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*J Immunol* 2003; 170:1870-1876; doi: 10.4049/jimmunol.170.4.1870

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Stat Signals Release Activated Naive Th Cells from an Anergic Checkpoint

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Activation of naive Th lymphocytes by the TCR and the costimulatory molecule, CD28, is believed to provide competent signals for differentiation to effector cells. Such activated cells proliferated and expressed IL-2, but arrested in an immature state maintained by CTLA-4. Although unresponsive to restimulation by TCR/CD28 alone, restimulation with TCR/CD28 and either Stat4- or Stat6-mediated cytokine signals rescued cells to proliferate and differentiate to the appropriately matched canonical Th subsets. Addition of IL-4 at defined periods revealed that naïve T cells were receptive to IL-4-mediated differentiation for up to 3 days after their initial priming. A Stat-dependent anergic checkpoint between clonal expansion and effector cell differentiation may defer the cytokine profile to be instructed at the site of infection, thus preventing the unregulated development of potentially damaging effector cells.


Individual helper CD4+ T cells have the capacity to differentiate into distinct effector subsets, allowing expansion of limited numbers of Ag-specific T cells to create a cytokine repertoire that best meets the needs of a given immune response (1, 2). Requirements for DNA synthesis or cell division have been reported in some, but not all studies (3–9), and activation is proposed to enable cells to express cytokine genes instructed by polarizing environmental cytokines. Evidence supporting such a model includes the ease with which T cells expressing a fixed pattern of cytokines can be expanded in vitro using distinct types of exogenous cytokines and the phenotype of cells prepared from various cytokine, cytokine-receptor, and cytokine signal-transducing Stat knockout mice (1, 2). Thus, naïve T cells can be differentiated into either of the most common T cell effector subsets, designated Th1 and Th2, using IL-12 and IL-4, respectively. With increasing cell divisions, the cytokine expression patterns of Th1 cells, characterized by IFN-γ production, and Th2 cells, characterized by IL-4 expression, become epigenetically fixed and no longer regulated by the environment (9–12).

Effective immunity requires both expression of the appropriate cytokine repertoire and selective expansion of such cells, although no functional linkage clearly exists between these events. Indeed, few studies have considered the apparent spatial and temporal dissociation between clonal expansion in lymph nodes and expression of effector function in peripheral tissues at sites of infection. Ag-specific CD4+ T cells accumulate in the lymph nodes—first in paracortical regions and later in parafollicular regions at the edge of B cell follicles—but peak in numbers around 3–4 days and subsequently decline (13, 14). CD4+ T cells activated using superantigens actively proliferated only a few days in lymph nodes before entering the circulation (15). T cells isolated from tissue sites of infection demonstrated substantially higher cytokine production as compared with activated cells isolated from the draining lymph nodes (16). Together, these studies suggest that mechanisms exist for compartmentalizing clonal expansion from effector function in differentiating T cells, although little is known regarding pathways that regulate this dichotomy. In this study, we provide evidence that signals transduced by cytokine receptors themselves establish a checkpoint between clonal expansion and the differentiation to canonical Th subsets, providing a mechanism for linking effector function with instructive cytokines present within an inflammatory focus.

Materials and Methods

Mice

BALB/c, Stat4-deficient (17) BALB/c, and Stat6-deficient (18) BALB/c mice (The Jackson Laboratory, Bar Harbor, ME), IL-4Rα-deficient BALB/c (19), tenth backcross TCR-Cα-deficient mice (20), IL-4 green-enhanced transcript (4get) IL-4 reporter mouse (21), and CTLA-4-deficient mouse (22) were kept in the University of California, San Francisco specific pathogen-free barrier facility in accordance with institutional guidelines.

