Choice of resident costimulatory molecule can influence cell fate in human naïve CD4+ T cell differentiation

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Abstract

With antigen stimulation, naïve CD4+ T cells differentiate to several effector or memory cell populations, and cytokines contribute to differentiation outcome. Several proteins on these cells receive costimulatory signals, but a systematic comparison of their differential effects on naïve T cell differentiation has not been conducted. Two costimulatory proteins, CD28 and ICAM-1, resident on human naïve CD4+ T cells were compared for participation in differentiation. Under controlled conditions, and with no added cytokines, costimulation through either CD3+CD28 or CD3+ICAM-1 induced differentiation to T effector and T memory cells. In contrast, costimulation through CD3+ICAM-1 induced differentiation to Treg cells whereas costimulation through CD3+CD28 did not.

Keywords

Costimulation; naive T cell differentiation; regulatory T cells; ICAM-1; CD28; Foxp3

1. Introduction

Naïve CD4+ T cells are quiescent, non-activated cells recently emigrated from the thymus with the ability to travel between blood and the lymphatic system in search of cognate antigen. Recognition of antigen triggers differentiation to any of several types of effector and memory cells. It is accepted [1,2] that naïve T cells are successfully activated by a series of signals delivered by Ag appropriately presented to the TCR (signal 1) plus a costimulatory signal (signal 2). Signal 2 is received by engagement of any of several proteins resident on the T cell surface and the best studied of these is CD28. Equally accepted is the concept that specific cytokines delivered to the intercellular milieu can influence outcome of the differentiation event. As examples, the Th1 cytokine IFNγ influences differentiation to Th1 cells whereas Th2 cytokines such as IL-4 foster Th2 cell differentiation [3]. So evidence to date suggests that full activation of the naïve T cell by the two signals, augmented by specific cytokines, guides the choice of differentiation pathway.

It also has become clear that a multiplicity of costimulatory molecules participates differentially to regulate activation of T cells as they become effector cells and during cell survival and outgrowth. As was reviewed recently [4], costimulatory proteins from the CD28 family (e.g. CD28 and ICOS), and the TNFR family (e.g. CD40/CD40L, 4-1BB,

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CD27/CD70, GITR/GITR-L) participate in activation of T cells and are opposed by co-inhibitory proteins (e.g. PD-1, CTLA-4). The participation of counter receptors on opposing cells in delivering these signals implicates the local cellular microenvironment as participating in differential regulation and evokes the possibility of specialized stimulation [reviewed in 5, 6]. The best studied example of this concept is the ability of engagement of the T cell surface protein CTLA-4 (CD152) to divert cell activation induced by costimulation through CD28 by competition for the same counter receptors (CD80 and CD86) [7] on antigen presenting cells. It is of interest to learn the degree to which additional proteins constitutively expressed on a naïve T cell contribute to the activation process and to learn which additional sets of stimuli might participate in determining the ultimate fate of the differentiating naïve T cell.

The surface phenotype of naïve T cells has been partly characterized in human and mouse with overlapping results. Human naïve T cells express TCR/CD3, CD4 or CD8, and the accessory molecules CD45RA, CD28, LFA-1, CCR7, CD62L, CD27, CD2, VLA-4, [reviewed, 8, 9] as well as ICAM-1 (Intercellular Adhesion Molecule-1, CD54), a subject of the present work. Mouse naïve T cells express these same surface molecules; however, mouse naïve T cells express the CD45RB isoform and are generally characterized as CD44^{dim}. After activation, the profile of cell surface molecules expressed by the naive T cell changes. Expression of, for example, ICOS, 4-1BB, OX40, CD40L, CTLA-4, and PD-1 is induced, some existing molecules such as CD28, LFA-1, and ICAM-1 are upregulated, some like CD27 can be downregulated, and CD45 isoform expression changes [reviewed, 8, 10]. Interactions between CTLA-4 or PD-1 and their ligands are generally thought to attenuate T cell activation.

Our laboratory is investigating the hypothesis that costimulatory proteins expressed on resting naïve T cells, can differentially influence cell fate as these cells differentiate after encountering cognate antigen. We previously reported that stimulation of T cells through resident ICAM-1 can serve as a legitimate costimulatory event [11] and that costimulation of human naïve CD4+ T cells through ICAM-1 induces formation of effector and memory T cells with the same efficacy as costimulation through CD28 in an *in vitro* system [12]. Because use of antigen presenting cells to provide signals 1 and 2 engages several additional surface proteins, we isolate stimulation to only the specific T cell surface proteins of interest by stimulating with immobilized Ab. The present manuscript uses this *in vitro* system of human naïve CD4+ T cell differentiation to suggest that the choice of costimulatory molecule can influence cell fate and indicates a new role for ICAM-1 as a co-inducer of human regulatory T cell differentiation.

2. Materials and Methods

2.1 Cell Purification

Human naïve CD4+ T cells were isolated from peripheral blood of healthy donors using Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) density gradient centrifugation followed by negative selection using Human Naïve CD4+ T Cell Enrichment Kits (StemCell Technologies, Vancouver, BC) as we have described previously [12]. Naïve cells for this study were defined as CD4+CD45RA+CD45RO(−)CD11a^{lo}CD27+CCR7+CD62L+ and routinely were >98% CD45RA+ as determined by flow cytometry (example in Fig. 1*A*). Cells were cultured in complete RPMI 1640 medium (Mediatech, Herndon, VA) containing 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine, 50 units/mL penicillin, and 50 µg/mL streptomycin (Invitrogen, Carlsbad, CA).

