Tolerance Induction by Anti-CD2 Plus Anti-CD3 Monoclonal Antibodies: Evidence for an IL-4 Requirement[1]

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Anti-CD2 mAb plus anti-CD3 mAb induce alloantigen specific tolerance. We sought to determine whether Th2 cytokines are involved in the induction of tolerance in this model. Addition of anti-IL-4 mAb or anti-IL-10 mAb to anti-CD2 plus anti-CD3 treatment abrogated tolerance and resulted in graft survivals of 26 ± 4 and 25 ± 5 days, respectively. Splenocytes from the anti-IL-4 mAb and anti-IL-10 groups had greater proliferation in response to alloantigen than either tolerant or naive groups. Cytokine analysis of MLR supernatants showed increased IL-10 in the tolerant group and increased IFN-γ in the anti-IL-4 mAb treated group. Donor-specific alloantibody responses in untreated immune animals had a predominantly Th1 (IgG2a) alloantibody response, while the tolerogenic regimen reduced the ratio of IgG2a:IgG1 titers. The addition of anti-IL-4 mAb to the tolerogenic regimen partly restored the Th1-related IgG2a response. Tolerance did not develop in IL-4 knockout animals treated with anti-CD2 plus anti-CD3 (mean graft survival, 27 ± 5 days). Restoration of IL-4 to IL-4 knockout animals by gene transfer with plasmid DNA resulted in prolongation of survival to 46 ± 7 days, while adoptive transfer of wild-type splenocytes into IL-4 knockout recipients resulted in indefinite graft survival (>60 days) and indefinite survival of second donor-type grafts. IL-10 gene transfer to IL-4 knockout recipients did not prolong graft survival (28 ± 4). These results demonstrate that tolerance in this model is mediated at least in part by Th2-type cells that secrete IL-4, promote IL-10 and IgG1 production, and inhibit alloantigen reactivity. *The Journal of Immunology,* 1998, 161: 1156–1162.

S uccessful human organ transplantation is complicated by the development of allograft rejection, and despite the use of immunosuppressive medications true graft tolerance is seldom realized. To improve results, the problem of inducing specific graft tolerance in man must be solved. In murine models, long lived graft acceptance has been achieved with short term courses of mAbs directed against several different receptors including CD2 plus CD3 (1), CD4 plus CD8 (2), ICAM-1 plus LFA-1 (3), and CD28 plus CD40 (4). However, the success in murine models has not yet been translated into improved results in clinical organ transplantation. To realize the goal of indefinite graft survival without long term immunosuppression in humans, an understanding of the specific cellular and molecular mechanisms of tolerance induction in these murine models is needed.

Our previous studies showed that mAbs directed against the CD2 and CD3 receptors produce Ag-specific tolerance only when given sequentially (5). Graft adaptation was excluded as a major determinant of tolerance, since adoptive transfer of naive recipient type cells breaks tolerance (1). Cellular studies showed T cell activation by anti-CD3 is inhibited by pretreatment with anti-CD2

mAb (6), yet tolerant animals displayed normal MLR and CTL reactivity toward donor type alloantigen. Therefore, the inhibition of T cell activation is not associated with clonal abortion or deletion, or ex vivo anergy in this model. We have also reported alterations in cell surface receptor expression induced by anti-CD3 mAb and anti-CD2 mAb treatment that may play a role in tolerance induction (7, 8). However, the precise cellular mechanisms by which sequential treatment with CD2 followed by CD3 mAb produces tolerance remain undefined.

It has been proposed that Th cells can be separated on the basis of the cytokine profile they produce into Th1 and Th2 subtypes (9). Furthermore, prevention of the Th1 response has been shown to be critical for neonatal tolerance, while an augmented Th1 response has been associated with graft rejection (10, 11). Conversely, Th2 cells may be able to suppress cellular immune responses to allografts and have been associated with graft tolerance (12). Other evidence that Th2 cells are important for allograft tolerance includes the observation that immunosuppression by CTLA4Ig blockade of B7 causes a shift to the Th2 phenotype and is associated with prolonged survival of rat renal allografts (13). Our previous studies in a hapten model demonstrated that anti-CD2 induced suppressor cells that secreted IL-4 and TGF-β and suppressed CTL responses (14). We therefore hypothesized that induction of Th2-type responses could provide a mechanism by which anti-CD2 plus anti-CD3 mAbs produce tolerance without causing clonal anergy or deletion. To test this hypothesis, we used IL-4 knockout recipients or inhibited IL-4 or IL-10 with neutralizing Abs to prevent the development and activity of Th2-type responses. Neutralization of IL-4 or IL-10 during tolerance induction with anti-CD2 plus anti-CD3 mAbs prevented tolerance induction. We also observed a shift from a Th2 toward a Th1 cytokine and alloantibody profile when IL-4 activity was inhibited. The effect could be reversed by IL-4 supplementation through gene transfer or adoptive cell transfer.

