

# Interleukin-10 Induces a Long-Term Antigen-specific Anergic State in Human CD4<sup>+</sup> T Cells

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## Summary

Human CD4<sup>+</sup> T cells, activated by allogeneic monocytes in a primary mixed lymphocyte reaction in the presence of exogenous interleukin (IL) 10, specifically failed to proliferate after restimulation with the same alloantigens. A comparable state of T cell unresponsiveness could be induced by activation of CD4<sup>+</sup> T cells by cross-linked anti-CD3 monoclonal antibodies (mAbs) in the presence of exogenous IL-10. The anergic T cells failed to produce IL-2, IL-5, IL-10, interferon  $\gamma$ , tumor necrosis factor  $\alpha$ , and granulocyte/macrophage colony-stimulating factor. The IL-10-induced anergic state was long-lasting. T cell anergy could not be reversed after restimulation of the cells with anti-CD3 and anti-CD28 mAbs, although CD3 and CD28 expression was normal. In addition, restimulation of anergized T cells with anti-CD3 mAbs induced normal Ca<sup>2+</sup> fluxes and resulted in increased CD3, CD28, and class II major histocompatibility complex expression, indicating that calcineurin-mediated signaling occurs in these anergic cells. However, the expression of the IL-2 receptor  $\alpha$  chain was not upregulated, which may account for the failure of exogenous IL-2 to reverse the anergic state. Interestingly, anergic T cells and their nonanergic counterparts showed comparable levels of proliferation and cytokine production after activation with phorbol myristate acetate and Ca<sup>2+</sup> ionophore, indicating that a direct activation of a protein kinase C-dependent pathway can overcome the tolerizing effect of IL-10. Taken together, these data demonstrate that IL-10 induces T cell anergy and therefore may play an important role in the induction and maintenance of antigen-specific T cell tolerance.

IL-10 has been shown to inhibit antigen-specific activation and proliferation of human peripheral blood T cells and T cell clones belonging to the Th0, Th1, or Th2 subsets (1, 2). These inhibitory effects were indirect and mediated through inhibition of the function of APCs (3–6). IL-10 regulates constitutive and IFN- $\gamma$ - or IL-4-induced class II MHC expression on monocytes, dendritic cells and Langerhans cells (2, 7). In addition, IL-10 inhibits the expression of CD54 (intercellular adhesion molecule-1, the ligand for LFA-1), CD80, and CD86 (ligands for CD28) which function as important costimulatory molecules for T cell activation (8–10). More recently, it has been shown that IL-10 also has a direct effect on CD4<sup>+</sup> T by suppressing IL-2 secretion (11, 12).

Similar to its inhibitory effects on T cell proliferation in response to soluble antigens, IL-10 strongly reduced the proliferation of human alloreactive cells in mixed lymphocyte reactions (MLR)<sup>1</sup>, and the levels of cytokines pro-

duced in these MLR were significantly reduced in the presence of exogenous IL-10 (13). In addition, IL-10 suppressed the proliferative responses of CD4<sup>+</sup> allogeneic T cell clones. In parallel with the reduced proliferation, reductions in the levels of IL-2, IL-5, GM-CSF, and IFN- $\gamma$  production by these T cell clones were observed (14).

Recently, we showed that in SCID patients successfully transplanted with HLA-mismatched hematopoietic cells, CD4<sup>+</sup> T cell clones specifically recognizing the host alloantigens produce very low levels of IL-2 after antigenic stimulation, but secrete high amounts of IL-10, which partially inhibits their proliferation in vitro (15). Furthermore, PBMC from these SCID patients express considerably higher levels of IL-10 transcript compared with PBMC of normal controls, especially in the non-T cell subset (15). These results suggest that the high expression of IL-10 detected in SCID human chimeras may play a key role in the maintenance of in vivo tolerance, by inducing an anergic state in donor-derived T cells specific for the host alloantigens.

Optimal activation and expansion of alloreactive T cells

<sup>1</sup> Abbreviations used in this paper: [Ca<sup>2+</sup>]<sub>i</sub>, intercellular calcium; MLR, mixed lymphocyte reaction.

requires, in addition to ligation of the TCR complex, costimulatory signals provided by one or more accessory molecules expressed on alloantigen-presenting cells. Engagement of the TCR by antigens without costimulation results in T cell anergy (16–19). This state of unresponsiveness may also be induced *in vitro* in long-term T cell clones by stimulation with agents that mimic TCR occupancy, in the absence of CD28 signaling, and/or by inhibition of IL-2 secretion by T cells (16–19). The observation that high levels of IL-10 are associated with transplantation tolerance, and that this cytokine has been shown to inhibit both the antigen-presenting and accessory function of monocytes and IL-2 production by T cells, suggest that IL-10 may be involved in the induction of anergy in CD4<sup>+</sup> T cells.

In the present study, we demonstrate that IL-10 is able to induce a long-lasting antigen-specific unresponsiveness against allogeneic antigens that cannot be reversed by IL-2 or CD28 stimulation.

## Materials and Methods

**Cells.** PBMC were prepared by centrifugation over Ficoll-Hypaque. CD4<sup>+</sup> T cells were purified by negative selection. Negative purification was performed using a cocktail of antibodies directed against non-CD4<sup>+</sup> T cells: CD8, CD14, CD16, CD19, CD20, CD56, and HLA-DR. Cells were incubated with saturating amounts of antibodies for 20 min at 4°C. After washing, Dynabeads (Dyna, Oslo, Norway) were added at a 10:1 bead/target cell ratio and incubated for 1 h at 4°C. Beads and contaminating cells were removed by magnetic field. The remaining cells were resuspended with the same amount of beads and a second incubation period of 1 h at 4°C was performed. After removing contaminating cells, CD4<sup>+</sup> T cells were analyzed by FACScan<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA) and revealed to be >90–95% positive. Monocytes were purified using the same procedure with an antibody cocktail containing CD2, CD3, CD8, CD16, CD19, CD20, and CD56. These monocytes were >95% CD14<sup>+</sup> by FACScan<sup>®</sup> analysis. In some experiments, CD4<sup>+</sup> T cells were purified by positive selection using magnetic beads directly coated with CD4 mAbs, according to the manufacturer's instructions (Dyna). With this procedure, cells were >95% pure.

**Reagents.** Purified recombinant IL-10 was provided by Schering-Plough Research Institute (Kenilworth, NJ). The anti-IL-2R mAb B-B10 (20) and anti-CD3 mAb SPV-T3 (21) were previously described. Nonconjugated, PE- or FITC-conjugated CD28, CD2, CD3, CD4, CD8, CD14, CD16, CD19, CD20, CD56, HLA-DR, and controls mAbs of the appropriate isotypes were purchased from Becton Dickinson & Co.