T cell purification and activation

Naïve, small, CD62Lhigh, CD4+ T cells were purified to >99% using flow cytometry (MoFlo MultiLaser; Cytomation, Fort Collins, CO) and labeled with CFSE (Molecular Probes, Eugene, OR) as described (23). Purified T cells were activated in vitro using 106 CD4+ T cells with 5 × 105 irradiated APCs developed from TCR-Cα-deficient mice, and mAbs to TCRβ (1 μg/ml) and CD28 (5 μg/ml) with 50 U/ml recombinant human IL-2 as described (23); omission of IL-2 had no effect on any of the described phenotypes. Th1 conditions included recombinant murine IL-12 (5 ng/ml) and anti-IL-4 Ab (11B11, 20 μg/ml); Th2 conditions included recombinant murine IL-4 (50 ng/ml) and anti-IFN-γ Ab (XMG1.2, 50 μg/ml); inclusion of anti-IL-12 Ab had no effect on any of the described phenotypes. Where indicated, T cells were activated in the presence of both anti-IL-4 and anti-IFN-γ, or with anti-IL-12 (10 μg/ml; R&D Systems, Minneapolis, MN), CTLA-4/Ig (4 μg/ml; Ancell, Bayport, ME), or IL-6 (10 ng/ml; R&D Systems). T cells activated in the absence of APC were stimulated using tissue culture plates precoated with anti-TCRβ and anti-CD28 as described (23).

Abbreviations used in this paper: 4get, IL-4 green-enhanced transcript; 7-AAD, 7-amino actinomycin D; eGFP, enhanced green fluorescent protein; IRES, internal ribosomal entry site; HPRT, hypoxanthine phosphoribosyltransferase.

How to cite this article: Mohrs M, Lacy DA, Locksley RM. Stat Signals Release Activated Naive Th Cells from an Anergic Checkpoint. The Journal of Immunology, 2003, 170: 1870–1876.

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Received for publication August 23, 2002. Accepted for publication December 11, 2002.

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0022-1767/03/$02.00
Cytokine analysis

Intracellular cytokine analysis was determined after activation with PMA and ionomycin as described (24). Mouse IL-2 ELISA using Abs JES6-1A12 and biotinylated JES6-5H4 (BD PharMingen, San Diego, CA) had no reactivity with human IL-2 and had a detection limit of 10 pg/ml. For analysis of cytokine transcripts, total cellular RNA was extracted using RNAzol B (Biotexx Laboratories, Houston, TX) and reverse transcribed using oligo(dT)15 priming (Clontech Laboratories, Palo Alto, CA). Semiquantitative RT-PCR used a competitor plasmid, pQRS, and densitometric quantitation of nonsaturated pixels (MultiAnalyst, Bio-Rad, Hercules, CA) as described (25).

Results

To examine the linkage between activation, clonal expansion and effector differentiation, purified naive CD4+ T cells from wild-type, IL-4Rα chain- and Stat6-deficient mice were activated in the presence of APCs using Abs to the TCR and the costimulatory molecule, CD28, under conditions that promote Th1 or Th2 cell development. The IL-4Rα chain mediates signal transduction by IL-4 and IL-13, and hence IL-4Rα-deficient cells remain ignorant of these cytokines that promote Th2 differentiation (19). Stat6-deficient T cells also show impaired Th2 development (18, 26, 27), but unlike IL-4Rα-deficient cells, retain signals through other adaptors associated with the IL-4R (28). Before stimulation, cells were labeled with the fluorescent marker CFSE, thus allowing an analysis of phenotype with processive cell division (29).

