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IL-2 and IL-15 Each Mediate De Novo Induction of FOXP3 Expression in Human Tumor Antigen-specific CD8 T Cells

Mojgan Ahmadzadeh, Paul A. Antony, and Steven A. Rosenberg

Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD

Summary

Although FOXP3 is primarily expressed by regulatory CD4 T cells (T_{reg}) in vivo, polyclonal activation of human CD8 T cells can result in the expression of FOXP3 in a fraction of CD8 T cells. However, the cellular lineage and mechanism of FOXP3 induction in CD8 T cells remain unclear. Here, we demonstrate that interleukin-2 (IL-2) induces FOXP3 expression in OKT3-stimulated or antigen-stimulated CD8 T cells, indicating that FOXP3 expression is neither limited to a unique subset of CD8 T cells nor dependent on the mode of T-cell receptor stimulation. In the absence of IL-2, antigen stimulation resulted in T-cell activation and acquisition of effector function without induction of FOXP3, indicating that acquisition of effector function is independent of induction of FOXP3 expression in CD8 T cells. Interestingly, IL-15, but not IL-7 or IL-21, also led to de novo induction of FOXP3 in antigen-specific CD8 T cells, suggesting that signaling by IL-2/IL-15R β chain is pivotal for induction of FOXP3 in human CD8 T cells. These findings indicate that induction of FOXP3 is intrinsic to CD8 T cells that are activated in the presence of IL-2 or IL-15, and in vitro-induced expression of FOXP3 cannot be simply interpreted as an indicator of T_{reg} activity or activation marker.

Keywords

FOXP3; memory T cells; regulatory T cells; IL-2; IL-15; human; tumor-Ag

A subset of CD4 T cells, known as regulatory CD4 T cells (T_{reg}), constitutively express interleukin-2 receptor α (IL-2R α) chain and possess potent suppressive activity in mice and humans.^{1,2} The forkhead winged-helix transcription factor, Foxp3, has been shown to be the lineage marker for murine CD4 T_{reg} ,³ but its role as a lineage marker in human T_{reg} is unclear.⁴ In mice, a frameshift mutation in Foxp3 gene leads to a fatal lymphoproliferative syndrome resulting in an early death shortly after birth,⁵ suggesting that Foxp3 expression is essential for the differentiation and function of CD4 T_{reg} to modulate immune responses. Mutations in FOXP3 gene have also resulted in the development of a similar syndrome in humans,⁶ which implies that the expression and normal function of FOXP3 protein is crucial for the function of human CD4 T_{reg} as it is in mice.

The role of FOXP3 expression in CD8 T cells is currently unknown. Circulating FOXP3⁺ CD8 T cells constitute a minuscule fraction of peripheral CD8 T cells in mice and humans^{7,8} and their ontogeny and function remain unknown owing to their scarcity in number. It has recently been reported that the polyclonal activation of peripheral blood mononuclear cells (PBMCs)

Reprints: Steven A. Rosenberg, Surgery Branch, National Cancer Institute, National Institutes of Health, CRC Building, Room 3W-3940, 10 Center Drive, Bethesda, MD 20892 (e-mail: sar@nih.gov).

Current address: Paul A. Antony, Laboratory of Immunology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD 21231.

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can lead to induction of FOXP3 expression in human CD8 T cells in vitro.⁸⁻¹⁰ However, the requirements for this induction and the cellular lineage of cells capable of expressing FOXP3 remain elusive. Here, we characterize the induction of FOXP3 in human antigen (Ag)-specific CD8 T cells and reveal that FOXP3 expression is induced by IL-2. Ag stimulation in the absence of exogenous IL-2 induces an effector phenotype and function independent of FOXP3 expression. Moreover, IL-15, but not IL-7 or IL-21, can also lead to induction of FOXP3 expression in CD8 T cells. These findings suggest that the ability to express FOXP3 is intrinsic to T cells that are activated in the presence of IL-2 or IL-15.

PATIENTS AND METHODS

Patients and PBMC Samples

PBMCs were obtained by leukapheresis from HLA-A*0201 positive melanoma patients (n = 4) with resected tumors who had completed 3 or 4 courses of immunizations with subcutaneous injections of 1 mg of the modified gp100:209-217 (g209-2M) peptide in IFA,¹¹⁻¹³ patients with metastatic melanoma with no prior immunotherapy treatment (n = 11), and healthy adults (n = 6), and were prepared over Ficoll-Hypaque gradient and cryopreserved until analyzed. All protocols were approved by the Institutional Review Board of the National Cancer Institute.