**Proliferation Assays.** In all proliferation assays, cells were cultured in Yssel's medium (22) supplemented with 10% FCS and 1% human serum. For MLR, purified CD4<sup>+</sup> T cells ( $5 \times 10^4$  cells/well) were stimulated with purified allogeneic monocytes ( $5 \times 10^4$  cells/well) or with irradiated PBMC ( $10^5$  cells/well) in 200- $\mu$ l flat-bottomed 96-well plates (Falcon; Becton Dickinson & Co., Lincoln Park, NJ). PBMC used as stimulators were irradiated at 4,000 rad.

For cross-linked anti-CD3 mAb activation, 500 ng/ml of anti-CD3 mAbs diluted in 0.1 M Tris buffer, pH 9.5, was incubated for 1 wk at 4°C in flat-bottomed 96- or 24-well plates. These experimental conditions were found to be optimal in experiments

in which different concentrations of antibodies and different incubation times were tested. After washing the plates three times, CD4<sup>+</sup> T cells were added at  $5 \times 10^4$  cells/well.

For activation with PMA plus Ca<sup>2+</sup> ionophore (A23187 Sigma Chemical Co., St. Louis, MO), cells were cultured at  $5 \times 10^4$  cells/well and activated for 3 d with PMA (1 ng/ml) and A23187 (500 ng/ml).

To measure the proliferation of T cells, cells were cultured for 72 h or 5 d for MLR experiments at 37°C in 5% CO<sub>2</sub>, subsequently pulsed with [<sup>3</sup>H]TdR for 12 h, and harvested as described previously (15). All tests were carried out in triplicate.

**Induction of Anergy.** To induce anergy, CD4<sup>+</sup> T cells were cultured at  $2.5 \times 10^5$  cells/ml in 24-well plates (Linbro; ICN Biomedicals, Inc., Aurora, Ohio) and activated either with purified allogeneic monocytes or cross-linked anti-CD3 mAbs in the presence of IL-10 (100 U/ml). After different incubation periods (ranging from 3 to 10 d, see below), cells were collected, layered on a Ficoll-Hypaque gradient to remove dead cells, washed twice, and restimulated with irradiated allogeneic PBMC or cross-linked anti-CD3 mAbs.

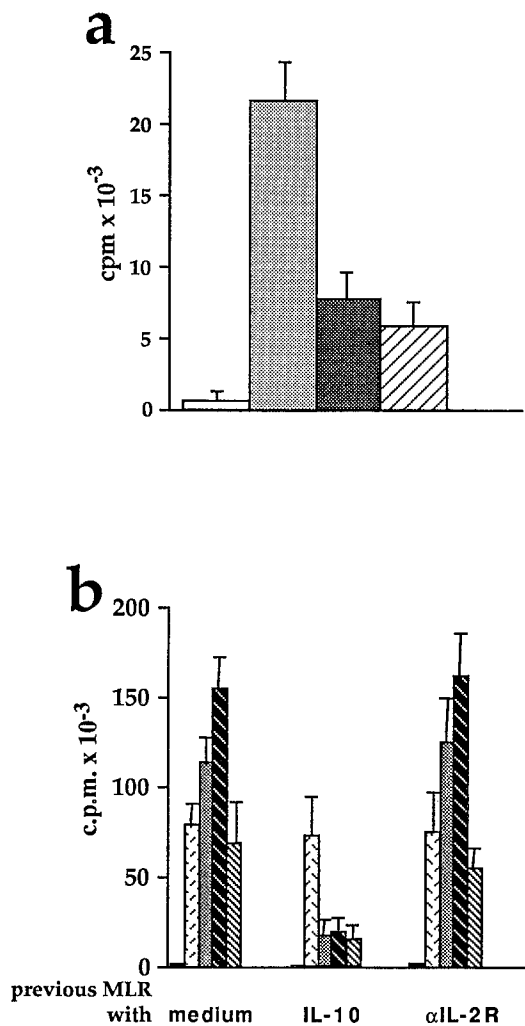
**Immunofluorescence Analysis.** For detection of cell surface antigens,  $10^5$  cells were labeled with PE- or FITC-conjugated mAbs. Cells were incubated for 30 min with the appropriate antibody at 4°C in PBS with 0.1% BSA and 0.02 mM NaN<sub>3</sub>. After three washes, the labeled cell samples were analyzed on a FACScan<sup>®</sup>.

**Determination of Lymphokine Production.**  $0.5 \times 10^6$  cells were stimulated by cross-linked anti-CD3 mAbs (10  $\mu$ g/ml), by PMA and Ca<sup>2+</sup> ionophore (A23187), or by allogeneic monocytes ( $1 \times 10^6$ ) for 24 h. The secretion of IL-2, IL-4, IL-5, IL-10, TNF- $\alpha$ , IFN- $\gamma$ , and GM-CSF was measured by immunoenzymetric assays performed as previously described (15, 23). The sensitivity of the various ELISAs was 20 pg/ml for IL-2, 40 pg/ml for IL-4 and IL-5, 50 pg/ml for GM-CSF and IL-10, and 100 pg/ml for IFN- $\gamma$  and TNF- $\alpha$ .

**Calcium Mobilization Studies.** Mobilization of intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>) in anergic cells loaded with indo-1/AM was assessed using standard fluorometry. Cells were loaded with 2  $\mu$ M indo-1/AM in complete growth medium at 20°C for 45 min. Cells were then washed, resuspended in Na-HBSS (in mM: 2 CaCl<sub>2</sub>, 145 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, D-glucose, and 20 Hepes, pH 7.3) containing 1% BSA and maintained at 20°C for up to 2 h. Approximately  $5 \times 10^5$  cells were then suspended in 2 ml Na-HBSS and maintained at 37°C in a constantly stirred acrylic cuvette. Anti-CD3 mAbs (1  $\mu$ g/ml) were added followed by goat anti-mouse Ig to cross-link the anti-CD3 mAbs on the cell surface. No Ca<sup>2+</sup> fluxes were detected in the absence of cross-linking, either in anergic or in control cells. Fluorescence measurements to determine [Ca<sup>2+</sup>]<sub>i</sub> were made using a Spectrofluorimeter (Photon Technologies Inc., South Brunswick, NJ).

