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Differentiation of Regulatory T Cells 1 Is Induced by CD2 Costimulation

Abdelilah Wakkach, Françoise Cottrez, and Hervé Groux

Induction and maintenance of peripheral tolerance is an important phenomenon for the control of homeostasis in the immune system. There is now compelling evidence for CD4+ T cells that prevent immune pathology, both in autoimmunity and in transplantation. However, the mechanisms involved in the specific differentiation of these T cells are unknown. We had previously shown that repetitive stimulations of naive T cells in the presence of IL-10 induce the differentiation of T regulatory cells. We further dissected the mechanism of IL-10 function and demonstrated that IL-10 acts by the down-regulation of most costimulatory molecules without modifying the expression of CD58. Using artificial APCs expressing various costimulatory molecules, we demonstrated that, in contrast to other costimulation patterns, costimulation via CD2 alone, in the absence of costimulations through CD28- or LFA-1, induced T cell anergy in an IL-10-independent pathway along with the differentiation of Ag-specific regulatory T cells. T regulatory cell-1 differentiation via CD2 was very efficient as both high IL-10 secretion and regulatory function were observed after the first stimulation of naive T cells with CD32-CD58 L cells. The possibility to rapidly induce the differentiation of Ag-specific regulatory T cells will certainly accelerate their characterization and their potential use as regulators of T cell-mediated diseases. The Journal of Immunology, 2001, 167: 3107–3113.

Successful human organ transplantation is complicated by the development of allograft rejection and despite the use of immunosuppressive medications true graft tolerance is seldom realized. One way to achieve this problem would be to specifically inhibit the expansion of alloreactive T cells or to induce the differentiation of regulatory T cells. T cell activation is a carefully orchestrated process that involves the TCR and a multitude of accessory molecules expressed on T cells and APCs. Operationally, two types of molecules that modulated TCR-mediated T cell activation can be distinguished: 1) accessory molecules such as LFA-1, which facilitate TCR triggering by promoting T cell-APC adhesion, and 2) costimulatory molecules, such as CD28, which enhance T cell activation without affecting the rate of TCR triggering (1, 2).

We have recently shown that both human and mouse CD4+ T cells, repeatedly stimulated in the presence of IL-10 differentiate into a new subset of CD4+ T cells different from the Th1 and Th2 cells (3). These cells, termed T regulatory cell 1 (Tr1), have a poor proliferative response, secrete high levels of IL-10, and regulate Th1 and Th2 immune responses in vivo (3, 4). IL-10 is a cytokine, produced by a variety of cells, with important anti-inflammatory and immunosuppressive properties. These properties are associated with a reduction of Ag-presenting capacity of APC. Indeed, IL-10 down-regulates the expression of costimulatory molecules CD80 (5) and CD86 (6) and the adhesion molecule CD54. However, IL-10 did not modify CD58/LFA-3 expression on APCs (7).

CD58/LFA-3, which is a ligand of CD2 (8), is a member of the Ig superfamily expressed widely by hemopoietic and nonhemopoietic cells. CD2, which is also a member of the Ig superfamily, is found on thymocytes, peripheral T lymphocytes, and NK cells. CD2-LFA-3 interaction is considered to contribute to T cell activation by strengthening the adhesion between T cells and APCs or target cells (9), thus helping the TCR to reinforce contact with its ligands. Besides these adhesion enhancer mechanisms, LFA3-CD2 interaction has also been reported to be an important regulator of T cell responses. Indeed, in murine models, sequential treatment with CD2 was shown to induce tolerance in vivo (10).

In this study, we dissected the functional mechanism of IL-10 on the differentiation of Tr1 cells and addressed the importance of the costimulatory role of CD2 in the induction of tolerance through the differentiation of regulatory T cells. Our data demonstrate that costimulation of TCR with the CD2 molecule specifically induced the differentiation of Ag-specific Tr1 cells. Moreover, Tr1 cell differentiation is not due to a lack of costimulation or poor stimulation as no regulatory T cells are induced after T cell stimulation in the absence of CD2 mobilization. However, the specific differentiation of Tr1 cells after CD2 and CD3 stimulation can be reverted by the mobilization of the CD28 molecule by its respective ligands.

