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IL-15 acts as a potent inducer of CD4⁺CD25^{high} cells expressing forkhead box protein P3

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Abstract

IL-15 is a member of the gamma c-dependent cytokines and known to affect innate and CD8⁺ adaptive immune responses. Despite a growing interest in the use of IL-15 as an immunotherapeutic agent, the broad spectrum of immunoregulatory functions exerted by IL-15 has not been fully elucidated. Here, we demonstrate that IL-15 increases expression of CD25 and FOXP3, a master transcriptional regulator of regulatory T cells, in human peripheral CD4⁺CD25⁻ T cells in the absence of antigenic stimulation. Comparisons involving IL-2 and IL-7 revealed that the induction of CD25^{high} and FOXP3 expression was most prominent with IL-15 and IL-2. More modest effects were seen with IL-7. Despite levels of FOXP3 expression comparable to that of conventional Tregs, cytokine-induced CD4⁺CD25^{high}FOXP3⁺ cells exerted only weak suppressor activity. Thus, the current study has demonstrated that IL-15 acts as a potent inducer of CD4⁺CD25^{high}FOXP3⁺ cells in the periphery, and suggests a potential role for IL-15 in blunting immune activation. This study has provided further insights into the pleiotropic nature of IL-15 beyond the regulation of CD8⁺ T cells.

Keywords

Cytokines; Regulatory T cells; T cells; Immune regulation

Introduction

IL-15 is a member of the family of cytokines that utilize the common γ chain (γ_c) as part of their receptor complex and activates signaling pathways leading to cellular activation, proliferation and survival [1,2]. While initially characterized as a T cell growth factor with similar in vitro properties as IL-2 [3,4], a large body of evidence has now demonstrated that IL-15 plays a multifaceted role in the development and control of the immune system, affecting a wide range of target cell population, including NK cells, NK T cells, certain subsets of intestinal epithelial lymphocytes as well as memory CD8⁺ T cells [5,6].

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Conflict of interest: H. Clifford Lane is a coinventor on a patent held by the U.S. Government on the use of intermittent IL-2 therapy ("Immunologic enhancement with intermittent interleukin-2 therapy", US Patent 6,548,055, issued April 15, 2003). All other authors have declared that no conflict of interest exists.

IL-2 and IL-7 are also γc -dependent cytokines. Consistent with sharing a common γc signaling component, IL-15, IL-2 and IL-7 all exhibit a range of similar activities *in vitro* including the activation of signaling pathways modulating cellular activation, proliferation, and survival [1,2]. However, their precise physiological roles remain unclear, as studies with knockout mice have demonstrated divergent phenotypes. Both IL-2 and IL-15 were initially characterized by their *in vitro* T-cell growth promoting properties [3,4,7]. However, while a primary role of IL-2 *in vivo* appears to restrain T cell activation and prevent autoimmunity [8,9,10], IL-15 is dedicated to the prolonged maintenance of memory T-cell responses [5,6]. IL-7 plays an important role at various stages of development and homeostasis of both B and T lymphocytes, and an important nonredundant role appears to be in support of the survival and proliferation of naïve T cells [11,12].

Among these three γc cytokines, IL-2 is the only FDA-approved drug. It is licensed for the treatment of metastatic melanoma and renal cell carcinoma [13,14] and also studied experimentally in a variety of conditions including HIV infection, common variable immunodeficiency, and as a vaccine adjuvant [15-18]. IL-7 is in phase I safety testing in humans [19]. Due to its biological activity as a lymphoid growth factor for both B and T cells, IL-7 is being considered for treatment in a variety of immunodeficiency diseases in humans including HIV infection. IL-15, to date, remains at the pre-clinical phase and has not been administered to humans. However, numerous *in vitro* and *in vivo* studies suggest that IL-15 may be useful in the treatment of cancer or as a vaccine adjuvant due to its ability to support the expansion and survival of memory CD8⁺ T cells [20,21]. In addition, blockade of the IL-15/IL-15 receptor system is being considered for treatment of inflammatory autoimmune diseases, as IL-15 induces pro-inflammatory cytokines such as TNF- α and IL-1 β [22].