## Results

**IL-10 Induces Alloantigen-specific T Cell Unresponsiveness.** As shown previously (13), IL-10 partially inhibits the proliferation of peripheral blood CD4<sup>+</sup> T cells in response to allogeneic monocytes in a primary MLR (Fig. 1 *a*). This inhibition was comparable with that induced by anti-IL-2R  $\alpha$  chain mAbs (anti-CD25 mAbs). To determine whether prolonged incubation of the alloantigen-stimulated CD4<sup>+</sup> T cells in IL-10 might also downregulate their proliferative responses after restimulation, the cells were kept in culture in the presence or absence of IL-10 for 10 d (Fig. 1 *b*). No



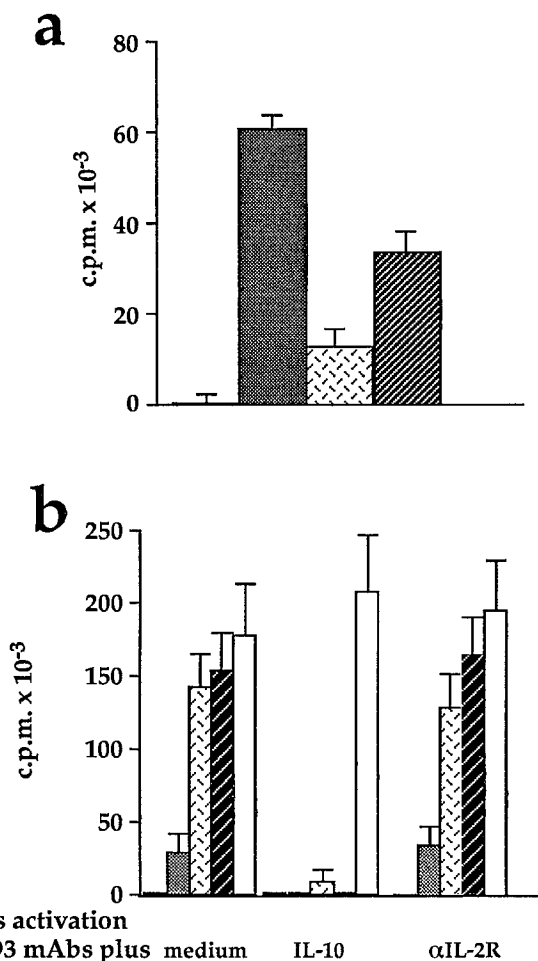
**Figure 1.** IL-10 induces alloantigen-specific anergy in CD4<sup>+</sup> T cells in MLR. (a) [<sup>3</sup>H]TdR incorporation of purified CD4<sup>+</sup> T cells cultured for 3 d in medium alone (white bar) or stimulated in a primary MLR with allogeneic purified monocytes (gray bar) in the presence of IL-10 (100 U/ml; black bar) or the anti-IL-2R $\alpha$  chain mAb B-B10 (hatched bar). (b) The three different bar sets represent cells that have been preactivated as described in a, kept in culture for 10 d, washed, and restimulated with either medium alone (black bars), allogeneic irradiated PBMC isolated from a third-party donor different from that of the monocytes used in the preactivation step (PBMC-2, crosshatched bars), allogeneic irradiated PBMC isolated from the same donor used in the preactivation step (PBMC-1, gray bars), PBMC-1 plus IL-2 (20 U/ml; black-hatched bars), or PBMC-1 plus anti-CD28 mAbs (10  $\mu$ g/ml; hatched bars). All measurements were done in triplicate; one representative experiment out of five is shown.

significant cell death was observed in either culture condition, and comparable cell numbers were harvested at termination of the assays (not shown). After this culture period, CD4<sup>+</sup> control T cells stimulated with alloantigens in the absence of IL-10 reached a resting state and could be fully reactivated by the same allogeneic irradiated PBMC in a secondary MLR, or by third-party allogeneic monocytes in a primary MLR (Fig. 1 b). In contrast, CD4<sup>+</sup> T cells incubated in the presence of IL-10 failed to proliferate in a secondary MLR in response to the same allogeneic PBMC,

but they retained their capacity to proliferate normally to third-party alloantigens in a primary MLR. This unresponsive state could not be reversed by saturating concentrations of exogenous IL-2 (20 U/ml) or by anti-CD28 mAbs (10  $\mu$ g/ml). Furthermore, this state of T cell unresponsiveness is specific for IL-10 and is not due to inhibition of the primary MLR, since CD4<sup>+</sup> T cells incubated in the presence of anti-CD25 mAbs (10  $\mu$ g/ml) for 10 d exhibited proliferative responses that were comparable with those of their untreated counterparts.

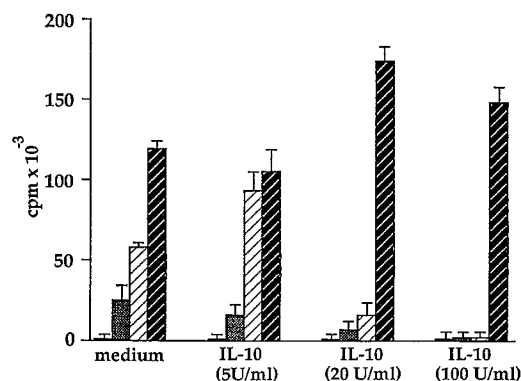
**IL-10 Induces an Anergic State in CD4<sup>+</sup> T Cells Activated by Anti-CD3 mAbs.** Next, we analyzed whether the IL-10-induced anergy reflected a direct effect of IL-10 on CD4<sup>+</sup> T cells. For this purpose, highly purified peripheral blood CD4<sup>+</sup> T cells were activated by cross-linked anti-CD3 mAbs in the presence or absence of exogenous IL-10 for 10 d. A direct effect of IL-10 on CD4<sup>+</sup> T cell proliferation was observed (11, 12), and this inhibitory effect was comparable with that induced by anti-CD25 mAbs (Fig. 2 a). Activation of the CD4<sup>+</sup> T cells in the presence of IL-10 resulted in a profound state of T cell unresponsiveness that could not be reversed by exogenous IL-2 or anti-CD28 mAbs (Fig. 2 b). However, anergic cells proliferated normally in response to stimulation by Ca<sup>2+</sup> ionophore and PMA. These results indicate that IL-10 induces an anergic state in CD4<sup>+</sup> T cells, but that these cells respond normally to signals circumventing TCR activation. This induction of anergy is IL-10 specific, similar to IL-10-induced T cell unresponsiveness to alloantigens, and not related to inhibition of cell proliferation, since CD4<sup>+</sup> T cells incubated in the presence of anti-CD25 mAbs for 10 d proliferated normally in response to restimulation with crosslinked anti-CD3 mAbs. The induction of anergy by IL-10 was dose dependent with maximal effects observed at 100 U/ml (Fig. 3). Cells that had been activated by anti-CD3 mAbs and cultured in the presence or absence of IL-10 for 10 d were in a resting state, and no apoptotic cell death was observed in the control or IL-10-treated cell cultures (not shown). Thus, IL-10 induces a comparable state of T cell anergy in alloantigen-specific and anti-CD3-stimulated CD4<sup>+</sup> T cells.

