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Human T Cell Activation by Costimulatory Signal-Deficient Allogeneic Cells Induces Inducible Costimulator-Expressing Anergic T Cells with Regulatory Cell Activity

Jan Vermeiren,* Jan L. Ceuppens,† Marijke Van Ghelue,* Peter Witters,* Dominique Bullens,† Hans Werner Mages,‡ Richard A. Kroczek,‡ and Stefaan W. Van Gool2*†

Although immunoregulation by several types of regulatory T cells is now clearly established in mice, the demonstration of such regulatory T cells in humans has been proven more difficult. In this study we demonstrate the induction of anergic regulatory T cells during an MLR performed in the presence of blocking mAb to the costimulatory molecules CD40, CD80, and CD86. Despite this costimulation blockade, which totally blocks T cell proliferation and cytokine production, a nonproliferating T cell subpopulation was activated to express inducible costimulator (ICOS). These ICOS+ cells were anergic when restimulated with unmanipulated allogeneic stimulator cells at the level of proliferation and Th1 and Th2 cytokine production, but they did produce IL-10. These ICOS-expressing cells also blocked the capacity of reciprocal ICOS-negative cells to proliferate and to produce cytokines. ICOS+ anergic cells could suppress allogenic responses of either primed or naive T cells through inhibition of IL-2 gene transcription. Suppression was not mediated by IL-10 and did not require ICOS-ICOS ligand interaction, but depended on cell-cell contact. Thus, a subtype of regulatory T cells in human blood can be activated in the absence of costimulatory signals from CD40, CD80, and CD86, and they can be identified by expression of ICOS after activation. The Journal of Immunology, 2004, 172: 5371–5378.

R egulatory T cells have an essential role in the control of immune responses, prevention of autoimmune diseases, and tolerance induction after transplantation (1–3). Several types of regulatory T cells have been described, including CD4+CD25+ natural suppressor T cells, Tr1 cells (producing IL-10) and Th3 (producing TGF-β) (4). Although CD4+CD25+ cells are generated in the thymus (1), specific subsets of dendritic cells (DC)3 might induce regulatory T cells in the periphery (5).

The crucial role of the costimulatory CD80/CD86-CD28 and CD40-CD154 interactions between DC and T cells have made them attractive tools for anergy or tolerance induction (6–8). It became clear that blocking the CD80/CD86-CD28 interaction alone did not induce permanent nonresponsiveness in vitro (9) or tolerance in vivo (10) unless combined with simultaneous transfer of donor cells or bone marrow (11, 12). Moreover, coadministration of soluble CTLA-4Ig and anti-CD154 mAb was shown to have synergistic effects and to induce long term graft acceptance in a non-human primate kidney model, but no permanent state of tolerance was achieved (13, 14). In contrast, when the combined costimulation blockade therapy was applied for induction of a state of stable chimerism, the animals acquired a state of tolerance, allowing skin transplants to be accepted without further need for therapy (15). The rationale for combining these blocking agents has been reviewed (16, 17). We and others reported that this approach might also be useful in humans (18, 19). Indeed, T cells activated in vitro by alloantigens in the presence of blocking anti-CD80, anti-CD86, and anti-CD40 mAb (further designated anti-CD40/CD80/CD86) as a method to accomplish complete costimulation blockade were functionally altered in an alloantigen-specific way, as evidenced upon restimulation by lack of proliferation, of cytotoxic T cell generation, and of IL-2, IL-5, and IL-13 production. IFN-γ production upon restimulation was reduced, whereas the production of IL-10 was markedly enhanced. We have previously interpreted the effects of costimulation blockade with anti-CD40/CD80/CD86 as being based on anergy induction (18). An alternative hypothesis, however, is that the costimulation blockade induces or activates a regulatory cell population, which suppresses the activity of the other cells. Nonproliferating, IL-10-producing anergic cells have also been found in other in vitro (20) and in vivo (21) models, and in addition, a regulatory role could be assigned to these IL-10-producing cells. In view of the recently described role of inducible costimulator (ICOS) in IL-10 induction (22), a potential role for this molecule in the high IL-10 induction after costimulation blockade was also considered.

