Cellular Basis of Skin Allograft Rejection across a Class I Major Histocompatibility Barrier in Mice Depleted of CD8+ T Cells In Vivo

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Summary

The present study was undertaken to define the cellular mechanisms involved in the rejection of major histocompatibility complex (MHC) class I disparate skin grafts by mice depleted of CD8+ T cells in vivo. Mice were effectively depleted of CD8+ T cells by adult thymectomy followed by in vivo administration of anti-CD8 monoclonal antibody (mAb) and then engrafted with allogeneic skin. We found that CD8 depleted mice did reject MHC class I disparate skin grafts, but only when the grafts also expressed additional alloantigens. Despite the marked depletion of CD8+ T cells in these mice, we found that their rejection of MHC class I disparate grafts was mediated by CD8+ cytolytic T lymphocyte (CTL) effectors that had escaped depletion. These CD8+ CTL effectors were unique in that: (*a*) their generation was dependent upon the injected anti-CD8 mAb and upon exposure to class I MHC alloantigens expressed on the engrafted skin, and (*b*) their effector function was resistant to blockade by anti-CD8 mAb. We observed that the additional alloantigens coexpressed on MHC class I disparate grafts that triggered graft rejection in CD8-depleted mice could be MHC-linked or not and that they functioned in these rejection responses to activate third party specific CD4+ T helper (Th) cells to provide helper signals for the generation of CD8+ anti-CD8 resistant CTL effector cells. Thus, mice depleted of CD8+ T cells by thymectomy and in vivo administration of anti-CD8 mAb harbor a unique population of anti-CD8 resistant, CD8+ effector cells that mediate anti-MHC class I responses in vivo and in vitro, but require help from third party specific Th cells to do so.

Rejection of skin allografts results from massive tissue injury inflicted by immunocompetent cytolytic T cells in response to allogeneic histocompatibility antigens expressed by the engrafted tissue (1–3). Studies in which isolated populations of CD8+ and CD4+ T cells were adoptively transferred into immunodeficient mice revealed that rejection of MHC class I disparate skin grafts was mediated exclusively by CD8+ T cells in the absence of CD4+ T cells (4–6). The importance of CD8+ T cells in rejection of MHC class I disparate skin grafts was confirmed in another experimental model in which normal mice were depleted of CD8+ T cells by in vivo administration of anti-CD8 mAb, and failed to efficiently reject mutant MHC class I K^{bm} skin grafts (7). This same experimental model was used to further investigate the rejection of MHC class I disparate grafts across both H-2K and H-2D barriers in many different strain combinations. Surprisingly, it was reported that in some strain combinations, CD8-depleted mice could still rapidly reject MHC class I disparate grafts, raising the possibility that cells other than CD8+ T cells might be able to effect rejection of some MHC class I disparate grafts (7). In considering the results of this previous study, we noted that mice depleted of CD8+ T cells by in vivo injection of anti-CD8 mAb efficiently rejected MHC class I disparate skin grafts primarily when the grafts also expressed additional histocompatibility antigens encoded by genes to the right of H-2D. The role that such additional antigens might have played in the rejection responses of CD8-depleted mice was not apparent.

We undertook the present study to identify the cellular mechanisms by which mice depleted of CD8+ T cells could reject MHC class I disparate skin grafts. In the present study, the experimental mice were depleted of CD8+ T cells by adult thymectomy (ATX)[1] followed by in vivo anti-CD8 mAb treatment, and are referred to as CD8− mice. Surprisingly, we found that rejection of MHC class I disparate skin grafts by CD8− mice was mediated by a small, unique subset of CD8+ T cells that escaped elimination. These sur-

[1] *Abbreviations used in this paper:* ATX, adult thymectomy; pCTL, precursor cytolytic T lymphocyte; RC′, rabbit complement.

viving CD8+ T cells were highly unusual in that they gave rise to CTL whose effector function was resistant to blockade by anti-CD8 mAb. The in vivo generation of such CD8+ anti-CD8 resistant CTL required engagement of MHC class I alloantigen expressed by the skin allografts and T cell help from third party CD4+ Th cells reactive against non-MHC encoded histocompatibility alloantigens expressed by the graft. Thus, this study demonstrates that rejection of MHC class I disparate skin grafts by mice depleted of CD8+ T cells is nonetheless mediated by CD8+ effector T cells, albeit a unique subset of helper dependent CD8+ T cells whose effector function is resistant to blockade by anti-CD8 mAb.

Materials and Methods

Mouse Strains. C57BL/6NCr (B6) female mice were purchased from the Frederick Cancer Research Facility (Frederick, MD). B6.C-H-2^{bm1} (bm1) (8) male and female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.K1 (9–11) and B6.Tla^a (10–12) mice were bred in our colony, from original breeding pairs generously provided by Dr. Lorraine Flaherty, New York State Department of Health (Albany, NY). The MHC, Qa-1, and Qa-2 phenotypes of these mice are listed in Table 1. The phenotypes of antigens encoded to the right of Qa-2 are poorly defined and are not listed.

