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Low-Dose Antigen Promotes Induction of FOXP3 in Human CD4+ T Cells

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Low Ag dose promotes induction and persistence of regulatory T cells (Tregs) in mice, yet few studies have addressed the role of Ag dose in the induction of adaptive CD4+FOXP3+ Tregs in humans. To this end, we examined the level of FOXP3 expression in human CD4+CD25+ T cells upon activation with autologous APCs and varying doses of peptide. Ag-specific T cells expressing FOXP3 were identified by flow cytometry using MHC class II tetramer (Tmr). We found an inverse relationship between Ag dose and the frequency of FOXP3+ cells for both foreign Ag-specific and self Ag-specific T cells. Through studies of FOXP3 locus demethylation and helios expression, we determined that variation in the frequency of Tmr+FOXP3+ T cells was not due to expansion of natural Tregs, but instead, we found that induction, proliferation, and persistence of FOXP3+ cells was similar in high- and low-dose cultures, whereas proliferation of FOXP3- T cells was favored in high Ag dose cultures. The frequency of FOXP3+ cells positively correlated with suppressive function, indicative of adaptive Treg generation. The frequency of FOXP3+ cells was maintained with IL-2, but not upon restimulation with Ag. Together, these data suggest that low Ag dose favors the transient generation of human Ag-specific adaptive Tregs over the proliferation of Ag-specific FOXP3- effector T cells. These adaptive Tregs could function to reduce ongoing inflammatory responses and promote low-dose tolerance in humans, especially when Ag exposure and tolerance is transient. The Journal of Immunology, 2011, 187: 3511–3520.

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Abbreviations used in this article: aTreg, adaptive regulatory T cell; GAD, glutamic acid decarboxylase; HA, hemagglutinin; IGRP, islet-specific glucose-6-phosphate catalytic subunit-related protein; MFI, mean fluorescence intensity; nTreg, natural regulatory T cell; Tg, transgenic; Tmr, tetramer; Treg, regulatory T cell; TSDR, Treg-specific demethylated region; TT, tetanus toxin.
in vitro induction and persistence of FOXP3 in foreign Ag-specific and self Ag-specific CD4 T cell populations using HLA class II tetramers. We found that Ag dose, as opposed to TGF-β or bystander activation, had a dominant impact on the generation of functional human Ag-specific aTregs. However, the frequency of FOXP3+ cells was reduced upon restimulation. This dose effect was observed with both foreign Ag-specific and self Ag-specific T cells. Together, these data suggest that low Ag dose favors the induction and proliferation of human Ag-specific FOXP3+ aTregs as opposed to FOXP3− effector T cells. Determining factors that promote the generation and persistence of self Ag-specific FOXP3+ aTregs while reducing FOXP3− T cell proliferation may lead to development of Ag-specific therapies that result in reduced immunogenicity and/or tolerance induction.

Materials and Methods

Human subjects and mice

PBMCs were derived from subjects participating in studies under the auspices of the Benaroya Research Institute-Juvenile Diabetes Research Foundation Center for Translational Research registry. Informed consent was obtained from all subjects according to institutional review board-approved protocols at Benaroya Research Institute (Seattle, WA). Control participants were selected based on lack of personal or family history of autoimmunity or asthma. Foxp3-GFP C57BL/6 mice were a gift from Dr. A. Rudensky. All mice were maintained in a specific pathogen-free Animal Care Facility at the Accreditation of Laboratory Animal Care-accredited animal facility at the Benaroya Research Institute and handled in accordance with institutional guidelines.

Cell preparation, culture, and phenotyping

Fresh PBMCs were prepared by centrifugation over Ficoll-Hypaque gradients. CD4+ T cells were purified with a CD4+ no-touch T cell isolation kit (Miltenyi) followed by negative selection with Miltenyi CD25 microbeads. Autologous APCs were obtained from the positive fraction of the CD4+CD25+ cell touch selection. FOXP3 expression in CD4+CD25 T cells was reduced upon restimulation. This dose effect lead to development of Ag-specific therapies that result in reduced (Miltenyi) followed by negative selection with Miltenyi CD25 microbeads.

GAD Tmr + T cells where a Tmr loaded with 555–567I was used for de-specificity of the peptide used to stimulate the culture with the exception of PD-1 from BioLegend, or allophycocyanin CTLA-4 or FITC CD103 from with PE GITR or PerCP–Cy5.5 LAP from R&D Systems, PerCP–Cy5.5 FITC helios (BioLegend), and in other experiments cells were costained described previously (28). In some experiments, cells were costained with (26–30). IL-2 (200 IU/ml; Chiron) was added at day 7. Cells were cultured → 6-phosphate catalytic subunit-related protein (IGRP) (247–259), Pre-islet-specific glutamic acid decarboxylase (GAD) (555–567), glucose-6-phosphate catalytic subunit-related protein (IGRP) (247–259), Preproinsulin [76–92(88K–85)], and tetanus toxin (TT) (674–693) peptides (26–30). IL-2 (200 IU/ml; Chiron) was added at day 7. Cells were cultured for 14 d, unless stated otherwise, and stained for expression of CD4, tetramer (Tm), FITC CD25, and Alexa 647 FOXP3 (BioLegend) as described previously (28). In some experiments, cells were costained with FITC helios (BioLegend), and in other experiments cells were costained with PE GITR or PerCP–Cy5.5 LAMP from R&D Systems, PerCP-Cy5.5 PD-1 from BioLegend, or allophycocyanin CTLA-4 or FITC CD103 from BD Biosciences. HLA DRB*0401 Tmr used for staining matched the specificity of the peptide used to stimulate the culture with the exception of GAD Tmr+ T cells where a Tmr loaded with 555–567I was used for de-tection as described previously (31). Positive Tm staining was determined to be responses at least 0.2% and 4-fold greater than those of irrelevant control Tm stains. FOXP3 isotype in conjunction with CD25 expression on activated T cells was used as FOXP3 staining controls as described previously (32) and shown in Supplemental Fig. 1.