The ICOS ligand (ICOSL)-ICOS interaction is an important costimulatory signal for cytokine and especially Th2 cytokine and IL-10 production. ICOSL, which belongs to the family of the B7 molecules, is expressed on monocytes and DC (23). ICOS is expressed on activated T cells, and its ligation up-regulates a number of membrane molecules, such as CD154, and enhances T cell proliferation, secretion of cytokines, and helper function for Ab secretion by B cells (22, 24–27). Unlike CD28 ligation, ICOS ligation does not up-regulate the production of IL-2, but superinduces
the synthesis of IL-10. The distinctive functional outcomes of co-stimulation via CD28 and ICOS are accompanied by differences in expression profiles (28). The functional role of ICOS was further characterized in gene-targeted, ICOS-deficient mice. These mice exhibit profound deficits in Ig isotype class switching accompanied by impaired germinal center formation and are prone to the development of experimental allergic encephalomyelitis (29, 30).

In the present study we explored whether the long-lasting effects of costimulation blockade in vitro are due to induction of regulatory T cells, and whether ICOS-ICOSL interaction plays a role in the activity of these cells.

Materials and Methods

Monoclonal Abs and reagents

Anti-CD80 mAb B7-24 (IgG2a) was produced at Innogenetics (Gent, Belgium) (31). Anti-CD86 mAb FUN-1 (IgG2a) was obtained from BD PharMingen (San Diego, CA). mAb 5D12 (IgG2a) (American Type Culture Collection, Manassas, VA) is a blocking mAb to CD40 (32). The anti-ICOS mAb F44 (IgG1), unlabeled and PE labeled, and the anti-ICOSL mAb HIL131 (IgG1) were produced by R.A.K. HIL131 is a blocking Ab (27). The mAb 2A11 (IgG1) was used as an id control Ab. A blocking anti-IL-10 mAb (37607.11) was obtained from R&D Systems (Minneapolis, MN). Anti-CD3, -CD25, -CTLA-4, -HLA-DR, -CD45RO, and -IL-10 or isotype control Abs were directly coupled to FITC, PE, PE-Cy5, or PerCP were purchased from BD PharMingen. rIL-2 (Boehringer Mannheim, Mannheim, Germany) was used where indicated at a concentration of 10 U/ml. To prevent IL-2 consumption, both anti-Tac mAb (anti-CD25; Zenapax; Roche Pharma, Reinach, Switzerland) and Mikb1 (anti-CD122; BD PharMingen) were used in some experiments at a final concentration of 5 μg/ml. This allows more accurate measurement of IL-2 production by T cells in the supernatants (33).

Mixed leukocyte reaction

All subjects donating blood for these experiments were healthy volunteers of both sexes, aged 20–50 years. PBMC were isolated by centrifugation on Ficoll-Hypaque (density, 1.077) gradients and resuspended in culture medium (RPMI 1640 (Boehringer Ingelheim, BioWhittaker, Heidelberg, Germany) supplemented with 2 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Autologous inactivated plasma was used at 10%. For the primary MLR, responder PBMC (PBMCr) and allogeneic irradiated (30 Gy) stimulator PBMC (PBMCs) were mixed at a ratio of 1:1 in culture flasks (Nunc, Roskilde, Denmark). The primary MLR was performed in the absence of mAb (i.e., control) or in the presence of 2 μg/ml anti-CD40, 2 μg/ml anti-CD80, and 2 μg/ml anti-CD86 mAb (anti-CD40/CD80/CD86) to generate costimulation-deficient APC (18). After 6 or 7 days, PBMCr were harvested, washed in PBS (Boehringer Ingelheim Bio-products), and resuspended in medium. After 2 days, cells were restimulated during 4 days (secondary MLR) with irradiated freshly isolated PBMCs at a responder/stimulator ratio of 1:1 in the absence or the presence of blocking mAb as further indicated. In some experiments the cells were again harvested after this secondary MLR, washed, and kept in culture for 2 days. After 2 days, these cells were restimulated with irradiated freshly isolated PBMCs in the absence of mAb during another 4 days (tertiary MLR). All restimulations were performed with fresh cells from the same donor as in the primary MLR.

