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Generation of Anergic and Potentially Immunoregulatory CD25+CD4 T Cells In Vivo After Induction of Peripheral Tolerance with Intravenous or Oral Antigen

Kristen M. Thorstenson and Alexander Khoruts

Immunoregulatory CD25+CD4 T cells are thought to arise from the thymus as a distinct lineage of CD4 T cells specific for self Ags. We used the DO11.10 TCR transgenic adoptive transfer system to show that cells of similar phenotype may also arise in the course of peripheral tolerance induction. Such cells emerged within 1 wk following Ag exposure and correlated negatively with the number of initial cell divisions. Limiting i.v. Ag dose or using an oral tolerance protocol yielded the greatest numbers of Ag-specific CD25+CD4 T cells. In contrast, immunogenic Ag exposure in the presence of an adjuvant did not lead to emergence of CD25+CD4 T cells. The profound anergic phenotype of these cells and their potential immunoregulatory properties make them an especially desirable population to induce in the course of immunotherapy in numerous clinical settings. This experimental system may be useful in future studies designed to optimize immunologic tolerance induction. The Journal of Immunology, 2001, 167: 188–195.

Materials and Methods

Mice and adoptive transfer protocol

The DO11.10 TCR transgenic mice (13), extensively backcrossed (>15 generations) onto the BALB/c background, were crossed with RAG-2-deficient mice purchased from Taconic (Germantown, NY). All donor mice were RAG-2-deficient DO11.10 TCR transgenic confirmed by staining peripheral blood cells for presence of KJ1-26+CD4 T cells and absence of B cells. Donor mice were maintained on autoclaved food and water in a specific pathogen-free facility in microisolator cages with filtered air according to National Institutes of Health guidelines. RAG-2-deficient DO11.10 CD4 T cells were adoptively transferred (2.5–5 × 106 cells per mouse) by i.v. injection into unirradiated BALB/c mice, as previously described (14). In some of the experiments, donor cells were labeled with CFSE (Molecular Probes, Eugene, OR) before transfer, using a technique previously described (15).

Abs and staining reagents

The following Abs and secondary reagents used for flow cytometry were purchased from BD PharMingen (San Diego, CA): CyChrome-labeled anti-CD4, PerCP-labeled anti-CD4, allophycocyanin-labeled anti-CD25, PE-labeled anti-CD25, PE-labeled anti-IL-2, PE-labeled anti-IFN-γ, PE-labeled anti-IL-4, PE-labeled anti-IL-5, PE-labeled anti-CTLA-4, and allophycocyanin-labeled streptavidin. The following Abs and secondary reagents were purchased from Caltag ( Burlingame, CA): PE-labeled KJ1-26 and PE-labeled streptavidin. Biotin-labeled KJ1-26 was generously provided by M. K. Jenkins (University of Minnesota, Minneapolis, MN). The following neutralizing Abs were used in the in vitro blocking studies: anti-IL-10, clone JES5-2A5 (PharMingen), and anti-TGF-β (R&D Systems, Minneapolis, MN).

Immunization and tolerance induction

A single i.v. injection of the OVA peptide 323–339, synthesized at Research Genetics (Huntsville, AL), at a dose of 5 μg per mouse, unless specified otherwise, was used to induce tolerance. LPS (25 μg), serotype...
**Results**

**Emergence of CD25+ CD4 T cells following peripheral tolerance induction with i.v. peptide Ag**

The DO11.10 TCR transgenic adoptive transfer system (14) was used to track the phenotype of Ag-specific CD4+ T cells in vivo following peripheral Ag exposure. It is well established that systemic, soluble Ag administration results in establishment of peripheral immunologic tolerance, but Ag administration accompanied by an adjuvant leads to immunologic priming (17–19). The DO11.10 TCR transgenic T cell adoptive transfer system was used previously to characterize the fate of Ag-specific CD4 T cells following these two different types of Ag exposure (14, 16, 20).