Materials and Methods

Cells, Abs, and other reagents

Mouse anti-human CD3 (clone X53.7), CD4 (clone O.516), CD8 (clone L533), and CD11b (cloneOM1), CD20 (clone 1F5) kindly provided by E. A. Clark (University of Washington, Seattle, WA); CD28 (clone 28.2), a gift from Dr. D. Olive (Institut National de la Santé et de la Recherche Médicale, Unité 119, Marseille, France); and CD58 (clone TS 2.9), a kind gift from Dr. B. Haynes (Duke University, Durham, NC) have been obtained in our laboratory and described previously (11, 12). Mouse anti-human IL-10 (clone JES5-9D7), antihuman IL-2R (BB-10), and human recombinant IL-2 and IL-10 were a kind gift from Dr. R. L. Coffman (DNAX Research Institute, Palo Alto, CA). The L cell line is a mouse fibroblast cell line. The L cells transfected with mouse CD32 or human CD80 were a gift from Dr. L. Lanier (DNAX Research Institute). The P815
is a mouse mastoyctoma cell line that expresses Fcγ receptors. DR1-EBV lymphoblastoid B cells were a generous gift from Dr. H. Yssel (Institut National de la Sante et de la Recherche Medicale, Unité 454, Montpellier, France).

Culture medium

DR1 EBV lymphoblastoid B cells and T cells were cultured in RPMI 1640 (Life Technologies, Cergy Pontoise, France) supplemented with 2 mM L-glutamine, 100 U/ml penicillin-streptomycin (both from Life Technologies), and 10% FCS (Boehringer Mannheim, Roche, France). L cell and P815 cell transfectants were cultured in F-12 medium (Life Technologies) supplemented with 10% FCS (Roche, Mylan, France), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin-streptomycin (Life Technologies), and 50 µg/ml 2-ME (Life Technologies).

Cell purification

PBMCs were prepared by centrifugation over Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). CD4+ T cells were purified by negative depletion. Negative purification was performed using a mixture of Abs directed against non-CD4+ T cells: mouse anti-human CD8, CD81, and CD20. Cells were incubated with saturating amounts of Abs for 20 min at 4°C in the presence of 10% Ater washing-dynabeads (Dynal, Compie`ne, France) and were added at 10:1 bead:target cell ratio and incubated for 1 h at 4°C. Beads and contaminating cells were removed by magnetic field. CD4+ T cells were analyzed by FACStar (BD Biosciences, Le Pont de Claix, France) and revealed to be 95% positive.

Generation and culture of mouse L cell and P815 transfectants

A full-length cDNA for human CD58 (LFA-3) was generated from PBMC RNA using primers flanked by Kmpl and NotI sites. This CDNA was cloned into pcDNA 3.1 hygro (Invitrogen, San Diego, CA), digested with Sccl (Roche), and transfected into CD32- and CD32-CD80-L cells using standard procedures of Lipofectamine (Life Technologies) to create CD32-CD58-, and CD32-CD58-CD80-L cells. Stable transfectants were purified by staining transfected cell populations with mAb against CD58 and then performing multiple sterile sorts using a FACS-Vantage SE (BD Biosciences). Similarly, HLA-DR1 α- and β-chain cDNA were generated from PBMC RNA isolated from a DR1+ individual, cloned into pcDNA3.1 (Invitrogen), and cotransfected into L cells as described above. DR1-L cells were purified by sorts using a FITC-conjugated anti-HLA-DR1 mAb (Dako, Trappes, France). DR1-CD58-L cells were obtained by transfection of the DR1-L cells with the cDNA of human CD58, and DR1-CD58-CD80-L cells were obtained by subcloning the cDNA of human CD80 into pcDNA3.1/scw and transfection of DR1-CD58-L cells. Stable transfectants were used to obtain CD54- and CD86-L cells as well as CD80- and CD58-P815 cells.