**Kinetics of Anergy Induction by IL-10.** To determine the kinetics of induction of T cell anergy, CD4<sup>+</sup> T cells were activated with cross-linked anti-CD3 mAbs in the presence or absence of IL-10 and restimulated at different time points after initiation of the cultures (Fig. 4). CD4<sup>+</sup> T cells cultured in the presence of IL-10 for only 3 d already failed to proliferate in response to reactivation by cross-linked anti-CD3 mAbs. Interestingly, the anergic state of these cells could still be reversed by exogenous IL-2 (20 U/ml) or anti-CD28 (10  $\mu$ g/ml). In contrast, incubation of the T cells with IL-10 for 9–10 d resulted in a complete state of anergy that could not be reversed by the addition of either IL-2 or anti-CD28 mAbs. These results indicate that T cells activated through their TCR/CD3 complexes in the presence of IL-10 acquire different degrees of unresponsiveness, depending on how long they have been exposed to IL-10.



**Figure 2.** IL-10 induces polyclonal anergy in CD4<sup>+</sup> T cells activated with CD3 mAb. (a) [<sup>3</sup>H]TdR incorporation of purified CD4<sup>+</sup> T cells cultured for 3 d in medium alone (white bar) or stimulated with cross-linked anti-CD3 mAbs (500 ng/ml; black bar) in the presence of IL-10 (100 U/ml; cross-hatched bar) or the anti-IL-2R $\alpha$  chain mAb B-B10 (hatched bar). (b) The three different bar sets represent cells that have been preactivated as described in a, kept in culture for 10 d, washed, and restimulated with either medium alone (black bars), cross-linked anti-CD3 mAbs (gray bars), anti-CD3 mAbs plus IL-2 (20 U/ml; cross-hatched bars), anti-CD3 mAbs plus anti-CD28 mAbs (10  $\mu$ g/ml; black-hatched bars) or PMA (1 ng/ml) plus Ca<sup>2+</sup> ionophore (A23187, 500 ng/ml) (white bars). All measurements were done in triplicate; one representative experiment out of five is shown.

**IL-10 Induced T Cell Anergy Is Long-lasting.** To determine the duration of the IL-10-induced anergic state in CD4<sup>+</sup> T cells after removal of IL-10, the cells were activated with cross-linked anti-CD3 mAbs in the presence or absence of IL-10 for 10 d. After this incubation period, the cells were washed and recultured for 24 d in the presence of low concentrations (2 U/ml) of exogenous IL-2 for optimal maintenance of the T cells over prolonged culture periods, but in the absence of IL-10. These T cells were collected every other day during the culture period, washed, and restimulated with anti-CD3 mAbs. T cells that previously had been incubated with IL-10 failed to proliferate even 24 d after removal of IL-10 (Fig. 5). In contrast, control CD4<sup>+</sup>

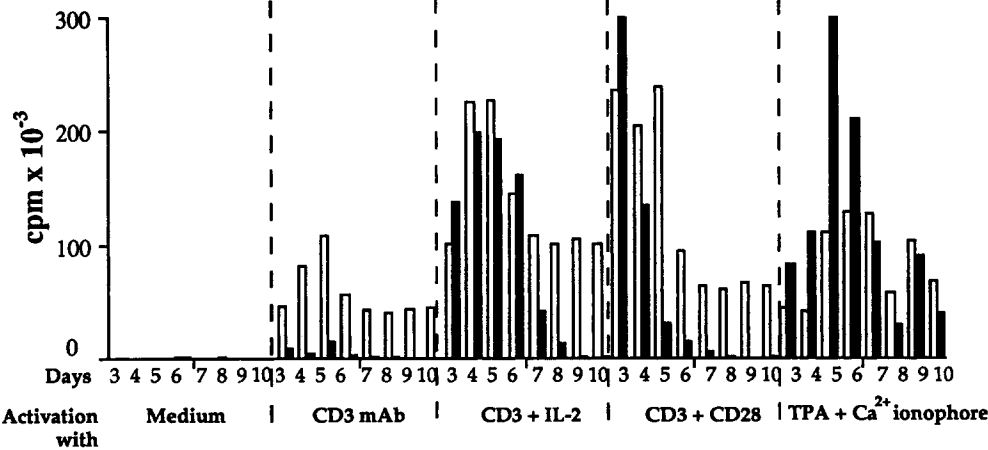


**Figure 3.** Dose-dependent effect of IL-10 on anergy induction in CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells were activated for 10 d with cross-linked anti-CD3 mAbs in the presence of medium alone, IL-10 (5 U/ml), IL-10 (20 U/ml), or IL-10 (100 U/ml), as indicated. After 10 d, cells were collected, washed, and restimulated with medium alone (white bars), cross-linked anti-CD3 mAbs (black bars), cross-linked anti-CD3 mAbs plus IL-2 (20 U/ml; hatched bars), or PMA plus Ca<sup>2+</sup> ionophore (black hatched bars). All measurements were made in triplicate; one representative experiment out of three is shown.

T cells that had been activated by anti-CD3 mAbs in the absence of IL-10, and maintained in the absence of IL-10 for 24 d, proliferated normally in response to stimulation with anti-CD3 mAbs. In addition, the unresponsiveness observed after culturing the IL-10-treated cells for 24 d could not be reversed by IL-2 (20 U/ml) or anti-CD28 (10  $\mu$ g/ml) mAbs. These results indicate that IL-10-induced T cell anergy is profound and long-lasting.