Intravenous administration of the OVA peptide 323–339 alone results in initial proliferation of Ag-specific CD4 T cells until peak clonal expansion is reached on day 3, which is then followed by clonal deletion and functional inactivation of the remaining Ag-specific CD4 T cells. The net result of expansion and contraction of the Ag-specific CD4 T cell population following a single wave of Ag in this model is a population that is smaller than the starting one and also functionally hyporesponsive to restimulation at the level of an average individual cell (14, 20). In contrast, while addition of LPS to the Ag does not change the overall kinetics of the T cell response, the end result is an expanded population of Ag-specific CD4 T cells that on restimulation produce large amounts of IL-2 and gain ability to produce effector cytokines such as IFN-γ (16, 20). The reported phenotypic characteristics of immunoregulatory CD25+ CD4 T cells resemble some of the phenotypic characteristics of CD4 T cells tolerized in vivo with i.v. peptide Ag. Thus, CD25+ CD4 T cells are unable to make IL-2 in vitro and proliferate poorly following TCR stimulation. Therefore, we wished to see whether a population of immunoregulatory CD25+ CD4 T cells emerges following peripheral tolerance induction with i.v. peptide Ag.

About 5% of the DO11.10 CD4 T cells in the wild-type transgenic mice coexpress CD25 (9, 11, and data not shown). Existence of these cells depends on expression of the second TCR resulting from endogenous rearrangement of the TCR α-chain. Therefore, to confine our investigation of origin of CD25+ CD4 T cells exclusively to differentiation following peripheral Ag exposure, we bred the donor mice onto the RAG-2-deficient background. Fewer than 1% of the RAG-2-deficient DO11.10 CD4 T cells taken directly from the intact transgenics stain positively for CD25 (9, and data not shown). Since the tolerant phenotype is established over the course of 1 wk following systemic Ag exposure (21), we looked at CD25 expression by DO11.10 T cells on day 8 or later following a single i.v. dose of Ag. Indeed, a subpopulation of CD25+ CD4 T cells was noted to be present, but its size was inversely correlated with the dose of the tolerizing peptide (Fig. 1). The inverse relationship between Ag dose and CD25 expression by the DO11.10 T cells at this time in the immune response argued against CD25 representing a mere transient marker of activation. Furthermore, coadministration of an adjuvant, LPS, along with the low dose
peptide Ag (5 μg) also did not lead to emergence of the Ag-specific CD25+CD4+ T cell population (Fig. 2). A more detailed kinetic analysis showed that transient expression of CD25 was seen on all Ag-specific T cells within 12 h of Ag administration regardless of adjuvant presence. However, Ag-specific CD4+ T cells expressing CD25 at later time points were seen only following a tolerizing protocol. In fact, there was an increase in the numbers of the KJ1-26+CD4+ T cells expressing CD25 between day 3 and day 8 following the tolerizing Ag encounter (Fig. 2) despite the fall in the size of the total KJ1-26+CD4+ T cell population. Therefore, it is unlikely that the increased proportion of CD25+ Ag-specific T cells seen following a tolerance protocol can be explained merely by preferential death of the CD25− Ag-specific T cells. The total number of CD25+ Ag-specific T cells remained relatively constant on days 16 and 23 following Ag exposure (data not shown).

Inverse correlation of the number of cell divisions following Ag exposure and numbers of Ag-specific CD4+ T cells coexpressing CD25

The number of cell divisions following Ag stimulation has been shown to be a major variable controlling CD4+ T cell differentiation (22). Thus, greater number of cell divisions in the presence of appropriate cytokine signals correlates with differentiation into polarized Th1 and Th2 cell populations and ability to produce IFN-γ and IL-4, respectively (22). Similarly, there is positive quantitative correlation between the number of cell divisions and ability to make IL-2 upon recall stimulation (23). The Ag dose dependence on the emergence of CD25+CD4+ T cells seen in our studies also suggested a relationship to cell division history. To test this hypothesis, we labeled the donor DO11.10 T cells with CFSE and compared three different Ag exposure conditions: peptide

**FIGURE 1.** The number of Ag-specific CD4 T cells persistently expressing CD25 is greatest following the lowest dose of tolerizing peptide Ag. Expression of CD25 by KJ1-26+CD4+ T cells in spleen was measured by flow cytometry 8 days after various doses of OVA peptide were injected i.v. Each point shows the mean value from two individual mice. This experiment is representative of three independent experiments. Similar results were also seen in the lymph nodes (data not shown).

