Published in final edited form as:

Semin Immunol. 2011 December ; 23(6): 410–417. doi:10.1016/j.smim.2011.06.004.

Extra-thymically induced regulatory T cells: do they have potential in disease prevention?

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Abstract

Foxp3⁺ Treg safeguard against autoimmune diseases and immune pathology. The extrathymic conversion of naïve T cells into Foxp3⁺ regulatory T cells can be achieved *in vivo* by the delivery of strong-agonist ligands under subimmunogenic conditions. Tolerogenic vaccination with strong-agonist mimetopes of self-antigen to promote self-antigen specific tolerance may represent the most specific and safest means of preventing autoimmunity. This review discusses the requirements for induction of dominant tolerance exerted by Foxp3⁺ Tregs in autoimmunity with special emphasis on their impact to interfere with T1D. The future goals are the understanding of self-non-self discrimination at the cellular and molecular level, which should then enable investigators to develop clinical vaccination protocols that specifically interfere with unwanted immune responses.

Keywords

regulatory T cells; Foxp3; conversion; vaccination; autoimmune disease

1. Introduction

The concept of self-non-self discrimination, and thereby immunological tolerance, is as old as our appreciation of the receptor-diversity in the immune system. The notion of repertoire selection through deletion of self-reactive lymphocytes was first put forward in the clonal selection theory by Burnet [1] and by Lederberg [2]. Negative selection in primary lymphoid organs, also called central or ‘recessive’ tolerance, was addressed by analyzing the fate of developing thymocytes in response to stimulation by conventional peptide MHC complexes in TCR-transgenic mice [3], or in response to superantigens in wild-type mice [4]. These studies concluded that there was a period during the ontogeny of lymphocytes when binding of receptors to antigen resulted in cell death or anergy [5] rather than proliferation and

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development into effector cells. Deletion of self-reactive lymphocytes was shown to be enhanced by promiscuous expression of ectopic antigens in the thymus [6;7] as well as by immigration of dendritic cells presenting antigen from the periphery [8]. It became clear, however, that central tolerance was only one of several mechanisms used to achieve non-responsiveness. Further 'recessive' tolerance mechanisms in secondary lymphoid organs such as deletion and/or anergy were described [9;10], but it became evident that even the presence of these additional passive mechanisms was insufficient to account for self-non-self-discrimination and that 'dominant' immunoregulation was of essential importance. Although central tolerance was the first tolerance mechanism to be described in depth, at the same time strong evidence for the existence of dominant tolerance was presented [11;12]. The identification of the transcription factor Foxp3, as the master regulator in Tregs, has led to the description of Foxp3 expressing cells that suppress the functions of other adaptive and innate immune cells [13;14]. Mutations in Foxp3 cause severe autoimmunity in man and mouse [15;16] as does acute depletion of Foxp3⁺ cells [17;18].

Further understanding of the mechanisms of immunological tolerance at the cellular and molecular level is required in order to deal with unwanted immune responses; in particular the development, activation and effector function of regulatory T cells needs to be studied in detail. These advances should then enable studies to precisely determine how tolerance fails in autoimmune diseases, and enable investigators to develop Foxp3⁺ Treg-vaccination protocols in order to interfere with autoimmunity, transplantation and allergy. This review discusses the requirements for the induction of dominant tolerance exerted by Foxp3⁺ Tregs with special emphasis on their capability to interfere with autoimmunity such as type 1 diabetes (T1D).

