Functional and Molecular Comparison of Anergic and Regulatory T Lymphocytes

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Tolerance in vivo is maintained by multiple mechanisms that function to prevent autoimmunity. An encounter of CD4+ T cells with a circulating self-Ag leads to partial thymic deletion, the development of CD25+ regulatory T cells (Tregs), and functional anergy in the surviving CD25− population. We have compared anergic and regulatory T cells of the same Ag specificity generated in vivo by the systemic self-Ag. Anergic cells are unresponsive to the self-Ag that induces tolerance, but upon transfer into a new host and immunization, anergic cells can induce a pathologic autoimmune reaction against tissue expressing the same Ag. Tregs, in contrast, are incapable of mediating harmful reactions. To define the basis of this functional difference, we have compared gene expression profiles of anergic and regulatory T cells. These analyses show that Tregs express a distinct molecular signature, but anergic cells largely lack such a profile. Anergic cells express transcripts that are associated with effector differentiation, e.g., the effector cytokines IL-4 and IFN-γ. Anergic cells do not produce these cytokines in response to self-Ag, because the cells exhibit a proximal signaling block in response to TCR engagement. Thus, anergy reflects an aborted activation pathway that can readily be reversed, resulting in pathologic effector cell responses, whereas Treg development follows a distinct developmental pathway that extinguishes effector functions. The Journal of Immunology, 2006, 176: 6473–6483.

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5 Abbreviations used in this paper: Tg, transgenic; HPRT, hypoxanthine phosphoribosyltransferase; mOVA, membrane-bound OVA; sOVA, soluble form of OVA; RIP, rat insulin promoter; RMA, robust multiarray average; Treg, CD25+ regulatory T cell.
Materials and Methods

Mice

All experimental mice were used at 6 – 12 wk of age. All mice were age and sex matched = 2 wk. BALB/c mice were purchased from Charles River Laboratory. Tg mice expressing the DO11 TCR specific for the chicken OVA peptide (OVA323-335) in the context of the MHC class II molecule I-A

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were obtained from K. Murphy (Washington University, St. Louis, MO). sOVA Tg mice express a soluble form of OVA in the serum under control of the metallothionein promoter I and were generated by cloning the OVA cDNA into the metallothionein locus control region expression vector 2999 (kindly provided by R. D. Palmiter (University of Washington, Seattle, WA) (19) and injecting the construct into blastocytes from FVB mice. Founders were screened for OVA expression by Southern blotting and PCR (ODA Research Diagnostics) and one founder, designated as 20 ng/ml OVA in the serum was selected. Mice were subsequently typed for the presence of OVA by PCR (forward primer, GCAAGTCTTTCA GAGTGA; reverse primer, GGCCTAATTCTCAGAGACG). sOVA Tg mice have normal life expectancy. The mice were backcrossed onto the BALB/c background for >10 generations and crossed with DO11 TCR Tg mice. Rat insulin promoter (RIP)-membrane-bound OVA (mOVA) Tg mice have been described (20, 21). For some experiments they were bred onto a Rag2

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background. All mice were bred and maintained in our pathogen-free facility in accordance with the guidelines of the Laboratory Animal Resource Center of the University of the California, San Francisco. All experiments were conducted with the approval of the Committee on Animal Research of the University of the California, San Francisco.

Abs and flow cytometry

CD4

+ cells were stained with the clonotypic Ab KJ1-26 (Caltag Laboratories), anti-CD4 ( GK1.5, H129.19, and RM4-5), and anti-CD25 (PC61, 7AD). All Abs were obtained from BD Pharmingen unless otherwise stated. Abs were used as FITC, PE, PE-Cy7, PE-Texas Red, allophycocyanin, or PerCP conjugates. Flow Cytometry analyses were done on a FACSCalibur with CellQuest software (both from BD Biosciences) or on a CyAn analyzer (DakoCytomation). Cells were sorted with a MoFlo cell sorter (DakoCytomation). For intracellular cytokine staining, transfected DO11 T cells recovered from the peripheral or pancreatic lymph nodes of RIP-mOVA Rag

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recipients were restimulated on mitomycin C-treated BALB/c splenocytes for 14 h in the presence of 1 mg/ml OVA-peptide. Brefeldin A (Epitope Biotechnologies) was added (10 mg/ml) for the last 2 h of stimulation. Cells were stained for the intracellular cytokines IL-2 and IFN-γ and analyzed by flow cytometry. Staining with appropriate isotype controls showed no detectable differences between experimental groups.