2.2 Antibodies and Reagents

Hybridoma producing anti-ICAM-1 (R6.5D6) was obtained from ATCC (Manassas, VA) and in early experiments antibodies were purified from serum-free hybridoma cultures; later Ab were obtained from BioXCell (West Lebanon, NH). Anti-CD3ε (OKT3) was either purified from serum-free hybridoma culture (ATCC) or was purchased from eBioscience (San Diego, CA). The anti-CD3 antibodies from both sources were used with similar results. Anti-CD28 (ANC28.1) from Ancell (Bayport, MN) or anti-CD28 (CD28.2) from BD Biosciences (San Diego, CA) were used with similar results. Anti-human Foxp3-FITC and anti-human Foxp3-PE (clone PCH101) were from eBioscience and used with the accompanying Fixation/Permeabilization reagents. In some cases, anti-Foxp3-PE (clone 3G3) was from MiltenyiBiotec (Auburn, CA). Anti-CD25-FITC, anti-CD25-TriColor, anti-CD11a-FITC, anti-CD27-PE, and anti-CD45RA-TriColor were from Caltag Laboratories (Burlingame, CA). Anti-CD152-PE (CTLA-4) and anti-CD127-PE were from BD Biosciences. Isotype control antibodies for anti-Foxp3 were Rat IgG2a-FITC (eBioscience) and Rat IgG2a-PE (Caltag) or mouse IgG1-PE (Caltag), and isotype control antibody for anti-CD45RA-Tri was Mouse IgG2b-TriColor (Caltag). Ab used for cytokine blocking were anti-IL-10 (eBioscience), anti-IL-2 and anti-TGF-β1 (R&D Systems Minneapolis, MN). Controls in these experiments were rat IgG1 and mouse IgG1 (eBioscience). CFSE (5-(and-6)-carboxyfluoresceindiacetate, succinimidyl ester) was from Molecular Probes (Carlsbad, CA) and used at 2.5 μM. Flow cytometry was performed using a FACScan (BD, San Jose, CA) or an Accuri C6 (AccuriCytometers, Ann Arbor, MI). Data analysis was performed using CellQuest software (BD), CFlow (Accuri) and FlowJo software (Tree Star, Inc., Ashland, OR).

2.3 Naïve CD4+ T Cell Stimulation

Stimulation of human naïve CD4+ T cells was performed using plate-bound antibodies. As we have described previously [12], all stimulating antibodies were first titrated to determine the lowest concentration that provides maximum stimulation. Antibodies in PBS were adhered to tissue-culture treated 96-well plates (Midwest Scientific, St. Louis, MO) by incubation at 37° for 2 hours. Wells were washed 3 times with PBS to remove unbound antibody. Antibodies were used at the minimum concentrations that resulted in maximum T cell proliferation (unpublished observations): anti-CD3 (1 μg/mL), anti-ICAM-1 (10 μg/mL), and anti-CD28 (2–5 μg/mL). Cells were stimulated at 1.5×10^6 cells/mL in 200 μL of complete RPMI 1640 with no exogenous cytokines added.

2.4 Cytokine ELISA

Cell culture supernates were collected from stimulated cultures and used after clarification by centrifugation. IL-10 production was measured using Human IL-10 ELISA Ready-Set-Go kits (eBioscience, San Diego, CA) or Human IL-10 Quantikine kits (R&D Systems, Minneapolis, MN). Levels of secreted TGF-β1 were determined using Human TGF-β-1 Quantikine kits (R&D Systems). Plates were analyzed using an Automated Microplate Reader (BioTek, Winooski, VT) and DeltaSoft software (BioMetallics Inc, Princeton, NJ).

2.5 Suppression Assay

Naïve CD4+ T cells were stimulated for 10 days using anti-CD3 plus anti-ICAM-1. On Day 10, the stimulated cells were spun over Ficoll-Paque (GE Healthcare, Piscataway, NJ) to remove dead cells. The CD4+CD25+ Treg cells were separated from the CD4+CD25(−) cells using CD4+CD25+ Regulatory T Cell Isolation Kits (MiltenyiBiotec, Auburn, CA). Also on Day 10, fresh peripheral blood was again obtained from the same donor and second bleed total T cells were isolated using Ficoll-Paque density centrifugation and a Human T Cell Enrichment Kit (StemCell Technologies, Vancouver, BC). The cultured CD4+CD25+

Treg cells, the cultured CD4+CD25(−) cells, and an aliquot of second bleed total T cells to be used as a control were each stained with PKH26 dye (Sigma, St. Louis, MO) at 2.5 μM concentration. An aliquot of second bleed total T cells to be used as responders was labeled with CFSE (Molecular Probes, Carlsbad, CA) at 2.5 μM concentration. Subsequently, the cells were cultured at Treg (or Control) Cell: Responder Cell ratios of 1:1, 1:2, and 1:4. Co-cultured cells were stimulated for 5 days using anti-CD3 plus anti-CD28 antibodies as described above (3×10^5 cells/well). Proliferation of the CFSE-labeled responder cell population was assessed using flow cytometry by gating out the PKH26-labeled Treg or control populations and analyzing proliferation of the CFSE-labeled responder population.

2.6 Human Subjects

Peripheral blood cells were obtained after informed consent of healthy volunteers. Procedures were approved by the University of Kansas Institutional Review Board.

3. Results

3.1 Costimulation through ICAM-1 but not CD28 differentiated naïve cells to a Treg phenotype

Naïve human T cells have been differentiated to Treg cells in culture using specific surface stimuli plus added cytokines [13–15], or using intercellular contact plus added cytokines [16–19]. It was important to our hypothesis regarding function of costimulatory molecules to avoid added cytokines and rely only on the cytokines induced by the stimulus. It was equally important to eliminate as far as possible, the possibility of apparently silent stimulation by other unknown proteins on an opposing cell surface during intercellular antigen presentation. Thus, we used plate-immobilized antibodies against proteins resident on the naïve T cell surface to explore induction of differentiation by specific stimuli in a more nearly defined manner.