Materials and Methods

Animals

CBA/J, C57BL/6, and BALB/c female mice (8-10 wk of age) and timed pregnant C57BL/6 and CBA/J mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). IL-4-deficient C57BL/6 mice (15) were obtained from The Jackson Laboratory (Bar Harbor, ME).

Reagents

Abs used for in vivo animal treatments were the 12-15 rat IgG1 anti-murine CD2 hybridoma (16), a gift from Dr. P. Altevogt (University of Heidelberg, Heidelberg, Germany); the 145-2C11 hamster anti-murine CD3ϵ hybridoma (17), a gift from Dr. J. A. Bluestone (University of Chicago, Chicago, IL); the S4B6 rat anti-mouse IL-2 hybridoma (18), a gift from Dr. T. R. Mossman (DNAX, Palo Alto, CA); the JES-2A5 rat IgG1 anti-murine IL-10 hybridoma, provided by Dr. R. Coffman (DNAX) (19), and the isotype control mAb YTS-259 rat IgG1 anti-Ras (American Type Culture Collection, Manassas, VA) (20). Hybridomas were grown in culture and purified over protein G (Pharmacia Biotech, Piscataway, NJ). Purified 11B11 rat IgG1 anti-mouse IL-4 (21) ascites, a gift from Dr. A. E. Chang (University of Michigan, Ann Arbor, MI), was also used. *Limulus* lysate tests (Sigma) confirmed the presence of <0.4 pg/ml of endotoxin in the mAb preparations. mAbs were diluted in 0.5-ml volumes with PBS and injected i.v. through the tail vein. Purified hybridoma supernatants from 11B11 and from the R4-6A2 rat IgG1 anti-murine IFN-γ hybridoma (American Type Culture Collection) were used in the ELISAs (22). Purified rat anti-murine IL-2 (JES6-1A12) and IL-10 (JES3-9D7) and biotinylated rat anti-murine IL-2 (JES6-5H4), IL-4 (BVDG-24G2), IL-10 (JES3-6B11), and IFN-γ (XMG1.2) were purchased from PharMingen (San Diego, CA).

Cardiac transplantation

The heterotopic, nonvascularized cardiac transplantation model was used. Briefly, donor neonatal C57BL/6 or CBA/J mice were sacrificed, whole hearts were removed and placed in the s.c. position of the ear pinnae of allogeneic recipients as previously described (23). Survival of cardiac allografts was followed with electrocardiogram monitoring (Polygraph 78 Series with preamp and filters, Grass Instruments, Quincy, MA) three times per week. The cessation of cardiac electrical activity was the determinant of rejection. There were at least four mice per group. Statistical comparison of results was performed using the Wilcoxon sign-rank test.

Gene transfer

A plasmid encoding the murine IL-4 gene under the CMV immediate early promoter, pCMV-A-mIL-4, was provided by Dr. Hideaki Tahara (University of Pittsburgh, Pittsburgh, PA). The pMP6AvIL-10 plasmid (viral IL-10 (VIL-10)[3] CMV promoter) was constructed by inserting vIL-10 insert cDNA into the pMP6A plasmid, provided by Dr. M. Philip (Applied Immune Sciences, Santa Clara, CA) (24). The control plasmid pCH110 (β-galactosidase with SV-40 promotor) was provided by Dr. James Norris (Medical University of South Carolina, Charleston, SC) (25). Plasmids were grown in *Escherichia coli* HB101, purified using cesium chloride ultracentrifugation, and tested to ensure they were endotoxin free by *Limulus* lysate (Sigma) before administration. Twenty micrograms of circular plasmid dissolved in 5 μl of PBS plus 5% sucrose was injected directly into the graft at the time of transplantation (26).

Mixed leukocyte reaction

Spleens were removed from transplanted animals at the indicated times after transplantation or allograft rejection and gently dissociated into single cell suspensions, and RBCs were removed from responder cells by centrifugation over a Ficoll-Hypaque gradient (Sigma). Stimulator cells were obtained in a similar fashion from donor-strain mice. Two $\times$ 10^5 responder spleen cells were cocultured in triplicate with 2 $\times$ 10^5 1500-rad gamma-irradiated stimulator cells. Eighteen hours before termination of 3-, 5-, and 7-day cultures the wells were pulsed with 0.5 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA), and incorporation was quantified on a scintillation counter. Results are expressed as the mean $\pm$ SEM. Statistical differences were assessed using Student's *t* tests, with $p < 0.05$ considered significant. Stimulation indexes are the ratio of counts per minuts of stimulate responder cells vs those of unstimulated responders.

[3] Abbreviation used in this paper: vIL-10, viral IL-10.