Generation of Tr1 cells

CD4+ T cells were plated in 96-well plates (BD Biosciences) at a density of 2 × 10^5 cells/ml in a total volume of 200 µl of RPMI 1640 supplemented and stimulated with irradiated CD32-CD58-, CD32-CD80-, CD32-CD54-, CD32-CD86-L cells, or CD80-, CD58-P815 (600 rad) at 5 × 10^5 cells/ml in the presence of soluble human anti-CD3 (10 µg/ml). For Ag-specific differentiation of Tr1 cells, CD4+ T cells were stimulated by irradiated DR1-CD58-L cells, DR1-CD58-CD80-L cells, or DR1-EBV lymphoblastoid cells (600 rad). Cytochrome synthesis was determined by the analysis of supermatants 48 h after stimulation, and proliferation was measured after a pulse with 0.5 µCi of [3H]thymidine during the final 18 h of a 3-day culture.

Test for regulatory function of T cells

CD4+ T cells were stimulated with irradiated PBMCs (600 rad) and soluble human anti-CD3 mAb (10 µg/ml) in the bottom compartment of a transwell system. The T cells were cocultured with CD4+ T cell populations (previously differentiated with different costimulatory molecules) contained in the upper transwell compartment. After 2 days, the basket was removed and the proliferative response of the bystander CD4+ T cells was measured after a pulse with 0.5 µCi of [3H]thymidine during the final 18 h of a 3-day culture.

Cloning of Tr1 cells

Human Tr1 cells were generated from CD4+ T cells stimulated with irradiated CD32-CD58-L cell transfectants or CD32-CD80-L cell transfectants used as controls. After 7 days, cells were labeled with an anti-CD4 FITC and anti-CD25 PE mAbs (BD Biosciences). The CD4+CD25+ T cells were cloned at one cell per well by flow cytometry (FACSVantage SE; BD Biosciences) in 96-wells precoated with anti-CD3 mAb (1 µg/ml) in 0.1 M Tris (pH 9.4; Sigma, St. Louis, MO). After sorting, a mixture of irradiated feeder cells (10^5/ml, and PBMCs, 10^5/ml) and 10 U/ml IL-2 in 100 µl was added. The clones were expanded with IL-2 (10 U/ml) and then analyzed for cytokine secretion after activation with immobilized anti-CD3 (1 µg/ml) and anti-CD28 mAb (1 µg/ml) for 48 h.

Determination of lymphokine production

Sandwich ELISAs were used to measure IL-2, IL-4, IL-10, and IFN-γ as previously described (13). In brief, ELISA plates (Polylab, Strasbourg, France) were coated with the appropriate anti-cytokine Abs (Mah IL-2, 8D4, 8, JES3-9D7, and A35, respectively) and incubated at 4°C overnight. After incubation, plates were blocked for 30 min at room temperature by adding 150 µl of 20% FCS/PBS (Life Technologies) containing 0.04% Tween 20 (Sigma) to each well. Supernatants from in vitro-stimulated CD4+ T cells or from activated T cell clones were added at a volume of 50 µl/well. Plates were incubated overnight at 4°C, then washed, and the second-step Ab:nitrophenyl acetic acid-conjugated mAb (Gall IL-2, MP4-25D2, JES312G8, 1, and B27 for IL-2, IL-4, IL-10, and IFN-γ, respectively) was added at 50 µl/well. Plates were incubated for 1 h at room temperature, then washed, and the anti-nitrophenyl acetic acid-HRP conjugate was added to each well. Plates remained at room temperature for 1 h, after which they were washed and 100 µl/well substrate containing 1 mg/ml 2.2'-azino-bis (Sigma, St. Quentin, Fallavier, France), 0.0005% H2O2, NaHPO4, and 0.05 µM citric acid was added. After the substrate was developed, applying 50 µl of 0.2 M citric acid solution to each well stopped the reaction. The plates were read on an ELISA reader (Lab-systems iEMS reader; Labsystems, Helsinki, Finland).