Peptide, Tetramers, Antibodies, and Reagents

The native gp100:209-217 (ITDQVPFSV) (g209) peptide and the modified peptide gp100:209-217 (210M) (IMDQVPFSV) (g209-2M), and the irrelevant peptide gp100:280-288 (YLEPGPVTA) (g280) were described previously.¹¹ Allophycocyanin (APC)-conjugated g209-2M tetramer and negative tetramer were purchased from Beckman Coulter, Inc (Fullerton, CA). The following mAb specific for human antigens were purchased from BD Biosciences (San Jose, CA): PerCP-conjugated anti-CD3 (SK7), FITC-conjugated anti-CD8 (SK1), PE-conjugated anti-CD27 (M-T271), anti-CD25 (2A3), anti-Ki67 (B56), anti-IFN- γ (25723.11), anti-CTLA-4 (BNI3), and APC-conjugated anti-CD4 (RPA-T4). Biotin-conjugated or APC-conjugated antihuman FOXP3 (PCH101) and its isotype control mAb were purchased from eBioscience. Recombinant human IL-2 was supplied by Chiron (Emeryville, CA). Neutralizing antihuman IL-2 (5334) and its isotype control mAb were purchased from Biospecific (Emeryville, CA). The following recombinant human cytokines were reconstituted and stored according to manufacturers' recommendations and used as indicated in the text: IL-15 (PeproTech Inc, Rocky Hill, NJ), IL-7 (Cytheris, France), IL-21 (Zymo-Genetics Inc, Seattle, WA), and IL-4 (PeproTech Inc).

CD8⁺ T-Cell Enrichment

CD8 T cells were negatively enriched using Miltenyi CD8 T-Cell Isolation Kit II (Miltenyi Biotec Inc, Auburn, CA) according to the manufacturer's instructions. Briefly, PBMCs were labeled with a cocktail of biotin-conjugated antibodies against CD4, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ , and Glycophorin A, followed by incubation with antibiotin MicroBeads. CD8⁺ T cells were enriched by the depletion of magnetically labeled non-CD8 cells. The purity was >90%.

T-cell Activation

Cryopreserved PBMCs were thawed into complete medium consisting of RPMI 1640 (Gibco) supplemented with 10% heat-inactivated human AB serum (Gemini Bio-products, Calabasa, CA), 100 U/mL penicillin and 100 μ g/mL streptomycin (Biofluids), 25 mM HEPES buffer (Biofluids), 2 mM L-glutamine (Biofluids), and 50 μ M 2-mercaptoethanol (Gibco) as described previously.¹⁴ For Ag activation, PBMCs (1.5×10^6 cells/mL) from immunized patients were cultured for 6 days with g209-2M peptide (1 μ M) alone or with IL-2 (300 IU/mL) added the

next day as previously described.¹¹ In some experiments, Ag stimulation was carried out in the presence of exogenous recombinant IL-7, IL-15, IL-21, or IL-4 at concentrations designated in the text. For polyclonal activation, PBMCs (1.5×10^6 cells/mL) from healthy volunteers were activated with soluble OKT3 (10 μ g/mL) antibody with titrated amounts of IL-2 for 4 days. Purified CD8 T cells (1×10^6 cells/mL) were activated using immobilized OKT3 (10 μ g/mL) with titrated amounts of IL-2 for 6 days. In some cultures, anti-IL-2 antibody (10 μ g/mL) or its isotype control antibody was added to cultures.

Intracellular Cytokine Induction

Activated CD8 T cells were washed once in media and cocultured with T2 cells previously pulsed with either 1 μ M g209 antigenic peptide or g280 irrelevant peptide as described previously¹¹ or phorbol myristate acetate (PMA) (2 ng/mL) and ionomycin (1 μ M) in the presence of 1 μ M monensin (GolgiStop, BD Biosciences) for 6 to 8 hours. Cells were then spun down and resuspended in complete medium plus 1% (10 μ g/mL) DNase (Pulmozyme, Genentech, Inc, CA) and stained for surface markers followed by fixation with Fix/Perm solution (eBioscience) for 40 to 50 minutes. Cells were washed once with staining buffer (phosphate-buffered saline, 3% fetal bovine serum) and stored at 4°C overnight before intracellular staining.