Naturally occurring regulatory T cells (nTreg) are a subset of CD4⁺ T cells that have been described in mice and humans [23,24]. They co-express high levels of CD25 and FOXP3 and exert strong suppressor activity following TCR stimulation. While these cells are thought to originate predominantly from the thymus [23-25], a series of *in vitro* studies have demonstrated that cells with similar characteristics can be generated from peripheral T cells under appropriate conditions that include TCR-mediated signaling [26-28]. Identification of the physiological factors that lead to induction of foxP3 expression remains elusive. However, the emergence of CD4⁺CD45RO⁻CD25⁺ T cells expressing high levels of FOXP3 in the context of *in vivo* administration of IL-2 in humans has raised the possibility of the direct induction of these cells by cytokines [29]. Given the commonality of immediate biochemical responses induced by IL-2, IL-7 and IL-15 *in vitro* [1,2], it is plausible that these cytokines dictate a common biological outcome. To examine this possibility, we have studied the effects of IL-15 on the induction of CD25 expression on CD4⁺ T cells, foxP3 expression, and suppressor cell activity, and compared them with those with IL-2 and IL-7. Our results have demonstrated that IL-15 induces high levels of CD25 and FOXP3 expression in human peripheral CD25⁺CD25⁻ T cells without antigenic stimulation. Cells directly induced by these three cytokines expressed levels of FOXP3 comparable to that of nTregs, however, were distinct from nTreg with respect to CD45 isoform expression, proliferative capacity, cytokine profile and suppressive activity.

Results

Induction of CD25, FOXP3 expression, and cell proliferation by IL-2, 7, and 15

While it has been shown that IL-2 is capable of direct induction of CD25 expression and cell proliferation on CD4⁺ and CD8⁺ T cells [30], little is known about this property of IL-15. After 9 days of culture, dose-dependent increases in total CD25 expression were seen with IL-15 as well as IL-2 or IL-7 on both CD4⁺ T cells and CD8⁺ T cells (Fig.1A). Similar levels of total CD25 expression were seen between CD4⁺ and CD8⁺ T cells, whereas a population of CD25^{high} cells was predominantly seen in the CD4⁺ subpopulation (Fig.1B, Supporting

information Table1). This was most pronounced with IL-2 or IL-15 stimulation and was only seen to a modest degree with IL-7.

IL-15 was capable of inducing CD4⁺ and CD8⁺ T cell proliferation without TCR-mediated signaling, as seen with IL-2 or IL-7 stimulation (Fig.1C, Supporting information Table1). The vast majority of proliferating CD4⁺ T cells were CD25⁺, while proliferating CD8⁺ T cells were seen in both CD25⁺ and CD25⁻ subsets, with the CD25⁻ subset representing the majority of proliferating cells.

To investigate the effects of IL-2, IL-7 and IL-15 on FOXP3 expression, the kinetics of FOXP3 expression and cell division were analyzed (Fig.1D). Stimulation of PBMCs with IL-15 led to upregulation of FOXP3 expression in CD4⁺ T cells and expansion of these cells. IL-2 had a similar effect. Only modest increases in FOXP3 expression were seen with IL-7. Consistent with previous reports [31-35], anti-CD3/anti-CD28 stimulation led to transient increase in FOXP3 expression in both CD4⁺ and CD8⁺ T cells with peak FOXP3 expression at 3-5 days, followed by downregulation. Of note, while cytokine stimulation led to similar percentages of CD4⁺ and CD8⁺ T cells expressing FOXP3, FOXP3^{high} cells were predominantly seen in the CD4⁺ T cell subpopulation.