Cell preparations, purification, and adoptive transfer

CD4

+ KJ1-26+CD25

− and CD25

+ cells from DO11, DO11 × sOVA Tg, or DO11 × RIP-mOVA Tg mice were recovered by cell sorting from lymph nodes or spleen. For adoptive transfer into wild-type recipients, sorted cells were labeled with 5 mg CFSE (Invitrogen Life Technologies) at 10 10

6

cells/ml for 10 min at 37°C and washed before injection. A total of 0.5 – 1 10

6

CD4

+ KJ1-26+CD25

− cells (purity 95%) were adoptively transferred by tail vein injection. In experiments using Rag

− recipients, a total of 2 10

4

CD4

+ KJ1-26+CD25

− or CD25

+ cells were adoptively transferred, and the mice were immunized with 200 mg OVA protein in IFA s.c. the following day. Activated DO11 cells for gene array were recovered by cell sorting from BALB/c or sOVA Tg mice that had been adoptively transferred with 5 10

6

CFSE-labeled DO11 cells and were immunized with 200 mg of OVA in IFA 4 days before sorting. Cells from up to four mice were pooled for each individual sample per group. Total RNA was extracted using the Absolutely RNA RT-PCR MiniPrep kit from Stratagene. To avoid contamination with DNA, samples were treated with DNase (Ambion) before amplification. A total of 56,76 ng of RNA per sample was used for linear in vitro amplification using Superscript II (Invitrogen Life Technologies) for reverse transcription and the MEGAscript T7 kit to generate cRNA (Ambion). The cRNA was used for a second round of amplification and finally labeled with biotinylated ribonucleotides using the Enzo BioArray Kit (Affymetrix).

Real-time RT-PCR

Quantitative RT-PCR was performed using real-time fluorogenic PCR (TaqMan) on a PE Biosystems ABI Prism 7700 Sequence BioDetector according to the manufacturer’s instructions (PerkinElmer). Total RNA was extracted as described above and reverse transcribed using Superscript II kit for RT-PCR (Invitrogen Life Technologies). No linear amplification was performed for RT-PCR. Primer and probe sequences for IL-4 and IFN-γ, and hypoxanthine phosphoribosyltransferase (HPRT) were used as published (23). GeneChip hybridization and analysis

Ten micrograms of cRNA was used to hybridize each MOE430A GeneChip array (Affymetrix). Hybridization was performed in a GeneChip Hybridization Oven 640 (Affymetrix) for 16 h at 45°C. The arrays were washed and stained on a GeneChip Fluidics Station 450 (Affymetrix), and scanned with a GeneChip Scanner 3000 (Affymetrix). Intensities of Perfect Match and Mismatch probes were generated by GeneChip operating software 1.2 (Affymetrix). Gene expression was adjusted with the robust multi-array average method using Bioconductor release 1.3. Robust multiarray average expression measurements of all probe sets were analyzed with one-way ANOVA using version R 1.8.1. Differentially expressed probe sets between groups were considered significant if their p values were < 0.01 and their fold-change > 2.0. For hierarchical clustering, differentially expressed probe sets were combined and clustered using GeneSpring 6.0 (Silicon Genetics). For identical genes, probe sets displaying the greatest fold change are shown.