2. Dominant tolerance

2.1. Intra-thymic differentiation of Foxp3⁺ Treg cells

Recognition of agonist TCR ligands appears to be a key event not only for negative selection, but also for the initiation of the developmental program leading to Treg generation. The lineage fate of such cells is instructed by the TCR binding to agonist ligands expressed within the thymus [19;20]. Cross-presentation of such ligands by hematopoietic cells is not required but can be sufficient [20]. TCR signaling induces expression of Foxp3 and of the high-affinity IL-2 receptor- α chain (CD25). In the thymus, the Treg cell lineage differentiation process can begin with CD4⁺CD8⁻ single-positive cells in the thymic medulla, but a contribution of Foxp3⁺-expressing double-positive cells in the thymic cortex is not entirely excluded [21]. It became clear that Foxp3 is not required for the initial Treg cell fate determination of developing thymocytes, but more likely required to complete Treg cell differentiation once the initial Treg cell fate is determined [22;23].

If engagement of TCR signaling instructs Treg cell development, one might expect that a TCR from a Treg-cell should instruct Treg cell development when expressed as a transgene [24;25]. However, recent studies pointed out that this is not the case due to saturable niches for Treg development: The reduction of the number of precursors with the specific transgenic Treg-cell derived TCR resulted in an increase of Tregs expressing that particular TCR [24-26]. Instruction of Treg-cell lineage fate results from TCR signals induced by intrathymic (i.t.) [27] and/or extrathymic (e.t.) [28;29] ligands. I.t. expression of peripheral antigens can be regulated by AIRE (autoimmune regulator) [30]. Given the fact, that i.t. ligands are able to instruct Treg development one might assume that such ligands include peripheral antigens. I.t. generation of Foxp3 Treg is most likely induced by autoantigens expressed within the thymus [31]. TCR repertoires of Treg cells and conventional CD4⁺ $\alpha\beta$ T cells are overlapping [26;32]. As discussed above saturable thymic niches for selection of

Treg cells could contribute to this finding, in that not all cells with an appropriate TCR can be selected into the Treg cell lineage [24].

Given that agonist TCR ligand recognition regulates the initiation of Treg generation, it has been hypothesized that environmental co-signals either support or prevent this cell fate decision. Especially CD28, IL-2 and TGF β have emerged as candidates. Genetic ablation of either CD28 or its ligands B7-1 and B7-2 results in severely reduced proportions and numbers of thymic Treg [27;33;34]. This scenario reflects a T-cell intrinsic function of CD28-signaling in Treg-differentiation, since bystander wild-type T cells do not restore Treg development of CD28-deficient thymocytes in mixed bone marrow chimeras [27]. Several mutually non-exclusive scenarios can be envisioned at which stage CD28 costimulation impacts i.t. Treg generation: lack of costimulation could result in quantitatively altered signal strength of the underlying TCR stimulus itself. Thus, the Treg-differentiation process could be hampered in the absence of CD28 costimulation. Additionally, the Ick-binding domain of the CD28 cytoplasmic tail appears critical for the induction of Foxp3 [27], thereby suggesting that binding of Ick to the CD28 cytosolic tail results in an enhancement of the intensity and duration of the antigen-specific TCR signal [35;36].

During i.t. Treg differentiation the IL-2 signaling pathway may cooperate with the instructive TCR stimulus. Ablation of either IL-2 or of IL-2 receptor subunits (CD25 or CD122) leads to severe impairment of dominant tolerance accompanied by massive autoimmunity [37-41]. Since IL-2 signaling is essentially required for the survival of Treg [42;43], the role of IL-2 as a true differentiation factor has been debated. The presence of Foxp3 expressing thymic CD4⁺ T cells in the context of IL-2 or IL-2R α deficiency underlines the conclusion that IL-2 is not absolutely required for i.t. Treg generation [44;45].

Besides its well established role for e.t. induction of Treg [46-48], the requirement of TGF β for the i.t. generation of Foxp3 Treg has been debated. Initially, T-cell specific ablation of the TGF β receptor type II did not significantly impact the i.t. development of Treg [49-51]. Subsequently, it was shown that T-cell specific deletion of TGF β RI [52] or TGF β RII [53] caused a reduction of the initial wave of thymically induced Treg. In support of this notion, ablation of TGF β RI subunit in DP thymocytes led to a profound but transient impairment in generation of Foxp3⁺ Treg cells during the first week of life followed by the recovery of Foxp3 thymocytes numbers to those seen in wild-type mice [52]. Another proposed scenario is that the requirement of TGF β for i.t. Treg induction was masked by IL-2, supporting survival and expansion of residual Treg. The absence of both TGF β signaling pathways results in a complete lack of thymic Treg [52], however, it is not clear whether this is a cell autonomous effect.

