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CD4⁺CD25⁺ T Cells Facilitate the Induction of T Cell Anergy

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T cell anergy is one of the mechanisms thought to act in the periphery to ensure tolerance to self. The term anergy was first used to describe T cell clones rendered unresponsive to subsequent restimulation by first activating them through the TCR (signal 1) without appropriate costimulation (signal 2) (1) or, more recently, using an altered peptide ligand for activation (2). The characteristic feature of this induced unresponsiveness was the inability of the anergic T cells to proliferate or produce IL-2 following subsequent optimal restimulation (3). Anergic T cells appeared to have a defect in signaling pathways upstream of transcription of the IL-2 gene (4–6). Additional studies demonstrated the presence of cis-dominant negative regulatory elements affecting IL-2 transcription (7, 8). The recent finding of increased general receptor of phosphoinositides-1 expression indicated that broader genetic changes may accompany the induction and maintenance of the anergic phenotype (9). Our laboratory identified a gene related to anergy in lymphocytes (GRAIL) as a novel gene that is preferentially expressed in anergized T cells. Its translated protein product has high homology to the Drosohila protein Golgi (10) and other zinc ring-finger-containing proteins. Overexpression of GRAIL in T cells dramatically reduced the transcription of IL-2 (2).

Anergy prevents the clonal expansion of T cells under stimulatory conditions. A similar block in proliferation was observed when activated murine CD4⁺CD25⁺ responder T cells were cocultured with activated CD4⁺CD25⁺ suppressor T cells in vitro. CD4⁺CD25⁺ suppressor T cells comprise 5–10% of CD4⁺ T cells in naive adult mice (11). Their special immunoregulatory properties were first described by Sakaguchi et al. (12), who showed that the adoptive transfer of CD4⁺CD25⁺ T cells could prevent autoimmune diseases that develop in mice thymectomized on day 3 of life. Similar findings were later obtained in adoptive transfer models of gastritis (13), diabetes (14), and colitis (15). Suppression of CD4⁺CD25⁺ T cells in vitro by CD4⁺CD25⁺ T cells required activation of the CD4⁺CD25⁺ T cells as well as direct cell-cell contact. Interestingly, suppression could be abrogated by increasing costimulation via CD28 or by the addition of exogenous IL-2 (16).

The close relationship of costimulation, IL-2 production, and proliferation in the CD4⁺CD25⁺ model of anergy was highly reminiscent of anergy systems and prompted us to look more closely at the suppressed CD4⁺CD25⁺ T cells. Using a system that substitutes beads coated with anti-CD3 and anti-CD28 Abs for APC function, we demonstrated that, upon activation and coculture in vitro, CD4⁺CD25⁺ T cells rendered CD4⁺CD25⁻ T cells anergic. Once unresponsiveness was established, the presence of the CD4⁺CD25⁺ T cells was no longer required. After removal of the suppressors from coculture, the CD4⁺CD25⁺ T cells proliferated only when exogenous IL-2 was added during restimulation. Suppression by CD4⁺CD25⁺ T cells thus appears to be an alternative mechanism for the induction of anergy in Ag-reactive CD4⁺ T cells in the presence of costimulation.

Materials and Methods

Mice

DBA/2J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were kept under specific pathogen-free conditions in the Department of Comparative Medicine at Stanford University School of Medicine (Stanford, CA).

The Abs

Purified anti-CD3 (145-2C11), anti-CD28 (37.51), anti-CTLA-4 (UC10-4F10-11), anti-CD80 (16-10A1), anti-CD86 (PO3), anti-FcR (2.4G2), and FITC-conjugated anti-CD4 (GK1.5) were purchased from BD PharMingen (San Diego, CA). Biotinylated anti-CD25 (PC61) and PE-conjugated streptavidin were purchased from Caltag Laboratories (Burlingame, CA).
Media

Cell preparation for FACS analysis and sorting was performed in Dulbecco’s PBS (Life Technologies, Gaithersburg, MD) plus 5% heat-inactivated FBS (HyClone Laboratories, Logan, UT). For proliferation assays, lymphocytes were cultured in RPMI-C, i.e., RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 1% nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin plus 100 µg/mL streptomycin, 2 mM l-glutamine (all obtained from Life Technologies), and 50 µM 2-ME (Sigma, St. Louis, MO).

Coating of latex beads

The 5-µm latex beads (Interfacial Dynamics, Portland, OR) were coated with 2.5 µg/mL anti-CD3 (145-2C11; BD Pharmingen) and varying amounts of anti-CD28 (37.5; BD Pharmingen) in PBS for 90 min at 37°C. The coated beads were then washed in RPMI-C, resuspended in RPMI-C, and stored at 4°C until use.

