Brief Definitive Report

CD28-mediated Costimulation of Interleukin 2 (IL-2) Production Plays a Critical Role in T Cell Priming for IL-4 and Interferon γ Production

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Summary

Naive T cells require interleukin 4 (IL-4) to develop into IL-4–producing T cells and IL-4 blocks development of such cells into interferon γ (IFN-γ) producers. Prior studies in accessory cell–independent priming systems using antireceptor antibodies as agonists have demonstrated that IL-2 is also necessary for the development of IL-4–producing cells under these culture conditions. Here we have examined the role of IL-2 and the CD28 costimulation pathway in priming for IL-4 and IFN-γ production using a more physiologic model. This involved antigen presentation by accessory cells to naive $CD4^+$ T cells from transgenic mice whose cells express a T cell receptor (TCR) specific for a cytochrome *c* peptide in association with I-E^k. With splenic antigen-presenting cells (APCs), inhibition of CD28 costimulation by the fusion protein CTLA4-immunoglobulin (Ig) blocked effective priming. Similarly, transfected fibroblasts expressing both MHC class II and the CD28 ligand B7 could prime for IL-4 production and such priming also was blocked by CTLA4-Ig. However, APCs deficient in CD28 ligands also could prime TCR transgenic T cells to become IL-4 producers if an exogenous source of IL-2, as well as IL-4, was provided, and the inhibition of priming seen with splenic or transfected fibroblast APCs in the presence of CTLA4-Ig could be reversed by addition of IL-2. Likewise, priming for IFN-γ production could be blocked by CTLA4-Ig and reversed by IL-2. Thus, we conclude that IL-2 plays a critical role in priming naive $CD4^+$ T cells to become IL-4 or IFN-γ producers. Engagement of the CD28 pathway, although normally important in such priming, is unnecessary in the presence of exogenous IL-2.

Lymphokine production by T cells plays an essential role in immune responses. After a short course of activation, naive T cells that produce only IL-2 can develop into "effector" cells capable of producing the key immunoregulatory cytokines IL-4 or IFN-γ (1, 2). The pattern of lymphokines produced by such effector cells plays a central role in determining the qualitative nature of the immune response to an infectious agent or vaccine, and thus, the control of this maturation of effector function has been of great interest. We and others (3–10) have demonstrated that lymphokines themselves can regulate this process. Initial studies, using an accessory cell–independent system, demonstrated that naive T cells stimulated by immobilized anti-CD3 Abs required both IL-2 and IL-4 for differentiation into IL-4–producing cells (3). Subsequent studies using naive T cells from mice transgenic for genes specifying TCR α and β chains confirmed the requirement for IL-4 for the differentiation of IL-4–producing cells (6, 7), but these studies did not address the role of IL-2.

Receptor–counter receptor costimulatory interactions also play an important role in controlling lymphokine production. In particular, the CD28 costimulatory pathway has been shown to regulate secretion of several lymphokines, especially IL-2 and to a lesser extent IFN-γ, by T cells that have been stimulated through their TCR (11–16). However, the role of the CD28 costimulatory pathway in accessory cell–dependent priming for IL-4 or IFN-γ production has not been addressed. Because of our evidence for an IL-2 requirement in priming for IL-4 in the anti-CD3 model, we wished to determine if CD28 engagement was important for such priming, and, if it was, whether its role was primarily to enhance IL-2 production, or to provide other essential signals necessary for the differentiation process. We report here that antigen and accessory cell–dependent priming for IL-4 and IFN-γ production in vitro is critically dependent on the activity of the CD28 costimulatory pathway, as assessed using inhibition by the fusion protein CTLA4-Ig (17). However, the role of this pathway seems limited to augmentation of the production of IL-2, and possibly other cytokines. If exogenous IL-2

is present, specific intracellular signals derived from CD28 engagement appear unnecessary for priming to occur. These data help define the contributions of both cell-associated costimulatory signals and secreted cytokines to regulation of T cell effector differentiation, with implications for understanding both the normal physiology of lymphokine-guided immune responses and the possible effects of exogenous IL-2 administration in the clinical setting.

