A critical role for interleukin 4 in activating alloreactive CD4 T cells

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To generate antigen-specific responses, T cells and antigen presenting cells (APCs) must physically associate with each other and elaborate soluble factors that drive the full differentiation of each cell type. Immediately after T cell activation, CD4 T cells can produce both interferon γ (IFN-γ) and interleukin 4 (IL-4) before polarization into distinct T helper subsets. Inhibition of IL-4 during mixed allogeneic lymphocyte culture resulted in a defect in the ability of APCs to generate sufficient costimulatory signals for activation of alloreactive T cells. *In vivo*, a deficiency in IL-4 production inhibited the activation of alloreactive IL-2–, IL-4– and IFN-γ–producing CD4 T cells in mice challenged with allogeneic skin grafts, resulting in prolonged skin graft survival. Thus, production of IL-4 by CD4 T cells helps activate alloreactive T cells by affecting APC function.

T cells recognize allogeneic major histocompatibility (MHC) antigens via two distinct pathways of antigen presentation[1]. The direct pathway involves the recognition of intact allogeneic MHC molecules on the surface of donor cells by host T cells, whereas the indirect pathway involves the recognition of donor MHC–derived peptides that are processed by host antigen presenting cells (APCs) and presented as peptides to T cells. In cases where host and donor are matched for MHC class II, CD4 T cells can also recognize donor MHC-derived peptides in the context of donor MHC class II, a second form of indirect recognition. CD4 T cells that recognize allogeneic antigens through the indirect pathway play a major role in allograft rejection[2]. However, the requirements for activating these CD4 T cell responses remain undefined.

In addition to MHC-restricted peptide recognition via the T cell receptor (TCR), T cells must receive additional costimulatory signals provided by APCs to become fully activated effector cells. Interaction of CD28 on T cells with B7.1 (CD80) or B7.2 (CD86) on APCs provides sufficient costimulatory signals to T cells to induce cytokine production and cell proliferation[3]. APCs such as B cells and dendritic cells express low B7.1 and B7.2 in their resting state, and expression can be up-regulated by many inflammatory stimuli including adjuvants, lipopolysaccharide, IL-2, IL-4 and signaling through the cytoplasmic tail of MHC class II[4–6]. B7.1 and B7.2 costimulate T cells, and the overexpression of these ligands increases the ability of APCs to activate T cells[7]. Disrupting the interaction of CD28 with B7.1 and B7.2 leads to donor-specific tolerance in several animal allograft models[8], demonstrating the importance of this interaction to transplant rejection.

It has been suggested that immediately after T cells are stimulated, CD4 T cells can produce both interferon γ (IFN-γ) and interleukin 4 (IL-4)[9]. Additional antigenic stimulation polarizes CD4 T cells into either T helper type-1 (T_H1) cells that produce IL-2, tumor necrosis factor α (TNF-α) and IFN-γ, or T_H2 cells that produce IL-4, IL-5 and IL-10[10]. To determine the function of IL-4 production we examined the possible role of IL-4 production during T cell activation. Our data suggest that IL-4 is critical for the up-regulation of costimulatory molecules on APCs, which in turn allows for activation and expansion of alloreactive CD4 T cells. Thus, production of IL-4 after antigen encounter ensures efficient costimulation of antigen reactive T cells.

Results

IL-4 in alloreactive CD4 T cell activation

To determine the function of IL-4 in T cell activation, the MHC class I–disparate congenic B10.AKM (*H-2K*^{k}, *I*^{k}, *D*^{q}) and B10.MBR (*H-2K*^{b}, *I*^{k}, *D*^{q}) mice were used to examine the role of IL-4 in activating alloreactive CD4 T cells *in vitro* using mixed allogeneic cultures and *in vivo* by skin graft rejection. CD8 T cell–depleted splenocytes from B10.AKM mice primed *in vivo* by grafting with B10.MBR skin were stimulated *in vitro* with T cell–depleted B10.MBR splenocytes in the presence of either the IL-4–neutralizing antibody 11B11 or normal rat IgG. After 5 days the frequency of IL-4– and IFN-γ–producing cells in the cultures was determined by enzyme-linked immunospot (ELISPOT) assays (**Fig. 1a**). The