Table I.   *Anti-IL-4 and anti-IL-10 mAbs inhibit tolerance induction[a]*

mAb Treatment	MST (primary graft)	n	MST (second graft)	n
Control mAb	11 $\pm$ 2	5	ND	ND
Anti-IL4	12 $\pm$ 2	6	ND	ND
Anti-CD2 + anti-CD3	>60	10	>60	5
Anti-CD2 + anti-CD3 + control mAb	>60	5	ND	ND
Anti-CD2 + anti-CD3 + anti-IFNγ	>60	5	>60	5
Anti-CD2 + anti-CD3 + anti-IL-2	>60	5	>60	5
Anti-CD2 + anti-CD3 + anti-IL-4	26 $\pm$ 4	15	ND	ND
Anti-CD2 + anti-CD3 + anti-IL-10	25 $\pm$ 5	16	ND	ND

[a] CBA/J recipients of primary and secondary C57BL/6 donor hearts were treated with the indicated mAbs as detailed in *Materials and Methods*. MST, mean survival time (days) $\pm$ SEM. ND, not done.

Adoptive transfer

Splenocytes were prepared as described for MLR above. Splenocytes (3 $\times$ 10^7) were injected into the tail vein in 1-ml volumes of PBS at the time of transplantation.

Cytokine assays

IL-2, IL-4, IL-10, and IFN-γ production were assayed using a two-antibody capture ELISA. Ninety-six-well flat-bottom plates were coated with 50 μl of the appropriate unconjugated capture Ab at 2 μg/ml in 0.1 M NaHCO$_3$ (pH 8.2) overnight at 4°C. Plates were washed twice with 0.05% Tween-20 in PBS and blocked with 200 μl of 3% BSA in PBS for 2 h at room temperature. Plates were then washed twice, and culture supernatants from the MLR of the appropriate groups were added and incubated overnight at 4°C. Plates were then washed four times, incubated with 100 μl of the appropriate biotinylated detecting Ab (1 μg/ml) for 45 min at room temperature, and washed six times, and then 100 μl of 1/1000 diluted avidin-peroxidase (PharMingen) was added to each well and incubated for 30 min at room temperature. Plates were washed eight times, and 100 μl of freshly prepared ABTS substrate (Kirkegaard & Perry, Gaithersburg, MD) was added to each well. The reaction was stopped after 5 min by adding 100 μl of 1% SDS (Sigma). Plate OD values were measured at 405 nm on a Biotek EL311s automated microplate reader (Windooski, VT). Cytokine concentrations were determined by comparison to a standard curve generated with recombinant human cytokines using the Biotek EL311 best curve-fit software. The lower limit of detectability for the cytokines was 0.1 U/ml for IL-4, 0.5 U/ml for IL-2 and IL-10, and 1.0 U/ml for IFN-γ. Results are expressed as the mean $\pm$ SEM. Statistical differences were assessed by Student's *t* tests, with $p < 0.05$ considered significant.

Alloantibody assay

Alloantibody isotypes were quantified as described previously (27). Briefly, 10^6 EL-4 cells (H-2^b) were incubated with 1/50 dilutions of sera collected from animals at the indicated times following transplantation, washed, and incubated with secondary FITC-conjugated, isotype-specific Abs. The dilution of sera used was determined to be in the linear range of the Ab titer curve by previous dose-response studies (not shown). FITC-conjugated, affinity-purified, sheep anti-mouse IgM, IgG1, and IgG2a (The Binding Site, San Diego, CA) were used at a 1/50 dilution. Data are reported as the mean channel fluorescence determined on a Becton Dickinson FACScan (Mountain View, CA). Statistical differences were assessed by Student's *t* test, with $p < 0.05$ considered significant.

Results

Anti-IL-4 and anti-IL-10 mAbs prevent induction of tolerance

Tolerance was induced in transplanted animals by treating them with anti-CD2 mAb (100 μg iv) on days 0 and 1 with respect to transplantation followed by anti-CD3 mAb (100 μg iv) on days 2, 3, 4, 5, and 10. As shown previously, this combination resulted in indefinite primary allograft survival (Table I) and acceptance of second donor-specific transplants without the need for further treatment. Previously published work from this laboratory has shown that these animals reject third party grafts normally (1). Animals that received an extended course of control mAb alone (YTS-259, 100 μg iv on days 0, 1, 2, 3, 4, 5, and 10) rejected the