Results

Co-stimulation with CD58 (LFA-3) alone induced the differentiation of Tr1-type cells

We have previously shown that repetitive stimulations of CD4+ T cells in the presence of IL-10 induces the differentiation of a novel subset of regulatory T cells (Tr1) with immunoregulatory properties (3). Preliminary studies have shown that differentiation of Tr1 cells in the presence of IL-10 could not be obtained in the absence of APCs (data not shown). To analyze whether IL-10 effects on the differentiation of Tr1 cells were due to a modification of the Ag-presenting capacity of APCs obtained through down-modulation of costimulatory molecules, we used CD32-L cells expressing different costimulatory molecules and analyzed their capacity to induce proliferation and cytokine production of purified CD4+ T cells in the presence of CD3 mAb. Proliferative response of CD4+ T cells was minimally induced by the cross-link of CD3 mAb on the surface of L cells expressing the CD32 molecule (Fig. 1A). In contrast, the expression of CD80 or CD86 (data not shown) on the cell surface of L cells induced maximal proliferative response as previously described (14). To a lesser extent, costimulation was also obtained by cell surface expression of CD54 (ICAM-1) and CD58 (LFA-3) as previously shown (15). Finally, the expression of CD58 and CD80 act in cooperation to induce proliferation (Fig. 1A) whereas minimal cooperation is observed between CD54 and CD80.

Analysis of cytokines released in the supernatants of stimulated CD4+ T cells (Table I) shows that stimulation of CD28 by CD80 or CD86 induces high levels of IFN-γ and IL-2 and some IL-10 and IL-4. Activation in the presence of CD58 induces solely the secretion of IL-2 as previously described (16). In contrast, activation in the presence of CD58 induces low levels of IL-2, no IL-4, some IFN-γ, and high levels of IL-10, a cytokine secretion profile.
similar to the one expressed by Tr1 T cell clones (3). Interestingly, this peculiar cytokine profile was lost upon coexpression of CD80 or CD54 molecules which triggered stimulation via their respective ligands to enhance IL-2 secretion (Table I).

To analyze whether the effect observed with LFA-3 stimulation could be linked to the cell line used to express the human molecule, similar experiments were performed with P815 cells transfected with CD80, CD54, and CD58. As shown in Fig. 1B and Table I, CD3 stimulation in the presence of CD80 induces maximum proliferation and secretion of IL-2, IFN-γ, and some IL-4 and IL-10. In contrast, activation with CD58 alone induced minimal proliferation and secretion of minimal amounts of IL-2 and IL-4, some IFN-γ, and high levels of IL-10, showing that this cytokine profile is related to activation through the CD2 molecule.

To confirm that CD2 costimulation specifically induced the differentiation of regulatory T cell populations, coculture experiments were performed to analyze the potential regulatory function of the different T cell populations obtained after activation with various costimulation conditions (Fig. 1, C and D). The proliferation of resting CD4+ T cells in response to CD3 mAb and irradiated PBMCs was dramatically reduced following coculture in the transwell system with CD3-activated Tr1 cell populations generated in the presence of CD58 alone. In contrast, CD3 stimulation of other T cell populations generated in the presence of other combinations of costimulatory molecules had either no significant effect or slightly enhanced bystander T cell proliferation presumably through the secretion of IL-2 and/or IL-4.

**CD2 costimulation-induced anergy of CD4+ T cells independently of the secretion of IL-10**

We had previously shown that IL-10 induces anergy in CD4+ T cells (13). To analyze whether stimulation of CD4+ T cells with CD58 alone that results in high levels of IL-10 secretion would induce anergy, CD4+ T cells were stimulated with soluble CD3 mAb (10 μg/ml) in the presence of L cells expressing CD80 or CD58 in the presence or absence of blocking anti-IL-10 mAb, blocking anti-IL-2R, or exogenous IL-2 (Fig. 2). Addition of anti-IL-10 Abs partially increases the proliferative response of CD4+ T cells stimulated with CD3 mAbs and L cells expressing CD58 alone. Blocking IL-10 did not further induce the proliferative response of CD4+ T cells stimulated with CD3 mAbs and L cells expressing CD80 alone. Blocking IL-10 did not further induce the proliferative response of CD4+ T cells stimulated with CD3 mAbs and L cells expressing CD58 alone (data not shown). To analyze whether anergy was induced in these cell culture conditions, the cells were kept in culture for 10 days. No significant cell death was observed in either culture condition and comparable cell numbers were harvested at termination of the assays (data not shown). After this culture period, similarly to culture performed in the presence of exogenous IL-10 (13), CD4+ T cells previously stimulated with CD58 alone failed to proliferate in response to cross-linked CD3 mAb stimulation. This unresponsive
Isolation and expansion of Ag-specific human Tr1 T cell clones

