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Cutting Edge: De Novo Induction of Functional Foxp3+ Regulatory CD4 T Cells in Response to Tissue-Restricted Self Antigen

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Naive CD4 T cells can differentiate into a number of functional subsets in response to Ag, including Foxp3+ induced regulatory T cells (iTregs). The in vivo development and function of iTregs has been primarily demonstrated in systems involving Ag encountered systematically or delivered via the intestinal mucosa. In this study, we demonstrate that de novo Foxp3 expression in naive CD4 T cells is a critical mechanism for establishing tolerance for a tissue-restricted neo-self Ag. Naive CD4 T cells lacking a functional Foxp3 gene cannot achieve tolerance, but can be suppressed in vivo in the presence of wild type naive CD4 T cells. Exposure to nonspecific inflammation during priming underestimates tolerance through impaired Foxp3 induction, suggesting that the microenvironment also has a role. These data show that de novo Foxp3 expression is an integral component of establishing and maintaining tolerance among naive peripheral CD4 T cells. The Journal of Immunology, 2011, 186: 4551–4555.

Naive CD4 T cells can develop into a variety of specialized effector or regulatory subsets (1). Some priming mechanisms result in tolerance, particularly to self Ag. Deletion and anergy can be achieved intrinsically by signals that lead to programmed death or inactivation in which cells are refractory to further stimulation (2). In addition, effective trans-suppression is mediated by regulatory T cells (Tregs) that express the transcription factor Foxp3 (3). The immune suppressive functions of persistent pathogens can work partly through induction of Foxp3 in microbe-specific CD4 T cells (4). Expression of Foxp3 is known to dampen CD4 T cell effector responses, suggesting that de novo Foxp3 expression may be both an intrinsic and extrinsic mechanism of Ag-specific tolerance.

It is well appreciated that TGF-β and retinoic acid are key mediators of peripheral Foxp3 induction, and these signals are enhanced in a subpopulation of APCs such as dendritic cells expressing CD103 and/or DEC-205 (5). Ag exposure in the context of chronic microbial infection, low-level peptide infusion, systemic soluble Ag, or oral administration can all induce Foxp3+ Tregs from naive CD4 T cell precursors (6). Inflammatory cytokines such as IL-6, IL-12, IL-27, and IFN-γ can undermine TGF-β-mediated induction of Foxp3, although IL-27 and IFN-γ can also enhance Foxp3 induction (7–10).

The significance of de novo Foxp3 expression in the maintenance of tissue-specific tolerance is unclear. The estimated frequency of peripherally generated Foxp3+ Tregs ranges from 30 (11) to <10% of total Tregs (12). Normal peripheral Treg conversion is thought to occur at mucosal surfaces in response to Ags derived from food, commensal bacteria, or inhaled particles. Peripheral de novo Treg conversion in response to tissue Ag is not well defined. In this study we demonstrate a model of islet tolerance in which de novo expression of Foxp3 is critical for prevention of autoimmunity. Peripheral Tregs are shown to be effective for trans-suppression of autoreactive effector T cells. These results provide compelling evidence of a role for de novo Foxp3 expression in the maintenance of tissue-specific CD4 T cell self tolerance.

Materials and Methods

Mice

DO11.10 mice and CD45.1 congenic B6.SJL-Ptprca Peprcb/BoyJ mice were purchased from the Jackson Laboratory. CD45.1 mice were backcrossed to BALB/c mice for at least 10 generations. Rat insulin promoter membrane-bound OVA transgenic/recombination activating gene-deficient (RO/RAG2) mice on the BALB/c background were provided by A. Abbas (University of California, San Francisco, CA). Foxp3-GFP mice were provided by A. Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY) and backcrossed to BALB/c mice for at least 10 generations. Influenza HA-specific TCR (HNT) transgenic mice were provided by S. Swain (University of Massachusetts, Worcester, MA). All mice were maintained in a specific pathogen-free facility. All protocols were conducted as approved by the Animal Care and Use Committee of the Benaroya Research Institute.

Diabetes monitoring and induction

Blood glucose levels in chimeric mice were monitored twice per week using an Ascensia Contour blood glucose monitoring device. Mice were considered...
diabetic after two consecutive blood glucose level readings above 250 mg/dl. As indicated, mice were treated with 100 μg OVA peptide 323-349 (New England Enzyme) emulsified in IFA and injected s.c. Mice treated with polyinosinic-polycytidylic acid (poly-IC; Invivogen) were given 100 μg poly-IC in PBS i.p. at the indicated time points.