**FIGURE 2.** Late emergence of Ag-specific CD25+CD4+ T cells in the primary response following Ag exposure occurs only in the absence of adjuvant. Mice were injected i.v. 5 μg OVA peptide alone or with 25 μg LPS. Spleens were harvested at different different time points of the immune response, and expression of CD25 by KJ1-26+CD4+ T cells in spleen was measured by flow cytometry. A, Representative histograms showing CD25 expression. Gray histograms show CD25 expression by naive KJ1-26+CD4+ T cells that were not exposed to Ag. Black, heavy histograms show CD25 expression at indicated times after Ag encounter. B, Left, total numbers of KJ1-26+CD4+ T cells in spleens of mice that received 5 μg peptide alone or with LPS at different times following Ag exposure; right, total numbers of CD25+KJ1-26+CD4+ T cells in the same mice. Each point shows the mean value from two individual mice. This experiment is representative of three independent experiments. Similar results were also seen in the lymph nodes (data not shown).
alone at either low or high dose, or low dose peptide with LPS.

Four-color flow cytometry was used to measure CD25 expression and cell division history of Ag-specific CD4+ T cells (Fig. 3).

Several conclusions can be drawn from the analysis of these data. In all experimental conditions, CD25+ CD4 T cells were seen to have gone through fewer cell divisions than CD25− CD4 T cells. Not surprisingly, higher Ag dose on average resulted in more cell divisions. Nevertheless, when the proportion of CD25+ T cells was measured within subpopulations of DO11.10 T cells that have undergone the same number of cell divisions, both high and low dose i.v. tolerance protocols were identical. Addition of adjuvant also led to more cell divisions for the total DO11.10 T cell population. However, the proportion of CD25+ T cells decreased more in the presence of adjuvant than would be predicted if cell division history were the sole variable controlling their induction.

Emergence of CD25+ CD4 T cells following induction of oral tolerance

Oral tolerance is a form of systemic, Ag-specific immunologic tolerance resulting from exposure to the Ag in the diet (24, 25). Many uncertainties remain about the mechanisms responsible for oral tolerance. Clonal deletion, clonal anergy, and active suppression have all been shown to participate in different experimental systems (25–28). Therefore, we tested the possibility that CD25+ CD4 T cells may arise following peripheral tolerance induction by oral Ag administration. Indeed, this proved to be the case (Fig. 4).

We allowed mice to consume ~25 mg OVA protein present in their drinking water in a single 18-h period, and examined the phenotype of adoptively transferred DO11.10 T cells at different time points after the feeding. The kinetics of the response (data not shown) as well as the extent of CFSE dilution suggests that the outcome of this oral tolerance protocol is qualitatively similar to the one that follows low dose peptide given i.v. In the oral tolerance protocol, there was relatively more dilution of the CFSE dye in the DO11.10 T cells taken from mesenteric lymph nodes as compared with sites more distant from the gut: axillary, brachial, inguinal lymph nodes, and spleen (Fig. 4).
Peripherally induced CD25+CD4+ T cells do not make IL-2 in vivo

One of the distinguishing features of the immunoregulatory CD25+CD4 T cells described in the literature is their inability to make IL-2 (11, 12). To evaluate cytokine production by Ag-specific CD25+ and CD25- T cells, we measured cytokine production in response to Ag restimulation within individual cells using flow cytometry. Ag-specific CD4 T cells were stimulated in vivo using an i.v. pulse of peptide Ag, and cells were stained for cytokine content directly ex vivo following fixation (16, 29). This assay takes advantage of the efficient and synchronous Ag presentation in vivo within undisrupted lymphoid tissues. Peak cytokine signal is seen at 1–2 h in Ag-experienced T cells (29), with identical kinetics for both tolerized and primed CD4 T cells (21). Peak production of IL-2 by naive CD4 T cells occurs at 4–6 h (29). Only CD25+ DO11.10 T cells were seen making IL-2 in vivo (Fig. 5). However, both CD25+ and CD25- DO11.10 T cells quickly up-regulated expression of CD69 in response to peptide rechallenge (data not shown). Both oral and i.v. tolerance protocols resulted in lesser ability of the DO11.10 T cells to make IL-2 upon peptide Ag rechallenge compared with DO11.10 T cells that experienced i.v. immunization with Ag and adjuvant. Staining for other cytokines, including IFN-γ, IL-4, IL-5, and IL-10, showed no detectable signal within either CD25+ or CD25- cell populations (data not shown).