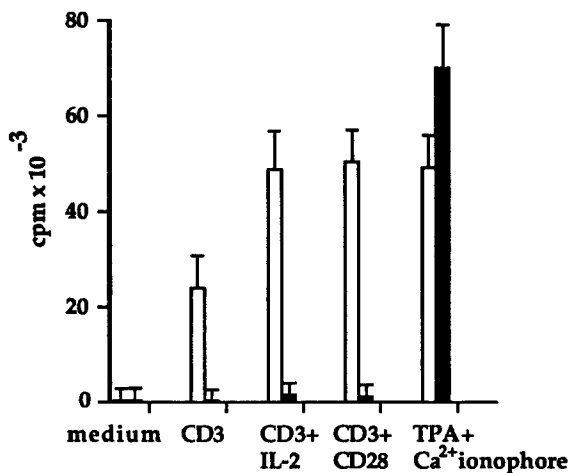
**Anergized T Cells Fail to Secrete Cytokines.** T cell anergy is generally defined as a failure of T cells to proliferate and to produce IL-2 in response to triggering of the TCR (18). To determine whether T cells rendered unresponsive after activation with either allogeneic monocytes or anti-CD3 mAbs in the presence of IL-10 retained their ability to secrete cytokines, we analyzed their cytokine production after restimulation with the same allogeneic monocytes or anti-CD3 mAbs, respectively. T cells that were rendered unresponsive after activation by either alloantigens plus IL-10 or anti-CD3 mAbs plus IL-10 failed to produce detectable amounts of IL-2, IL-4, IL-5, IL-10, TNF- $\alpha$ , IFN- $\gamma$ , or GM-CSF at 24 h (Table 1) or 48 h (not shown) after restimulation with the relevant allogeneic monocytes or anti-CD3 mAbs. In contrast, the untreated control cells produced levels of IL-2, IL-5, IL-10, IFN- $\gamma$ , TNF- $\alpha$ , and GM-CSF that were comparable with those of a panel of CD4<sup>+</sup> T cells derived from different donors and stimulated in similar fashion (not shown). However, these cells did not secrete detectable levels of IL-4 (Table 1).

As shown in Fig. 2, activation with PMA and Ca<sup>2+</sup> ionophore completely reversed IL-10-induced unresponsiveness of anti-CD3-activated T cells. In Table 1, it is shown that activation of anergic T cells by PMA and Ca<sup>2+</sup> ionophore for 24 h also resulted in levels of IL-2, IL-5, IL-10, IFN- $\gamma$ , and TNF- $\alpha$  production that were comparable with those of their nonanergized counterparts.



**Figure 4.** Kinetics of anergy induction by IL-10 in CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells were activated with cross-linked anti-CD3 mAbs in the absence (white bars) or presence (black bars) of IL-10 (100 U/ml). After different incubation periods, ranging from 3 to 10 d as indicated, cells were collected, washed, and restimulated with either medium alone, cross-linked anti-CD3 mAbs, cross-linked anti-CD3 mAbs plus IL-2 (20 U/ml), cross-linked anti-CD3 mAbs plus anti-CD28 mAbs (10 μg/ml), or PMA plus Ca<sup>2+</sup> ionophore. All measurements were done in triplicate and one representative experiment out of three is shown.

*Anergic T Cells Fail to Express CD25 after Activation.* Phenotypic analysis of T cells rendered anergic after activation by anti-CD3 mAbs in the presence of IL-10 revealed no major differences as compared with their untreated counterparts, with the exception of a decrease in the IL-2Rα chain (CD25) expression on the anergic T cells (Figs. 6 and 7, *a* and *b*). No modulation of CD3 or CD28 expression was observed on anergic cells, and activation by cross-linked anti-CD3 mAbs enhanced CD3, CD28, and class II MHC expression to the same extent as on control cells (Fig. 6). These data exclude the possibility that the T cell unresponsiveness was related to defective TCR/CD3 or CD28 expression and show that signaling through the



**Figure 5.** Anergy induced by IL-10 in CD4<sup>+</sup> T cells is long-lasting. CD4<sup>+</sup> T cells were activated with cross-linked anti-CD3 mAbs in the absence (white bars) or presence (black bars) of IL-10 (100 U/ml) for 10 d. After 10 d, cells were collected, washed, and cultured in the presence of IL-2 (2 U/ml). 24 d later, cells were collected and reactivated with cross-linked anti-CD3 mAbs, cross-linked anti-CD3 mAbs plus IL-2 (20 U/ml), cross-linked anti-CD3 mAbs plus anti-CD28 mAbs (10 μg/ml), or PMA plus Ca<sup>2+</sup> ionophore. The results show one representative experiment out of three.

TCR/CD3 complex, which is insufficient for T cell proliferation, still occurs in these anergic T cells.

However, in contrast to the control cells, the anergic T cells failed to upregulate IL-2Rα chain expression, indicating that inhibition of IL-2Rα chain expression is a specific property of anergized T cells (Fig. 7 *b*). The defect in upregulation of CD25 expression was observed also in the presence of exogenous IL-2, and correlated with the failure of IL-2 to reverse T cell anergy induced by 8–10 d of incubation with IL-10 (Fig. 4). In contrast, T cells rendered unresponsive after incubation with IL-10 for 3 d still expressed considerable levels of CD25 (Fig. 7 *a*). Slight upregulation of CD25 was also observed after restimulation by anti-CD3 mAbs, but the levels of expression were still reduced as compared with untreated cells. However, addition of IL-2, which was able to reverse the anergic state, resulted in comparable levels of CD25 expression on control T cells and T cells incubated in the presence of IL-10 for 3 d (Fig. 7 *a*).

*Anti-CD3 mAbs Induce Normal Ca<sup>2+</sup> Fluxes in Anergic T Cells.* The notion that signaling through the TCR/CD3 complex occurs in anergic T cells was confirmed by measuring the induction of Ca<sup>2+</sup> fluxes in these cells. After loading with Indo-1, cells were activated with anti-CD3 mAbs cross-linked by goat anti-mouse Ig, as indicated in Fig. 8. Ca<sup>2+</sup> fluxes induced in anergized T cells after activation with cross-linked anti-CD3 mAbs were comparable with those in untreated control T cells (Fig. 8). The loading of the anergic and control cells with Indo-1 was equivalent, as shown by the comparable rise of Ca<sup>2+</sup> fluxes induced in the cells by addition of Ca<sup>2+</sup> ionophore (Fig. 8).