Adult Thymectomy (ATX) and Antibody Depletions. Mice were thymectomized under direct visualization by sternotomy at 8–12-wk-of-age. They were rested for 2 wk and then given three consecutive daily i.p. doses of 2.43 mAb (1 ml/dose) and an additional dose 1 wk later. Depletion of T cell subsets was monitored by immunofluorescence and flow cytometry.

Monoclonal Antibodies Used In Vivo Depletions. Anti-CD8 mAb was used as an ascitic fluid of the hybridoma cell line 2.43 (13), a rat IgG_{2b} antibody. Ascites was diluted 1:10 in PBS, filtered and injected intraperitoneally. Anti-CD4 mAb was used as an ascitic fluid of the hybridoma cell line GK 1.5 (14), a rat IgG_{2b} antibody. GK 1.5 ascites was diluted 1:4 in PBS, filtered and injected intraperitoneally.

Monoclonal Antibodies Used in In Vitro Depletions, Blocking, and Immunofluorescence Studies. Monoclonal anti-CD4 antibody was either a culture supernatant of the hybridoma cell line H129.1a (15) or RL 172/4 (16). Monoclonal anti-Lyt2.2 antibody was a culture supernatant of the cell line 83-12-5 (17) or 2.43 (13). Anti-Fc receptor mAb, 2.4G2 (18) was used to block nonspecific uptake of antibody by FcR^+ cells in immunofluorescence studies. FITC-conjugated MAR 18.5 (mouse anti-rat Ig) was purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA).

Skin Grafting. Recipient mice were engrafted within 3 wk of antibody depletion on the left flank with one or two tailskin grafts from donor animals, according to an adaptation of the method of Billingham and Medawar (19), as previously described (20). Bandages were removed on day 7 and the grafts scored daily until rejection or the endpoint of the experiment (60 d). Grafts were considered rejected when ≥80% of the grafted tissue was destroyed.

Flow Cytometric Analysis. Cells were indirectly stained with anti-CD8 mAb followed by fluoresceinated mouse anti-rat Ig (MAR 18.5) as indicated, before in vitro culturing and samples were analyzed on a modified dual laser (488 nm, 590 nm) FACS II® (Becton Dickinson Immunocytometry Systems). Fluorescence data were collected using three-decade logarithmic amplification on viable cells as determined by forward light scatter intensity and propidium iodide exclusion.

In Vitro Cytolytic T Cell (CTL) Responses. Spleen cells (4–5 × 10^6) from either normal untreated or antibody injected mice were cocultured with 3,300R-irradiated (1 R = 0.258 mC/kg) spleen stimulator cells (4–5 × 10^6) in 2-ml cultures for 5 d as described (21) and assayed for cytolytic activity on ^{51}Cr-labeled lipopolysaccharide induced blast cells as target cells in a 4-h ^{51}Cr-release assay. Percent specific lysis = 100 × ([experimental-spontaneous release]/[maximum-spontaneous release]).

Antibody-plus Complement Treatment of Effector Cells. Depletion of CD4+ cells or CD8+ cells was accomplished by incubating spleen cells at a density of 10^7 cells/ml with anti-CD4 mAb (1:2 dilution of culture supernatant RL172), anti-CD8 mAb (1:2 dilution of culture supernatant 83-12-5), or medium alone, respectively, for 30 min on ice. Cells were then pelleted, resuspended, and incubated with rabbit complement (RC′) (Pel-Freeze, Brown Deer, Wisconsin) diluted 1:10 at a density of 10^7 cells/ml for 30 min at 37° C. Control cultures were treated with RC′ alone. Treated cells were washed three times and reconstituted, without recounting, to the desired concentration based on cell counts for the RC′ alone treated culture.

Antibody Blocking of Effector Cells. Blocking of CTL effectors was accomplished by preincubating effector cells with 25% mAb culture supernatant for 30 min at 37°C. Then, ^{51}Cr-labeled targets were added leaving the antibody in the cultures during the 4-h ^{51}Cr-release assay.

Results

Construction of CD8⁻ Mice. To investigate the rejection of MHC class I disparate skin grafts in mice depleted of CD8+ T cells in vivo, we constructed "CD8⁻" mice according to the following protocol. Female mice were thymectomized at 8–12-wk-of-age to prevent the emergence of new T cells, rested for 2 wk, and then given three consecutive daily i.p. injections of anti-CD8 mAb. An additional dose of anti-CD8 mAb was given 1 wk later. Treated animals were skin grafted within 3 wk of the final antibody treatment.

To assess the efficacy of CD8+ T cell depletion, T cell populations present in treated mice were assessed by immunofluorescence and flow cytometry. As can be seen in Table 2, < 1% of splenocytes were CD8+, indicating that effective depletion of CD8+ T cells was achieved using this protocol.

Table 1. *Phenotype of Mice Used in this Study*

	H-2 alleles					
Strain	K	IA	D	L	Qa-1	Qa-2
B6	b	b	b	b	b	(+)
B6-Tla^a	b	b	b	b	a	(+)
B6.K1	b	b	b	b	b	(−)
bm1	bm1	b	b	b	b	(+)
B10.YBR*	b	b	d	d	a	(+)

* Strain type is inferred from the typing of B10.AQR from which this recombinant strain was derived.

