Indirect Recognition by Helper Cells Can Induce Donor-specific Cytotoxic T Lymphocytes In Vivo

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Summary

In vitro studies have revealed that help for cytotoxic T lymphocyte (CTL) induction can be mediated through several pathways, including direct recognition of allogeneic class II antigens by CD4+ cells, direct recognition of allogeneic class I antigens by "CD4-independent" CD8+ cells, and "indirect" recognition of peptides of alloantigens presented in association with self class II molecules. Whereas good evidence for the two direct pathways is available in vivo, there is relatively little evidence to show that indirect recognition can initiate graft rejection. This study examined the role of indirect allorecognition during the generation of CTLs in mice as they rejected major histocompatibility complex (MHC) class II–deficient skin after depletion of CD8+ T cells in vivo. Recipients were depleted of CD8+ T cells by in vivo treatment with anti-CD8 monoclonal antibody and then grafted with allogeneic skin lacking MHC class II antigens. The mice rejected the skin grafts rapidly. Although flow cytometry showed marked depletion of CD8+ T cells in these mice, we found that (a) CD8+ CTLs were generated and sensitized to MHC class I antigens of the donor; (b) the generation of the CD8+ CTLs required the help in vivo of CD4+ cells, as well as priming with the allogeneic skin graft; and (c) the CD4+ T helper cells were sensitized indirectly to donor peptides presented in association with class II antigens on recipient antigen-presenting cells. These results provide evidence that indirect recognition can provide effective help for CTL induction during graft rejection, even when the cytotoxic T cells are sensitized by determinants expressed only on the donor graft.

Indirect recognition describes the stimulation of recipient T cells by allogeneic donor antigens presented in association with self MHC antigens on recipient APCs. Although indirect recognition represents the ordinary process by which T cells are sensitized during normal immune responses, its contribution to graft rejection is obscured by the powerful direct stimulation of T cells by donor APCs in allogeneic responses. Although it has been known for a long time that indirect recognition can occur during rejection of foreign tissue, and that it can play an effective role in allogeneic responses in vitro (1–3), it has not been clear whether this pathway alone can actually initiate graft rejection in vivo. This uncertainty has been especially important when the donor and recipient have been MHC mismatched, such that the determinants formed on recipient APCs would not be expressed on the cells of the donor graft.

The recent availability of mice lacking MHC class II antigens has provided a new opportunity to examine the role of indirect recognition. Lacking class II antigens on their own APCs, grafts from these animals to normal recipients would be expected to stimulate CD4+ T cells only by the presentation of donor peptides in association with recipient class II molecules. However, because these grafts still express class I antigens, they can sensitize alloreactive CD8+ cytotoxic cells without the help of CD4+ cells. Thus, a direct pathway for rejection of a MHC-disparate graft can still exist even when the donor lacks class II antigens, making it still difficult to demonstrate that indirect recognition alone can initiate graft destruction.

To overcome this problem, we have recently performed and reported a series of in vivo experiments with class II–deficient donor skin grafts using two strategies to eliminate the direct pathway of donor antigen recognition (4). First, we used grafts without MHC antigen disparities and only minimal minor antigen differences to eliminate the direct CD8+ stimulation by donor class I antigens. Second, we treated mice with anti-CD8 antibody in vivo, forcing rejection of the class II–deficient grafts to depend on CD4+ cells. The results of these studies suggested that indirect recognition could effectively initiate rejection.

In this paper we provide in vitro evidence to support the conclusion that indirect recognition can lead to rejection even when the donor and recipient are mismatched for their MHC antigens. We examined T cell responses in mice that had been treated with anti-CD8 antibody in vivo and that had rejected class II–deficient but class I–mismatched grafts. As expected from the previously published work of Rosenberg et al. (5), we found that despite the in vivo treatment with anti-CD8 antibody, CD8$^+$ CTLs were present in these mice after graft rejection. We found that these CTLs were specific for donor class I antigens and depended on CD4$^+$ helper cells that had been sensitized by donor peptides presented by recipient class II molecules. These results confirm that indirect recognition can play an effective role in graft rejection. They also show that CD4$^+$ cells (sensitized indirectly by recipient APCs) can provide help for CD8$^+$ CTLs (sensitized directly by donor cells), suggesting that four different cell types can work together to produce cytotoxic T cells.

Materials and Methods

Animals. BALB/cBy (H-2^d), C57BL/6J (H-2^b) (B6), B10.D2 (H-2^d), and [BALB/c × B6]F1 (H-2$^{d/b}$), (CB6F1) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). C3H/Sed (H-2^k) mice were supplied by the Edwin L. Steele Laboratory at Massachusetts General Hospital (Boston, MA). The generation of [129 × B6]F3 MHC class II knockout mice (H-2^b), (II$^-$) has been reported elsewhere (6). Briefly, the A$_\beta^b$ gene was disrupted in the D3 embryonic stem cell line of 129/Sv origin using the technique of homologous recombination. Cloned embryonic stem cells expressing the mutant gene were injected into B6 blastocysts and implanted in foster mothers. A chimeric male founder animal was bred with a normal B6 female to select for germline transmission of the defective gene. Their offspring were intercrossed and those of their offspring which were homozygous for the mutant gene were selected as founders for further breeding. Mice of the next (and subsequent) generations expressed the class II deficiency and a random assortment of B6 and 129 genes, some homozygous and others still heterozygous. These mice are referred to as F3 class II–deficient mice (II$^-$). For use in certain in vitro experiments, BALB/c mice were bred with F3 II$^-$ mice to generate [BALB/c × II$^-$]F1 mice and 8th generation B6 class II–deficient mice were generated by backcrossing onto a B6 background.

