Anti-CD4 Monoclonal Antibody-Induced Tolerance to MHC-Incompatible Cardiac Allografts Maintained by CD4⁺ Suppressor T Cells That Are Not Dependent upon IL-4

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Anti-CD4 Monoclonal Antibody-Induced Tolerance to MHC-Incompatible Cardiac Allografts Maintained by CD4+ Suppressor T Cells That Are Not Dependent upon IL-4

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Anti-CD4 mAb-induced tolerance to transplanted tissues has been proposed as due to down-regulation of Th1 cells by preferential induction of Th2 cytokines, especially IL-4. This study examined the role of CD4+ cells and cytokines in tolerance to fully allogeneic PVG strain heterotopic cardiac allografts induced in naive DA rats by treatment with MRC Ox38, a nondepleting anti-CD4 mAb. All grafts survived >100 days but had a minor mononuclear cell infiltrate that increased mRNA for the Th1 cytokines IL-2, IFN-γ, and TNF-β, but not for Th2 cytokines IL-4 and IL-6 or the cytolytic molecules perforin and granzyme A. These hosts accepted PVG skin grafts but rejected third-party grafts, which were not blocked by anti-IL-4 mAb. Cells from these tolerant hosts proliferated in MLC and produced IL-2, IFN-γ, and IL-4 at levels equivalent to naive cells. Unfractionated and CD4+ T cells, but not CD8+ T cells, transferred specific tolerance to irradiated heart grafted hosts and inhibited reconstitution of rejection by cotransferred naive cells. This transfer of tolerance was associated with normal induction of IL-2 and delayed induction of IFN-γ, but not with increased IL-4 or IL-10 mRNA. Transfer of tolerance was also not inhibited by anti-IL-4 mAb. This study demonstrated that tolerance induced by a nondepleting anti-CD4 mAb is maintained by a CD4+ suppressor T cell that is not associated with preferential induction of Th2 cytokines or the need for IL-4; nor is it associated with an inability to induce Th1 cytokines or anergy. The Journal of Immunology, 1998, 161: 5147–5156.

Tolerance to transplanted tissue in adult rodents can be induced by a variety of therapies that inhibit T cell function during the initial period of engraftment (1). These include therapy with mAb that blocks critical ligands in the T cell-APC interaction such as anti-CD4 (2–5) and anti-CD3 (6, 7), as well as ligands that block the second signal pathway of B7 and CD28 activation (8), especially when combined with blocking of CD40 (9). One of the most studied mechanisms of tolerance induction is anti-CD4 therapy, as the CD4+ cell is central to the development of both rejection (10–12) and induction of tolerance (13–15). Anti-CD4 mAb therapy can work either by depletion of CD4+ T cells, allowing regeneration of new tolerized T cells (2–4), or by blocking the function of CD4+ T cells without significant depletion (5, 15–18). The most popular hypothesis to explain the long term induction of transplant tolerance with anti-CD4 mAb is that Th1 responses are preferentially blocked, which allows the development of non-graft-destructive Th2 responses that, in turn, negatively regulate Th1 responses (19–23). This Th2 dominance has been related to infectious tolerance, in which CD4+ T cells can adoptively transfer specific tolerance to a naive host by suppressing the ability of host cells to effect allograft rejection (19, 21).

Other mechanisms proposed include: induction of anergy, so that on re-exposure to alloantigen the CD4+ T cells fail to produce IL-2; or that the poorly functioning anergic cells consume IL-2 required for the activation of normal alloreactive cells (24, 25). Clonal deletion is not considered a mechanism in this form of tolerance (26, 27).

Th1 cells are activated by IL-12; they produce IL-2, IFN-γ, and TNF-β but not Th2 cytokines; and their activation is inhibited by IL-4 and IL-10 (28, 29). Th2 cells are activated by IL-4; they produce IL-4, IL-5, IL-6, and IL-10, and IL-13 but not Th2 cytokines; and their activation is inhibited by IFN-γ (28, 29). IL-4 has been the most studied Th2 cytokine, and many of the effects of Th2 cells have been attributed to this cytokine alone or in combination with IL-10. It is thought that naive CD4+ T cells (Th0) can mature into either Th1 or Th2 cells depending upon the environmental stimuli they are exposed to on activation. In infections such as Leishmania (30) and in autoimmunity (31, 32), development of Th2 responses can lead to chronic infection or protect against autoimmunity because these cells inhibit Th1 responses. These observations have been adapted to allogeneic responses; however, the data supporting a prime role for Th2 in all forms of transplant tolerance are limited.

