Activated Self-MHC-Reactive T Cells Have the Cytokine Phenotype of Th3/T Regulatory Activated Self-MHC-Reactive T Cells

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Activated Self-MHC-Reactive T Cells Have the Cytokine Phenotype of Th3/T Regulatory Cell 1 T Cells

Atsushi Kitani, Kevin Chua, Kazuhiko Nakamura, and Warren Strober

In the present study, we show that human self-MHC-reactive (autoreactive) T cell clones are functionally distinct from Ag-specific T cell clones. Self-MHC-reactive T cells exhibited helper function for B cell Ig production when cultured with non-T cells alone, and they exhibited suppressor function when cultured with PWM- or rCD40 ligand (rCD40L)-activated non-T cells, whereas tetanus toxoid (TT)-specific clones exhibited only helper function in the presence of TT with or without PWM or rCD40L. Addition of neutralizing Abs to the cultures showed that the suppression was mediated by TGF-β but not by IL-10 or IFN-γ. The self-MHC-reactive clones also inhibited proliferation of primary CD4+ T cells and TT-specific T cell clones, but in this case the inhibition was mediated by both IL-10 and TGF-β. In further studies, the interactions between self-MHC-reactive T cell clones and non-T cells that led to suppressor cytokine production have been explored. We found that prestimulation of non-T cells for 8 h with PWM or for 48 h for rCD40L results in non-T cells capable of inducing self-MHC-reactive T cell to produce high levels of TGF-β and IL-10. In addition, these prestimulation times coincided with peak induction of HLA-DR and costimulatory B7 molecule (especially CD86) expression on B cells. Finally, addition of CTLA-4/Fc or blocking F(ab’)_2 anti-CTLA-4 mAb, plus optimally stimulated non-T cells, to cultures of self-MHC-reactive clones inhibited the induction of TGF-β but not IL-10 or IFN-γ production. In summary, these studies show that activated self-MHC-reactive T cells have the cytokine phenotype of Th3 or T regulatory cell 1 and thus may be important regulatory cells that mediate oral and peripheral tolerance and prevent the development of autoimmunity. The Journal of Immunology, 2000, 165: 691–702.

The T cells stimulated by class II self-MHC Ag in the apparent absence of exogenous or nominal peptide Ag (autoreactive T cells or self-MHC-reactive T cells) are of considerable interest because such T cells may play an important role in the induction and maintenance of immune homeostasis and may act as a bulwark against the development of autoimmunity (1–3). In previous studies conducted about a decade ago, we showed that repeated stimulation of peripheral T cells with tetanus toxoid (TT)-pulsed APCs leads not only to the induction of Ag-specific (TT-specific) clones but also to the appearance of self-MHC-reactive clones that undergo vigorous proliferation when stimulated with cells bearing self-MHC in the absence of TT (4–6). In further studies, we demonstrated that such self-reactive (SR) T cell clones possess the unique ability to help and suppress B cell Ig production under different circumstances. If cocultured with B cells in the absence of an exogenous B cell stimulant (PWM), they helped B cells to produce Ig. In contrast, if cultured with B cells in the presence of PWM, they suppressed B cell Ig synthesis. These earlier studies of self-MHC-reactive T cells, for the most part, were performed before the acquisition of our present knowledge of cytokine production and function; thus, the mechanism of the help and suppression mediated by self-MHC-reactive T cells remained largely unexplained.

It is now well-established that immune responses driven by Th1 T cells and Th2 T cells (7–10) are sometimes also influenced by a third T cell type whose main function is counterregulation or suppression of immune responses mediated by Th1 and Th2 (11, 12). It has been shown, for instance, that the induction of oral tolerance by the feeding of relatively low amounts of myelin basic protein leads to the induction of immunoregulatory T cells, which prevent the development of experimental autoimmune encephalitis (11, 12). Such T cells have a unique cytokine production profile in that they produce high levels of TGF-β without necessarily producing either Th1 or Th2 cytokines; therefore, they have been dubbed Th3 T cells (12). Th3 cells producing TGF-β have also been shown to occur in experimental models of colitis or diabetes or in HgCl2-induced autoimmune disease, and in these instances it is thought that such T cells play an important role in disease prevention or cure (13–16). Recently, another type of regulatory T cell has been identified (in both mice and humans) that may be related to the aforementioned Th3 T cell. This cell, termed T regulatory cell 1 (Tr1), is induced in vitro by stimulation of T cells in the presence of IL-10 and is a T cell that produces high levels of IL-10 (17).

The recognition that self-MHC-reactive T cells can act as suppressor T cells as well as the recent interest in Th3/Tr1 T cells prompted us to re-examine self-MHC-reactive T cells to more completely characterize the mechanism of the suppressive capability. To this end, we developed self-MHC-reactive T cell clones as in previous studies and then showed that stimulation of these clones by activated B cells leads to production of high levels of TGF-β and IL-10 and that such production accounts for their suppressor activity. We also showed that such cytokine production depended on interaction between B7 molecules on the activated B cell and CTLA-4 on the stimulated self-MHC-reactive T cell.
These studies thus characterize a unique T cell subset that may play an important immunoregulatory role during inflammation.

Materials and Methods
FITC-conjugated anti-CD4 (Leu 3a + 3b), anti-CD19 (B43), anti-HLA-DR (G46–6), PE-conjugated CD86 (B7–2, FUN1), CD152 (CTLA-4, BN13), anti-HLA-DR, and FITC- or PE-conjugated isotypic control mAbs were obtained from PharMingen (San Diego, CA). PE-conjugated anti-CD80 (B7–1, L307.4) was purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). Anti-CD3 (OKT3) by hybridoma from the American Type Culture Collection (Manassas, VA), and mAb was subsequently purified from ascites. Anti-CD28 mAb (9.3) was kindly provided by Dr. C. June (Bethesda Naval Hospital, Bethesda, MD). F(ab’)2 anti-CD4–B8 (HS) was obtained from Ancell (Bayport, MN). Neutralizing anti-TGF-β1, -β2, and -β3 mAb was purchased from Genzyme (Cambridge, MA); and anti-IL-2, anti-IFN-γ, and anti-IL-10 mAb and recombinant human CTLA-4/Fc were obtained from R&D Systems (Minneapolis, MN). PWM and recombinant human IL-2 were obtained from Life Science Technologies (Baltimore, MD). TT was purchased from List Biological Laboratories (Campbell, CA) and was provided by Wyeth-Lederle Vaccines and Pediatrics (Pearl River, NY). Recombinant CD40 ligand (rCD40L)-trimer was kindly provided by Immunex (Seattle, WA).