Skin Grafting and Adult Thymectomy. Skin grafts were placed on mice according to the technique of Billingham and Medawar (7). Mice were anesthetized with chloral hydrate supplemented with ether. Grafts were placed on the lateral thoracic area and held in place with vaseline gauze and plaster bandages. The bandages were removed on the seventh or eighth day. Rejection was recorded when there was >90% destruction of the tissue. Thymectomies were performed on mice using the suction pipette technique with ether anesthesia.

mAbs and In Vivo T Cell Depletion. Anti-CD8 (2.43) (8), anti-CD4 (GK1.5) (9), and anti-Thy 1.2 (H0 13-4) (10) ascites were prepared from hybridomas obtained from the American Type Culture Collection (Rockville, MD). Anti-CD3 (YCD3-1) (11) ascites was prepared from a hybridoma kindly donated by K. Bottomly (Yale University, New Haven, CT). CD8$^+$ T cells were depleted using the 2.43 (rat anti–mouse CD8) antibody, and CD4$^+$ T cells were depleted using the GK 1.5 (rat anti–mouse CD4) antibody, as we have described previously (12). All treated mice were thymectomized

before receiving 0.1 ml i.p. of unpurified ascites of mAb on days −6, −3, and −1 before skin transplantation.

Anti–mouse Surface Marker Reagents. FITC-conjugated rat anti–mouse Thy 1.2 and PE-conjugated rat anti–mouse Lyt 2 (mouse CD8), purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA), were used to stain T cells.

Staining and Flow Cytometric Analysis. FACS® medium (Becton Dickinson) containing 1× PBS, 0.1% fetal bovine albumin, and 0.1% sodium azide was used. 3 × 10^5 cells were stained per well in 96-well, U-bottomed plates. Mouse FcγR were blocked with 0.5 μg of 2.4G2 (13), a rat anti–mouse FcγR mAb. Cells were then incubated with staining reagents for 30 min at 4°C. The cells were washed twice with FACS® medium. All cells were fixed with 2% paraformaldehyde for 30 min. Flow cytometric analysis was performed using a FACScan® flow cytometer (Becton Dickinson). Indirect staining was performed using a FITC-conjugated mouse antibody specific for rat IgG, H and L chain (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). For indirect staining, no blocking antibody was used.

In Vitro Cytolytic Assay. Responder spleen cells (7.5–25 × 10^6) were cocultured with irradiated (2,000 cGy) spleen stimulator cells (25 × 10^6) in 10–20-ml cultures of tissue culture medium consisting of RPMI-1640 supplemented with 10% FCS, 20 mM Hepes, 2 mM L-glutamine, 0.1 mM nonessential amino acids (all from GIBCO BRL, Gaithersburg, MD), 100 μg/ml gentamicin sulfate, and 0.05 mM 2-ME (both from Sigma Chemical Co., St. Louis, MO). After 5 d, cultures were assayed for cytolytic activity against ^{51}Cr-labeled Con A (Sigma Chemical Co.) or LPS (Difco Laboratories, Detroit, MI) induced lymphoblasts as targets in a 4-h ^{51}Cr-release assay. Specific lysis was determined using the formula: [100 × (Experimental release − spontaneous release)/(maximum release − spontaneous release)]. In some assays 50 μl of appropriate mAb (dilution of 1/200–1/400 ascites) was added to wells at the time of incubation with the targets.

Mixed Lymphocyte Reaction (MLR)[1]. Untreated or antibody plus complement–treated responder spleen cells (2 × 10^5) and stimulator spleen cells (4 × 10^5) (2,000 cGy) were added in a final vol of 200 μl of tissue culture medium to U-bottomed wells in triplicate. The cultures were incubated at 37°C in a humidified air containing 5% CO$_2$ for 3–4 d. [^{3}H]TdR (1 μCi/well; New England Nuclear, Boston, MA) was added 12–14 h before the end of culture. The samples were harvested onto glass fiber filters, and [^{3}H]TdR uptake was measured by β scintillation counting on a RackBeta counter (model 1209; Pharmacia, Gaithersburg, MD). Results are expressed as mean counts per minute ± standard error of the mean. In certain assays, 50 μl of the appropriate mAb in ascites (1/50 to 1/100 dilution) or culture supernatants (1/2–1/16 dilution) was added to the culture wells for blockade.

Antibody Plus Complement Depletion. Spleen cells were resuspended at a density of 50 × 10^6 cells/ml and incubated with appropriate antibody dilution or medium alone for 30 min on ice. Cells were washed with medium, pelleted, resuspended, and incubated with rabbit C′ (C-6 Diagnostic, Inc., Mequon, WI) at a dilution of 1/5, at a density of 40 × 10^6 cells/ml, for 30 min at 37°C. Control cultures contained complement alone. The cells were then washed twice and resuspended.

Results

Mice Treated With Anti-CD8 Antibody In Vivo Rejected Skin Grafts Lacking MHC Class II Antigens. We have previously

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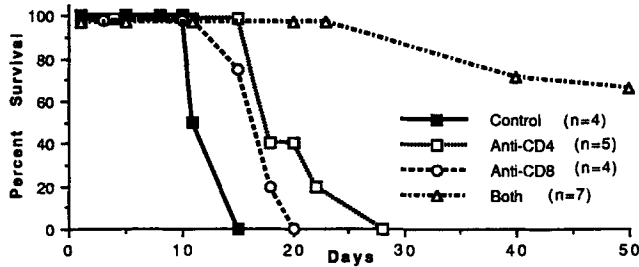[1] *Abbreviation used in this paper:* MLR, mixed lymphocyte reaction.