Costaining for BrdU and FOXP3 was performed as described previously (14). Briefly, after overnight incubation with BrdU, cells were fixed and permeabilized using the BioLegend FOXP3 fixation buffer and then the BD Cytofix/Cytoperm and BD Cytoperm Plus buffers. Permeabilized cells were treated with DNase for 1 h at 37°C prior to staining with FITC anti-BrdU Ab. Costaining for intracellular IL-10 was performed with BD CytoFix/Perm reagents per the manufacturer’s instructions after 5-h stimulation with PMA (50 ng/ml) and ionomycin (1 μg/ml) in the presence of 1 μM GolgiStop. All phenotype data were acquired on a FACS Calibur and analyzed using FlowJo 7.6 software.

Methylation analysis

All methylation analysis was performed on cells isolated from male donors. Genomic DNA was isolated by DNeasy Blood and Tissue Kit (Qiagen) and bisulfite converted using the EpTect Bisulfite Kit (Qiagen) according to the manufacturer’s instructions. The Treg-specific demethylated region (TSRD) was amplified using bisulfite forward and reverse primers: AmpA1 forward, 5′-TTTGGGGTJAGAGTTTJAGAG-3′; AmpA1 reverse, 5′-CCACCTAAACCAACTACTCAA-3′ [modified from Baron et al. (33)]. The region of the FOXP3 promoter immediately upstream of the transcription start site was amplified using bisulfite forward and reverse primers: PROM forward, 5′-GTGAATGGGGATGATGAGAGAGAAT-3′; PROM reverse, 5′-CTTTGTTATTTTCCATAATCGGAAATG-3′. PCR was performed in 25 μl containing 1X PCR buffer, 1 U ZyMoTaq DNA polymerase (Zymo Research), bisulfite-converted genomic DNA, deoxyribonucleotide triphosphates at a final concentration of 1 mM, and forward and reverse primers at 1 μM each. PCR conditions were 95°C for 10 min, 35–40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 7 min. Product PCR products were Exo-SAP purified (USB Corp) and subcloned using a TOPO TA Cloning Kit (Invitrogen), and the DNA from individual bacterial colonies was sequenced with M13 forward primer using Big Dye Terminator v1.1 chemistry (Ap-plied Biosystems).

Functional assays

For polyclonal assays, experiments were performed as described previously (34). In brief, autologous CD4+CD25+ responder T cells were thawed, CFSE labeled, and cultured in a round-bottom 96-well plate with or without CD25+ sorted Tregs at a 1:4 ratio (Tregs/responder cells) Cells were stimulated with M-280 Tosylactivated Dynabeads (Invitrogen), which were preincubated with anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml). Beads were used at a ratio of 2:1 (beads/responder cells). Analysis was performed on day 4 by flow cytometry.

For Ag-specific assays, thawed Tm*CD25+T cells (2.5 × 10^6), thawed CD4+ Tm− cells (2.5 × 10^6) isolated from autologous PBMCs, or both were incubated with irradiated APCs (2.5 × 10^6), TT, and 5 μg/ml peptide Ag in a 96-well round-bottom plate as described previously (32, 35), [3H]thymidine (1 μCi) was added during the final 16 h of a 6- to 7-d assay, and proliferation was measured by a scintillation counter. All culture conditions were performed in triplicate. Percent inhibition was determined based on the percentage of dividing responders in the coculture compared with that when cultured alone.

Statistics

For analysis of experiments comparing a single variable, statistical signif-icance was analyzed using a two-sample Student t test unless otherwise noted. For analysis of multiple variables, a one-way ANOVA was per-formed or linear regression as noted in the legends to figures that ac-company this article. Comparisons required a p value <0.05 for the data to be significantly different.

Results

Both level of TCR stimulation and TGF-β contribute to induction of FOXP3 expression

To dissect the relative contribution of Ag dose and cytokines on FOXP3 induction, we measured Foxp3 expression upon activation in the presence or absence of cytokines that promote Foxp3 expression in a well-defined murine system where Foxp3 and GFP are genetically linked. As shown in this study and by others (36), in the absence of costimulation, TGF-β and IL-2 are required for Foxp3 expression in mice regardless of the level of TCR activation (Fig. 1A). In the presence of TGF-β and IL-2, a greater percentage of murine GFP+ FOXP3+ T cells were generated from GFP+ FOXP3+ T cells when stimulated with low concentrations of anti-CD3 Ab. Thus, both cytokines and the level of TCR engagement contribute to the expression of FOXP3 in mice, and the impact of Ag dose can be observed in the presence of cytokines that promote FOXP3 expression suggesting a dominant effect of Ag dose, consistent with two very recent reports (15, 16).