A role for Th2 cells in the maintenance of tolerance is best described in neonatal tolerance (33, 34) and in post-total lymphoid irradiation tolerance (35, 36), in which a predominance of IL-4-producing cells as well as blocking of tolerance induction by anti-IL-4 mAb therapy (37, 38) have been described. In allograft tolerance induced in adults, Th2 responses have been described as predominates when either depleting anti-CD4 mAb (19, 20) or CD28-B7 blockade (39) is used to induce tolerance. The mechanisms maintaining long term tolerance are less well described. It is a common finding from a variety of models that specific tolerance can be adoptively transferred by CD4+ T cells (15, 40–42). The
Infectious tolerance with CD4
strain graft when IL-4 was blocked, the in vitro and in vivo reac-
tions within the grafts, their capacity to accept a second donor
had developed tolerance to their grafts. The ongoing immune re-
responses were not inhibited (47), a finding that differs from sev-
that Th2 responses were inhibited by the anti-CD4 therapy. Th1
with a complete inhibition of alloantibody responses, confirming
IL-13 in the first week after transplantation (47). This is associated
and a specific down-regulation of Th2 cytokines IL-4, IL-5, and
inHeymann nephritis (S.T.S. and B.M.H., unpublished data). To confirm

diminished. Transfer of anti-CD4-induced tolerance with CD4
blocked by an anti-IL-4 mAb. There was no evidence that prefer-
erential up-regulation of Th2 cytokines, especially IL-4, was re-
quired for the maintenance or transfer of tolerance in this model.

Materials and Methods
Animals and procedures
DA (RT1\(^*\)), PVG (RT1\(^*\)), Lewis (RT1\(^*\)), F344 (RT1\(^{1+1}\)), and Sprague Daw-
ley rats and BALB/c mice were bred and maintained as previously de-
scribed (47). Operative procedures including heterotopic heart grafts, neo-
aternal heart grafts, irradiation, preparation of single-cells suspensions from
lymph node and spleen, and enrichment for CD4\(^+\) T cells have all been
previously described in detail (11, 51). All experiments were conducted as
approved by the animal ethics committee of the University of New South
Wales, Sydney, New South Wales, Australia.

Production and administration of mAb
The clone for MRC Ox38 (IgG1), an anti-rat CD4 mAb, and for MRC
Ox81 (IgG1), a mAb that blocks rat IL-4 function (52), were a kind gift of
Dr. Don Mason (Medical Research Council Cellular Immunology Unit,
Oxford, U.K.). The isotype control mAb was A6 (IgG1), which reacts with
human but not mouse CD45RO (53). For functional and in vitro studies,
mAb was grown in ascites of BALB/c mice primed by i.p. injection of IFA
(Sigma-Aldrich, St. Louis, MO) 3 days before injection of clones. Ascites
were purified on a DEAE-Sepharose column (Pharmacia, Uppsala, Swe-
den), and Ab concentration was determined by radial immunodiffusion
(54). Animals were treated with four doses of MRC Ox38, MRC Ox81, or
A6 at 7 mg/kg i.p. on the day of transplantation and on days 3, 7, and 10.
MRC Ox38 therapy given from the day of transplantation leads to indef-
inite graft acceptance in all DA recipients compared with normal the re-
jection time of 6–8 days; it coats peripheral CD4\(^+\) cells but does not de-
pend on IL-4 (5, 47).

MRC Ox81 therapy given as described inhibits IL-4 function in vivo; it
reduces inflammation in experimental uveoretinitis (55), blocks IgG iso-
type switch in alloimmune response (45), and alters the immune response
in Heymann nephritis (S.T.S. and B.M.H., unpublished data). To confirm
the presence of active MRC Ox81 mAb in DA rats treated with one dose
of 7 mg/kg, serum was collected at 4 h and 1, 2, 3, and 8 days. This serum
was tested for its ability to block rat rIL-4 up-regulation of class II MHC
on B cells in vitro, as described (45). In this assay, rIL-4 was used at
100-fold above the minimum required to induce class II MHC expression
in the assay; 8 \(\mu\)g/ml of MRC Ox81 was the minimum concentration re-
duced block rIL-4 in this assay. All samples of serum taken from
MRC Ox81-treated rats fully blocked class II MHC induction by rIL-4
(Fig. 1). In addition, in DA rats grafted with PVG hearts, the in vivo effect
of MRC Ox81 on the IgG1 isotype of the alloantibody responses was
examined at day 7 using an indirect flow cytometric assay and specific
isotype antiserum, as described (45). The IgG1 isotype was assayed be-
cause it is dependent on IL-4 for induction of class shift (56). In sera from
rats with normal rejection, an IgG1 response was observed that was en-
hanced in rats treated with rIL-4, as described (45). This response was
entirely inhibited in rats treated with MRC Ox81 (Fig. 2). Controls treated
with isotype-matched mouse mAb (A6) had similar IgG1 alloantibody re-
ponses to normal rejection (data not shown).

Immunostains
Indirect immunofluorescence staining of single cells and immunoperox-
dase staining of tissue with mAb were performed as described (57). The
anti-rat mAb used included G4.18 (CD3), R7.3 (TCR \(\alpha\) chain Ig), W3/25 (CD4),
MRC Ox8 (CD8), MRC Ox6 (MHC class II), and MRC Ox12 (\(\kappa\) light
chain Ig) and were either purchased from Pharmingen (San Diego, CA) or
produced in our laboratory.

Cell preparation and purification
Single-cell preparations from spleen and lymph nodes were resuspended in
PBS with calcium and magnesium salts (DAB; Oxoid, Oxford, U.K.) and
10% FCS (Life Technologies, Gaithersburg, MD) as described (5). Cells
were separated by an indirect panning technique to deplete unwanted sub-
populations with mAb MRC Ox8 or W3/25 as previously described (5).
Enriched populations had a purity of 97–99%.