De novo induction and expansion of FOXP3⁺ cells by IL-15 or IL-2, but not IL-7

When purified CD4⁺ and CD8⁺ T cells instead of PBMCs were employed, induction of CD25⁺ and FOXP3⁺ cells were still observed with all three cytokines (Fig.2A-B). Among the three cytokines, IL-15 was demonstrated to be the most potent inducer of FOXP3 in CD4⁺CD25⁻ T cells. Indeed, when compared to IL-2 or IL-7 treated cells at the end of a 9-day culture, IL-15 treated CD4⁺CD25⁻ T cells contained approximately 4- and 11-fold more FOXP3⁺ cells, respectively. Of note are the distinct kinetic profiles seen between anti-CD3/CD28 stimulated and cytokine-stimulated cells. While FOXP3 induction by anti-CD3/CD28 stimulation was a transient phenomenon with peak expression at day 3, followed by quick downregulation, stimulation of CD4⁺CD25⁻ cells with IL-15 or IL-2 led to a sustained increase in FOXP3 expression.

The addition of neutralizing antibodies to HLA-DR, DP and DQ had no impact on cytokine-induced increases in CD25 or FOXP3 expression, indicating little, if any, for TCR-mediated signaling in these effects.(data not shown). Furthermore, the contribution of pre-existing CD25⁺ cells to the expansion of the CD25⁺ pool and appearance of FOXP3⁺ cells appears to be minimal, as greater degrees of FOXP3 expression (Fig.2B) and expansion of FOXP3⁺ cells (Fig.2C) were seen in CD4⁺CD25⁻ cell cultures compared to total CD4⁺ cell cultures.

While the percentages of cytokine-induced CD25⁺ cells in the CD8⁺ T cell pool were comparable to those in the CD4⁺ T cell subsets (Fig.2A), lower levels of FOXP3 induction and expansion of FOXP3⁺ cells were seen in CD8⁺ T cells (Fig.2B-C). This observation highlights a fundamental difference between direct cytokine stimulation of CD4⁺ and CD8⁺ T cells.

To investigate the relationship between FOXP3 expression and cell division, we labeled CD4⁺CD25⁻ T cells with CFSE and stimulated them with IL-2, IL-7 or IL-15 (Fig.2D). Stimulation of CD4⁺CD25⁻ T cells with IL-15 or IL-2 led to not only *de novo* induction of FOXP3⁺ cells but also expansion of these FOXP3⁺ cells as illustrated by dilution of CFSE dye in the FOXP3⁺ gate. While IL-7 stimulation led to high levels of total CD25 expression and proliferation of CD25⁺ cells, very little FOXP3 expression was observed in the proliferating cells. Stimulation of CD4⁺CD25⁻ T cells with anti-CD3/CD28 led to proliferation of cells that were CD25⁺. While these proliferating cells remained largely CD25⁺, there was little FOXP3 expression in the proliferating cells at day 9. This observation underscores qualitative differences between biological signals induced by antigen and cytokine stimulation.

High levels of FOXP3 expression in cytokine-induced CD4⁺CD25^{high} cells

To more precisely compare the CD25^{high} cells derived from *in vitro* cytokine stimulation to CD4⁺CD25^{high} regulatory T cells freshly isolated from the peripheral blood (nTreg), a series of studies were carried out to compare the two populations (Fig.3). Upon bead-separation of nTregs from PBMC of normal volunteers, a single population of CD4⁺ T cells that expressed high levels of CD25 was obtained with a high purity (average 96%, range: 90-99%). These nTregs were found almost exclusively in the CD45RO⁺CD27⁺ central memory phenotype (average 87%, range: 79-95%, n=10) (Fig.3A). In addition, nTregs purified from PBMCs on the basis of CD25 expression were on average 80% FOXP3⁺ (range 76-89%, n=5), and thus consistent with previous reports [33,34].

The cytokine-induced CD25^{high} cells were comprised of a mixture of CD45RO⁺CD27⁺ central memory and a CD45RO⁺CD27⁺ naïve phenotype (Fig.3B), while nTregs predominantly consist of the CD45RO⁺CD27⁺ central memory phenotype. In contrast to the case for activated CD4⁺ T cells receiving persistent antigen stimulation, which has been associated with up-regulation of cell surface CD25 but down-regulation of CD27 expression [36-38], there appeared to be very little, if any, down-regulation of CD27 seen in these CD25⁺ subsets induced by cytokine alone.

