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CD4+ CD25\textsuperscript{high} Regulatory Cells in Human Peripheral Blood\textsuperscript{1}

Clare Baecher-Allan,\textsuperscript{2*} Julia A. Brown,\textsuperscript{†} Gordon J. Freeman,\textsuperscript{†} and David A. Hafler\textsuperscript{*}

Thymectomy in mice on neonatal day 3 leads to the development of multiorgan autoimmune disease due to loss of a CD4+ CD25\textsuperscript{+} T cell regulatory population in their peripheral lymphoid tissues. Here, we report the identification of a CD4+ population of regulatory T cells in the circulation of humans expressing high levels of CD25 that exhibit in vitro characteristics identical with those of the CD4+ CD25\textsuperscript{+} regulatory cells isolated in mice. With TCR cross-linking, CD4+ CD25\textsuperscript{high} cells did not proliferate but instead totally inhibited proliferation and cytokine secretion by activated CD4+ CD25\textsuperscript{−} responder T cells in a contact-dependent manner. The CD4+ CD25\textsuperscript{high} regulatory T cells expressed high levels of CD45RO but not CD45RA, akin to the expression of CD45RB\textsuperscript{low} on murine CD4+ CD25\textsuperscript{+} regulatory cells. Increasing the strength of signal by providing either costimulation with CD28 cross-linking or the addition of IL-2 to a maximal anti-CD3 stimulus resulted in a modest induction of proliferation and the loss of observable suppression in cocultures of CD4+ CD25\textsuperscript{high} regulatory cells and CD4+ CD25\textsuperscript{−} responder cells. Whereas higher ratios of CD4+ CD25\textsuperscript{high} T cells are required to suppress proliferation if the PD-L1 receptor is blocked, regulatory cell function is shown to persist in the absence of the PD-1/PD-L1 or CTLA-4/B7 pathway. Thus, regulatory CD4 T cells expressing high levels of the IL-2 receptor are present in humans, providing the opportunity to determine whether alterations of these populations of T cells are involved in the induction of human autoimmune disorders. The Journal of Immunology, 2001, 167: 1245–1253.

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\textsuperscript{†} Abbreviation used in this paper: NOD, nonobese diabetic.
to those of murine CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells. This CD4<sup>+</sup>CD25<sup>high</sup> T subset in humans comprises ~1-2% of circulating CD4<sup>+</sup> T cells, unlike that in rodents where 6–10% of CD4<sup>+</sup> T cells demonstrate regulatory function. Whereas the entire population of CD4<sup>+</sup>CD25<sup>+</sup> T cells expressing both low and high CD25 levels exhibit regulatory function in the mouse, only the CD4<sup>+</sup>CD25<sup>high</sup> population (CD4<sup>+</sup>CD25<sup>high</sup>) exhibits a similarly strong regulatory function in humans. These CD4<sup>+</sup>CD25<sup>high</sup> cells inhibit proliferation and cytokine secretion induced by TCR cross-linking of CD4<sup>+</sup>CD25<sup>+</sup> responder T cells in a contact-dependent manner. Although higher numbers of CD4<sup>+</sup>CD25<sup>high</sup> T cells are required, these regulatory cells can still suppress proliferation in the absence of the PD-1/PD-L1 or CTLA-4/B7 pathways. Thus, regulatory CD4<sup>+</sup> T cells expressing high levels of the IL-2 receptor exist in human peripheral blood, providing the opportunity to determine whether alterations in this population of T cells are involved in the induction of human autoimmune disorders.

Materials and Methods

Cell culture reagents

Cells were cultured in RPMI 1640 supplemented with 2 nM l-glutamine, 5 mM HEPES, and 100 U/ml penicillin/g/ml streptomycin (all from Bio-Whittaker, Walkersville, MD), 0.5 mM sodium pyruvate, 0.05 mM non-essential amino acids (both from Life Technologies, Gaithersburg, MD), and 5% human AB serum (Gemini Bio-Products, Woodland, CA) in 96-well U-bottom plates (Costar, Corning, NY). The anti-CD3 (clone d1C7) (mouse for plate-bound assays and clone H13a for soluble conditions) and anti-CD28 (clone 3D10) were provided by Genetics Institute (Cambridge, MA). In subsequent assays, the UCHT1 anti-CD3 mAb gave the same results as the Hit3a mAb when tested in soluble form; in the mouse, the anti-CD25 mAb gave the same results as the anti-CD25<sup>+</sup> mAb when tested in soluble form; and past work demonstrated that a strong regulatory function in humans. These CD4<sup>+</sup>CD25<sup>high</sup> T cells are required, these regulatory cells can still suppress proliferation in the absence of the anti-CD4-CyChrome (IgG1, BD PharMingen) and either anti-CD25-PE (IgG1, BD PharMingen) or anti-CD25-PE (IgG2b, BD PharMingen), both of which were purchased from Immunotech. As the third color, the samples stained with anti-CD4-CyChrome and anti-CD25-PE were also stained with either IgG1-FITC or anti-CD25-PE (IgG1) from Immunotech, IgG2b-FITC (Caltag, San Francisco South, CA), or anti-CD45RA-FITC (IgG2b; BD PharMingen). As the third color, the samples stained with anti-CD4-CyChrome and anti-CD25-PE were also stained with either IgG1-PE or anti-CD25-PE (IgG2a), anti-CD58(LFA-3)-PE (IgG2a), or anti-CD71-PE (IgG1) from Immunotech, or IgG2b-PE from Beckman Coulter (Miami, FL). The samples were run on an EPICS flow cytometer, collecting data on 2 × 10<sup>5</sup> lymphocytes gated by forward and side scatter properties, and analyzed using CellQuest software (BD Biosciences). Although appropriate isotype controls were run for each sample, because they gave similar results, only the IgG1 isotype third color stain is shown.