Cell culture
RPMI 1640 (Life Technologies) was supplemented with 100 ng/ml of pen-
cillin and 100 U/ml of streptomycin (Life Technologies), 2 mM glutamine,
5 \(\times\) 10\(^{-5}\)M 2-ME (Sigma-Aldrich), and either 5–10% FCS or 20%
Sprague Dawley rat serum. Of the sera from a variety of different rat strains, 20% Sprague Dawley serum was identified as best supporting MLC because it produced low background stimulation and allowed cytokine mRNA analysis with no background in autologous controls.

**Mixed lymphocyte culture**

Spleen and thymus cells were isolated from rats given 9.5 Gy whole body irradiation 24 h earlier. These cells were used as stimulator cells and were predominantly dendritic cells, containing <1% lymphocytes. This method of preparing stimulators eliminated background levels of mRNA for T cell cytokines for the RT-PCR assays. These stimulators were as effective as in vitro-irradiated spleen cells at one-tenth the number of cells. Lymph node cells from naïve rats or allograft recipients treated with MRC Ox38 and sacrificed >100 days posttransplant were cultured at room temperature overnight in 5% FCS-supplemented medium. These cells were then washed and resuspended in 20% Sprague Dawley serum-supplemented medium, as were the stimulator cells. Responder cells were seeded in 96-well U-bottom plates at 2 × 10^5 cells/ml and stimulators at 2 × 10^5 cells/ml in a total volume of 200 μl. Quadruplicate wells were cultured for each sample at 37°C in humidified air containing 5% CO₂. Proliferation was assessed by pulsing with 0.5 μCi [³H]Tdr (Amersham, Arlington Heights, IL) 18 h before harvesting using a Harvester 96 (Tomtec, Orange, CT) and counting in liquid scintillation fluid on a Microbeta Plus scintillation counter (Wallac Oy, Turku, Finland). The stimulation index was calculated as specific response/response to autologous stimulators. Relative response was calculated as specific response − response to autologous stimulators/response to third party minus response to autologous stimulators.

**RT-PCR**

The methods of mRNA extraction, cDNA synthesis, and PCR have been described in detail (47, 57). Semiquantitative PCR was performed either by terminating the reaction every 3–5 cycles or by diluting the starting cDNA sample 10-, 100-, and 1000-fold. Results were scored as the cycle or dilution of cDNA at which specific PCR product was first detected. All samples were standardized by quantitating the RNA by spectrophotometry before RT-PCR and by PCR of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to confirm intact RNA and consistency of cDNA synthesis. The primers and optimal conditions for the cytokines IL-2, IL-4, IL-5, IL-10, IFN-γ, TNF-α, TNF-β (lymphotoxin), cytolsin (homologue of mouse perforin), and granzyme A have been described (45, 47). A five-cycle interval detects ~10-fold differences, while a three-cycle interval detects ~5-fold differences; this has been previously demonstrated for IL-2, IFN-γ, and IL-10 (47), and IL-4 (45). In the assay range tested, increases and decreases in mRNA for these cytokines can be demonstrated (45, 47, 57). Similar dilutional studies have confirmed the sensitivity of the assays for TNF-α, TNF-β, cytolsin, and granzyme A (our unpublished data).

**Statistical analyses**

Data were analyzed with ANOVA, and significance was examined by the Bonferroni-Dunn post hoc test (p < 0.005 for significance); for nonparametric unpaired tests, Mann-Whitney U tests were performed (p < 0.05 for significance) using the Statview program for Apple Macintosh.

**Results**

**Tolerance in the host with long surviving grafts**

The grafted hearts of MRC Ox38-treated recipients were examined >100 days posttransplant, all having contracted normally with no evidence of acute rejection on palpation. Macroscopically, the hearts appeared normal. Histology and immunopathology revealed a diffuse light infiltrate of mononuclear cells as well as scattered small foci of these cells. The infiltrate include CD4⁺ and CD8⁺ T cells and macrophages. Vessels in some grafts showed mild intimal hyperplasia and infiltrate of T cells and macrophages consistent with early chronic rejection.

To examine the cytokine profile of this cellular infiltrate, RT-PCR of mRNA from transplanted heart tissue was undertaken and compared with that in the recipients own heart and normal heart tissue (Fig. 3). As a positive control, normal rejecting hearts at day 6 posttransplant were compared. All cDNA preparations were standardized and when amplified by PCR had the same level of GAPDH mRNA, detectable at 15 cycles. The long-surviving hearts had an increase in mRNA for all three Th1 cytokines examined. IL-2, IFN-γ, and TNF-β were not significantly different from normal rejection levels; levels of IFN-γ and TNF-β in rejecting hearts were significantly above those found in the recipient’s own heart and normal heart tissue. Each five-cycle difference represents a 10-fold difference in mRNA. In this assay, low levels of nearly all cytokines are detected in normal tissue, presumably due to the presence of circulating activated T cells and to the sensitivity of the RT-PCR technique used. The levels of Th1 cytokines had fallen from that observed during the induction of tolerance by anti-CD4 therapy (day 6 posttransplant), but were not back to background levels (data not shown) as previously published (47). In tolerant grafts, mRNA for the Th2 cytokines IL-4 and IL-6 were not increased above the level observed in the recipient’s own heart or in normal hearts. The level of IL-4 was not different from that seen in normal rejection but was above that seen during the induction phase with anti-CD4 mAb, when IL-4 and IL-5 expression in the transplant was below that in normal or rejecting hearts (previously published data (47)). In tolerant grafts, IL-6 was rarely detected and was significantly below the level found in both rejecting grafts as well as that observed during induction of tolerance, when this cytokine was increased to levels seen in normal rejection. IL-10 expression in tolerant grafts was increased above the level found in the recipient heart and normal heart, but appeared less than the levels found during acute rejection or the induction phase of tolerance, although not reaching significance. The mRNA for effector molecules TNF-α and cytolsin in tolerant hearts were at the background levels found in normal and recipient