In some experiments responder T cells were further purified before start of the primary stimulation using a complement-mediated depletion of all non-T cells with lympho-KiWi-T (One λ, Los Angeles, CA) as previously described (34).

T cell proliferation assay

Prestimulated PBMCr (105) were mixed with 105 irradiated PBMCs in 200 μl of medium in a flat-bottom, 96-well plate (Nunc) for 4 days (secondary and tertiary MLR) or 7 days (primary MLR). During the last 8 h of the culture period, the cells were pulsed with 1 μCi/well 3H-thymidine (ICN Biomedicals, Costa Mesa, CA). The mean cpm of quadruplicate wells was calculated. Proliferation of T cells was expressed as the stimulation index: SI = cpm(responder × stimulator)/(cpm(responder) + cpm(stimulator)).

Alternatively 5 μM CFSE (Molecular Probes, Eugene, OR) was used to label T cells before the start of the MLR (35). The occurrence of cell divisions was evaluated on day 6 on the FACSort (BD Biosciences, Mountain View, CA).

Cytokine production and ELISA

One million prestimulated PBMCr were mixed with 108 irradiated PBMCs in 1 ml of medium in a flat-bottom, 24-well plate. Supernatants were taken on day 4. Cytokines were determined by sandwich ELISA technique, using combinations of unlabeled and biotin- or enzyme-coupled mAb to different epitopes of each cytokine (IL-2, IL-5, IL-13, IL-10, and IFN-γ; mAb from BD PharMingen).

Transwell experiments

Transwell experiments were performed in 24-well plates. Responder T cells (105) were stimulated with 106 irradiated PBMC. In addition, 1 × 105 ICOS+ or ICOS− cells derived from priming conditions in the presence of blocking Abs were either added directly to the culture or placed in Transwell chambers (cell culture insert, 1 μm; BD Biosciences) with an additional 1 × 105 irradiated stimulator cells.

Cell sorting

Cells were stained directly with PE-labeled F44 (anti-ICOS) or isotype control mAb for 20 min at 4°C at optimal dilution. After staining, cells were washed and resuspended in PBS at 3 × 106 cells/ml. ICOS+ and ICOS− lymphocyte cell fractions were sorted with FACSVantage (BD PharMingen, San Jose, CA). After sorting, the cells were washed with PBS and resuspended in culture medium. The purity of the populations obtained was confirmed on a FACSort (using CellQuest software for analysis of the data; BD PharMingen).

Intracellular staining

During the last 24 h of the priming T cell cultures, 3 mM monensin (Sigma-Aldrich, St. Louis, MO) was added to the cultures. Additionally PMA (Sigma-Aldrich; 1 ng/ml) and ionomycin (Sigma-Aldrich; 0.5 μg/ml) were added during the last 4 h. Cells were collected and, after membrane stabilization, fixed with 500 μl of 2% paraformaldehyde (Janssen Pharmaceutica, Beerse, Belgium) in PBS at 4°C for 10 min. For permeabilization, cells were washed with PBS containing 0.5% BSA (Sigma-Aldrich) and 0.2% saponin (Sigma-Aldrich). Cells were then resuspended in 100 μl of this permeabilization buffer, and 10 μl of normal mouse serum was added for 5 min at room temperature. Then 10 μl of either PE-conjugated control IgG1 Ab (BD PharMingen), PE-Cy5-conjugated IgG2a Ab (BD PharMingen), PE-labeled anti-human IL-10 (18555A), or PE-Cy5 labeled anti-CTLA-4 were added. Cells were gently mixed for 30 min in the dark at room temperature. Cells were resuspended, washed twice with permeabilization buffer, and resuspended in PBS. Cells were analyzed with a FACS flow cytometer (BD PharMingen).