Preparation of CD4+CD25+ cells

Female mice, 6–8 wk old, were sacrificed, spleen and lymph nodes (inguinal, axillary, brachial, submandibular, mesenteric, pancreatic, and paraaortic) were harvested, and single-cell suspensions were prepared. CD4+ cells were then enriched using anti-CD4 magnetic MicroBeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. After staining with FITC-conjugated anti-CD4 and biotinylated anti-CD25 followed by PE-conjugated streptavidin, cells were sorted into CD4+CD25− and CD4+CD25+ populations.

Cell sorting

Cells were sorted on a FACStar cell sorter (BD Biosciences, Mountain View, CA) in the Shared FACS Facility, Center for Molecular and Genetic Medicine at Stanford University.

In vitro proliferation assay

A total of 12,500 sorted cells/well was incubated with equal numbers of Ab-coated beads in RPMI-C in a 96-well U-bottom plate (BD Biosciences). Beads were coated with 2.5 µg/mL anti-CD3 and 1.25 µg/mL anti-CD28, unless stated otherwise. Cells were pulsed with 1 µCi [3H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) per well for the last 15 h of the 72-h culture period. Cells were then harvested onto filter membranes using a Wallac harvester (PerkinElmer Life Sciences, Gaithersburg, MD), and the amount of incorporated [3H]thymidine was measured with a Wallac Betaplate counter (PerkinElmer Life Sciences). For coinubication experiments, CD4+CD25− and CD4+CD25+ cells were mixed at a 1:1 ratio and stimulated with equal numbers of Ab-coated beads. Where indicated, recombinant murine IL-2 (R&D Systems, Minneapolis, MN) was added at 50 U/mL.

Labeling of cells

For experiments requiring the separation of CD4+CD25− and CD4+CD25+ cells after coinubication, the CD4+CD25+ cells were labeled with CFSE (Molecular Probes, Eugene, OR). Sorted cells were incubated in PBS with 5 µM CFSE for 10 min at 37°C, washed, and resuspended in RPMI-C.

Analysis of resorted cell populations

Coincubated CD4+CD25− and CFSE-labeled CD4+CD25+ cells (50,000 cells each type with equal numbers of beads per well of a 96-well U-bottom plate) were harvested, washed, resuspended in FACs buffer with propidium iodide (0.1 µg/mL, Sigma), and sorted based on their CFSE signal. CD4+CD25− cells stimulated alone were treated identically and served as control. The resorted cells were frozen for subsequent PCR analysis or counted and resuspended in RPMI-C for functional studies (IL-2 secretion, proliferation). In some experiments, the resorted populations were rested for 2 days at 25,000 cells/well together with 200,000 T cells depleted (using anti-CD4 plus anti-CD8 magnetic MicroBeads) DBA/2 splenocytes irradiated with 3000 rad. After the rest period, the cells were washed and restimulated with soluble anti-CD3 (0.5 µg/mL), murine IL-2 (10 U/mL), or medium. Proliferation was measured after 72 h as described.

IL-2 ELISA

Samples and serial dilutions of recombinant murine IL-2 were incubated overnight at room temperature on Maxisorb ELISA plates (Nalge Nunc International, Rochester, NY) coated with 1 µg/mL anti-mouse IL-2 (1JES6-1A12; BD Pharmingen). This was followed by incubation with 0.5 µg/mL biotinylated anti-mouse IL-2 (1JES6-5H4; BD Pharmingen) for 3 h at 4°C and extravidin/peroxidase (Sigma, St. Louis, MO) for 30 min at room temperature. The plate was washed extensively between all steps with PBS plus 0.1% Tween 20 (Fisher Scientific, Pittsburgh, PA). Finally, tetramethylbenzidine liquid substrate system (Sigma) was added for 20 min, the reaction was stopped with 1 N HCl (Mallinckrodt, St. Louis, MO), and extinction at 450 nm was read on a Victor 1420 ELISA reader (PerkinElmer Wallac, Gaithersburg, MD). Analysis was performed using Microsoft Excel (Microsoft, Redmond, WA). The sensitivity of the assay was 4 mU/mL.

Real-time quantitative PCR

The mRNA was extracted from frozen cell pellets with a QIAGEN RNeasy mini kit (QIAGEN, Valencia, CA). DNase treated (DNA-free, Ambion, Austin, TX), and reverse transcribed with MultiScribe reverse transcriptase in the presence of random hexamers (PerkinElmer Applied Biosystems, Foster City, CA). Real-time quantitative PCR (17) for GRAIL and ribosomal RNA for normalization was performed using the ABI Prism 7700 Sequence Detection System that contains a Gene-Amp PCR System 9600 (PerkinElmer Applied Biosystems). Primers and probes were synthesized by PerkinElmer Applied Biosystems. All samples were analyzed in triplicates. A cDNA pool from DBA/2 CD4+ lymphocytes served as reference standard. The arbitrary units used to express results are multiples of this standard.