Materials and Methods

Animals. Mice transgenic for TCR α and β chains yielding a receptor specific for cytochrome *c* and the MHC class II molecule $E\alpha^kE\beta^k$ were obtained from Barbara Fazekas de St. Groth and Mark Davis (Stanford University, Stanford, CA) and maintained by crossing to B10.A mice in our animal facilities (7). All mice used were homozygous for I-E^k and expressed the TCR transgenes. Splenic APCs were prepared from virus-free 8–12-wk-old B10.A female mice obtained from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD).

Tissue Culture Medium. DMEM supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 2 ME (50 μM) was used for all stimulation assays.

Peptide. A peptide with the sequence corresponding to residues 88-104 of pigeon cytochrome C was synthesized and purified by the National Institute of Allergy and Infectious Diseases (NIAID) Biological Resources Branch (Bethesda, MD), as previously described (7).

Recombinant Lymphokines. Human rIL-2 was a gift of the Cetus Corp. (Emeryville, CA). IL-2 activity was defined in terms of "Cetus Units". One Cetus unit is equivalent to 0.3 ng. Mouse rIL-4 was obtained by a baculovirus expression system, utilizing a vector into which the IL-4 gene had been inserted by C. Watson (Laboratory of Immunology, NIAID). One unit of IL-4 is equivalent to 0.5 pg.

Antibodies. Purified monoclonal rat anti–mouse IL-4 (11B11) (18) was prepared by Verax Corp. (Lebanon, NH). Anti-IL-2 (S4.B6) (19) was purchased from Pharmingen (San Diego, CA). The CTLA4-Ig fusion protein contains the extracellular domain of human CTLA-4 joined to the human IgCγ1 segment (17). The chimeric monoclonal antibody L6 was the gift of Drs. I. Hellstrom and K. E. Hellstrom (Bristol-Meyers Squibb, Seattle, WA). Anti-CD28 antibody was a gift of Dr. James Allison (University of California, Berkeley, CA) (20).

Preparation of T Cells. Purified $CD4^+$ LN T cells were prepared as previously described (7). Briefly, LN cells were first passed through a nylon wool column and then depleted of residual I-E^{k+} and $CD8^+$ cells by negative selection using magnetic beads. The purity and transgene expression of the $CD4^+$ T cells were verified by flow cytometric analysis using two-color immunofluorescence with PE-labeled anti-CD4 and FITC anti-Vα11(RR8-1) Abs obtained from Pharmingen. In most experiments, >90% of the T cells expressed CD4 and Vα11.

Preparation of Accessory Cells. L cells expressing $E\alpha E\beta^k$ (DCEK cells) were prepared as previously described (21, and Miller, J., and R. N. Germain, unpublished results). COS-7 cells stably expressing $E\alpha E\beta^k$ were prepared by Dr. Jerry Tanner, Federal Drug Administration (Bethesda, MD), using Eα and E^k_β cDNAs inserted into pCMVIE-AK1 vector (22) and transfected into COS.7 cells, followed by selection in G418 and cloning of high expressing cells. T-depleted spleen cells were prepared from B10.A mice by removing T cells from splenocytes by treatment with a cocktail containing anti-Thy1.2 (HO134; ATCC TIB 99, American Type Culture Collection, Rockville, MD) (23), anti-CD4 (GK1.5) (24), and anti-CD8 (3.155) (25) Abs plus Low-tox M rabbit complement (Cedarlane, Westbury, NY).