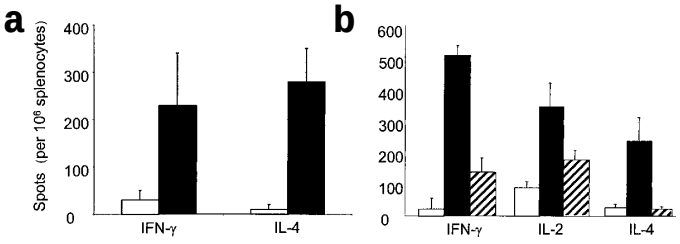

Figure 1. (a) Neutralization of IL-4 in mixed allogeneic cultures inhibits activation of cytokine-producing CD4 T cells. CD8-depleted B10.AKM splenocytes were cultured with T cell–depleted B10.MBR splenocytes in the presence of 100 μg/ml of either 11B11 (open bars) or control normal rat IgG (filled bars). After 5 days, the frequency of IFN-γ– or IL-4–producing cells was determined by ELISPOT assays. Results shown are an average of two independent representative experiments. **(b) Absence of IL-4 in mixed allogeneic culture inhibits activation of cytokine-producing CD4 T cells.** CD8-depleted C57BL/6 splenocytes were cultured with T cell–depleted B10.D2-*H2*^{d7} splenocytes in the presence of 100 μg/ml of either 11B11 (open bars) or control normal rat IgG (filled bars). CD8-depleted IL-4$^{-/-}$ splenocytes (hatched bars) were cultured with T cell–depleted B10.D2-*H2*^{d7} splenocytes. After 3 days, the frequency of IFN-γ–, IL-2– or IL-4–producing cells was determined by ELISPOT assays.

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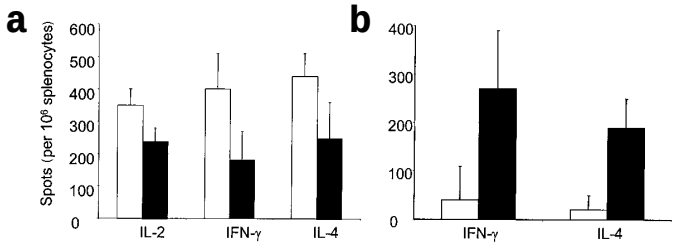


Figure 2. (a) Neutralization of IL-4 in mixed allogeneic culture does not lead to T cell anergy. CD8-depleted B10.AKM splenocytes were stimulated with T cell–depleted B10.MBR splenocytes in the presence of 100 μg/ml of either 11B11 (open bars) or control normal rat IgG (filled bars) for 3 days. The cells were then collected and CD4 T cells purified by magnetic cell sorting. The T cell preparations were then stimulated with fresh T cell–depleted splenocytes from B10.MBR mice in ELISPOT assays. One representative experiment of three is shown. **(b) Neutralization of IL-4 inhibits the ability of APC to stimulate alloreactive T cells.** CD8-depleted B10.AKM splenocytes were stimulated with T cell–depleted B10.MBR splenocytes in the presence of 100 μg/ml of either 11B11 (open bars) or control normal rat IgG (filled bars) for 5 days. The cells were then collected and T cells depleted to purify APC. The T cell–depleted APC preparations were then used to stimulate fresh CD8 T cell–depleted splenocytes from primed B10.AKM mice in ELISPOT assays. One representative experiment of three is shown.

frequency of IL-4–producing cells was significantly lower in 11B11-treated cultures (10±10 per 10^6 splenocytes) than in normal rat IgG–treated controls (280±70 per 10^6 splenocytes, $P<0.01$), consistent with the observation that IL-4 supports differentiation of T_H2 cells[10]. The frequency of IFN-γ–producing cells was also significantly decreased in 11B11-treated cultures (30±20 per 10^6 splenocytes) when compared with controls (230±110 per 10^6 splenocytes, $P<0.01$). Similar results were observed after 3 and 4 days of stimulation with T cell–depleted B10.MBR splenocytes (not shown). Detection of cytokine-producing cells required the presence of alloantigen during the stimulation phase of the assay and, in control studies, T cell–depletion of stimulated cells led to a significant decrease in the frequency of cytokine producing cells (not shown).

To examine whether the inhibition of alloreactive T cell activation after IL-4 neutralization was strain specific, we also examined the response of primed CD8 T cell–depleted splenocytes from C57BL/6 (*H-2*^{b}) mice to T cell–depleted splenocytes from MHC class I–mismatched B10.D2-*H2*^{i7} mice in the presence of 11B11 or normal rat IgG. C57BL/6J and B10.D2-*H2*^{i7} are not congenic strains, and are mismatched at *H-2D* and *H-2L* as well as multiple minor antigens. Thus, both the precursor frequency of alloreactive T cells and the speed of skin allograft rejection in this strain combination should be greater than in the congenic B10.AKM and B10.MBR strains that are mismatched at only the *H-2K* locus. The frequency of C57BL/6 alloreactive IL-4– (25±5 *versus* 240±75 per 10^6 splenocytes, $P<0.01$), IFN-γ– (25±35 *versus* 515±30 per 10^6 splenocytes, $P<0.01$) and IL-2– (90±20 *versus* 350±75 per 10^6 splenocytes, $P<0.02$) producing cells was significantly reduced in 11B11-treated cultures when compared with control cultures containing normal rat IgG (**Fig. 1b**), suggesting that the requirement for IL-4 in activating alloreactive T cells is not strain specific.