Table 2. *Flow Cytometric Analysis of Spleen Cells from Mice Treated in Vivo with anti-CD8 mAb*

Mice	Treatment	Skin graft	% Cells staining positive for: CD4	CD8	Thy1.2
6	None	none	19 ± 1.1[§]	12.8 ± 0.8	32.6 ± 2.0
4	ATX + anti-CD8 mAb	pre*	14.2 ± 1.0	0.1 ± 0.07	16.3 ± 0.3
10	ATX + anti-CD8 mAb	post‡	15 ± 1.1	0.9 ± 0.1	19.4 ± 1.3

* T cell subsets were assessed 2 wk before skin engraftment.
‡ T cell subsets were assessed 10 wk after skin engraftment (after rejection).
§ Mean ± SEM.

Rejection of H-2D and H-2K Disparate Skin Grafts by CD8⁻ Mice. To test whether CD8⁻ mice responded differently to skin grafts across an H-2K versus an H-2D disparity, individual CD8⁻ B6 mice were simultaneously engrafted with two MHC class I disparate skin grafts: a bm1 skin graft expressing K^{bm1} allodeterminants and a B10.YBR skin graft expressing D^d and L^d allodeterminants. Unlike normal B6 mice which reject bm1 skin grafts, CD8⁻ B6 mice did not reject bm1 skin allografts but did efficiently reject B10.YBR skin grafts (Fig. 1). To explain why CD8⁻ mice rejected B10.YBR grafts, we considered that CD8⁺ T cells might have expanded to significant numbers in vivo following engraftment. Therefore, we analyzed splenocytes from CD8⁻ mice that had rejected B10.YBR skin grafts. As shown in Table 2, spleen cell populations from animals that had rejected B10.YBR skin grafts were still depleted of CD8⁺ T cells. We next considered that CD8⁻ B6 mice rejected B10.YBR skin grafts because B10.YBR skin grafts expressed additional histocompatibility disparities to the right of H-2D that would be recognized as foreign by the B6 host. To investigate this possibility, we used B6.K1 and B6.Tla^a mice, which are H-2^b but differ from B6 mice in expression of antigens to the right of H-2D. So, whereas bm1 skin grafts express only a K^{bm1} disparity to B6 host mice, bm1 skin grafts express both K^{bm1} and additional alloantigenic differences to the right of H-2D to B6.K1 and B6.Tla^a host mice. We therefore engrafted individual B6.K1 and B6.Tla^a mice with both bm1 and B10.YBR skin grafts. As can be seen in Fig. 2, CD8⁻ B6.K1 and B6.Tla^a mice efficiently rejected bm1 skin grafts whereas CD8⁻ B6 mice failed to do so. In contrast, all three strains rejected B10.YBR skin grafts. Thus, these results suggested that rejection by CD8⁻ mice of MHC class I disparate skin grafts requires the expression by the graft of additional histocompatibility disparities and that antigens encoded by genes to the right of H-2D provide these additional disparities.

MHC Class I Specific CTL Effector Cells in CD8⁻ Mice. We next investigated the phenotype of the effector T cells mediating anti-MHC class I responses in CD8⁻ mice. As can be seen in Fig. 3 (*left*), bm1-specific effector cells from untreated B6.K1 animals are CD8⁺, as they are eliminated by treatment with anti-CD8 mAb and C′ (Fig. 3, *lower left*). Surprisingly, CTL mediating anti-bm1 responses in CD8⁻ B6.K1 mice (Fig. 3, *right*) are also CD8⁺, as the cytolytic effector cells are also eliminated by treatment with anti-CD8 mAb and C′ (Fig. 3, *lower right*), but not by anti-CD4 mAb and C′ (data not shown). Importantly, and in marked contrast to the normal animal (Fig. 3, *top left*), bm1 specific CTL in CD8⁻ mice were not blocked by addition of anti-CD8 mAb to the effector culture (Fig. 3, *top right*). Thus, these