Costimulation of human naïve CD4+ T cells through CD3+ICAM-1 induced differentiation to Treg cells whereas costimulation through CD3+CD28 did not. Human naïve T cells were isolated to greater than 98% purity and were CD45RA+ (Fig. 1*A*) CD45RO(−) (Fig. 1*B*) CD11a^{lo} CD27+ (Fig. 1*C*) CD62L+CCR7+ (Fig. 1*D*). Our naïve T cell populations (Fig. 1*E*) routinely harbored less than 3% of CD25+Foxp3^{lo} cells (MFI = 29, mean of 15 samples), and no Foxp3^{hi} cells (defined in other samples, below, as MFI = 236, mean of 11 samples). Stimulations were as follow: nonstimulated, anti-CD3 alone, anti-CD3+anti-ICAM-1, and anti-CD3+anti-CD28. No exogenous cytokines were added.

Some inducible Treg cells are characterized phenotypically as CD4+CD25+ CTLA-4+ CD62L+ Foxp3^{hi} and CD127^{lo} [20–23]. Beginning after 5 days of stimulation using anti-CD3+anti-ICAM-1, we observed a subset of cells with a Treg phenotype. Expressing high levels of Foxp3 (Fig. 2*A*), they were CD25+ CTLA-4+ CD127^{lo} and retained CD62L (Fig. 2*B*) and CCR7 (unpublished observations). In general, cells costimulated through CD3+CD28 did not display this phenotype (Fig. 2*A, B*). In agreement with published work [14], we did not observe Foxp3^{hi} Treg type cells in the anti-CD3 stimulated, or anti-CD3+anti-CD28 costimulated cells (Fig. 2*A, B*). Some cells expressed intermediate levels of Foxp3. This likely indicates the slight transient increase in Foxp3 expression without prolonged Treg cell formation proposed by others [24–27].

3.2 Proliferation of differentiating human naïve T cells and kinetics of expression of Foxp3

Proliferation of differentiating cells was assessed by staining the naïve CD4+ T cells with CFSE before stimulation (Fig. 3*A*). After 7 days, the Foxp3^{hi} population routinely included both undivided cells and cells that had undergone cell division. This suggested that at least

some of the naïve cells were induced to express Foxp3 without dividing, and leaves open the question whether cells divided and then activated Foxp3, activated Foxp3 then divided, or both. Stimulation through either CD3 alone or ICAM-1 alone did not generate either naïve T cell proliferation or a Treg population, indicating that co-stimulation is essential in this system. Although cells proliferated robustly with costimulation through CD28, Foxp3hi cells were minimally present. Analyzed kinetically, the mean percentage of Foxp3hi cells was highest at day 7 of costimulation through CD3+ICAM-1 (Fig. 3*B*), and expression remained high at 10 days. In contrast, stimulation through CD3+CD28 did not achieve notable Foxp3hi expression at any time.

3.3 IL-2 was, and IL-10 and TGFβ were not necessary for differentiation to Treg in this system

One mechanism of suppression by Treg cells is production of the immunosuppressive cytokine IL-10 which inhibits IL-2 production and proliferation of T cells [28, 29]. IL-10 also can promote differentiation of Treg cells [13]. Culture supernates from naïve CD4+ T cells stimulated through CD3+ICAM-1 or through CD3+CD28 were examined for IL-10 expression. High concentrations of IL-10 were found in supernates from cells costimulated through ICAM-1 (Fig. 4A). Interestingly, in this composite of 3 experiments, the mean concentration of IL-10 peaked on day 7, corresponding to the day of the highest mean Foxp3+ percentage in ICAM-1 costimulated cultures (Fig. 3*B*). Error for the cells costimulated through CD3+ICAM-1 is greater than optimal because, typical of human samples, the temporal peak was not always on the same day with each subject. Cells that had been costimulated through CD28 produced comparatively small amounts of IL-10 and little error. In some systems using human cells, TGF-β participates in differentiation to Treg [15, 17] but in others, TGFβ is not able to induce cells that exhibit suppressive activity [30]. In the present system, peak secretion of TGF-β by both CD28- and ICAM-1-costimulated cells varied from 1 ng/ml to 6 ng/ml among 4 human subjects, and was not selectively induced by costimulation through either CD3+ICAM-1 or CD3+CD28 (Fig. 4B) over the same time course of 10 days. Thus, equal TGF-β secretion occurred in response to each form of costimulation, regardless of success in differentiation to Treg.

Participation of cytokines in ICAM-1-induced differentiation to Treg was further investigated by introducing anti-cytokine Ab into the cultures to remove the cytokine from participation. Figure 5 shows that removal of IL-2 prevented differentiation to Treg (Fig. 5*A*, upper right panel), whereas removal of IL-10 (lower left panel) or TGF-β (lower center) had no effect on differentiation. Isotype control Ab had no effect (lower right panel). Combined results of four independent experiments are shown in Fig. 5*B* and support the assertion that removal of IL-2 interfered with differentiation to Treg but removal of IL-10 or TGFβ did not. Attempts at removal of IL-10 and TGF-β from the cell supernates were verified by ELISA (unpublished observations). IL-10 levels were effectively reduced to zero whereas with TGF-β, a minimal ELISA TGFβ reactivity level of 0.7 ng/ml was not removed by mAb in several attempts using high Ab concentrations. So the question of the participation of TGF-β in ICAM-1 induction of differentiation to Treg remains formally open for this system. However, stimulation through either CD3+CD28 or CD3+ICAM-1 provided equal access to TGF-β during differentiation as seen in Fig. 4B, but only costimulation through CD3+ICAM-1 produced Treg cells. Thus, it is clear that costimulation through ICAM-1 induced additional function with respect to Treg differentiation that CD28 did not provide.