To examine whether the effect of IL-4 neutralization was due to a non-specific inhibitory effect of the neutralizing antibody 11B11, we next examined the response of CD8 T cell–depleted splenocytes from primed C57BL/6 IL-4 knockout mice[11] when stimulated with MHC class I mismatched B10.D2-*H2*^{i7} T cell–depleted splenocytes. The frequency of alloreactive CD4 T cells from IL-4–deficient mice that produced IFN-γ (140±45 *versus* 515±30, $P<0.02$) and IL-2 (180±30 *versus* 350±75, $P<0.02$) were significantly reduced when compared with the frequency observed for C57BL/6 controls (**Fig. 1b**). The frequency of alloreactive IFN-γ–, IL-2– and IL-4–producing cells observed using T cells from IL-4–deficient mice was statistically the same as the frequency observed in cultures containing C57BL/6 T cells and 11B11 ($P>0.08$ in all cases). Collectively, these data suggest that IL-4 is involved in activation of alloreactive CD4 T cells.

IL-4 and APC-stimulation of alloreactive CD4 T cells

To further examine the role of IL-4 in the activation of alloreactive CD4 T cells, CD8 T cell–depleted splenocytes from primed B10.AKM mice were stimulated *in vitro* with T cell–depleted splenocytes from B10.MBR mice for 5 days in the presence of either 11B11 or normal rat IgG. CD4 T cells were then purified from the cultures by magnetic cell sorting, and cytokine ELISPOT assays were done after stimulation with freshly isolated T cell–depleted B10.MBR splenocytes. CD4 T cells collected from 11B11-treated cultures were readily able to produce IFN-γ (400±11 per 10^6 splenocytes), IL-2 (350±50 per 10^6 splenocytes) and IL-4 (440±70 per 10^6 splenocytes) when stimulated for 24 h with T cell–depleted allogeneic stimulators (**Fig. 2a**), indicating that IL-4 neutralization did not result in T cell anergy or death. There was no statistically significant difference in the frequency of cytokine-producing CD4 T cells collected from 11B11 or normal rat IgG–treated cultures (**Fig. 2a**).

To determine whether the effect of IL-4 neutralization on T cell activation was due to changes in APC function, we examined the ability of APCs collected from similar cultures to stimulate freshly isolated CD4 T cells from primed B10.AKM mice. When analyzed by flow cytometry, no significant difference in the number of CD45R$^+$ (B220) B cells or CD11c$^+$ dendritic cells was observed in 11B11-treated cultures when compared with normal rat IgG–treated cultures (not shown). APCs collected from normal rat IgG–treated cultures could stimulate both alloreactive IFN-γ– (270±120 per 10^6 splenocytes) and IL-4–producing CD4 T cells (190±70 per 10^6 splenocytes) (**Fig. 2b**). In contrast, APCs collected from 11B11-treated cultures could not stimulate alloreactive IFN-γ– (40±60 per 10^6

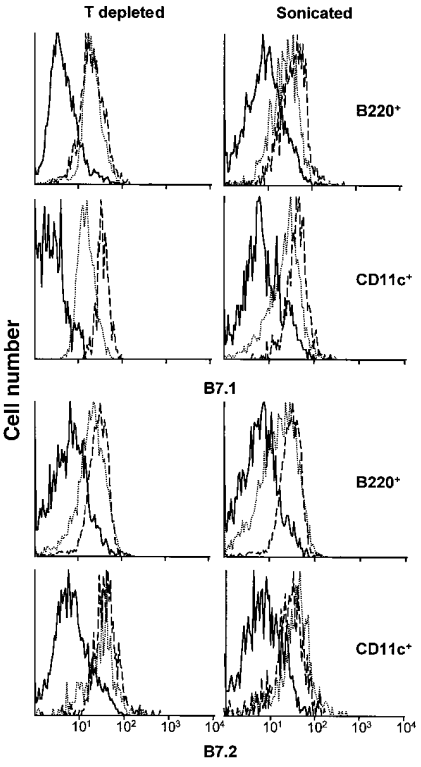


Figure 3. Neutralization of IL-4 *in vitro* prevents up-regulation of B7.1 and B7.2 surface expression on APC. Splenocytes were collected from primed B10.AKM mice, CD8 T cell–depleted and then cultured for 7 days with either T cell–depleted B10.MBR splenocytes (T depleted) or sonicated B10.MBR splenocytes (sonicated). The cells were cultured in media alone (dashed line), media containing 100 μg/ml 11B11 (solid line) or media containing 100 μg/ml 11B11 and 5 ng/ml recombinant murine IL-4 (dotted line). To analyze B7.1 and B7.2 expression by flow cytometry, the cells were gated on either B220$^+$ or CD11c$^+$ cells. One representative experiment of four is shown.

splenocytes, $P<0.01$) and IL-4–producing CD4 T cells (20±30 per 10^6 splenocytes, $P<0.01$). Thus, neutralization of IL-4 caused defects in the ability of APCs to stimulate alloreactive CD4 T cells.