allografts at 11 ± 2 days. To determine whether the Th2 cytokines IL-4 and IL-10 are involved in tolerance induction by the anti-CD2 plus anti-CD3 combination, animals were treated with anti-IL-4 mAb (100 μg iv) or anti-IL-10 mAb (100 μg iv) on days 1, 3, 5, 7, and 9 in addition to the standard anti-CD2 plus anti-CD3 regimen described above. The addition of either anti-IL-4 or anti-IL-10 mAb prevented the development of tolerance (Table I). Animals treated with anti-IL-4 mAb rejected their grafts at 26 ± 4 days, while animals treated with anti-IL-10 rejected their grafts at 25 ± 5 days (Table I). This graft survival is similar to the graft survival of 27 ± 3 days observed with anti-CD3 mAb alone (6). To determine the specificity of the anti-cytokine mAb treatment, anti-IL-2 and anti-IFN-γ mAb (100 μg iv on days 1, 3, 5, 7, and 9) were added to the standard anti-CD2 plus anti-CD3 regimen. In contrast to anti-IL-4 and anti-IL-10 treatment, neither anti-IL-2 nor anti-IFN-γ mAb prevented the development of tolerance (Table I). The addition of isotype control mAb (YTS-259) treatment to the anti-CD2 plus anti-CD3 regimen also did not prevent the development of prolonged graft survival. Since IL-4 is required for Th2 induction, these results suggest that Th2 cells may be required for tolerance induction in this model. These results also demonstrate that anti-cytokine mAbs do not interfere with tolerance induction in a nonspecific way. Since IL-2 and IFN-γ are required for Th1 development, this result shows that inhibition of the Th1 subset does not affect tolerance induction in this model.

Anti-CD2 plus anti-CD3 does not induce tolerance in IL-4-deficient mice

To confirm the importance of IL-4 in the induction of tolerance by anti-CD2 plus anti-CD3 mAb, IL-4 knockout C57BL/6 were transplanted with hearts from CBA/J donors. Previously published work from this laboratory has shown that anti-CD2 plus anti-CD3 mAb treatment normally produces tolerance with this strain combination (1). Two IL-4 knockout mice did not receive mAb treatment and rejected the grafts at 10 and 13 days, similar to wild-type C57BL/6 animals that received control mAb (Table I). IL-4 knockout mice that received anti-CD2 plus anti-CD3 mAb treatment rejected their grafts at 27 ± 5 days ($n = 5$), similar to the graft survival of 24 ± 3 days observed in wild-type animals treated with the tolerogenic regimen plus anti-IL-4 mAb (Table I). These data further confirm that IL-4 is required for the induction of tolerance by anti-CD2 plus anti-CD3 mAb. Attempts to test whether tolerance could be induced by anti-CD2 plus anti-CD3 mAb in IL-10 knockout were unsuccessful, since they died following the administration of anti-CD3 mAb as a result of the anti-CD3-mediated cytokine syndrome. It has similarly been reported that the lethal dose of LPS is 20-fold lower in IL-10-deficient mice than in wild-type mice due to increased TNF-α production following T cell activation in the absence of IL-10 (28).

Restoration of IL-4 prolongs graft survival in IL-4-deficient mice

It was necessary to confirm that the inability to generate tolerance in IL-4-deficient mice with anti-CD2 plus anti-CD3 was related to the deficiency of IL-4 and was not due to an artifact of immunologic development in the absence of IL-4. The experiment was repeated with additional groups of animals that, at the time of engraftment and tolerance induction, received exogenous IL-4 transfer gene using a technique we have previously shown to be efficacious in this model (26, 29). A third group of IL-4 knockout recipients received adoptive transfer of normal, IL-4-sufficient C57BL/6 splenocytes at the time of transplant. Again, IL-4 knockout animals that received the tolerogenic regimen of anti-CD2 plus anti-CD3 mAbs did not develop tolerance and rejected their grafts at about 27 days (Table II). Animals that received control gene

Table II. *Anti-CD2 plus anti-CD3 mAbs do not produce tolerance in IL-4 knockout animals*[a]

Treatment	Mean Graft Survival (days)	n
Anti-CD2 + anti-CD3	27 ± 5	9
Anti-CD2 + anti-CD3 + control plasmid	22 ± 4	4
Anti-CD2 + anti-CD3 + IL-4 gene transfer	46 ± 7	5
Anti-CD2 + anti-CD3 + vIL-10 gene transfer	28 ± 4	5
Anti-CD2 + anti-CD3 + normal C57BL/6 splenocytes	>60	5

[a] IL-4-deficient C57BL/6 recipients of CBA/J donor hearts were treated with the indicated mAbs. Gene and cellular adoptive transfer were performed as described in *Materials and Methods.*

transfer (β-galactosidase) had a similar graft survival of 22 ± 4 days, while animals that received IL-4 gene transfer had significantly prolonged graft survival to 46 ± 7 days. It is likely that indefinite survival was not achieved because IL-4 gene expression was probably low and transient (26, 29). Gene transfer using vIL-10 did not affect graft survival, suggesting that IL-4 was not functioning solely through the induction of IL-10, but probably also through other mechanisms, including inhibition of Th1 cytokines (10). Adoptive transfer of wild-type C57BL/6 splenocytes at the time of transplantation restored indefinite graft survival (>60 days). These animals received secondary donor-type grafts in the opposite ear 60 days after primary grafting without further modification. Indefinite secondary graft survival was observed (>60 days). These data additionally confirm the importance of IL-4 for the induction of tolerance by anti-CD2 plus anti-CD3 mAb.