Quantification of cytokine mRNA expression

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). The Netherlands) according to the manufacturer’s instructions. A constant amount of 2 μg of target RNA was used for cDNA synthesis (Ready-to-Go Kit; Pharmacia Biotech, Upplasa, Sweden). After 90 min at 37°C, the reverse transcriptase was inactivated by incubating the cDNA samples for 5 min at 95°C. The cDNA samples were then subjected to real-time quantitative PCR, performed in the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) as previously described (36). The sequences used for the IL-2 PCR were AACTCAACAGGATGCTCA CATTTA for the forward primer and TCCCTTGGTCCTTTAGTGA AAGTTT for the reverse primer, resulting in a 128-bp amplicon. The sequence of the IL-2 probe was TTTTTACATGCCCAAGGGCCACAG AACT. The sequences of the primers and probe for β-actin have been previously reported (36). All primers and probes were designed with the assistance of the computer program Primer Express (Applied Biosystems) and were purchased from Eurogentec (Seraing, Belgium). The 5′-nuclease activity of the Taq polymerase was used to cleave a nonextendable, dual-labeled, fluorogenic probe. Fluorescent emission was measured continuously during the PCR. PCR amplifications were performed in a total volume of 25 μl containing 5 μl of cDNA, 12.5 μl of Universal PCR Master Mix (Applied Biosystems, AB), 100–300 nM of each primer, and 200 nM of the corresponding detection probe. Each PCR amplification was performed in triplicate wells using the following conditions: 94°C for 10 min, followed by 40 or 45 cycles at 94°C for 15 s and 60°C for 1 min. cDNA plasmid standards, consisting of purified plasmid DNA specific for each individual target, were used to quantify the target gene in the unknown samples, as previously described (36). All results were normalized to β-actin to compensate for differences in the amount of cDNA in all samples.
Results

ICOS induction and function after allogeneic stimulation in the absence of costimulatory molecules

The results of in vitro stimulation by costimulation-deficient APC in MLR (18) is shown in Fig. 1. PBMCs were stimulated with irradiated allogeneic PBMCs either without added Abs (control stimulation) or in the presence of anti-CD40/CD80/CD86 (costimulation-deficient condition). A combination of all three Abs was required for the induction of nonresponsiveness (18). Addition of anti-CD40/CD80/CD86 completely blocked proliferation and inhibited cytokine production by >90% (data not shown). After this primary MLR, cells were restimulated with PBMCs (from the same donor as in the primary MLR) in the absence of any Ab. At the end of the secondary MLR, the proliferative response, and the productions of Th1 (IFN-γ) and Th2 (IL-5 and IL-13) cytokines and of IL-10 were measured. As shown in Fig. 1, restimulation of T cells after the control primary MLR induced proliferation (Fig. 1A) and production of IFN-γ (Fig. 1B), IL-5 (Fig. 1C), and IL-13 (Fig. 1D), whereas restimulated T cells after the costimulation-deficient primary MLR failed to proliferate and failed to produce cytokines except IL-10 (Fig. 1E). Thus, allogeneic triggering with costimulation blockade results not only in inhibition of T cell proliferation and cytokine production, but also in a failure of T cells to mount a normal response upon restimulation.

The expression of ICOS on the T cells after different stimulation conditions in the primary MLR was evaluated by staining with labeled mAb. Before stimulation, 0–3% (n = 6) of T cells expressed ICOS. At the end of the control primary MLR, 19–44% (n = 6) of T cells expressed ICOS. Surprisingly, after the costimulation-deficient MLR, 3–18% (n = 9) of the T cells expressed ICOS. Almost all (98 ± 1.6%) of ICOS+ cells expressed CD45RO, and the majority (82 ± 5.6%) expressed HLA-DR. In addition, all the ICOS+ T cells expressed CD25. The up-regulation of ICOS in the costimulation-deficient condition demonstrates that a subset of T cells was at least partly activated despite costimulation blockade.