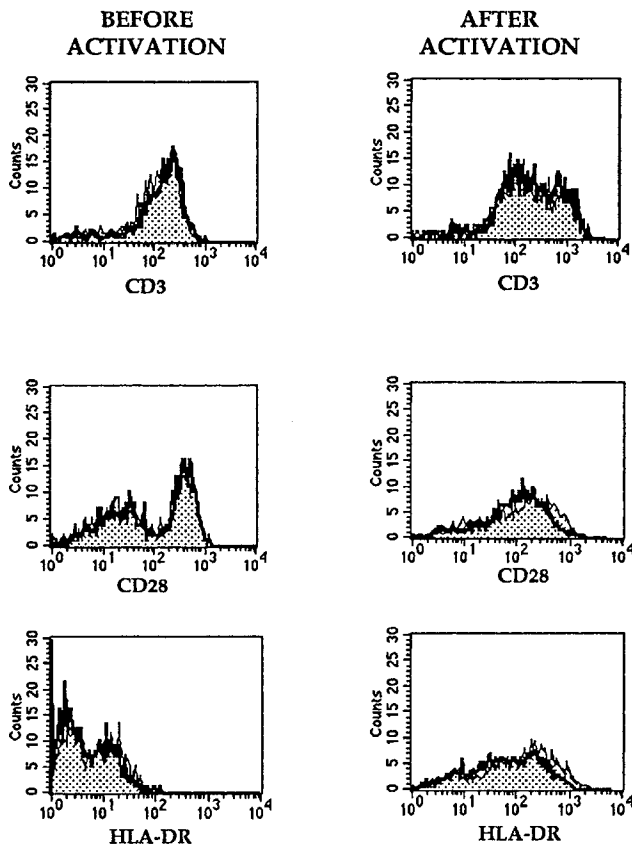
## Discussion

In the present study, we show that human peripheral blood CD4<sup>+</sup> T cells activated by allogeneic monocytes in the presence of IL-10 for 10 d were rendered unresponsive in an antigen-specific fashion. These unresponsive CD4<sup>+</sup> T

**Table 1.** Cytokine Profile of IL-10-induced Anergic T Cells

		cpm	IL-2	IL-4	IL-5	IL-10	TNF- $\alpha$	IFN- $\gamma$	GM-CSF
			pg/ml	pg/ml	pg/ml	pg/ml	ng/ml	ng/ml	pg/ml
CD3	Control								
	cells	42,369	875 $\pm$ 59	<40	80 $\pm$ 23	1,067 $\pm$ 226	10.5 $\pm$ 0.9	5.5 $\pm$ 0.6	1,245 $\pm$ 687
PMA + Ca <sup>2+</sup>	Control								
	ionophore								
cells	cells	2,635	<40	<40	<20	<50	<0.1	<0.1	<50
	Anergic								
cells	cells	147,217	2,121 $\pm$ 317	<40	841 $\pm$ 127	51 $\pm$ 27	21.6 $\pm$ 5.1	7.6 $\pm$ 3.1	2,721 $\pm$ 635
	Anergic								
cells	cells	169,321	1,921 $\pm$ 427	<40	1,517 $\pm$ 415	117 $\pm$ 4.3	18.4 $\pm$ 3.6	8.4 $\pm$ 2.6	1,615 $\pm$ 317
	Anergic								
Monocytes	Control								
	cells	32,236	617 $\pm$ 42	<40	64 $\pm$ 15	897 $\pm$ 125	9.2 $\pm$ 0.5	2.3 $\pm$ 0.1	987 $\pm$ 98
cells	cells	869	<40	<40	<20	<50	<0.1	<0.1	<50

CD4<sup>+</sup> T cells were activated with either cross-linked anti-CD3 mAbs, allogeneic monocytes in the absence (control cells) or presence (anergic cells) of IL-10 (100 U/ml). Cells were kept in culture for 10 d, washed, and restimulated with cross-linked anti-CD3 mAbs (10  $\mu$ g/ml) or PMA (1  $\mu$ g/ml) and Ca<sup>2+</sup> ionophore (A23187, 500 ng/ml) or allogeneic PBMC, respectively. Supernatants were harvested after 24 h and the levels of cytokines were analyzed by ELISA. To measure cell proliferation, cells were pulsed with [<sup>3</sup>H]TdR for 12 h at the end of day 3 for cells activated with cross-linked anti-CD3 mAbs, or at the end of day 5 for cells activated with allogeneic monocytes. Results are from one representative experiment out of three.

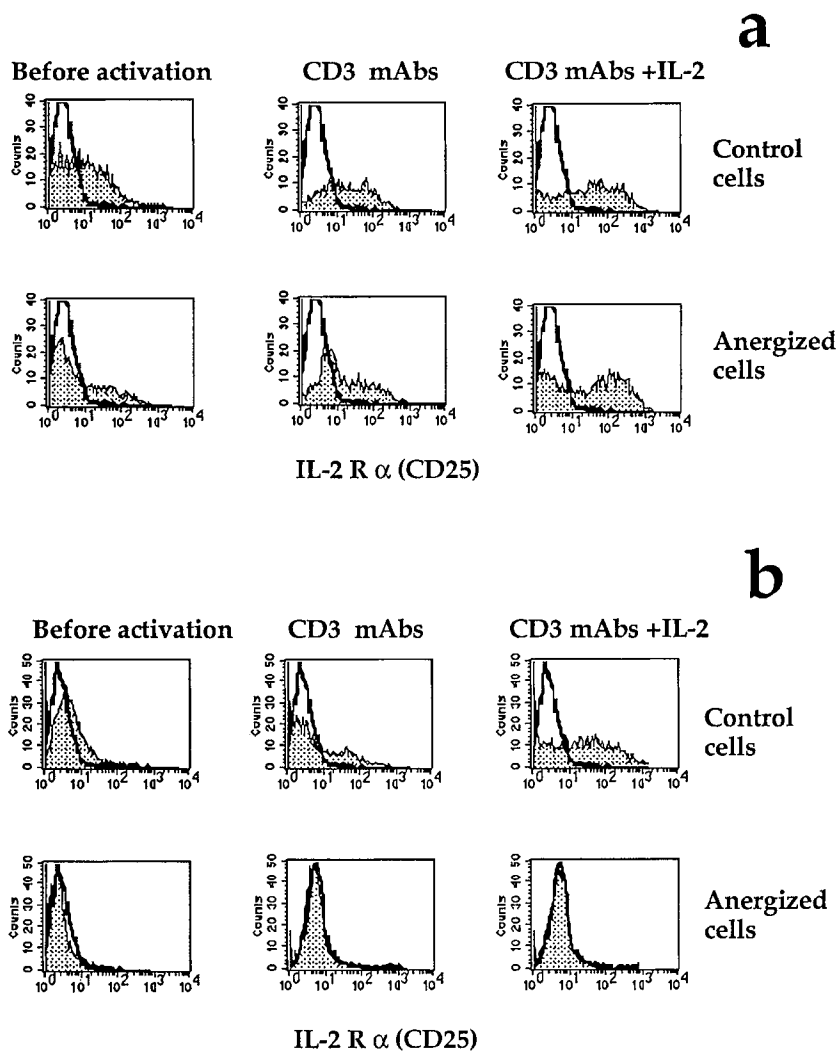


**Figure 6.** Cytofluorometric analysis of anergic T cells. CD4<sup>+</sup> T cells were activated with cross-linked anti-CD3 mAbs in the absence or presence of IL-10 (100 U/ml) for 10 d and the expression of CD3, CD28,

and HLA-DR was analyzed before and after reactivation with cross-linked anti-CD3 mAbs for 24 h. (Black lines) Control T cells cultured in the absence of IL-10; (dotted histograms) anergic T cells cultured in the presence of IL-10.