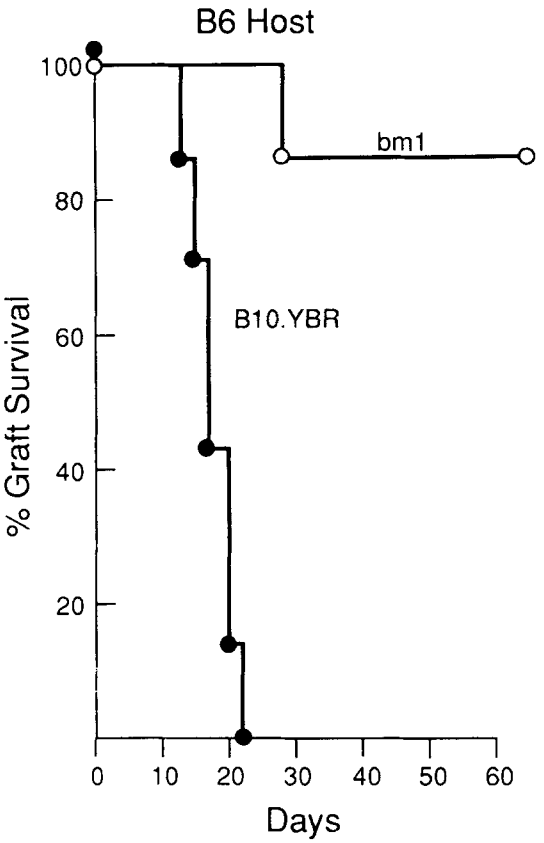


Figure 1. CD8⁻ B6 mice reject B10.YBR skin allografts but not bm1 skin allografts. On day 0, individual CD8⁻ B6 mice were simultaneously engrafted with B10.YBR (●) and bm1 (○) tail skin grafts. The grafts were scored daily. Survival curves reflect rejection responses of seven animals. Median Survival Time (MST) were 17 d for B10.YBR skin grafts and >60 d for bm1 skin grafts.

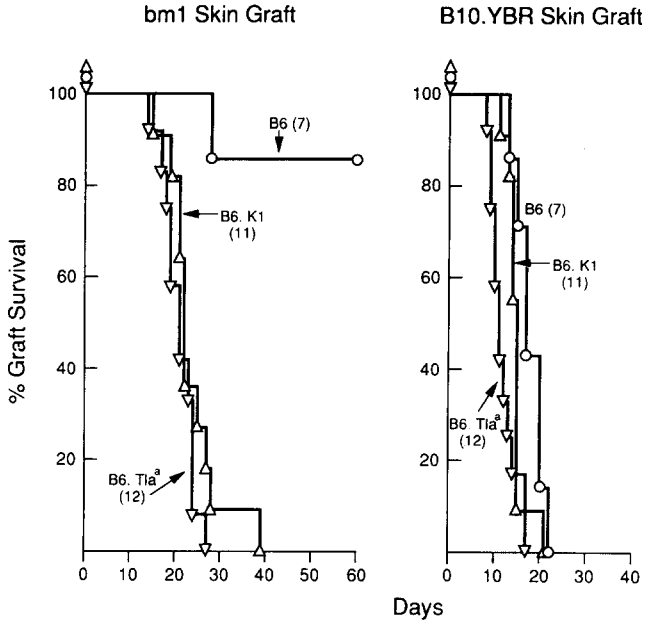


Figure 2. Rejection of bm1 and B10.YBR skin allografts by H-2^b congenic B6, B6.Tla^a, and B6.K1 mice. On day 0, individual B6 (○), B6.Tla^a (▽) and B6.K1 (△) mice were simultaneously engrafted with bm1 and B10.YBR tail skin grafts. The grafts were inspected daily. The number of experimental animals in each responder group is shown in parentheses. MST for bm1 grafts: B6 mice >60 days; B6.Tla^a mice=21 d; B6.Kl mice=22 d. MST for B10.YBR grafts: B6 mice=17 d; B6.Tla^a mice=11 d; B6.K1 mice=15 d.