As has been previously shown by others [39,40], nTregs isolated from PBMCs expressed approximately 100-fold more foxP3 mRNA than CD4⁺CD25⁻ cells from normal PBMCs (Fig. 3C). Among the cytokine-induced CD25^{high} cells, the highest levels of foxP3 mRNA expression were detected in the IL-2 or IL-15 induced CD25^{high} cells. These levels were comparable to that of nTregs. Significantly lower levels of foxP3 expression were noted in IL-7 induced CD25^{high} cells. Western blot analysis of FOXP3 protein expression confirmed the findings at the mRNA level and demonstrated in nTregs as well as in the *in vitro* stimulated cells the presence of full-length FOXP3; the smaller FOXP3Δ2, which lacks exon2 [26,41, 42]; and a third band yet to be more precisely defined, (Fig.3D).

Proliferative capacity and cytokine profile of cytokine-induced CD25^{high} cells

We next investigated whether the high levels of FOXP3 expression seen in cytokine-induced CD25^{high} cells was associated with a phenotype similar to nTregs. In line with previous reports demonstrating an anergic state of nTregs *ex vivo* [43,44], nTregs were hyporesponsive upon *in vitro* stimulation of IL-2, IL-7 or IL-15 at the doses used (Fig.4). This refractory state of nTregs could only be reversed by the addition of anti-CD3/CD28 beads. On the contrary, populations of cytokine-induced CD25^{high} cells showed a considerably high proliferative capacity in response to IL-2, IL-7, or IL-15 stimulation. In particular, IL-2 or IL-15-induced CD25^{high} cells showed a strong proliferation capacity. In addition, spontaneous proliferation was observed with the IL-2 or IL-15-induced CD25^{high} cells, indicating that these cells represent a population of cells that were proliferating upon their purification from the CD4⁺CD25⁻ culture. Compared to IL-2 or IL-15-induced CD25^{high} cells, IL-7-induced CD25^{high} cells exhibited lower rates of proliferation. Of note, in the absence of IL-2, IL-7 or IL-15, nTregs showed reduced cell survival and downregulation of CD25 and FOXP3 expression during the 4-day culture period (supplementary Fig.1), indicating that these γ cytokines may play some roles in the maintenance of optimal nTreg phenotype.

Cells were activated *in vitro* with PMA and ionomycin to analyze and compare the intracellular cytokine profiles of CD4⁺CD25⁺ Tregs freshly isolated from PBMCs and cytokine-induced CD4⁺CD25^{high} (Fig.5). CD4⁺CD25⁻ T cells freshly isolated from PBMCs were also included in the comparison panel as a control for IL-2 and IFN-γ production. In line with the lack of proliferation shown in Fig.4, nTreg showed little IL-2, or IFN-γ production upon activation with PMA and ionomycin. In contrast, cytokine-induced CD4⁺CD25^{high} cells expressed

substantial amounts of IL-2 and IFN- γ following PMA/Ionomycin stimulation, indicating a distinct difference between these two CD4⁺ T cell populations. While production of IL-10 from activated CD4⁺CD25⁺ Tregs has been reported, the ability of these cells to produce IL-10 appears to be dependent on the strength and types of stimulus employed [43-45]. Under the current condition, no measurable amount of IL-10 production was observed in any of the cell subsets analyzed.