Peripherally induced CD25+CD4+ T cells have regulatory properties in vitro

Naturally occurring CD4 T cells constitutively expressing CD25 can inhibit IL-2 production by CD25-CD4 T cells in vitro (11, 12). This suppressive effect requires Ag stimulation of the CD25+CD4 T cells, although the Ag specificity may differ from the responding CD25+CD4 T cell population. Therefore, we compared IL-2 concentrations in supernatants of Ag-stimulated cultures containing or lacking CD25+ T cells (Fig. 6). As shown above, only lymphoid tissues of adoptively transferred mice tolerized with i.v. administered low Ag dose contain significant numbers of DO11.10 CD25+ CD4 T cells. In contrast, lymphoid tissues of adoptively transferred mice that were not exposed to Ag, or received Ag accompanied by LPS, contain only few DO11.10 CD25+CD4 T cells. All lymphoid tissues also contain endogenous, polyclonal CD25+CD4 T cells of unknown specificity. Single cell suspensions of lymph nodes taken from adoptively transferred mice 8 days following 5 μg peptide dose administered i.v. with or without LPS or mice that received no Ag. CD25 depletion using magnetic beads was performed on one half of the lymph node cells, whereas the other half experienced mock depletion. The absolute number of CD25+ T cells in undepleted and depleted cultures was identical. Left, IL-2 production expressed in concentration found in the 24-h supernatants divided by the number of CD25+ KJ1-26+CD4+ T cells present in the well; right, percentage of improvement in IL-2 production achieved by depletion of the CD25+ cell fraction. Cultures were set up in duplicate with (1 μM) or without OVA peptide, and the error bars represent the range between the two samples. Results of this experiment are representative of three independent experiments.

**FIGURE 5.** Ag-specific CD25+CD4+ T cells do not make IL-2 upon Ag restimulation, and tolerized CD25+CD4 T cells have a relative defect in IL-2 production. Mice were primed with i.v. 5 μg OVA peptide and 25 μg LPS or tolerized orally with i.v. 5 μg OVA peptide alone. Eight days after initial Ag exposure, mice were given an i.v. pulse of peptide Ag or left untreated. After 1.5 h following peptide rechallenge, mesenteric lymph nodes (LN’s) and spleens were taken from the animals. Density plots show IL-2 content and CD25 expression of gated KJ1-26+CD4+ T cells.

**FIGURE 6.** Suppression of IL-2 by CD25+ cells in vitro. Axillary, brachial, inguinal, and cervical lymph nodes were taken from adoptively transferred mice 8 days following 5 μg peptide dose administered i.v. with or without LPS or mice that received no Ag. CD25 depletion using magnetic beads was performed on one half of the lymph node cells, whereas the other half experienced mock depletion. The absolute number of CD25+ T cells in undepleted and depleted cultures was identical. Left, IL-2 production expressed in concentration found in the 24-h supernatants divided by the number of CD25+ KJ1-26+CD4+ T cells present in the well; right, percentage of improvement in IL-2 production achieved by depletion of the CD25+ cell fraction. Cultures were set up in duplicate with (1 μM) or without OVA peptide, and the error bars represent the range between the two samples. Results of this experiment are representative of three independent experiments.
DO11.10 transgenic mice. Thus, all cultures contained equal numbers of endogenous polyclonal CD25⁺ T cells, while the second and third groups also contained equal numbers of either peripherally generated CD25⁺ DO11.10 T cells or CD25⁺ DO11.10 T cells from the wild-type transgenic animals. OVA-specific CD25⁺CD4 T cells, group 2 contained DO11.10 CD25⁺ T cells generated following peripheral tolerance induction with low dose i.v. Ag, and group 3 contained naturally occurring DO11.10 CD25⁺ T cells from wild-type transgenics. Cultures were set up in duplicate with (5 × 10⁵) or without OVA peptide, and the error bars represent the range between the two samples.