Figure 1. Survival of MHC disparate tail skin grafts without class II antigens on recipients depleted of selected T cells. Tail skin grafts from F3 class II–deficient mice were transplanted to thymectomized BALB/c mice that had been treated with nothing (--■--), anti-CD4 antibody (--□--), anti-CD8 antibody (--O--), or both antibodies (--△--).

published results similar to those in Fig. 1 showing the survival of MHC class II–deficient skin grafts placed on otherwise normal mice after depletion of $CD4^+$, $CD8^+$, or both T cell subpopulations (4). Grafts placed on recipients depleted of either subpopulation alone were rejected with only slight delay, whereas those on recipients depleted of both subpopulations showed prolonged survival. We were interested in examining more carefully the rejection observed after $CD8^+$ T cell depletion. Whereas this rejection would seem to depend entirely on $CD4^+$ T cells, even though the grafts lacked MHC class II antigens, we were aware that Rosenberg et al. (5) had found cytotoxic T cells derived from $CD8^+$ cells even in mice that had been treated in vivo with anti-CD8 antibody. We therefore tested mice from this group to determine whether they had CTLs and how they had been sensitized.

Anti-CD8–treated and Skin-grafted Mice Had Few $CD8^+$ Cells But Nonetheless Generated $CD8^+$ CTLs Specific for Donor Class I Antigens. Fig. 2 shows the results of flow cytometric analysis of mice with or without anti-CD8 treatment in vivo. Normal mice have ~10% $CD8^+$ Thy1.2$^+$ spleen cells whereas those treated with anti-CD8 antibody had <1%. Indirect staining showed no evidence for anti-CD8 antibody coating of the cells (data not shown). Even after rejection of grafts, treated mice had <1% $CD8^+$ Thy 1.2$^+$ T cells.

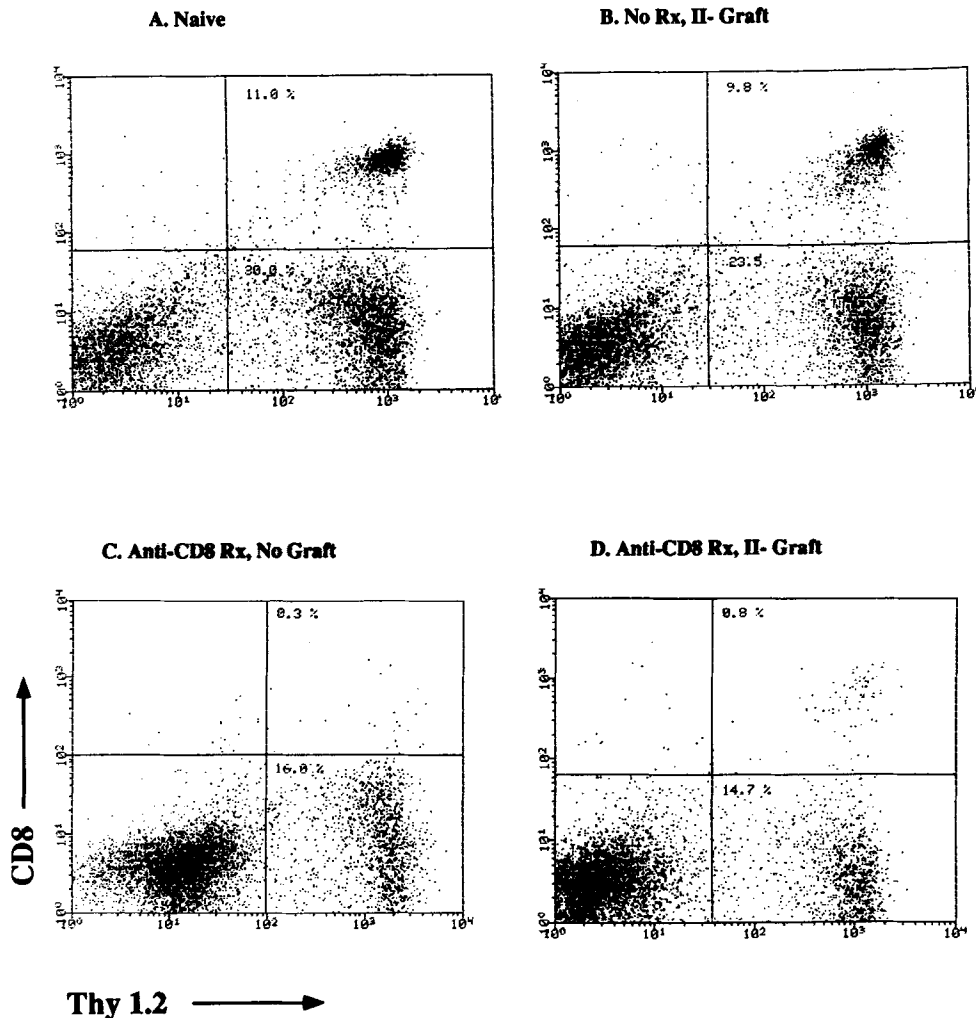Despite the apparent effectiveness of CD8 depletion, we