Cytokine-induced CD25^{high}FOXP3⁺ cells have only weak suppressive activity

It has been reported that FOXP3 is necessary for the generation of CD4⁺CD25⁺ Tregs in mice, and expression of FOXP3 was found to be correlated with the suppressive activity of these cells [39,46,47]. To investigate whether CD4⁺CD25^{high} cells, arising from *in vitro* cytokine stimulation, also possess suppressor cell activity, we stained CD4⁺CD25⁻ T cells from autologous donors with CFSE and used them as responder cells to determine the suppressive activity of nTreg and cytokine-induced CD4⁺CD25^{high} cells (Fig.6). In this system, nTregs, consistent with their high FOXP3 expression, displayed strong suppressor activity and were able to suppress the anti-CD3 proliferative response of CD4⁺CD25⁻ cells by 97.8% (range: 93-100%) at a 1:1 of responder:suppressor ratio and by 93% at a 2:1 ratio (range: 87-100%). On the contrary, despite their similar levels of FOXP3 expression, cytokine-induced CD25^{high} cells exerted only modest suppression compared to nTregs. In a series of six experiments using different donors, the levels of suppression conferred by the cytokine-induced cells at a 1:1 ratio were approximately 50% of that seen with nTregs (Supporting Information Table2). Taken together, these data demonstrate that high levels of FOXP3 expression induced by cytokine stimulation did not lead to acquisition of high-level suppressor activity in the CD25^{high} cells derived from *in vitro* cytokine stimulation. A separate experiment using CD25^{high} cells from anti-CD3/CD28-stimulated CD4⁺CD25⁻ cells as suppressor cells revealed that these cells also exhibited mild suppression similar to that of the cytokine-induced CD25^{high} cells, suggesting that one mechanism behind the mild suppression may be competition for IL-2 (supplementary Fig.2).

Discussion

The present study has clearly demonstrated that IL-15 is capable of inducing proliferation, high levels of CD25 and FOXP3 expression in human peripheral CD4⁺CD25⁻ T cells without TCR-mediated signaling. Despite these similarities to Tregs, these cells exhibited only modest levels of suppression. A similar set of effects was seen with IL-2; and a more modest effect was seen with IL-7. While predominantly a direct product of thymic output, data from a series of *in vitro* studies have demonstrated that Treg/Treg-like cells can be generated in the periphery as well [26-28]. Previous studies have demonstrated a requirement for TCR-stimulation in the *in vitro* generation of Treg. The current study has demonstrated that *de novo* generation of Treg-like cells can be achieved by cytokine stimulation alone and that the TCR-mediated signaling is not a prerequisite.

Despite the fact that IL-2, IL-7 and IL-15 share the γ_c as a common receptor chain, their physiological roles, inferred from studies with knockout mice, are quite distinct. IL-2^{-/-} and IL-2R α ^{-/-} mice demonstrate lymphoid hyperplasia [8,9], while IL-7 and IL-15- deficient mice exhibit lymphopenia [5,6,11,12]. Thus, given the divergent roles that IL-2 and IL-15 play in lymphoid homeostasis *in vivo*, it is somewhat surprising to find that similar levels of FOXP3 were induced by these cytokines. Studies with IL-2R β ^{-/-} mice have revealed that IL-2R β ^{-/-} deficiency leads to abnormal expansion of activated T cells and lethal autoimmunity [48]. The homeostatic abnormalities appear to be attributed from the absence of Tregs in these mice [48,49]. Given the fact that both IL-2 and IL-15, but not IL-7, use IL-2R β as a component of their heterotrimeric receptor complex, the similarities between IL-2 and IL-15 and the

differences between IL-7 and these two with respect to FOXP3 and CD25^{high} induction is likely explained by the differences in their usage of the IL-2R β . A recent report demonstrating that IL-2R β -dependent STAT5 activation is required for the development of FOXP3⁺ Tregs further supports this notion [50]. IL-15 has been noted as a lymphoid growth factor that works predominantly on the proliferation and survival of CD8⁺ T cells. The current study has demonstrated that the scope of immunoregulatory functions exerted by IL-15 is far beyond and not restricted to the regulation of CD8⁺ T cells. Further studies are necessary to fully delineate the broad spectrum of biological functions that can be activated through IL-15 signaling in multiple cell types.

The high proportion of FOXP3⁺ cells seen following prolonged (a 9 day) culture of CD4⁺CD25⁻ T cells with IL-2 or IL-15 could have reflected a relative enrichment of these cells merely due to a decrease in the number of FOXP3⁻ cells. The <1% of FOXP3⁺ cells present at the start of culture coupled with the fact that the FOXP3⁺ cells had proliferated, as evidenced by CFSE dye dilutions, argue against this hypothesis. Of particular note is the observation that both the percentages and absolute numbers of FOXP3⁺ cells continued to increase in conditions of IL-2 or IL-15 stimulation. It remains to be seen whether IL-15 acting alone or in combination with additional co-factors is responsible for the induction of FOXP3⁺ cells. Studies are currently underway to test the hypothesis.