cells failed to proliferate and to produce cytokines when restimulated by the same allogeneic monocytes. However, these T cell populations proliferated normally in response to third-party alloantigens. A comparable state of T cell anergy was induced after activation of CD4<sup>+</sup> T cells by anti-CD3 mAbs in the presence of IL-10. The IL-10-induced anergy was dose dependent and lasted for more than 24 d after withdrawal of IL-10, which was the maximal time period analyzed. Furthermore, the anergic state could not be reversed by exogenous IL-2 or the addition of anti-CD28 mAbs. In addition, T cell anergy could not be induced by stimulation with allogeneic monocytes or anti-CD3 mAbs in the presence of anti-IL-2R mAbs. These results indicate that T cell anergy is specifically induced by IL-10 and is not simply related to downregulation of IL-2 production by these cells (16, 24). Recently it was shown that T cell unresponsiveness to alloantigens induced in MLR in the absence of costimulation could be reversed by exogenous IL-2 and stimulation of the CD2 pathway (25). Whether IL-10-induced T cell anergy can be restored in this way remains to be determined.

IL-10-induced T cell anergy differs clearly from T cell unresponsiveness induced by incubation of mouse or human T cells with antigenic peptides in the absence of pro-



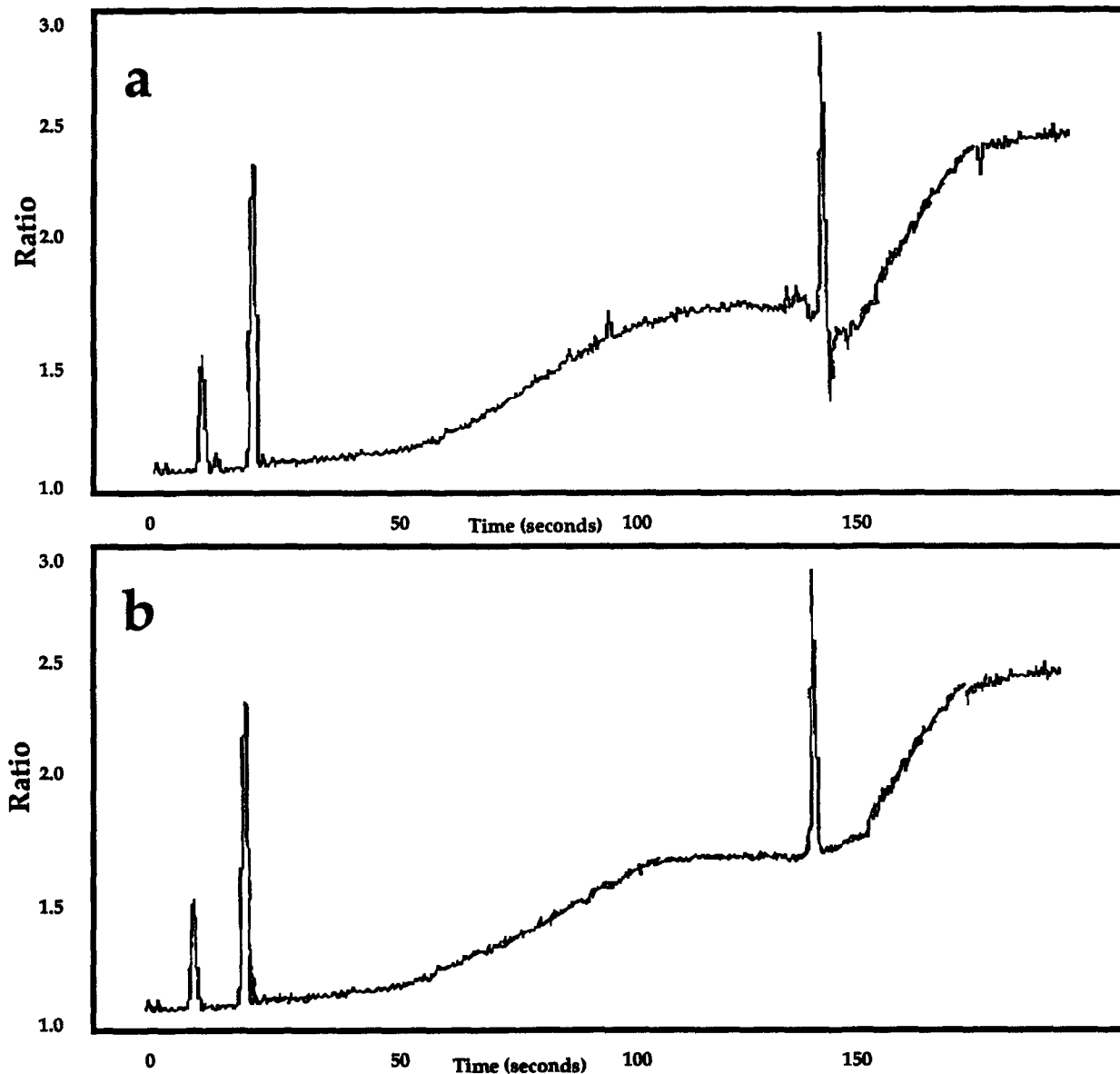
**Figure 7.** IL2R $\alpha$  chain expression on anergic T cells. CD4<sup>+</sup> T cells were activated with cross-linked anti-CD3 mAbs in the presence or absence of IL-10 (100 U/ml) for 3 d (*a*) or 10 d (*b*) and restimulated with either cross-linked anti-CD3 mAbs alone or with cross-linked anti-CD3 mAbs plus IL-2 (100 U/ml). The expression of the CD25 molecule was analyzed 24 h after activation by cytofluorometry. (Black lines) Control isotype; (dotted histograms) labeling with anti-CD25 mAbs.