To determine the role of IL-4 in APC function, CD8 T cell–depleted splenocytes from primed B10.AKM mice were stimulated *in vitro* with T cell–depleted splenocytes from MHC class I–disparate B10.MBR mice in the presence of 11B11, and cells were then examined for B7.1 and B7.2 expression by flow cytometry. B7.1 and B7.2 were up-regulated on the surface of B cells and dendritic cells in mixed lymphocyte cultures containing CD4 T cells from primed B10.AKM mice and T cell–depleted B10.MBR splenocyte stimulators. In contrast, neutralization of IL-4 in culture with 11B11 prevented up-regulation of B7.1 and B7.2 on both B cells and dendritic cells, consistent with the observation that IL-4 affects expression of B7.1 and B7.2 on APCs[12] (**Fig. 3**). The effect of 11B11 on B7.1 and B7.2 expression was concentration-dependent, and required allogeneic stimulators (not shown). Addition of rat IgG to control cultures had no effect on B7.1 and B7.2 up-regulation (not shown). The effect of IL-4 neutralization was apparent on both syngeneic B10.AKM and allogeneic B10.MBR APCs (not shown). Addition of exogenous IL-4 to 11B11-treated cultures restored B7.1 and B7.2 up-regulation in culture, suggesting that the inhibitory effects of 11B11 on B7.1 and B7.2 surface expression were specific to the action of IL-4.

Because alloreactive CD4 T cells can recognize processed alloantigen presented by MHC class II on self-APCs, we also examined the effect of IL-4 neutralization on B7.1 and B7.2 expression after stimulation of CD8 T cell–depleted splenocytes from primed B10.AKM mice with sonicated B10.MBR splenocytes. Neutralization of IL-4 with 11B11 in these cultures prevented the up-regulation of B7.1 and B7.2 to a similar degree as that observed using intact B10.MBR stimulators (**Fig. 3**).

Table 1. Cytokine gene expression in allogenic and syngeneic skin grafts from Thx-CD8$^-$ B10.AKM and untreated B10.AKM mice

	Thx-CD8$^-$ B10.AKM Skin graft				Untreated B10.AKM Skin graft			
Cytokine	B10.MBR		B10.AKM		B10.MBR		B10.AKM	
TNF-α	6/8[a]	75%[b]	8/8	100%	3/4	75%	4/4	100%
IL-2	5/8	62%	5/8	62%	3/4	75%	3/4	75%
IL-10	8/8	100%	8/8	100%	4/4	100%	4/4	100%
IFNγ	6/8	75%	6/8	62%	2/4	50%	3/4	75%
IL-4	5/8	62%	0/8	0%	2/4	50%	0/4	0%

[a]Number of grafts expressing cytokine mRNA. [b]Percentage of grafts expressing cytokine mRNA.

IL-4 expression in allografts undergoing rejection

To determine whether IL-4 has a role in activating alloreactive CD4 T cells *in vivo*, we first examined cytokine gene expression in allogeneic skin grafts undergoing CD4 T cell–mediated rejection. To this end, thymectomized and CD8 T cell–depleted (Thx-CD8$^-$ mice) or untreated B10.AKM mice were grafted with both B10.MBR and syngeneic tail skin. Grafts were collected for RNA isolation 12 days later and intragraft expression of cytokine genes was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). IL-2–, IL-10–, TNF-α– and IFN-γ–transcripts were expressed in both allogeneic and syngeneic grafts collected from both Thx-CD8$^-$ and normal B10.AKM mice, suggesting that expression of these cytokines was not specific to allograft rejection.

Although IL-4 transcripts were detected in 62% of rejected B10.MBR allografts, we were unable to detect expression of IL-4 in syngeneic B10.AKM grafts collected from the same mice (**Table 1**, $n=8$, $P=0.02$). IL-4 transcripts were also detected in 50% of allogeneic B10.MBR grafts collected from normal B10.AKM mice but not in the syngeneic grafts (**Table 1**, $n=4$, $P>0.05$). Expression of IL-4 in rejecting grafts was still apparent at later timepoints in both Thx-CD8$^-$ and normal B10.AKM mice (not shown). Thus, IL-4 is produced during the specific rejection response and not simply produced as a result of nonspecific inflammation.

IL-4 in CD4 T cell–mediated allograft rejection

We next examined whether neutralization of IL-4 *in vivo* could affect allograft survival. Thx-CD8$^-$ B10.AKM mice were injected intraperitoneally with either 11B11 or normal rat IgG 1 day before skin grafting and then every other day until the grafts were rejected. Survival of class I–disparate grafts from B10.MBR congenic mice on B10.AKM mice treated with 11B11 (**Fig. 4a**) was significantly prolonged—median survival time (MST)=30 days—when compared to survival of B10.MBR grafts on mice treated with normal rat IgG (MST=17 days, $P=0.001$). Survival of B10.D2-*H2*^{i7} skin grafts was also significantly prolonged on Thx-CD8$^-$ C57BL/6 IL-4 knockout mice (MST=19 days) when compared with survival on Thx-CD8$^-$ C57BL/6 controls (MST=11 days, $P=0.0001$) (**Fig. 4b**), suggesting that the effect of 11B11 treatment was neither strain-specific nor the result of a nonspecific inhibitory effect of 11B11. After rejection of the B10.MBR skin grafts, the percentage of peripheral blood CD4 T cells in Thx-CD8$^-$ B10.AKM mice treated with saline (8.0±0.42%) or normal rat IgG (7.2±0.43%) was increased when compared with the percentage observed in the blood of Thx-CD8$^-$ mice that never received a skin graft (3.4±0.19%, $P<0.001$) (**Fig. 5a**). These data suggest that CD4 T cells undergo significant expansion in response to alloantigen.