2.2. Extrathymic generation of Foxp3⁺ Treg cells

Although the physiological relevance of e.t. Tregs is much less certain than that of i.t. induced Treg, e.t. induced Treg are being studied, as they can be easily generated and bear therapeutic potential. It became clear that i.t.- versus e.t. generated Tregs display close similarity, with regard to their regulatory function, surface phenotype, stability as well as their global gene-expression pattern. Transcriptional profiling of Foxp3⁺-expressing Tregs in comparison with naïve or activated T cells revealed a distinct subset of differentially expressed genes comprising some genes normally up-regulated in activated T cells, such as IL2ra (CD25), Ctla4 (CTLA4) and Tnfrsf18 (glucocorticoid-induced TNF receptor; GITR), representing signature target genes for Foxp3 [54;55].

E.t. induced Treg can be formed in peripheral lymphoid organs; they can be generated by sub-immunogenic delivery of strong-agonist ligands under conditions that avoid the

functional activation of antigen-presenting cells [28;29;56-58]. These studies confirmed anecdotal evidence of generating 'dominant' tolerance by sub-immunogenic antigen delivery [59]. Best conversion is seen in T cells that undergo only limited proliferation, whereas higher doses of TCR agonists result in more extensive proliferation and diminished conversion. Such high doses of TCR ligands can lead to the activation of the PI3K/Akt/mTOR pathway [60] that could interfere with e.t. Foxp3 induction [61]. Robust T cell activation was shown to negatively impact on the e.t. generation of Treg based on a cell cycle dependent maintenance of a silenced state of the Foxp3 locus [62].

With regard to lifespan it became clear, that e.t. Treg can persist for months as resting cells in an intermitotic stage independent of further supply of the agonist ligand that induced their formation. This is an important feature since it permits the prospective induction of such cells to suppress unwanted immune responses. The e.t. Foxp3⁺ Treg generated by subimmunogenic antigen delivery are stable [63] and maintain their function even in an immunogenic context [64] since re-encounter of antigen under immunogenic conditions does cause neither loss of Treg cells nor loss of their activity [65] but results in expansion of Tregs [29].

2.3 Signals controlling Foxp3 expression and stability

Major inducers of Foxp3 expression in peripheral T cells as studied *in vitro* include TCR signaling in the presence of TGFβ [46], the downstream signaling transducers Smad2 and Smad3 as well as retinoic acid [66-69]. It should be noted, however, that *in vitro* generation of Foxp3⁺ cells in short term cultures yields unstable Tregs that do not resemble Tregs generated *in vivo* [63]. The expression of Foxp3 is tightly regulated by multiple transcription factors, chromatin and by cis-regulatory elements. Upon TCR activation the binding of transcription factors such as AP1, NFAT, CREB and ATF to the Foxp3 promoter and enhancer elements are induced [70;71]. In addition to the promoter, at least three conserved non-coding sequence elements (CNS) have been identified and contribute to regulation of the Foxp3 locus as well as to the maintenance of Foxp3 expression [70-73]. The CNS being important for the maintenance of Foxp3 expression contains a region rich in CpG dinucleotides, which are targets for DNA methylation [63;74;75]. While this region is methylated in conventional T cells, it is completely demethylated in Tregs, induced i.t. or generated e.t. *in vivo* [63]. In contrast, these elements remain methylated in Tregs generated *in vitro* that do not stably express Foxp3 [63;74;75]. One possible consequence of continuous methylation of these CpG-containing elements in *in vitro* generated Tregs is that transcription factors like the methyl-sensitive CREB fail to bind to this region. Accordingly, demethylation of this particular CNS appears to facilitate binding of transcription factors that mediate stable heritable maintenance of Foxp3 expression. In fact, a demethylated state of this region was shown to correlate with a stable Treg phenotype [72]. Recent studies demonstrated that loss of DNA methylation at this region promotes the binding of CREB, ATF, c-rel and Foxp3 itself, supporting the scenario that Foxp3 could form part of a positive feedback loop, maintaining its own expression [76].