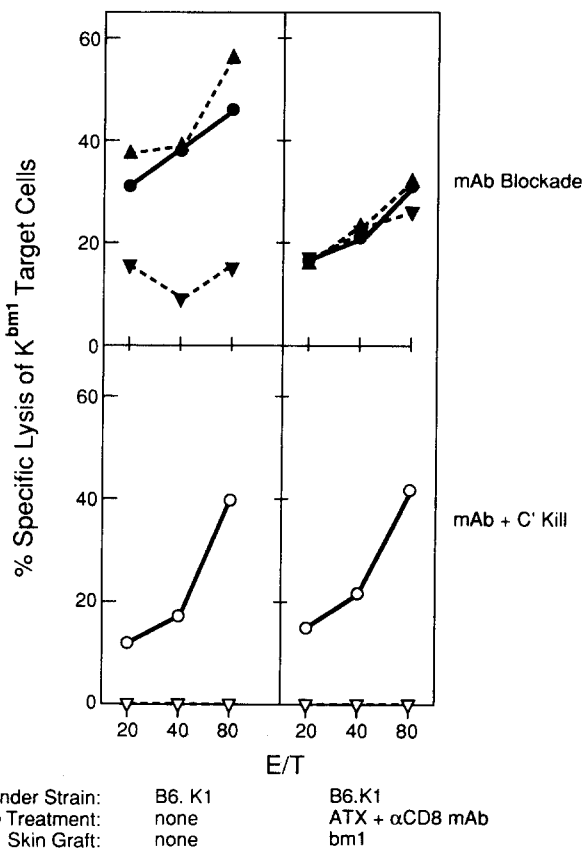


Figure 3. CD8$^-$ B6.K1 mice that rejected bm1 skin grafts generate K^{bm1} specific CTL effector cells that are CD8$^+$ but not inhibited by anti-CD8 mAb. Spleen cells from CD8$^-$ B6.K1 mice that had rejected bm1 skin allografts (*right*) and spleen cells from normal unengrafted B6.K1 mice (*left*) were cultured with irradiated bm1 stimulator cells. After 5 d in culture, effector cells were harvested and assessed for generation of K^{bm1} specific CTL. The phenotype of K^{bm1} specific CTL effector cells was assessed by depletion of T cell subpopulations before their incubation with bm1 target cells by treatment with anti-CD8 mAb + RC' (▽), or with RC' alone (○) (*bottom*). To assess the ability of mAb to inhibit K^{bm1} specific CTL effector function, unfractionated effector cells were incubated with bm1 target cells in the presence of anti-CD4 mAb (▲), anti-CD8 mAb (▼), or no mAb (●) (*top*). Target cells in this and all subsequent experiments are ^{51}Cr-labeled LPS blasts from spleen cell precursors. Lysis of syngeneic B6.K1 target cells was always <2%.

results demonstrate that bm1 specific effector cells in CD8$^-$ B6.K1 mice are CD8$^+$, but anti-CD8 resistant in that their effector function is not blocked by anti-CD8 mAb.

To establish whether CD8$^-$ mice generated H-2D allospecific CTL, as well as H-2K allospecific CTL, we analyzed D^d and K^{bm1} specific CTL from CD8$^-$ B6.TLa^a mice. As shown in Fig. 4, CD8$^-$ mice generated CD8$^+$ effector cells specific for both B10.YBR and bm1 target cells as both effector cell populations were eliminated by treatment of effectors with anti-CD8 mAb and RC' (Fig. 4, *lower right*). However, bm1 and B10.YBR specific CTL effectors were not blocked by addition of anti-CD8 mAb to effector cultures (Fig. 4, *upper right*). Thus, even though CD8$^-$ mice are markedly depleted of CD8$^+$ T cells (Table 2), the effector cells mediating anti-MHC class I responses in these mice are a distinct subset of CD8$^+$ CTL whose precursors are not depleted by in vivo anti-CD8 mAb treatment and whose effector function is not inhibited by anti-CD8 mAb.

Generation of Anti-K^{bm1} CTL in CD8$^-$ Mice Requires Engraftment with Allogeneic bm1 Skin. We next determined if K^{bm1} specific, anti-CD8 resistant precursor CTL (pCTL) had been generated in CD8$^-$ B6.K1 mice before engraftment with skin allografts, or if they only were generated subsequent to in vivo exposure to K^{bm1} alloantigen expressed on bm1 skin grafts. To distinguish between these two possibilities, we assessed the same individual CD8$^-$ B6.K1 mice (#195 and #199) before and after engraftment with allogeneic skin. Thus, individual CD8$^-$ mice underwent hemisplenectomy before skin grafting and again following rejection of bm1 skin allografts. Before skin grafting, splenocytes from CD8$^-$ B6.K1 mice contained very few CD8$^+$ T cells and K^{bm1} CTL were not detected, whereas splenocytes from the normal undepleted B6.K1 mouse generated a strong CTL response to K^{bm1} allodeterminants (Fig. 5). However, following rejection of bm1 skin grafts, splenocytes from the same CD8$^-$ B6.K1 mice did generate anti-K^{bm1} CTL despite the persistent depletion of CD8$^+$ T cells (Fig. 5). Thus, failure to detect anti-CD8 resistant K^{bm1} specific CTL effectors in CD8$^-$ mice before engraftment indicates that their generation is contingent on activation of pCTL in vivo by alloantigen expressed on skin grafts.

Recruitment of Additional Th Cells Triggers Rejection of MHC Class I Disparate Skin Grafts in CD8$^-$ Mice. Because rejec-

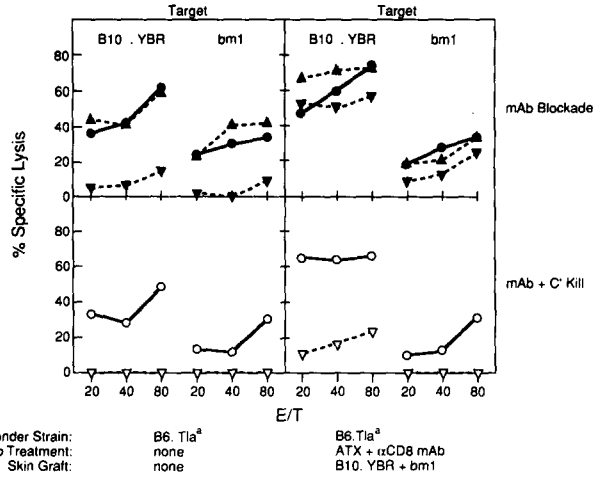


Figure 4. CD8⁻ B6.Tla^a mice generate CD8⁺ anti-CD8 resistant CTL effectors to both H-2K and H-2D alloantigens. Spleen cells from CD8⁻ B6.Tla^a mice that had rejected bm1 and B10.YBR skin grafts (*right*) and from normal unengrafted B6.Tla^a mice (*left*) were cultured with irradiated B10.YBR or bm1 stimulator cells. After 5 d, effector cells were harvested, and tested for their ability to lyse bm1 and B10.YBR target cells. CTL effectors were phenotyped by depletion of effector cells before incubation with bm1 and B10.YBR target cells with anti-CD8 mAb + RC' (▽) or with RC' alone (○) (*bottom*). mAb inhibition of CTL effector function was tested by culture of unfractionated effector cells with bm1 or B10.YBR target cells in the presence of anti-CD8 mAb (▼), anti-CD4 mAb (▲), or no mAb (●) (*top*). Lysis of syngeneic B6.Tla^a target cells was always <2%.