Cytokine analyses by ELISA

The supernatants that were removed before addition of [<sup>3</sup>H]thymidine and were diluted and analyzed on Immunulon 4 ELISA plates (Dynex Technologies, Chantilly, VA) using the Ab pairs IFN-γ (M-700A, M-701-B, bionin; Endogen, Woburn, MA), IL-10 (18551D, 18562d-biotinylated; BD PharMingen), and IL-13 (554570, biotinylated 555054; BD PharMingen), developed with avidin-peroxidase conjugate (U.S. Biological, St. Louis, MO) and tetramethylbenzidine peroxide substrate (Kirkegaard & Perry Laboratories, Gaithersburg MD). Instead of IL-4, IL-13 was assayed as a prototypical Th2 cytokine due to limitations in the detection of IL-4 in culture supernatants of human T cells likely due to its consumption and the fact that these assays were set up with very low numbers of T cells per well.

IL-2 mRNA analyses by reverse transcription-semiquantitative PCR

Sixty wells of cultured CD4<sup>+</sup>CD25<sup>+</sup>, CD4<sup>+</sup>CD25<sup>high</sup>, or cocultured cells were stimulated with soluble anti-CD3/anti-CD28 and (and cell-depleted accessory cells) as before. At 5 days, RNA was isolated from the collected cells by solubilization in TRIzol reagent and converted into cDNA via the Superscript II Reverse Transcriptase protocol using oligo(dT)12-18 (all reagents purchased from Life Technologies). Actin and IL-2 messages were amplified using the actin primers: 5′: AACCCCAAGGCCAACGGCGGA GAGATGACC and 3′: GTGTGATACGCCGTCAGGCACCTG GTA and the IL-2 primers: 5′: TACAGGATGAACTTCCGGTAGT CATTGCA and 3′: GTTGGTCGCTCTGATCATCATTAGACATG ATG. PCR (100 μl) was performed using 2.5 U Taq DNA polymerase, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 μM primers, and cycling parameters of 94°C for 2 s, then the indicated number of cycles of: 94°C for 20 s, 60°C for 30 s, 72°C for 1 s. On combination of all reagents, the reaction PCR was performed in 20 wells, one for every three cycles within the desired range. (Additional PCR controls (not shown), and past work demonstrated that a three-cycle delay in the appearance of a PCR product under these conditions were indicative of a 4-fold difference in input template (18)). Reaction products were analyzed on 2% agarose, Tris-buffered EDTA TBE gels.

Results

CD25<sup>+</sup> regulatory cells comprise ~1–2% of CD<sup>+</sup> T cells in human peripheral blood

Approximately one-half of the circulating human peripheral blood lymphocytes express CD4<sup>+</sup>, and of these roughly 10% express the IL-2 growth factor receptor α-chain, CD25. Peripheral blood lymphocytes do not stain very strongly for CD25. Unlike what is seen in the mouse, the CD25<sup>+</sup> population in the human is not as clearly discernable (Fig. 1a) (11, 14). Rather, the CD4<sup>+</sup> T cells with the...
highest level of CD25 (CD4⁺CD25high) appear as a tail to the right from the major population containing both CD4⁺CD25low and CD4⁺CD25⁻ cells. The CD25high cells represent 1–2% of the total CD4⁺ T cell population, whereas the CD25low cells can represent up to 16% of CD4⁺ T cells.

CD4⁺CD25high, CD4⁺CD25low, and CD4⁺CD25⁻ T cells were sorted using the gates shown (Fig. 1a) to address whether either population exhibited regulatory cell characteristics such as hyporesponsiveness and suppression of proliferation as described in the murine system. The CD4⁺CD25high (high or low) cells, CD4⁺CD25⁻ cells, or a 1:1 mixture (cocultures) were stimulated by submaximal cross-linking of the TCR (plate-bound anti-CD3 at 0.05 μg/ml) and monitored for proliferation by [³H]thymidine incorporation. The CD4⁺CD25low cells responded to TCR cross-linking with a strong proliferative response and did not suppress the proliferation of the cocultured CD4⁺CD25⁻ cells at either 5 (data not shown) or 7 days (Fig. 1b). In striking contrast, the CD4⁺CD25high cells cultured alone did not respond to this submaximal TCR stimulation. Moreover, the CD4⁺CD25high T cells were able to strongly inhibit the proliferation of CD4⁺CD25⁻ responder T cells (Fig. 1b, bottom). This inhibition was highly significant because the CD4⁺CD25high T cells reproducibly reduced proliferation of CD4⁺ cells by 69% at day 5 and >98% by day 7, compared with the response of the CD4⁺CD25⁻ cells cultured alone.