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**FIGURE 2.** Detection of IgG1 alloantibody in serum from allograft recipients. A, Negative control of normal DA rat serum (NEG) compared with serum from a DA rat with rejection of a PVG allograft (REJ), which develops IgG1 alloantibody. B, Serum from an rIL-4-treated allograft recipient (IL-4) with increased IgG1 compared with serum from an allograft recipient treated concurrently with IL-4 and Ox81 (IL-4/Ox81), which has a low IgG1 response. MRC Ox81 treatment alone also blocked IgG1 alloantibody, and A6 isotype-matched control mAb therapy had no effect on IgG1 alloantibody (data not shown).
hearts, and granzyme A was not detected in tolerant hearts. These findings demonstrated that within the tolerated graft there is a persistent infiltration of Th1 cells with no evidence of preferential Th2 cell activation or an increase in cytotoxic cell molecules.

Acceptance of donor strain skin grafts even if IL-4 is blocked

DA rats with long surviving PVG allografts after MRC Ox38 therapy were tested for specific tolerance by application of donor and third-party Lewis skin grafts. All third-party grafts were rejected in first set tempo of 10–13 days (data not shown). If PVG skin grafts were applied 84–100 days postcardiac transplantation, skin graft survival was prolonged; however, some grafts were rejected in a delayed fashion (Table I). After 100 days posttransplant, all PVG grafts skin were accepted and grafts appeared normal up to 100 days later. To determine whether IL-4 was essential for the establishment of tolerance to the second grafts, MRC Ox81, a mAb that blocks IL-4 function in vitro and in vivo, was administered (see Materials and Methods). This therapy failed to prevent acceptance of the new graft or affect the function of the long-surviving cardiac allograft.

Table I. Survival of PVG skin grafts on DA rats with long-surviving PVG cardiac allografts after treatment with anti-CD4 mAb therapy

<table>
<thead>
<tr>
<th>Host</th>
<th>Graft Survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
</tr>
<tr>
<td>Naive</td>
<td>12</td>
</tr>
<tr>
<td>Tolerant 84–100 days</td>
<td>37</td>
</tr>
<tr>
<td>Tolerant &gt;100 days</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Tolerant given anti-IL-4</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*All tolerant heart grafts were not rejected even though donor strain skin was rejected.

*Time since tolerance was induced; either 84–100 days or >100 days.

*Recipients were given MRC Ox81 (anti-IL-4 mAb) on the day of skin grafting and 3, 7, and 10 days later.

Function of lymphocytes from rats with long-surviving grafts in vitro

Lymph node cells from rats with long-surviving grafts (>100 days) when stimulated in MLC against specific or third-party Lewis responded by both proliferation and induction of Th1 cytokines. The response from five separate tolerant hosts was examined, and the kinetics of proliferation to PVG and Lewis was identical, starting at day 3 and peaking at days 4 to 5. The proliferative response of cells from tolerant hosts was half that of naive cells, but the relative responses and stimulation indices were similar (Fig. 4, A and B) because naive cells have higher autologous proliferation. These studies demonstrate there was no loss in the capacity of cells from tolerant animals to proliferate against specific donor Ag. RT-PCR was performed to assess cytokine mRNA in MLC 48 h after stimulation, using a 10-fold dilution series of cDNA. These results show that while there was some variation between animals, there was comparable induction of IL-2, IFN-γ, and IL-4 mRNA in tolerant cells against syngeneic, specific donor, and third-party Lewis stimulators. These levels were not significantly different from those in control naive cells (Fig. 4C). IL-10 was not increased above high background expression in autologous controls (data not shown). Taken together, these in vitro experiments demonstrate that lymphocytes from tolerant hosts are not deleted, rendered anergic, or diverted to a Th2 response.

Examination of capacity of lymphocytes from tolerant hosts to transfer tolerance

For these studies, an adoptive transfer assay with whole body irradiated DA hosts was used. Initial studies transplanted donor-specific PVG neonatal heart grafts into one ear and third-party Lewis grafts in the other; thus, each animal had its own internal third-party control. Irradiation delays rejection to 31 (27–36) days (median range) for PVG grafts and 26 days (13–36) for Lewis compared with normal rejection times of 13 (11–15) and 12 (10–16) days, respectively (Table II). Naive spleen and lymph node cells partially restore rejection of both grafts to normal. Cells from
rats with long-surviving grafts after MRC Ox38 therapy were unable to restore rejection of donor strain PVG grafts but did effect rejection of third-party Lewis grafts, similar to naive cells. A role for an active inhibitory cell was shown by cotransfer of cells from tolerant hosts with naive cells. These experiments were done with a ratio of tolerant cells to naive cells of 4:1, as this ratio has been shown necessary in other models of tolerance transfer (40, 58). In these experiments, cells from tolerant animals inhibited the co-transferred naive cells capacity to effect rejection of PVG grafts, but third-party Lewis grafts were rapidly rejected.