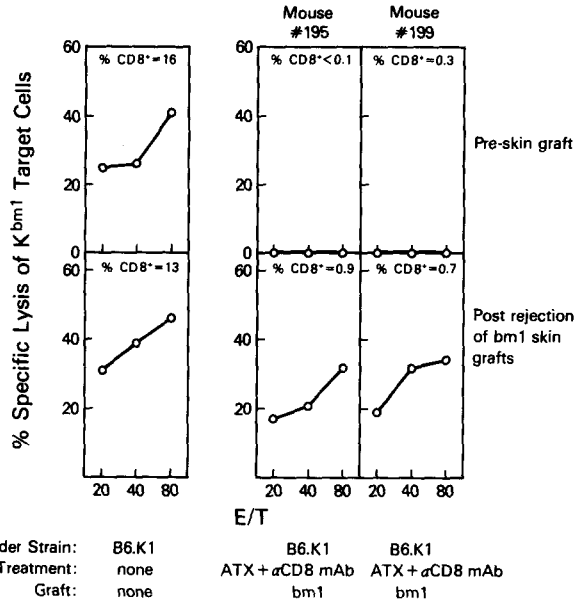


Figure 5. Generation of anti-K^{bm1} specific CTL effector cells in CD8⁻ mice requires in vivo exposure to bm1 skin grafts. Spleen cells from two individual CD8⁻ B6.K1 mice (#195 and #199) were obtained by hemisplenectomy and tested before engraftment (*top right*) and following rejection of bm1 skin grafts (*bottom right*) for the presence of K^{bm1} specific CTL effector cells and for the percentage of CD8⁺ T cells. Responder cells were incubated with irradiated bm1 stimulator cells for 5 d and then tested for lysis of bm1 target cells. Percentage of CD8⁺ T cells was assessed by immunofluorescence and flow cytometry. Spleen cells from normal unengrafted B6.K1 animals served as positive controls (*left*). Lysis of syngeneic B6.K1 target cells was <2%.

tion of MHC class I disparate skin allografts by CD8⁻ mice appeared to be contingent on the expression of additional histocompatibility disparities to the right of H-2D, we wished to determine why expression of such additional antigens was necessary. It was possible that these additional antigens were the true target specificities of effector cells or, alternatively, that they were helper specificities that were necessary for the in vivo activation of distinct populations of Th cells.

Because alloantigens encoded by genes to the right of H-2D are relatively poorly defined, we wished to determine if a well-defined non-MHC encoded alloantigen could substitute for them. For example, H-Y alloantigen is a male specific minor-histocompatibility antigen which is not encoded by H-2 but which does activate CD4⁺ Th cells in B6 female mice (6, 22). Thus, we asked whether CD8⁻ mice could reject an isolated MHC class I disparate graft that did not express additional differences to the right of H-2D, but instead expressed the male antigen H-Y. CD8⁻ female B6 mice were engrafted simultaneously with two bm1 skin grafts: an "inducer" bm1 male graft that expressed both K^{bm1} and H-Y alloantigens, and an "indicator" bm1 female graft which expressed only K^{bm1} alloantigen. As shown in Table 3, CD8⁻ B6 mice engrafted with a bm1 female "inducer" graft did not reject their bm1 female "indicator" grafts (group 1). In contrast, CD8⁻ B6 mice engrafted with a bm1 male "inducer" graft did efficiently reject their bm1 female indicator grafts (group 2), indicating that CD8⁻ B6 mice can reject skin allografts expressing an isolated MHC class I K^{bm1} disparity without any additional differences, so long as the mice were engrafted with an inducer graft capable of additionally activating third party Th cells.