Human CD4⁺CD25high cells express CD45RO and MHC class II (HLA-DR)

The CD4⁺CD25neg/low/high T cells were analyzed for expression of surface Ags to gain insight into their mechanism of action and to more fully characterize this regulatory population in humans. The different levels of surface Ag expression were compared among the CD4⁺CD25neg, CD4⁺CD25low, and CD4⁺CD25high cell subsets (Fig. 2). CD45RO, which can be associated with proliferative responses to recall Ags, was expressed at significantly higher levels by the CD4⁺CD25high population (99%) than the CD4⁺CD25low (89%) or CD4⁺CD25⁻ (33%) subset. This high expression of CD45RO on human CD4⁺CD25high T cells is akin to the expression of CD45RBlow on the CD4⁺CD25⁺ regulatory cells in mice (9). In contrast, the expression of CD45RA, considered a marker for naive T cells, showed the opposite expression profile. Greater than 50% of the CD4⁺CD25⁻ subset and 25% of the CD4⁺CD25high subset expressed CD45RA in contrast to only 4% of the CD4⁺CD25high T cells.

These three T cell populations defined by varying levels of CD25 expression exhibited marked differences in a number of surface Ags that have been used to define functionally distinct populations (Fig. 2). The CD4⁺CD25n/high cells expressed the highest levels of the peripheral lymph node homing receptor, L-selectin (CD62 ligand) (19). The CD4⁺CD25high cells also expressed the highest frequency and intensity of the IL-2R β-chain (CD122) and LFA-3 (CD58) compared with the other T cell subsets. The IL-2R β-chain was expressed by only 6% of CD4⁺CD25⁻ cells, by 28% of the CD4⁺CD25low cell subset, and by >85% of the CD4⁺CD25high cells. Unlike mouse T cells, activated human T

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Human peripheral blood contains both CD4⁺CD25high regulatory cells and CD4⁺CD25low nonregulatory cells. a, Mononuclear cells from freshly drawn human blood were stained with different combinations of anti-CD4-CyChrome (Cy), mlgG1-CyChrome, mlgG2-anti-PE, and anti-CD25-PE. The cells in these analyses were gated on lymphocytes via their forward and side scatter properties. The CD4⁺CD25neg, CD4⁺CD25low, and CD4⁺CD25high populations were sorted using the indicated sorting gates. b, The CD4⁺CD25low (top) and the CD4⁺CD25high (bottom) T cells were stimulated alone at 3 × 10⁵ cells/well and in coculture with 3 × 10⁵ CD4⁺CD25⁻ responder T cells in the presence of 3 × 10⁵ T cell-depleted accessory cells. The CD4⁺CD25⁻ cells were also stimulated alone. Data are representative of three independent experiments and are presented as the mean of proliferation at day 7 ± SEM. The stimulus used for activation was plate-bound anti-CD3 at the submaximal concentration of 0.05 μg/ml.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Human CD4⁺CD25high cells are CD45RO⁺, CD45RA⁻, and express surface Ags associated with activated cells. Human PBMC were stained with either anti-CD25-PE, anti-CD4-CyChrome, and anti-CD45RA-FITC or anti-CD62 ligand (CD62L)-FITC; or anti-CD25-FITC, anti-CD4-CyChrome, and anti-CD45RO-PE, anti-CD58-PE, anti-CD71-PE, anti-CD122-PE, or eDR-PE. Control samples stained with IgG2b-FITC, IgG2a-FITC, or IgG2b-PE and IgG2a-PE as the third color are not shown because they were identical with the staining pattern for the IgG1 isotype control. Gates were set as described in Fig. 1. The histogram analysis of the CD4⁺CD25⁻ and CD4⁺CD25low populations were scaled to 150, and the scale for the analysis of the CD4⁺CD25high population was set at 30. The analysis was performed with CellQuest software.
cells express HLA class II molecules on their surface allowing them to assume the role of APC (20). Importantly, the low level of HLA-DR expression observed in human blood was found to be limited to the CD4+CD25<sub>high</sub> cells (~20%) compared with ~2% of the CD4+CD25<sup>−</sup> and CD4+CD25<sub>low</sub> populations. The CD4+CD25<sub>high</sub> subset also exhibited preferential expression of the transferrin receptor (CD71, 46%), which is usually expressed on the surface of activated lymphocytes and all cells entering proliferation (21). Thus, the CD4+CD25<sub>high</sub> regulatory cells express a number of surface Ags associated with activation, migration, and Ag presentation.