Under Th2 conditions, both IL-4Rα- and Stat6-deficient T cells abruptly ceased dividing after 3 days (Fig. 1A). Impaired proliferation of deficient cells was associated with a small forward scatter profile that contrasted with the development of large blasts by wild-type T cells (Fig. 1, B and C). Further, IL-4Rα- and Stat6-deficient cells acquired a similar phenotype, characterized by incomplete up-regulation of activation markers like CD25, CD44 (data not shown), or CD69, and incomplete down-regulation of the lymph node homing receptor, CD62L. Arrested proliferation was not due to increased cell death, as established by 7-AAD incorporation, by annexin V staining and by direct enumeration of viable cells by trypan blue exclusion (Fig. 1C, and data not shown), despite robust expression of the apoptosis-mediating Fas molecule, CD95 (Fig. 1B). Impaired activation and proliferative arrest were not likely due to limiting amounts of anti-TCRβ Ab because the same results were observed with 10-fold higher (10 μg/ml) or lower (0.1 μg/ml) concentrations (data not shown). Under Th1 conditions, the forward scatter profile, proliferation, cell death, and surface phenotype of Stat6-deficient cells were no different from wild-type cells (Fig. 1C). Thus, naive CD4+ T cells activated under Th2 conditions in the absence of signals transmitted by the IL-4Rα chain or Stat6 demonstrated arrested division and differentiation and were maintained in culture with minimal cell loss over the week of the assay (Fig. 1A, and data not shown).

We next analyzed Th1 development using Stat4-deficient T cells that are unable to signal from the Th1-polarizing cytokine, IL-12 (17, 30). The phenotype of wild-type T cells was different when activated under Th1 conditions, as compared with Th2 conditions. Cell division was less robust, cell death was more evident, and CD62L was not comparably down-modulated, as previously noted (31, 32). Despite these differences, Stat4-deficient cells demonstrated incomplete activation under Th1 conditions that resembled that of Stat6-deficient cells under Th2 conditions. Thus, CD25 expression was incompletely up-regulated and the forward scatter profile was compromised (Fig. 1C). Stat4-deficient cells demonstrated a normal phenotype when activated using Th2 conditions (Fig. 1C). Compromised activation was not due to unusual development of the Stat-deficient cells because wild-type CD4+ T cells demonstrated a Stat-deficient phenotype when activated with TCR/FIGURE 1. Phenotype of activated naive CD4+ T cells. Naive CD4+ T cells from wild-type, Stat4−/−, Stat6−/− and IL-4Rα−/− mice were labeled with CFSE and primed with anti-TCR/CD28 under indicated conditions with irradiated APC. A, Cells from designated mice were activated under Th2 conditions (IL-4 and α-IFN-γ), and progressive cell division was assessed by CFSE dilution on indicated days after priming. B, Cells from designated mice were stimulated under Th2 conditions and analyzed on day 4 for forward scatter (FSC) as a measure of activation, and for expression of designated cell surface markers, each as a function of cell division. Electronic gates for cell surface markers depict isotype control Abs. C, Cells from designated mice were activated under Th2 (top) or Th1 (bottom) conditions and were analyzed on day 4 by flow cytometry for dead cells (7-AAD incorporation), and for FSC and CD25 expression, each as a function of cell division. Electronic gates for 7-AAD discrimination and CD25 isotype controls are shown.
CD28 Abs in the absence of IFN-γ, IL-12, and IL-4 (Fig. 2, A and B), or functional blockade of the IL-4R by the mAb, M1 (data not shown). Conversely, activation of IL-4-deficient CD4+ T cells in the presence of exogenous IL-4 revealed a wild-type phenotype (data not shown).

IL-6 has been previously implicated in promoting Th2 differentiation through both IL-4-dependent and -independent mechanisms (33, 34). IL-6 can also promote T cell survival (35, 36), raising the possibility that limiting amounts of IL-6 might contribute to the arrested phenotype. Addition of IL-6 to naive T cells induced to proliferate in the absence of IL-4, IFN-γ, and IL-12, however, was unable to overcome these cytokine-dependent requirements for full activation (Fig. 2A) and progression through cell division (Fig. 2B).

The arrest of cells stimulated by TCR/CD28 in the absence of polarizing cytokines was puzzling given the competent nature of these signals. Indeed, the expression and secretion of IL-2, which depend on TCR/CD28 signals (37), did not require IL-4Rα-mediated signals (Fig. 3, A and B). In fact, more IL-2 was present in supernatants under these conditions than from wild-type cells, possibly reflecting impaired CD25 expression required for high-affinity IL-2 receptors. Despite high levels of IL-2 and CD95, these cells did not undergo apoptosis during the period of arrested division (Fig. 1C).