Anti-IL-4 and anti-IL-10 increase alloreactivity

To determine whether anti-IL-4- and anti-IL-10 mAb-mediated inhibition of tolerance was accompanied by altered alloreactivity, splenic lymphocytes were obtained from the various tolerant and nontolerant groups 48 to 52 days after transplantation and assayed for their responses to donor alloantigen. At this time point, the animals that received anti-IL-4 and anti-IL-10 (in addition to anti-CD2 and anti-CD3) had rejected their grafts. The proliferative responses in MLR of splenocytes from tolerant mice and those from untreated (naive) mice was similar and significantly less than those of anti-IL-4 mAb-treated animals that had rejected their grafts (Fig. 1A). That naive and tolerant cells were not significantly different from each other in their responses to donor-specific Ag is a demonstration of the split tolerance of this model, previously reported (1, 30, 31). Cells from anti-IL-4 mAb-treated animals demonstrated mild nonspecific activation, with slightly higher proliferative responses than tolerant or naive cells against third-party BALB/c stimulators, although the stimulation index relative to that of naive cells was greater for donor-specific responses to C57BL/6 (7.4) than for third-party responses to BALB/c (1.9; $p = 0.04$; data not shown). Cells from animals that received anti-IL-10 treatment also had significantly increased proliferative responses to donor alloantigen than cells from either naive or tolerant animals or cells from animals that received anti-IFN-γ, all of which had similar proliferative responses (Fig. 1B).

Anti-IL-4 alter cytokine production toward a Th1 profile

To determine whether the change in alloreactivity was accompanied by changes in cytokine production, supernatants from 7-day MLR cultures were assayed for IL-2, IL-4, IL-10, and IFN-γ contents by ELISA. There were no significant differences in any of the measured cytokine levels on days 3 and 5 (data not shown). IL-2 levels were similar in naive, tolerant, and anti-IL-4 mAb-treated
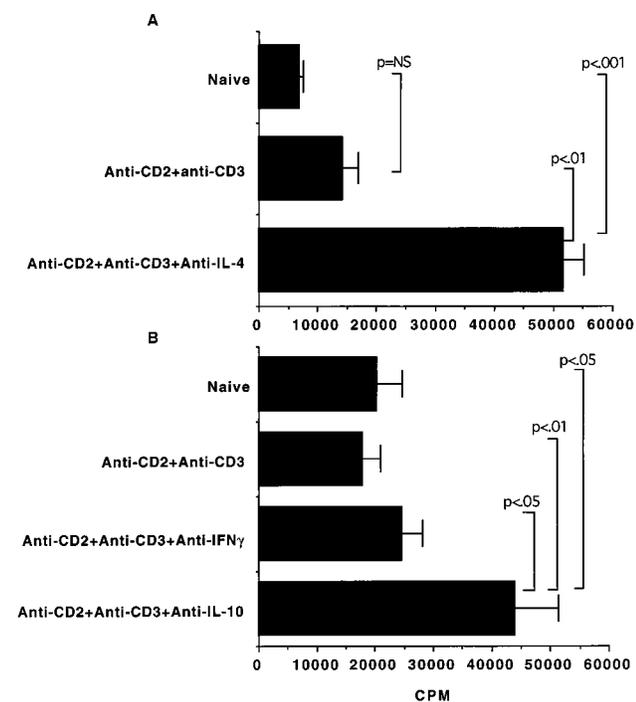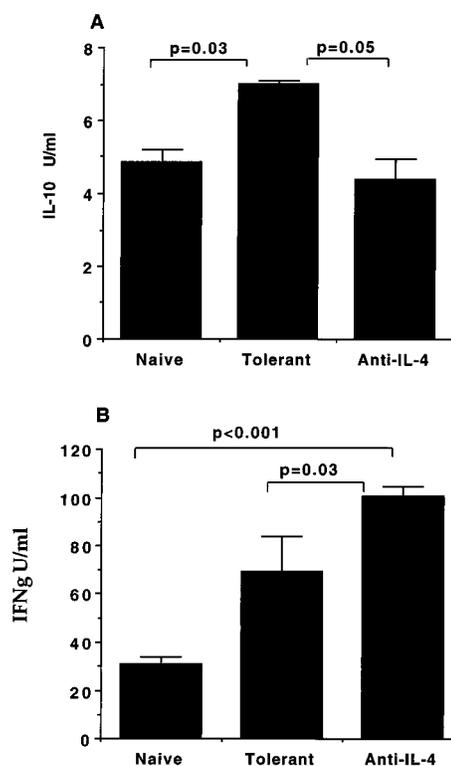
FIGURE 1. *A*, Abrogation of tolerance with anti-IL-4 is associated with an increased MLR response. Splenocytes from naive (untransplanted), tolerant (transplanted and treated with anti-CD2 plus anti-CD3 mAb), and anti-IL-4 mAb-treated (transplanted and treated with anti-CD2 plus anti-CD3 plus anti-IL-4 mAb) CBA/J animals were obtained 48 days after transplant and stimulated in culture with donor (C57BL/6) splenocytes. Results of a 5-day MLR are shown. $n = 3$ or 4 animals in each group. Similar data were confirmed in two additional experiments. *B*, Abrogation of tolerance with anti-IL-10 but not anti-IFN-γ is associated with an increased MLR response. Splenocytes were obtained and assayed as described in *A*. The data were confirmed in additional experiments.