TGFβ was shown to cooperate with TCR signals to induce Foxp3 in part by antagonizing cell-cycle dependent recruitment of maintenance DNA methyltransferase I (DNMT1) to the Foxp3 locus resulting in its inactivation [62]. Not mutually exclusive is the scenario that signals capable of inducing Foxp3 initiate chromatin remodeling and establish a poised state of the Foxp3 locus. Robust proliferation accompanied by propagation of CpG methylation by DNMT1 may prevent establishment of this permissive state [77]. TGFβ seems to mediate Treg induction through both direct and indirect mechanism, with Smad3/2 and NFAT binding to the Foxp3-CNS [71].

Recent studies providing genetic and pharmacological evidence indicate that the PI3K-Akt-mTOR signaling pathway interferes with Foxp3 induction *in vitro* and *in vivo*. In the absence of mTOR, naïve CD4⁺ T cells differentiate into Treg [78]. That is, T cell activation in the absence of mTOR leads to a default Treg pathway. Until recently, the mechanisms that link PI3K-Akt-mTOR signaling to Foxp3 expression have been unknown. Merckenschlager and colleagues have shown that early withdrawal of TCR signaling through pharmacological inhibition of PI3K-Akt-mTOR signaling results in robust induction of Foxp3 [61]. Efficient induction of Foxp3 expression requires proteins that limit the activity of PI3K such as Cbl-b [79] or PTEN phosphatase [61], which antagonizes PI3K function, consequently inhibiting downstream signaling through Akt. In line with these observations, a constitutive activation of Akt was shown to interfere with stable induction of Foxp3 [80]. mTORC2 activates Akt by phosphorylating it on Ser473 [78]. Recently, Foxo transcription factors have been shown to link PI3K-Akt-mTOR to Foxp3 [81;82]. Foxo transcription factors possess import functions in the homeostasis of the immune system [83]. Activity of Foxo factors was shown to be regulated at multiple levels, most strikingly by Akt-mediated phosphorylation. Phosphorylation by Akt was demonstrated to inactivate Foxo proteins by excluding them from the nucleus. Additionally, mTORC2-dependent phosphorylation of Foxo1 and Foxo3a leads to their inactivation by similarly promoting their sequestration into the cytoplasm [60]. Both of these transcription factors play a role in Foxp3 induction. Therefore, in the presence of mTORC2 activation, Foxo1- and Foxo3a-mediated Foxp3 expression is diminished. Foxo binding motifs in the Foxp3 promoter region were identified [81] in accordance with several conserved Foxo consensus sequences in the Foxp3 locus [82]. Reporter gene assays suggested the importance of a site close to the Foxp3 transcription start site [81;82]. The proposed scenario is that PI3K-Akt-mTOR signaling pathway limits the induction of Foxp3 expression but does not impinge on the maintenance of Foxp3 Tregs [60]. These signals are, at least in part, integrated by Foxo transcription factors.