Intermittent administration of IL-2 in HIV-infected patients leads to substantial and sustained expansion of CD4⁺ T cell pool [51,52]. The mechanism of CD4⁺ T cell expansion in the setting of IL-2 administration is a prolongation of cell survival [53]. Despite IL-2 causing increases in CD25 and enhancing proliferation in both CD4⁺ and CD8⁺ T cells, it only leads to expansion of CD4⁺ T cell pool *in vivo* [54]. In the present study, we demonstrated that expansion of FOXP3⁺ cells by IL-15 or IL-2 could be predominantly seen in CD4⁺ T cells and only minimal increase of FOXP3⁺ cells was detected in CD8⁺ T cells by these cytokines. This dichotomy in foxp3 induction in CD4⁺ and CD8⁺ T cells parallels the dichotomy in cell expansion in these two cell subsets seen *in vivo* following the administration of intermittent cycles of IL-2. This association of prolonged CD4⁺ T cell survival with FOXP3 induction suggests that FOXP3 may also play a role in T cell homeostasis either through prolongation of cell survival and/or blunting persistent immune activation.

Materials and Methods

Isolation of PBMC and separation of CD4⁺ T cell subsets by magnetic beads

PBMC were separated by Ficoll-Hypaque centrifugation of leukopheresis pack from normal healthy volunteers as previously described [30]. CD4⁺ and CD8⁺ T cells were purified from PBMCs using the CD4 MultiSort or CD4⁺ T cell Isolation Kit II and CD8⁺ T cell Isolation Kit II (Miltenyi Biotec), respectively, according to the manufacturer's protocols. Separation of CD25⁻ and CD25^{high} fractions from CD4⁺ T cells was performed using CD25 microbeads (Miltenyi Biotec) as previously described [29]. Cells were cultured at a cell density of 2×10^6 and $3-4 \times 10^6$ cells/ml, respectively for PBMC and purified CD4⁺, CD8⁺, CD4⁺CD25⁻ T cells. Reagents used were: IL-2 (Proleukin; kindly provided by Chiron), IL-7 (R&D), IL-15 (R&D), or anti-CD3 mAb with/without anti-CD28 mAb (1 μ g/ml, BD Biosciences), Dynabeads CD3/CD28 T cell expander (Invitrogen). Approximately 10 % of culture medium was replaced with fresh medium (with the appropriate cytokine amount) on days 3, 5 and 7 of culture. Cytokine-induced CD4⁺CD25^{high} cells were separated using 5 μ l of CD25 beads/ 10^7 cells as previously described [28].

Flow cytometry

The monoclonal antibodies used for staining were: CD3-FITC (SK7); CD25-PE (2A3, M-A251); CD27-FITC (M-T271), CD69-FITC/PE (FN50), IL-2-FITC (MQ1-17H12), IFN- γ -FITC (B27), IL-10-PE (JES3-19F1) from BD Biosciences; CD4-PC5 (13B8.2); CD8-PC5 (B9.11); CD45RO-ECD (UCHL1); CD3-ECD (UCHT1), from Beckman Coulter. Staining for FOXP3 was performed with 236A/E7 (eBioscience) according to the manufacturer's instructions. For analysis of intracellular cytokine production, cells were stimulated with 10 ng/ml PMA and 1 μ g/ml Ionomycin for 6 h in the presence of 10 μ g/ml brefeldin A (Sigma, for IL-2 or IFN- γ production) or GolgiStop (BD Biosciences, for IL-10 production).

Real-time quantitative PCR for measurement of levels of foxP3 mRNA expression

Real-time PCR was performed using the foxP3 and 18S rRNA primer/probe mix (TaqMan Gene Expression Assays, Applied Biosystems) as previously described [29]. Levels of foxP3 mRNA expression were determined by a standard curve generated by serial dilutions of a foxP3 encoding plasmid and normalized to levels of 18S rRNA expression in each sample.