A. Naive

B. No Rx, II- Graft

C. Anti-CD8 Rx, No Graft

D. Anti-CD8 Rx, II- Graft

CD8

Thy 1.2

Figure 2. Anti-CD8–treated and II$^-$ skin-grafted BALB/c mice possess very few CD8$^+$ T cells. Representative two-color flow cytometric analysis of splenocytes is shown. (*A*) Naive BALB/c mice have 11.0% CD8$^+$, Thy 1.2$^+$ splenocytes. (*B*) II$^-$ Skin-grafted BALB/c mice have 9.8% CD8$^+$, Thy 1.2$^+$ splenocytes. (*C*) Anti-CD8–treated, ungrafted, BALB/c mice have 0.3% CD8$^+$, Thy 1.2$^+$ splenocytes. (*D*) Anti-CD8–treated and II$^-$ skin-grafted BALB/c mice have 0.8% CD8$^+$, Thy 1.2$^+$ splenocytes. 4–6 wk after treatment, splenocytes were stained with PE-anti-CD8 and FITC-anti-Thy 1.2 antibodies, analyzed as described, and presented in log-log scatter plots.

found evidence for the presence of cytotoxic cells in anti-CD8–treated BALB/c mice after rejection of MHC class II⁻ skin. When spleen cells from anti-CD8–treated and skin-grafted mice were boosted in vitro with B6 stimulators, significant lysis was detected against B6 Con A blasts that express H-2ᵇ class I but not class II molecules (Fig. 3).

The CD8⁺ CTLs primed by skin grafting could have been sensitized directly by the donor H-2ᵇ class I antigens expressed on donor APCs or indirectly by peptides of donor antigens presented by recipient H-2ᵈ class I antigens expressed on recipient APCs. To examine these two possibilities, we tested the specificity of the CD8⁺ CTLs primed in vivo. As shown in Fig. 3 B, the CTLs killed B6 (H-2ᵇ) targets well but not C3H (H-2ᵏ) targets at all, an unlikely event if they were sensitized to peptides plus H-2ᵈ MHC antigens. In addition, Fig. 3 C shows that the CTLs killed B6 targets expressing H-2ᵇ class I antigens but were unable to kill B10.D2 targets after in vitro boosting with B6 or B10.D2 or [BALB/c × II⁻]F1 stimulators (which would offer peptides of F3 antigens in association with self, H-2ᵈ class II molecules but would not express native H-2ᵇ class II mole-

cules). B10.D2 targets present B10 minor antigens in association with H-2ᵈ class I antigens. The complete absence of kill on both B10.D2 and C3H targets suggests that the CTLs were primed in vivo by direct sensitization to donor class I antigens and not to self MHC antigens plus donor peptides.

The data in Fig. 4 show that the CTLs mediating anti-class I responses in anti-CD8–treated mice were CD8⁺, as the cytotoxicity was eliminated with anti-CD8 mAb plus C′ treatment (Fig. 4 A) or anti-CD8 mAb blockade but not by anti-CD4 mAb blockade (Fig. 4 B). Thus, despite the substantial depletion of CD8⁺ T cells by antibody treatment in vivo, anti-CD8–treated, skin-grafted mice did possess CD8⁺ CTLs that were specific for donor classes I antigens.

Generation of Anti-class I CTLs in Anti-CD8–treated Mice Required In Vivo Priming With a Skin Graft and the Help of CD4⁺ Cells. We next investigated whether generation of the anti-class I CTLs required in vivo sensitization. We tested splenocytes from BALB/c mice that were thymectomized and treated in vivo with anti-CD8 mAb but that had not received a skin graft. We found no CTL activity after in vitro stimulation with B6 splenocytes (Fig. 5 A). Thus, the ability to
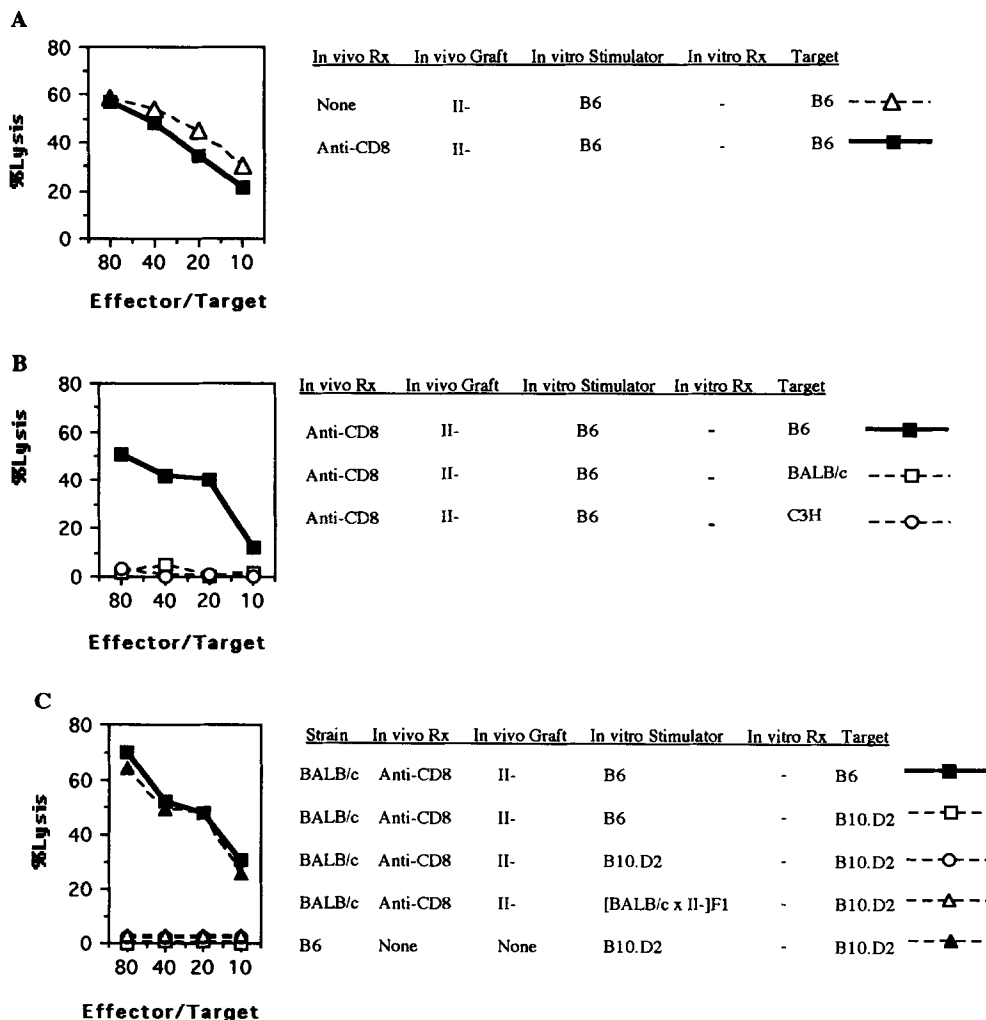
A