Ca2+ flux analysis by flow cytometry

For Ca2+ analysis, lymph node cells or splenocytes were enriched for CD4+ cells by depletion of B220+ and CD8+ cells (Dynal), stained for CD4 and KJ1-26, and labeled with 5 mg/ml indo-1 acetoxyethyl ester (Invitrogen Life Technologies). Ca2+ flux was induced by adding soluble anti-CD3 (2C11; BD Pharmingen). Soluble anti-CD3 was used at 5 mg/ml in experiments with unpurified DO11 × sOVA Tg T cells and at 40 mg/ml for adoptively transferred DO11 cells because of the higher cell density per sample and low frequency of DO11 cells. Ionomycin (0.5 - 1 mg/ml; Sigma-Aldrich) was used as a positive control. Ca2+ flux was analyzed on a Cyan flow cytometer with an Enterprise 621 UV laser by gating on adoptively transferred, CFSE-labeled KJ1-26+CD4+, KJ1-26+CD4+CD25+ T cells in the TCR Tg mice, respectively. The ratio of emission at 400:40 and 450:50 nm over time is shown.

Results

Encounter with self-Ag leads to T cell deletion, anergy, and the development of regulatory T cells

The initial experiments were designed to establish the fate of DO11 T cells that encountered OVA throughout development as a ubiquitously secreted “self-Ag” expressed systemically. To do this, we have generated Tg mice that express a soluble form of
The numbers of DO11 cells were measured by cell counting, and the percentage of DO11/H11003 cells responds to self-Ag.

KJ1-26, CD4, and CD25 to determine the percentage of CD25 in two individual experiments with two mice each are shown.

Thymic deletion and development of CD25

FIGURE 1. Thymic deletion and development of CD25+ T cells in response to self-Ag. A, Lymph nodes (LN) and thymus (THY) of DO11, DO11 x sOVA Tg, and DO11 x RIP-mOVA Tg mice were harvested, total numbers of DO11 cells were measured by cell counting, and the percentage of KJ1-26 CD4+ and CD25 to determine the percentage of CD25+ cells. Each symbol represents one individual mouse. B, FACS plots show the expression of CD25 and CD62L in KJ1-26 CD4+ cells from lymph nodes. Data for a representative mouse from four experiments with at least two mice per group are shown. C, Thymi from DO11 Rag−/− or DO11 x sOVA Tg Rag−/− mice were harvested and stained as described in A. Lower graphs are gated on CD4+ single positive thymocytes. Numbers refer to the percentage of gated cells in the upper right quadrant. Data from one representative mouse from two independent experiments with two mice each are shown.

Anergic CD25− cells do not have suppressive activity

In the next series of experiments, we asked whether the anergic CD25− populations had any suppressive activity. In coculture assays, CD25− cells from DO11 x sOVA Tg mice profoundly suppress the responses of normal DO11 T cells in a dose-dependent manner (Fig. 3). For comparison, we included CD25+ T cells from DO11 x RIP-mOVA Tg mice, which have previously been shown to have potent suppressive activity (26). The suppression is Ag dependent, because CD25− CD4+ cells from normal BALB/c animals do not inhibit the responses of normal DO11 cells to OVA. In contrast to the CD25− cells, CD25+ T cells from either DO11 x sOVA or DO11 x RIP-mOVA Tg mice are not suppressive (Fig. 3). The conclusion of these experiments is that self-Ag encounter induces two populations of T cells: CD25+ regulatory cells and CD25− anergic, but not regulatory, cells.
Gene expression profiles of anergic and regulatory T cells

Because anergic and regulatory T cells that are chronically exposed to self-Ag show quite different functional response potentials, we asked whether their functions are reflected in the patterns of genes expressed in these cells. We isolated CD25\(^{-}\)/H11002 (anergic) and CD25\(^{+}\)/H11001 (regulatory) DO11 cells from the DO11/H11003sOVA Tg mice as well as DO11 cells that had been transferred into BALB/c recipients and activated by immunization. We compared their gene expression profiles with naive DO11 CD25\(^{-}\)/H11002 T cells using Affymetrix gene chips displaying 22,000 probe sets. At the statistical thresholds described in Materials and Methods, we found a total of 511 individual genes that are differentially up- or down-regulated at least 2-fold in any of the anergic, activated, or regulatory T cell population when compared with naive. Table I shows a list of selected genes. The complete listing of differentially regulated genes corresponding to the hierarchical clustering shown in Fig. 4 can be found in the supplemental data Table I.