In contrast, the percentage of CD4 T cells in 11B11-treated Thx-CD8$^-$ B10.AKM mice that rejected a B10.MBR skin graft was significantly lower (4.4±0.45%, $P=0.004$) than the percentage of CD4 T cells in mice receiving normal rat IgG, and did not differ significantly from the percentage of CD4 T cells in the blood of naïve Thx-CD8$^-$ mice ($P=0.09$). Similarly, after rejection of the B10.D2-*H2*^{i7} skin grafts, the percentage of CD4 T cells in the blood of Thx-CD8$^-$ C57BL/6 mice (15.7±1.7%) was increased when compared to the percentage observed before skin grafting (12.5±1.2%, $P=0.02$) (**Fig. 5b**). In contrast, after rejection of the B10.D2-*H2*^{i7} skin grafts, the percentage of CD4 T cells in the blood of Thx-CD8$^-$ C57BL/6 IL-4 knockout mice was unchanged ($P=0.8$) when

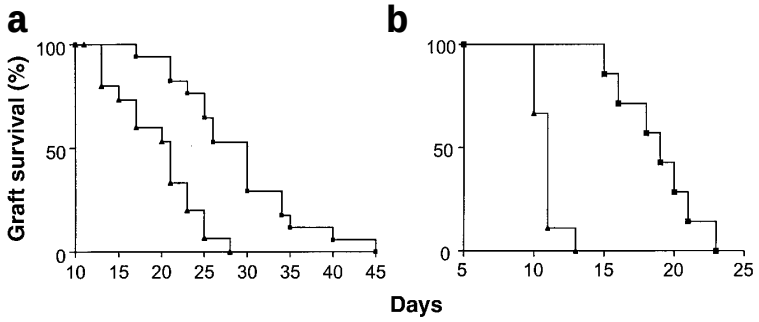


Figure 4. Neutralization or absence of IL-4 *in vivo* results in prolonged skin allograft survival. (**a**) Survival of B10.MBR skin grafts on Thx-CD8$^-$ B10.AKM mice treated with 11B11 (■, $n=18$) or control normal rat IgG (▲, $n=11$). One representative experiment of three is shown. (**b**) Survival of B10.D2-*H2*^{i7} skin grafts on Thx-CD8$^-$ IL-4$^{-/-}$ mice (■, $n=7$) or C57BL/6 mice (◆, $n=9$).

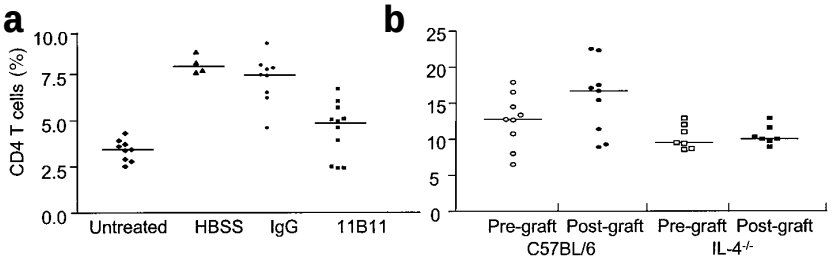


Figure 5. (a) Neutralization of IL-4 *in vivo* prevents expansion of CD4 T cells. Blood was collected from Thx-CD8$^-$ B10.AKM mice 35 days after skin grafting, and stained with saturating concentrations of anti-mouse CD4. Shown is the percentage of CD4 T cells in the blood of control Thx-CD8$^-$ mice that were not treated, that is they did not receive a skin graft or antibody treatment (♦, *n*=9), Thx-CD8$^-$ mice treated with HBSS and grafted with B10.MBR skin (▲, *n*=4), Thx-CD8$^-$ mice treated with normal rat IgG and grafted with B10.MBR skin (●, *n*=9), and Thx-CD8$^-$ mice treated with 11B11 and grafted with B10.MBR skin (■, *n*=11). Values for individual mice are plotted. The horizontal lines show the mean values for each group. **(b) Absence of IL-4 *in vivo* prevents expansion of CD4 T cells.** The percentage of CD4 T cells in the blood of control Thx-CD8$^-$ C57BL/6 mice before receiving a skin graft (○, *n*=9), Thx-CD8$^-$ C57BL/6 mice grafted with B10.D2-*H2*^{i7} skin (●, *n*=9), Thx-CD8$^-$ IL-4$^{-/-}$ mice before receiving a skin graft (□ *n*=7), and Thx-CD8$^-$ IL-4$^{-/-}$ mice grafted with B10.D2-*H2*^{i7} skin (■, *n*=7) is shown. Values for individual mice are plotted. The horizontal lines show the mean values for each group.

compared to the percentage before skin grafting. There was no statistically significant difference in the number of total leukocytes per ml of blood between any groups (not shown). Together, these data suggest that IL-4 is directly involved in CD4 T cell–mediated allograft rejection, and that neutralization of IL-4 *in vivo* prevents expansion of CD4 T cells.