Generation of SR T cell clones and Ag-specific T cell clones
Self-MHC-reactive human T cell lines and clones were derived from peripheral blood T cells repetitively stimulated with TT in the presence of culture supernatants of PWM or rCD40L, as previously described (4, 5). In brief, using previously described Ficoll gradient and SRBC rosetting techniques, T cell and non-T cell populations were isolated from the peripheral blood of a normal volunteer who had previously undergone TT immunization. The T cell population contained >95% T cells, and the non-T cell population contained <0.5% T cells. A CD4+ T cell subset was then isolated from the T cell population by negative selection using a human CD4 subset minicolumn (R&D Systems); a population containing more than 93% CD4+ T cells was obtained. Equal numbers of CD4+ T cells and non-T cells were then cocultured at a concentration of 2 × 10^6 cells/ml in complete RPMI 1640 medium (Bio-Whittaker, Walkersville, MD) supplemented with 10 mM HEPES, penicillin (100 U/ml), streptomycin (10 μg/ml), and 10% FCS (Biologics, Rockville, MD) in 24-well culture plates (Costar, Corning, NY) with TT stimulation (2 μg/ml). In some experiments, heat-inactivated autologous serum was used instead of FCS in the induction phase of these cultures (during the first 8 days of cultures). Eight to 10 days later, viable cells were isolated by density gradient centrifugation on lymphocyte separation medium (ICN Biomedicals, Aurora, OH), and restimulated with fresh TT, non-Tx cells, and IL-2 (2–4 μg/ml). TT and non-Tx cells were repurified every 8–10 days, and IL-2 was replenished every 3–4 days. Eight to 10 days after the last restimulation, the stimulated CD4+ T cells were cloned by limiting dilution at one cell per well in the presence of 1 × 10^4 non-Tx cells with TT and IL-2 in round-bottom 96-well microplates (Nalge Nunc International, Rochester, NY). Wells containing expanding colonies of ~5 × 10^5 cells on days 14–28 were transferred to larger wells and expanded by further exposure to TT, non-Tx cells, and IL-2. Cloned T cells were assessed for their proliferative response to non-Tx cell alone or to TT and non-T cells to determine their status as SR or Ag-reactive (TT-reactive) T cells. The three self-MHC-reactive T cell clones (SR-1, SR-2, and SR-3) and two TT-specific T cell clones (TT-1 and TT-2) were subsequently maintained for long periods of time.

Assay of self-reactivity and Ag-reactivity of cloned T cells
Each T cell clone was cocultured at a concentration of 1–2.5 × 10^5 cells/well with non-Tx or TT at a concentration of 5 × 10^5 cells/well in the presence or absence of TT (2 μg/ml) for 72 h in 0.2 ml 10% FCS containing complete RPMI 1640 medium or in 15% autologous serum containing X-VIVO 20 medium (BioWhittaker), which is free from FCS, in 96-well round-bottom microplates (in triplicate). The cells were pulsed with 1 μCi/well of [3H]Thymidine (Amersham Pharmacia Biotech, Chicago, IL) for the last 8 h of culture, and then they were harvested and assessed by thymidine deoxyribose incorporation in a liquid scintillation counter.

Cell culture and ELISA for Ig production
SR or Ag-reactive T cell clones were cocultured at a concentration of 5 × 10^5 cells/well with an equal concentration of non-T cells with or without PWM (20 μg/ml), rCD40L (2 μg/ml), or TT (2 μg/ml) and with or without one of several neutralizing anti-cytokine mAbs (anti-TGF-β1, anti-IL-10, anti-IFN-γ, anti-IL-2, or IgG1 control MOPC-21; all added to achieve a concentration of 10 μg/ml) for 8 days. In some experiments, B cells were positively selected by magnetic beads (Dynal, Lake Success, NY), and then they were cultured with Staphylococcus aureus Cowan I bacteria (SAC) (Pansorbin; Calbiochem, La Jolla, CA) and IL-2 (18) in the presence or absence of supernatants of self-MHC-reactive T cell cultures. At the end of culture, supernatants were collected, and IgG in the supernatant was assessed. ELISA was performed (18) using affinity-purified (mouse Ig-absorbed) goat anti-IgG Ab and alkaline phosphatase-conjugated goat anti-IgG Ab (Sigma, St. Louis, MO) in 96-well ELISA plates (Immulon 1; Dynatech, Chantilly, VA). After washing, colorimetric substrate p-nitrophenyl phosphate (Sigma) was added and A405 was determined using a microplate ELISA reader (MR5000; Dynatech).

Suppressor activity of T cell proliferative response
To examine the capacity of the SR T cell clones to suppress T cell proliferation, culture supernatants were obtained from cultures containing equal numbers (5 × 10^5 cells/well each in 24-well plates) of SR T cell and non-Tx cell clones, which had been preincubated with or without PWM for 8 h and washed three times to remove PWM. These supernatants were then added (at a 50% final concentration) to cultures containing fresh autologous or allogeneic CD4+ T cells (5 × 10^5 cells/well) under stimulation with immobilized anti-T cell clone and soluble anti-CD28 in 96-well plates. Alternatively, the supernatants were added to cultures of TT-1 cells (2.5 × 10^5 cells/well) under stimulation with TT in the presence of an equal number of fresh non-Tx cells. Cultures were terminated at 72 h, and cell proliferation was measured by the incorporation of 3H during the last 8 h of cultures.