**Increasing the TCR strength of signal for CD4+CD25<sub>high</sub> T cells induces proliferation and loss of regulatory function**

We next examined whether alterations in the TCR strength of signal could overcome either the nonresponsiveness or the suppression mediated by CD4+CD25<sub>high</sub> cells. Cultures were stimulated with two different doses of plate-bound anti-CD3 to compare the effect of varying the quantity of the same quality of TCR signal (i.e., that delivered by plate-bound anti-CD3). TCR stimulation through plate-bound anti-CD3 at 5 μg/ml (maximal response) or 0.05 μg/ml (submaximal) alone (Fig. 3) did not reverse the nonresponsive state of the CD4+CD25<sub>high</sub> cells. Stimulation of cocultures with maximal plate-bound anti-CD3 stimulation resulted in only 69% inhibition at a 1:1 ratio of CD4+CD25<sub>high</sub> to CD4+CD25<sup>−</sup> cells. In contrast, cocultures stimulated with the submaximal dose of plate-bound anti-CD3 exhibited >99% inhibition of proliferation. Increasing the strength of signal by providing either costimulation with CD28 cross-linking or the addition of IL-2 to the maximal anti-CD3 (5 μg/ml) stimulus resulted in both CD4+CD25<sub>high</sub> proliferation (albeit at a low level) and the complete loss of regulation. Thus, signaling through the TCR with a strong stimulus caused either the target cell population to become refractory to inhibition or the regulatory cell population to lose its effector function. Interestingly, the ability of IL-2 to break the nonresponsiveness of the CD4+CD25<sub>high</sub> population was strongest if the cells were stimulated with submaximal anti-CD3.

The results were somewhat different if there was less TCR cross-linking using soluble anti-CD3 stimulation. The very low proliferation on soluble anti-CD3 stimulation are not surprising in light of the paucity of the signal transduced by the soluble Ab, likely due to low levels of accessory cell cross-linking. Soluble anti-CD3 stimulation resulted in both CD4+CD25<sub>high</sub> cell nonresponsiveness and >95% inhibition of coculture proliferation similar to what was observed in cultures stimulated with submaximal plate-bound anti-CD3. As predicted, the addition of IL-2 to soluble anti-CD3 stimulated cocultures resulted in a loss of regulatory function. Surprisingly, however, the suppression apparent in soluble anti-CD3 stimulated cocultures could not be reversed by providing anti-CD28 costimulation. A second anti-CD28 mAb (3D10) was also unable to abrogate suppression in conjunction with soluble anti-CD3 stimulation, demonstrating that this phenomenon was not reagent specific (data not shown). It appears that the signal generated by the soluble anti-CD3 condition is so weak that even with anti-CD28 cross-linking, suppression can still occur.

The effect of the CD4+CD25<sub>high</sub> T cells on cytokine secretion in the coculture conditions was next examined (Fig. 4). We analyzed supernatants taken from the cultures depicted in Fig. 3 for levels of IFN-γ (Th1 cytokine) and IL-13 (Th2 cytokine). We also measured secretion of the suppressive cytokine, IL-10 as it is known to inhibit T cell proliferation and is secreted by other types of regulatory T cells such as Tr1 cells as well as non-T cell populations (22, 23). The samples were diluted and analyzed by ELISA as described. The level of IFN-γ secretion mirrored the level of proliferation with each stimulus (Fig. 4a). In general, when the proliferation was inhibited by coculture with the CD4+CD25<sub>high</sub> cells, the secretion of IFN-γ was also decreased. However, the soluble anti-CD3 alone coculture condition was an exception because there was no decrease in IFN-γ secretion in light of a striking inhibition of proliferation.

IL-13 was only secreted by CD4+CD25<sup>−</sup> under conditions of strong TCR signaling by maximal plate-bound anti-CD3 or under conditions of weaker TCR signals augmented with exogenous IL-2 or anti-CD28 costimulation (Fig. 4c). In all conditions in which the CD4+CD25<sup>−</sup> cells secreted IL-13, the corresponding coculture resulted in a significant decrease in IL-13 secretion regardless of whether proliferation was inhibited. Notable is the complete suppression of IL-13 in cocultures stimulated with soluble anti-CD3 plus IL-2 even though there was little to no inhibition of proliferation. Thus, it appears that IL-13 secretion may be more sensitive than IFN-γ secretion or proliferation to the effects of coculture with regulatory cells under conditions of different strengths of signal.