CD2 costimulation induces the differentiation of Tr1 T cell clones

state could not be reversed by saturating concentrations of exogenous IL-2 (200 U/ml) or by anti-CD28 mAbs (10 μg/ml; Fig. 2B).

Interestingly this anergic state was also induced in CD4+ T cells stimulated with CD58 alone in the presence of anti-IL-10 Abs. These results suggest that anergy induced in CD4+ T cells by IL-10 was due to an indirect effect of IL-10 that modulates APC costimulatory molecules and not to a direct effect of IL-10 on T cells. In contrast to regulatory T cell populations obtained after CD2 costimulation, CD4+ T cell populations obtained after CD28 costimulation could be fully reactivated by cross-linked CD3 mAb alone or in the presence of soluble CD28 mAb or IL-2 (Fig. 2B).

To analyze the importance of IL-2 in anergy induction, experiments were performed in the presence of exogenous IL-2 or blocking anti-IL-2R Abs. The data presented in Fig. 2 clearly show that anergy induction cannot be explained by the lack of IL-2 secretion after CD2 costimulation as anergy was obtained even after exogenous IL-2 addition. Similarly, even after stimulation in the presence of blocking anti-IL-2R Abs, T cells stimulated in the presence of CD80 were fully responsive. Taken together, these results suggest that the unresponsiveness observed in CD4+ T cells stimulated in the presence of CD58 alone is due to the differentiation of regulatory T cells (Tr1) that actively regulated the proliferative response of the T cell population.

CD2 costimulation induces the differentiation of Tr1 T cell clones

Isolation and expansion of Ag-specific human Tr1 T cell clones have proven to be difficult. To analyze whether CD58 costimulation could be used to easily induce the differentiation of Tr1 T cell clones, human purified CD4+ T cells were stimulated with CD3 mAb in the presence of mouse CD32-CD85-L cells in the presence or absence of anti-IL-10 mAb or with CD32-CD80-L cell transfectants. Seven days later, T cells were collected, washed, stained with CD4 and CD25 mAbs, and CD4+CD25+ T cells were cloned at 1 cell/well in 96-well plates and expanded in the presence of IL-2. After expansion the different clones were differentiated with cross-linked CD3 and soluble CD28 mAbs and their cytokine profiles were analyzed by ELISA after 48 h (Fig. 3). Approximately half (48%) of the T cell clones stimulated with CD3 and CD58 displayed the cytokine profile of Th0 T cell clones with high IL-10, intermediate IFN-γ, and low to no IL-4 secretion, whereas T cell clones stimulated with CD3 and CD80 displayed a cytokine profile of Th0 or Th1 T cell clones with high to moderate IFN-γ and some IL-4 secretion (Fig. 3). Addition of blocking anti-IL-10 mAb did not modify the differentiation of Tr1 cells induced by CD2 costimulation (Fig. 3).

Tr1 cells induced by CD2 costimulation suppress proliferation of naive T cells

To analyze the functional properties of Tr1 T cell clones induced after stimulation with CD2 and CD3 mobilization, coculture