Cell culture and ELISA for cytokine production
For assessing the capacity to produce cytokines, each T cell clone at a concentration of 5 × 10^5 cells/well was stimulated with immobilized anti-CD3 (10 μg/ml) and soluble anti-CD28 (1 μg/ml) or was cocultured with an equal number of resting or prestimulated non-Tx cells for varying periods with PWM (20 μg/ml) or CD40L (2 μg/ml) in a total volume of 1 ml in 24-well plates. In some experiments, non-Tx cells were further separated by magnetic beads into B cells using positive selection with anti-CD19-coated beads or into monocyte/macrophages (Mφ) using negative selection with mAbs to CD2, CD7, CD16, CD19, and CD56 (Monocyte Negative Isolation Kit; Dynal), and then they were prestimulated with PWM. The purified B cells obtained were >95% CD19+ cells, and the monocyte-Mφ were >90% CD14+ positive. In other experiments, T cell clones were stimulated with immobilized anti-CD3 (5 μg/ml) and anti-CTLA-4 (2 μg/ml) or MOPC-21 (IgG1; 5 μg/ml) and anti-CD40L (2 μg/ml). Culture supernatants were collected after 48 h or, in the case of TGF-β1, after 72 h. In cultures set up for the assay of TGF-β1 production, the FCS concentration was reduced to 3%, and wells containing culture medium without cells were included to measure “background” TGF-β1. Cytokines secreted into culture fluid were assayed by commercial ELISA kits according to the manufacturer’s protocol. IL-2, IFN-γ, IL-4, IL-5, and IL-10 were measured by Quantikine Immunoassay Kits (R&D Systems). TGF-β1 was measured with the TGF-β1 Emax immunoassay kit (Promega, Madison, WI). The total amounts of TGF-β1 (latent and bioactive TGF-β1) produced by cells were assessed by conversion of latent TGF-β1 into bioactive TGF-β1 by acid treatment. Briefly, culture supernatants were diluted with four volumes of Dulbecco’s PBS and then were acidified with 1 μl of 1N HCl for each 50 μl of diluted sample. Samples were incubated for 15 min at room temperature, neutralized by adding 1 μl of 1N NaOH per 50 μl of sample, and processed for sandwich ELISA according to the manufacturer’s instructions. The amounts of total TGF-β1 produced by cells were determined by subtracting the amount of TGF-β1 contained in the 3% FCS in the medium (−90–110 pg/ml) that had been incubated in wells without cells. In some experiments, non-T cells prestimulated for an indicated period with or without PWM or rCD40L were exposed to brefeldin A (5 μg/ml; Sigma) treatment for the last 3 h. The non-T cells prestimulated in this way were then irradiated and cocultured with SR or TT-specific clones in the presence or absence of rCTLA-4/FC (5 μg/ml) or rFab’2 anti-CTLA-3 (5 μg/ml).

Flow cytometric analysis
After prestimulation with PWM or rCD40L as described above, non-T cells were enriched for CD19+ B cells by magnetic beads (Dynal, Lake Success, NY) (18) and stained with the combinations of FITC anti-CD19 vs PE anti-HLA-DR, FITC anti-CD4 vs PE anti-HLA-DR, FITC anti-HLA-DR vs PE anti-CD80, and FITC anti-HLA-DR vs PE anti-CD86, respectively.
In addition, cells in cocultures of a SR T cell clone and non-T cells stimulated with PWM, CD40L, or immobilized anti-CD3 and anti-CD28 for 8 h were stained with FITC anti-CD4 vs PE anti-CTLA-4. The stained cells were then analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) using Cellquest software.

### Results

#### Proliferative characteristics of SR and Ag-reactive human T cell clones

Using previously described methods (4–6), SR and Ag (TT)-reactive T cells were generated in cultures of CD4+ T cells (from a TT preimmunized HLA-DR9+ individual) repetitively stimulated by TT in the presence of irradiated non-T cells (non-Tx cells). Subsequently, the expanded T cells were cloned by limiting dilution, and the specificity of each clone was characterized by proliferation assay. As shown in the experiment displayed in Table I (which is representative of four similar experiments), this procedure gives rise to an array of T cell clones, as defined by proliferative responses to non-Tx alone or to non-Tx plus TT. Eight of 18 of the generated clones, exemplified by clone AT-1, exhibited some proliferation when restimulated with non-Tx cells alone as well as enhanced proliferation when stimulated with non-Tx plus TT; these clones thus appeared to be both SR and Ag-reactive to varying degrees. In contrast to these “mixed” clones, two clones of the 18 clones generated, exemplified by clones TT-1 and TT-2, exhibited little or no proliferation when restimulated with non-Tx cells, but they exhibited robust proliferation when restimulated with non-Tx cells plus TT; these cells were thus strictly Ag-reactive T cells. In addition, another two clones of the 18 clones generated, exemplified by clones SR-1 and SR-2, exhibited equally strong proliferation when restimulated with non-Tx cells or with non-Tx cells plus TT; therefore, these cells were self-MHC-reactive cells. Finally, six of 18 clones, exemplified by BT-2, responded poorly to either non-Tx cells or non-Tx cells plus TT, and they eventually died out. Overall, these data, plus those obtained from three other similar experiments, indicate that ~11% of the clones generated under these culture conditions were self-MHC-reactive clones similar to SR-1 and SR-2.

Important confirmation of the view that the self-MHC-reactive clones exemplified by SR-1 and SR-2 are indeed self-MHC-reactive clones came from the fact, shown in Table II, that these clones also proliferate well when stimulated by non-T cells in a FCS-free medium (X-VIVO 20), which contains human serum albumin and 15% autologous human serum rather than FCS. In addition, as also shown in Table II, a self-MHC-reactive T cell clone, exemplified by SR-3, could be generated in cultures of cells repetitively stimulated by TT in which the first two cycles of stimulation were conducted in FCS-free medium (X-VIVO 20). Finally, the self-MHC-reactive T cell clones SR-1 and SR-2 did not proliferate when cocultured with non-Tx cells obtained from three individuals with different HLA-DR types (allogeneic T cells), and proliferation induced by self non-Tx cells was completely inhibited by the presence of anti-HLA-DR in the culture (data not shown).