As discussed above, it was important mechanistically to further examine whether IL-10 was secreted by the CD4+CD25<sub>high</sub> regulatory cells in coculture assays, because this cytokine is inhibitory to T cell activation (24, 25). No culture stimulated with soluble anti-CD3 secreted IL-10, even though these cultures exhibited

**FIGURE 3.** The strength and quality of the TCR signal affect the ability of the CD4+CD25<sub>high</sub> cells to suppress the proliferation of the cocultured CD4+CD25<sup>−</sup> cells. CD4+CD25<sub>high</sub> and CD4+CD25<sup>−</sup> cells (2.5 × 10<sup>5</sup>/well) were cultured alone or together in the presence of the indicated stimuli and 2.5 × 10<sup>3</sup> T cell-depleted accessory cells. The cells were stimulated in wells that had been coated with either 5 μg/ml (maximal) or 0.05 μg/ml (submaximal) anti-(α)CD3 mAb or by the addition of soluble anti-CD3 at 5 μg/ml. In some cases as noted, additional stimulatory signals were provided by anti-CD28 or recombinant human IL-2 at 50 U/ml. Proliferation was determined at day 6, with [3H]thymidine added for the last 16 h of culture. These stimuli had similar effects on the CD4+CD25<sup>−</sup> and CD4+CD25<sub>high</sub> populations in three multiple experiments.
marked suppression (Fig. 4b). In addition, although IL-10 was produced in all the cultures of CD4+CD25+ cells alone and cocultures stimulated with plate-bound anti-CD3 supplemented with IL-2 or anti-CD28, it was not secreted by stimulation of the CD4+CD25high cells (alone) under any condition. There also was no correlation between suppression and the secretion of IL-10, suggesting that regulation by CD4+CD25high cells was independent of IL-10. This interpretation was further supported by Transwell analysis (Fig. 5) which demonstrated that contact is required for the CD4+CD25high cells to exert their regulatory function on CD4+CD25+ T cells. Stimulation of CD4+CD25high cells in the upper chamber had little effect on the growth of the CD4+CD25+ cells in the lower chamber. In contrast, when the two populations were cocultured in the same lower well, there was a marked inhibition of proliferation.

**Kinetics of CD4+CD25high T cell regulatory function**

A series of experiments were performed to examine both the kinetics and the degree of suppression mediated by CD4+CD25high T cells. Using soluble anti-CD3/anti-CD28 stimulation, different numbers of CD4+CD25high cells (serial 3-fold dilutions) were cocultured with a constant number of CD4+CD25+ responder cells. Proliferation was monitored at days 3, 5, and 7 (Fig. 6a). Although there was almost no detectable proliferation on day 3, by day 5 there were barely detectable levels of proliferation which showed little inhibition. In contrast, the inhibitory effect of the CD4+CD25high T cells was striking by day 7. The CD4+CD25high T cells inhibited the proliferative response of the cocultured CD4+CD25+ cells in a dose-dependent manner (Fig. 6a). These data show that 93 and 80% suppression occurs at the 1:1 and at the

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**FIGURE 4.** The CD4+CD25high cells do not secrete cytokine but can suppress the secretion of IFN-γ and IL13 by cocultured CD4+CD25+ cells in a dose-dependent manner. Culture supernatants were removed from the proliferation cultures depicted in Fig. 3 before the addition of [3H]thymidine incorporation (at day 5). Levels of IFN-γ (a), IL10 (b), and IL-13 (c) were determined from culture supernatants by ELISA. All data represent the mean ± SEM of duplicate assays. α-, Anti-.
of proliferation mediated by CD4+CD25high T-cell-mediated suppression on cell contact. CD4+CD25high and CD4+CD25− cells (5 × 10^5/well) were stimulated in the lower chamber of a Transwell plate in the absence of additional T cells or in the presence of CD4+CD25high cells that were stimulated either in the same lower well or in the separate upper chamber of the Transwell. Data represent total cpm from the cultures at day 5.

The kinetics of cytokine secretion in these cocultures were also monitored from supernatants removed just before [3H]thymidine addition. CD4+CD25high T cells stimulated with soluble anti-CD3/anti-CD28 did not secrete IFN-γ, IL-10, or IL-13, whereas CD4+CD25− responder cells secreted only IFN-γ. On titrating the regulatory cells into the coculture, there was a dose-dependent inhibition of IFN-γ secretion (Fig. 6b) which was apparent by the fifth day of culture and more prominent by day 7. Thus, the suppression of IFN-γ secretion was observable well before suppression of proliferation.

**CD4+CD25high cells inhibit IL-2 mRNA levels in cocultures**

We addressed whether coculture with human CD4+CD25high cells resulted in a decrease in the levels of IL-2 mRNA as has been shown in the analysis of mouse CD4+CD25− cells (11). RNA samples of anti-CD3/anti-CD28 (soluble)-stimulated cultures of CD4+CD25−negative, CD4+CD25high, or cocultures, were analyzed for their levels of actin and IL-2 message by semiquantitative RT-PCR (18). As shown in Fig. 7, the levels of IL-2 message were significantly decreased in the cocultures, even though the coculture sample contained twice the amount of CDNA as in the CD4+CD25− only sample, as indicated by the levels of actin product.