Table I.  Cytokine profile of CD4+ T cells stimulated with different APCs

<table>
<thead>
<tr>
<th></th>
<th>IL-2 (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L cells</td>
<td>24 ± 3</td>
<td>ND</td>
<td>46 ± 20</td>
<td>ND</td>
</tr>
<tr>
<td>L-CDS4</td>
<td>1,897 ± 216</td>
<td>186 ± 31</td>
<td>800 ± 48</td>
<td>476 ± 9</td>
</tr>
<tr>
<td>L-CDS8</td>
<td>251 ± 20</td>
<td>200 ± 29</td>
<td>6,666 ± 209</td>
<td>1,349 ± 17</td>
</tr>
<tr>
<td>L-CDS80</td>
<td>4,788 ± 325</td>
<td>825 ± 360</td>
<td>2,132 ± 123</td>
<td>7,447 ± 428</td>
</tr>
<tr>
<td>L-DC58</td>
<td>3,987 ± 541</td>
<td>936 ± 57</td>
<td>2,057 ± 59</td>
<td>6,212 ± 238</td>
</tr>
<tr>
<td>L-DC58CD80</td>
<td>5,102 ± 329</td>
<td>809 ± 67</td>
<td>4,325 ± 65</td>
<td>7,015 ± 718</td>
</tr>
<tr>
<td>L-DC58CD80</td>
<td>4,879 ± 297</td>
<td>769 ± 59</td>
<td>3,643 ± 85</td>
<td>4,226 ± 86</td>
</tr>
<tr>
<td>P815</td>
<td>20 ± 2</td>
<td>210 ± 59</td>
<td>2,033 ± 30</td>
<td>3,070 ± 35</td>
</tr>
<tr>
<td>P815-CDS4</td>
<td>1,472 ± 124</td>
<td>112 ± 41</td>
<td>2,302 ± 99</td>
<td>3,959 ± 220</td>
</tr>
<tr>
<td>P815-CDS8</td>
<td>43 ± 3</td>
<td>124 ± 39</td>
<td>10,658 ± 471</td>
<td>4,730 ± 100</td>
</tr>
<tr>
<td>P815-CDS80</td>
<td>6,459 ± 827</td>
<td>1,025 ± 257</td>
<td>5,728 ± 225</td>
<td>8,241 ± 70</td>
</tr>
</tbody>
</table>

a CD4+ T cells (10⁶ cells/ml) were activated with cross-linked anti-CD3 mAbs (10 μg/ml) in the presence of L cells (2 × 10⁶/ml) or P815 (10⁵/ml) expressing different costimulatory molecules as indicated. Supernatants were harvested after 24 h and the levels of cytokines were analyzed by ELISA. Results represent mean ± SD of triplicate experiments and are from one representative experiment of six.

b ND, Not detected.
experiments with resting T cells in a transwell system were performed using two different Tr1 T cell clones obtained after differentiation with L-CD58 cells. To this end, syngeneic naive CD4+ T cells were stimulated with CD3 and CD28 mAbs in the presence of Tr1, Th0, or Th1 T cell clones placed in the top basket. As shown in Fig. 4, Tr1 T cell clones suppressed the proliferation of bystander resting T cells in response to CD3 and CD28 mAb stimulation. Addition of a combination of anti–IL-10 and anti–TGF-β mAbs partially restored the proliferation of the bystander T cells as previously described (3). In contrast, coculture experiments with Th0 or Th1 T cell clones induced the proliferation of the naive T cells as expected (Fig. 4).

Ag-specific differentiation of Tr1 T cell clones
To determine whether this protocol could be used to differentiate in vitro Ag-specific Tr1 T cell clones, we generated L cells expressing the two chains of HLA-DR1 along with CD58. Purified CD4+ T cells from a non-DR1 donor were stimulated with L cells expressing HLA-DR1 in the presence of CD58 alone or with CD80. As a control, T cells were also stimulated with an HLA-DR1-positive EBV-B cell line. After 7 days, CD4+CD25+ T cells were cloned as described above. The several Ag-specific T cell clones obtained after stimulation with HLA-DR and CD58 displayed a cytokine profile of Tr1 T cell clones with high IL-10 secretion. In contrast, all of the T cell clones obtained after stimulation with the HLA-DR1+ EBV-B cell line or L cells expressing CD80 (data not shown) displayed a cytokine profile of the Th0 or Th1 T cell clones (Table II).

