing a possible (only) mechanism for its effect, since by itself in the absence of TGF-β retinoic acid does not affect conversion. Thus, there is an interesting difference between intra-thymic and extra-thymic Treg generation: While the former requires costimulation and takes

place even in the absence of TGF-β, the latter is essentially dependent on TGF-β and takes place in the absence of costimulation. It is worthwhile pointing out that even in the presence of retinoic acid, the TGF-β-dependent conversion works effectively only with naïve T cells while preactivated T cells, perhaps because of their high AP-1 content, are relatively resistant to a TCR-induced conversion process.

At present one can only speculate why costimulation and AP-1 generation interferes with the upregulation of Foxp3 in naïve T cells: it may be that AP-1 interferes with regulation of the Foxp3 locus by TCR and TGF-β-dependent signals that eventually results in stable Foxp3 expression. Whether it does so by interfering with the action of TGF-β–induced Foxp3 regulation or autoregulation by Foxp3 is unknown. It is also possible that AP-1 interferes somehow with demethylation of the Foxp3 locus. In this context it is of interest to note that the slow in vivo conversion process generates long-lived and stable Foxp3-expressing Tregs whereas the conversion process in vitro utilizing costimulation and TGF-β often results in cells with unstable Foxp3 expression, at least when these cells are analyzed during antigenic stimulation.

Lifestyle of Tregs

As pointed out above, Tregs can survive for relatively long periods of time as resting cells at an intermitotic stage but as soon as they encounter their TCR agonist ligand they will express activation markers and begin to home to antigen-draining lymph nodes and undergo considerable expansion [21,22,37]. This is usually accompanied by loss of CD62L and acquisition of CD44 expression and followed by expression of the α_E integrin (CD103) (at least in the mouse). Such activated cells extravasate and accumulate together with other T effector cells in inflamed tissue [10]. It is in fact the co-recruitment of CD4 and/or CD8 effector cells with activated Tregs in draining lymph nodes and/or inflamed tissue which determines the specificity of immunosuppression [37]: since Tregs suppress neighboring T cells in a “bystander” fashion it can only be effective when most antigen-specific effector cells are co-recruited to the same anatomical location, which depends on presentation of TCR ligands in these places, such as antigen-draining lymph nodes [20]. Thus while Tregs may suppress “innocent” bystanders that happen to be in their vicinity this will not result in general immunosuppression because the majority of such “innocent” cells will be distributed throughout the body and not recruited by antigen such that they will not be subject to suppression. It is for this reason that injection of Tregs specific for a pancreas-derived antigen is far more effective in suppressing diabetes than polyclonal Tregs that will all not accumulate in pancreatic lymph nodes [20].

“Bystander suppression” is well documented by the fact that for instance $CD4^+$ Tregs recognizing a class II MHC-presented epitope from one particular protein can suppress CD8 T cells recognizing a different class I MHC-presented epitope from the same protein [44]. Thus the antigen specificity of Tregs and effector T cells does not need to match in order for effective immunosuppression to occur: it is sufficient

that the two cell types are co-recruited to the same tissue. This of course is good news since this will permit a Treg of one particular specificity to suppress a variety of effector cells with different specificity as long as all these different epitopes are present within the same draining lymph node or anatomical site.

Since many intrathymically generated Tregs are specific for self-antigen it is perhaps not surprising that normally there are always "activated" Tregs present in the organism [45] and some of these Tregs may be engaged in locally preventing autoimmunity. In fact neonatal removal of Tregs will result in the "scurfy" phenotype associated with multi-organ-specific autoimmunity [46,47]. Other Tregs are apparently not "in action" and patrol the body by exhibiting a phenotype of naïvec T cells that do not divide [31,45].