#### Regulatory function of self-MHC-reactive T cells with respect to autologous B cell Ig production

In previous studies it was shown that SR T cells have dual regulatory function with respect to their capacity to regulate (help or suppress non-T cell (B cell)) Ig production. This property of SR T cells was confirmed in the present study. Thus, as shown in Fig. 1A–C, in the absence of a cell stimulant such as PWM, the self-MHC-reactive T cell clones induced autologous B cells present in the same culture to produce IgG, whereas in the presence of PWM, such induction of B cells was greatly decreased. That this decrease of Ig production was in fact a result of suppression (rather than lack of help) was shown in previous studies demonstrating that appropriately stimulated self-MHC-reactive T cell clones suppress Ig production in cultures of allogeneic T and B cells stimulated by PWM (5, 6) and that, in the present study, help is restored in cultures of SR T cell clones and autologous B cells cultured with PWM by addition of Abs to suppressor cytokines (see below). Another polyclonal B cell stimulant with a more defined mechanism of action, CD40L-trimer, also induced non-T cells that elicited self-MHC-reactive T cell clone suppressor activity. This was shown best in the studies depicted in Fig. 2, in which non-T cells alone were prestimulated with CD40L-trimer for 48 h, irradiated, and cocultured with self-MHC-reactive T cell clones (SR-2 and SR-3) and fresh autologous B cells for 8 days. The need for preincubation in this instance probably relates to the fact that CD40L require more time to induce expression of stimulatory surface molecules on non-T cells than does PWM (see below). Finally, as shown in Fig. 1, D and E, in contrast to the above findings with the SR T cell clones, the Ag-reactive T cell clones TT-1 and TT-2 did not induce autologous B cells to produce Ig unless TT was added to the culture and, perhaps more importantly, these clones did not significantly suppress Ig production when PWM (or CD40 ligand-trimer) was added to the culture.

In an initial investigation of the mechanism of the suppressor effect of self-MHC-reactive T cells, we added several neutralizing anti-cytokine mAbs to cultures of self-MHC-reactive T cells and non-T cells containing PWM. As also shown in Fig. 1, A–C, we found that

### Table I. Proliferative responses of self-MHC-reactive T cell clones stimulated by non-Tx cells in non-FCS-containing medium

<table>
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<th>TT and non-Tx</th>
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<tbody>
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<td>276</td>
<td>437</td>
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<tr>
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<td>531</td>
<td>409</td>
<td>334</td>
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<tr>
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<tr>
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<td>377</td>
<td>299</td>
<td>31,349</td>
</tr>
<tr>
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</tr>
<tr>
<td>Non-Tx alone</td>
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<td>344</td>
<td>23,668</td>
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<table>
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<th>Non-Tx</th>
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<tbody>
<tr>
<td>SR-1</td>
<td>510</td>
<td>437</td>
</tr>
<tr>
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<tr>
<td>SR-3</td>
<td>443</td>
<td>410</td>
</tr>
<tr>
<td>Non-Tx alone</td>
<td>307</td>
<td>276</td>
</tr>
</tbody>
</table>

* T cell clones (2.5 × 10⁴ cells/well) were cocultured with non-Tx cells (5 × 10⁴ cells/well) in the presence or absence of TT (2 μg/ml) for 72 h.
* [3H]Thymidine incorporation was measured on day 3. Results are expressed as the mean cpm of triplicate cultures.
addition of anti-TGF-β completely restored the helper function of the self-MHC-reactive T cell clones SR-1, SR-2, and SR-3 in cultures with non-T cells containing PWM. In contrast, anti-IL-10 and anti-IFN-γ did not reverse the suppression, and in fact, addition of anti-IL-10 (in cultures stimulated with TT alone) led to decreased Ig production even in the absence of suppression, possibly because IL-10 is necessary for B cell differentiation. The addition of anti-IL-2 to cultures of the same cells in the absence of PWM abolished the helper effect, indicating that the helper function was IL-2 dependent. Similarly, as shown in Fig. 2, addition of anti-TGF-β to cultures of self-MHC-reactive T cell clones stimulated by non-T cells pre-exposed to rCD40L-trimer also abolished the suppressor activity of the clones, in this case with respect to fresh non-T cells. Taken together, these studies show that self-MHC-reactive, but not Ag-reactive, T cells act as suppressor cells (with respect to B cell Ig production) in the presence of activated B cells and that such suppression is mediated by the secretion of TGF-β.

In a final series of studies, we also determined whether B cells are the target of the suppressive activity of the self-MHC-reactive T cell clones. In these studies, culture supernatants were collected from cocultures of self-MHC-reactive T cell clones SR-1 or SR-3 cells prestimulated with or without PWM and added to cultures of highly purified PBL B cells or a B cell line (CL-1) in which the B cells were stimulated by SAC and IL-2. As shown in Fig. 3, the supernatant obtained from SR-1 or SR-3 cells suppressed Ig synthesis by activated purified B cells, indicating that the self-MHC-reactive SR T cell clones have a direct suppressive effect on B cells. This conclusion was supported by the fact that a similar suppressive effect was observed in cultures of CL-1 B cells (data not shown).