CFSE labeling of the responder cells was therefore used as a method to simultaneously evaluate proliferation and marker expression. In proliferating cells, CFSE is diluted, and thus the proliferating cells can be distinguished from nonproliferating cells on the basis of fluorescence intensity (35). Our results confirmed that in the absence of costimulation, no T cell proliferation occurred in a primary culture, although the expression of ICOS and CD25 was induced on a subset of the cells (Fig. 2A). In the control MLR, the expression of ICOS and CD25 was predominant on the proliferating T cells (Fig. 2A).

Whereas intracellular IL-10 could be detected in up to 30% of the T cells after costimulation-deficient MLR, no intracellular IL-10 was detectable in T cells stimulated in a control MLR (Fig. 2B). These T cells are most likely the source of the IL-10 found after restimulation (Fig. 1E). Of note, T cells in the experimental condition after costimulation-deficient MLR did not express intracellular CD152, in contrast to T cells stimulated in the control MLR (Fig. 2B).

We then studied whether during restimulation, ICOSL-ICOS interactions have any functional role in IL-10 induction or in the suppression of the other cytokines. To this end, blocking anti-ICOSL or anti-CD80/CD86 mAbs were added in the secondary MLR after a primary MLR in the presence of anti-CD40/CD80/CD86. Blocking the ICOSL-ICOS interaction in the secondary MLR did not restore the proliferation or the production of IL-5, IL-13, and IFN-γ, but significantly reduced the superproduction of IL-10 (Fig. 1E), showing that ICOS acts as the major costimulatory receptor for IL-10 induction in these cultures. Blocking the CD80/CD86-CD28 interaction in these secondary MLRs also partially affected IL-10 production, and the effect was additive to that of anti-ICOSL, resulting in complete inhibition of IL-10 production.

We can conclude from these data that after stimulation of T cells by costimulation-deficient APC, ICOS is induced on a nonproliferating T cell subset. The latter subset, upon restimulation, is apparently able to produce IL-10 in a secondary MLR, depending on...
ICOS and ICOSL-ICOS and, to a lesser degree, CD80/CD86-CD28 interactions.

**ICOS+ cells are anergic and block the primary and secondary MLR**

We have previously interpreted defective T cell restimulations after costimulation blockade in a primary MLR as being due to anergy induction in responder cells (18). An alternative interpretation was now raised by the data presented above and in other reports (2, 3, 20), namely, the induction of a regulatory cell subset. Therefore, after the primary MLR with costimulation-deficient APC, ICOS+ and ICOS− cells were separated on a FACSVantage. The purities of this selection procedure were >80 and >98%, respectively. Both subsets were then restimulated in a secondary MLR. There was a marked difference between ICOS+ vs ICOS− cells in their functional response. Whereas the total (nonsorted) cell population and sorted ICOS+ cells did not proliferate and did not produce IFN-γ, IL-5, and IL-13 upon restimulation, ICOS− cells had a normal proliferative response in a secondary MLR (Fig. 3), and they produced cytokines in amounts comparable to the production by control primed T cells. Purified ICOS+ and ICOS− cells sorted after a control MLR, both depicted a normal secondary response upon restimulation (data not shown). We conclude that allogeneic T cell stimulation with costimulation-deficient APC induces an ICOS+ anergic T cell subset that prevents the response of ICOS− T cells. Removal of ICOS+ cells restores the responsiveness of ICOS− cells.