fessional APC (19, 25, 26). In this latter case, the expression of the TCR/CD3 complex was downregulated and no mobilization of  $[Ca^{2+}]_i$  was observed (26, 27), unlike the findings with IL-10-induced anergy. On the other hand, CD4<sup>+</sup> T cells anergized by IL-10 share many characteristics with anergic T cells described in murine models of anergy induced by lack of costimulatory signals (16, 18, 28, 29). These anergic cells failed to proliferate and to produce IL-2, but they had a normal expression of CD3 or CD28 surface molecules, and normal calcium fluxes after mobilization of the TCR/CD3 complex (18). However, T cell anergy induced by IL-10 is much more profound than that described in these murine models, since the proliferative response of anergic T cells cannot be restored by addition of IL-2 or anti-CD28 mAbs. Furthermore, not only IL-2 production, but also the production of IFN- $\gamma$ , IL-5, IL-10, TNF- $\alpha$ , and GM-CSF by these anergic cells was impaired. Therefore, induction of T cell anergy by IL-10 is not due simply to inhibition of IL-2 production and prevention of productive CD28-CD80/CD86 interactions. IL-10-anergized T cells are also different from T cells that undergo apoptosis

or anergized T cells able to secrete cytokines in the absence of proliferation (24, 30). In fact, no significant cell loss by apoptosis was observed in the anergic T cells cultured in IL-10, and the anergic T cells were viable as proven by the normal proliferation after stimulation with  $Ca^{2+}$  ionophore and PMA.

Overall, our data indicate that signaling through the TCR/CD3 complex is selectively impaired in IL-10-anergized T cells. This is not due to downregulation of the TCR or CD28 molecules, since the anergic T cells expressed levels of TCR/CD3 and CD28 that were comparable with those of untreated T cells. Furthermore, although stimulation of the anergic T cells through the TCR/CD3 complex did not result in cell proliferation and cytokine production, a clear increase in CD3, CD28, and class II MHC expression was observed after restimulation of the cells with anti-CD3 mAbs, indicating that some degree of TCR activation occurred in these anergic cells.

This notion was further supported by the observation that  $Ca^{2+}$  fluxes in anergized T cells were normal after CD3 activation, demonstrating that calcineurin-mediated



**Figure 8.** Calcium mobilization analysis in anergic T cells. CD4<sup>+</sup> T cells were activated with anti-CD3 mAbs in the absence (a) or presence (b) of IL-10 (100 U/ml) for 10 d and loaded with indo-1/AM. As indicated, anti-CD3 mAbs (10 μg/ml), goat anti-mouse IgG (1 μg/ml), or Ca<sup>2+</sup> ionophore (500 ng/ml) were sequentially added into the cuvette and the rise of [Ca<sup>2+</sup>]<sub>i</sub> was analyzed by spectrofluorimetry and measured by the ratio of emission at 405/485. One representative experiment out of three is shown.

signaling is not affected. Importantly, a complete reversal of anergy was observed after activation with PMA and Ca<sup>2+</sup> ionophore. These stimuli, which circumvent TCR activation, completely restored proliferation and cytokine secretion by anergic cells. These findings suggest that IL-10 interferes with proximal events in the TCR signaling pathway, probably at the level of Ras-microtubule-associated protein (MAP) kinase activation, as is suggested for mouse models of anergy induction (31–33). Alternatively, although the signaling cascade downstream of p21ras seems to be intact, it cannot be excluded that IL-10 induces a negative regulator of this signaling pathway.

T cells rendered anergic by activation and incubation with IL-10 for 9–10 d failed, in contrast to their untreated counterparts, to upregulate the IL-2Rα chain expression when restimulated with anti-CD3 mAbs. It is therefore tempting to speculate that this defect in upregulation of IL-2Rα chain expression accounts for the failure of exogenous IL-2 to reverse anergy. This hypothesis is supported by the observation that T cells rendered anergic by activation and incubation with IL-10 for 3 d, and which still expressed CD25, could be rescued by exogenous IL-2. A comparable, but less profound, defect in IL-2 use has been reported for superantigen-induced T cell unresponsiveness in TCR-



transgenic mice (34). Taken together, these data suggest that anergy induction may be permanent if induced properly.

Transplantation tolerance, the long-term acceptance of grafted tissue in the absence of continuous immunosuppression, remains an elusive goal in humans, but it has been achieved in animal models using various approaches (29, 35, 36). Among the mechanisms responsible for induction of tolerance to alloantigens, a nondeletional mechanism that results in functional inactivation of the appropriate alloreactive T cells has been proposed. This functional inactivation can be achieved by blocking costimulatory signals provided by accessory molecules expressed on APC and T cells (37–40). Prevention of the interaction between CD28 and either CD80 or CD86 appears to be critical for this induction of anergy, but blockade of other costimulatory molecules such as CD2 or ICAM-1 may also be involved (25, 39–41). However, the fact that the tolerance induced in these experimental models is long-lasting and potentially irreversible suggests underlying mechanisms that may be more complex than the mere lack of a second signal to the Th cell upon recognition of alloantigens. It is possible that some form of active suppression is mediated by cytokines. These cytokines may contribute to the induction or maintenance of T cell anergy not only by downregulating co-

stimulatory molecules, but also by inducing the expression of negative regulators, such as CTLA-4 (42). In humans, SCID patients are one of the few examples in which *in vivo* tolerance is obtained after HLA-mismatched transplantation. This tolerance is due to a nondeletional mechanism that is responsible for the functional inactivation of T cells specifically recognizing the host alloantigens (43, 44). These host-reactive T cells secrete high levels of IL-10 *in vitro*, and high IL-10 levels have been observed *in vivo*, suggesting that IL-10 may play a role in the induction and maintenance of tolerance (15). Based on the present results, it is tempting to conclude that the high levels of IL-10 observed in these patients render the host-reactive T cells anergic *in vivo*. High levels of IL-10 secretion before transplantation have been also shown to correlate with a successful outcome of the transplant (45), further supporting the hypothesis that IL-10 might play a role in the induction of tolerance.

Collectively, our data suggest that IL-10 might have an important role in transplantation tolerance by inducing anergy to donor- and/or host-specific alloantigens. In addition, they indicate that IL-10 may have a potential clinical use in preventing or reducing GVHD and allograft rejection.

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The authors thank Lewis Lanier for critical review of the manuscript, René de Waal Malefyt for discussions and comments, Kevin Bacon for help with Ca<sup>2+</sup> flux experiments, and JoAnn Katheiser for secretarial assistance.

DNAX Research Institute of Molecular and Cellular Biology is supported by Schering-Plough Corporation.

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*Received for publication 16 January 1996 and in revised form 15 April 1996.*

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