In vivo Rx	In vivo Graft	In vitro Stimulator	In vitro Rx	Target	
None	II-	B6	-	B6	--△--
Anti-CD8	II-	B6	-	B6	──■──

B

In vivo Rx	In vivo Graft	In vitro Stimulator	In vitro Rx	Target	
Anti-CD8	II-	B6	-	B6	──■──
Anti-CD8	II-	B6	-	BALB/c	--□--
Anti-CD8	II-	B6	-	C3H	--○--

C

Strain	In vivo Rx	In vivo Graft	In vitro Stimulator	In vitro Rx	Target	
BALB/c	Anti-CD8	II-	B6	-	B6	──■──
BALB/c	Anti-CD8	II-	B6	-	B10.D2	--□--
BALB/c	Anti-CD8	II-	B10.D2	-	B10.D2	--○--
BALB/c	Anti-CD8	II-	[BALB/c x II-]F1	-	B10.D2	--▲--
B6	None	None	B10.D2	-	B10.D2	--▲--

Figure 3. Anti-CD8–treated and skin-grafted BALB/c mice have CTLs specific for donor class I antigens. Splenocytes from anti-CD8–treated and skin-grafted BALB/c mice were cultured with irradiated stimulator cells. After 5 d in culture, effector cells were harvested and assessed for generation of CTLs. (*A*) CTLs from anti-CD8–treated and skin-grafted BALB/c mice (-■-) versus control-untreated but skin-grafted BALB/c mice (--△--). (*B*) Effector cells from anti-CD8–treated and skin-grafted BALB/c mice tested against con A B6 (H-2ᵇ) targets (-■-), self BALB/c (H-2ᵈ) (--□--), and third-party C3H (H-2ᵏ) (--O--) targets. (*C*) Responders from anti-CD8–treated and skin-grafted BALB/c mice were cultured with stimulators from B6 (-■-, --□--), B10.D2 (--O--, --▲--), and [BALB/c × II⁻]F1 (--△--) mice and tested against B6 (-■-) and B10.D2 (--□--, --O--, --△--) targets. Control CTL activity of B6 responders cultured with B10.D2 stimulators and tested against B10.D2 targets (--▲--) is shown.

A
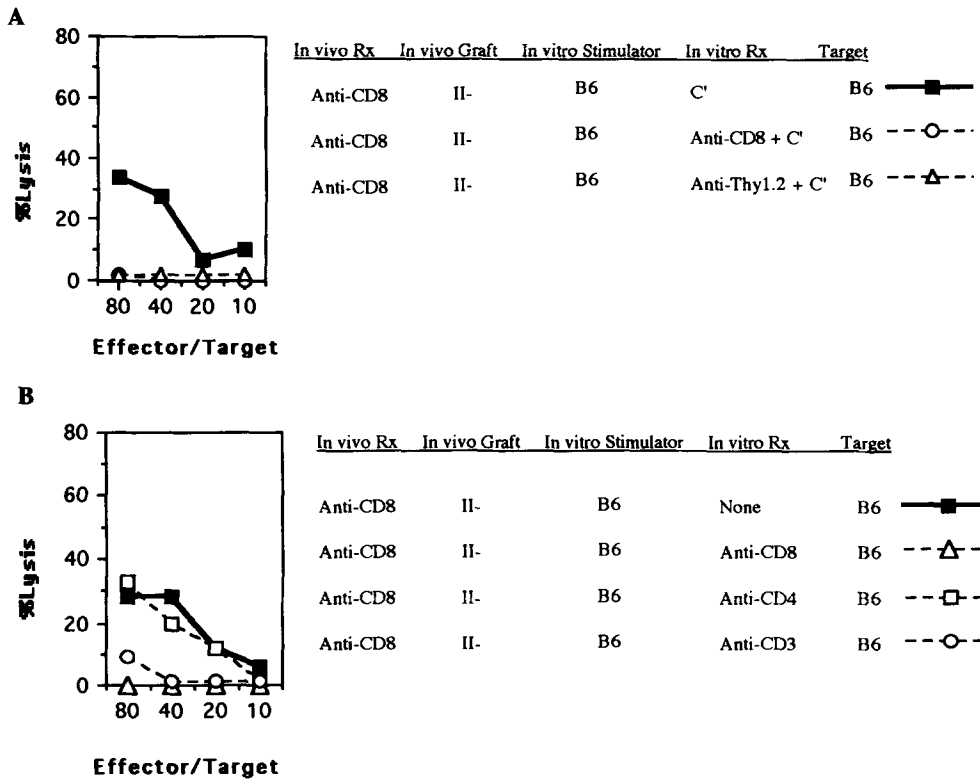

In vivo Rx	In vivo Graft	In vitro Stimulator	In vitro Rx	Target	
Anti-CD8	II-	B6	C'	B6	■
Anti-CD8	II-	B6	Anti-CD8 + C'	B6	-O-
Anti-CD8	II-	B6	Anti-Thy1.2 + C'	B6	-Δ-