Flow Cytometry Analysis

Cells were resuspended in staining buffer (phosphate-buffered saline containing 3% fetal bovine serum) and blocked with mouse Ig (Caltag Labs, Burlingame, CA) for 15 to 30 minutes at room temperature. Cells were stained with tetramer and surface antibodies for 30 minutes at 4°C in the dark and subsequently washed in staining buffer twice before fixation for intracellular staining. Cells were analyzed on a FACSCalibur or FACSCanto flow cytometry instruments (BD Biosciences).

Statistical Analysis

Statistical comparisons of the frequencies of FOXP3⁺ T cells in healthy individuals and melanoma patients were calculated using the Wilcoxon Rank Sum test. *P*2 values less than 0.05 were considered significant.

RESULTS

Circulating CD8 T Cells in Melanoma Patients Primarily Lack FOXP3 Expression

Initially, we investigated FOXP3 expression in peripheral CD8 T cells from healthy individuals and melanoma patients. FOXP3 expression was primarily limited to a fraction of CD4 T cells in both healthy donors (Fig. 1A, 6.4%; median 5.2, range 3.7 to 7.2, *n* = 6) and melanoma patients (Fig. 1A, 9.6%; median 6.9, range 3.2 to 9.6, *n* = 12), consistent with previous reports in healthy volunteers⁸ and mice.^{7,15,16} FOXP3 was expressed by a minuscule fraction of circulating human CD8 T cells, indicating that FOXP3⁺CD8 T cells exist *in vivo* but are not prevalent in the peripheral blood of either healthy donors (0.5%; median 0.6, range 0.2 to 3.1, *n* = 6) or melanoma patients (1.5%; median 0.6, range 0.2 to 1.9, *n* = 12) (Fig. 1A). At present, FOXP3⁺CD8 T cells represent a small fraction of CD8 T cells *in vivo* and their functional significance, origin, and survival factors remain largely unknown.

There was no significant difference in the frequency of circulating FOXP3⁺CD4 (*P*2 = 0.2) or FOXP3⁺CD8 (*P*2 = 0.8) T cells between healthy individuals and patients with melanoma. Although several studies have indicated an elevated frequency of CD4 T_{reg} cells in cancer patients (Refs. 17, 18 and reviewed in Ref. 19), we did not find a significant increase in the frequency of circulating FOXP3⁺CD4 T cells in our melanoma patients compared with healthy

adults. As we analyzed the patients' PBMCs before any chemotherapy or immunotherapy which may alter the homeostasis of regulatory T cells,^{17,20} we suggest this may explain why we did not detect any significant increase in FOXP3 expressing CD4 T cells in immune intact patients with cancer.

IL-2 Induces FOXP3 Expression in CD4 and CD8 T Cells in Activated PBMC Cultures

Although peripheral CD8 T cells primarily lack FOXP3 expression *in vivo*, anti-CD3 activation of PBMC led to a small induction of FOXP3 expression in CD8 T cells isolated from healthy individuals (Fig. 1B, OKT3, 6%), consistent with a recent report.⁸ To validate that the detection of FOXP3 in CD8 T cells by anti-FOXP3 monoclonal antibody was specific, we purified CD8 and CD4 T cells from 2 patients whose lymphocytes were activated *in vitro* with OKT3 and high-dose IL-2 and quantified their FOXP3 mRNA levels by quantitative RT-PCR as previously described.¹⁴ We found that the high levels of FOXP3 mRNA were detectable in purified activated CD8 T cells (data not shown), indicating that FOXP3 expression was up-regulated at gene level and validated the detection of FOXP3 protein expression by the antibody.

We next investigated the requirements for FOXP3 induction in CD8 T cells that had not been previously characterized. Because IL-2 is essential for the maintenance of FOXP3 expression by CD4 T_{reg} in mice²¹ and man,²² we investigated the potential role of IL-2 in the induction of FOXP3 in human CD8 T cells. The addition of IL-2 substantially increased the frequency of FOXP3⁺CD8 T cells by 6-fold (Fig. 1B, OKT3/IL-2³⁰⁰ IU, 36%). In fact, IL-2 increased the percentage of FOXP3-expressing CD8 T cells in a dose-dependent manner from 1% to 38% (upper right quadrants, Fig. 2A) that was consistent among different donors (Fig. 2B). In addition to CD8 T cells, IL-2 resulted in FOXP3 expression by more than half of CD4 T cells (Fig. 1B, 53%) and this induction was also IL-2 dose-dependent (Fig. 2C). The level of FOXP3 expression was comparable between CD4 T_{reg} and CD8 T cells stimulated with OKT3 and IL-2 (300 IU). In fact, low doses of exogenous IL-2 did not lead to a high level of FOXP3 expression as compared with higher doses (300 IU) (Fig. 2A, comparing upper left quadrants vs. upper right quadrants).