**Regulatory function of SR T cell clones with respect to T cell function**

In further studies of the regulatory function of self-MHC-reactive T cell clones, we determined whether these clones could down-regulate T cell proliferation as well as B cell Ig production. Because effects on the proliferation of indicator T cells cannot be reliably measured in cocultures of regulatory T cells and indicator T cells if the regulatory T cells must themselves be activated (and thus induced to proliferate)
to manifest regulatory function, we determined whether supernatants obtained from cultures of activated SR T cell clones could inhibit T cell proliferation in separate cultures of various indicator T cells. This experimental design was based on the observation that the stimulation of the SR T cell clones with PWM-prestimulated non-T cells for optimal time periods results in the secretion of cytokines with suppression activity (see further discussion below). As shown in Fig. 4, A and B, supernatants harvested from cultures of the self-MHC-reactive T cell clone SR-1 stimulated by PWM or CD40L-prestimulated autologous non-Tx cells led to moderate (but highly reproducible) inhibition of proliferation of anti-CD3/anti-CD28-stimulated allogeneic and autologous CD4⁺ T cells, whereas the same cell clone activated by PWM-prestimulated allogeneic non-Tx cells did not inhibit. Furthermore, as shown in Fig. 4, C and D, the supernatants generated from cultures of the self-MHC-reactive T cell clone SR-1 with the PWM or CD40L-prestimulated autologous non-Tx cells also inhibited TT-induced proliferation of the Ag-reactive T cell clone TT-1, and such inhibition was reversed by the addition of anti-IL-10, anti-TGF-β, or the combination of anti-IL-10 and anti-TGF-β to the culture. These data show that self-MHC-reactive T cell clones also down-regulate T cell responses, albeit not as completely as they down-regulate B cell responses. In addition, they show that such down-regulation is mediated via the secretion of both IL-10 and TGF-β. In separate studies, we found that the proliferation of the self-MHC-reactive T cell clone SR-1 was also decreased when cocultured with prestimulated autologous non-Tx cells compared with proliferation when cocultured with unstimulated non-T cells, suggesting that self-MHC-reactive T cells are also sensitive to the autocrine TGF-β and IL-10 production (data not shown).

**Cytokine production patterns of SR and Ag-reactive T Cell clones**

In further studies related to the mechanism of the suppressor effect of self-MHC-reactive T cells, we determined the cytokine production of such cells as well as the cellular interactions necessary for such cytokine production. Initially, we determined the cytokines produced by the various T cell clones after stimulation with the polyclonal stimulants anti-CD3/anti-CD28 (see Materials and Methods). As shown in Table III, both of the Ag-reactive T cell clones TT-1 and TT-2 produced substantial amounts of IL-2 and IFN-γ, but not IL-4. On the other hand, although both of the self-MHC-reactive T cell clones, SR-1 and SR-2, exposed to the same stimulants produced comparable amounts of IFN-γ, they produced considerable amounts of IL-2 when stimulated with non-Tx/PWM but not when stimulated with anti-CD3/anti-CD28. Next, we determined cytokine production by SR T cell clones and Ag-reactive T cell clones after stimulation with non-Tx cells prestimulated by PWM or CD40L, i.e., stimulants similar to those encountered by self-MHC-reactive and Ag-reactive T cell clones in cultures in which the former manifested suppressor activity. In these studies, the length of time the non-Tx cells were prestimulated before coculture with the T cell clones was varied from 0 to 24 h (or in some cases from 0 to 48 h) to determine the time of peak expression of stimulatory Ags on the surface of cells in the non-Tx cell population. As shown in Fig. 5, stimulation of Ag-reactive TT-1 T cells with prestimulated non-Tx cells led to only a slight increase in IL-10 or TGF-β production, whereas stimulation of self-reactive SR-1 and SR-2 cells with prestimulated non-Tx cells led to substantial increases in both IL-10 and TGF-β production, with the former peaking with non-Tx cells preactivated for 4 h and the latter peaking with non-Tx cells prestimulated for 8 h. Similar studies of cytokine production were conducted with CD40L-prestimulated non-Tx cells, but in this case, neither the Ag- nor the self-MHC-reactive T cell clones produced IL-10 or TGF-β. However, when we prestimulated non-Tx cells for 48 h with CD40L, the non-Tx cells now stimulated self-MHC-reactive T cell clones to produce high levels of TGF-β1 and IL-10 (data not shown).

A possible reason for the different cytokine secretion patterns exhibited by self-MHC-reactive and Ag-reactive T cell clones noted above is that two types of clones are being subjected to different levels of stimulation. To address this possibility we determined the capacity of Ag-reactive clones to secrete TGF-β1 when stimulated by a range of TT concentrations. As shown in Fig. 6, TT-1 and TT-2 secreted low levels of TGF-β1 (compared with SR-1, SR-2, and SR-3) when stimulated over a wide range of TT

**FIGURE 2.** Suppression of IgG production of fresh autologous B cells cocultured with the self-MHC-reactive T cell clones (SR-2 or SR-3) and non-Tx cells prestimulated with rCD40L. Non-T cells were prestimulated for 48 h with rCD40L (5 μg/ml), washed, irradiated, added to equal numbers of fresh autologous B cells (5 × 10⁶ cells) and SR-2 or SR-3 (5 × 10⁴ cells), and then incubated for 8 days in the presence of anti-TGF-β mAb or control IgG1 (10 μg/ml). IgG production in culture supernatant was determined by ELISA. The results shown represent the mean ± SD (ng/ml) of triplicate cultures.

**FIGURE 3.** Effect of culture supernatants of self-MHC-reactive clones (SR-1 or SR-3) cocultured with non-Tx cells or non-Tx/PWM on IgG production by B cells stimulated with SAC and IL-2. Non-T cells were prestimulated with or without PWM for 8 h, washed, irradiated, and cocultured with or without SR-1 or SR-3 T cells for another 48 h. Culture medium alone or supernatants from the above cultures were then added to the fresh CD19⁺ B cells at a concentration of 50% and were cultured for 8 days in the presence of SAC (1:20,000) and IL-2 (30 U/ml). IgG production in culture supernatant was determined by ELISA. The results shown represent the mean ± SD (ng/ml) of triplicate cultures.
concentrations that induce a level of proliferation equal to or greater than exhibition by SR-1, SR-2, and SR-3 (compare with Tables I and II). Thus, we conclude that the difference in TGF-β1/IL-10 between self-MHC-reactive and Ag-reactive clones is not due to inadequate activation of the latter.

In a final series of studies relating to cytokine production by self-MHC-reactive T cells, we determined which of the cells in the non-T cell population was responsible for stimulation of the self-MHC-reactive T cells. As shown in Fig. 7, cocultures of both the self-MHC-reactive T cell clones SR-1 and SR-2 with PWM-stimulated non-T cells as well as with purified B cells led to substantial TGF-β1 secretion, whereas coculture with purified monocytes/Mφ did not. Therefore, these studies show that B cells themselves are capable of inducing self-MHC-reactive T cells (20) to produce TGF-β1. Whether monocytes/Mφ also have this property awaits studies in which the Mφ are stimulated with a range of known Mφ stimulants.