To determine whether the overall decrease in the percentage of CD4 T cells in 11B11-treated mice reflected a specific decrease in alloreactive cells, Thx-CD8$^-$ B10.AKM mice were allowed to reject B10.MBR grafts while receiving 11B11 or normal rat IgG. Four weeks after graft rejection, the mice were killed and cytokine ELISPOT assays were done to determine the frequency of IL-2–, IFN-γ– or IL-4– producing alloreactive T cells after stimulation for 24 h *in vitro* with either T cell–depleted syngeneic splenocytes or T cell–depleted allogeneic B10.MBR splenocytes. The frequency of cytokine-producing cells was expressed as the ratio of cytokine producing cells detected after stimulation with allogeneic *versus* syngeneic splenocytes to normalize for potential differences in the cell composition of the spleens of the 11B11- and normal rat IgG–treated mice.

As expected, the ratio of IL-4–producing T cells capable of responding to allogeneic *versus* syngeneic splenocytes was significantly reduced (1.3±0.15) in 11B11-treated mice when compared to those in normal rat IgG–treated controls (3.0±0.7, $P \leq 0.01$) (**Fig. 6a**). The ratio of cells producing IL-2 or IFN-γ was also reduced in 11B11-treated mice. The ratio of IL-2–producing cells was 1.2±0.07 in 11B11-treated mice compared with 2.6±0.7 (P=0.01) in normal rat IgG–treated controls, and the ratio of cells producing IFN-γ was 1.1±0.07 in 11B11-treated mice and 4.0±0.96 ($P \leq 0.01$) in normal rat IgG–treated mice. Similarly, four weeks after rejection of B10.D2-*H2*^{i7} skin grafts the ratio of cytokine-producing T cells capable of responding to allogeneic *versus* syngeneic splenocytes was significantly reduced in Thx-CD8$^-$ C57BL/6 IL-4 knockout mice when compared with Thx-CD8$^-$ C57BL/6 controls (**Figure 6b**). Thus, as suggested by our *in vitro* experiments, neutralization of IL-4 impairs formation of alloreactive CD4 T cells.

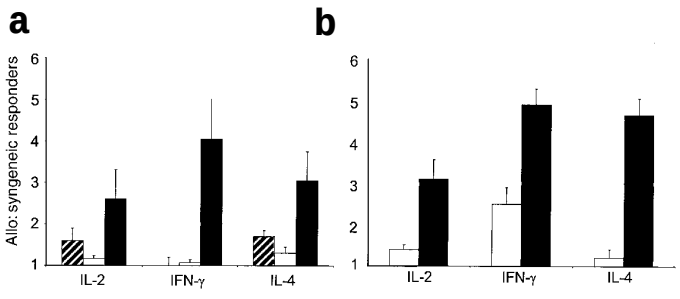
Figure 6. (a) Neutralization of IL-4 *in vivo* inhibits the activation of alloreactive CD4 T cells. Thx-CD8$^-$ B10.AKM mice were grafted with B10.MBR skin and treated with either 11B11 (open bars) or normal rat IgG (filled bars). Five weeks after skin graft rejection, spleens were collected and ELISPOT assays done on splenocytes to measure IL-4, IFN-γ and IL-2 production after stimulation with either T cell–depleted B10.MBR or B10.AKM splenocytes. Control mice (hatched bars) were thymectomized and CD8 T cell–depleted as described, but did not receive B10.MBR skin grafts. Results are expressed as the ratio of alloreactive to autoreactive cells. A ratio of 1 indicates no difference over background observed using syngeneic stimulators. Values shown represent the average of six mice in each group. **(b) Absence of IL-4 *in vivo* inhibits the activation of alloreactive CD4 T cells.** Thx-CD8$^-$ IL-4$^{-/-}$ (open bars, *n*=7) or Thx-CD8$^-$ C57BL/6 (filled bars, *n*=5) mice were grafted with B10.D2-*H2*^{i7}. Four weeks after skin graft rejection, spleens were collected and ELISPOT assays done on splenocytes to measure IL-4, IFN-γ and IL-2 production after stimulation with either T cell–depleted C57BL/6 splenocytes or B10.D2-*H2*^{i7} splenocytes. Values shown represent an average of two independent experiments.