In humans, addition of IL-2 and TGF-β augmented induction of polyclonal FOXP3+ T cells through stimulation with anti-CD3/anti-CD28–coated beads in the absence of APC (Ref. 37 and data not shown). However, in cultures containing APC and soluble anti-CD3, addition of exogenous TGF-β did not alter the frequency of aTregs (Fig. 1B) most likely due to TGF-β produced by or
bound to the APC (38). To determine whether the FOXP3+ T cell populations induced in these cultures resemble aTregs or nTregs, we measured methylation of the FOXP3 locus and function of the sorted CD25+ populations. Demethylation at the promoter and TSDR of the FOXP3 locus marks stable FOXP3+ nTregs, whereas human TGFβ–induced aTregs are only demethylated at the promoter region (33, 39, 40). Culture of CD4+CD25− T cells using anti-CD3/anti-CD28 beads resulted in effector cells that expressed little FOXP3 protein, whereas culture of the same cells with irradiated APCs and soluble anti-CD3 Ab increased FOXP3 expression with a concomitant decrease in promoter methylation (Fig. 1C). This pattern of FOXP3 protein expression and promoter demethylation are characteristic of aTregs (39). In fact, CD25+ cells sorted from the APC plus soluble anti-CD3 cultures bound to the APC (38). To determine whether the FOXP3+ T cell populations induced in these cultures resemble aTregs or nTregs, we measured methylation of the FOXP3 locus and function of the sorted CD25+ populations. Demethylation at the promoter and TSDR of the FOXP3 locus marks stable FOXP3+ nTregs, whereas human TGFβ–induced aTregs are only demethylated at the promoter region (33, 39, 40). Culture of CD4+CD25− T cells using anti-CD3/anti-CD28 beads resulted in effector cells that expressed little FOXP3 protein, whereas culture of the same cells with irradiated APCs and soluble anti-CD3 Ab increased FOXP3 expression with a concomitant decrease in promoter methylation (Fig. 1C). This pattern of FOXP3 protein expression and promoter demethylation are characteristic of aTregs (39). In fact, CD25+ cells sorted from the APC plus soluble anti-CD3 cultures were functionally suppressive, whereas CD25+ cells from bead-activated cultures were not (Fig. 1D). Thus, we established an in vitro culture system using APC in which we can generate functional human CD4+CD25+FOXP3+ T cells and address the effects of Ag dose on the generation of this population. Low Ag dose promotes an increased frequency of FOXP3+ cells in human influenza-specific CD4 T cells CD4+CD25− T cells were stimulated with autologous irradiated APCs and varying doses of peptide Ag of known affinity (28, 29) as shown in Fig. 2A. IL-2 was added on day 7 of culture to support T cell survival and proliferation. Induction of Ag-specific FOXP3+ T cells was measured by flow cytometry for Tmr, CD25, and FOXP3 expression on day 14. When analyzing the frequency of FOXP3+ T cells within the HLA DRB*0401 HA Tmr+ population on day 14, the highest frequency of influenza Ag-specific FOXP3+ cells was observed in the low HA Ag dose (0.1 μg/ml) culture as opposed to the high HA Ag dose (10 μg/ml) culture (Fig. 2B). This increased frequency in FOXP3+ cells at lower Ag doses was consistently observed in multiple HLA DRB*0401 subjects stimulated with HA peptide (n = 8) (Fig. 2C). Comparable results were observed in HLA DRB*0301 and DRB*0404 subjects with influenza-specific peptide stimulation (data not shown) demonstrating that the percentage of FOXP3-expressing cells 14 d after activation is enhanced with low Ag dose. These data show that Ag dose can influence the relative frequency of in vitro induction and the persistence of FOXP3+ cells within the HA-specific human CD4 T cell population.

nTreg expansion does not contribute to the increased frequency of Tmr+ FOXP3+ cells in low-dose cultures Both nTregs and aTregs are characterized by expression of FOXP3 yet differ in their affinity, expression of helios, and demethylation (1, 2). We used independent measures to determine whether low Ag dose promoted selective expansion of nTregs that cross-react and bind HLA DRB*0401 HA Tmr with a high affinity. Using Tmr mean fluorescence intensity (MFI), a surrogate marker of TCR affinity, we found that FOXP3+ and FOXP3− T cells from low Ag dose cultures express similar levels of Tmr (Fig. 3A) suggesting that the FOXP3+ T cells are not contained within a high-affinity
Treg subpopulation. In addition, the frequency of bystander FOXP3+ Tmr− T cells, a population likely to contain self-reactive nTregs, did not differ between cultures (Fig. 3B). Last, we used molecular signatures of nTregs to confirm whether low Ag dose promoted selective expansion of nTregs in our culture system. Where enough cells could be obtained, we observed >75% methylation of the TSDR in Tmr−CD25hi sorted populations regardless of Ag dose (n = 2, data not shown), suggesting an absence of nTregs that are demethylated at the TSDR. Recently, helios expression was shown to be selectively expressed in FOXP3+ nTregs, but not in aTregs or effector CD4+ T cells (41). CD4+CD25− T cells were cultured as shown in Fig. 2A, and single-cell analysis was performed by flow cytometry for Tmr, FOXP3, and Helios expression on day 14. Consistent with data in Fig. 2, activation of CD4+CD25− T cells with low-dose peptide resulted in a higher frequency of FOXP3+ cells in the Tmr− population compared with that in high-dose cultures (Fig. 3C). The level of FOXP3 expression in the low Ag dose aTregs was less than that of nTregs in freshly isolated PBMCs but higher than that of Tmr+FOXP3+ cells from low-dose cultures (Supplemental Fig. 2). However, helios expression was detected in neither the lower nor high-dose cultures, whereas FOXP3helios+ cells were detected in CD4+ T cells of PBMCs prior to activation and when CD25+ enriched T cells were activated in a similar manner with peptide and irradiated APCs (Fig. 3D), as shown previously by others (41, 42). Together, these data support the hypothesis that activation with low Ag dose leads to induction of aTregs via de novo expression of FOXP3 in this in vitro culture system and not via selective outgrowth of nTregs.