Finally, a direct comparison of the endogenous polyclonal CD25⁺ T cells with Ag-specific CD25⁺ T cells was similar to that of the naturally occurring CD25⁺ T cells noted in the past that is different from type 1 regulatory T cells or Th3 cells.

FIGURE 7. Suppressive potency of peripherally generated DO11.10 CD25⁺ T cells is similar to that of naturally occurring DO11.10 T cells generated following peripheral tolerance induction with low dose i.v. Ag, and group 3 contained naturally occurring DO11.10 CD25⁺ T cells from wild-type transgenics. Cultures were set up in duplicate with (5 μM) or without OVA peptide, and the error bars represent the range between the two samples.

FIGURE 8. Suppression of IL-2 and proliferation of responder cells are seen only if CD25⁺ T cells are stimulated by Ag. Positively selected CD25⁺ T cells from adoptively transferred i.v. tolerized mice were sorted into DO11.10⁺ and DO11.10⁻ using their CFSE content as a marker. They were then titrated to cultures containing 5 μM OVA peptide, irradiated CD4-depleted APCs, and naive RAG-2-deficient DO11.10 responder T cells. IL-2 concentration was measured in the supernatants at 48 h (A), and proliferative response of the responder cells was measured using flow cytometry to separate CFSE-positive suppressor and CFSE-negative responder DO11.10 T cells (B). Samples were run in triplicate, and error bars represent SDs.

Therefore, fold expansion of the responder cells was determined by counting their numbers using FACS. In these experiments, only Ag-specific CD25⁺ T cells could significantly reduce IL-2 measured in culture supernatants and the proliferative response of the responder cells (Fig. 8).

Discussion
There is ample evidence that a subpopulation of immunoregulatory CD4 T cells contributes to immunologic tolerance (30). Constitutive coexpression of CD25 defines at least a subset of this immunoregulatory CD4 T cell population. However, relatively little is understood about the origin of the CD25⁺CD4 T cells, their Ag specificity, and mechanisms of action. Existing evidence suggests that CD25⁺CD4 T cells arise directly from the thymus following a unique lineage of CD4 T cell development. Thus, appearance of autoimmunity in mice thymectomized 3 days after birth correlates with reduced numbers of immunoregulatory CD25⁺CD4 T cells found in the peripheral lymphoid tissues. In addition, suppressive CD25⁺CD4⁺CD8⁻ thymocytes can be shown to appear in the fetal thymic organ cultures in vitro, and emerge in vivo following direct thymic inoculation by CD4⁺CD8⁻ precursors distinguished by congenic markers (9).

The goal of this study was to test the possibility that CD25⁺CD4 T cells with similar immunosuppressive phenotype

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can also be generated in the course of peripheral tolerance induction to a known Ag. Indeed, we found that to be possible, but only when Ag dose was low, such that cell cycle progression was limited. As anticipated, CD25 was promptly, but only transiently, expressed by Ag-specific CD4 T cells following Ag exposure. Late expression of CD25 was seen only if Ag exposure was tolerogenic. The later expression of CD25 is unlikely to reflect acute activation because of the kinetically biphasic pattern of CD25 appearance, inverse correlation with Ag dose, and relative absence of CD25°CD4 T cells at these time points following priming with Ag and adjuvant. Phenotypically, peripherally generated Ag-specific CD25°CD4 T cells were indistinguishable in a number of assays from endogenous immunoregulatory CD25°CD4 T cells. They were unable to make any detectable IL-2, expressed high levels of CTLA-4 (data not shown), and suppressed IL-2 in the supernatants and proliferation of naive responder cells of the same Ag specificity in vitro cultures. The Ag-specific CD25°CD4 T cells were indistinguishable from Ag-specific CD25°CD4 T cells by cell size, which was equal to that of resting naive T cells, and levels of CD5 (data not shown). Clearly, our mechanistic analysis of suppression seen in in vitro cultures was limited in this study. Future studies will be done to determine whether the peripherally generated CD25°CD4 T cells mediate their suppression in vitro according to the same rules that were defined for the naturally occurring polyclonal CD25°CD4 T cells, dependent on direct contact between responder and suppressor T cells, dependent on Ag stimulation, independent of regulatory effects on the APCs, and Ag nonspecific (11, 31). Nevertheless, at the minimum, there are sufficient similarities in the phenotypes of these cells to raise the possibility that a subpopulation of the naturally occurring polyclonal immunoregulatory CD25°CD4 T cells may originate in the periphery rather than the thymus.