We next set up experiments to directly demonstrate that ICOS+ anergic T cells have a suppressive effect on the response of either primed or naive T cells during allogeneic stimulation. ICOS+ anergic T cells, generated and sorted as explained above, were added to a secondary or a primary MLR. The proportion of ICOS+ anergic cells added was 10%, which is similar to the proportion of ICOS− cells found in the total cell population after the costimulation-deficient primary MLR. As shown in Fig. 4, ICOS+ anergic cells...
T cells strongly suppressed the proliferative response in a secondary MLR. They similarly suppressed T cell proliferation when added in a primary MLR. We therefore conclude that ICOS+ anergic cells have properties of regulatory T cells. Addition of purified ICOS+ cells derived from a control primary MLR did not suppress the naive T cell response (data not shown).

**Suppression by ICOS+ requires cell-cell contact and inhibits IL-2 mRNA induction**

From previous experiments, the mechanism by which ICOS+ anergic T cells exert their regulatory cell activity was not yet clear. Because suppressive soluble factors might play a role, Transwell experiments were performed. To this end, primed T cells were restimulated with allogeneic PBMC. ICOS+ anergic T cells were added in the same culture, either directly in contact with the primed T cells or separated by Transwell only. In the former culture condition were ICOS+ anergic T cells able to suppress the response of primed T cells (Table I). Thus, cell-cell contact is required for suppression to occur. In accordance with this interpretation, addition of a blocking anti-IL-10R mAb during restimulation in a secondary MLR after a costimulation-deficient primary MLR did not restore T cell proliferation or cytokine production.

One particular mechanism that has been proposed to play a role in regulatory T cell activity is ligand competition (37). The CD25 expressed on ICOS+ anergic T cells might indeed be responsible for competitive uptake of IL-2, thereby preventing the proliferation and cytokine production of responder T cells. To exclude this mechanism, we measured IL-2 mRNA and IL-2 production during allogeneic stimulations of primed T cells in the presence or the absence of ICOS+ anergic T cells. To exclude IL-2 consumption by regulatory T cells, both anti-Tac (anti-CD25) and anti-CD122 mAbs were added to those cultures used thereafter only to measure IL-2 production (33). As shown in Fig. 5, control primed T cells, when restimulated, produce IL-2, and IL-2 mRNA is up-regulated. In contrast, T cells that have been primed in the absence of costimulation did not produce IL-2 and did not express IL-2 mRNA when restimulated. Most importantly, the ICOS+ anergic subset, but not the ICOS- subset, completely prevented both IL-2 production and IL-2 mRNA expression during restimulation of primed T cells. Thus, the lack of response in the presence of ICOS+ anergic cells is not caused by preferential uptake of IL-2 by anergic regulatory T cells. These data show that ICOS+ anergic T cells exert their suppressive effect via cell-cell interaction thereby preventing cytokine (IL-2) gene transcription.

**Blocking of ICOS does not alter the suppression by ICOS+ cells**

To study the role of ICOSL-ICOS interaction for function and maintenance of regulatory cell activity, we performed tertiary MLR experiments. Cells that had been primed in the presence of anti-CD40/CD80/CD86 were restimulated in a secondary MLR without or with added anti-ICOSL mAb (to block the ICOSL-ICOS interaction and the IL-10 production) and were then restimulated again in a tertiary MLR in the absence of any blocking mAb. As shown in Fig. 6, proliferation and Th1 and Th2 cytokine production during this tertiary MLR were not restored by the presence of anti-ICOSL mAb during the secondary MLR. Moreover, the high IL-10 production also reappeared in these tertiary MLR cell cultures. These data show that regulatory T cell activity induced by costimulation blockade is a long-lasting functional state and that the maintenance of it does not depend on the ICOSL-ICOS interaction.

**Discussion**

The present experiments reveal that T cell activation by allogeneic costimulation-deficient APC results in the expression of ICOS on

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**Table I. Regulatory function of ICOS+ cells is dependent on cell-cell contact**

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<th>Cytokine Production (% inhibition)a</th>
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<tr>
<td></td>
<td>IFN-γ (pg/ml)</td>
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<tr>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>None</td>
<td>−</td>
</tr>
<tr>
<td>ICOS+ sorted cells added</td>
<td>−</td>
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<td>ICOS+ sorted cells added</td>
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a ICOS+ cells were either in direct contact with the responder cells or separated by a Transwell membrane.

b T cells were primed with allogeneic PBMCs and then restimulated with PBMCs from the same donor in the absence or the presence of autologous ICOS+ T cells (induced and sorted as explained). Cytokine production was measured by ELISA.
secondary MLR. After 4 days, proliferation (A; expressed as SI) and proliferation of the stimulator cells used for restimulation was 471 cpm.