**IL-2 complex treatment**

Recombinant murine IL-2 (eBioscience) was incubated with anti–IL-2 mAb clone JES6-1 (University of California, San Francisco Monoclonal Antibody Core) in sterile PBS at 25°C for 30–60 min. After incubation, the complex was injected into mice (1.5 μg IL-2, 50 μg IL-2 mAb per mouse) i.p. every other day for a total of three treatments. Five days after the first treatment, spleens were harvested and CD4 T lymphocytes were analyzed for intracellular Foxp3 expression.

**Flow cytometry and cell sorting**

Fluorochrome-conjugated mAbs for murine CD4 (RM4-5), DO11.10 TCR (KJ1-26), Foxp3 (FJK16), CD45.1 (A20), IFN-γ (XMG1.2), and IL17A (TC11-18H10.1) were purchased from eBioscience and Biologend. Intracellular detection of Foxp3 was achieved using fixation and permeabilization protocol and buffers purchased from eBioscience. To purify CD4 T cells, no-touch magnetic bead purification was conducted using the Miltenyi Biotec CD4 T cell isolation kit.

**Histology**

Pancreata were snap-frozen on dry ice in O.C.T. (Tissue-Tek), and frozen sections were sectioned into 5-μm slices. Slides were fixed in acetone, dried, and stained with H&E. Analysis used Leica DM2500 light microscope, and images were acquired with an attached SPOT Insight 4 digital camera with SPOT 4 Advanced imaging software. No image modifications were made after acquisition, other than the incorporation of scale bars.

**Intracellular cytokine production**

Single cell splenocyte suspensions were incubated at 37°C in complete RPMI 1640 medium (cRPMI) alone or in cRPMI with 1 μg/ml OVA 323-349 peptide. For CD3/CD28 stimulation, tissue-culture plates were coated with 3 μg/ml anti-CD3 (145.2C11) and 1 μg/ml anti-CD28 (P-V1) in plain PBS overnight. Abs from the University of California, San Francisco Monoclonal Antibody Core. After 1 h of incubation, Golgi Stop (BD Bioscience) was added and cells were incubated for an additional 4 h.

**Results and Discussion**

To generate Ag-specific CD4 T cells that lack endogenous Foxp3 regulatory T cells, DO11.10 mice were crossed onto a RAG<sup>-/-</sup> background—referred to as DR mice or cells (13, 14). These mice contained monoclonal naive CD4 T cells with specificity restricted to an epitope from OVA (OVA<sub>323-339</sub>). To determine the ability of these cells to promote autoimmunity in the absence of endogenous Tregs, naive DR CD4 T cells were transferred into RAG<sup>0</sup> mice expressing a transgene encoding membrane-bound OVA under control of the rat insulin promoter (RO/RAG<sup>0</sup>). As a control, DR T cells were transferred into RAG<sup>0</sup> mice lacking the RO transgene. By day 80 after transfer, none of the control mice had become diabetic, and >80% of the RO/RAG<sup>0</sup> recipients also remained free of diabetes (Fig. 1A, and data not shown). The low incidence of autoimmunity in the RO/RAG<sup>0</sup> recipients indicates that even in the absence of endogenous regulatory T cells, naive tissue-specific Foxp3-negative CD4 T cells have an intrinsic mechanism for long-lasting tolerance. To determine the role of Foxp3 in tolerance generation, we crossed DR mice with Scurfy (SF) mutant mice (SF/DR), which harbor a loss-of-function mutation of the foxp3 gene (15). Because the DR cognate Ag is not expressed in these mice, they remain healthy and viable (13, 16, and L.J. Thompson and S.F. Ziegler, unpublished observations). When SF/DR donor CD4 T cells were transferred into RO/RAG<sup>0</sup> mice, 100% of the chimeric mice became diabetic within 40 d of the adoptive transfer (Fig. 1A). Consistent with a robust effector T cell response, SF/DR T cells accumulated more in draining pancreatic lymph nodes than the wild type (WT) DR T cells (Fig. 1B). These data clearly demonstrate that naive CD4 T cell tolerance to a self-tissue Ag is dependent on the ability to express functional Foxp3.

An examination of the donor CD4 T cells showed that a subset of both WT DR and SF/DR CD4 T cells had differentiated into Th1 effector cells, as shown by intracellular expression of IFN-γ upon restimulation with OVA peptide in vitro (Fig. 1C), with a trend toward greater frequencies of IFN-γ<sup>+</sup> cells in mice with SF/DR cells. In addition, donor CD4 T cells from healthy mice expressed no detectable IL-10, and TGF-β expression was unchanged (data not shown). Upon histologic examination of pancreata from chimeric mice, Ag-dependent islet infiltration was seen in both healthy and diabetic mice (Supplemental Fig. 1). These data show that in this system, Th1 effector populations develop and are capable of infiltrating the islets, but the presence of induced Tregs (iTregs) prevents autoimmune disease.