Primary and Secondary Stimulation of Transgenic T Cells. Primary stimulation of transgenic T cells was carried out by adding 3×10^5–1×10^6 $CD4^+$ T cells to individual wells of 24-well plates in a total volume of 1 ml together with accessory cells, peptide, and lymphokines as described in Results. After 36–48 h, cells were transferred for an additional 48 h to 5-ml dishes supplemented with fresh medium to allow further expansion. Cultures were washed three times and 5×10^4–1×10^5 T cells were restimulated with peptide and APC in a total volume of 200 μl in wells of 96-well plates for 36 h. Supernatants were collected and assayed for lymphokine.

Measurement of Lymphokine Production. An IL-4–dependent line (CT.4S) (26), and an IL-2–dependent cell line (CTLL) (27), were used to measure IL-4 and IL-2 production, respectively, using serial dilutions of supernatants and comparing responses to those elicited by known amounts of murine rIL-4 and human rIL-2 as standards. IFN-γ was assayed by a specific two-site ELISA (28, 29), using reference standard curves prepared using known amounts of rIFNγ.

Results

COS/I-E^k Fail to Stimulate T Cell Proliferation in the Absence of Exogenous IL-2 or Anti-CD28. To assess the respective roles of direct CD28 intracellular signaling vs. CD28-mediated augmentation of IL-2 availability on T cell priming for IL-4 production in an antigen and MHC class II–dependent system, we utilized COS/I-E^k cells as APCs. COS/I-E^k express little to no CTLA4-reactive protein as assessed by FACS® (Becton Dickinson & Co., Mountain View, CA) analysis with human CTLA4-Ig chimeric protein (data not shown), and thus should fail to activate the CD28 costimulation pathway. $CD4^+$ transgenic T cells stimulated with either cytochrome *c* peptide, IL-2, or anti-CD28 in the absence of COS-I-E^k showed minimal uptake of [^{3}H]thymidine (Fig. 1). When $CD4^+$ transgenic T cells were stimulated with COS/I-E^k and peptide alone, little stimulation of [^{3}H]thymidine uptake occurred above that seen with COS/I-E^k and peptide without T cells (Fig. 1). However, when IL-2 was added to these cultures, there was a substantial increase in DNA synthesis. The addition of anti-CD28 also caused an increase in DNA synthesis. The CD28-mediated increase was completely inhibited by anti-IL-2. Moreover, adding CTLA4-Ig did not diminish DNA synthesis in response to IL-2. Thus, COS/I-E^k cells are effective at peptide presentation to transgenic $CD4^+$ T cells, but do not induce IL-2 production without independent provision of T cell costimulatory signals.

IL-2 or Anti-CD28 Is Required for Priming for IL-4 Production in the Absence of a Source of CD28 Ligand. COS/I-E^k cells were then used as APCs to study the roles of IL-2 and CD28 stimulation in priming naive TCR transgenic $CD4^+$ T cells to develop into IL-4 producers. Fig. 2 demonstrates that, in the presence of COS/I-E^k and peptide, addition of IL-4 alone did not result in priming TCR transgenic T cells to produce IL-4 upon restimulation. However, adding either IL-2 or anti-CD28 induced the cells to produce substantial