Discussion
This study demonstrates that CD2-CD58 interaction induces the differentiation of nonproliferating regulatory T cells secreting high levels of IL-10 (Tr1). This function is specific for CD2 signaling as it is not obtained and even inhibited by mobilization of other costimulatory molecules like CD80 or LFA-1.

We had previously shown that repetitive stimulations of human or mouse T cells in the presence of IL-10 led to the differentiation of a regulatory subset of CD4+ T cells secreting high levels of IL-10 (3). These Ag-specific Tr1 cells suppress the proliferation of CD4+ T cells in response to Ag and prevent experimental colitis induced in SCID mice by pathogenic CD4+CD45RBhigh splenic T cells (3). We also demonstrated that IL-10 induces anergy in both CD4+ (13) and CD8+ T cells (7). We further dissected the functional role of IL-10 in inducing anergy and regulatory T cell differentiation. Although it has been shown that IL-10 has immunosuppressive activities directly on purified T cells (17), preliminary experiments have shown that no regulatory T cells were obtained after stimulation of purified CD4+ T cells with CD3 and CD28 mAbs in the presence of IL-10. These results suggest that IL-10 primarily acts by modifying APCs. Indeed, IL-10 displays immunoregulatory functions mainly through the down-regulation of costimulatory molecules. We (7) and others (18–20) have shown that IL-10 addition on activated human monocytes decreases MHC class I and class II expression as well as the expression of costimulatory molecules like CD80, CD86, or CD54 (5, 21, 22).
However, expression of CD58 (LFA-3) was not affected by IL-10 addition (7). Using artificial APCs expressing selective costimulatory molecules, we mimicked the effect of IL-10 on APCs. We demonstrated that stimulation of T cells using APCs expressing only the CD58 molecule, in the absence of CD80, CD86, and CD54, induced the differentiation of regulatory T cells able to suppress the proliferation of bystander T cells through the secretion of IL-10 and TGF-β. This mechanism has been indirectly confirmed in two recent manuscripts (23, 24) where the authors demonstrated that blockade of CD28/CD80-CD86 and CD40-CD154 interactions during primary allogeneic stimulation resulted in the differentiation of alloantigen-specific regulatory T cells secreting high levels of IL-10 and maintaining anergy. There have been other studies showing that the combination of certain costimulatory molecules can induce selective cytokine patterns. In agreement with our results, it has been reported by Parra et al. (25) that costimulation with CD80, on T cells stimulated with superantigen (staphylococcal enterotoxin A), resulted in a vigorous response with production of high levels of IL-2 and IFN-γ and prolonged proliferation. In contrast, transient proliferation and low levels of IL-2 were seen after CD58 costimulation. CD54 costimulation was characterized by a high proliferative response and high levels of IL-2 secretion, whereas secretion of IFN-γ was not induced (16). Moreover, in recent experiments (26), distinct IL-10 and TNF-α profiles depending on either ICAM-1 (CD54), ICAM-2, or ICAM-3 were used to costimulate human T cells with anti-CD3 mAb were observed (26). ICAM-1 costimulation induces higher IL-10 and lower TNF-α secretion as compared with ICAM-2 and ICAM-3 (26).