Although OKT3 stimulation in the absence of exogenous IL-2 was sufficient to induce low levels of FOXP3 expression in both CD4 and CD8 T cells, this induction was attributed to IL-2 produced by activated T cells as anti-IL-2 antibody reduced the induction of FOXP3 in both subsets by half (Fig. 1B). In the absence of T-cell receptor (TCR) stimulation, high-dose IL-2 (6000 IU/mL) was sufficient to modestly enhance the proportion of FOXP3-expressing cells in CD4 and CD8 T cells (Fig. 1C). Therefore, IL-2-mediated induction of FOXP3 in T cells is enhanced by TCR stimulation presumably because of up-regulation of IL-2R.

IL-2 Induces FOXP3 Expression in Purified CD8 T Cells in the Absence of CD4 T_{reg}

We have thus far demonstrated that IL-2 induced FOXP3 expression in CD8 and CD4 T cells in OKT3-activated PBMC cultures. However, this induction could not be ruled out to be independent of endogenous CD4 T_{reg} present in PBMC cultures since CD4 T_{reg} can induce suppressive phenotype and function to non-regulatory T cells.²³ To elucidate whether IL-2 can independently mediate the induction of FOXP3 in CD8 T cells, purified CD8 T cells (purity >90%) were activated with immobilized OKT3 with or without exogenous IL-2. Similar to the activation of PBMC, addition of exogenous IL-2 to OKT3-stimulated CD8 T-cell cultures (OKT3/IL-2³⁰⁰ IU) increased the percentage of FOXP3⁺ cells nearly 7-fold, whereas anti-IL-2 antibody impaired the induction of FOXP3 expression (Fig. 3A). Expression of FOXP3 in OKT3-stimulated CD8 T cells was not due to the lack of costimulation since combination of OKT3 and CD28 stimulation also resulted in FOXP3 expression that was profoundly increased

by exogenous IL-2 (data not shown). Collectively, these findings indicate that IL-2 can mediate de novo induction of FOXP3 in CD8 T cells independent of CD4 T_{reg} cells.

We also found that induction of FOXP3 correlated with CD25 expression (Fig. 3A), suggesting that the ability to express FOXP3 corresponds to T-cell activation and up-regulation of CD25 under these activation conditions. Furthermore, FOXP3⁺CD8 T cells expressed cytolytic T lymphocyte-associated antigen-4 (CTLA-4) (Fig. 3B), a marker that can be expressed by CD4 T_{reg} and activated T cells.¹ Although expression of CD25 and CTLA-4 on FOXP3⁺CD8 T cells was similar to the phenotype of conventional CD4 T_{reg}, FOXP3⁺CD8 T cells expressed the reduced levels of CD27 (Fig. 2B) unlike CD4 T_{reg} that express high levels of CD27.^{14, 24} FOXP3 expression was also associated with expression of Ki67 (Fig. 3B), thus indicating that induction of FOXP3 expression in CD8 T cells correlated with proliferation.

IL-2 Induces FOXP3 Expression in Ag-stimulated Memory CD8 T Cells

Although our results clearly demonstrate a crucial role for IL-2 to mediate induction of FOXP3 in both CD4 and CD8 T cells, we could not determine whether the induction of FOXP3 was limited to a precommitted T-cell subset capable of expressing FOXP3 as previously suggested⁸ or was intrinsic to T cells that were activated in the presence of IL-2. To address this issue, we used a tumor antigen-specific CD8 T-cell population that was generated in vivo following multiple courses of immunization with a modified g209-2M peptide derived from gp100, a melanoma antigen.²⁵ We had previously established that the peptide vaccination resulted in an increased frequency of g209-2M tetramer⁺ CD8 T cells, which exhibited phenotypic and functional characteristics of memory CD8 T cells.¹¹⁻¹³ These tumor-Ag specific (g209-2M tetramer⁺) CD8 T cells expressed CD45RO, lacked expression of CD62L and CCR7,^{11,13} and produced interferon- γ (IFN- γ) and lysed target cells in an Ag-specific manner.^{12,13} Although this vaccine-induced g209-2M tetramer⁺ CD8 T-cell population may be comprised of effector memory and central memory subsets, we refer to them as memory CD8 T cells to include both subsets. Before in vitro stimulation, the g209-2M tetramer⁺ memory CD8 T cells lacked FOXP3 expression and also CD25, CTLA-4, and Ki67 expression ex vivo (Fig. 4, No Stim). Although these Ag-specific CD8 T cells were FOXP3⁻ before in vitro activation, IL-2 led to the induction of FOXP3 in the majority of Ag-stimulated memory CD8 T cells, whereas Ag activation alone resulted in a minimal FOXP3 expression (Fig. 4). Similar to OKT3-stimulated CD8 T cells shown earlier, the induction of FOXP3 in tetramer⁺ CD8 T cells was also IL-2 dose-dependent (data not shown). In addition to memory CD8 T cells, IL-2 also led to induction of FOXP3 expression in purified naive (CD45RA⁺ CD45RO⁻) CD8 T cells (data not shown). Collectively, these results reveal that the de novo induction of FOXP3 is not due to preferential expansion of a preexisting regulatory T cells, rather CD8 T cells, naive or memory, were capable of expressing FOXP3 when stimulated in the presence of IL-2. Furthermore, these results suggest that the induction of FOXP3 was dependent on the presence of IL-2 but independent of the mode of TCR stimulation.