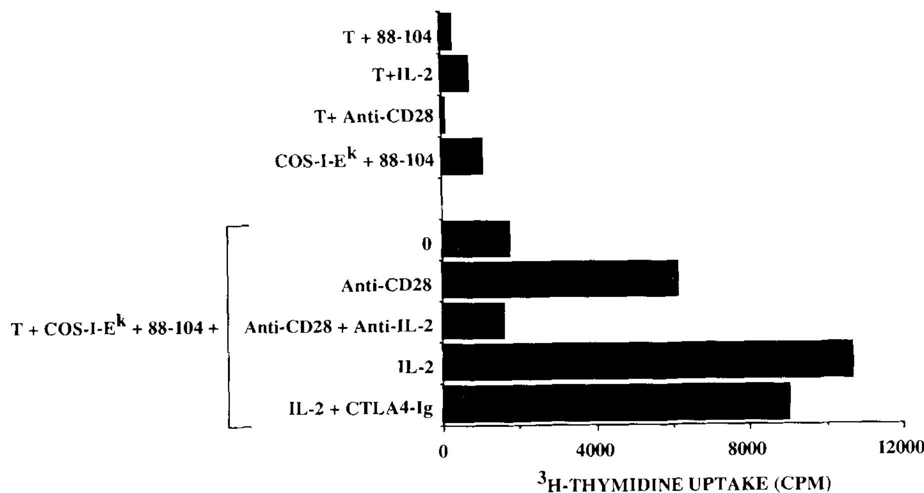

Figure 1. COS/I-E^k cells, in the presence of peptide, do not stimulate T cells to proliferate in the absence of exogenous IL-2 or anti-CD28. CD4$^+$ transgenic T cells (5×10^4) were stimulated with COS/I-E^k (2×10^4) and peptide (1 μM), in the presence of varying combinations of anti-CD28 (1/250 dilution), anti-IL-2 (10 μg/ml), and CTLA4-Ig (10 μg/ml) in triplicate microcultures (200 μl/well). After 3 d, cultures were pulsed for 8 h with [^{3}H]thymidine and harvested for counting. As controls, T cells were incubated with either peptide, IL-2 or anti-CD28, or COS/I-E^k were incubated with peptide in the absence of T cells.

amounts of IL-4 when restimulated. The addition of CTLA4-Ig to the priming culture when both IL-2 and IL-4 were present did not significantly diminish the subsequent production of IL-4, indicating that blocking potential stimulation of the CD28 pathway does not affect priming for IL-4 production, provided exogenous IL-2 is present.

Anti-IL-2 Inhibits Priming for IL-4 in the Presence of APCs that Express CD28 Ligands. To examine the role of more physiologic engagement of the CD28/CTLA-4 pathway in the priming process, we used as APCs either T-depleted spleen or transfected L cells that express I-E^k together with B7 and possibly other CD28 ligands (DCEK cells). Fig. 3 demonstrates that IL-4 is produced only from cells exposed to both IL-4 and IL-2 in the priming culture. If either IL-2 or IL-4 is neutralized by the addition of mAbs, no IL-4 is produced upon restimulation.

IL-2 may act as a positive differentiative stimulus or it might function simply to sustain cell viability. However, priming cultures containing IL-4 and anti-IL-2 have a cell yield of 30–40% of cultures carried in IL-2 plus IL-4. Nonetheless, restimulation of the same number of viable T cells results in IL-4 production only from cells primed in the presence of IL-2. Thus, it appears that priming for IL-4 production requires the activity of IL-2 in a system in which the APCs possess the ability to activate the CD28 dependent costimulatory pathway.

IL-2 Production Is Inhibited by CTLA4-Ig in a Primary Response. The data generated using COS/I-E^k as well as normal or costimulator-positive transfectants all were consistent with the necessary role of CD28-dependent costimulation in IL-4 priming being solely through augmented production of IL-2. We therefore used CTLA-4-Ig to inhibit the activation of the CD28 pathway, to further evaluate whether signaling via this pathway was simply required to generate IL-2 or was providing an additional signal independent of IL-2 that was required for cells to differentiate into IL-4 or IFN-γ-producing cells. Table 1 illustrates the effect of CTLA4-Ig on production of IL-2 or IL-4 after primary