The hierarchical clustering of targets expressed in the various cell populations makes several important points (Fig. 4, A and B). First, regulatory T cells have a distinct molecular signature that is completely different from that of activated or anergic T cells. We found 123 individual genes that were exclusively regulated in Treg (Fig. 4B). Many of the genes that are uniquely regulated in Treg have been previously described (27, 28); these include Foxp3, Cd25, Cita4, and a number of inhibitory cytokines, chemokines, and chemokine receptors (Table I and supplemental data Table I) (29–31). Similarly, a distinct gene expression profile can be found in activated cells (Fig. 4, A and B). The expression level of 149 genes is uniquely regulated in activated cells, many of which play a role in cell division and cell cycle control (Table I and supplemental data Table I).

In contrast, anergic T cells do not express a clear molecular signature (Fig. 4, A and B). Only 24 individual genes are uniquely regulated in anergic cells at a significant level when compared with naive. Many of these have been described as aiding in peripheral deletion (e.g., FasL) or maybe altering the strength of signal transduction, such as Mapk12 (32, 33). Importantly, none of the genes that have been described as being responsible for regulatory T cell function, like Foxp3, are expressed in anergic cells. It is also noteworthy that several genes that are up-regulated in activated T cells are also altered similarly or to a lower extent in anergic cells e.g., members of the TNF superfamily such as RANKL (Tnfsf11) or LIGHT (Tnfsf14) (supplemental data Table I).\(^{6}\) Surprisingly, some of the genes expressed in anergic cells (such as IFN-\(\gamma\) and IL-4) are thought to be characteristic of activated and effector T cells. These findings suggest that anergy may reflect a partial activation phenotype.

\(^{6}\) The online version of this article contains supplemental material.
Several genes are regulated in the same fashion in activated, regulatory, and anergic T cells (85 genes). These genes may be markers of Ag recognition (Table I and supplemental data Table I). Among these genes are the chemokine receptors CCR9 and CXCR3, known to be expressed on intestinal homing lymphocytes and activated Th1 cells, respectively (34–36), and the costimulatory molecule ICOS (37, 38). In contrast, genes that are shared between regulatory and anergic but not activated cells most likely reflect exposure to self-Ag rather than regulatory potency and include surface receptors that have been associated with signaling modification in anergic cells and regulatory T cells (e.g., CD5, GITR, CD38, and neuropilin) (Table I) (28, 39–44).

Therefore, within the groups of genes that are regulated in the same fashion in anergic and activated cells, anergic and Treg cells, or in all three groups, the great majority of the genes is regulated to the same or greater degree in activated or regulatory cells when compared with anergic cells (Fig. 4B and supplemental data Table I). This means that the magnitude of gene regulation in anergic cells, for the most part, lies between naive and regulatory or naive and activated cells. The gene expression profile of anergic cells may therefore reflect partial activation or Ag recognition in general, but failure to fully commit to either the effector or Treg lineage.

**Anergy induction in vivo is associated with partial effector differentiation and proximal signaling defects**

The genes that are regulated in anergic cells, when compared with naive T cells, appear to fall into two expression patterns. Anergic cells express some genes such as IFN-γ and IL-4, which are typically found in effector cells, and also express other genes such as PD-1, which are shared with regulatory T cells and thus most likely represent genes that are induced by continuous exposure to tolerogenic Ag. We therefore hypothesized that the induction of anergy is accompanied by simultaneous differentiation along an aborted effector pathway and a tolerogenic pathway, with decreased responsiveness to an antigenic stimulus. To test this hypothesis, we used an adoptive transfer model in which CD4-purified DO11 T cells were transferred into sOVA Tg or WT BALB/c recipients (18). After 5 days, the DO11 cells were isolated from the lymph node and spleen by cell sorting and restimulated on Ag ex vivo. Fig. 5A shows that the DO11 cells that had encountered the Ag were defective in proliferation and IL-2 production and did not show any detectable IFN-γ production (data not shown) upon restimulation with Ag and splenocytes ex vivo. Thus, they are considered hyporesponsive and are functionally tolerant. To determine the expression of IFN-γ and IL-4 mRNA, quantitative real-time PCR was performed for DO11 cells that had encountered the Ag in sOVA Tg mice or naive DO11 cells. Fig. 5B shows that self-Ag recognition leads to strong expression of IFN-γ and IL-4 mRNA 4 days after transfer. Thus, anergy induction is accompanied by partial effector differentiation in vivo, and the anergic cells seem to be poised to turn into effector cells but are incapable of secreting the cytokine in a tolerogenic environment.