Overall, these cytokine production studies established that self-MHC-reactive and Ag-reactive T cell clones are phenotypically different with respect to cytokine production. Thus, although the former exhibits the cytokine profile of a Th3 or Tr1 T cell, the latter exhibits the cytokine profile of a Th1 T cell.

Expression of costimulatory molecules on the surface of non-T cells preactivated by PWM or rCD40L and on SR T cell clones activated by PWM or rCD40L

In the next set of studies, we focused on the cellular interactions between activated non-Tx cells and SR T cell clones that resulted in suppressor cytokine production. In this regard, it is known that PWM activates purified non-T cells (as well as purified B cells) (21, 22) so that the question arose as to whether such activation results in increased expression of B7-1 (CD80) and B7-2 (CD86) (23), which then facilitates interaction with T cells via CTLA-4, a recently described signaling pathway of TGF-β production (24). In an initial exploration of this question, we performed flow cytometric analyses of non-Tx cells (particularly B cells) after preactivation with PWM or rCD40L (25) as well as parallel analyses of T cell clones stimulated with PWM or CD40L to determine the surface expression of B7 and CTLA-4 on these cells at various times after stimulation.

As shown in Fig. 8A, the expression of B7-1 (CD80) or B7-2 (CD86) on CD19-positive B cells after PWM stimulation was highest at 8 h after initiation of stimulation and then declined to nearly baseline levels at 24 h after initiation of stimulation. Thus, as shown in Fig. 8B, the time of maximum coexpression of HLA-DR and B7-1/B7-2 corresponds to time that non-Tx cells prestimulated with PWM are most capable of inducing TGF-β production. As shown in Fig. 8C, similar studies conducted on rCD40L-stimulated non-T cells showed that in this case expression of CD80 and, more prominently, expression of CD86 occurred more slowly and did not peak until 48 h after initiation of stimulation. This finding explains why non-Tx cells preactivated with rCD40L-trimer for only 8 h did not induce suppressor cytokine production in SR T cell clones, whereas the addition of rCD40L-trimer into longer-term cultures of SR T cells and non-Tx cells did lead to suppression of PWM-stimulated Ig secretion.
In parallel studies, we determined the expression of CTLA-4 (and CD28) on T cell clones after PWM or CD40L stimulation. As shown in Fig. 9 and in data not shown, whereas before stimulation none of the T cell clones expressed CTLA-4 with or without non-T cell coculture (during 0–72 h), after 8 h of stimulation with PWM, 24 h of stimulation with anti-CD3/anti-CD28, and 48 h of stimulation with CD40L, the self-MHC-reactive T cell clones SR-1 and SR-2 induced CTLA-4 expression. On the other hand, the Ag-reactive T cell clone TT-1 expressed CTLA-4 after stimulation with anti-CD3/anti-CD28 after 24 h but did not express CTLA-4 after stimulation with PWM or rCD40L (in the absence of TT). In contrast, CD28 was present on the cell surfaces of the self-MHC-reactive T cell clones and Ag-specific clones before stimulation and was not increased by stimulation (data not shown). We also observed that the CD40L was not expressed on SR-1 and SR-2 cells unless it was stimulated with PWM or with anti-CD3/anti-CD28 (data not shown) as previously reported (26).

The effect of CTLA-4/Fc on production of IL-10 and TGF-β by SR T cell clones

Having established that PWM-preactivated B cells express B7-2 and SR T cell clones express CTLA-4 at a time course consistent with suppressor cytokine production, it remained for us to explore whether the interaction between these costimulatory molecules was essential for such cytokine production. Accordingly, we determined the ability of PWM-prestimulated non-Tx cells to induce IL-10 and TGF-β1 when these cells were cocultured in the presence of soluble CTLA-4/Fc, a molecule with a high binding affinity for both forms of B7, which therefore blocks B7 interaction with cell-bound CTLA-4 or CD28. As shown in Fig. 10, whereas coculture of PWM-prestimulated non-Tx cells with either of the SR T cell clones, SR-1 or SR-2, in the absence of CTLA-4/Fc again led to the production of substantial amounts of TGF-β1, coculture in the presence of CTLA-4/Fc led to a marked inhibition of such production. However, somewhat surprisingly, addition of CTLA-4/Fc to the coculture did not inhibit production of IL-10. Finally, coculture of the Ag-reactive T cell clone TT-1 with PWM-preactivated non-Tx cells or TT and non-T cells led to only marginal TGF-β1 production that was only slightly affected by the addition of CTLA-4/Fc.

In the final set of studies, we examined the mechanism of induction of TGF-β1 as it relates to interactions between the B7 costimulatory molecules and CTLA-4 or CD28. In an initial series of studies, we added F(ab')2 anti-CTLA-4 (8H5), a mAb which blocks the binding of B7-1 and B7-2 to CTLA-4 without affecting CD28 signaling, to cultures of self-MHC-reactive T cell clones and non-T cells prestimulated by PWM. As shown in Fig. 11, we found that this blocking mAb prevented TGF-β1 production but had no effect on IL-10 production. Furthermore, down-regulation of TGF-β1 production by this mAb was accompanied by a reciprocal up-regulation of IFN-γ production. In further studies, we cultured self-MHC-reactive T cells (SR-1 and SR-2) or Ag-reactive T cells (TT-1) in wells coated with anti-CTLA-4 mAb (BN13) and anti-CD3 to determine whether direct stimulation of these clones via CTLA-4 leads to TGF-β1 production. As shown in Fig. 12, CD28 induces marginal TGF-β1 production in both types of T cell clones.