3. Enhancement of extrathymic Treg generation

We and others have shown that the mTOR-inhibitor rapamycin and its analogue everolimus can specifically promote the generation of Treg *in vitro* [56;84] and *in vivo* [56;57]. The rapamycin-mediated increase in Foxp3 Treg generation can be attributed to its interference with T-cell costimulation, to its ability to reduce cell division and thereby to reduce activation of DNMT1 as well by reducing T-cell activation through the ATP-gated P2X7 receptor controlling Ca²⁺ influx [56]. Rapamycin-induced Treg generation was shown to be associated with histone H3K4me2 and 3 methylation near the Foxp3 transcriptional start site [61]. Initially, it was hypothesized that rapamycin specifically inhibited mTORC1 without affecting mTORC2 activity. The ability of rapamycin to promote Treg cell generation was therefore attributed to its ability to inhibit mTORC1. However, more recent studies confirmed that mTOR inhibitors as rapamycin or everolimus do inhibit mTORC2 as well, thereby causing a reduction of Akt activity *in vitro* and *in vivo* [85-88]. This fact appears to be in line with recent studies on the role of Foxo factors in regulating Foxp3 expression. As discussed above, phosphorylation by Akt was demonstrated to inactivate Foxo proteins by excluding them from the nucleus. Rapamycin- or everolimus-mediated inhibition of mTORC1, mTORC2 and thereby Akt should support the maintenance of active Foxo factors within the nucleus in order to promote Foxp3 expression. mTOR-inhibitors as rapamycin or everolimus were shown not only to increase the conversion rate into Treg but also to stabilize Foxp3 expression [56]. The use of other drugs like inhibitors of DNMT1 as 5-Aza-deoxycytidine is often limited due to high toxicity and these compounds offer less enhancement of Treg induction. The ability of mTOR inhibition to block CD4⁺ T cell effector differentiation and to promote the generation of Treg has important clinical implications for autoimmune diseases and transplantation.

Recent studies by others and us have documented that specific expansion of Treg *in vivo* can be achieved by application of IL-2/IL-2ab complexes [56;89]. The achieved expansion of Treg is however transient and expanded Treg were shown to survive for only 1-2 weeks. Characterization of CD25⁺Foxp3⁻ cells increased after application of IL-2/IL-2ab complexes revealed that these cells do not represent cytokine producing effector cells but anergic cells. Some of these cells were shown to produce IL-10 as well. Therefore, the combined approach of rapamycin/everolimus application followed by administration of IL-2/IL-2ab complexes makes it feasible to achieve highly efficient Treg-conversion and their expansion [56].

Further advances in the mechanistic understanding of signals that determine Foxp3 induction will facilitate the development of novel approaches and drugs to increase Treg generation for preventive and therapeutic interventions. As discussed above Foxo factors, especially the kinases that regulate their activity represent promising new drug targets.

4. Tolerogenic vaccination by self to prevent autoimmunity Characteristics of autoreactive T cell epitopes and induction of Foxp3

In the past we performed several studies exploring the optimal conditions for Foxp3⁺ Treg conversion in order to induce tolerance to antigens, including transplantation antigens [19;29;56;58]. In those models unrelated to autoimmunity, we were successful in using optimized Treg-vaccination protocols for instance in order to induce prospective tolerance in female mice to a variety of male tissues, including skin and hematopoietic grafts [58]. However, our initial attempts to prospectively induce tolerance in the NOD mouse model of autoimmune T1D by subimmunogenic application of the natural insulin B chain epitope failed. Several reasons might account for this observed scenario.

There is compelling evidence that autoantigens are presented and recognized by autoreactive T cell receptors in a unique way [90-96]. Insulin was shown to represent an essential autoantigen in T1D that must be recognized by the immune system in order to initiate the disease [97;98]. In early experiments Wegmann et al. [99] and Daniel et al. [100] cloned T-cells directly from islets of pre-diabetic NOD mice and reported that the majority of isolated CD4 clones reacted with insulin. More than 90% of such insulin-reactive clones were shown to respond specifically to insulin B:9-23. Besides this well-established knowledge, there has been considerable difficulty in identifying the relevant epitope(s) for the T-cells within the insulin B:9-23 peptide, supporting the notion that this peptide may bind to the MHC class II molecule I-A^{g7} and be recognized by T cells in more than one way. Recent work by John Kapplers group provided convincing evidence that the relevant insulin B chain epitope is presented by MHCII I-A^{g7} in an unfavored binding register, resulting in weak agonist activity of the peptide-MHC-complex [96]. The poor binding is the result of an incompatibility between the p9 amino acid of the insulin epitope and the unique I-A^{g7} p9 pocket polymorphisms that are strongly associated with disease susceptibility [96]. In this unfavored reading frame an arginine residue confronts another arginine in the positively charged p9 pocket of I-A^{g7}.