In view of these findings, we hypothesized that tolerogenic stimuli must use other signaling pathways in parallel that prevent effective cytokine production. To test this hypothesis, we isolated DO11 cells that had been transferred into sOVA Tg or BALB/c recipients and measured Ca²⁺ mobilization in response to TCR cross-linking. As shown in Fig. 6A, DO11 cells that have encountered the self-Ag show a defect in Ca²⁺ flux when compared with naive cells. Although cell cycling seems to further promote this signaling block, defective Ca²⁺ mobilization can already be seen in cells that have not divided. Thus, the induction of anergy is simultaneously associated with the development of a proximal signaling defect as well as with partial effector differentiation. A similar Ca²⁺ mobilization defect can be seen in CD25⁺ DO11 cells from DO11 × sOVA Tg mice (Fig. 6B). Therefore, it should be possible for anergic cells to acquire full effector function if the antigenic stimulus is strong enough to overcome the signaling block.
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*GeneChip processing and statistical analysis were done as described in Materials and Methods and the Fig. 5 legend. Fold change values of anergic, activated, and regulatory T cells versus naive DO11 cells were calculated using robust multiarray average data and considered as significant with a cutoff of >2.0 and a p value of <0.01 determined by one-way ANOVA. Genes with a known function in T cells and those that were increased over naive were chosen. The complete listing of known differentially regulated genes can be found in supplemental data Table I.*
CD25− anergic cells but not CD25+ regulatory cells can induce autoimmunity

If responses of anergic cells can be restored with strong stimulation (Fig. 2B), it is possible that anergic cells retain the capacity for causing harmful reactions. It is not known whether the same is true of CD25+ Treg.

To compare the pathogenic potential of these cell populations, we used an experimental system that we have described previously (45) in which DO11 cells induce diabetes when transferred into lymphopenic (Rag−/−) RIP-mOVA recipients and immunized. CD25− or CD25+ DO11 cells were purified from DO11 × sOVA Tg mice and transferred into RIP-mOVA Rag−/− recipients. The recipient mice were immunized with OVA in adjuvant and followed for the development of diabetes. The CD25+ cells failed to induce diabetes. In contrast, all mice that had been transferred with anergic CD25− cells and immunized developed diabetes within 3 wk (Fig. 7A). DO11 T cells were isolated from these mice 4 wk after transfer and analyzed for cytokine production by intracellular cytokine stains. Consistent with diabetes development, the CD25− cells produced IL-2 and IFN-γ, whereas CD25+ regulatory T cells lacked cytokine production (Fig. 7B). Thus, immunization in a model of autoimmunity will make anergic cells autoaggressive, although the same anergic cells do not respond to systemic self-Ag alone (Fig. 2A). In contrast to anergic cells, transfer into RIP-mOVA Rag−/− recipients followed by immunization does not break the unresponsiveness of regulatory T cells.

Discussion

The studies in this paper were designed to analyze the properties of anergic and regulatory T cells induced in the same lymphocyte population by a known systemic Ag. By crossing a TCR Tg mouse with a strain that expresses a circulating form of the Ag recognized by the TCR, we have generated both anergic and regulatory T cells with the same Ag specificity in the population that survives thymic deletion. It is therefore possible to compare the functional responses and biochemical properties of these cells in a way that is not feasible in most experimental systems.