Discussion

Our data suggest that IL-4 plays a critical role in activating alloreactive CD4 T cells. Neutralization of IL-4 *in vitro* led to a defect in the ability of APCs to stimulate cytokine-producing CD4 T cells. Although T cells collected from cultures in which IL-4 had been neutralized responded to fresh APCs in the presence of IL-4, APCs collected from these cultures were unable to stimulate fresh alloreactive CD4 T cells. Our analysis of APCs collected from these cultures showed that the up-regulation of at least two key costimulatory molecules, B7.1 and B7.2, was inhibited after IL-4 neutralization. *In vivo*, in the absence of IL-4, the ability to activate alloreactive CD4 cells was inhibited, resulting in prolonged skin allograft survival. Although IL-4 knockout mice can reject allografts[13] and generate T_H1 responses under appropriate conditions, we suggest that rejection in these cases may be mediated by CD8 T cells that can reject skin grafts in the absence of CD4 T cells[14,15]. Although neutralization of IL-4 does not result in permanent graft survival in the models we tested, APC maturation can be induced by many stimuli other than IL-4. We suggest that stimuli, such as the shedding of necrotic or apoptotic cells from the graft, are likely to occur *in vivo* and could overcome the requirement for IL-4[16,17]. Nevertheless the ability to prolong graft survival, a rigorous test of T cell function, by inhibiting IL-4 shows that this cytokine plays a major role in mediating a rejection response.

Although a role for IL-4 in activating IL-2– or IFN-γ–producing CD4 T cells may seem contrary to the current findings in IL-4 knockout mice, IL-4 knockout mice do not generate normal CD4 T cell responses under all conditions[18,19]. Experiments in IL-4 knockout mice have suggested that IL-4 may be required for the generation of tumor-specific T_H1 cells[20]. Elimination of IL-4–producing cells after stimulation *in vitro* prevents production of IFN-γ–producing T_H1 cells and T_H2 cells[21]. Together, these data support the hypothesis that IL-4 is critically involved in priming of T_H1 and T_H2 responses.

Our results suggest that inhibition of IL-4 leaves T cells in a state of clonal ignorance, consistent with the observation that preventing the interaction with B7 molecules leaves T cells unactivated but functional[22]. In some systems, it has been suggested that peripheral T cell tolerance to allografts may be IL-4–dependent[23]. Because the induction of T cell



anergy requires costimulation through B7 molecules[22], our findings suggest that IL-4–mediated up-regulation of B7.1 and B7.2 may play a role in promoting T cell anergy in some systems through its action on APCs. Because in our model alloreactive CD4 T cells must recognize processed allogeneic peptides rather than intact cell surface allogeneic MHC class I, antigen recognition in this system is similar to that observed in normal T cell responses to protein antigens. The requirement for IL-4 in CD4 T cell responses to nontransplantation antigens is not yet known. Based on our data, we suggest that IL-4 helps ensure productive T cell–APC interaction in some systems.

Methods

Mice. 6 to 12-week-old female B10.AKM/SnJ, B10.MBR/Sx, C57BL/6J, C57BL/6J-IL-4$^{tm/cgn}$ (IL-4 knockouts) and male or female B10.D2-*H2*^{i7} mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained under microisolator conditions on autoclaved feed and water. C57BL/6J-IL-4$^{tm/cgn}$ mice were backcrossed to C57BL/6 mice for 12 generations. All animal procedures were approved by the institutional review board at Massachusetts General Hospital.

Mixed allogeneic culture. To increase the frequency of alloreactive T cells, B10.AKM mice were primed by grafting with B10.MBR skin. Two weeks after graft rejection, the mice were killed. Splenocytes were then collected and depleted of CD8 T cells using the monoclonal antibody to CD8, 2.43 (rat IgG2b)[24], and goat anti-rat microbeads (Miltenyi Biotech., Sunnyvale, CA) in conjunction with the MACS magnetic cell sorting system according to the manufacturer's specifications (Miltenyi Biotech., Auburn, CA). Splenocytes were collected from naïve B10.MBR mice and T cell–depleted using 2.43 and the monoclonal antibody to CD4, GK1.5 (rat IgG2b)[25], in conjunction with MACS cell sorting as above. Similarly, C57BL/6 and IL-4 knockout mice were primed by grafting with B10.D2-H2[17] skin. All magnetically purified splenocytes were checked for complete depletion by FACS using antibodies not blocked by those used for depletion (53-5.8 anti-CD8, RM4-5 anti-CD4, Pharmingen). CD8 T cell–depleted B10.AKM and T cell–depleted B10.MBR splenocytes were plated at a 1:1 ratio, at a concentration of 3×10^6 cells/ml in DMEM media containing 15% fetal calf serum and either 100 μg/ml 11B11 (rat anti-mouse IL-4)[26] or 100 μg/ml normal rat IgG (Jackson ImmunoResearch, West Grove, PA). All normal rat IgG preparations were adsorbed on B10.AKM mouse thymocytes to remove antibodies reactive against unknown mouse epitopes. Purified CD4 T cells were prepared by collecting splenocytes from mixed allogeneic cultures containing CD8-depleted B10.AKM and T-depleted B10.MBR splenocytes after 3 days as described above. CD4 T cells were purified using GK1.5 in conjunction with magnetic cell sorting according to the manufacturers specifications (Miltenyi Biotech.). Similarly, APCs were prepared by collecting splenocytes from mixed allogeneic cultures containing CD8-depleted B10.AKM and T-depleted B10.MBR splenocytes after 3 days as described above. Splenocytes were then further T cell–depleted by using GK1.5 and 2.43 in conjunction with magnetic cell sorting according to the manufacturers specifications (Miltenyi Biotech.).