Various roles for CD2 in T cell activation have been proposed, including function as an adhesion molecule (15, 27), thereby reducing amounts of Ag required for T cell activation (15), as a costimulatory molecule (28), or as a direct promoter of T cell activation (29). Moreover, CD2 has been implicated in the induction of T cell anergy (30) and has been reported to modulate cytokine production by T cells (31, 32) and to regulate positive selection (33). Surprisingly, however, CD2-deficient mice did not show an obvious phenotype and could efficiently cope with viral infections (34), undermining the view that CD2 plays a major role in T cell activation. When stimulated by appropriate mAbs, CD2 elicits proliferative responses comparable to those of CD3 mAb and mitogenic lectins (35, 36). A distinctive feature of CD2 mAb-mediated activation of T cells is the requirements that pairs of mAbs be used (37). For human CD2, one of the mAbs must be directed against the CD2R epitope, which is poorly expressed by resting T cells but is induced following binding to CD2 of the second Ab of the pair (35). None of the known physiologic CD2 ligands alone delivers an activation signal, but the combination of anti-CD2R mAb and CD58 induces T cell proliferation (35). Whereas ligation of the CD2R epitope is linked to the delivery of activation signals, perturbation of other regions of CD2 can inhibit T cells, apparently through a mechanism more complex than simply the disruption of the adhesion function of CD2 (30, 38). For example, T cells obtained from mice treated with a nondepleting CD2 mAb display a markedly reduced proliferative response to various stimuli (30). This polyclonal T cell unresponsiveness persists long after the full recovery of CD2 cell surface expression and the apparent clearance of the mAb and thus cannot be due to the disruption of the interaction of CD2 with its ligands (30). Moreover, the use of CD2 mAb has been shown to induce long-term tolerance in both mouse and rat models of transplantation. These observations could be explained by the specific differentiation of regulatory T cells induced by the specific mobilization of the CD2 molecule. However, in a completely different system using anergic T cell clones, Boussiotis et al. (39) have shown that CD2 costimulation restored responsiveness to TCR engagement. This discrepancy could be explained by the fact that we analyzed on naive T cells the importance of CD2 costimulation on the differentiation of T cells toward a Tr1 phenotype, whereas in that study the role of CD2 costimulation was analyzed on already anergic fully differentiated T cells. Therefore, it cannot be excluded that CD2 costimulation in the absence of other costimulatory signals has an important role in the differentiation of Tr1 whereas in conjunction with other costimulatory signals it can induce the reversal of T cell anergy.

Interestingly, differentiation of T cells induced by CD2 costimulation did not require multiple stimulations, as both high IL-10 secretion and regulatory function were observed after the first stimulation of naive T cells with anti-CD3 and CD32-CD58-L cells. This is in contrast with previous studies showing that the differentiation of Tr1 cells needs repetitive stimulations by immature dendritic cells (40) or IL-10-10 (3). This discrepancy further suggests that Tr1 differentiation is primarily dependent on the stimulation of naive T cells with TCR and CD2 mobilization in the absence of other costimulatory signals.

Finally, the ability to rapidly differentiate in vitro Ag-specific regulatory T cells opens new therapeutic perspectives for the use of Tr1 in autoimmune/inflammatory diseases and allogeneic transplantation. Indeed, in vitro pulsing of modified APCs with self or alloantigens followed by in vivo injection of the T cell populations could lead to down-regulation of self/alloreactivity mediated by both Th1 (3) or Th2 T cells (4).

References

Table II. Differentiation of Ag-specific Tr1 cells after stimulation with HLA-DR/CD58 L cells

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Donor 1</th>
<th></th>
<th>Donor 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10 (pg/ml)</td>
<td>IFN-γ (pg/ml)</td>
<td>IL-10 (pg/ml)</td>
<td>IFN-γ (pg/ml)</td>
<td></td>
</tr>
<tr>
<td>DR1-L cells</td>
<td>326 ± 40</td>
<td>13,372 ± 1,500</td>
<td>3,657 ± 324</td>
<td>9,638 ± 63</td>
</tr>
<tr>
<td>DR1-CD58L cells</td>
<td>2,315 ± 183</td>
<td>19,403 ± 975</td>
<td>6,402 ± 272</td>
<td>914 ± 322</td>
</tr>
<tr>
<td>DR1-B cell line</td>
<td>&lt;40</td>
<td>16,005 ± 123</td>
<td>174 ± 59</td>
<td>4,930 ± 685</td>
</tr>
</tbody>
</table>

* Purified CD4+ T cells from two different DR1+ donors were stimulated for 7 days with L cells expressing HLA-DR1 either alone or in combination with CD58. As a control, CD4+ T cells were stimulated with DR1+ transformed B cells. After 7 days, cells were washed and labeled with CD4-PE and CD25-FITC and cloned at one cell per well using a FACS sorter. T cell clones were expanded as described above and 10 T cell clones in each group were stimulated with DR1+ transformed B cells. Cytokine levels were measured by ELISA in supernatants collected 48 h after stimulation. Results represent mean ± SEM for 10 clones in each group of one representative experiment of four.