The elucidation of the crystal structures of human susceptibility alleles HLA-DQ8 and DQ2 and the homologous molecule in the NOD mouse, I-A^{g7}, revealed striking structural similarities in the peptide binding pockets [101]. The one unique feature that confers disease susceptibility was identified in the peptide-binding groove of HLA-DQ8 and I-A^{g7}: Most human HLA-DQ and mouse I-A alleles harbor an aspartic acid residue at position 57 in the β chain that points into the peptide-binding groove [102;103]. In most HLA-DQ and I-A alleles, this aspartic acid forms an electrostatic salt bridge with arginine at position 76 in the α chain of the molecule. It was demonstrated that both HLA-DQ8 and I-A^{g7} lack aspartic acid at position 57 in the β chain. This variation was shown to disrupt the electrostatic

interaction and to leave the positive charge of the arginine unopposed [101;104]. This results in a positive surface charge of the peptide binding groove. The described polymorphisms therefore strongly implicate a decisive role of peptide presentation in disease susceptibility.

Recently the peptide WE14 from chromogranin A was identified as the antigen for the BDC2.5 T cell clone and other related NOD-derived T cells [105]. Earlier work had indicated that the antigen for those clones was contained within the secretory granules of islet β cells [106]. Peptide truncation and extension analysis now revealed that WE14 binds to MHCII I-A^{g7} in an atypical way, occupying only the carboxy-terminal half of the MHC binding groove [105]. The findings for chromogranin A further supports the notion that in autoimmune disease such as T1D autoreactive T cells respond to unusually presented self-ligands [96;105].

Similar findings of atypical antigen presentation were demonstrated by Garcia's group in the murine experimental allergic encephalomyelitis EAE system: The immunodominant epitope of myelin basic protein MBP1-11 was shown to bind weakly to I-A^u[93]. Crystal structures of the I-A^u/MBP1-11 complex revealed that the MBP1-11 peptide only partially occupies the peptide binding groove. In addition, the fourth residue of MBP1-11 was shown to be located in a structurally incompatible p6 pocket of I-A^u, accounting also for the short half-life of the peptide-MHC complex [93]. Identified structures of the tri-molecular complex for TCRs recognizing the MBP1-11 peptide demonstrated that partial occupancy of the peptide binding groove also impaired TCR interactions with this self-epitope. The observed scenario was that the partial occupancy of the peptide-binding groove not only affected the stability of the peptide-MHC complex, but also resulted in diminished potential contacts with the TCRs.