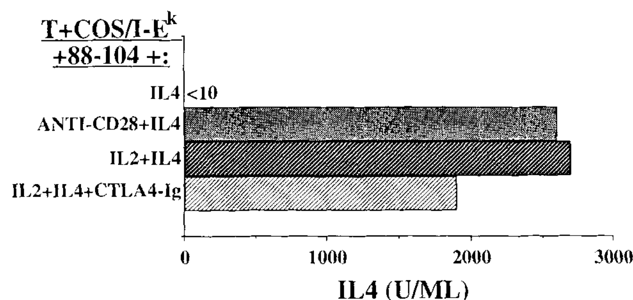

Figure 2. IL-2 or anti-CD28 is required for priming for IL-4 production in the absence of a source of CD28 ligand(s). CD4$^+$ transgenic T cells (1.2×10^6) were stimulated for 4 d with COS/I-E^k (4×10^5), peptide (1 μM), IL-4 (1,000 U/ml) ± anti-CD28 (1/250 dilution), or IL-2 (10 U/ml) + IL-4 (1,000 U/ml) ± CTLA4-Ig (10 μg/ml). Cells were washed and recultured at 5×10^4/200 μl with COS/I-E^k (2.5×10^4) and peptide (1 μm) for 36 h. Supernatants were harvested and IL-4 content measured. Results are reported as U/ml of IL-4.

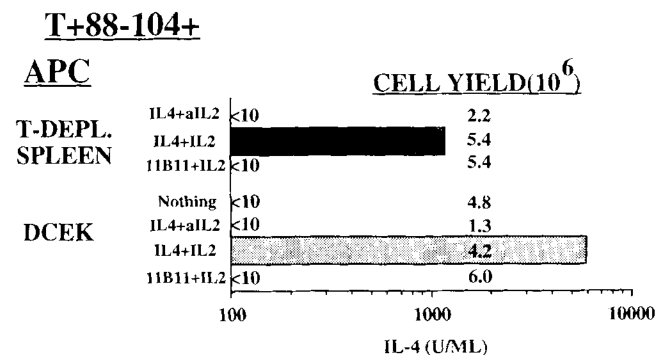

Figure 3. Anti-IL-2 inhibits priming for IL-4 in the presence of costimulatory APCs. CD4$^+$ transgenic T cells (10^6) were stimulated for 4 d with T-depleted spleen (5×10^5) or DCEK (5×10^5) and peptide (1 μM) in the presence of nothing, IL-4 (1,000 U/ml) plus IL-2 (10 U/ml) or anti-IL-2 (10 μg/ml), or anti-IL-4 (11B11) plus IL-2. Cells were washed and recultured at 10^5/200 μl with T-depleted spleen (10^5) and peptide (1 μm) for 36 h. Supernatants were harvested and IL-4 content was measured as described in Fig. 2.

Table 1. *IL-2 Production Is Inhibited by CTLA4-Ig in a Primary Response*

T + APC + 88 − 104 +	IL-2 (U/ml)	IL-4 (U/ml)
0	19	0
CTLA4-Ig	0.6	0
L6	20	0

$CD4^+$ transgenic T cells (10^5) were stimulated for 36 h with T-depleted spleen cells (10^5), and peptide (1 μm) in the presence of no additions, CTLA4-Ig (10 μg/ml), or L6 (10 μg/ml). Supernatants were assessed for IL-2 and IL-4 content by utilizing indicator cell lines sensitive to IL-2 and IL-4, respectively. Results are reported as U/ml of IL-2 or IL-4.

stimulation of $CD4^+$ T cells with T-depleted spleen cells and peptide 88-104. In this experiment, $CD4^+$ T cells were stimulated for only 36 h with APCs and peptide and lymphokine production was assessed. In the absence of CTLA4-Ig, 19 U/ml of IL-2 was made. When CTLA4-Ig was added, this was decreased 30-fold to 0.6 U/ml. L6, a control human Ig fusion protein, caused no inhibition of IL-2 production.