**Role of CTLA-4 and PDL1 in T-T cell regulation**

We then tried to identify a receptor-ligand interaction important for CD4+CD25high-mediated suppression of CD4+ T cells. We chose to examine the PD-1, PD-L1, and CTLA-4 receptors on CD4+CD25high T cells, because their engagement is known to induce cell cycle arrest. Specifically, PD-1 was identified as a receptor that is induced on activated T cells and involved in programmed cell death (26, 27). The ligand for PD-1, PD-L1, was recently shown to deliver a negative signal through the PD-1 receptor, which down-regulates T cell proliferation and cytokine production in response to suboptimal TCR stimulation (28, 29). Furthermore, both PD-L1 and PD-1 are expressed by subsets of activated T cells (27, 28). CTLA-4 is similarly expressed by activated T cells and can deliver a negative signal that results in down-regulation of T cell activation (30). Thus, we asked whether inhibitory anti-PD-L1 or anti-CTLA-4 mAbs could reverse the suppression induced by CD4+CD25high T cells (Fig. 8). When analyzed directly from the blood, CD4+CD25high T cells did not express CTLA-4 or PD-L1 on the cell surface (data not shown). This is similar to the mouse system, in which CTLA-4 is constitutively expressed in the cytoplasm of murine CD4+CD25− regulatory T cells but is not detected on the cell surface (9, 14). However, due to the longer kinetics of suppression and the fact that peak levels of CTLA-4, PD1, and PD-L1 expression can be induced on T cells by 2–3 days postactivation (27, 30, 31), it was still possible that these regulatory molecules could be involved in the suppression mediated by the CD4+CD25high regulatory cells. Adding anti-CTLA-4 Fab mAb to CD4+CD25high/CD4+CD25− cocultures did not alter the functional suppression of proliferation (Fig. 8a). Similarly, anti-CTLA-4 Fab mAb had no effect on IFN-γ secretion (Fig. 8b) while inducing a paradoxical decrease in the secretion of IL-13 by CD4+CD25− T cells, as previously described (17) (Fig. 8c).
Consistent with the engagement of PD-1 on the surface of T cells leading to inhibition of proliferation (29), there was a marked increase in proliferation of the CD4<sup>+</sup> CD25<sup>+</sup> T cells on PD-L1 blockade. However, the CD4<sup>+</sup> CD25<sup>high</sup> T cells could still suppress proliferation in the presence of anti-PD-L1, although significantly more regulatory T cells were required to attain similar percent inhibition of proliferation (Fig. 8a, inset). These data suggest that PD-L1/PD-1 interactions may mediate only a small part of the T-T cell interaction that results in inhibition of the target CD4 T cell. Adding the anti-PD-L1 mAb to these culture conditions strongly enhanced the secretion of both IFN-γ and IL-13, which were both inhibited by the CD4<sup>+</sup> CD25<sup>high</sup> regulatory T cells in a dose-dependent manner. The addition of both anti-CTLA-4 and anti-PD-L1 together mirrored that of anti-PD-L1 cultures alone for proliferation and IFN-γ secretion. Again, the blocking CTLA-4 enhanced the secretion of IL-13 (Fig. 8c). However, blocking these two inhibitory surface molecules had little effect on the ability of the CD4<sup>+</sup> CD25<sup>high</sup> cells to inhibit the proliferation of the cocultured responder cells.

**Discussion**

Here, we describe the isolation and characterization of the human counterpart to the murine CD4<sup>+</sup> CD25<sup>+</sup> regulatory subset from human peripheral blood. These CD4<sup>+</sup> CD25<sup>high</sup> T cells were hyporesponsive to TCR engagement yet were able to totally inhibit [<sup>3</sup>H]-thymidine incorporation or cytokine secretion by cocultured poresponsive to TCR engagement yet were able to totally inhibit proliferation and IFN-γ secretion. Again, the blocking CTLA-4 enhanced the secretion of IL-13 (Fig. 8c). However, blocking these two inhibitory surface molecules had little effect on the ability of the CD4<sup>+</sup> CD25<sup>high</sup> cells to inhibit the proliferation of the cocultured responder cells.

**FIGURE 8.** Blocking αCTLA-4 Fab or αPD-L1 Ab in the inhibition of regulatory function of the CD4<sup>+</sup> CD25<sup>high</sup> cells. CD4<sup>+</sup> CD25<sup>high</sup> and CD4<sup>+</sup> CD25<sup>+</sup> cells (5 × 10<sup>4</sup>/well) were cultured alone or together at the indicated ratios in the presence of soluble anti-α<sub>-</sub>CD3 (5 µg/ml), soluble anti-CD28 (5 µg/ml), and 5 × 10<sup>4</sup> T cell-depleted accessory cells. These cells were also cultured in the presence of mlgG ( ● 5 µg/ml), anti-CTLA-4 Fab ( ●, at 5 µg/ml), anti-PD-L1 ( ●, at 10 µg/ml), or anti-CTLA-4 and anti-PD-L1 ( ●). Proliferation (a) was determined after 7 days of culture and the concentration of IFN-γ (b) and IL-13 (c) were assayed from supernatants removed on day 6, as above. Inset, data as percent proliferation, where the mean of proliferation of cocultures supplemented with the indicated blocking reagents was divided by the different baseline mean of proliferation of the cultures of CD4<sup>+</sup> CD25<sup>+</sup> cells only supplemented with the same Ab reagents.