Tmr+FOXP3+ cells arise upon activation of naive and memory cells with high and low doses of Ag, whereas high Ag dose selectively promotes FOXP3+ T cell expansion

To address whether differences in the kinetics of activation explain dose-related variation in the frequency of FOXP3+ expression in our culture system, we measured the frequency of FOXP3+ T cells in the Tmr+ population at earlier time points in cultures where Tmr+ could be detected. Similar to analysis at day 14, we observed a decrease in the frequency of FOXP3+ T cells within the Tmr+ population (Fig. 4A). This is consistent with our previous observation that the frequency of FOXP3+ cells in the Tmr+ population for a single Ag dose was similar between day 10 and day 14 (32). We directly assessed the proliferation rate of FOXP3+ cells, a population containing Tmr+ cells, by measuring BrdU incorporation. Comparing the rate of proliferation of FOXP3+ T cells in low and high Ag dose cultures, we found no difference in the kinetics of FOXP3+ T cell proliferation (Fig. 4B). Thus, increased frequency of FOXP3+ cells with low Ag dose was not due to delayed kinetics of FOXP3 expression upon activation.

Equivalent proliferation rates of FOXP3+ cells in low and high Ag dose cultures suggest that differences in the frequency of FOXP3+ cells may occur through variation in FOXP3+ T cell proliferation or death. To address this hypothesis, equal numbers of sorted naive (CCR7+, CD45RO−), central memory (CCR7+, CD45RO−), and effector memory (CCR7+, CD45RO+) cells were stimulated with low (0.1 μg/ml) and high (10 μg/ml) doses of HA peptide. Universally, high-dose cultures resulted in a greater absolute number of Tmr+ cells (Fig. 4C) reflecting both the relative frequency and proliferative capacity of Ag-specific cells in each sorted population. When stratified by FOXP3 expression, the absolute number of FOXP3+ cells did not increase with Ag dose, consistent with equivalent rates of proliferation observed with BrdU incorporation (Fig. 4D). In contrast, the absolute number of FOXP3− T cells increased in high Ag dose cultures resulting in a lower frequency of Tmr+FOXP3+ T cells. Together, these data suggest that FOXP3+ cells originate primarily from memory cells after activation with all doses of Ag tested, and a decreased frequency of HA-specific FOXP3+ T cells with high Ag dose results from increased proliferation of FOXP3− T cells.

Increased frequency of influenza-specific FOXP3hi T cells in low Ag dose cultures correlates with suppressive function

Transient FOXP3 expression occurs upon activation of human CD4+CD25− T cells. After transient activation, a subset of T cells retains FOXP3 expression and function as Tregs (43). Previously, we demonstrated that stimulation of CD4+CD25− T cells for 14 d with a single dose of Ag resulted in an Ag-specific Tmr+CD25hi population that stably expressed FOXP3 while not coexpressing IFN-γ. These cells functioned in an Ag-specific manner, and the potency of suppression correlated directly with the frequency of FOXP3+ T cells in the sorted Tmr+CD25hi population (32). We tested whether FOXP3+ cells induced by stimulation with either low or high Ag dose function as Tregs. We activated CD4+CD25− T cells with a single dose of Ag resulted in an Ag-specific Tmr+CD25hi population that stably expressed FOXP3 while not coexpressing IFN-γ.
T cells isolated from the same subject with different doses of Ag, assessed FOXP3 and Tmr content on day 14, sorted Tmr\(^+\)CD25\(^{hi}\) T cells, and then measured inhibition of proliferation in an Ag-specific manner as done previously (32, 35). HA-specific Tmr\(^+\)CD25\(^{hi}\) T cells were isolated from low (0.1 \(\mu\)g/ml) and high (10 \(\mu\)g/ml) Ag dose cultures and assessed for FOXP3 content as shown in Fig. 5A. Sorted Tmr\(^+\)CD25\(^{hi}\) T cells were coincubated with thawed, autologous CD4\(^+\)CD25\(^{hi}\) T cells and activated with TT alone or in combination with HA, the Ag for which the CD25\(^{hi}\) Tmr\(^+\) cells were specific. In the absence of HA (TT alone cultures), addition of CD25\(^+\)Tmr\(^+\) cells had no significant effect on proliferation, regardless of the dose of HA used to generated the sorted cells (Fig. 5B). Addition of HA-specific Tmr\(^+\)CD25\(^+\) cells generated from both doses of Ag led to suppression of CD4\(^+\)CD25\(^{hi}\) T cell proliferation in response to TT when HA was also present (TT plus HA). As has been observed previously (32, 35), Tmr\(^+\)CD25\(^+\) T cells sorted from the same cultures failed to suppress proliferation of autologous CD4\(^+\)CD25\(^{hi}\) T cells stimulated with either TT alone or TT plus HA, whereas Tmr\(^+\)CD25\(^+\) cells suppressed proliferation in response to both TT and TT plus HA responses (data not shown). By titrating the concentration of aTregs relative to responders, we found that sorted CD25\(^{hi}\) Tmr\(^+\) T cells generated with low Ag dose were more potent at all ratios tested (Fig. 5C).