3.4 Differentiated Treg exhibited inhibitory function

To determine whether the CD4+CD25+Foxp3hi population generated after ICAM-1 costimulation could function as Treg cells, we examined whether the cells could suppress proliferation of autologous T cells. Naïve CD4+ T cells were differentiated to a Treg

phenotype using anti-CD3+anti-ICAM-1. Responder T cells were obtained 10 days later in a second bleed as described in the legend to Fig. 6. When CD4+CD25+ cells that had differentiated in ICAM-1-costimulated cultures were added to responder cells, responder cell proliferation was greatly diminished supporting the supposition that Treg cells had been generated. Figure 6*A* shows a suppression assay performed at a nominal Treg: Responder cell ratio of 1:4. In actuality, the Foxp3^{hi} cells only represented a percentage of these cells, so the actual functional ratio contained a much lower number of Treg. In this experiment, when only responder cells (1st panel) were measured or when control T cells (2nd panel) or CD4+CD25(−) cells (4th panel) were added to the responder cell culture, over 50% of the responder cells divided and multiple rounds of cell division were observed. In contrast, only 15% of the responder cells divided when CD4+CD25+ Treg cells (3rd panel) were added to the culture and only one round of cell division occurred. The CD4+CD25+ Treg cells inhibited responder cell proliferation at each Treg: Responder ratio, and a dose-dependent effect was observed (Fig. 6*B*). The CD4+CD25(−) cells also mildly inhibited responder cell proliferation in some experiments. This type of response has been observed by other investigators (e.g. 31), and could be due to the presence of CD25(−) cells with suppressive capabilities, or to contamination of CD4+CD25+ cells in the CD4+CD25(−) population [purity of the CD4+CD25(−) population was >90%, (unpublished observations)].

4. Discussion

We report that costimulation of human naïve CD4+ T cells through CD3+ICAM-1 without exogenously added cytokines induced differentiation to Treg cells, in contrast to results obtained by costimulation through CD3+CD28. The cells expressed the Treg cell markers CD25, CTLA-4, CD62L and Foxp3 and were CD127^{lo}. The culture produced the immunosuppressive cytokine IL-10, and the cells suppressed proliferation of autologous responder T cells in co-culture experiments. Previous studies have hinted at a role for ICAM-1 in Treg cell differentiation or function. Murine and human regulatory T cells have higher levels of ICAM-1 expression than non-Treg cells [32, 33] and ICAM-1$^{−/−}$ mice exhibit fewer Treg cells and a heightened immune response to *Mycobacterium tuberculosis* [34]. Although one study reported that ICAM-1 is not required for murine Treg cell activation [35], a direct and active role for ICAM-1, resident on human naive T cells, in Treg cell differentiation has not been investigated until now.

Induction of regulatory T cells after costimulation through ICAM-1 is consistent with what would be beneficial in controlling an immune response, where signaling of T cells through resident ICAM-1 may provide an important sensing mechanism. In addition to T cells, ICAM-1 is expressed on diverse cell types, but the various primary ligands of ICAM-1, [LFA-1 (CD18/CD11a), MAC-1 (CD18/CD11b) and gp150/95 (CD18/CD11c)] are expressed only on leukocytes and various types of dendritic cells. For a strong interaction to occur between ICAM-1 and LFA-1, the heterodimers of LFA-1 must be in an activated conformation [36]. Thus, signaling through ICAM-1 resident on a T cell may provide a mechanism by which the T cell perceives that it is interacting with an activated leukocyte or specialized dendritic cell. Perhaps this type of interaction might signal the presence of a mature immune response and that the time may be appropriate to induce Treg differentiation. Breakdown in localized antigen presenting cells of the ability to costimulate through ICAM-1 might conceivably lead to a reduction of Treg cells at a crucial time, a process that would favor onset of autoimmune disease.

We have as yet obtained no data regarding the differentially accessed mechanism by which ICAM-1 costimulation induces differentiation to Treg cells whereas CD28 costimulation does not. The most logical mechanism is that each costimulatory combination induces expression of its own specific set of genes and that these differ according to stimulus, with

ICAM-1 favoring differentiation to Treg. It also is likely that differential induction of chemokine or cytokine receptors provides the differentiating cells selective access to these signal inducing molecules. This would be supported by the possibility of differential induction of specific cytokines or chemokines. In recent years it has become apparent that autophagy plays a role in maintenance and activation of naïve T cells [reviewed, e.g. 37] although it is not completely clear whether autophagy might provide a selective advantage to cell survival or might participate in induction of apoptosis. In our previous studies [12] we noted that the level of apoptosis induced under conditions studied herein is the same when the same naïve T cell population is costimulated through CD28 or through ICAM-1. Full elucidation of the differential mechanism must include study of these plus potentially other possibilities.

It seems most likely that contributions of resident ICAM-1 toward cell fate decisions will be made in the context of multiple counter receptor interactions during intercellular contact. However, based on results presented here, it does seem reasonable to speculate that at least some naïve T cells may find themselves in a microenvironment where they can be activated by an Ag-TCR signal coincident with ligation of their resident ICAM-1, and that this signal is delivered by counter receptors such as activated LFA-1 found on dendritic cell subsets capable of antigen presentation in this type of specialized manner.