In DO11 × sOVA Tg mice, a large fraction of the DO11 cells is deleted in the thymus; among the survivors, ~30% express CD25 (Fig. 1). CD25− cells isolated from these mice are hyporesponsive to the same circulating form of OVA in vivo or to stimulation with the Ag ex vivo (Fig. 2), thus fulfilling the essential criteria for T cell anergy. Interestingly, these anergic cells can be induced to respond at high Ag concentrations (Fig. 2B). More significantly, the anergic cells are able to elicit pathologic reactions against tissue (islet) OVA if the cells are removed from the systemic tolerogen and exposed to an immunogenic form of the Ag (Fig. 7). It has long been suspected that anergy can be broken by strong Ags or by an encounter with microbes, and this loss of the tolerant state results in autoimmune reactions (4, 46–49). Our studies formally demonstrate that T cells that are chronically exposed to a circulating Ag are unresponsive to the same Ag in vivo but can react strongly to other forms of that Ag. Thus, anergy
appears to be a state of "desensitization" that is not permanent and can be overcome by strong external stimuli (17, 50–52).

The CD25⁺ cells that develop in the DO11 × sOVA Tg mice have the functional characteristics of Tregs because they inhibit the responses of normal DO11 cells in coculture assays (Fig. 3). In this experimental situation, CD25⁺ DO11 cells have no regulatory function. This is different from other double Tg models in which CD25⁺ cells also show suppressive function, and it may be related

FIGURE 5. Recognition of tolerogenic Ag by naive T cells promotes expression of effector cytokine mRNA. CD4⁺-purified DO11 T cells were transferred into WT BALB/c or sOVA Tg animals. Lymphocytes were harvested from peripheral lymph nodes on day 5 (A) or day 4 (B), and CD4⁺ KJ1-26⁺ cells were isolated by cell sorting. A, Sorted CD4⁺ KJ1-26⁺ cells were restimulated with APCs and OVA at the indicated doses. [³H]Thymidine incorporation was assayed on day 3, and IL-2 secretion by ELISA was measured on day 2. B, mRNA was isolated from sorted cells and analyzed for the expression of IFN-γ, IL-4, and HPRT by real-time fluorogenic RT-PCR. The abbreviation n.d. represents the amounts of transcript that are not detectable. Transcript abundance is represented as the ratio of cytokine to HPRT. Data are from one representative experiment of three.

FIGURE 6. Anergic CD4⁺ cells have a cell cycle-independent defect in Ca²⁺-mobilization. A, CFSE-labeled CD4⁺ T cells from DO11 mice were adoptively transferred into BALB/c (a) or sOVA Tg recipients on day 0. Splenocytes were recovered 3 days later, and the Ca²⁺ flux of KJ1-26⁺ CD4⁺ cells gated on cells that had divided (c) or not divided (b) was measured after stimulation with anti-CD3 as described in Materials and Methods. The table summarizes data from day 3 (d3) to day 5 (d5) with two mice per group. B, Splenocytes from DO11 × sOVA Tg mice or DO11 Tg mice were isolated, stained for KJ1-26, CD4, and CD25, and labeled with indo-1 dye. Ca²⁺ flux was measured in gated KJ1-26⁺ CD4⁺ CD25⁻ cells after stimulation with soluble anti-CD3. The table summarizes the percentage of KJ1-26⁺ CD4⁺ CD25⁻ cells that flux Ca²⁺. Data from one of five experiments is shown.
to the amount of the Ag or to the way the Ag is presented (16). As expected, the CD25<sup>+</sup> Tregs are incapable of mediating pathologic reactions. By this criterion, Tregs are fundamentally different from anergic T cells.

To examine the molecular basis of T cell anergy, we have initiated a study of gene expression profiles in anergic T cells. An important aspect of our studies is that, for the first time, we have compared anergic and regulatory T cells expressing the same Ag receptor and induced by the same self-Ag. This analysis reveals interesting differences between the two cell populations.