Ag-stimulated FOXP3⁻CD8 T cells expressed phenotypic markers that were associated with T-cell activation and proliferation (ie, CD25, CTLA-4, and Ki67) at similar levels as FOXP3⁺CD8 T cells (Fig. 4), indicating that T-cell activation and proliferation is independent of induction of FOXP3. Similar to earlier results (Fig. 3B) and unlike CD4 T_{reg}, CD27 expression was down-regulated on memory CD8 T cells that were activated in the presence of IL-2 (Fig. 4). However, CD27 expression was retained on Ag-stimulated CD8 T cells in the absence of IL-2 (Fig. 4). These observations are consistent with a recent report that demonstrated IL-2 led to down-regulation of CD27 on human CD8 T cells.²⁶ Thus, in vitro-generated FOXP3⁺CD8 T cells share expression of CD25 and CTLA-4, but not CD27, with conventional CD4 T_{reg}. Taken together, these findings indicate that T-cell activation and proliferation can be independent of induction of FOXP3 expression.

FOXP3⁺ Memory CD8 T Cells Produce Effector Cytokine

Next, we sought to examine whether expression of FOXP3 inhibits the effector function of FOXP3⁺CD8 T cells. Activated memory CD8 T cells derived from in vitro-stimulated cultures were restimulated with either Ag or PMA and ionomycin (PMA/ION). High intracellular expression of IFN- γ was detected in both FOXP3⁺ and FOXP3⁻ tetramer⁺ CD8 T cells derived from Ag-stimulated IL-2 cultures at similar levels in response to restimulation with either Ag, PMA/ION (Fig. 5), or tumor (data not shown). These results indicate that induction and expression of FOXP3 in memory CD8 T cells does not impair production of IFN- γ by activated memory CD8 T cells. Furthermore, Ag-stimulated memory CD8 T cells (in the absence of exogenous IL-2) that lacked FOXP3 expression were able to produce IFN- γ upon restimulation with Ag or PMA/ION (Fig. 5, bottom row). These results further indicate that lack of FOXP3 expression in these cells after antigen stimulation alone (Ag) does not correspond to the lack of activation, because these cells exhibited not only the phenotype of activated T cells as demonstrated by the up-regulation of CD25, CTLA-4, and Ki67 (Fig. 4) but also produced effector cytokine (Fig. 5). Therefore, FOXP3 expression is not a prerequisite for acquisition of effector function, suggesting that the mechanism leading to differentiation of effector function is independent of the one leading to expression of FOXP3.

IL-15, But not IL-7 and IL-21, Induces FOXP3 Expression in Ag-activated Memory CD8 T Cells

We have demonstrated here that IL-2 is crucial for the induction of FOXP3 expression in human CD8 T cells. Because IL-2R complex uses the common γ chain (γ_c) receptor, we asked whether other γ_c cytokines can propagate signals sufficient to result in FOXP3 expression in CD8 T cells. To investigate this question, we again used Ag-specific memory CD8 T cells, as they produced undetectable levels of endogenous IL-2 (data not shown). As illustrated in Figure 6, Ag stimulation in the presence of IL-15 (100 U/mL) resulted in a substantial induction of FOXP3 expression (71.8%), whereas IL-7 (2.7%) and IL-21 (4.4%) induced minimal FOXP3 expression in tetramer⁺ CD8 T cells. Addition of IL-4 resulted in a small (12.5%) induction in FOXP3 expression in Ag-specific cells. Addition of one log lower concentration of IL-15 (10 U/mL) reduced FOXP3 expression by half in Ag-specific CD8 T cells (data not shown), suggesting that induction of FOXP3 by IL-15 is also dose-dependent similar to IL-2. As tetramer⁺ memory CD8 T cells expressed IL-7R α chain (data not shown), we concluded that inability of IL-7 to induce FOXP3 expression in these cells was an intrinsic signaling property of IL-7R complex. However, similar conclusions cannot be drawn for IL-21 owing to the lack of commercially available monoclonal antibody specific for human IL-21R α chain to confirm the expression of its receptor on tetramer⁺ memory CD8 T cells. Collectively, these data demonstrate that FOXP3 expression can be mediated by either IL-2 or IL-15, both of which share the common IL-2R β chain.