The mechanism of suppression by these CD4<sup>+</sup> CD25<sup>high</sup> regulatory cells appears to be independent of the inhibitory cytokines, IL-10 and TGF-β. Although IL-10 was secreted by CD4<sup>+</sup> CD25<sup>+</sup> cells, the presence or absence of IL-10 did not correlate with suppression in cocultures. To rule out the possibilities that IL-10 was consumed or was in very low abundance, additional experiments demonstrated that the addition of blocking anti-TGF-β or anti-IL-10 Abs had no effect on the ability of CD4<sup>+</sup> CD25<sup>high</sup> cells to suppress the proliferation of cocultured CD4<sup>+</sup> CD25<sup>+</sup> cells (data not shown). Thus, the cytokine-independent suppression by CD4<sup>+</sup> CD25<sup>high</sup> cells is an important distinction given that secretion of these two cytokines has been found to be integral to the function of other types of regulatory T cells (23).

Immune regulation is highly complex, and the mechanisms of suppression is not as yet understood. The identification of two major CD4<sup>+</sup> T cell subsets by Mosmann and Coffman (32) was a major advance, providing the insight that cytokines secreted by CD4<sup>+</sup> T cells may regulate immune responses. However, it was clear that populations of T cells could also mediate immune responses by cell contact in the absence of cytokine secretion. Experiments demonstrating that CD4<sup>+</sup> CD25<sup>+</sup> T cells function as key regulatory effectors in mice have provided important information about a specific cellular population that performs immune regulation through suppression of self responses (6). In those studies, it was demonstrated that thymectomy on neonatal day 3 that resulted in a multiorgan autoimmune disease (gastritis, thyroiditis, and insulinitis) was associated with the loss of this CD4<sup>+</sup> CD25<sup>+</sup> population (4, 10, 33). Mason et al. (34) have also demonstrated the existence of similar regulatory T cells in the rat, further suggesting the importance of CD4<sup>+</sup> CD25<sup>+</sup> T cells across species in regulating immune responses. The mechanism of action by which CD4<sup>+</sup> CD25<sup>+</sup> T cells so effectively inhibit proliferation of CD4 T cells remains unknown. The work by Thornton and Shevach (11) establishing an in vitro model system that mimics the function of CD4<sup>+</sup> CD25<sup>+</sup> T cells in vivo is critical in directing the investigation of regulatory T cells in humans. Those experiments demonstrated that although murine CD4<sup>+</sup> CD25<sup>+</sup> cells failed to proliferate after TCR stimulation alone, they could proliferate quite well if exogenous IL-2 was also provided. Yet this addition of IL-2 or anti-CD28 abolished the suppression of the proliferation of the cocultured cells. Interestingly, in humans, we found that although anti-CD28 costimulation also inhibited suppression, it did so only in the context of certain TCR signals. Yet suppression by both murine and human CD4<sup>+</sup> CD25<sup>+</sup> regulatory cells has been shown to require cell contact. Thus, because the features of the murine CD4<sup>+</sup> CD25<sup>+</sup> and the human CD4<sup>+</sup> CD25<sup>high</sup> regulatory cell populations are essentially identical, we conclude that they represent homologous populations.
Human CD4 regulatory function was observed only when the cells expressed high levels of CD25 and were isolated apart from the CD25low T cells. The mouse CD4+CD25+ regulatory subset is isolated from all CD25-expressing cells regardless of their level of CD25 expression (7, 11, 12). If similar criteria are followed to isolate these cells from human blood, the resulting CD25+ cells (high and low together) did not exhibit a hyporesponsive phenotype or significant suppressive function. CD4 T cells expressing low levels of the IL-2 receptor (CD25) strongly proliferated to submaximal TCR stimulation and showed no suppressive ability. Furthermore, CD25high cells derived from in vitro stimulation of CD4+CD25− cells, and large activated CD4+CD25+ T cells isolated directly ex vivo also did not demonstrate suppressor activity (data not shown). Thus, as suggested by Thornton and Shevach (11), these data together support the concept that the CD4+CD25+ T cells may represent a distinct lineage of professional suppressor cells. Moreover, our experiments indicate the need to re-evaluate data on IL-2 receptor expression on CD4 T cells in the peripheral blood and inflammatory compartments of human diseases, as CD4+CD25+ T cells may represent either activated or regulatory T cells.