B

In vivo Rx	In vivo Graft	In vitro Stimulator	In vitro Rx	Target	
Anti-CD8	II-	B6	None	B6	■
Anti-CD8	II-	B6	Anti-CD8	B6	-Δ-
Anti-CD8	II-	B6	Anti-CD4	B6	-□-
Anti-CD8	II-	B6	Anti-CD3	B6	-O-

Figure 4. The CTLs from anti-CD8–treated and skin-grafted BALB/c mice are CD8+. After 5 d of MLC (A) effector cells were depleted of T cell subpopulations before their incubation with B6 targets by treatment with anti-CD8 (2.43) mAb + C' (--O--), anti-Thy 1.2 (HO-13.4) + C' (--Δ--), or with C' alone (--■--); or (B) effector cells were incubated with anti-CD8 (2.43) mAb (--Δ--), anti-CD4 mAb (GK1.5) (--□--), anti-CD3 (YCD3-1) (--O--), or with no mAb (--■--) during the 4-h ^{51}Cr-release assay.

detect anti-class I CTLs in anti-CD8–treated mice required that these CTLs be primed in vivo by the MHC class II⁻ skin graft.

These CD8⁺ CTLs might have been derived from the so-called "helper-independent" CD8⁺ population that does not require help from CD4⁺ T cells (14). However, as shown earlier in Fig. 1, BALB/c mice treated simultaneously with anti-CD4 and anti-CD8 mAb before skin grafting retained
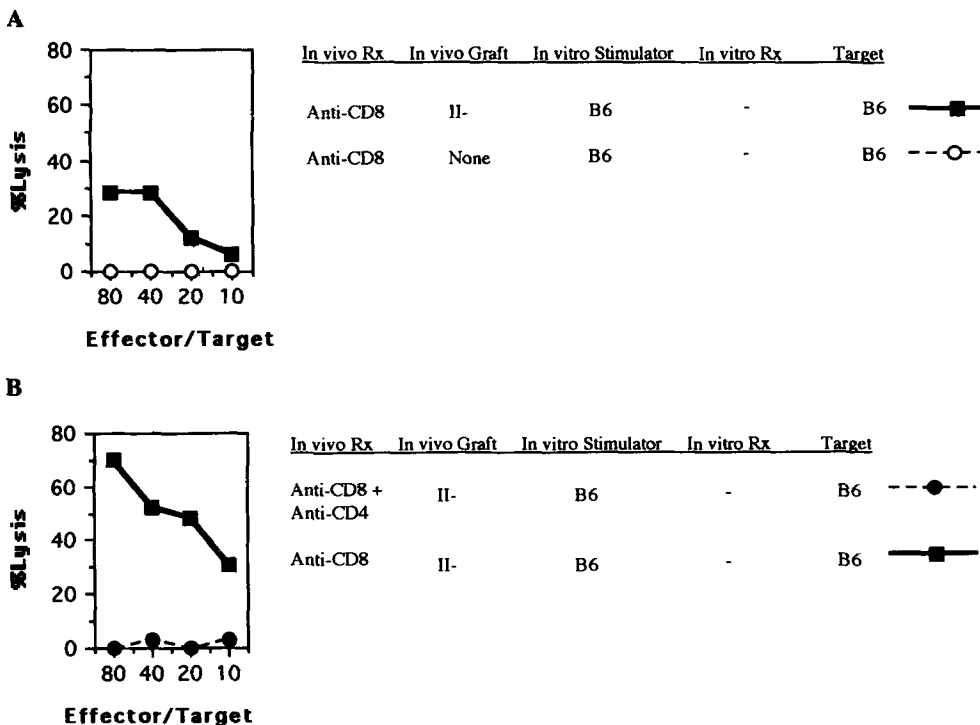
A

In vivo Rx	In vivo Graft	In vitro Stimulator	In vitro Rx	Target	
Anti-CD8	II-	B6	-	B6	■
Anti-CD8	None	B6	-	B6	-O-

B

In vivo Rx	In vivo Graft	In vitro Stimulator	In vitro Rx	Target	
Anti-CD8 + Anti-CD4	II-	B6	-	B6	-●-
Anti-CD8	II-	B6	-	B6	■

Figure 5. Generation of CTLs after anti-CD8 treatment in vivo requires in vivo priming and CD4⁺ T cells. (A) Splenocytes from anti-CD8–treated, ungrafted, thymectomized BALB/c mice (--O--) and from anti-CD8–treated, skin-grafted BALB/c mice (--■--) were cultured with B6 stimulators and tested for lytic activity against B6 Con A blasts. (B) Splenocytes from anti-CD8 + anti-CD4–treated BALB/c mice that were grafted with II⁻ skin (--●--) and from anti-CD8–treated and skin-grafted mice (--■--) were tested for anti-B6 kill.

their grafts for more than 50 d, suggesting that CD4$^+$ cells are required for graft rejection in CD8-depleted mice. In addition, as shown in Fig. 5 B, splenocytes from anti-CD4 plus anti-CD8-treated mice did not show cytotoxic activity against B6 Con A targets. Thus, CTL generation in anti-CD8-treated and skin-grafted mice was dependent upon CD4$^+$ helper cells in vivo.