Continued Presence of IL-2 Sustains FOXP3 Expression in Ag-IL-2-stimulated CD8 T Cells

It has been reported that activation-induced expression of FOXP3 is transient.^{8,24} We examined the role of IL-2 in maintenance of FOXP3 expression in the Ag/IL-2-induced tetramer⁺ CD8 T cells by washing day 6 activated cells to remove residual cytokines and reculturing them in IL-2 or media for an additional 48 or 72 hours. Removal of IL-2 from FOXP3⁺ tetramer⁺ cultures resulted in substantially lower levels of FOXP3 expression in this population (Media, Fig. 7), whereas addition of IL-2 sustained FOXP3 expression in tetramer⁺ CD8 T cells (+ IL-2, Fig. 7). Therefore, IL-2 retains FOXP3 expression in CD8 T cells.

DISCUSSION

Although induction of FOXP3 in primary CD4²⁷ and CD8 T cells⁸⁻¹⁰ have recently been reported, both the mechanism regulating FOXP3 induction and the cellular lineage of FOXP3

expressing cells have been unclear. In this report, we demonstrate that the ability to express FOXP3 is inherent to CD8 T cells, including memory T cells, and it is independent of the mode of TCR activation yet critically dependent on IL-2 or IL-15 signaling. Our results clearly demonstrate a novel role for IL-2 family cytokines to mediate the induction of FOXP3 in human CD8 T cells that was not previously described.

IL-2 has been implicated for the development and function of CD4 T_{reg} cells in mice,²⁸ and it has been demonstrated to enhance FOXP3 expression in CD4⁺CD25⁺ T_{reg} cells in humans.²² Our results expand the role of IL-2 beyond CD4 T_{reg} cells by defining a novel role for IL-2 in the de novo induction of FOXP3 in human CD8 T cells. In addition to IL-2, we found that IL-15, but not the other γ_c cytokines, can also result in the induction of FOXP3, suggesting that IL-2/IL-15R β chain propagates a signal to mediate the induction of FOXP3 in T cells. In mice, the development and maintenance of Foxp3-expressing CD4 T cells in the absence of IL-2 were attributed to other γ_c cytokines.^{21,29} On the basis of our results, we speculate that in the absence of IL-2, IL-15 may provide signals sufficient to induce FOXP3 expression in CD4 T cells in these mice as it has in human CD8 T cells (our study).

Although addition of exogenous IL-2 was sufficient to induce FOXP3 expression in CD8 T cells, maximum IL-2-induced FOXP3 expression was detected when CD8 T cells were stimulated. We attribute this dependency on TCR stimulation, on the lack of IL-2R α chain (CD25) expression on nonstimulated CD8 T cells. Upon TCR stimulation, however, CD25 is up-regulated, resulting in the expression of high affinity IL-2R complex on the activated T cells. This is supported by our data that demonstrates Ag stimulation resulted in CD25 up-regulation but did not result in FOXP3 expression in the absence of IL-2 or IL-15. Additionally, we speculate that cell division, initiated by TCR stimulation, results in chromatin remodeling and demethylation, allowing FOXP3 regulatory elements to be accessible. In support of this hypothesis, it has been shown that addition of a demethylating agent resulted in IL-2-mediated induction of FOXP3 in NK cells.²² We also speculate that IL-2 signaling may not necessarily need to be concomitantly delivered with TCR signaling to induce FOXP3 expression, because IL-2 was added to cultures 24 hours after Ag stimulation of tetramer⁺ CD8 T cells. Furthermore, addition of IL-2 was sufficient to retain FOXP3 expression even 6 days postpeptide stimulation whereas removal of IL-2 resulted in down-regulation of FOXP3 expression by tetramer⁺ CD8 T cells (Fig. 7). These results also provide a plausible explanation for transient expression of FOXP3 in TCR-stimulated cells^{8,24} that we speculate it is due to consumption of endogenous IL-2 in those cultures. In fact, FOXP3 expression was detectable in our tetramer⁺ CD8 T cells even 2 weeks after Ag stimulation if IL-2 was available (data not shown). Given that removal of IL-2 signaling leads to down-regulation of FOXP3 expression in T cells, we raise caution for therapeutic values of in vitro-generated FOXP3⁺ T cells for the treatment of autoimmune disorders.