It has therefore been proposed that the unfavorable manner in which self-reactive epitopes interact with the disease-promoting MHC molecule constitutes a scenario in which autoreactive T cells are able to efficiently evade central tolerance. This observation is well in line with studies demonstrating that T-cells with specificity for poorly agonistic self-peptides escape negative selection in the thymus and mount an autoimmune attack in peripheral tissue [90-92;94;96;107]. We argue that in autoimmune disease poor agonistic ligands not only permit escape from 'recessive' tolerance -- i.e. negative selection in the thymus but that such ligands also fail to efficiently induce 'dominant' tolerance i.e. Foxp3⁺ Treg that can control the disease in peripheral tissue. Our recent work with the insulin B: 9-23 peptide showed that in autoimmune T1D the intrinsic properties of self-reactive epitopes critically impact the e.t. induction of Foxp3⁺ Treg: Our results revealed that dominant tolerance to essential insulin epitopes fails in T1D. We demonstrated that the generation and subimmunogenic delivery of a strongly agonistic insulin mimetope was suited to convert naïve T cells into Foxp3⁺ Treg with high efficacy and thereby to prevent T1D. Subimmunogenic doses of the insulin mimetope protected NOD mice for 40 weeks and longer from development of diabetes. Mice vaccinated with the insulin mimetope exhibited significantly lower insulinitis scores in comparison to untreated mice or mice that had received the natural insulin epitope. As seen from immunohistochemistry analyzing Foxp3 expression in pancreatic cryosections, in contrast to the natural insulin epitope, tolerogenic vaccination with the strong-agonist mimetope resulted in high numbers of Foxp3⁺ cells that suppress the proliferation of IFN γ and IL-17 cytokine-secreting [108] insulin-specific effector cells. Natural insulin epitopes were found to be ineffective irrespective of the applied dose.

In accordance with these findings, conducted in short-term experiments unrelated to autoimmunity, Allison's group showed that TCR ligand affinity determined peripheral Foxp3 induction *in vivo* [109]. Time course experiments demonstrated that Foxp3⁺ Tregs

induced by weak agonist ligands did not persist. We and others demonstrated that low doses of strong-agonist ligands are suited to generate stable Foxp3⁺ Tregs with high efficacy [29;56;63;109], whereas even high doses of poorly agonistic ligands fail to induce stable Foxp3⁺ Treg. These data support the notion that TCR ligand density cannot compensate for diminished ligand affinity in determining the efficacy and persistence of Foxp3⁺ Tregs. It was therefore suggested that qualitative differences in TCR stimulation are relevant for stable induction of Foxp3⁺ Tregs [109]. The proposed scenario could be that a strong TCR-peptide-MHC interaction specifically triggers survival signals, as opposed to pro-apoptotic signals which are induced by triggering of many complexes with weaker interactions [109]. It was suggested that cell-intrinsic mechanisms of ligand distinction could play a role in the discrimination of self- and foreign ligands [110]. In addition, it became clear that cell-extrinsic mechanisms could account for the discrimination between high-potency and high density ligands. Cell-extrinsic mechanisms seem especially relevant in the context of T-cell-APC interactions. It became evident that the nature of the DC-T cell interactions highly impinges on the outcome of the immune response, with prolonged stable interactions being essential for effective proliferation and induction of effector functions as visualized by live imaging within lymphoid tissues [111;112]. The maturation state of APCs was shown to be of high importance for the T cell fate under tolerizing conditions [113]. Moreover, ligand dose and potency were indicated to impact on the kinetics and type of effector T-cell APC contact formation [114;115]. Thus, peptide MHC ligand density was shown to critically influence the duration and quality of DC-T cell interactions *in vivo*, and thereby also to impact the threshold for *in vivo* induction of T cell proliferation versus tolerance. Accordingly, it was assumed that low doses of peptides could lead to short interactions, which, given a strong-agonist ligand, could be sufficient to provide the subimmunogenic stimulation necessary for efficient and stable Foxp3 induction in the absence of strong cell division *in vivo*.

Given that many aspects of how TCR-peptide MHC binding are communicated from the extracellular portions of the TCR to signaling pathways across the T cell membrane remain unresolved [116], it is though accepted that quantitative and qualitative differences in the strength of TCR-peptide MHC interactions critically influence signaling events far downstream of TCR recognition and thereby the type of cell fate decision instructed in peripheral T cells [116].