Regulatory cells have a clear and distinct molecular signature, as is now widely accepted (27, 28). We found many of the genes that have been described and would be expected to be regulatory based on various methods of detection, the most abundantly expressed of which are *Cd25*, *Foxp3*, and *Ctla4* (29–31). Furthermore, it has recently been shown that Treg efficiency can be triggered directly via TLR stimulation (53). The NF-κB-related genes (*RelB*, *Traf1*, and *NfkB2*) that are up-regulated in Treg may be important in mediating this effect.

Activated cells present a unique gene expression profile that distinguishes them from both anergic and regulatory T cells. In contrast, anergic cells do not have such a clear signature (Fig. 4). We find that anergic cells regulate many genes in the same fashion as activated or regulatory T cells or both groups. However, the great majority of these genes are regulated at the same or higher magnitude in the activated or regulatory population as compared with anergic cells. This finding suggests that anergic cells have responded to self-Ag but failed to become fully activated or to develop into regulatory cells. It is interesting to note that many genes are regulated in a similar fashion in both anergic and regulatory T cells (Fig. 4 and supplemental Table I). These overlapping genes, such as *CD5*, *GITR*, *CD38*, and *neuropilin*, may reflect Ag encounter in a tolerogenic rather than immunogenic fashion and may be necessary for preventing the response of T cells, e.g., by varying activation thresholds, but do not confer full commitment to the regulatory T cell lineage. CD5 has been suggested to play a role in down-regulation of TCR.

**FIGURE 7.** Anergic cells can induce autoimmune diabetes. KJ1-26<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> and KJ1-26<sup>+</sup>CD4<sup>+</sup>CD25<sup>-</sup> cells from DO11 × sOVA Tg mice or KJ1-26<sup>+</sup>CD4<sup>+</sup>CD25<sup>-</sup> cells from DO11 mice were isolated by cell sorting, and 2 × 10<sup>5</sup> cells were transferred into RIP-mOVA Rag<sup>−/−</sup> mice. The recipients were immunized with OVA/IFA the following day. 

**A**, Blood glucose readings of recipient mice from week 0 to week 6 are shown. All mice transferred with CD25<sup>+</sup> cells remained normoglycemic for >5 wk. Data are from one representative experiment of two.

**B**, Peripheral and pancreatic lymph nodes (LN) of recipients were harvested after 4 wk, restimulated with OVA and APCs, and stained for intracellular cytokines. The average percentage of cytokine-positive KJ1-26<sup>+</sup>CD4<sup>+</sup> cells from two individual mice per group is shown. One representative experiment of two is shown.
responses by recruitment of SHP-1 protein phosphatase (54), and CD5 protein surface expression in our model correlates with RNA levels (data not shown).

How a tolerogenic stimulus is different from an immunogenic stimulus and how these stimuli result in strikingly different functional consequences are fundamental questions. Our results suggest that lymphocyte activation and anergy are associated with surprisingly overlapping cellular responses. For instance, even anergic T cells express abundant transcripts for cytokines thought to be typical of effector responses (IFN-γ and IL-4). The reason why anergic cells do not continuously produce these cytokines when exposed to a self-Ag is because anergy is associated with a proximal signaling block in response to the Ag. This phenomenon has been called “turning” of the activation threshold of T cells (17). Clearly, however, anergic cells retain their capacity to develop into effector cells, even effector cells capable of causing disease (Fig. 7).

The situation appears to be fundamentally different in regulatory T cells, which show functional responses reflected in the patterns of gene expression that are very different from those in anergic cells. These differences suggest that anergic and regulatory cells do not share a common lineage or developmental pathway.

In summary, by using a simple Tg experimental system with a true self-Ag, our results demonstrate that anergy as a mechanism of tolerance is a fundamentally different process from the development of regulatory T cells. It is important to determine whether either or both of these control pathways are triggered by exposure to different forms of Ags, including self-Ags and chronic microbial infections. Gene expression profiling may be an approach for comparing the relative development of these control mechanisms and how they can be manipulated.

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Disclosures

The authors have no financial conflict of interest.

References