FIGURE 2. *A*, Tolerance is associated with increased IL-10. Cells were stimulated with donor splenocytes as detailed in Figure 1, and supernatants were harvested after 7 days of culture and assayed by ELISA. *B*, Abrogation of tolerance is associated with increased IFN-γ. Cells were stimulated, and supernatants were analyzed by ELISA as described in *A*. The data were confirmed in additional experiments.

groups on day 7, while IL-4 levels were very low (<0.1 U/ml) and were not significantly different in all groups at this time point (data not shown). Levels of the Th2 cytokine IL-10 measured on day 7 were significantly higher in tolerant derived cells than in naive ($p = 0.03$) and anti-IL-4 mAb-treated groups ($p = 0.05$), while IFN-γ levels were higher in the anti-IL-4 mAb-treated group compared with those in both naive (p 0.001) and tolerant groups at this time ($p = 0.03$; Fig. 2). These results suggest a relative shift from a Th0 phenotype in naive cells to a Th2 phenotype in the tolerant group and a Th1 phenotype in the anti-IL-4 mAb-treated group.

Anti-IL-4 alters alloantibody production toward a Th1 profile

To further investigate the role of Th1 and Th2 cytokines in channeling immune responses, alloantigen-specific Ab was quantified in sera from transplanted animals. The mean channel fluorescence ratios of IgG2a:IgG1 at 2 and 3 weeks after transplantation were 2.5 and 3.4, respectively, in unmodified rejecting control animals, indicating that the Th1-associated IgG2a response predominated (Table III). Treatment with anti-CD2 plus anti-CD3 mAbs resulted in suppression of both the absolute titers and the ratios of IgG2a to IgG1 at 2 and 3 weeks, indicating a suppression of both Ab isotypes, but an almost 3-fold greater suppression of the Th1 (IgG2a) isotype (67%) compared with the Th2 (IgG1) isotype (28%). The anti-CD2-, anti-CD3-, plus anti-IL-4 mAb-treated animals had increased absolute titers and IgG2a:IgG1 ratios of 3.4 and 2.9 at 2 and 3 weeks posttransplant. These values are similar to unmodified control ratios, indicating that the predominantly Th1 response as-

sociated with unmodified animals was partially restored by anti-IL-4 mAb treatment. T cell-independent Ab production, as measured by IgM, was minimally affected by each of the Ab treatments (data not shown).

Discussion

Studies attempting to define the cellular and molecular events that characterize the induction of tolerance have yet to fully explain graft acceptance. Previous studies using the anti-CD2 plus anti-CD3 mAb induced tolerance model demonstrated that both anti-CD2 and anti-CD3 mAbs were required or tolerance did not result (5). Investigations into the mechanism of tolerance showed that clonal deletion and T cell anergy were not present, since the splenocytes from tolerant animals were able to respond in vitro to donor-type Ag, yet in vivo the animals will accept a second graft of donor type (1). A possible explanation for this finding is the IL-4-dependent induction of alloreactive regulatory cells by anti-CD2 plus anti-CD3 treatment. The data in the current study support this hypothesis, since anti-IL-4 mAb, anti-IL-10 mAb, and the use of IL-4 knockout recipients prevented the induction of tolerance. A problem with the interpretation of the mAb blocking experiments is that it has been reported that mAbs that neutralize the effects of IL-4 in vitro may not always inhibit the cytokine effects in vivo (32). However, tolerance similarly could not be induced in IL-4 knockout animals with anti-CD2 plus anti-CD3 mAb treatment, and these animals rejected allografts at a similar time point as wild-type animals that were treated with anti-IL-4, anti-CD2, and anti-CD3 mAbs together. Furthermore, we observed a shift from Th2 to Th1 cytokine and alloantibody responses with

Table III.   *Tolerance is associated with predominant Th2 Ab responses; abrogation of tolerance is associated with restoration of Th1 Ab responses*[a]

| | Mean Channel Fluorescence | | | | | |
| | 2 wk | | | 3 wk | | |
Treatment	IgG2a	IgG1	Ratio	IgG2a	IgG1	Ratio
No mAb	62.2	25.2	2.5	58.4	27.2	3.4
Anti-CD2 + anti-CD3	20.8	18.2	1.1	12.1	7.8	1.6
Anti-CD2 + anti-CD3 + anti-IL-4	37.5	11.0	3.4	23.7	8.1	2.9

[a] Groups of four animals each were transplanted and treated as indicated; sera were obtained 2 and 3 wk after transplantation and assayed for isotype-specific antidonor alloantibody. The mean channel fluorescence ratio for Th1-specific (IgG2a) to Th2-specific isotypes (IgG1) was calculated.

anti-IL-4 mAb treatment, suggesting that the effect of IL-4 was inhibited, not augmented.