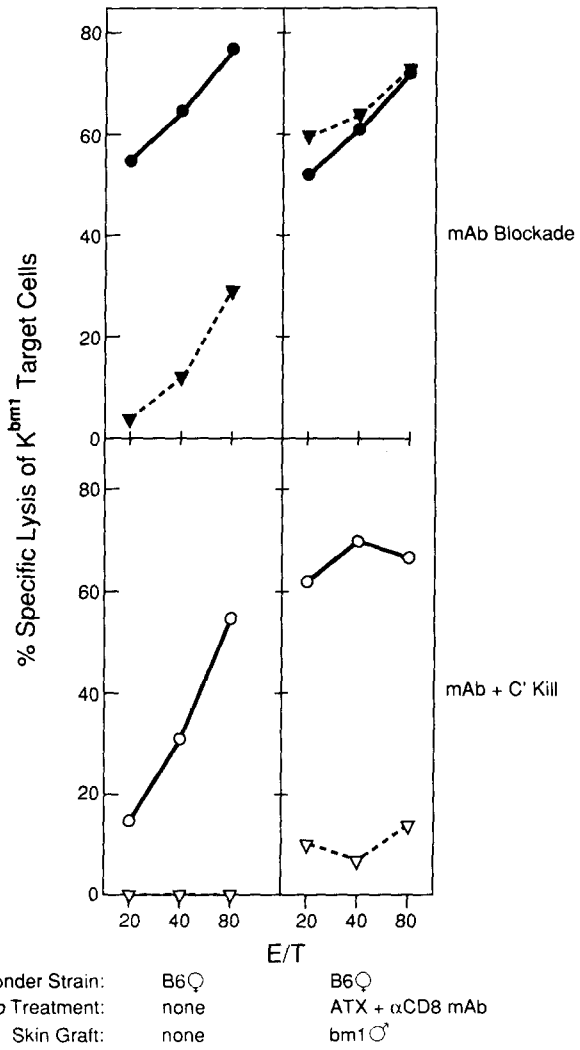
We then characterized the anti-K^{bm1} effector cells generated in CD8⁻ B6 female mice in response to bm1 male skin grafts to see if they resembled the anti-K^{bm1} effector cells generated in CD8⁻ B6.K1 and B6.Tla^a mice (Fig. 3 and 4). As can be seen in Fig. 6, anti-K^{bm1} CTL were generated from the spleens of CD8⁻ B6 female mice engrafted with bm1 male inducer grafts, and these CTL were indistinguishable from those generated in CD8⁻ B6.K1 and B6.T1a^a mice (to which the bm1 skin allograft expressed additional alloantigens encoded by genes to the right of H-2D), in that the anti-bm1 CTL were CD8⁺ but anti-CD8 resistant (compare Figs. 3 and 4 and Fig. 6, *right*). Thus, the generation of anti-bm1 rejection responses mediated by CD8⁺ anti-CD8 resistant CTL in CD8⁻ B6 mice required expression by the bm1 skin graft of additional alloantigens, but the precise identity of the additional alloantigens expressed by the skin allograft was unimportant in that they could be encoded by genes linked to the MHC or not.

To determine if the required expression of H-Y alloantigen on inducer bm1 male grafts triggered rejection of bm1 female indicator grafts by CD8⁻ B6 female mice because generation of anti-H-Y CTL effector cells was required, we assessed the ability of splenocytes from rejector CD8⁻ B6

Table 3. *Rejection of bm1 Skin Grafts by Mice Depleted of T Cell Subsets in Vivo*

Group	ATX B6F host* treatment	Inducer skin graft: Strain	Antigen disparity	# Rejected	MST‡ (d)	Indicator skin graft: Strain	Antigen disparity	# Rejected	MST (d)
1	anti-CD8	bm1 F§	K^{bm1}	0/8	>60	bm1 F	K^{bm1}	0/8	>60
2	anti-CD8	bm1 M‖	K^{bm1} + H-Y	8/8	19	bm1 F	K^{bm1}	8/8	22.5
3	anti-CD8 + anti-CD4	bm1 M	K^{bm1} + H-Y	1/5	>50	bm1 F	K^{bm1}	1/5	>50
4	anti-CD4	bm1 M	K^{bm1} + H-Y	5/5	8	bm1 F	K^{bm1}	5/5	8
5	PBS	bm1 F	K^{bm1}	4/4	22	bm1 F	K^{bm1}	4/4	23

* Thymectomized B6 female mice were treated with four doses of the indicated mAb(s) and engrafted with skin from the indicated donor mice.
‡ MST-Median Survival Time.
§ F = female.
‖ M = male.



mice to generate anti-H-Y as well as anti-K^{bm1} CTL. In contrast to their ability to generate anti-K^{bm1} CTL (Fig. 7, *right*), splenocytes from $CD8^-$ B6 mice were unable to generate anti-H-Y CTL despite the fact that the mice had been engrafted with bm1 male inducer grafts expressing both K^{bm1} and H-Y alloantigens (Fig. 7, *left*). Thus, this result demonstrates that $CD8^-$ mice are limited in the alloantigens against which they are able to generate CTL. More importantly, this result indicates that the requirement for H-Y alloantigen expression by the bm1 inducer graft is not as an additional CTL target specificity.

To document the possibility that expression of the H-Y alloantigen on bm1 male inducer grafts functioned to activate $CD4^+$ Th cells in vivo, we treated mice in vivo with anti-CD4 mAb. It can be seen that in vivo anti-CD4 mAb treatment of mice that were thymectomized but not T cell depleted, did not itself interfere with the in vivo generation of anti-bm1 effector cells mediating bm1-specific rejection responses (Table 3, group 4). However, in vivo anti-CD4 mAb treatment of $CD8^-$ mice did block the generation of bm1-specific rejection responses in $CD8^-$ B6 female mice engrafted with B6 male inducer grafts (Table 3, group 3).