Logically, activation of naïve T cells occurs in at least two waves. An immediate wave of stimulation occurs through receptors that pre-exist on the resting naïve T cell surface at the time of interaction with the cognate-antigen presenting cell. These can be cytokine and chemokine receptors as well as cell surface proteins including CD28, LFA-1, ICAM-1, CD2 or VLA-4 that may be engaged by counter receptors during the intercellular contact with the APC. A secondary wave of stimulation is received by receptors that are induced to appear on the naïve T cell surface as a result of the immediate wave of stimulation. These include induced receptors for cytokines and chemokines as well as targets for counter receptor interaction such as ICOS, 4-1BB, OX40 or CD40L. Classically, activation initiates when the TCR, CD28, LFA-1 and several other proteins resident on the resting naïve T cell form a synapse and, among other things, initiate signals to the nucleus for a change in gene expression that will permit access to the secondary wave of stimulation. It is of interest to learn which of the proteins constitutively expressed on a naïve T cell contribute to the immediate activation process and to learn which of these might participate in determining the ultimate fate of the differentiating naïve T cell. Such proteins will be directly reflective of specific micro-environments.

Previously, we reported that costimulation *in vitro* of human naïve CD4+ T cells through ICAM-1 could induce formation of Teffector and Tmemory cells with the same efficacy as costimulation through CD28 [12], suggesting a surprising commonality of function. However, in that study, costimulation of naïve T cells through CD28 led to both Th1 and Th2 cells where costimulation through ICAM-1 led to Th1 cells but not Th2, and costimulation through LFA-1 led to Teffector but not T memory cells. Here, we expand these observations to include differentiation to Treg cells. Under defined conditions, the naïve T cells were induced to differentiate into Treg by costimulation through ICAM-1 but, consistent with published work by others [14], not by costimulation through CD28. The most parsimonious explanation of our data is that costimulation through ICAM-1 induced a set of genes to express that were not accessible to costimulation through CD28, leading to differential differentiation pathways. Thus, it is reasonable to suggest that under certain circumstances, specific costimulatory signals might participate in cell fate determination.