The CD4$^+$ Helper T Cells in Anti-CD8-treated, Skin-grafted Mice Responded to Peptides of Donor Antigen Presented in Association With Recipient Class II Antigen. Since the skin grafts in these experiments lacked class II antigens, we presumed that the CD4$^+$ helper population responded to donor antigens presented by the class II antigens on recipient APCs. Further in vitro experiments were performed to test this hypothesis.

To examine CD4$^+$ T cell responses, we used anti-CD8-treated BALB/c mice that had been grafted with skin from B6 class II-deficient mice. We reasoned that if CD4$^+$ cells were sensitized indirectly to donor antigens by recipient class II molecules, these mice would respond to B10.D2 stimulators in an MLR. Fig. 6 shows that CD4$^+$ cells from anti-CD8-treated and skin-grafted BALB/c responders responded to donor antigens presented in association with self, H-2^d class II molecules. They did not respond, even after graft rejection, to B6 class II-deficient stimulators. Naive BALB/c responders did not respond to B10.D2 stimulators, as expected in the case of minor antigen disparities. Variations of this experiment have been performed five times. CD4$^+$ cells from mice that had rejected class II-deficient grafts always responded to donor antigens presented by cells expressing recipient MHC antigens, and never responded to cells from the original class II-deficient donor. Thus, these in vitro data suggest that the CD4$^+$ cells in these recipients were sensitized by indirect recognition.
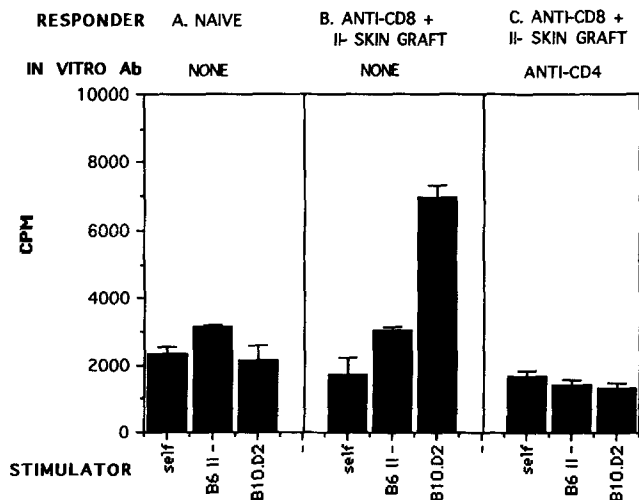

Figure 6. MLR of BALB/c mice after anti-CD8 treatment and skin grafting. (*A*) Naive BALB/c, (*B*) anti-CD8-treated and 8th generation B6 II$^-$ skin-grafted BALB/c, and (*C*) anti-CD8-treated and skin-grafted BALB/c responders plus in vitro anti-CD4 mAb (GK 1.5) tested against self, B6 II$^-$, and B10.D2 irradiated stimulators. Proliferation was measured by [^{3}H]TdR uptake.

Discussion

The primary conclusion from the in vitro evidence in this paper is that indirect recognition alone can sensitize CD4$^+$ helper cells that effectively initiate graft rejection. Whereas this conclusion has long been suspected, until now the powerful direct stimulation provided by donor class II antigens has made it difficult to examine the independent importance of the indirect pathway. The availability of the MHC class II knockout mice has provided a remarkable tool for studying this aspect of the mechanism of graft rejection.

The second conclusion from these data is that indirect recognition sensitizing CD4$^+$ helper cells to determinants expressed on recipient APCs can provide help for CD8$^+$ cytotoxic cells sensitized to determinants expressed only on donor cells. Thus, four different cell types can be involved in the development of mature CTLs: CD4$^+$ cells, recipient APCs, CD8$^+$ cells, and donor APCs. This conclusion is important because most previous evidence has suggested that helper cells can only interact effectively in vivo with precursor effector cells if they are brought physically together in a three-cell cluster by a single APC.

Mitchison and O'Malley (15) first characterized the need for a "three cell-type cluster" to generate in vivo cytolytic responses. The principle has also been suggested as a requirement for other helper T cell interactions with effector populations (16–18). Furthermore, the idea that helper and cytotoxic determinants must be expressed on the same APC to bring cooperating T cells together is consistent with several previous observations regarding in vivo allogeneic responses. For example, Keene and Forman (19) have shown that in vivo generation of anti-Qa1 CTLs requires that the H-Y helper determinant and the Qa-1 CTL determinant be presented on the same immunizing cell, and Rosenberg et al. (20) showed that the helper determinant on bm12 antigens had to be coexpressed with cytotoxic determinants of bm6 on the same graft in order to produce rejection of a bm6 graft by B6 mice. They found similar results with respect to Qa-1 and H-Y antigens (20).

The finding in this paper that four cell types can participate in CTL priming seems to contradict the evidence for a physical linkage of helper and cytotoxic determinants and suggests a model that would seem to be inherently unstable. If CD4$^+$ T cells, interacting with self APCs in one part of the body, can provide help for CD8$^+$ T cells, interacting with donor APCs in another location, then there should be no requirement that the peptides presented by the self APCs need even be derived from the graft. Any immune stimulus should be capable of helping the CTL response, a situation that would seem highly chaotic, not only with respect to graft rejection, but for all quiescent T cell responses.