Upon ex vivo examination of donor DR cells, RO/RAG<sup>0</sup> hosts had substantial populations of Foxp3<sup>+</sup> cells in spleen and pancreatic lymph nodes, with only a nominal Foxp3<sup>+</sup> population in RAG<sup>0</sup> hosts (Fig. 2A). As expected, both WT DR and SF/DR cells proliferated after adoptive transfer, with the WT DR exhibiting a somewhat diminished proliferation compared with SF/DR (Fig. 2B). The enhanced proliferation and expansion in the absence of a functional Foxp3 protein is consistent with the notion that effector cell responses may be curtailed by a suppression of cell division by Foxp3<sup>+</sup> Tregs.
Spleens (SPL) and pancreatic lymph nodes (PLN) from RAG0 or RO/RAG0 hosts were harvested, and donor DR cells were analyzed by FACS by gating on CD45.1-negative cells that received only SF/DR cells (SF/DR only) or the 1:1 mix of WT DR and SF/DR cells (data not shown). A significant trend was seen toward increased percentages of Foxp3 positive (upper) and Foxp3 negative (lower) donor DR as determined by intracellular staining. Representative FACS plots depicted, n = 3 for RAG0 recipients and n = 4 for RO/RAG0 recipients. B, CFSE-labeled WT DR or SF/DR CD4 T cells were adoptively transferred into RO/RAG0 hosts and donor DO11.10 cells were analyzed by FACS for CFSE dilution and intracellular Foxp3 expression. C, DR and SF/DR CD4 T cells were mixed at either a 1:1 or 1:19 ratio, and 2 × 10^5 total CD4 T cells were transferred into RO/RAG0 mice. Diabetes induction was monitored as previously described. The data show diabetes-free survival of indicated chimeric mice, 1:1 mix n = 9, 1:19 mix n = 7, for two independent experiments. D and E, SF/DR CD4 T cells were transferred alone or at equal ratios with CD45.1 WT DR CD4 T cells and transferred into RO/RAG0 mice. D, Total numbers of intracellular IFN-γ+ SF/DR cells from splenocytes stimulated with anti-CD3/CD28. CD45.1-negative cells were from chimeric mice that received only SF/DR cells (SF/DR only) or the 1:1 mixed SF/DR and WT DR donors (SF/WT mix). E, Analysis of mixed donor WT DR and SF/DR CD4 T cells. The left panel shows mixed donor populations prior to adoptive transfer (Pretransfer). The middle and right panels show ex vivo mixed donor populations from spleen (SPL) and pancreatic lymph node (PLN) 20 d after transfer. Representative FACS plot are depicted; n = 3 to 4 mice per group.

These data demonstrate that an expanded Foxp3+ Treg population responding to cognate Ag expressed in self tissues is an important factor in establishing self tolerance.

Transfer of purified Foxp3+ DO11 CD4 T cells was sufficient to inhibit autoimmunity when mixed at a 1:1 ratio with SF/DR cells (data not shown), demonstrating that Tregs can control this autoreactive T cell population. To directly determine the capacity of the de novo Foxp3+ iTreg population to control autoimmunity, naive DR CD4 T cells were mixed with SF/DR CD4 T cells and transferred into RO/RAG0 mice. When transferred in equal numbers, the DR CD4 T cells established tolerance and mice did not develop disease, but this could be overcome with higher frequencies of SF/DR donor cells (Fig. 2C). To determine whether protection by naive WT DR CD4 T cells was simply the ability of those cells to out-compete the SF/DR CD4 T cells, SF/DR cells were cotransferred with monoclonal CD4 T cells from mice expressing an HA-specific TCR (HNT) (17) into RO/RAG0 hosts, and these were not protected from diabetes (data not shown). A nonsignificant trend was seen toward increased total expansion of SF/DR cells in the absence of WT DR cells (data not shown). When total numbers of IFN-γ+ SF/DR cells were calculated, a significant difference emerged, indicating that the de novo Tregs in the mixed populations may suppress disease by inhibiting overall expansion of Th1 effector cells (Fig. 2D). Ex vivo analysis confirmed that a substantial fraction of DR cells had converted into Foxp3+ Tregs (Fig. 2E). Notably, the pretransfer ratio of WT to SF donor cells was similar at day 20 after transfer. These results establish that Tregs that arise from the donor WT DR population are capable of exerting a dominant state of tolerance in a dose-dependent manner.