The ability of cells primed in the absence of these cytokine signals to be maintained in a quiescent state suggested that such cells might constitute precursors for either Th1 or Th2 cells upon subsequent activation in the appropriate cytokine environment. When sorted after cell division, arrested T cells retained the capacity to develop into polarized effector cells of either subset upon secondary activation under Th1- or Th2-inducing conditions as assessed by surface phenotype, clonal expansion and cytokine profiles (Fig. 4A). Polarization and acquisition of an effector surface phenotype required signals administered through both TCR/CD28 and the appropriate cytokine receptor. Restimulation with IL-4 (but not IL-12) alone engendered up-regulation of CD25 but minimal cell division and no IL-4 expression. Reactivation with TCR/CD28 alone, which induced proliferation among naive T cells, neither resulted in further proliferation nor induced effector cytokines (Fig. 4A). Comparable results were obtained using cells sorted from any of divisions 1–4 after priming (data not shown). Thus, despite arrested development and failure to undergo apoptosis, naive T cells primed in the absence of these Stat-mediated signals could be reactivated for further clonal expansion and differentiation upon restimulation with TCR/CD28 when administered together with the appropriate cytokine-polarizing signal.

The absence of proliferation upon restimulation with TCR/CD28 (Fig. 4A) suggested a state of anergy (38). Indeed, consistent with an anergic phenotype, restimulation with high concentrations of IL-2 (>1000 U/ml) induced proliferation upon restimulation in the absence of additional cytokine costimulation. Although typically induced by TCR activation in the absence of CD28 costimulation, recent findings indicate that activation of the inhibitory ligand, CTLA-4, can maintain this nonreactive state (39, 40). Further, CTLA-4 is expressed maximally on naive CD4+ T cells 72 h after activation (41) during the period of arrested proliferation (Fig. 1A). Indeed, when cells were sorted from an arrested state after division and restimulated with TCR/CD28 in the presence of CTLA4/Ig fusion protein that masks B7 molecules on APC, proliferation was restored (Fig. 4A). In contrast, cytokine production remained dependent upon the appropriate cytokine receptor-mediated signals; neither IL-4 nor IFN-γ production was induced after TCR/CD28 stimulation in the presence of CTLA4/Ig. Similarly, when naive T cells were activated in the presence of CTLA-4/Ig, the arrested phenotype was bypassed, even in the absence of cytokine-mediated signals (Fig. 4B). Cells demonstrated an augmented forward scatter profile, up-regulated CD25, and progressed through cell division. Proliferative arrest and impaired activation could also be prevented when naive T cells were primed in the

**FIGURE 2.** IL-6 does not rescue requirements for Th1 or Th2 conditions. A, Naive CD4+ T cells from wild-type mice were labeled with CFSE and primed with anti-TCR/CD28 with irradiated APC under Th2 conditions (left) or without (middle) or with (right) 10 ng/ml IL-6 in the presence of neutralizing IL-4, IFN-γ, and IL-12 Abs. Cells were analyzed on day 6 for cell death (7-AAD incorporation), forward scatter (FSC), and CD25 expression as a function of CFSE dilution. B, CFSE division profile of cells activated under the indicated conditions.