To examine the subpopulation of cells that transferred tolerance, enriched subpopulations of CD4\(^+\) and CD8\(^+\) T cells were tested. The capacity to transfer tolerance was solely in the CD4\(^+\) T cell subset (Table III). CD4\(^+\) T cells from tolerant animals failed to restore PVG graft rejection but did effect third-party F344 rejection. In this model, naive CD4\(^+\) T cells were all that was required to restore graft rejection (11). Mixing CD4\(^+\) T cells from tolerant rats with naive CD4\(^+\) T cells lead to inhibition of the naive cell’s capacity to effect PVG graft rejection. CD8\(^+\) T cells lack the capacity to restore rejection in irradiated hosts unless sensitized (11, 59). CD8\(^+\) T cells from tolerant hosts failed to effect either PVG or F334 graft rejection and, when mixed with naive CD4\(^+\), did not inhibit their capacity to effect rejection (data not shown). Thus, it was concluded that tolerance was associated with a CD4\(^+\) T cell that could transfer tolerance and had an inhibitory function on naive CD4\(^+\) T cells.

To determine whether the transfer of tolerance was associated with an up-regulation of Th2 cytokines, adoptive hosts were sacrificed at 3, 6, 9, and 12 days postirradiation. Groups reconstituted with either tolerant or naive cells, as well as a group of

Table II. Comparison of the capacity of unfractionated spleen and lymph node cells from normal and MRC Ox38-treated rats to adoptively restore neonatal heart graft rejection in irradiated hosts

<table>
<thead>
<tr>
<th>Restorative Innocula</th>
<th>PVG grafts</th>
<th>Lewis grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Source</td>
<td>Number of cells</td>
</tr>
<tr>
<td>Naive</td>
<td></td>
<td>2 \times 10^7</td>
</tr>
<tr>
<td>Naive</td>
<td>5 \times 10^6</td>
<td>11</td>
</tr>
<tr>
<td>MRC Ox38 Rx(^a)</td>
<td>2 \times 10^7</td>
<td>&gt;100*</td>
</tr>
<tr>
<td>MRC Ox38 Rx(^a) + Naive</td>
<td>2 \times 10^7</td>
<td>&gt;100*</td>
</tr>
<tr>
<td>MRC Ox38 Rx(^a)</td>
<td>5 \times 10^6</td>
<td>&gt;100*</td>
</tr>
</tbody>
</table>

\(^a\) MRC Ox38 Rx = DA rats grafted with PVG hearts and treated with MRC Ox38 to induce tolerance. Recipients were sacrificed >100 days posttransplantation for collection of cells.

\(^*p < 0.05\) compared with all groups restored with naive cells alone; **\(^p < 0.05\) compared with PVG grafts in rats restored with MRC Ox38 Rx cells.
unreconstituted controls, were examined, with three animals at each time point. The transplanted neonatal heart and the lymph node draining the site of implantation were removed and mRNA extracted for RT-PCR of IL-2, IFN-γ, IL-4, and IL-10. GAPDH expression was comparable for all samples. These studies failed to demonstrate any up-regulation of IL-4 in hosts given tolerant cells (Fig. 5). There was no significant difference in the up-regulation of IL-4 in heart grafts and lymph nodes of hosts reconstituted with naive cells compared with tolerant cells. IFN-γ was significantly less at early time points in the grafted hearts of hosts given tolerant cells compared with those given naive cells, but there was no difference in expression within the draining node. IFN-γ levels were also reduced compared with unreconstituted controls at day 9, although by day 12 there was no significant difference between the treatment groups. This effect was seen only within specific donor (PVG) grafts, with no difference in IFN-γ levels between tolerant and naive cell reconstituted groups for Lewis grafts (data not shown). IL-2 was rarely detected in heart grafts. IL-10 was readily detected in all samples at all time points and was not discriminatory between groups (data not shown). With the exception of IFN-γ in grafted hearts, the cytokine expression profiles were similar in both PVG and Lewis allografted hosts (data not shown). The samples available from neonatal grafts were small, not permitting analysis of further cytokines, so experiments with adoptive transfer to hosts with heterotopic adult heart grafts were also examined.

Adoptive transfer of tolerance to irradiated hosts with heterotopic adult heart grafts

Irradiated hosts with PVG heterotopic grafts were reconstituted with either $5\times10^7$ naive CD4+ T cells or with $2\times10^7$ tolerant CD4+ T cells and $5\times10^6$ naive CD4+ T cells. Controls were not reconstituted with cells. Graft survival was the same as for the neonatal heart graft model (Table IV). In this model, as few as $5\times10^6$ CD4+ T cells was an optimal inoculum that induced significant prolongation of graft survival (Table IV).