Western blotting

Ten micrograms of protein were loaded per lane, separated on 10% NuPAGE Novex Bis-Tris Gel (Invitrogen), and transferred to a nitrocellulose membrane. The membrane was probed with 1 μ g/ml of mouse anti-human FOXP3 monoclonal antibody (eBio7979, eBioscience). Detection of FOXP3 protein was performed with the use of the WesternBreeze Chemiluminescent kit (Invitrogen). After the detection of FOXP3 protein, the membrane was stripped and reprobed with mouse anti-beta Actin antibody (ab8226, Abcam) to assess loading equivalency.

CFSE labeling and *in vitro* suppression assay

CFSE (Molecular Probes) labeling of PBMC or purified CD4⁺CD25⁻ cells was performed as previously described [30]. The ability of CD4⁺CD25⁺ cells isolated from PBMCs and cytokine-induced CD4⁺CD25⁺ cells to suppress the proliferation of a responder population of peripheral blood CD4⁺CD25⁻ T cells was assessed by coculture of CFSE-labeled responders with autologous suppressors (4 $\times$ 10⁵ cells/well) in 200 μ l of complete medium without IL-2, at responder:suppressor ratios of 1:1 or 2:1. Cultures were performed in 96-well flat bottom plates pre-coated with anti-CD3 antibody (BD Biocoat Anti-Human CD3 T-cell Activation 96-well Assay Plates, BD Biosciences) or in uncoated 96-well plates for unstimulated controls. Proliferation of the CFSE-stained fraction was evaluated by CFSE dye dilution at day 5.

Statistical analysis

Statistical analysis was performed with StatView 5.0.1 (SAS Institute, Inc.). Significant differences when comparing two groups were determined by using the two-tailed unpaired nonparametric Mann-Whitney test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FoxP3

forkhead box transcription factor p3

nTreg

naturally occurring Treg

γ cthe common γ chain**CD25**

IL-2R alpha

C

Figure 1. Induction of CD25 expression, cell proliferation and FOXP3 expression in CD4⁺ and CD8⁺ T cells by IL-15

PBMCs were isolated from normal healthy volunteers and cultured for up to 9 days in complete medium with IL-2, IL-7 or IL-15. Stimulation via the TCR using soluble anti-CD3/CD28 antibodies served as a control. (A) The percentages of CD4⁺ or CD8⁺ T cells positive for CD25 were plotted over time. Mean $\pm$ s.e.m values were calculated from 10 independent experiments. (B) Representative FACS plots of CD25 expression on CD4⁺ and CD8⁺ T cells after 9 days of culture with IL-2, IL-7 or IL-15. Gating was done on CD3 positive cells in the lymphocyte gate. Levels of total CD25 expression were calculated as percentages of cells with a CD25 expression level above the black dotted lines. CD25^{high} was defined as a CD25 expression level that exceeded that of all CD4⁺ cells in the lymphocyte gate at day 9. The population defined as CD25^{high} is highlighted in red circles for the CD4⁺ subpopulation and dotted squares for the CD8⁺ subpopulation. (C) PBMCs isolated from normal healthy volunteers were stained with CFSE and cultured in the presence of IL-2, IL-7 or IL-15. Stimulation via the TCR using soluble anti-CD3/CD28 served as a positive control. Cells were gated as CD3⁺CD4⁺ or CD3⁺CD8⁺ in the lymphocyte gate. Cells were analyzed at day 9 of culture. One representative

set of FACS plots from eight independent experiments is shown. **(D)** Kinetics of proliferation and FOXP3 expression were monitored overtime. One representative set of FACS plots from five independent experiments is shown.