It should be noted that stimulation of self-MHC-reactive T cells by anti-CD3 induces relatively low TGF-β production compared with stimulation by non-T cells, and in addition, under these conditions there was no significant difference between the amounts of

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### Table III. Cytokine production by self-MHC-reactive and TT-specific Th1 clones

<table>
<thead>
<tr>
<th>T Cell Clone Stimulated with</th>
<th>IL-2</th>
<th>IFN-γ</th>
<th>IL-4</th>
<th>IL-5</th>
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<tr>
<td><strong>Self-MHC-reactive clones</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD3/anti-CD28</td>
<td>20</td>
<td>6230</td>
<td>&lt;40</td>
<td>&lt;40</td>
</tr>
<tr>
<td>Non-Tx PWM</td>
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<td>4400</td>
<td>&lt;40</td>
<td>&lt;40</td>
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<tr>
<td>SR-2</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>4630</td>
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<td>&lt;40</td>
</tr>
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<td>Non-Tx PWM</td>
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<td>6810</td>
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<td>&lt;40</td>
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<td></td>
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<td>&lt;40</td>
<td>680</td>
</tr>
<tr>
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<td>8360</td>
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<td>&lt;40</td>
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<tr>
<td><strong>Ag-specific clones</strong></td>
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</tr>
<tr>
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</tr>
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<td>&lt;40</td>
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<td>Non-Tx/PWM alone</td>
<td>20</td>
<td>&lt;40</td>
<td>&lt;40</td>
<td>&lt;40</td>
</tr>
</tbody>
</table>

* T cell clones were stimulated with immobilized anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) mAbs or with PWM-prestimulated (8 h) non-Tx cells. Supernatants were collected after 48 h of culture.  
* Cytokine production was determined by ELISA. Results are expressed in pg/ml from duplicate experiments.
TGF-β1 produced by self-MHC-reactive T cell clones and the Ag-reactive T cell clones. This result is parallel to previous studies by Chen et al. (24), which showed that Ag-reactive T cells can also be costimulated via CTLA-4 to produce TGF-β1, albeit not at the high level of TGF-β1 produced by self-MHC-reactive T cells stimulated by non-T cells (see further discussion below).

In summary, these CTLA-4 blocking and stimulation studies, taken in conjunction with the above-described data on B7 and CTLA-4 expression, establish that the induction of TGF-β1 production by self-MHC-reactive T cells is at least in part dependent on costimulation via CTLA-4.

Discussion
As shown in previous studies, self-MHC-reactive (autoreactive) T cells regularly appear in cultures of human T cells being stimulated
with exogenous Ag (in this case, TT) (4, 5). This indicates that T cells with self-reactivity presumably reacting to an as yet undefined cohort of self-Ags in the MHC groove are a normal part of the T cell population (27–30) and are unintentionally expanded during ordinary Ag-driven T cell priming. However, the present studies indicate that these T cells are very different from the exogenous Ag-specific T cells with which they codevelop, in that they have the properties of regulatory T cells (Th3 T cells or Tr1 T cells) that have been shown in previous studies to regulate autoimmune inflammation (11, 12, 17).

In our initial studies of the function of the self-MHC-reactive T cell clones, we found that during or after culture with activated

**FIGURE 9.** Flow cytometric analysis of CTLA-4 expression on SR-1, SR-2, and TT-1 T cell clones (5 × 10⁵ cells/ml) stimulated with immobilized anti-CD3 and soluble anti-CD28 without non-T cells for 24 h, with PWM for 8 h, or with rCD40L for 48 h in the presence of non-T cells. T cell clones were detected as CD4⁺ because non-T cells alone contained <2% CD4⁺ cells.

**FIGURE 10.** Effect of CTLA-4/Fc on the production of TGF-β1 (A) or IL-10 (B) by SR T cell clones (SR-1 and SR-2) or TT-specific (TT-1) T cell clones (in absence or presence of TT) cocultured with or without (open bar) autologous non-Tx cells. Non-T cells (5 × 10⁵ cells/ml) were stimulated with PWM for 8 h (A) or 4 h (B, hatched bar), were stimulated with PWM for 8 h (A) or 4 h (B) in the presence of rCTLA-4/Fc (5 μg/ml; filled bar), or were unstimulated (gray bar), washed, irradiated, and then cocultured with an equal number of the indicated T cell clones. Cells were cultured for 72 h for TGF-β1 production and for 48 h for IL-10 production. TGF-β1 and IL-10 in cell supernatants were determined by ELISA. The data shown are representative of three independent experiments.
non-T cells, these clones suppress both B cell Ig production and T cell proliferation. With respect to B cell suppression, such suppression is directly attributable to the secretion of TGF-β because inclusion of anti-TGF-β into the culture converts the SR T clones into helper cells that support (help) Ig production. On the other hand, the suppression of T cell proliferation is attributable not only to TGF-β but also to IL-10, a cytokine also secreted in large amounts by the self-MHC-reactive T cells. These results with self-MHC-reactive T cells contrast with those obtained with Ag-reactive T cells. Thus, although both self-MHC-reactive and Ag-reactive T cells produce large amounts of IFN-γ when activated by anti-CD3/CD28, Ag-reactive T cells produce only modest (non-suppressive) amounts of TGF-β1 or IL-10 when stimulated by specific Ag in a manner analogous to self-MHC-reactive T cell stimulation by non-T cells. This lack of TGF-β1 production cannot be attributed to lack of stimulation because it was not seen at Ag concentrations inducing optimal proliferation, i.e., proliferation that was equivalent to or exceeded that seen in the stimulation of self-MHC-reactive T cells.

In further studies, we established that the suppressor function of self-MHC-reactive T cells, at least that because of TGF-β secretion, was mediated by stimulation of the T cells via CTLA-4. This was shown by the fact that maximal TGF-β secretion correlated with expression of B7 (particularly B7-2) and, more importantly, that TGF-β secretion was blocked by the presence of a nonstimulatory F(ab)2 anti-CTLA-4 mAb. Contrariwise, cross-linking (stimulatory) anti-CTLA-4 Ab (but not anti-CD28 Ab) induced anti-CD3-activated self-MHC-reactive T cells to produce TGF-β1 in the absence of activated B cells. It is of interest that IL-10 secretion by the SR T cell clones was not similarly related to CTLA-4 signaling, because IL-10 secretion was not blocked by CTLA-4/Fc or anti-CTLA-4 mAb. Recently, an inducible costimulator molecule termed ICOS has been identified that is involved in the superinduction of IL-10 (31). This costimulator molecule does not contain a structural motif that is involved in the CD28/CTLA-4 interaction with B7-1/B7-2 and therefore may be a discrete co-stimulator involved in the induction of IL-10 secretion by self-MHC-reactive T cells.