Neither anti-IL-4 mAb treatment, anti-IL-10 mAb treatment, nor the use of IL-4 knockouts reduced graft survival to that of unmodified control animals (11 days), indicating that anti-CD2 and/or anti-CD3 mAb treatment has additional immunosuppressive effects that are independent of Th2-type cytokine induction. The observed graft survival when Th2 responses are inhibited was approximately 25 days, which is similar to the graft survival that is seen when either anti-CD2 mAb (22 days) or anti-CD3 mAb (27 days) is given alone (6). Since we also know that tolerance only results when anti-CD2 and anti-CD3 mAbs are given sequentially (1), these findings suggest that it is the sequential combination of anti-CD2 followed by anti-CD3 mAbs that triggers the induction of Th2-type cytokines, which prolong graft survival beyond 25 days and ultimately induce a state of Ag-specific tolerance.

A central issue is how IL-4 induces tolerance and how this relates to Th2 cells. Since both anti-IL-4 mAb and anti-IL-10 mAb prevent tolerance, this suggests that the anti-CD2 plus anti-CD3 regimen induces Th2 cells that secrete IL-4 and IL-10, which are both necessary for tolerance. Since IL-10 gene transfer did not prolong graft survival in IL-4 knockouts, this also suggests that IL-4 does not function solely through induction of IL-10, but probably though other mechanisms, including inhibition of Th1 cytokines (10). It is possible, however, that anti-IL-4 mAb or IL-4 insufficiency promotes the generation of Th1 that over-ride a tolerogenic mechanism unrelated to IL-4 or Th2, although there is no further evidence for this alternative interpretation. Other data also support our interpretation that Th2 cells were generated by our tolerogenic regimen. Thus, MLR results confirmed that alloreactivity was altered by anti-IL-4 and IL-10 mAb treatments, since tolerant animals had lower proliferative responses than anti-IL-4- and anti-IL-10 mAb-treated animals that rejected their grafts. The predominant alloantigen-specific Abs produced by tolerant animals were the Th2-associated isotype (IgG1), while the addition of anti-IL-4 mAb changed this response to a Th1-associated isotype (IgG2a). Similarly, the cytokines produced in response to alloantigenic stimulation also demonstrated a predominance of the Th2-type cytokine IL-10 and a decrease in the Th1 cytokine IFN-γ in the tolerant groups compared with those in the anti-IL-4 mAb-treated group. This indicates that anti-CD2 plus anti-CD3 mAbs induced a Th2-related response, while the addition of anti-IL-4 shifted this response back to Th1. In sum, these results suggest that Th2 cytokines, and perhaps Th2 cells, are instrumental in altering multiple aspects of the immune response, preventing rejection, and promoting tolerance.

Experiments using adoptive transfer of Th1 or Th2 cells have attempted to clarify the issue of whether Th2 cells per se, as opposed to Th2-related cytokines, produce rejection and autoimmu-

nity or protect the animal from developing these phenomena. Maeda et al. observed prolonged skin graft survival with adoptive transfer of a Th2-like cell line (33). In contrast, Orosz and colleagues adoptively transferred acutely generated Th2-type cell populations into SCID recipients and were able to induce acute graft rejection, presumably in the absence of Th1 cells, although the Th2 cell population produced substantial amounts of IL-2 (34, 35). It is possible that the model used in these experiments is actually too rigorous, in that Th2 cells cannot effectively function in a regulatory capacity in the absence of normal Th1 effects. Alternatively, subtle differences in the experimental models, including strain-specific differences in Th2 cytokine production, may explain these apparently opposite demonstrations of the effects of Th2 cells on allogeneic tissues (36). Our present results do not distinguish between the importance of Th2 cells vs Th2-related cytokines. However, the adoptive transfer of cell subsets into IL-4 knockouts may permit a definitive answer in the future.