Figure 2. IL-15 or IL-2 stimulation leads to *de novo* induction and expansion of FOXP3⁺ cells without TCR-mediated signaling

CD4⁺, CD8⁺ and CD25-depleted CD4⁺ T cells were purified from PBMCs of normal volunteers and cultured in the presence of IL-2, IL-7, IL-15 or anti-CD3/CD28 beads. The percentages of expressing (A) CD25 or (B) FOXP3 were plotted over time. Mean $\pm$ s.e.m values were calculated from five independent experiments. (C) The absolute numbers of FOXP3⁺ cells induced by IL-2 (red square), IL-7 (green triangle), IL-15 (blue circle) or anti-CD3/CD28 (open square) were calculated by multiplying the percentages of FOXP3⁺ cells by the total number of cells. (D) CD4⁺CD25⁻ T cells purified from PBMCs of normal volunteers were labeled with CFSE and cultured for 9 days in the presence of IL-2 (100 IU/ml), IL-7 (100 ng/ml), IL-15 (100 ng/ml), or anti-CD3/CD28 beads. The percentages of CD25⁺ or FOXP3⁺ cells are depicted in each plot. One representative set of FACS plots from five independent experiments is shown.

Figure 3. Phenotypes of CD4⁺CD25^{high} T cells freshly isolated from the peripheral blood (nTreg) and cytokine-induced CD4⁺CD25^{high} cells

(A) CD4⁺CD25^{high} T cells freshly isolated from PBMC (nTreg). (B) Cytokine-induced CD4⁺CD25^{high} cells. Cytokine-induced CD25^{high} cells were bead-separated from CD4⁺CD25⁻ cells cultured for 9 days in the presence of IL-2 (100 IU/ml), IL-7 (100 ng/ml) or IL-15 (100 ng/ml). The purity of CD25^{high} cells was typically $\geq 99\%$, and the cell viability of the bead-separated fractions was also $\geq 99\%$ (determined by trypan blue exclusion method). The data presented are from one of 12 independent experiments using cells from different donors. (C) Levels of foxP3 mRNA were measured in uncultured CD4⁺CD25⁻, nTregs, and in cytokine-induced CD4⁺CD25^{high} cells. (D) Western blot analysis of FOXP3 protein expression. Levels of FOXP3 protein expression in the cytokine-induced CD25^{high} cells were compared with those detected in nTregs and CD4⁺CD25⁻ cells. Results are representative of seven independent experiments using cells from different donors.

Figure 4. Proliferative capacity of cytokine-induced CD25^{high} cells

nTreg and cytokine-induced CD25^{high} cells were stained with CFSE and cultured for 4 days in 96-well plates in the presence of IL-2 (100 IU/ml), IL-7 (100 ng/ml), IL-15 (100 ng/ml) or anti-CD3/CD28 beads. The extent of cell proliferation was assessed by the pattern of CFSE dye dilution. One of six similar experiments from independent donors is shown.

Figure 5. nTreg and cytokine-induced CD25^{high} cells exhibit different cytokine profile
 nTreg and cytokine-induced CD4⁺CD25^{high} cells were stimulated with PMA/Ionomycin for 6 h in the presence of a protein transport inhibitor. No measurable amount of cytokine production was observed in the absence of PMA/Ionomycin stimulation in any of the cell subsets analyzed. The percentage of cells expressing IL-2, IFN- γ or IL-10 were determined with intracellular cytokine staining. Lack of detection of IL-10 production was not associated with lower activation, since CD69 upregulation was noted in each subset upon stimulation. Cells were gated as CD4⁺ in the lymphocyte gate. One of three independent experiments with similar results is shown.

Figure 6. Suppressive activity of cytokine-induced CD4⁺CD25^{high} cells

CD4⁺CD25⁻ responder cells were stained with CFSE and stimulated for 5 days in 96-well plates coated with anti-CD3 antibody with or without the addition of unlabeled autologous suppressors: CD25⁻, nTreg or cytokine-induced CD25^{high} cells. Cells were added at the responder:suppressor ratios of 1:1 or 2:1. The patterns of CFSE dye dilution for an unstimulated control (highlighted in grey) and a positive control (solid line) are shown in the left upper panel. Gating was done on CD4⁺CFSE⁺ cells. One of six similar experiments from independent donors is shown.