**Table III.** Comparison of the capacity of unfractionated or CD4+ T cells from spleen and lymph nodes of normal and MRC Ox38-treated rats to adoptively restore neonatal heart graft rejection in irradiated hosts

<table>
<thead>
<tr>
<th>Restorative Inocula (number of cells)</th>
<th>Graft strain</th>
<th>Rejection Time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Naive MRC Ox38 Rx</td>
<td></td>
</tr>
<tr>
<td>$2 \times 10^7$</td>
<td>PVG</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>F344</td>
<td>18</td>
</tr>
<tr>
<td>$2 \times 10^7$</td>
<td>PVG</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>F344</td>
<td>22</td>
</tr>
<tr>
<td>$5 \times 10^6$</td>
<td>PVG</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>F344</td>
<td>21</td>
</tr>
<tr>
<td>$2 \times 10^7$</td>
<td>PVG</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>F344</td>
<td>12</td>
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<tr>
<td>$5 \times 10^6$</td>
<td>PVG</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>F344</td>
<td>14</td>
</tr>
<tr>
<td>$2 \times 10^7$</td>
<td>PVG</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>F344</td>
<td>26</td>
</tr>
</tbody>
</table>

$^a$ MRC Ox38 Rx = DA rats grafted with PVG hearts and treated with MRC Ox38 to induce tolerance. Recipients were sacrificed >100 days posttransplantation for collection of cells.

FIGURE 5. Semiquantitative RT-PCR analysis of mRNA for IL-2, IFN-γ, and IL-4 in neonatal (NN) heart grafts and their draining (popliteal) lymph nodes (LN). Comparison of irradiated adoptive hosts reconstituted with cells from either naive nongrafted rats (NAIVE CELLS) or from MRC Ox38-treated tolerant hosts sacrificed at $>100$ days posttransplantation (MRC Ox38 Rx CELLS). Controls were irradiated adoptive hosts not reconstituted with cells (NO CELLS). Samples were taken at 3, 6, 9, and 12 days posttransplantation. All samples had similar starting concentration of cDNA as assessed by GAPDH PCR product (data not shown). $^a, p < 0.0005$ compared with naive cell reconstituted at the same time point; $^b, p < 0.0005$ compared with no cell control at the same time point. Horizontal axis shows cDNA dilution factor at which specific PCR product was detected (neat = 1–1/1000 dilution = $10^3$). Results are presented as the median ± range. Product detected at a higher cDNA dilution factor indicates a higher concentration of specific mRNA in the starting sample.
$10^7$ CD4$^+$ T cells effect graft rejection (unpublished data), but 10 times more naive CD4$^+$ T cells combined with CD4$^+$ T cells from rats with long-surviving grafts failed to effect specific donor graft rejection. They did effect third-party rejection (data not shown). As in the neonatal heart graft model, tolerant cells mixed with naive cells inhibited the reconstitution of rejection by the naive cells. To examine whether IL-4 was required to inhibit the naive cell response this cytokine was blocked with MRC Ox81. This did not inhibit transfer of tolerance.

The cytokine mRNA induced within the grafted hearts of adoptive host were examined 12 days after grafting and reconstitution. There was induction of Th1 cytokines, including IL-2, IFN-γ, and TNF-β, similar to that seen in rats restored with naive cells as well as in nonreconstituted hosts (Fig. 6). This suggests that regenerating cells in the host contribute to the allograft response. Th2 cytokines IL-4 and IL-10 were not increased in hosts restored with suppressor cells compared with those restored with naive cells; but these cytokine levels appeared slightly higher then in the irradiation controls, although this difference did not reach significance. Surprisingly, there was also induction of the effector molecules TNF-α, cytolsyn, and granzyme A. Despite this identification of an effector Th1 response and cytolytic T cell molecules, there was no evidence of graft damage in the hosts restored with suppressor cells or in the irradiation controls.

**Discussion**

The model of transplant tolerance in this study differs from the other reports on tolerance induced by anti-CD4 mAb, which have identified CD4$^+$ suppressor T cell as mediating long term tolerance and have concluded that IL-4 may mediate this process (17, 21, 42). These other studies have all used depleting anti-CD4 mAb to reduce the CD4$^+$ T cell population to <15% of normal. In addition, studies done in mice by Waldmann’s groups (15, 21, 60) used adult thymectomy to limit T cell regeneration, as well as anti-CD8 mAb to deplete this subset of T cells. In rats, a depleting anti-CD4 mAb was used to prolong kidney graft survival after the recipient had been specifically sensitized with a skin graft (42). In our studies, using naive rats, anti-CD4 mAb does not deplete the majority of T cells (5, 47), and prior adult thymectomy or concurrent use of depleting anti-CD8 mAb prevents induction of tolerance (Ref. 4; and our unpublished data). Thus, this study is the first to examine the mechanisms that maintain long term tolerance when it is induced by anti-CD4 mAb alone in immunologically naive recipient. It was demonstrated that specific tolerance can be adoptively transferred by CD4$^+$ T cells, but not CD8$^+$ T cells, and that a third party graft can be rejected in the same host that acquires tolerance. Further, tolerant cells when mixed with naive cells in a ratio of 4:1 inhibit the ability of naive cells to effect rejection. A requirement for an appropriate ratio of suppressor to naive cells has also been described by others (21), and the transferred cells have the ability to infect the naive cells to effect rejection. A requirement for an appropriate ratio of suppressor to naive cells has been also described by others (21), and the transferred cells have the ability to infect the naive cells to effect rejection. A requirement for an appropriate ratio of suppressor to naive cells has been also described by others (21), and the transferred cells have the ability to infect the naive cells to effect rejection.