There are, however, at least two reasonable hypotheses to explain our findings without contradicting previous observations. First, the four-cell phenomenon may only be observable when there are large numbers of antigen disparities during graft rejection. Most previous in vivo studies suggesting a physical linkage have used only a single H-Y (19, 20), Qa (19, 20), mutant class I or II antigen (20) disparity. Perhaps the large number of minor antigen disparities in-
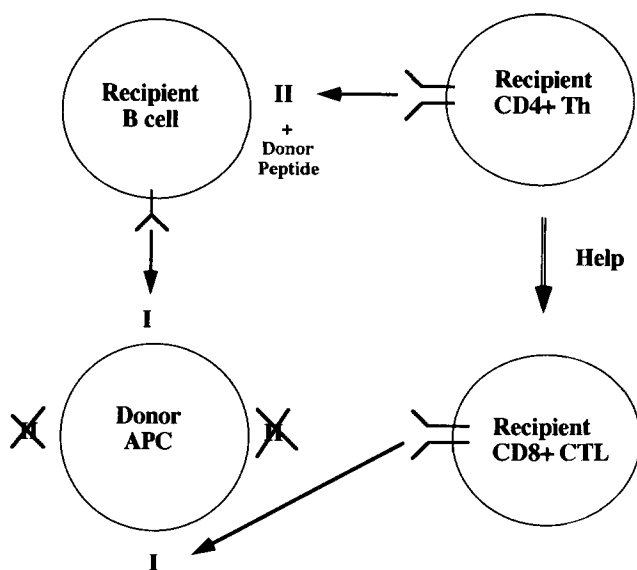
Figure 7. A four-cell cluster model of recipient CD4+ T cell, recipient CD8+ T cell, recipient B cell, and donor APC for CTL sensitization in vivo. A responding B cell binds through its surface Ig receptor to the class I antigen on the donor APC and presents donor antigens to a CD4+ T cell. The CD4+ T cell provides help to a nearby CD8+ T cell that is being primed to the class I antigen on the donor APC. Three sets of cognate interactions maintain the four-cell cluster.

volved in our studies provided such a huge indirect immune stimulus in the draining LNs of a graft bed that CTLs contacting donor APCs in the same location were able to receive sufficient help from nearby CD4+ T cells that were primed to donor antigens processed on recipient MHC molecules. Mitchison and O'Malley (15) have reported that epitope linkage in a three-cell cluster is not required in the setting of high concentrations of stimulating antigens, a finding that is consistent with this explanation. In their adoptive transfer studies, high concentrations (10^7) of a one-to-one mixture containing one cell type expressing helper determinants and another cell type expressing a cytotoxic determinant generated CTLs in vivo.

Alternatively, the principle of physical linkage would be maintained, even if four cells were involved in T cell priming, if the recipient APC must be a B cell. As diagrammed in Fig. 7, a recipient B cell, bound to a donor-stimulating cell by a surface receptor for donor class I antigen, could present donor peptides in association with its own class II antigens to recipient CD4+ cells to form a four cell-type cluster. This

would hold the CD4+ T cell in close proximity to a recipient CD8+ CTL recognizing the donor antigens. This model would thus prevent nonspecific T cell activation. It would also predict that four cells could lead to T cell priming only under circumstances in which a B cell response was possible, which is in keeping with the available data. No B cell response is measurable in the case of H-Y, Qa, or the narrow antigenic disparity generated by the mutant bm6 antigen, but anti-H-2^b class I antibodies are generated in anti-CD8–treated BALB/c mice after rejection of class II–deficient skin grafts (Lee, R., unpublished observations). We are currently attempting to determine whether CTLs can be generated by mice that have no B cells.

Whatever the mechanism by which four cells together lead to CTL activation, the evidence that they can do so has important implications for our understanding of graft rejection. For years there has been ongoing uncertainty about whether T cells primed indirectly by donor antigens presented by recipient APCs could play a role in generating donor-specific CTLs if the donor was MHC disparate. If they cannot, then the indirect pathway for initiating rejection could not play a role in a cytotoxic rejection mechanism whenever the donor was MHC mismatched. Our findings, however, suggest that the indirect pathway is always available for generating donor-reactive CTLs under some circumstances.

Another implication is that APC depletion from a donor graft may not always prevent CTL generation because the indirect pathway is still available to generate help. Several early studies have suggested a role for indirect recognition in the rejection of APC-depleted grafts (21–24), such as La Rosa and Talmage's (21) suggestion that indirect recognition of minor histocompatibility antigens from APC-depleted thyroid grafts might be responsible for graft rejection. In light of our data, it would appear that neither depletion of donor APCs before transplantation, nor their replacement by recipient APCs after transplantation, can permanently remove the stimulus for productive CD4+ helper T cell activation. Donor APC depletion may temporarily diminish indirect presentation if the donor APCs are initially required as the vehicles to transport donor antigens to draining LNs. Alternatively, the effectiveness of donor APC depletion may depend primarily on its effect on CD8+ T cells. If APC depletion induces anergy of CD8+ T cells, then graft rejection may not occur, but if any stimulus for CD8+ activation remains, perhaps by "semi-professional" APCs (25), then CD4+ helper cells, sensitized through the indirect pathway, should be available to help these CD8+ cells cause graft rejection.

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