The functional consequence of induced FOXP3 expression in CD8 T cells is currently unknown. Polyclonal activation of CD8 T cells with a modified OKT3 antibody has been reported to give rise to FOXP3-expressing cells that exhibited a suppressive function in vitro⁹; however, that study did not evaluate induction of FOXP3 and potential suppressive function by nonmodified OKT3-stimulated CD8 T cells. We used not only OKT3 but also Ag and found that IL-2 and IL-15 can mediate the induction of FOXP3 in CD8 T cells independently of the mode of TCR stimulation. Because IL-2-induced FOXP3 expression did not correlate with a unique surface phenotypic marker that could be used to isolate FOXP3⁺ CD8 T cells for suppression assay, the potential suppressive ability of in vitro-generated FOXP3⁺CD8 T cells could not be assessed. However, in contrast to peripheral CD4 T_{reg}, we found that FOXP3⁺CD8 T cells produced IFN- γ comparable with their FOXP3⁻ counterparts in response to Ag restimulation. Thus, the induction of FOXP3 in Ag-activated memory CD8 T cells was not sufficient to suppress the acquisition and production of IFN- γ , consistent with

a recent report using OKT3-activated polyclonal T cells.⁸ It is known that FOXP3 protein attenuates activation-induced cytokine production and proliferation in CD4 T cells by suppressing the activity of NFAT and NF- κ B.³⁰ In mice, ectopic expression of FOXP3 confers regulatory and suppressive phenotype and function in both CD4 and CD8 T cells.^{5,16} It is possible that long-term expression of FOXP3 by primary T cells leads to repression of cytokine genes, whereas short-term expression is not sufficient to have a measurable impact on cytokine secretion.

Although IL-2, as a T-cell growth factor, is historically known to enhance activation and proliferation of T cells, it can also enhance FOXP3 expression in CD4 T_{reg}²² and lead to its de novo induction in CD8 T cells (this report). IL-2 is critical for suppressive function and expression of FOXP3 and CD25 by CD4 T_{reg} in mice,^{29,31,32} and leads to expansion of circulating FOXP3 expressing CD4 T_{reg} in cancer patients.^{14,20,22} As FOXP3 negatively regulates the IL-2 gene,^{33,34} we speculate that IL-2-mediated induction of FOXP3 during T-cell activation in CD8 T cells may provide an autoregulatory mechanism for IL-2 to regulate its own production.

The significance of FOXP3⁺CD8 T cells in vivo is currently unknown. Although FOXP3⁺CD8 T cells represent a small fraction of circulating CD8 T cells in peripheral blood, it is plausible that certain cytokines such as IL-2 may expand these cells in vivo, as it has been reported for FOXP3⁺CD4 T cells.¹⁴ Furthermore, the frequency of FOXP3⁺CD8 T cells outside circulation, such as in a tumor, may differ from peripheral blood. We currently investigate the frequency and function of FOXP3⁺CD8 T cells found in tumors isolated from patients with melanoma. At present, FOXP3⁺CD8 T cells represent a small fraction of CD8 T cells in vivo and their functional significance and also their origin and survival factors remain largely unknown.

In conclusion, we report here that the de novo induction of FOXP3 expression in CD8 T cells is mediated by IL-2 or IL-15, and FOXP3 expression is not required for acquisition of effector function. Our findings further demonstrate that the ability to acquire FOXP3 expression is an intrinsic property of T cells that are activated in the presence of IL-2 or IL-15 and it is not limited to a precommitted subset. Although FOXP3 expression correlates with regulatory phenotype and function in vivo, our results suggest that in vitro-induced FOXP3 expression cannot be simply interpreted as an indicator of T_{reg} activity or activation marker. This report provides novel findings pertaining to IL-2-dependent T-cell activation and expansion of tumor-reactive CD8 T cells and CD4 T_{reg} used for the treatment of patients with cancer and autoimmune diseases, respectively.

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FIGURE 1.