To test the effect of inhibiting the CD28 pathway on the generation of IL-4–producing cells, $CD4^+$ T cells were cultured for 4 d with T-depleted spleen cells, peptide, and IL-4 in the presence of anti-IL-2, CTLA4-Ig, or IL-2 plus CTLA4-Ig (Fig. 4). Cells primed in the presence of IL-4 produced substantial amounts of IL-4 upon challenge. However, cells primed in the presence of both IL-4 and either anti-IL-2 or CTLA-4-Ig had little or no capacity to produce IL-4 upon restimulation, demonstrating that inhibiting the CD28 pathway directly diminishes the ability of cells to be primed for IL-4 production. Moreover, the addition of IL-2 to a priming culture containing IL-4 and CTLA-4-Ig overcame the inhibition mediated by CTLA-4-Ig. This reemphasizes the conclusion that CD28 costimulation is required for priming for IL-4 production only through its capacity to regulate IL-2 production.

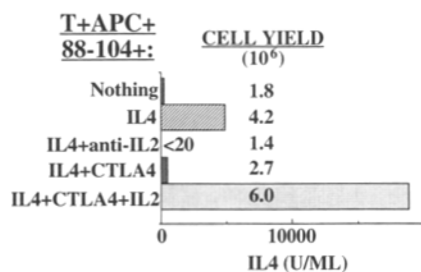

Figure 4. IL-4 production is inhibited in the absence of IL-2 and substantially diminished in the presence of CTLA4-Ig. $CD4^+$ transgenic T cells (4×10^4) were stimulated for 4 d with T-depleted spleen cells (4×10^4), peptide (1 μm), nothing, or IL-4 (1,000 U/ml) in the presence or absence of anti-IL-2 (10 μg/ml), CTLA4-Ig (10 μg/ml), or CTLA4-Ig + IL-2 (10 U/ml). Cells were washed, recultured and IL-4 content was assessed as described in Fig. 3.

Priming for IFN-γ Requires IL-2 and Can Be Achieved in the Presence of CTLA4-Ig if IL-2 Is Present. The role of IL-2 and CD28 in the development of IFN-γ–producing cells was examined by culturing naive T cells with APC, peptide, and anti-IL-4 (11B11) in the presence or absence of IL-2 and CTLA4-Ig (Fig. 5). Cells cultured with 11B11 alone were able to produce >1,000 U/ml of IFN-γ upon restimulation. IFN-γ production was markedly diminished in the presence of CTLA4-Ig. The addition of exogenous IL-2 to the priming cultures increased IFN-γ production three to four-fold. This enhancing effect of IL-2 was not diminished by CTLA4-Ig, demonstrating that blocking the CD28 pathway in the presence of exogenous IL-2 does not prevent priming for IFN-γ production. Whether IL-2 is absolutely required for naive T cells to develop into IFN-γ producers is not directly evaluatable here, since in the presence of anti-IL-2 and the absence of IL-4 (a condition necessary for priming for IFN-γ production), there is no cell yield.

Discussion

Previous work (1–9) has demonstrated that lymphokines themselves are important regulators of differentiation of naive T cells into cells capable of producing IL-4 and IFN-γ. These experiments were carried out by culturing naive T cells for short periods with polyclonal mitogens or antigen and accessory cells in the presence of various combinations of cytokines. In such studies, IL-2 was present either as a result of endogenous production or of addition. We had previously shown in an accessory cell–independent system that T cells activated by immobilized anti-CD3 in the presence of IL-4 but in the absence of IL-2 failed to secrete IL-4 upon restimulation (3). Recent work (11, 12) has shown that ligand–receptor interactions such as those mediated through the CD28 costimulatory pathway substantially influence cytokine production by T cells. This particular pathway is notable for its striking ability to enhance the production of IL-2 (13–16).