Variation in function may be due to differences in the phenotype of Tmr\(^+\)FOXP3\(^+\) cells generated with high and low doses of Ag and/or the FOXP3 content of CD25\(^{hi}\)Tmr\(^+\) cells. Comparing expression of known Treg markers in the Tmr\(^+\)FOXP3\(^+\) population generated by stimulating CD4\(^+\)CD25\(^{hi}\) T cells from the same subject with either high or low Ag dose, we found a subtle yet significant increase in FOXP3 expression and increased CTLA-4 expression in three of the four subjects studied in the low Ag dose cultures (Fig. 5D). However, we found no difference in the expression levels of PD-1, CD39, or CD95 (Supplemental Fig. 3A). We also found no difference between Ag doses in TGF-\(\beta\) (as measured by LAP expression) and IL-10 secretion (Fig. 5D) consistent with the observation that sorted low-dose aTreg function was contact dependent and was not inhibited by blocking Abs against IL-10 and TGF-\(\beta\) (Supplemental Fig. 4) as was found previously with high Ag dose aTregs (32). To examine whether the composition of the CD25\(^{hi}\)Tmr\(^+\) population contributed to function, we correlated FOXP3 content with suppression. Similar to previous studies in humans with both polyclonal and Ag-specific stimulation of CD25\(^{hi}\)FOXP3\(^+\) T cells (14, 32), the frequency of FOXP3\(^+\) cells in the sorted population directly correlated with the degree of inhibition of proliferation (Fig. 5E). To determine whether culture with high and low Ag dose also altered the composition of cells as measured by cytokine profiles of Tmr\(^+\) cells as has been reported by others (44, 45), we measured cytokine secretion upon restimulation in the Tmr\(^+\) cells but found no significant differences in the frequency of IFN-\(\gamma\), IL-17, or IL-5 secretion, representative cytokines secreted by Th1, Th17, and Th2 cells, respectively (Supplemental Fig. 3B). Thus, Ag dose influences the frequency of FOXP3\(^+\) cells in the CD25\(^{-}\)T cell population and expression of FOXP3. Both of these measures correlate with suppressive function of these Ag-specific aTregs.

**FOXP3 expression in human islet Ag-specific CD4 T cells is enhanced through stimulation with lower Ag dose**

Variation in the induction of FOXP3 may be due to intrinsic factors but also due to T cell extrinsic factors. To test whether the generation of Ag-specific aTregs was influenced by cytokines induced through bystander activation, we performed mixed cultures with multiple different peptides known to stimulate Tmr\(^+\) populations. We found no difference in the frequency of HA-specific FOXP3\(^+\)
cells when comparing cultures in which cells of other specificities were activated (as monitored by Tmr+ cells) with cultures stimulated only with HA peptide (data not shown). To test whether intrinsic factors may influence the frequency of FOXP3+ cells, we analyzed responses to well-defined self Ag-specific peptides (27, 29). Self Ag-specific T cells are generally low-affinity cells and are less frequent in peripheral blood than foreign Ag-specific cells (46, 47). Thus, induction of FOXP3 expression with low doses of self Ag may differ from that of foreign Ag-specific T cells. On average, 1.13% (range 0.14–6.6%) islet Ag-specific T cells were detected in the CD4 T cell cultures (data not shown). Two representative HLA DRB*0404 islet-specific Tmr and FOXP3 stains are shown in Fig. 6A. When assessing multiple subjects (n = 12) with two islet Ags, we observed a significant increase in the frequency of islet-specific FOXP3+ Tmr+ cells in low Ag dose (0.1 μg/ml) cultures compared with that in higher Ag dose (50 μg/ml) cultures (Fig. 6B). Together, these data suggest that lower Ag dose may promote an increased frequency of human Ag-specific FOXP3+ T cells regardless of TCR specificity.

Tmr+FOXP3+ aTregs frequencies were maintained with IL-2, but not upon restimulation with Ag

Recently, there has been greater appreciation for the plasticity of FOXP3+ populations, which is influenced, in part, by the source of the FOXP3+ cells and the inflammatory milieu (48). To address the stability of Ag-specific aTreg populations, we assessed the change in FOXP3 content of sorted aTregs after restimulation with low and high doses of Ag and with anti-CD3/anti-CD28 stimulation in a functional assay. Sorted low-dose Tmr+CD25+ aTregs cultured with IL-2 alone maintained FOXP3 expression (Fig. 7A). However, FOXP3 expression was not maintained upon restimulation of the same sorted cells with irradiated autologous APCs and HA peptide, regardless of the dose of Ag used in the restimulation cultures. Polyclonal activation with anti-CD3/anti-CD28–coated beads also resulted in a loss of FOXP3+ cells (data not shown). Similarly, aTregs generated with either low (0.1 μg/ml) or high (10 μg/ml) doses of HA peptide lost FOXP3 expression when placed in a functional assay with autologous responder cells (Fig. 7B). This suggests that higher frequencies of aTregs are not maintained upon in vitro restimulation with Ag, whereas IL-2 alone maintains aTregs.