They were then restimulated (tertiary MLR) with freshly isolated and irradiated PBMCs each time from the same donor as in the primary and secondary MLR. After 4 days, proliferation (A; expressed as SI) and production of IFN-γ (B), IL-5 (C), IL-13 (D), and IL-10 (E) in this tertiary MLR was measured. For proliferation, the mean basal cpm of the responding cells are, respectively, 1141, 1085, 723, and 457 cpm. The mean basal proliferation of the stimulator cells used for restimulation was 471 cpm. The results of three individual experiments are shown. The black bar represents the mean of the condition.

FIGURE 6. Effect of anti-ICOSL mAb added during secondary MLR on T cell responses in the tertiary MLR. PBMCs were prestimulated during 7 days with PBMCs in the absence or the presence of anti-CD40/CD80/CD86 as described in Fig. 1. After 2 days of rest, PBMCs were restimulated during 4 days with PBMCs in the absence or the presence of anti-ICOSL mAb or control mAb (20 μg/ml) as indicated. After 4 days the cells were washed and resuspended in medium alone and rested again for 2 days. They were then restimulated (tertiary MLR) with freshly isolated and irradiated PBMCs each time from the same donor as in the primary and secondary MLR. After 4 days, proliferation (A; expressed as SI) and production of IFN-γ (B), IL-5 (C), IL-13 (D), and IL-10 (E) in this tertiary MLR was measured. For proliferation, the mean basal cpm of the responding cells are, respectively, 1141, 1085, 723, and 457 cpm. The mean basal proliferation of the stimulator cells used for restimulation was 471 cpm. The results of three individual experiments are shown. The black bar represents the mean of the condition.

a nonproliferating subpopulation of T cells. These ICOS− cells, upon restimulation, appear to be anergic (i.e., nonproliferating, no Th1 and Th2 cytokine production), but they produce IL-10 and they suppress the response of the reciprocal ICOS+ cells. Moreover, this particular regulatory T cell subpopulation also suppresses the activation of primed or naive T cells in response to allogeneic triggering. Neither IL-10 nor ICOS triggering is required for the suppressive activity of these cells, which inhibit IL-2 mRNA formation and IL-2 production by responder T cells in a cell contact-dependent manner. The relation between anergy and regulatory T cell activity is not surprising. Regulatory T cell activity has previously been demonstrated for other types of anergic T cells, generated by repetitive cycles of stimulation with immature DC (20) or stimulation in the presence of IL-10 (2). Moreover, naturally occurring regulatory T cells that express CD4+CD25+ are also anergic and unable to produce IL-2.

Costimulation-deficient APC in our experiments were generated by adding blocking mAb toward three important costimulatory signals (CD80, CD86, and CD40) in the primary MLR, and the rationale for this has previously been discussed extensively (16, 17). The advantage of this system is that it is well defined and reproducible as a model. The activation of ICOS+ regulatory T cells under costimulation-deficient conditions implies that these T cells do not require signals from B7 or CD40. Other studies have also suggested that the result of stimulation combined with costimulation blockade is based on the induction of regulatory T cells (17, 19, 38). This does not yet imply that costimulation could not be required for the generation of these cells in either the thymus or the periphery. The issue remains controversial, as interruption of B7-CTLA-4 interactions with anti-CTLA-4 blocks regulatory T cell activity (39), and B7 expression is needed for induction of natural regulatory T cells (40–42). In accordance with the lack of B7 requirement, the ICOS+ anergic cells did not express CTLA-4.