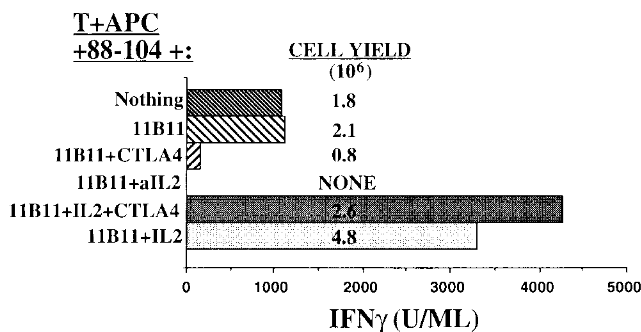

Figure 5. Priming for IFN-γ production requires IL-2 and can be achieved in the presence of CTLA4-Ig if IL-2 is present. $CD4^+$ transgenic T cells (4×10^4) were stimulated for 4 d with T-depleted spleen (4×10^4), peptide (1 μm), nothing, or 11B11 (10 μg/ml) alone or in the presence of CTLA4-Ig (10 μg/ml), anti-IL-2 (10 μg/ml), or IL-2 (10 U/ml) ± CTLA4-Ig. Cells were washed and recultured at 10^5/200 μl) with T-depleted spleen (10^5) and peptide (1 μm) for 36 h. Supernatants were harvested and IFN-γ content was measured as described in Materials and Methods.

Using an accessory cell–dependent model to stimulate T cells, we examined the role of IL-2 in priming for IL-4 production and also evaluated whether the CD28 costimulatory pathway had an essential, IL-2–independent role in this process.

Stimulation of transgenic T cells with APCs lacking ligands for CD28 defined by reactivity with the CTLA4-Ig fusion protein failed to permit priming for IL-4 production. This defect could be corrected using anti-CD28 Ab or by addition of exogenous IL-2. Similarly, blocking stimulation through CD28 using CTLA4-Ig with splenic APCs or B7-expressing fibroblasts also prevented priming for IL-4 or IFN-γ production, an inhibitory effect that also could be overcome by addition of IL-2 to the cultures. These data indicate that the CD28 costimulatory pathway can play a major role in the development of T cells secreting the polar cytokines IL-4 or IFN-γ. At least in this model system, the role of the CD28 pathway seems limited to ensuring adequate production of IL-2, rather than providing necessary unique intracellular signals for T cell differentiation and IL-4/IFN-γ production. It remains possible that a broadly expressed costimulatory ligand present on COS cells or the T cells themselves and not reactive with CTLA4-Ig might also play a significant direct signaling role during IL-4 or IFN-γ priming.

The complete neutralization of IL-2 in these antigen-stimulated cultures prevented development of IL-4–producing cells. Heinzel et al. (30) have reported that treatment of mice infected with *Leishmania major* with anti-IL-2 Ab decreased the amount of IL-4 produced and allowed a majority of the mice to heal, suggesting a need for IL-2 in the development of IL-4 producing cells in vivo. Thus, independent of the issue of undetected costimulatory signals, these results suggest that IL-2 itself plays a substantial role in the physiologic development of effector cytokine responses, especially of IL-4. However, because these experiments are done under controlled conditions in vitro and substantial redundancy exists in the effects of various cytokines, we cannot exclude the possibility that other cytokines may replace IL-2 in the priming process under certain conditions. Studies are underway to examine this possibility by utilizing T cells from mice in which the IL-2 gene has been deleted and evaluating whether IL-4 or IFN-γ production is normal after priming and restimulation. In addition, experiments are underway to evaluate whether T cell growth factors such as IL-7 can be used to maintain cell viability so that cells primed in the absence of IL-4 and IL-2 can be assessed for IFN-γ production upon restimulation.

The absence of a requisite intracellular signal from the CD28 pathway in priming for effector cytokine production implies that in the presence of IL-2, APCs that lack CD28/CTLA4 ligands may nonetheless act to prime naive T cells to become IL-4 or IFN-γ producers. In situations in which high levels of IL-2 are present, either as a result of endogenous production or pharmacologic intervention, such priming might lead to increased immunologically mediated tissue damage or graft rejection. Alternatively, it might enhance the protective value of an immune response to viral or tumor antigens. The findings presented here thus may have relevance to the use of IL-2 in the clinic, and to the possible benefit of IL-2 inhibition in treatment for conditions in which effector cytokine production results in pathology rather than protection.

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