IL-2 induces FOXP3 expression in both CD4 and CD8 subsets in activated PBMC cultures. A, PBMCs from a healthy adult and a vaccinated melanoma patient were stained for CD8, CD3, and FOXP3. B, PBMC from a healthy donor was activated with soluble OKT3 in the absence (OKT3) or presence of exogenous IL-2 (OKT3/IL-2³⁰⁰ IU), or neutralizing anti-IL-2 (OKT3/anti-IL-2). C, IL-2 was added to PBMC cultures (healthy donors) in the absence of TCR stimulation. All cultures were analyzed after 4 days. The dot plots were gated on CD3⁺ T cells and the number in upper left quadrant represents %FOXP3⁺ in CD4 T cells and the number in upper right quadrant represents %FOXP3⁺ in CD8 T cells. No Stim represents freshly thawed cells without activation. A, Representative of FOXP3 staining in 6 different healthy individuals, and 12 melanoma patients. B and C, Representatives of 3 independent experiments using 3 different healthy donors.

FIGURE 2.

IL-2 induces FOXP3 expression in CD4 and CD8 T cells in a dose-dependent manner. PBMCs from healthy donors were stimulated with OKT3 and different doses of IL-2 for 4 days. Activated cells were stained for expression of CD3, CD8, and FOXP3. A, The dot plots were gated on CD3⁺ T cells and the number in upper left quadrant represents %FOXP3⁺ in CD4 T cells and the number in upper right quadrant represents %FOXP3⁺ in CD8 T cells. Results are representative of 3 separate experiments using 3 healthy donors. The percent FOXP3⁺ cells for CD8 T cells (B) and CD4 T cells (C) from 3 independent experiments from 3 healthy donors were enumerated. For CD4 T cells, CD3⁺CD8⁻ T cells were considered as CD4 T cells. Each symbol represents one individual.

FIGURE 3.

Induction of FOXP3 expression in purified CD8 T cells by IL-2. Purified CD8 T cells were isolated from PBMCs (healthy donors) and were stimulated with immobilized OKT3 antibody for 6 days in the presence or absence of IL-2. Cultured cells were costained for CD8, CD3, and FOXP3, with either (A) CD25, or (B) CTLA-4, CD27, and Ki67 antibodies. The dot plots were gated on CD3⁺CD8⁺ T cells. This result is representative of three separate healthy donors. No Stim represents freshly thawed cells without activation.

FIGURE 4.

IL-2 induces FOXP3 expression in antigen-stimulated memory CD8 T cells. PBMCs from immunized melanoma patients were stimulated with Ag in the presence or absence of exogenous IL-2 (300 IU/mL) for 6 days. Freshly thawed cells (No Stim) were used as control. The dot plots were gated on tetramer⁺ CD8⁺ T cells. This result is representative of 5 independent experiments using 4 different vaccinated patients.

FIGURE 5.

FOXP3⁺ memory CD8 T cells produce effector cytokine. In vitro-generated, Ag-stimulated memory CD8 T cells (as described in Fig. 4) were restimulated with either native g209 peptide (Ag) or irrelevant peptide (not shown) pulsed on T2 cells or PMA and ionomycin (PMA/ION) for 6 to 8 hours. The dot plots were gated on tetramer⁺ CD8 T cells. This result is representative of the 4 independent experiments.

FIGURE 6.

IL-15 induces FOXP3 expression in antigen-specific memory CD8 T cells. PBMCs from immunized melanoma patients ($n = 2$) were stimulated with Ag alone or with IL-2 (300 IU/mL), IL-15 (100 U/mL), IL-7 (10 ng/mL), IL-21 (10 ng/mL), or IL-4 (100 U/mL) as indicated for 6 days. Freshly thawed cells (No Stim) without activation were used as control. The dot plots were gated on tetramer⁺ CD8⁺ T cells. The quadrants were set on the basis of isotype control antibodies for each culture. This result is representative of the 3 independent experiments.

FIGURE 7.

Removal of IL-2 abrogates FOXP3 expression in IL-2-induced FOXP3⁺ CD8 T cells. CD8 T cells were activated with Ag and IL-2 (300 IU/mL) for 6 days as described in Methods. Cultured cells were washed in complete medium 3 times and recultured with (+IL-2) or without (Media) exogenous IL-2 (6000 IU/mL) for an additional 48 or 72 hours before staining for FOXP3 expression. The dot plots were gated on tetramer⁺ CD8 T cells. This result is representative of the 2 independent experiments. The quadrants were set on the basis of isotype control antibodies for each culture.