Discussion

Both effector T cells and FOXP3+ aTregs may be generated upon antigenic exposure. Thus, it is important to understand the mechanisms that promote aTreg generation as opposed to those that promote effector cell generation. We observed an inverse relationship between Ag dose and FOXP3 expression in human CD4 T cells activated with either foreign or self Ag, as has been shown recently in mice with foreign Ag (11, 15, 16). Using demethylation and helios expression, we established that low Ag dose did not preferentially expand nTregs in vitro from CD4+ CD25+ T cells, but instead induced generation of functional FOXP3+ aTregs in which the frequency of FOXP3+expressing cells positively correlated with suppressive function. These Tmr+FOXP3+ cells proliferated equivalently with both low and high Ag dose. Yet, with high Ag dose stimulation, FOXP3+ T cells proliferated to a greater extent resulting in a decreased frequency of FOXP3+ cells. Of note, the frequency of sorted HA-specific aTregs was maintained with IL-2 alone but not upon

FIGURE 4. FOXP3+ T cells generated in high- and low-dose cultures proliferate at equivalent rates. A, CD4+CD25− T cells from the same subject were activated as in Fig. 2 with different doses of HA peptide and assayed for Tmr and FOXP3 content on different days after activation when Tmr was detectable. Statistical significance was determined using a paired Student t test. B, Cells were activated with low (0.1 μg/ml) and high (10 μg/ml) doses of HA peptide as in Fig. 2, and proliferation was assessed by pulsing overnight with BrdU and staining for BrdU incorporation in FOXP3+ cells as described in Materials and Methods. Average ± SEM (n = 3 subjects) is shown. Statistical significance was determined using a paired Student t test at each time point. C and D, Naive (CCR7+CD45RO+), effector memory (EM; CCR7−CD45RO−), and central memory (CM; CCR7−CD45RO−) cells were sorted from the same individual, and equal numbers (1 × 10⁶) of cells were placed in a 48-well plate with APC and different doses of HA peptide. The absolute number of Tmr+ and FOXP3+ populations was determined using total cell counts and flow cytometry for Tmr and FOXP3. Closed circles denote 0.1 μg/ml HA peptide, and open squares denote 10 μg/ml HA peptide.
restimulation with Ag. Thus, the frequency of human aTregs may be one consequence of exposure to low doses of Ag thereby promoting poor immunogenicity and transient tolerance.

The potency and duration of peptide–MHC–TCR interaction can influence the nature of the CD4 T cell response, as is well documented with high- and low-dose Ag driving Th1 and Th2 responses, respectively (reviewed in Refs. 44, 45), and low Ag dose promoting Th17 cells (49) from naive T cells. Less is known about the role of Ag dose on the induction, persistence, and stability of FOXP3 expression in human aTregs. In mice, it has been shown that TCR engagement, costimulation, and cytokines may impact the induction and persistence of FOXP3 expression, which is required for generation of aTregs (8, 11, 13, 15–17). In this study, we established a culture system in which we limit the impact of costimulation and non-T cell-derived cytokines by holding the APC population constant for all doses of Ag tested for each subject. In addition, exogenous TGF-β was not required for FOXP3 expression and thus was not added to our cultures, as was also found by Turner et al. (17) using murine BDC2.5 TCR Tg T cells activated in the presence of dendritic cells. This lack of a requirement for TGF-β may be due to sufficient amounts of biologically active TGF-β produced by or bound to the APC (38, 50). In comparison, stimulation with anti-CD3 anti-CD28 beads (Fig. 1) induced far less FOXP3 expression. Whether this is due to the quality of the stimulation through the TCR or the absence of additional signals provided by the irradiated APC is not yet known. However, increasing frequencies of FOXP3+ cells occur in both murine (Fig. 1 and Ref. 17) and human CD4 T cells (51) early upon activation when low levels of anti-CD3 are used to activate the cells. Likewise, in our Ag-specific culture system, we found that the rate of proliferation of FOXP3+ cells from both 0.1 (closed symbols) and 10 μg/ml (open symbols) cultures were generated.