The expanded Foxp3+ donor CD4 T cell population could have originated either from a small undetectable precursor population of preexisting Tregs, or de novo from a fraction of the naive CD4 T cells. Recent studies using BDC2.5 TCR+ T cells have concluded that the islet-specific Treg population arises from expansion of thymically generated “natural Treg” precursors (18, 19). However, in the DR model the cognate ligand is not expressed in the donor mouse, whereas the natural ligand for the BDC2.5 TCR, chromogranin, is present in the RAG0/BDC2.5 mice as a potential Treg-selecting Ag (20). To address these possibilities, two approaches were taken.

First, DR mice were crossed to mice containing a Foxp3- GFP functional reporter gene (16). Bone-marrow chimeric mice were generated using Foxp3-GFP DR donor bone marrow engrafted into lethally irradiated RAG0 or RO/RAG0 recipients, to generate monoclonal Treg-deficient and sufficient DR donors, respectively. FACS-sorted GFP-negative cells were then transferred into RO/RAG0 hosts (Supplemental Fig. 2). In this way, donor cells could be obtained from an environment in which the Treg-selecting ligand is absent (RAG0) or present (RO/RAG0). Transfer of sorted
GFP− DR CD4 T cells (Foxp3−) into RO/RAG0 hosts resulted in long-term tolerance and the appearance of similar levels of Foxp3+ donor cells (Figure 3A, 3B). These data demonstrate that tissue-specific tolerance can be achieved and Tregs can arise from a population that has been rigorously selected to exclude precursor Foxp3+ cells. In addition, tolerant CD4 T cells exhibit similar levels of Foxp3+ populations, indicating that even when the cognate ligand is present to select Treg precursors, the naive Foxp3-negative donor population retains the potential to give rise to Foxp3+ cells upon encountering Ag in the RO/RAG0 host.

Second, DR mice were treated with an IL-2–IL-2mAb complex that selectively expands Tregs (21). After treatment, Foxp3+ cells were not detectable in the DR mice, whereas WT BALB/c mice displayed robust Treg expansion (Fig. 3C). This result contrasts with recent studies concluding that monoclonal TCR transgenic mice harbor a normally undetectable population of Foxp3+ cells, again using the BDC2.5 model. These data demonstrate that the transfer of naive, Foxp3-negative DR T cells into RO/RAG0 hosts results in differentiation of these T cells into iTregs through de novo expression of Foxp3. Transfer of naive DR CD4 T cells, followed by immunization with OVA peptide in IFA, causes diabetes in the RO/RAG0 host (Fig. 4A) (22). Therefore, with Ag-mediated activation, naive monoclonal CD4 T cells develop effector function and drive autoimmunity. To determine whether nonspecific inflammatory stimulation in the absence immunization could trigger autoimmunity, chimeric mice were treated with the viral mimetic poly-IC to induce a systemic inflammatory response. When poly-IC was given at day 1 following transfer of the DR cells, tolerance was undermined and 100% of the mice became diabetic within 30 d (Fig. 4A). However, if poly-IC was delivered once tolerance was established at day 80 after DR transfer, tolerance was maintained and the mice remained free of diabetes (Fig. 4A). Upon ex vivo analysis at day 18 after transfer, the fraction of Foxp3+ donor cells was drastically reduced in mice that received day 1 poly-IC treatment compared with control mice (Fig. 4B). Furthermore, donor DR cells from day 1 poly-IC–treated chimeras developed into Th1 effectors (Supplemental Fig. 3). This finding would suggest that when naive CD4 T cells encounter a normally tolerizing self Ag in the context of nonspecific systemic inflammation, signals that lead to tolerance and de novo Foxp3 expression are subverted and effector differentiation proceeds. Furthermore, the lack of tolerance breakdown when systemic inflammation is encountered at a late point would suggest that the initial tolerizing program is durable once it is established.

These concepts provide an important insight into the mechanisms of CD4 T cell tolerance. The results shown in this study suggest a model in which de novo expression of Foxp3 is a critical determinant of the tolerogenic response to tissue Ag. Importantly, if tissue-specific naive CD4 T cells cannot express Foxp3, they will not establish tolerance. The heterogeneity of outcomes achieved by the naive Ag-specific CD4 T cell precursors shown in this study demonstrates that the outcome of tolerance or effector differentiation is likely achieved by a balance of the signals that act on the naive T cells as they undergo priming. The key to this balance is the relative proportion of CD4 T cells that can upregulate Foxp3, and the nature of the environment in which they encounter Ag. In the presence of inflammatory signals, Foxp3 fails to be induced and effector CD4 T cell differentiation dominates, resulting in autoimmunity. However, under normal homeostatic condition, de novo induction of Tregs limits the damage that effector T cells can inflict upon self tissues. These findings lend an important insight into an important cell-intrinsic mechanism for tolerogenic CD4 T cell responses.
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