**FIGURE 3.** IL-2 production by activated naive CD4+ T cells. A, Naive CD4+ T cells from wild-type and IL-4Rα−/− mice were activated under Th2 conditions, and supernatants were analyzed after 4 days for murine IL-2 using ELISA. B, CFSE-labeled naive CD4+ T cells from designated mice were activated under Th2 conditions and sorted after 3 days based on CFSE intensity into cells divided 0, 1, 2, or 3 times. IL-2 and HPRT transcripts were analyzed using semiquantitative RT-PCR.
presence of high concentrations of IL-2 (>1000 U/ml), an observation that is consistent with anergy (data not shown). Activation of naïve T cells using plate-bound Abs in the absence of APC (and of CTLA-4 ligands) also resulted in progression through the checkpoint. Finally, activation of CFSE-labeled CTLA-4-deficient naïve T cells with TCR/CD28 in the absence of cytokine-mediated signals resulted in continued proliferation after 6 days that did not occur among comparably activated wild-type CD4 T cells (Fig. 4C). Together, these data suggest a CTLA-4-mediated anergic checkpoint that can be bypassed or released by concomitant stimulation using TCR/CD28 and Stat-activating cytokines.

The requirement for cytokine receptor-mediated signals to alleviate the primary arrested phenotype was further demonstrated using cells deficient in IL-4Rα or Stat6. After activation under neutralizing conditions, wild-type, IL-4Rα- and Stat6-deficient CD4+ T cells arrested with a similar phenotype (Figs. 5 and 2A). When restimulated under Th2 conditions, wild-type T cells promptly entered cell cycle and completed differentiation to Th2 cells (Figs. 5 and 4A), whereas IL-4Rα- and Stat6-deficient T cells remained arrested, demonstrating an irrevocable block in the absence of Stat6 signals through this receptor (Fig. 5). To assess when the block in IL-4-mediated signals became established in Th2 differentiation, we used T cells from mice with a bicistronic IL-4/IRES/eGFP knockin reporter gene (21). T cells from these mice, designated 4get, faithfully report IL-4 expression in vitro and in vivo. Naïve CD4+ 4get T cells were stimulated with TCR/CD28 under nonpolarizing conditions, and at designated days, IL-4 was added exogenously to the culture media. After 6 days, the cells were analyzed for eGFP fluorescence as a marker of IL-4 expression, and cultures were restimulated using TCR without addition of exogenous cytokines. In agreement with prior findings (21), the majority of 4get T cells primed from the beginning with IL-4 expressed eGFP by day 6, and over 85% of these cells expressed eGFP after neutral restimulation with anti-CD3 (Fig. 6). Delaying the addition of IL-4 by 24 h caused substantial reduction in the percentage of cells expressing IL-4 by the end of the 6-day priming period. However, a delay in exposure to exogenous IL-4 for up to 3 days after priming continued to induce substantial IL-4 expression upon neutral restimulation, whereas later exposure was no longer effective (Fig. 6).

FIGURE 4. Analysis of the arrested checkpoint in activated naïve T cells. A, CFSE-labeled naïve CD4+ T cells were activated with anti-TCR/CD28 and anti-IFN-γ and anti-IL-4 Abs and sorted after 4 days from division 3 (left panels) and 2 (right panels), as indicated. Cells were restimulated using the designated conditions, and 4 days later analyzed for division, using CFSE dilution, CD25 expression, and for expression of IL-4 and IFN-γ by intracellular cytokine staining as compared with isotype control Ab. In three experiments, analysis was comparable using primed cells recovered from divisions 1–4. B, CFSE-labeled naïve CD4+ T cells were activated with anti-TCR/CD28 in the presence of APC, APC plus CTLA-4/Ig fusion protein, or with plate-bound anti-TCR/CD28 and no APC. After 4 days, cells were analyzed for forward scatter (FSC) and CD25 expression as a function of CFSE. Isotype control gates for CD25 are indicated. C, CFSE-labeled naïve CD4+ T cells from wild-type or CTLA-4−/− mice were activated with anti-TCR/CD28 in the presence of APC. Progressive cell division of CD4+ cells was assessed by CFSE dilution on days 4 and 6 after priming.
Another 4 days. eGFP-primed cells were restimulated with fresh APC under neutral conditions for exogenous IL-4 was added on indicated days of priming. After 6 days, the fluorescence of CD4+ cells was assessed by CFSE dilution on day 6 after priming (gray histograms) and day 4 after restimulation (open histogram).