Function of Tregs

One of the questions that has remained rather elusive concerns the molecular mechanisms by which Tregs control other T cells. There are probably several not mutually exclusive mechanisms, some of which may dominate in certain situations [26]. In vitro data have emphasized the role of close cell-to-cell contact and dispensable cytokines such as IL-10 or TGF-β. All in vivo data published so far have emphasized the crucial role of the TGF-βRII on suppressed cells since a dominant negative form of that receptor is usually associated with ineffective Treg suppression and with generalized autoimmunity. It is still not clear whether this results from the fact that Tregs produce TGF-β (which they do but only in moderate amounts) or whether in general TGF-β-induced signaling "conditions" effector cells for more stringent suppression by a mechanism that does not involve increased TGF-β production but depends on specific Treg activation [26]. A good example for such a scenario is the suppression of tumor-specific CD8 T cells by CD4 Tregs that crucially depends on an intact TGF-βRII receptor on the CD8 T cells: In this particular model the suppression affects the function of fully differentiated cytotoxic T lymphocytes (CTL), notably the secretion of cytolytic granules. However, in vitro experiments with fully differentiated CTL have shown that TGF-β does not have any negative impact on cytolysis when added during the effector phase. This is consistent with the hypothesis that TGF-β-dependent signaling "conditions" the CD8 T cells for Treg suppression rather than representing the sole suppressor mechanism [44].

These experiments also make another important point, namely that it is apparently never too late to interfere with an immune response by Treg suppression since the experiments show that suppression can affect fully differentiated effector cells. This is good news in the sense that the obviously effective suppression late during an immune response can revert rather than prevent unwanted immunity, a concept that may become extremely useful in the clinic.

Different experiments attempting to reverse rather than prevent diabetes are fully consistent with that view: CD4 T cells specific for an islet-derived antigen of unknown nature could be activated in vitro and retrovirally transduced with Foxp3

such that within 24 hours they assumed a phenotype of Tregs. When 10^5 of such converted cells were injected into NOD mice that had become just diabetic because of beginning destruction of their islet cells, these islet-specific Tregs cured the mice of diabetes and they remained diabetes-free for at least three months when the experiment was terminated. Again this experiment suggests that Tregs can silence already fully developed effector cells [20].

Additional controls make important points with regard to the role of Treg antigen receptors in this process and hence the specificity of immunosuppression: while the injection of 10^5 cells with islet-antigen specificity was sufficient to abolish disease, the injection of even 10^6 Tregs with specificity for a large variety of different antigens or the injection of Tregs with specificity for an antigen not present in the pancreatic lymph node did not have any effect and the animals died several days later from complete destruction of β cells and resulting diabetes that obviously at this point could be no longer reversed by Tregs [20]. These results and similar results by others employing in vitro expanded Tregs [21,22] are very encouraging since they suggest that by adoptive Treg therapy early-diagnosed diabetes may be cured, in spite of the fact that the generation of sufficient numbers of islet-antigen-specific Tregs still represents a staggering logistic problem.

Thus in spite of our ignorance concerning molecular mechanisms of Treg-mediated suppression (even though a variety has been proposed [26]) we have promising evidence from murine models of disease that Tregs have the capacity to interfere with unwanted immunity early and/or late during the immune response in an antigen-specific way since they interfere with such immunity in a local milieu only while leaving the rest of the immune system intact.

There is also no compelling reason why the findings made in the somewhat popular models of type 1 diabetes should not be extended to other autoimmune diseases such as rheumatic diseases provided that there are clues about relevant antigens that are presented in local lymphoid tissue.

Concluding Remarks

The described properties of Tregs i.e. the possibility to generate them extrathymically in vivo or in vitro with any desired antigen specificity, their ability to co-home with T effector cells into antigen-draining lymph nodes and/or sites of inflammation, their potential to suppress effector cells at early and late stages of differentiation and last but not least to suppress neighboring T effector cells of any antigenic specificity, make these cells an ideal tool to intervene with unwanted immunity in an antigen-specific way. Thus one would eventually hope that the exploitation of evolutionarily selected mechanisms to deal with unwanted immune responses against self will replace indiscriminatory immunosuppression by drugs with potentially deadly side effects. This is not to say that such drugs may be completely useless: their transient application may help to set the immune system to a stage where Tregs can be more effective in dealing specifically with unwanted immunity. What should be

avoided, however, is the long-term indiscriminatory use of the drugs that eventually will ruin the protection against infections and malignant disease afforded by the immune system.

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Regulatory T Cells and Clinical Application
Jiang, S. (Ed.)
2008, XVIII, 590 p. 71 illus., 32 illus. in color., Hardcover
ISBN: 978-0-387-77908-9