**Table IV.** Survival of PVG heterotopic cardiac allografts in irradiated DA recipients restored with cells from either naive DA or DA rats with long-surviving heart allografts after MRC Ox38 treatment$^a$

<table>
<thead>
<tr>
<th>Restorative Inocula (number of cells)</th>
<th>MRC Ox38 Rx</th>
<th>Other Therapy</th>
<th>Rejection Time (days)</th>
<th>Individual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive $5 \times 10^7$</td>
<td>$5 \times 10^7$</td>
<td></td>
<td>11</td>
<td>10, 11 (3), 12, 13</td>
</tr>
<tr>
<td>MRC Ox38 Rx $5 \times 10^7$ CD4$^+$</td>
<td>$5 \times 10^7$</td>
<td></td>
<td>&gt;100</td>
<td>&gt;100 (5)</td>
</tr>
<tr>
<td>MRC Ox38 Rx $2 \times 10^7$ CD4$^+$</td>
<td>$2 \times 10^7$</td>
<td></td>
<td>&gt;100</td>
<td>&gt;100 (6)</td>
</tr>
<tr>
<td>MRC Ox81$^*$</td>
<td></td>
<td>MRC Ox81$^*$</td>
<td>&gt;100</td>
<td>&gt;100 (3)</td>
</tr>
</tbody>
</table>

$^a$ MRC Ox38 Rx = DA rats grafted with PVG hearts and treated with MRC Ox38 to induce tolerance. Recipients were sacrificed >100 days posttransplantation for collection of cells.

$^b$ Adoptive hosts were given MRC Ox81 (anti-IL-4 mAb) on the day of grafting and 3, 7, and 10 days later.
these cells in all models are similar and independent of the original tolerizing immunosuppressive therapy.

Differences between the models of transplant tolerance revolve around the reactivity of cells from tolerant hosts in vitro and the cytokines that they may produce or be associated with. Three mechanisms have been examined to explain tolerance; these are clonal deletion, clonal anergy, or clonal dysregulation of cytokines (in particular, Th2 dominance over Th1). In our model, alloreactive Th1 but not Th2 cells are activated during induction (47). This study demonstrated that Th1 activation persists in the graft >100 days and was not accompanied by a late activation of Th2 cells. Further, the response of tolerant cells in vitro was similar against a specific donor and a third-party alloantigen, and mRNA for Th1 cytokines IL-2 and IFN-γ were induced, indicating that the cells were not anergic. There was no heightened induction of IL-4 and IL-10 by these cells, indicating that Th2 cells were not increased. These results are similar to those of most other studies of transplant tolerance induced in adults in that cells can proliferate to the donor in MLC (7, 17, 27, 36, 66); only a few studies show diminished proliferation (42). When cytokine activity of in vitro-stimulated cells has been assayed, no loss of IL-2 or enhancement of IL-4 response has been observed (17, 36). Why essentially normal alloreactivity in vitro does not translate into rejection effectors in vivo remains an unresolved paradox of this form of tolerance. Our in vivo studies accord with our in vitro data in that within the graft, both in the original tolerant host and in the adoptive hosts rendered tolerant by transfer of cells, there was evidence of Th1 cell activation, albeit IFN-γ in the graft of adoptive hosts, but not in the regional lymph node, was delayed compared with naive cell reconstituted, unreconstituted, and third-party grafts. There was no enhanced induction of mRNA for IL-4 or other Th2 cytokines. This situation is similar to the induction phase of tolerance in this model, when there is activation and infiltration of Th1 cells as well as cytolytic cells and activated macrophages, but there in no graft injury nor induction of cells with mRNA for IL-4 and IL-5 (47). Why there is no graft injury is not known, but these findings suggest that the regulation of tolerance may occur at the final effector phase. The delayed induction of IFN-γ in the graft but not in the regional node suggests there may be a reduced infiltration of Th1 effector cells, which may account for the failure of the adoptive host to effect rejection. The mechanism by which there is reduced infiltration of IFN-γ-producing cells was not ascertained by these studies. Although our studies did not identify anergy as a mechanism, anergy in CD8+ T cells has been described with anti-CD4 mAb-induced tolerance (67). This possibility could not be explored in DA rats because in this strain CD8+ T cells alone do not proliferate or produce IL-2 without help from CD4+ T cells (5, 68).