ELISPOT. ELISPOT assays were done with Millipore HA plates (Millipore, Bedford, MA) according to the manufacturer's specifications. Monoclonal antibodies BVD-1D11 (anti–IL-4), JES6-1A12 (anti–IL-2) and R4-6A2 (anti–IFN-γ) (Caltag Laboratories, Burlingame, CA) were used at concentrations of 10 μg/ml to coat plates. 5×10^5 T cell–depleted allogeneic or syngeneic stimulator cells were added to each well. Responder cells were assayed at 10^6, 10^5 and 10^4 cells per well. Cytokine production was detected after 24 h using concentrations of 10 μg/ml of biotinylated monoclonal antibodies BVD-24G2 (anti–IL-4), JES6-5H4 (anti–IL-2) and XMG1.2 (anti–IFN-γ).

Flow cytometry. B10.AKM mice were primed by allografting as described above and killed 5 weeks after the grafts were rejected. Splenocytes were collected from primed B10.AKM and naïve B10.MBR mice. 3×10^6 CD8 T cell–depleted B10.AKM splenocytes were then placed in culture with either 3×10^6 T cell–depleted B10.MBR cells or an equivalent number of sonicated B10.MBR splenocytes. B10.MBR splenocytes were sonicated for 5 mins using a W-380 ultrasonic processor (Heat Systems, Farmingdale, NY). No viable cells were observed by Trypan blue exclusion. Cells were cultured in DMEM media containing 15% fetal calf serum or media containing either 100 μg/ml 11B11 or 100 μg/ml normal rat IgG with or without 5 ng/ml exogenous IL-4. The culture media, including the antibodies indicated, was replaced every 2 days. After 5–7 days in culture at 37 °C, 5% CO_2 the cells were collected and examined for B7.1 and B7.2 expression by flow cytometry using anti-CD80 (RMMP-1) and anti-CD86 (RMMP-2) purchased from Caltag Laboratories. All cell surface staining and flow cytometry was performed as described previously[14].

***In vivo* neutralization of IL-4.** B10.AKM mice were thymectomized at 4 to 6 weeks of age. Two weeks after thymectomy, the mice were injected intraperitoneally with 100 μg of purified 2.43 (anti-CD8) or 100 μl of 2.43 ascities fluid at days 7 and 14 after thymectomy to produce Thx-CD8$^-$ mice[14]. Before skin grafting, T cell depletion was confirmed by flow cytometry. One day before skin grafting, mice were injected intraperitoneally with 100 μg purified 11B11 or 100 μg of purified normal rat serum IgG. The following day, tail skin grafts from 4 to 8-week-old B10.MBR mice were placed on the flank of control and experimental mice. Skin grafting and thymectomy were performed as described[14]. 11B11 or control rat IgG was administered every other day until the grafts were rejected. C57BL/6 and C57BL/6J-IL-4$^{tm/cgn}$ mice were thymectomized and CD8-depleted as described, and grafted with tail skin from 4 to 6-week-old B10.D2-*H2*^{i7} mice. Grafts were monitored every day until rejection

Intragraft cytokine PCR. Skin grafts were collected 12 days after grafting and total RNA was isolated as described[27]. 1 μg of RNA was then used to generate cDNA using an oligo dT primer and the SuperScript pre-amplification system (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's specifications. PCR primers specific for each cytokine were obtained from Clontech (Palo Alto, CA). All cytokine primers were designed either to not amplify genomic DNA or allow amplification of cDNA and genomic DNA to be distinguished based on the size of the PCR product obtained. Amplification of β-actin or glyceraldehyde 3-phosphate dehydrogenase transcripts was used to control for the quality and amount of cDNA amplified in each experiment.

Statistics. All statistical calculations were performed using GraphPad Prism 2.01 software (GraphPad Software Inc., San Diego). Comparison of proportions was performed by the Fisher exact test. The Kaplan and Meier method with a 95% confidence interval was used for the calculation of survival curves. Comparison of survival curves was performed using the log rank test. Comparisons of CD4 T cell levels before and after graft rejection were performed using a paired *t*-test. All other statistics were calculated using a two-tailed Student's *t*-test.

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