The association between Th2 cytokines and graft acceptance has been reported by other investigators. Intragraft IL-4 peptide prolongs cardiac allograft survival in rats treated with donor-specific transfusions and cyclosporine (37), and murine transgenic IL-4 cardiac allografts exhibit prolonged survival (38). CTLA4Ig treatment is associated with long term survival of islet allografts (13), increased IL-4 production, and delayed rejection of lung allografts (39). Anti-CD4 mAb suppresses Th1, but not Th2, responses in association with long term survival of renal allografts (40), improved survival of cardiac allografts (41), and cardiac allograft tolerance (42). Th2 responses induced by oral Ag have also been reported to prolong allograft acceptance (43). In experiments similar to our own, Davies et al. showed that anti-IL-4 mAb inhibits the suppression of rejection induced by anti-CD4 plus anti-CD8 mAb, although interestingly these investigators were not able to demonstrate the presence of IL-4-secreting cells in tolerant animals (44). Anti-IL-4 and anti-IL-10 mAbs also inhibit the acceptance of allogeneic bone marrow and skin grafts in a model of tolerance induced by anti-CD8 mAb and dimethylmyeleran (45). Evidence from a model of improved allograft survival induced by gene transfer of the Th2 cytokine IL-10, which promotes Th2 and inhibits Th1 cells, provides further support for the idea that both the absolute amount and the ratio of Th1 and Th2 cytokines determine graft acceptance (26, 46). Finally, preliminary data using in situ RT-PCR to evaluate cytokine responses during graft acceptance and rejection in the anti-CD2- plus anti-CD3-mediated tolerance model also support the contention that graft acceptance is associated with a decrease in the ratio of IL-2- to IL-4-transcribing cells within the graft (J. D. Punch, unpublished observations).

Despite clear evidence that Th2 responses can be critical for tolerance induction in some models, it is also apparent that Th2 cells are not always associated with graft tolerance and that graft

tolerance is not always associated with Th2 cytokines. Chan et al. depleted CD8$^+$ cells and observed a shift to Th2 cytokines and alloantibody isotypes, but rather than observing tolerance, generated alternate mechanisms of allograft rejection (47). However, it is possible that their method of producing Th2 responses may not have been potent enough to adequately suppress Th1 responses in their system, explaining why their findings differ from the ones presented here. A recent report by Kupar et al. showed that Ag-specific tolerance induced by CD2 ligation alone was associated with decreased IL-10 mRNA in a murine pancreatic islet cell model (48). However, other Th1 and Th2 cytokines were not reported in that communication, and the mRNA level may not strictly correlate with the type of Th response (49). Lakkis et al. found that long term cardiac allograft acceptance could be induced by CTLA4Ig in IL-4-deficient mice (50). This finding suggests that the mechanism of CTLA4Ig-mediated allograft acceptance is not through induction of a Th2 response despite the association of CTLA4Ig treatment with Th2 cytokine secretion (39). Therefore, all models of tolerance induction through receptor ligation or blockade may not share a common mechanism.

Similar paradoxes regarding the effects of Th2 cells on autoimmune disease have been reported. Th1 cells and cytokines have been associated with increased immunoreactivity, while a shift to predominantly Th2 responses has been associated with abrogation of autoimmunity in the nonobese diabetic mouse model (51, 52) and in experimental autoimmune encephalomyelitis (53). However, in both of these systems, Th2 cells have also been shown to induce rather than inhibit the development of autoimmune disease. Th2 cells can induce diabetes in immune compromised nonobese diabetic mice (54), and immuodeficient mice are not protected against the development of experimental autoimmune encephalomyelitis by Th2 cells. Therefore, the protective, regulatory effects of Th2 cells may not be operative under some conditions.

A possible mechanism underlying the induction of Th2 responses after CD2 receptor ligation may be CD2-associated responsiveness to IL-12. Gollob et al. described inhibition of IL-12-induced proliferation and IFN-γ production by activated human T cells treated with mAb directed against either CD2 or its human ligand CD58 (55). Since IL-12 plays a central role in the development of cells with the Th1 phenotype, inhibition of IL-12 responsiveness would shift the ratio of Th1:Th2 toward the Th2 phenotype (56). Another report demonstrated that CD2 and the costimulatory receptors CD28 and CD40 influenced the Th phenotype in a murine model of mercury chloride-induced autoimmune murine disease (57). In this report, soluble CTLA4Ig and mAb directed against CD40 ligand inhibited autoimmunity, while anti-CD2 mAb augmented autoimmunity, as well as autoantibody and IL-4 production. The authors interpreted these findings as showing that CD2 was important for Th1 generation and that anti-CD2 inhibited Th1 generation and promoted Th2 responses and Th2-dependent autoimmune disease. Similarly, in a previous report of hapten-specific immunity we demonstrated that CD2 ligation alone induced Th2 cells and cytokines (14). However, CD2 ligation is not sufficient to produce tolerance when administered alone (23). Since anti-CD3 mAb can also inhibit Th1 responses (58), these reports suggest that tolerance induction by the combination of anti-CD2 plus anti-CD3 mAbs may be the result of a shift in the Th1-Th2 balance. The data in the present communication support this assertion, since inhibition of the Th2 arm by eliminating IL-4 with mAb or by using IL-4-deficient animals blocked tolerance induction by CD2 plus CD3 receptor ligation. The additive effect of Th2 stimulation and Th1 inhibition by CD2 ligation plus Th1 inhibition by anti-CD3 mAb may explain why the combination of CD2 and CD3 ligation is particularly efficacious in

tolerance induction and why others have failed to prolong graft survival with maneuvers that influence Th2 cells or cytokines alone.

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