The CD4+CD25high cells may exist in a semiactivated state in vivo, expressing a number of surface Ags that are usually associated with activated T cells. Interestingly, the CD4+CD25high T cells we identified expressed high levels of both IL-2 receptor α-chain, and (CD122) IL-2 receptor β-chain, making up the high affinity IL-2R. Thus, these regulatory T cells may be poised for a quick response or alternatively, constantly turned on and performing continual low level regulatory activity.

The mechanism of suppression mediated by CD4+CD25high cells appears to be linked in part to the strength of signal delivered through the TCR. Our data demonstrate that the addition of co-stimulatory signals did not abrogate regulatory function if the coincident TCR signal strength was low, thus linking the mechanism of suppression to TCR strength of signal. In contrast, the addition of IL-2 to cocultures ablated suppression in all cases regardless of the strength of the TCR stimulus. This suggests that suppression by regulatory cells at the initiation of significant inflammatory responses in vivo would be kept in check by the secretion of IL-2 by Ag-responsive T cells. With time, as activated T cells no longer secrete IL-2, the CD4+CD25high cells can manifest their function. Conversely, it appears that the regulatory CD4+CD25high cells require their own activation signal to then feed back the suppression of Ag-activated T cells. The mechanism underlying these initial signaling event are not as yet elucidated.

Whereas IL-2 ablated the suppressor function of CD4+CD25high cells under all stimulatory conditions, regulatory cells could function in the presence of CD28 costimulation depending on the strength of the TCR signal. This may be of importance in relationship to the nature of T cell-stimulatory signals delivered in association with responses to self Ag (low strength of signal) in which suppression of T cell responses is desirable as compared with responses to foreign microbial Ags (stronger strength of signals) where suppression could be detrimental. It is also possible that either B7-1 or B7-2 expressed on the surface of activated human T cells with activation may provide important costimulatory signals that ablate the suppression by CD4+CD25high cells (30, 35). We are currently determining whether the strength of the TCR signal alters the sensitivity of the responder cell to suppression or whether it inactivates the regulatory cell.

In our experiments, blocking engagement of CTLA-4 did not alter the functional suppression by regulatory CD4+CD25high T cells. In the mouse model, differing results were obtained with blocking CTLA-4 in in vitro cultures stimulated with soluble anti-CD3 mAb (11, 14). Sakaguchi et al. demonstrated that anti-CTLA-4 at 300 µg/ml was able to completely inhibit suppression. Because both the regulatory and responder T cells could express CTLA-4 in the coculture, it was important that they demonstrated that the ability of CTLA-4 Fab to inhibit regulatory cell function did not require CTLA-4 expression on the responder T cell. In contrast, Shevach et al. found no effect on inhibition of coculture proliferation with anti-CTLA-4 mAb when added at 10 µg/ml. Yet blocking CTLA-4 in vivo via anti-CTLA-4 treatment as well as blocking signaling through CTLA-4/CD28/B7 pathways with CTLA-4Ig abrogated any protection offered by cotransfer of the CD4+CD25+ population in the in vivo models of autoimmunity of diabetes (NOD) and intestinal inflammation (8, 9).

Somewhat expected as a result of its inhibitory effect on proliferation (29), blocking PD-1 engagement by anti-PD-L1 increased [3H]thymidine incorporation by target CD4 T cells. In this situation, significantly more regulatory CD4+CD25high T cells were required to suppress the proliferative response, although with a high enough ratio, the proliferative response could still be totally abolished. However, because a second receptor for PD-1 has just been identified, PD-L2, combined blockade of both PD-1 ligands might have a more pronounced effect (16). We interpret these data to imply that regulation is a complex phenomenon where the relative activation states of the different T cell populations are critical in determining the outcome of TCR engagement.

It has been long known that a subpopulation of human T cells from normal adult subjects express class II MHC (20). A unique and perhaps important aspect of this work is the observation that the CD4+CD25high subset expressed HLA-DR. T cell expression of class II MHC allows T-T cell presentation of Ag, and this results in a profound state of anergy (36). The suppression resulting from T-T cell presentation of Ag is similarly not blocked by inhibiting the B7/CTLA-4 pathway (36). Because we found that it is the CD4+CD25high and not the CD4+CD25low population that expresses class II MHC ex vivo, it is tempting to speculate that presentation of some as yet undefined Ag or even invariant TCR to the target CD4 cell is responsible for suppression of proliferation. Experiments to test this hypothesis are in progress.

In summary, we report the identification of a CD4+CD25+ population of regulatory T cells in the circulation of humans that exhibit practically identical in vitro characteristics to the CD4+CD25+ regulatory cells isolated in mice. With TCR cross-linking, CD4+CD25high cells did not proliferate but instead totally inhibited proliferation and cytokine secretion by activated CD4+CD25− responder T cells in a contact-dependent manner. Thus, regulatory CD4 T cells expressing high levels of the IL-2 receptor and class II MHC are present in humans, providing the opportunity to determine whether alterations of these populations of T cells are involved in the induction of human autoimmune disorders.

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References