Discussion
These studies describe a novel mechanism by which naive Th cells expand but accumulate in an arrested, uncommitted state maintained by CTLA-4 until relieved by the simultaneous delivery of both TCR/CD28 and a Stat signal from either of the polarizing cytokines, IL-4 or IL-12. Initial activation of cells with TCR/CD28 in the absence of Stat4- or Stat6-mediated signals led to IL-2 production and progression through several rounds of division. These cells sustained a small forward scatter profile and ultimately arrested with an altered surface phenotype, including attenuated CD25 expression, continued CD62L expression, and increased CD95 expression. Although unresponsive to TCR/CD28 alone, restimulation with TCR/CD28 in addition to polarizing cytokines was associated with further proliferation and differentiation to either of the canonical Th subsets. The role of Stat4 and Stat6 in relieving CTLA-4-mediated arrest reveals a novel checkpoint that ensures that expansion of phenotypically mature effector cells becomes tightly linked with the cytokine repertoire.

The role for CTLA-4 in maintaining an anergic checkpoint is consistent with the spontaneous activation demonstrated by CTLA-4-deficient T cells in vitro and in vivo (22, 42). In mice, the activated phenotype was CD4+ T cell-dependent (43) and was consistent with direct inhibitory effects of CTLA-4 on cell-cycle progression (44). Crossing CTLA-4-deficient animals to the B7-1/B7-2 double-deficient background abrogated the phenotype, suggesting CTLA-4-mediated dominant regulation of CD28-B7 activation (45). CTLA-4 becomes optimally induced 3–4 days after TCR/CD28 stimulation (41), consistent with the period of arrest demonstrated in the absence of Stat signals (Fig. 1A), implicating a physiologically relevant checkpoint during the normal activation of naive Th cells in vitro and in vivo (40). The novel finding in this study is the demonstration that signals from Stat4 or Stat6, when provided together with TCR-mediated activation, were capable of bypassing or releasing CTLA-4-mediated arrest, thus linking secondary proliferation and differentiation with effector cytokine expression. In agreement with our studies, Doyle et al. (46) have shown a CTLA-4-mediated proliferative arrest of primed CD4+ T cells in the absence of polarizing cytokines. Our present data extend this finding by demonstrating that these cells are refractory to TCR/CD28 restimulation in the absence of polarizing cytokine signals (Figs. 4A and 5). This anergic state can be released by TCR/CD28 activation when CTLA-4 interactions are blocked simultaneously or cytokine costimulation is provided (Fig. 4A). Thus, expanded populations of nonpolarized Th cells are not irreversibly anergized, but can be reactivated under polarizing cytokine conditions. Moreover, our data demonstrate that polarization of Th cells can be temporally and spatially dissociated from primary TCR/CD28 activation. Richter et al. (6) have shown that polarizing IL-4 signals can affect the differentiation of naive T cells even when administered 1 day before TCR activation. Conversely, we demonstrate that T cells activated in the absence of polarizing cytokines can be polarized even several days after primary activation when anergic arrest is released (Figs. 4A and 6). Further studies will be required, however, to determine whether these findings can be extended to more physiologic stimulation using peptide-specific T cell activation or to defined in vivo conditions.

Uncommitted Th cells can also be generated and expanded by active suppression of polarization in the presence TGF-β and limiting amounts of Ag (47–50). Release of TGF-β-mediated suppression resulted in loss of pluripotency. In contrast, we demonstrate that primed, nonpolarized T cells can be generated by the absence of polarizing cytokine costimulation (rather than active suppression) and are subsequently rendered anergic. The anergic state was not due to limited stimulation because it was observed even using 100-fold higher concentrations of TCR-cross-linking Ab. In addition, the absence of polarizing cytokines during priming generated expanded populations of pluripotent precursors. In contrast, pluripotency is rapidly lost when T cells are primed under polarizing conditions (9).