Acknowledgments

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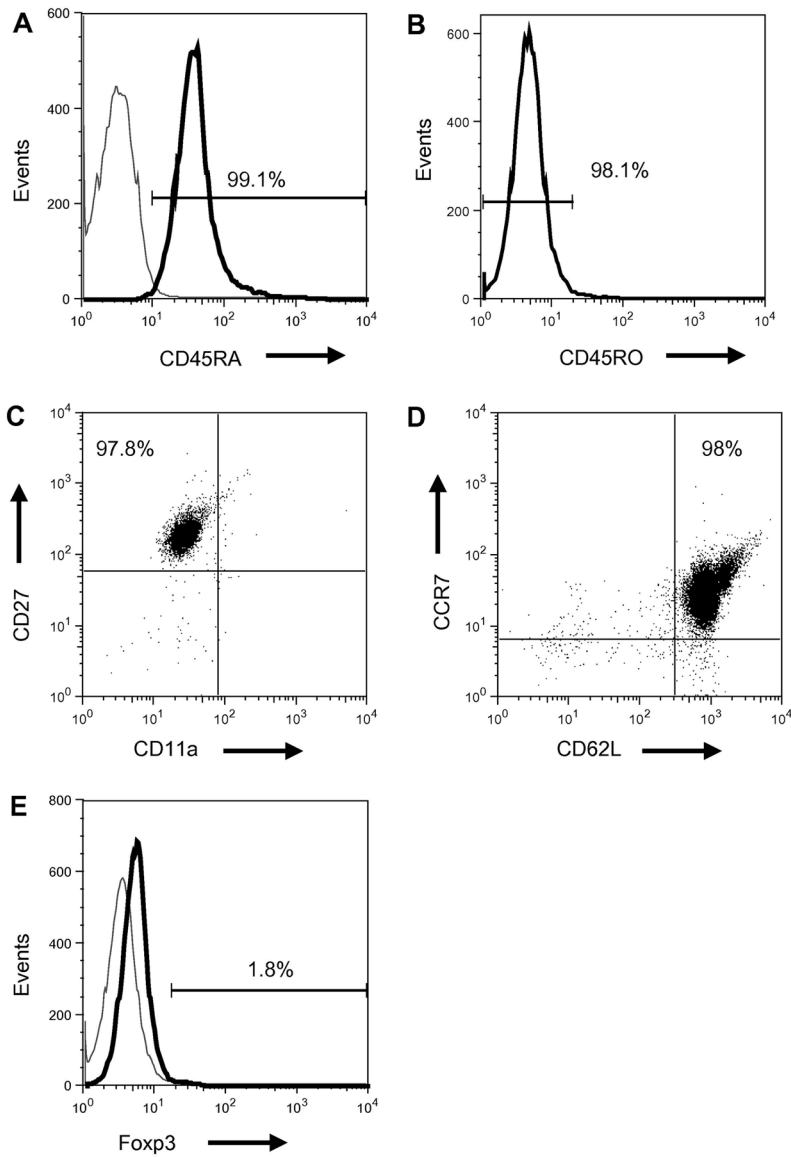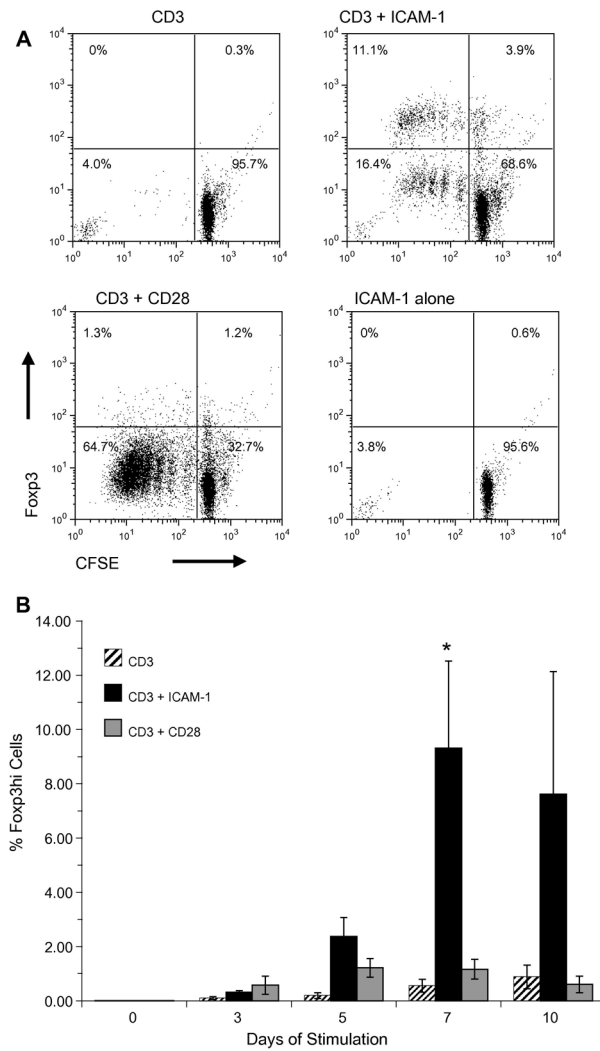
Figure 1.
The initially purified cell population displayed a naïve phenotype. Panel *A*, Purified human naïve CD4+ T cells were CD45RA+ (cells stained with anti-CD45RA are shown in bold line and cells stained with isotype control are shown in thin gray), Panel *B* CD45RO(−), Panel *C* CD11a^{lo}CD27+, and Panel *D* CD62L(+) CCR7(+). Panel *E*, A small percentage of the naïve cells were weakly Foxp3+ (cells stained with anti-Foxp3 are shown in bold and cells stained with isotype control are shown in thin gray). Panels A, C and E are representative of greater than 10 experiments; panel B represents 6 experiments; and panel D represents 4 experiments.
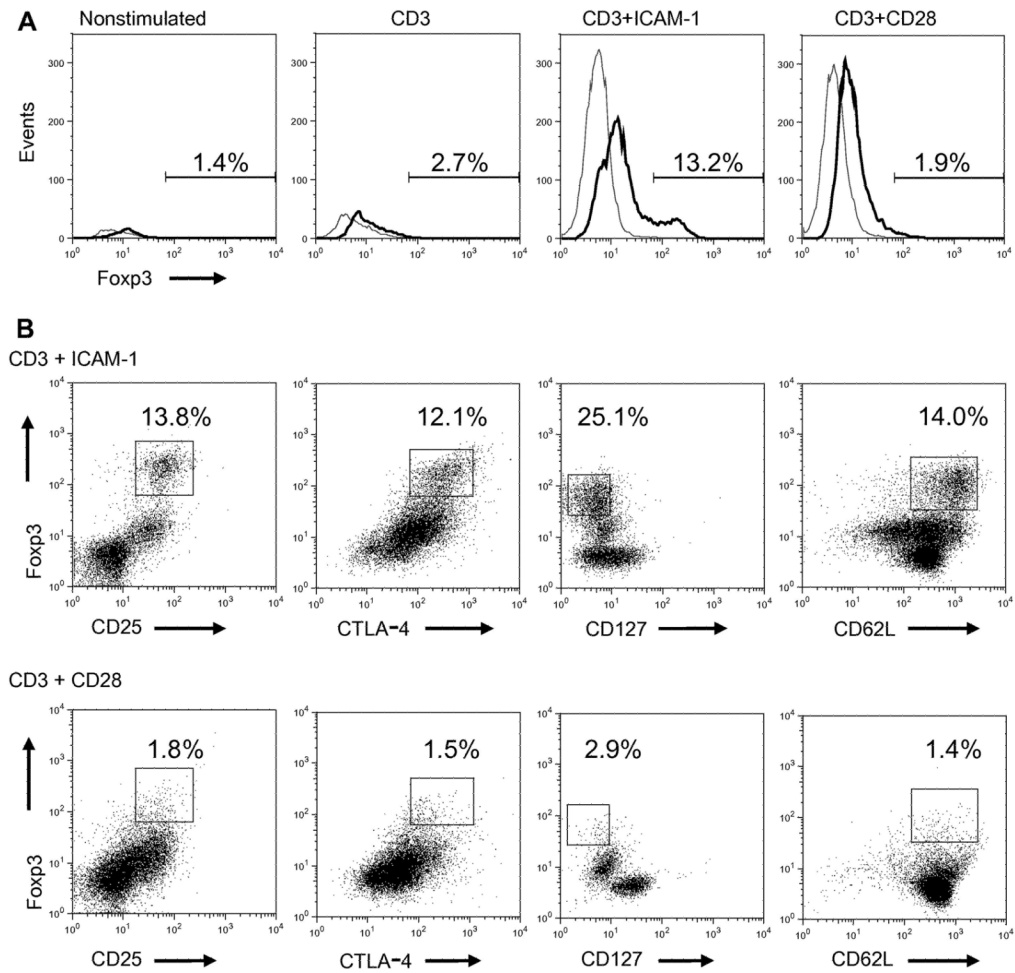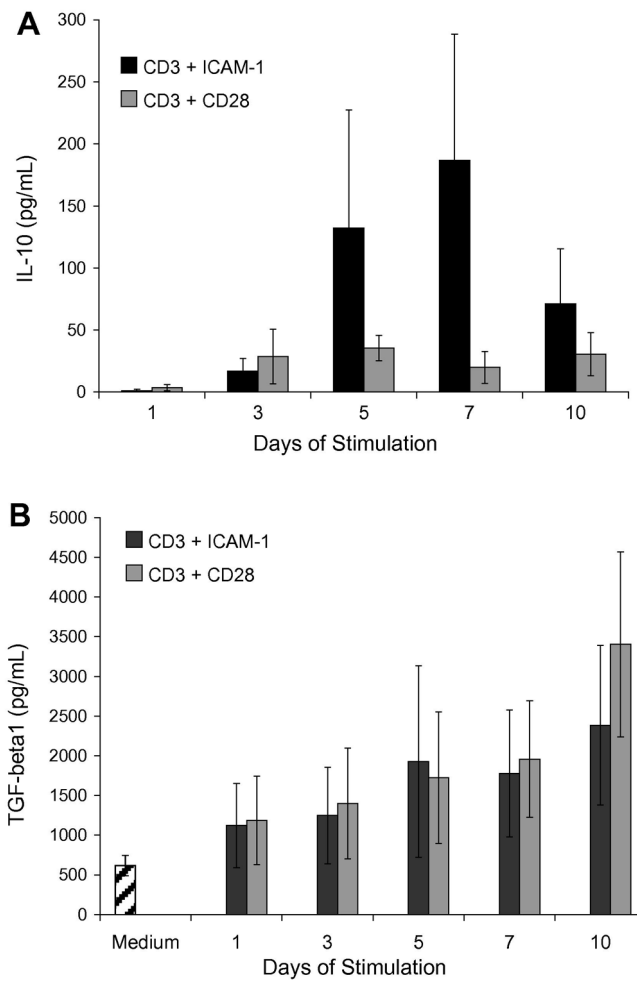
Figure 2.
T cells with a CD25+Foxp3+ regulatory phenotype were induced following costimulation of naïve CD4+ T cells through ICAM-1 but not CD28. *A*, naïve CD4+ T cells were stimulated as indicated for 14 days and then analyzed using flow cytometry. The cells stained with anti-Foxp3 are shown in bold whereas cells stained with isotype control are in thin gray. Representative of greater than 10 experiments. *B*, naïve CD4+ T cells were stimulated with anti-CD3 plus either anti-ICAM-1 or anti-CD28 and analyzed by flow cytometry for expression of CD25 at 7 days, CTLA-4 at 14 days, and CD127 or CD62L at 10 days. Representative of greater than 10 experiments (CD25 and CD127) or 3 experiments (CTLA-4 and CD62L).