The role of the CTLA-4 costimulatory molecule in the negative regulation of immune responses is now well-established (32). It has been shown that cross-linking of this molecule leads to suppression of IL-2 production and inhibition of cell cycle progression, probably through the activation of a CTLA-4-associated phosphatase, Src homology 2-containing tyrosine phosphatase 2, which dephosphorylates TCR-ζ (33). On this basis, costimulation...
of cells via this molecule is now thought to be a prerequisite for the induction of T cell anergy and/or T cell deletion. A second function of CTLA-4 in negative regulation is inherent in recent studies and in the present study showing that cross-linking of this molecule on T cells leads to TGF-β production (24, 34). Thus, in the present study we showed that Ag-reactive T cells, when stimulated by anti-CD3 and cross-linking anti-CTLA-4 Ab, produce increased TGF-β1 even though they do not produce TGF-β1 under normal stimulation by APC and Ag. A somewhat reversed situation occurs with self-MHC-reactive T cells, where anti-CD3/anti-CTLA-4 results in increased TGF-β1 production, but such production is considerably lower than that obtained by stimulation by self-Ag. Thus, the picture that emerges is that CTLA-4 provides signaling pathways for TGF-β1 production but that this pathway has a higher capacity to induce TGF-β1 in self-MHC-reactive T cells compared with Ag-reactive T cells (in our study, TT-specific T cell clones).

In addition to its role as the initiator of a cellular program leading to suppressor cytokine production through interaction with CTLA-4, B7 costimulatory ligands may have a more fundamental role as activators of normally anergic SR T cells. As shown in our previous studies of the autoreactive T cell clone known as MTC-1 as well as in the present study of a similar clone, SR-1, the ability of the cell both to provide help for B cells and to suppress B cells is MHC-restricted, at least in the inductive phase, because neither of these functions is observed with respect to allogeneic B cells, even in the presence of PWM. Thus, there can be no question that the self-MHC-reactive T cell clones under study are in fact reacting with self-MHC, most likely a self-MHC containing a self-peptide in the MHC class II groove (30, 35). Recent studies of the origin of SR (autoreactive) T cells show that these cells recognize Ag with sufficiently low affinity to escape negative selection in the thymus. They are functionally anergic when released into the periphery and thus do not cause disease unless stimulated under particular conditions (36, 37). One such condition relative to the present findings may be the presence of cells expressing high levels of B7 that are thus capable of providing a potent costimulatory signal to T cells via CD28 (38). The latter possibility is suggested by studies showing that transgenic mice carrying a B7-2 transgene under the control of an insulin promoter (and thus expressing B7-2 in islet cells) ultimately develop a lymphoid infiltrate in the vicinity of the islets, whereas mice transgenic for B7-1 under the control of the same promoter do not. Furthermore, if the intensity of stimulation of the autoreactive cells, i.e., islet cell Ag-reactive cells, is increased by increasing their level of TCR signaling by the presence of a second transgene giving rise to MHC-class II expression in the islet cell, this distinction between B7-2 and B7-1 disappears (39). Thus, in this instance as well as in other instances (20), the level of B7 expression may determine whether an autoreactive clone will respond to a self-Ag.

The possible relation of self-MHC-reactive T cells producing suppressor cytokines to autoimmune disease has recently been explored in several animal models of autoimmunity (15, 16, 40). In one such model in which Th2 T cell-mediated autoimmunity is elicited in Brown Norway rats by administration of HgCl2, the T cells inducing disease have been shown to be reactive to self-MHC class II molecules and/or a self-peptide presented by the latter. In contrast, treatment of Lewis rats with HgCl2 also elicited SR T cells, but in this case the T cells prevented rather than caused autoimmune disease when administered to rats undergoing induction of experimental autoimmune encephalitis or HgCl2-induced autoimmunity (40). Although the autoreactive T cells mediating disease in the HgCl2 autoimmune model produced Th2 cytokines, the autoreactive T cells preventing disease produce IFN-γ and high levels of TGF-β, and it is the latter cytokine that was responsible for disease prevention because the administration of anti-TGF-β along with the autoreactive cells abrogated prevention (16). These and similar studies in animal models of diabetes establish that self-MHC-reactive T cells producing TGF-β can act as counter-regulatory cells in autoimmune states (15).

A second context in which SR T cells producing suppressor cytokines may relate to autoimmunity (and in this case inflammation as well) relates to recent findings concerning the regulatory activity of T cells arising in the mucosal immune system after oral Ag administration (11, 12, 41, 42). In these studies it has been shown that cells termed Th3 cells and producing high amounts of TGF-β are generated by oral administration of Ag and that such cells are active in the prevention of experimental allergic encephalitis and other autoimmune states. In addition, it has been shown in the SCID adoptive transfer model of chronic colonic inflammation as well as in the chronic colonic inflammations developing in trinitrobenzene sulfonic acid-colitis or the colitis associated with IL-2 deficiency that cells producing TGF-β can also prevent or even reverse the colitis (14, 43). Finally, it has been shown that T cell clones developed in the presence of IL-10, now termed Tr1 cells, produce IL-10 and/or TGF-β and prevent colonic inflammation in SCID mice reconstituted with naive CD4+ T cells that otherwise induce colitis (17). Taken together, these studies draw attention to the possibility that counterregulatory cells controlling autoimmunity (15, 16, 39, 44) or inflammation of the gastrointestinal tract are actually self-MHC-reactive T cells. However, further work will be necessary to substantiate this possibility, including the isolation of regulatory cells occurring during (after) the induction of autoimmune inflammation of the specificity of such cells.

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References