Tolerance induction was demonstrated by the acceptance of donor skin grafts and rejection of third-party grafts. We reasoned that if IL-4 was to play a major role in the regulation of the Th1 cells and in preventing rejection in this model, the rechallenge with skin, which is a more difficult organ to which to induce tolerance, would provide adequate antigenic challenge and overcome the lack of APC in the original graft. In this circumstance, regulator cells producing IL-4 would be most active in preventing rejection of the new graft. However, administration of a large dose of an anti-IL-4-blocking mAb had no effect, and the new skin graft and the original heart grafts continued to function normally for an additional 100 days. This same batch and dose of mAb has blocked IL-4 function in another model of transplant tolerance induction (45) and has altered the response to autoimmune nephritis in rats (S.T.S. and B.M.H., unpublished data). In the current study, it was shown that significant circulating inhibitory levels of MRC Ox81 could be detected up to 8 days after injection of a single dose of MRC Ox81; thus, experimental hosts given 4 doses over a 10-day period would have had sufficient mAb to block any IL-4 function. The capacity of MRC Ox81 to block IL-4 function in vivo was demonstrated by its ability to block IgG1 alloantibody responses even in hosts treated with large doses of IL-4. In experimental autoimmune uveoretinitis, a similar dose of MRC Ox81 blocked the proinflammatory effect of rIL-4 on this disease and alone lessened the severity of the disease (55). Taken together, these findings suggest that this dose of MRC Ox81 should have blocked IL-4 function in the skin-grafted tolerant host and should have prevented adoptive transfer of tolerance to irradiated hosts if IL-4 played an essential role in the maintenance of tolerance. In the transfer of anti-CD4/ anti-CD8-induced tolerance in mice, blocking anti-IL-4 mAb only partially impaired transfer of tolerance in situations in which the ratio of tolerant to naive cells was marginal (21). Anti-IL-4 mAb more readily blocks induction of neonatal transplant tolerance (38, 69), as well as restoring effector responses to Leishmania (30). Combined with the failure to detect increased mRNA for IL-4 and other Th2 cytokines in adoptive hosts given tolerant cells, these data suggested that IL-4, in particular, and Th2 cells were not necessary for the maintenance of tolerance in this model.

This conclusion is in variance with many other studies on transplant tolerance, which report that Th2 cells and IL-4 in particular are central to maintenance of transplant tolerance (19, 20, 39, 42, 70, 71). Many of these studies have observed heightened levels of IL-4 during induction, usually in models in which there was major T cell depletion or during the neonatal period. These observations may be due to the predominance of Th2 responses early during ontogeny. Nondepleting anti-CD4 mAb in MLC also diverts the response to Th2 (72), which suggests that depletion of CD4+ T cells is not the only factor and that species, strain, prior exposure to Ag, and protocol differences may affect whether Th2 responses are induced. However, few others have examined the change in cytokines during the transfer of tolerance. Transfer of posttotal lymphoid irradiation tolerance was associated with activation of both Th1 and Th2 cells, which was similar to that observed in rats restored with naive cells (36). In our studies, the methods of RT-PCR used have the capacity to detect as little as a fivefold increase or decrease in mRNA for IL-4 (45, 47, 57); thus, biologically significant changes in Th1/Th2 cell function could be assayed and would have been detected. Combined with the failure of anti-IL-4 mAb therapy to affect maintenance or transfer of tolerance, these studies suggest that IL-4 is not an essential cytokine for the maintenance of tolerance. IL-4 itself has mixed effects as an agent to induce transplant tolerance, with some reports showing that IL-4 can prolong graft survival (43–45), while others report no effect (22, 47, 73). Further, Th2 cells have been proposed as effectors of rejection (48, 49), and in IL-4 knockout mice, transplant tolerance can be induced but is prevented by the administration of rIL-4 (74).

The understanding of the mechanisms that maintain tolerance has major potential to promote clinical organ graft acceptance without the need for long term immunosuppression. From the findings of this and other reports, we conclude that the maintenance of tolerance is due to a CD4+ T cell that can suppress the normal alloreactive cell’s capacity to mount an effector response that destroys the allograft. The mechanisms by which these cells mediate their inhibitory effect remain a matter of speculation. One theory is that the cells act as nonproductive alloreactive cells that consume IL-2, thereby starving the potentially active effector cells from this or other cytokines (17). In support of this possibility is the finding that the maintenance of suppressor cell function in vitro is IL-2 dependent (41) and the cells express IL-2R (62). The current study did not examine whether there was a relative deficit of IL-2, but
cells from tolerant animals could produce mRNA for IL-2 when stimulated in vitro and did not induce production of mRNA for IL-2 in adoptive hosts. The second theory involves Th1 cell inhibition by Th2 cells or other immunoregulatory cells. It has recently been reported that there may be other immunoregulatory cells, including Th3 cells, that have different cytokine profiles than Th2 cells and that produce TGF-β; these are thought to regulate tolerance in experimental allergic encephalitis (65) and in Tr1 cells that inhibit autoimmune colitis and produce IL-10 and IL-5 but not IL-4 (75).

Our findings are consistent with the possibility of either of these regulatory cells playing a role in transplant tolerance, and these possibilities are currently under investigation. The data reported here provide evidence that classical Th2 cells and IL-4 are not essential for the maintenance of tolerance. It is possible that IL-4 is a major immunoregulatory cytokine; in this model, however, it was not essential and may have been replaced by other cytokines with similar actions.

**Acknowledgment**

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**References**