**FIGURE 5.** Increased frequency and expression of FOXP3 in low-dose cultures correlates with increased suppressive function. Fourteen days after activation with 0.1 μg/ml or 10 μg/ml HA peptide, CD25-Tmr+ cells were sorted and assayed for specificity and function with 5 μg/ml HA and 1 μg/ml TT as described previously (32, 35) and in Materials and Methods. A, An aliquot of HLA DRB*0401 HA Tmr and CD25 stained cells were costained with FOXP3 to determine FOXP3 content. Percent FOXP3 of Tmr is shown in bold in representative dot plots. B, Function and specificity for high and low Ag dose sorted CD25-Tmr+ cells generated from one representative sample of two is shown. Bars represent means ± SEM for triplicates within an experiment. *p < 0.05 (significant difference from CD4+CD25 responder cells alone as measured by a two-sample Student t test). The level of suppression conferred by 0.1 μg/ml aTreg and 10 μg/ml aTreg did not differ (p > 0.05). C, Potency of function was measured by titrating aTregs from low and high Ag dose sorted CD25-Tmr+ cells generated from the same individual. Percent inhibition of proliferation with TT plus HA peptide stimulation was compared between aTregs. One representative sample of two is shown. D, Tmr+FOXP3+ T cells from low and high HA peptide cultures from the same individual were costained for CTLA-4 and LAP. MFI of these markers on Tmr+FOXP3+ cells is shown for four subjects. IL-10 was detected by intracellular flow cytometry after 5-h stimulation with PMA and ionomycin as described in Materials and Methods. Statistical significance was determined using a paired Student t test. E, Correlation between FOXP3 expression in the sorted CD25-Tmr+ population and function was determined by linear regression for three subjects (circles = subject 1, squares = subject 2, triangles = subject 3) for which CD25-Tmr+ cells from both 0.1 (closed symbols) and 10 μg/ml (open symbols) cultures were generated.
preferential activation and proliferation of FOXP3− T cells with high Ag dose. Together, these data suggest that the quality of the TCR signal may contribute to the frequency of Ag-specific aTregs.

We and others (32, 52) have demonstrated that functional self Ag-specific aTregs can be generated from CD4+CD25+ T cells in vitro. In this study, we further demonstrate that an increased frequency of islet-specific FOXP3+ T cells was generated in vitro with low Ag dose (1 μg/ml) compared with that for high Ag dose (50 μg/ml). This suggests that generation of a greater frequency of FOXP3+ Tmr cells is an inherent consequence of all human CD4 T cells activated with lower concentrations of Ag, not just high-affinity foreign Ag-specific T cells. Notably, higher concentrations of self peptide compared with foreign peptide were required to detect low-affinity self Ag-specific aTregs (1 μg/ml GAD versus 0.1 μg/ml HA) consistent with both potency and density of peptide affecting the percentage of FOXP3+ cells in a population as has been shown by others in a mouse model (11). Together, these data demonstrate that exposure to limited, but detectable, foreign or self Ag may influence the frequency of FOXP3+ cells in human CD4 T cells.

In humans, the plasticity of some FOXP3+ populations is highlighted by the kinetics of FOXP3 expression in different cell subsets: FOXP3 expression is constitutively expressed in nTregs, is induced in aTregs, and is transiently expressed in activated T cells (2, 53). In our Ag-specific cultures, FOXP3 expression was induced and maintained with similar kinetics in low- and high-dose cultures indicative of an aTreg subset, and the frequency of FOXP3+ cells remained stable upon further exposure to IL-2 (Fig. 7)—both characteristics of aTreg. However, upon restimulation through the TCR, the frequency of aTregs decreased. Although some of the loss in FOXP3+ cell frequency may occur through activation-induced cell death of FOXP3+ cells, in all experiments the number of cells recovered after activation far exceeded the number of FOXP3− cells in the sorted populations (data not shown) suggesting that some FOXP3+ cells may have lost FOXP3 expression and represent a plastic cell population. In this experiment, FOXP3− T cells could have preferentially proliferated upon restimulation, thereby resulting in a decreased frequency of FOXP3+ cells on a population level. However, the frequency of FOXP3+ cells was similar after restimulation with low and high Ag doses. Whether restimulation in vitro with low-dose Ag is a potent enough stimulus to result in enhanced proliferation of FOXP3− cells observed upon primary stimulation

FIGURE 6. Lower doses of Ag favor an increased frequency of FOXP3+ cells in islet Ag-specific T cells. CD4+CD25− T cells were activated with different doses of islet Ag peptides as in Fig. 2. A, Tmr and FOXP3 staining is shown for one subject stimulated with different doses of GAD peptide. Frequency of FOXP3+ in the Tmr+ population is noted in bold in each dot plot. B, Frequency of islet-specific FOXP3+ cells was determined by enumerating HLA DRB*0401 GAD and IGRP Tmr+FOXP3+ T cells as a percentage of total Tmr+ T cells. Analysis of GAD-specific T cells from nine subjects is noted by open squares, and analysis of IGRP-specific T cells from three subjects is noted by open circles. Horizontal lines show the means. Statistical significance was determined using a two-sample Student t test. in which FOXP3 expression is induced upon activation with high and low Ag dose and this population persists. Decreased frequency of FOXP3+ cells in high Ag dose cultures occurred due to

FIGURE 7. The frequency of FOXP3+ cells in aTregs is not maintained upon restimulation. A, On day 14, CD25+ Tmr+ T cells from low (0.1 μg/ml) Ag dose cultures were sorted and stained to assess FOXP3 content (black bars). Sorted Tmr+CD25+ cells were then placed in culture with IL-2 alone (gray bars) or irradiated APCs and 0.1 or 10 μg/ml HA peptide. FOXP3 content of the Tmr+ population was assessed by flow cytometry 5 d after reactivation. Two control subjects with a high (>5%) frequency of Tmr staining, which allows for accurate analysis of restimulation cultures, are shown. B, CD25+ Tmr+ T cells from low (0.1 μg/ml) and high (10 μg/ml) dose cultures were stained to assess FOXP3 content, sorted, and placed in a functional assay with CFSE-labeled CD4+CD25− responder cells stimulated with anti-CD3/anti-CD28 beads at a 4:1 responder to Treg ratio as described in Materials and Methods. FOXP3 content of CFSE-negative aTregs was assessed by flow cytometry on day 4 of the functional assay. FOXP3 content prior to placement in the functional assay and on day 4 was compared and plotted as \( \Delta (d-0) \) for each culture. Different subjects are noted by different symbols (circles = subject 1, squares = subject 2, upward triangles = subject 3, downward triangles = subject 4).
exclusively in the high Ag dose culture or other mechanisms are involved is not known to date. Lastly, addition of IL-2 to these restimulation cultures or ongoing production of high levels of IL-2 in vivo may result in maintenance of these aTregs even after TCR ligation, as shown in a murine adoptive transfer model (54). We clearly demonstrated that nTregs were not expanded in our culture system. Thus, although our data do not exclude the involvement of nTregs in low-dose tolerance in vivo, they do strongly suggest that Ag dose influences the frequency of aTreg generation in vitro and that human Ag-specific aTregs could play an effective role in low-dose tolerance. This may especially be evident clinically when low-dose tolerance is transient.