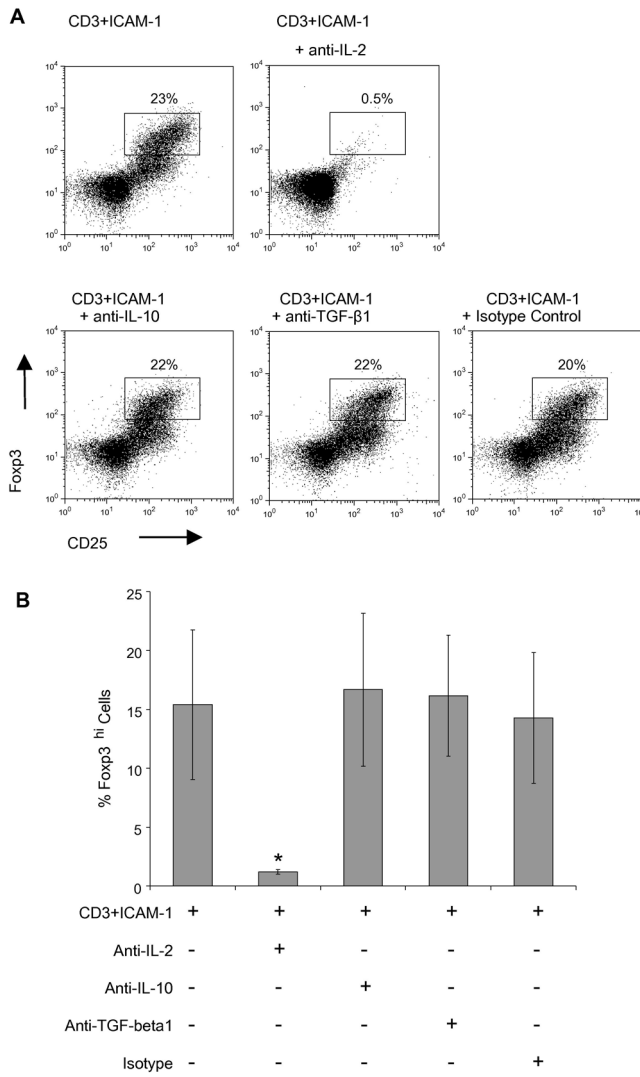
Figure 3.
Cells undergoing differentiation to Treg cells were proliferative and high levels of Foxp3 expression were maintained for at least 10 days. *A*, naïve CD4+ T cells were stained with CFSE and stimulated as indicated. Cell division and Foxp3 expression were analyzed after 7 days. Representative of 4 experiments. *B*, Kinetics of Foxp3^{hi} induction were measured for cells stimulated through CD3 (hatched bars), CD3+ICAM-1 (closed bars), or CD3+CD28 (gray bars). The mean percentage of Foxp3+ cells in 5 separate experiments is shown for each time point +/− SEM. The asterisk indicates a statistically significant difference between the percentage of Foxp3^{hi} cells after costimulation through ICAM-1 and the percentage of Foxp3^{hi} cells after costimulation through CD28 (paired t-Test, one tail $p<0.05$). There were no other significant differences.
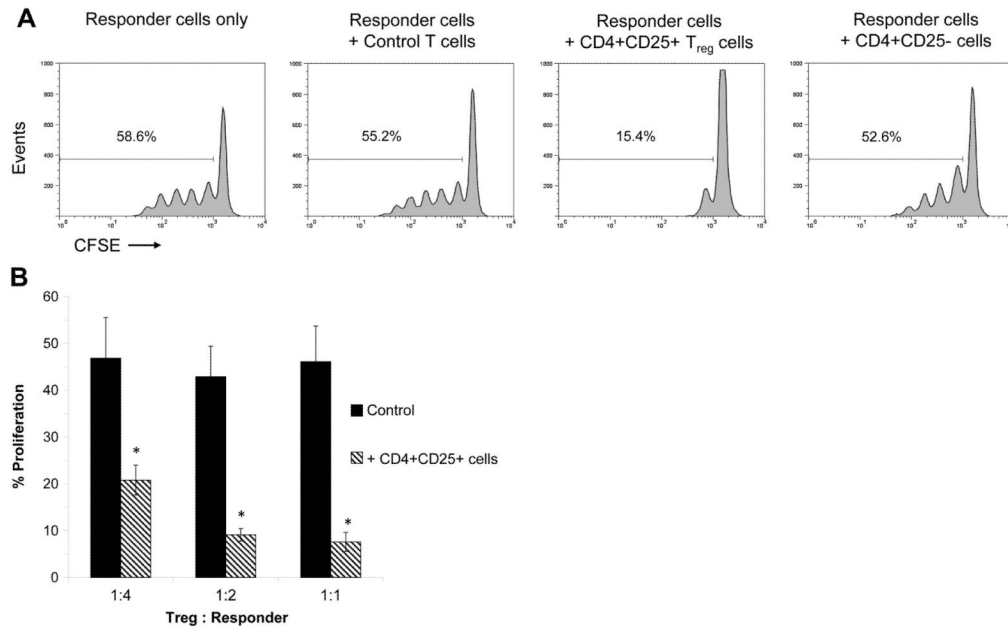
Figure 4.
IL-10 production differed between cells costimulated through ICAM-1 and cells costimulated through CD28, while TGF-β1 production was similar. *A*, Human naïve CD4+ T cells were stimulated with anti-CD3+anti-ICAM-1 or anti-CD3+anti-CD28. Cell culture supernates from indicated times were assayed in duplicate using IL-10 ELISA. Data are the means of duplicate samples from three experiments ± S.E.M. *B*, Cell culture supernates from indicated times were assayed in duplicate using TGF-β1 ELISA. Data are the means of duplicate samples from four experiments ± S.E.M. The hatched bar indicates the mean concentration of TGF-β1 detected in the medium alone.