The data presented in this work are particularly relevant to the study of oral tolerance. Multiple uncertainties remain with regard to the mechanisms of oral tolerance as well as uptake and presentation of dietary Ags. Previous reports showed that orally administered Ag is encountered systemically within all lymphoid tissues (32), although detailed analyses of various feeding schedules are probably needed to further clarify this point. In this study, we attempted to compare an oral tolerance protocol with an i.v. peptide Ag protocol. Clearly, it is very difficult to estimate the amount of class II MHC/peptide complex seen by Ag-specific T cells following i.v. peptide Ag vs oral protein administration. Furthermore, the two routes of tolerance induction may differ in the types of APCs and costimulatory molecules provided. Nevertheless, the pattern of CFSE dilution in DO11.10 T cells suggested that our oral tolerance protocol is qualitatively similar to the low dose tolerance achieved with i.v. peptide Ag.

There is abundant literature suggesting that mechanisms of oral tolerance differ at different Ag doses (33). Relatively high Ag doses have been described to cause clonal deletion and anergy, while repeated administration of low Ag doses leads to development of suppressor T cells. Our results raise the possibility that CD25°CD4 T cells may indeed represent these suppressor cells and fit the Ag dose relationship noted in oral tolerance. If that were true, we would argue that peripheral induction of immunoregulatory T cells is not unique to oral tolerance. It may be that physiologic amounts of absorbed dietary Ags are typically in the low range; therefore, immunoregulatory mechanisms of tolerance may be more prominent in various oral tolerance protocols. Furthermore, the gastrointestinal tract along with the liver is a very efficient filter of LPS. Orally administered Ag delivered to distant lymph nodes is adjuvant free. In fact, commercial OVA protein given i.v. is relatively poor for tolerance induction. However, once LPS is extracted away, its potency is similar to that of synthesized peptide (A. Khoruts, unpublished observations). It is interesting to note that the percentage of CD25°CD4 Ag-specific T cells was greatest in the peripheral lymph nodes most distant from the gut, irrespective of cell division history. It is possible that some baseline exposure to bacterial products is present in the mesenteric lymph nodes, preventing optimal induction of the CD25°CD4 T cell subpopulation.

Ability to induce Ag-specific immunologic tolerance may be a strategy to treat autoimmune diseases and transplant rejection. The profound anergic phenotype of CD25°CD4 T cells and their potential immunoregulatory properties make them a very desirable subpopulation to be able to generate in the course of tolerance induction. The results presented in this work suggest that Ag encounter accompanied by minimal or no cell division are optimal conditions for peripheral induction of these cells from naive precursors. A number of clinically useful immunosuppressive agents (e.g., rapamycin, purine metabolites, cyclophosphamide, etc.) inhibit cell division. In addition, signals inhibitory to cell division are mediated through multiple endogenous immunoregulatory molecules (e.g., CTLA-4, TGF-β, and IL-10). Future studies may test whether any of these agents can be used as adjuncts for optimal induction of Ag-specific immunologic tolerance. In addition, it will be clinically important to determine whether differentiated effector or memory CD4 T cells can also be driven to acquire an anergic and possibly immunoregulatory phenotype in vivo.

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References