Results and Discussion

Ab-coated beads can replace APC function in suppression assays in vitro

CD4+CD25+ T cells have been demonstrated to inhibit the prolife-ration of CD4+CD25 T cells in vitro. This suppressor activity required activation of both the CD4+CD25+ and the CD4+CD25− T cells as well as direct cell contact between these cell types and APCs (16, 18). To address the role of the APCs in the interaction between CD4+CD25+ and CD4+CD25− T cells, we stimulated FACS-sorted cell populations with latex beads coated with anti-CD3 and anti-CD28 Abs. These beads could replace APCs without changing the characteristics of the system (Fig. 1 and Refs. 16 and 18). CD4+CD25− T cells, but not CD4+CD25+ T cells, proliferated when stimulated with Ab-coated beads alone. Coincubation of both populations led to inhibition of the CD4+CD25+ T cell response. Exogenous IL-2 rescued the proliferation of the CD4+CD25+ T cells stimulated alone as well as of cocultured CD4+CD25− and CD4+CD25+ T cells (Fig. 1A). The amount of costimulation provided to the T cells was easily controlled by changing the concentration of anti-CD28 during the coinubication procedure. Stronger costimulation enhanced the proliferative response of CD4+CD25+ T cells and abrogated suppression in the coinubication assay (Fig. 1B), as previously described (16).

CD4+CD25+ T cells suppressed the CD4+CD25− T cell proliferative response with Ab-coated beads as surrogate APCs (Fig. 1A); thus, suppression was the result of a direct interaction between the two T cell populations and was not mediated by APCs. These results confirm findings obtained with chemically fixed APCs (19) but do not rule out additional indirect effects. It has been reported that the interaction of CD4+CD25− T cells with dendritic cells resulted in decreased expression of B7 molecules on the dendritic cells (20). In contrast, CD86 expression on B220+ APCs was not affected by incubation with CD4+CD25+ T cells (19). These conflicting results might be due to differences in the cell types studied; further investigations are required to resolve this issue. Conceptually, it would make sense if the CD4+CD25+ T cells could “deactivate” APCs. The amount of CD28 costimulation present correlates negatively with the capacity of the CD4+CD25+ T cells to suppress CD4+CD25− T cells (Fig. 1B); thus, CD4+CD25− T cell-APC interaction could lower the expression
of costimulatory molecules to a level at which direct suppression can operate.

Blocking signaling through CTLA-4 does not abrogate suppression

Freshly isolated CD4+CD25+ T cells have high intracellular levels of CTLA-4 (14, 21). Takahashi et al. (21) abrogated suppression of the CD4+CD25+ response by CD4+CD25+ T cells in vitro with Abs against CTLA-4. The same result was obtained with CD4+CD25- responders from CTLA-4−/− mice, suggesting that the anti-CTLA-4 Ab affected the CD4+CD25+ suppressors. These authors proposed that CD4+CD25+ T cells might suppress by competing with CD4+CD25− T cells for B7 ligands on the surface of the APCs (as CTLA-4 has a higher affinity for CD80/CD86 than CD28) and/or that the balance between signals received through CD28 and CTLA-4 might influence the suppressive capacity of the CD4+CD25+ T cells. However, our experiments using Ab-coated beads for stimulation show that an APC-derived signal via CTLA-4 is not required for the suppressive activity of the CD4+CD25+ T cells. Interestingly, CD4+CD25+ T cells express higher levels of B7 molecules than CD4+CD25− T cells (data not shown). To exclude the possibility that CTLA-4 signals were provided by neighboring T cells, we blocked all potential B7-CTLA-4 interactions by adding anti-CTLA-4 (clone 4F10) at high doses with additional anti-FCR Ab or a combination of Abs against CD80 and CD86 to the culture. In our system, anti-B7 Abs do not interfere with T cell activation. As shown by data presented in Fig. 2, neither anti-CTLA-4 nor the mixture of anti-CD80 and anti-CD86 Abs abrogated suppression. This argues against a role for CTLA-4 in the suppressive mechanism in our system.

CD4+CD25+ T cells are rendered anergic by CD4+CD25+ T cells

A block in T cell proliferation in vitro due to insufficient IL-2 production that can be overcome by the addition of exogenous IL-2 is the hallmark of T cell anergy. The suppressed CD4+CD25+ T cells demonstrated all of these characteristics. However, anergy is an acquired endogenous phenotype of T cells resulting from insufficient activation and has not been demonstrably dependent on the presence or actions of other cells. To ask whether the “suppressed” CD4+CD25− T cells had become anergic, we FACS sorted CD4+CD25+ T cells after 24 h of coincubation with CD4+CD25+ T cells and analyzed their functional response to restimulation. The CD4+CD25− T cells were labeled with CFSE to allow differentiation of the two populations by FACS, as virtually all cells expressed CD25+ by that time (data not shown). In contrast to control CD4+CD25− T cells that had been stimulated alone, CD4+CD25− T cells initially coincubated with CD4+CD25+ T cells showed defective IL-2 production upon restimulation and proliferated only when exogenous IL-2 was added (Fig. 3A). In a subsequent set of experiments, we rested the cells for 2 days before restimulation. Data presented in Fig. 3B show that under these experimental conditions, the previously co-incubated CD4+CD25− T cells also showed an anergic phenotype. They did not respond to restimulation through the TCR but proliferated well to exogenous IL-2.