The exact mechanism of how ICOS+ regulatory T cells exert their regulatory function on alloantigen-responding T cells is partly unraveled. IL-10 has been shown to block alloantigen responses during MLR and is considered as the main cytokine for the induction and maintenance of anergy or tolerance (43–45). Our experimental model, however, did not reveal a functional effect of the high IL-10 production by ICOS+ anergic cells. In the experiments with allogeneic T cell restimulation after costimulation blockade, interruption of ICOSL-ICOS interaction reduced the production of IL-10, but did not restore proliferation (Fig. 1A) or cytokine production (IFN-γ, IL-5, and IL-13) to normal levels (Fig. 1, B–D). Similarly, when a blocking anti-IL-10R mAb was added during the restimulation, proliferation and cytokine production remained low (data not shown). Several other mechanisms of suppression can be considered. First, ICOS+ anergic T cells can produce other cytokines with regulatory properties such as TGF-β, although the role of TGF-β in regulatory T cell function remains controversial (3). However, Transwell experiments did not support a functional role of any suppressive soluble cytokine in preventing the T cell responses and instead suggests the need for cell-cell contact. ICOS+ anergic T cells can directly affect responder T cell through cell contact-dependent mechanisms (similar to CD4+CD25+ cells). Importantly, we could demonstrate that the anergic regulatory cells do not simply compete for IL-2 with responding T cells. Using real-time RT-PCR we indeed found that they suppress the formation of IL-2 mRNA. Moreover, IL-2 concentrations in the supernatants of responding T cells cocultured with ICOS+ regulatory cells were strongly reduced compared with the situation without ICOS+ regulatory cells. These experiments were set up in the presence of mAb that block IL-2 consumption by IL-2R+ cells. The results therefore exclude IL-2 consumption as a mechanism, and at the same time they demonstrate that the regulatory cells do not need IL-2 for their activity. Alternatively, they can exert contact-dependent inhibitory or deactivating effects on APC (3), or they can use the APC surface as a platform on which they interact physically with responding T cells. Competitive inhibition of alloantigen binding based on enhanced affinity of the TCR on ICOS+ T cells also cannot be excluded (37).

The regulatory cells described in this paper thus have the anergic phenotype in common with other regulatory cells, but the exact relation between these different subtypes of regulatory T cells awaits further study. Nevertheless, we can conclude that, similar to
CD4+/CD25+ cells, ICOS+ regulatory T cells suppress by cell-cell contact and suppress IL-2 mRNA formation and IL-2 production by responder cells, rather than consuming IL-2 from the responder cells (46). A final reason to believe that these ICOS+ cells are similar or related to CD4+/CD25+ cells is that those cells were apparently present in blood, and they only needed an in vitro activation step before their suppressive role became manifest. These cells were thus most likely circulating in a resting state. Tr1 cells, in contrast, could only be induced by prolonged culture in the presence of IL-10 in vitro or by stimulations with a specific subset of DC (5, 47). Moreover, Tr1 cells are thought to act predominantly through production of soluble cytokines and are also in that respect different from the ICOS+ cells described in this study (2).

It should be stressed that ICOS is expressed on different T cell populations. ICOS expression is dependent on cell activation and ICOS is a marker for effector T cells (48). ICOS-L-ICOS interaction is important in the activation and function of effector T cells in general, and induces CD28-independent T cell proliferation and cytokine production, especially IL-10 production, along with production of Th1 and Th2 cytokines (22, 25–27). Most likely and comparable to its function in other types of effector cells, ICOS might only be a marker for the state of activation of this regulatory cell type and/or an amplifier of the function of regulatory T cells. ICOS therefore cannot be considered a marker for regulatory T cells. In contrast, ICOS+ regulatory T cells have recently also been described in an allergen-induced airway hyper-reactivity model (49). In this in vivo model, there is indirect evidence for the in vivo generation and puriﬁcation of T cells described in this study (2).

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References