Persistent expression of FOXP3 is associated with suppressive function and increased demethylation of the FOXP3 promoter region (4, 10, 32, 55, 56), whereas transient and lower expression of FOXP3 upon activation is not (53). In our cultures, we found that the frequency of FOXP3+ cells in the Tnr+ population positively correlated with suppressive function, resulting in greater suppression by CD25hi Tnr+ cells sorted from low Ag dose cultures where FOXP3 content and level of expression was increased. Notably, the frequency of FOXP3+ cells correlated with function for both low- and high-dose aTregs even though the level of expression of FOXP3 was higher in the low Ag dose aTregs, a phenotype typical of more potent Tregs. Moreover, even though high Ag dose aTregs expressed lower levels of FOXP3, a phenotype associated with unstable FOXP3 expression in activated effector cells, cytokine production was similar between high and low Ag dose aTregs. Together, these data suggest that the level of expression of FOXP3 in Ag-specific human aTregs does not significantly impinge upon their suppressive function. This is in contrast to the culture systems of others in which cells were activated with anti-CD3 and anti-CD28 Abs and/or assayed earlier in culture for suppressive function, and a lack of suppression was observed (51, 57, 58). Together, these data suggest that stimulation with low Ag dose in APC-peptide cultures results in an increased frequency of functional Tnr+ aTregs in which FOXP3 expression persists and is associated with suppression.

Differential of FOXP3+ T cells from naive T cell subsets is well established. In this study, we demonstrate that FOXP3+ T cells can arise from naive, central memory, and effector memory CD4 T cell subsets. This may be particularly important in providing a mechanism to control the magnitude of memory responses when Ag is limited. We propose a model in which a high frequency of FOXP3+ cells induced with low Ag limits perpetuation of immune responses by transiently suppressing proliferation of other cells responding to the same Ag. In contrast, activation with high Ag dose results in a greater frequency of FOXP3+ cells that could overwhelm the suppressive effects of the FOXP3+ cells resulting in immunogenic responses to Ag. Hence, FOXP3 expression upon activation may function to place a transient brake on low Ag dose immune responses while not limiting the magnitude of future immune responses to higher doses of the same Ag. These studies further suggest that aTregs could play a role in low-dose tolerance in vivo.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figure 1: Tmr and FOXP3 gating strategy. (A) Positive Tmr staining was determined to be responses at least 0.2% and 4-fold greater than irrelevant control Tmr stains. (B) FOXP3 and CD25 expression on unactivated (lower left quadrant) and activated (upper quadrants) T cells was used as a FOXP3 staining control as described previously [30].
Supplemental Figure 2: Relative FOXP3 expression in aTreg and nTreg. FOXP3 expression was compared between CD25hi gated PBMC (nTreg, red), Tmr+FOXP3+ aTreg from day 14 of a 0.1 mg/ml culture (low dose aTreg, dark blue) and Tmr+FOXP3+ aTreg from day 14 of a 10 mg/ml culture (high dose aTreg, light blue).
Supplemental Figure 3: Characterization of Tmr+ cells in low and high antigen dose cultures. Tmr+ cells from low (0.1 mg/ml) and high (10 mg/ml) dose cultures were generated from the same individual. (A) Tmr+FOXP3+ cells were co-stained for PD-1, CD39 and CD95. Level of expression of each marker in the Tmr+FOXP3+ population was compared between low (grey) and high (black) antigen dose cultures. One representative of 4 subjects is shown. (B) Cells were re-stimulated on day14 with PMA and ionomycin in the presence of GolgiStop for 5 hours and stained for Tmr and intracellular cytokines. Statistical significance was determined using a paired student’s t-test.
Supplemental Figure 4: Characterization of the function of low antigen dose CD25+Tmr+ aTreg. CD25+Tmr+ cells from low dose (0.1 mg/ml) cultures were sorted and placed in a functional assay with irradiated APC and autologous CD4+CD25- responder cells at a 1:2 aTreg:Responder ratio. Responders alone and co-cultures of responders and aTreg were stimulated with TT+5μg/ml HA peptide in the presence or absence of blocking anti-IL-10 and anti-TGFβ antibodies or in a transwell. Proliferation was measured by ³H incorporation as described in Materials and Methods. Averages ± SD of triplicates in one representative experiment of 2 is shown. * denote significance as compared to responders alone using a student’s t-test.