Figure 5.
Treg differentiation required IL-2, but not IL-10 or TGFβ. *A*, Cells were stimulated with anti-CD3+anti-ICAM-1 for 10 days with the blocking Ab indicated added at 20 μg/ml on day 0 and again on day 5. Representative of 4 experiments. *B*, Summary of the creation of Foxp3^{hi} cells in the presence of cytokine-directed Ab. Data are mean of 4 experiments ± SEM. Asterisk indicates significant difference between sample with cytokine Ab and sample without (paired t-Test, one tail $P \leq 0.05$).

Figure 6.
CD4+CD25+ cells induced after costimulation through ICAM-1 suppressed responder cell proliferation. Human naïve CD4+ T cells were stimulated with anti-CD3+anti-ICAM-1 to induce differentiation to cells with a Treg phenotype. After 10 days, cells were separated into CD4+CD25+ and CD4+CD25(−), and labeled with PKH26 to allow them to be gated out of the flow cytometry profile facilitating analysis of proliferation by only responder cells. Also on day 10, total T cells to be used as responders were collected by a second bleed of the original donor. One aliquot of responder cells was labeled with PKH26 to use as control. The remaining cells were labeled with CFSE and used as responders. Thus, the populations of cells used in the suppression assay were: 1) CFSE-labeled Responder T cells (newly isolated), 2) PKH26-labeled Control T cells (newly isolated), 3) PKH26-labeled CD4+CD25+ Treg cells (from stimulated cultures), and 4) PKH26-labeled CD4+CD25(−) cells (from stimulated cultures). The cells were cultured at Treg (or Control): Responder cell ratios of 1:4, 1:2 and 1:1 and stimulated with anti-CD3+anti-CD28 for 5 days to induce proliferation. Proliferation of the responder cell population was measured by flow cytometry after gating-out the PKH26-labeled Treg or control cells. *Panels A*, CD4+CD25+ Treg cells [or control cells or CD25(−) cells] at a Treg: responder ratio of 1:4. The percentage of cells undergoing cell division is shown for each treatment. Representative of 3 experiments. *Panel B*, Proliferation data from all three Treg: responder ratios are presented for Treg cells (CD4+CD25+, hatched bars) as well as the control T cells (closed bars). The data are shown as the mean % proliferation of responder cells from 3 separate experiments. Asterisks indicate statistically significant differences between control T cell samples and samples with added CD25+ cells (normalized to samples containing CFSE-labeled responder cells only, paired t-Test, one tail $p<0.05$).