In terms of molecular aspects impacting T cell activation it is of interest that a strong agonist TCR-ligand is required for conversion of naive T cells into Treg even though it must be delivered under sub-immunogenic conditions and by avoiding activation of the PI3K/Akt/mTOR pathway [28;29;109]. At a first glance this appears somewhat counter-intuitive considering the most popular models of T cell activation by TCR cross linking and costimulation [117]. However conformational changes induced in single receptors by TCR-ligands are likewise considered as a mode of T cell activation [118] and perhaps it is this mode that leads to conversion of T cells into Treg rather than to induction of effector function such as cytokine secretion. Clearly further studies are required to explore the molecular mechanisms involved in the *in vivo* Treg-conversion process.

5. Conclusions

In the setting of autoimmune T1D, it became clear that subimmunogenic application of a strong-agonist insulin mimotope converts naïve insulin-specific T cells into stable Foxp3⁺ Treg with high efficacy resulting in the most efficient diabetes prevention recorded to date. Contrary, immunogenic high doses of antigens were found to result in T-cell proliferation and activation of the PI3K/Akt/mTOR pathway [60] that both could interfere with e.t. Foxp3 induction [61],[29]. At present the described tolerogenic vaccination strategy is limited to

prevention rather than treatment of disease, because it converts naïve T cells only. It is assumed that treatment may become possible when drugs are developed that specifically interfere with the silencing of the Foxp3 locus in activated T cells.

In T1D, previous studies attempted the induction of immunological tolerance using natural insulin or insulin-derived peptides via mucosal exposure or in the presence of adjuvant such as cholera toxin B subunit or IFA [119-125]. Some of these approaches resulted in reduced incidence of T1D in NOD mice but were not able to completely prevent disease. From our recent work it became clear that subimmunogenic treatment with a strong-agonist insulin mimotope resulted in a reduction of insulin autoantibody levels, while immunization trials with insulin in the presence of IFA were shown to increase titers of insulin autoantibodies [126]. These results therefore suggested that different mechanisms of tolerization are at work and that perhaps a Th1 to Th2 shift accounted for the previously published results [127].

In NOD mice insulin was shown to represent an essential target of the autoimmune attack [97;98]. The understanding of the scenario in humans is less advanced even though in humans i.t. expression levels of insulin control T1D [128]. Thus insulin has a major influence on human disease as well. At present our proposed strategy is preventive only and therefore an envisioned clinical protocol aims at inducing insulin-specific Foxp3⁺ Tregs in pre-disposed individuals at genetic risk of developing disease and in the presence of moderate levels of insulin autoantibodies. Foxp3⁺ Treg vaccination was shown to require only a single epitope because the induced Treg suppress immune responses of T cells to antigen from the same source through bystander suppression[58], whereby the vicinity to antigen presenting cells but not the specificity of the suppressed cells is relevant. It is the distinct advantage of this protocol that Foxp3⁺ Tregs of one specificity can suppress effector cells with specificity for any antigen from the same source.

Given the promise of Foxp3⁺ Treg generation in the immunotherapy of autoimmunity and immune pathology, the development of clinical protocols aiming at the specific prevention of unwanted immune response should result in the future replacement of indiscriminate immunosuppression that often has fatal consequences.

Acknowledgments

The studies were supported by National Institutes of Health Grant NIH-AI-53102 (to H.v.B.). C.D. was supported by a Leopoldina research fellowship (BMBF-LPD 9901/8-184) and by the LOEWE (LipidSignaling Forschungszentrum Frankfurt) program of the Federal State of Hessen, Germany.

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Highlights

- TCR ligands of strong-agonist activity applied under subimmunogenic conditions convert naïve but not activated T cells into stable Foxp3⁺ Treg.
- Tolerogenic vaccination with strong-agonist mimetopes of self-antigen may represent the most specific and safest means of preventing autoimmunity.
- Autoantigens are presented and recognized by autoreactive T cell receptors as weak agonists.
- Weakly agonistic autoantigens recognized by autoreactive T cells fail to efficiently induce extrathymic Tregs.
- Tolerogenic vaccination with strongly-agonistic mimetopes of insulin prevents T1D in mice.