Our laboratory recently described GRAIL, a novel gene that appears to be involved in the induction and maintenance of CD4+ T cell anergy by negatively regulating IL-2 transcription.1 The induction of anergy in the CD4+CD25− T cells coincubated with CD4+CD25+ T cells was accompanied by marked up-regulation of GRAIL (Fig. 4B). The increased expression of GRAIL correlated inversely with the ability of the cells to produce IL-2 (Fig. 4A). Interestingly, the CD4+CD25− cells (isolated from the same coculture at 24 h) did not proliferate or produce IL-2 upon restimulation, but expressed low GRAIL levels that were comparable to the nonsuppressed CD4+CD25− cells (data not shown). This could indicate that the IL-2 block in the CD4+CD25+ T cell subset

[FIGURE 1. CD4+CD25+ T cells can suppress the proliferation of CD4+CD25− T cells in vitro with latex beads coated with anti-CD3 and anti-CD28 Abs used as surrogate APCs. A, A total of 12,500 FACS-sorted CD4+CD25− and/or CD4+CD25+ T cells per well was incubated for 72 h with equal numbers of beads (coated with 2.5 μg/ml anti-CD3 and 1.25 μg/ml anti-CD28). Murine rIL-2 (50 U/ml) was added as indicated. Proliferation was measured as incorporated radioactivity after a 15-h pulse with [3H]thymidine at the end of the 72-h culture period. B, Beads were coated with a constant amount of anti-CD3 (2.5 μg/ml) and titrated amounts of anti-CD28 (0.62, 1.25, 2.5, and 5 μg/ml). The proliferation assay was performed as in A. Results are representative of at least three experiments performed.]

[FIGURE 2. CTLA-4 blockade does not abrogate the suppression of CD4+CD25− by CD4+CD25+ T cells in vitro. CD4+CD25− T cells and/or CD4+CD25+ T cells were stimulated with Ab-coated beads in the presence or absence of Abs blocking the interaction between B7 molecules and CTLA-4. Anti-CTLA-4 (UC10-4F10-11) was added at 100 μg/ml, and anti-CD80 (16-10A1), anti-CD86 (PO3), and anti-FCR (2.4G2) were used at 10 μg/ml. Proliferation was measured as incorporated radioactivity after a 15-h pulse with [3H]thymidine at the end of the 72-h culture period. Results are representative of five experiments performed. The Abs were shown to be effective in a primary T cell activation assay (data not shown).]
involves either a different pathway(s), or reflected different kinetics of gene expression.

**Conclusion**

We conclude that CD4⁺CD25⁻ T cells suppressed in vitro by CD4⁺CD25⁺ T cells are rendered anergic. They remain viable but do not produce the IL-2 needed for proliferation. This constitutes a novel mechanism for the induction of anergy as it occurs in vivo (22, 23). However, the apparent exposure of naive T cells to B7 signals under tolerizing conditions in vivo (23) and the loss of suppression/anergy with higher degrees of costimulation through CD28 in our system suggest that the need for CTLA-4 signaling in anergy induction might be relative rather than absolute. In this scenario, CTLA-4 signals derived from APCs and the unknown negative signal(s) provided by CD4⁺CD25⁺ T cells would be complementary in opposing positive costimulatory signals.

The relevance of our in vitro findings for the in vivo situation remains to be shown. It is interesting to speculate about the potential role of CD4⁺CD25⁺ T cells and the requirements for suppression or control of responses to self in noninflammatory conditions (low expression of costimulatory products), vs immune responses to foreign proteins in inflammatory conditions (up-regulation of costimulatory products). The fact that CD4⁺CD25⁺ T cells from normal mice can induce autoimmune diseases when adoptively transferred into T cell-deficient hosts does not contradict the model proposed here. The apparent lack of anergy in autoreactive cells in these models could be due to transfer of not yet anergized recent thymic emigrants and/or to limited duration of the anergic phenotype in vivo, requiring the repeated interaction with CD4⁺CD25⁺ T cells.

In any event, it has been demonstrated that CD4⁺CD25⁺ T cells act to suppress CD4⁺CD25⁻ T cells by inducing anergy in the suppressed population in the presence of costimulation in vitro, creating unresponsiveness to restimulation and expression of the anergy-related gene GRAIL.

**References**