The mechanisms by which Stat-transduced cytokine signals promote progression through the CTLA-4-mediated checkpoint require further study. In part, failure to regulate efficient CD25 expression, a pathway that may be promoted by Stat4- and Stat6-induced signals

![FIGURE 5. Release of proliferative arrest. CFSE-labeled naïve CD4+ T cells from designated mice were activated with anti-TCR/CD28 and ant-IFN-γ and anti-IL-4 Abs in the presence of APC for 6 days. Then, cells were washed and subsequently restimulated with fresh APC under Th2 polarizing conditions for 4 days. Progressive cell division of CD4+ cells was assessed by CFSE dilution on day 6 after priming (gray histograms) and day 4 after restimulation (open histogram).](http://www.jimmunol.org/)

![FIGURE 6. A temporal window for delayed Th2 polarization. Naïve CD4+ IL-4 reporter (4get) cells were primed under neutral conditions and exogenous IL-4 was added on indicated days of priming. After 6 days, the primed cells were restimulated with fresh APC under neutral conditions for another 4 days. eGFP fluorescence of CD4+ cells was determined by FACS analysis after 6 days of priming (left panels) and 4 days of restimulation (right panels).](http://www.jimmunol.org/)
(51–54) could contribute to inefficient Stat5 activation and interruption of progressive cell division (55). Indeed, proliferation of primed CD4+ T cells is not limited by the presence of IL-2 but rather the surface expression of the high affinity IL-2R constituted by CD25 (56). In fact, anergy can be prevented or released when IL-2 concentrations are used that overcome the requirement for increased CD25 expression (data not shown). Inhibition of CTLA-4 engagement by CTLA-4/Ig significantly increases CD25 expression and allows IL-2-induced proliferation (Fig. 4, A and B). Interestingly, Zhu et al. (57) recently identified Gfi-1 as a Stat6-dependent gene promoting proliferation and preventing apoptosis of Th2 cells. Retroviral expression of Gfi-1 increases the surface expression of CD25, an effect we also observe when Stat6-dependent IL-4 costimulation is provided during priming (Fig. 1, B and C) or release of anergy (Fig. 4A). As shown, IL-4 was able to induce CD25 expression in cells with proliferative arrest even in the absence of TCR/CD28 signals (Fig. 4A). Despite the cross-talk between cytokine receptors and alleviation of CTLA-4-mediated blockade as revealed by these various studies, we find CTLA-4 levels on day 4 to be higher after activation under polarizing conditions than under cytokine-receptor-deficient conditions, suggesting complexities in regulation of these pathways that extend beyond simple modulation of CTLA-4 expression (M. Mohrs, unpublished data).

Current models of CD4+ T cell differentiation envision bifurcating developmental pathways instructed by Stat-dependent signals from the environment. In contrast, our data suggest a model by which naive T cells expand in lymph nodes without establishing their effector profiles, thereby increasing the precursor frequency to a given Ag. Although initially activated to divide and secrete IL-2, these data suggest that activated cells arrest over 3 days as CTLA-4 levels accumulate at the membrane, and exit the lymph node, a period consistent with in situ observations (13, 15). Upon re-exposure to Ag within an inflammatory, cytokine-rich site, primed cells would be released to undergo effector cell differentiation with an appropriately matched cytokine repertoire. Although clonal expansion occurs infrequently in nonlymphoid tissues (58), cytokine expression does not require cell division (6, 21, 58). Thus, Stat4 and Stat6 signals can link expansion of a preactivated, expanded Ag-specific, repertoire with the acquisition of a stabilized cytokine and effector program. In the absence of Stat signals, activated T cells remain arrested by CTLA-4 in an anergic state, thus preventing the mischievous development of an unregulated effector response. Indeed, competent signals provided inappropriately inflamed tissues might release the anergic program established within pre-activated cells populations and contribute to the development of autoimmunity (59).