Taken together these data indicate that, in $CD8^-$ B6 fe-

Figure 6. $CD8^-$ B6 female mice generate $CD8^+$ anti-CD8 resistant CTL specific for K^{bm1} following rejection of bm1 male skin grafts. Responder cells from the spleen of an individual $CD8^-$ B6 mouse that had rejected a bm1 skin graft (*right*) or from a normal unengrafted B6 mouse (*left*) were cultured with irradiated bm1 stimulator cells for 5 d. Effector cells were phenotyped by treatment with anti-CD8 mAb + RC' (▽) or RC' alone (○) prior to incubation with bm1 target cells (*bottom*). mAb inhibition of CTL effector function was assessed by incubation of effector cells with bm1 target cells in the presence of anti-CD8 mAb (▼) or absence of anti-CD8 mAb (●) (*top*). Lysis of syngeneic B6 female target cells was <2%.

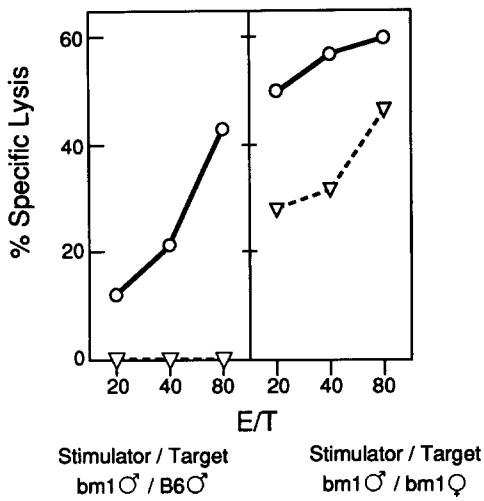


Figure 7. CD8⁻ B6 female mice generate anti-K^{bm1}, but not anti-H-Y CTL following rejection of bm1 male skin grafts. Responder cells from a CD8⁻ B6 mouse that had rejected a bm1 male skin graft or from control ATX mice that had rejected B6 male skin grafts were cultured with bm1 male stimulator cells. After 5 d, effector cells were harvested and tested for their ability to lyse both B6 male targets (*left*) and bm1 female target cells (*right*). (▽) CD8⁻ responder (○) control ATX mouse. Lysis of syngeneic B6 female target cells was <2%.

male mice, the H-Y alloantigen expressed by the bm1 male inducer graft functioned to activate CD4⁺ Th cells which, in turn, were required for the generation of CD8⁺ anti-CD8 resistant CTL specific for K^{bm1} determinants expressed by the bm1 inducer and indicator grafts.

Discussion

The elimination of T cell subsets in vivo by treatment of animals with specific mAbs is a powerful tool for dissecting cellular mechanisms involved in immunologic responses. However, the present study demonstrates that despite marked depletion of CD8⁺ T cells in mice treated in vivo with anti-CD8 mAb, a unique subpopulation of CD8⁺ T cells remains which mediates rejection of MHC class I disparate skin grafts in vivo and generates allospecific CTL responses in vitro.

The unique CD8⁺ T cell population that is not depleted by anti-CD8 mAb in vivo and mediates anti-MHC class I responses is distinguished from CD8⁺ T effector cells normally mediating such responses in that (*a*) their cytolytic effector function is not inhibited by anti-CD8 mAb in vitro and so they are anti-CD8 "resistant" and (*b*) they are exquisitely dependent on an exogenous source of T cell help for activation. The generation of CD8⁺ anti-CD8 resistant K^{bm1} specific CTL effector cells in vivo is contingent on three factors: exposure of pCTL to anti-CD8 mAb, exposure of pCTL to alloantigen, and provision of T cell help from Th cells specific for third party alloantigens expressed by the graft. Thus, in these respects the cells appear identical to in vitro generated CD8⁺ anti-CD8 resistant CTL which have been previously described (23–24).

The mechanism by which anti-CD8 mAb induces anti-CD8 resistant CD8⁺ T cells in vivo is likely to be similar to that by which it induces such cells in vitro. However, anti-CD8 resistant CTL were generated in vitro only when the CD8 determinants on CD8⁺ pCTL were multivalently crosslinked by mAb, a process which induced the down modulation of cell surface CD8 on these cells (23–24). Indeed, in the present study, bivalently bound IgG anti-CD8 mAb would be multivalently crosslinked in vivo by further binding to Fc receptors present in vivo on FcR⁺ cells. Importantly, the reduced surface expression of CD8 induced by multivalent anti-CD8 crosslinking might explain why anti-CD8 resistant CD8⁺ T cells escape depletion in vivo.

A further requirement for generation of anti-CD8 resistant CD8⁺ CTL effector cells is exposure of precursor cells to alloantigen, indicating that generation of these cells is an antigen specific event requiring engagement of TCR. Interestingly, however, anti-CD8 resistant H-Y specific CTL were not generated despite administration of anti-CD8 mAb and engraftment with an H-Y expressing allograft, indicating that the repertoire of anti-CD8 resistant CD8⁺ effector cells is not as extensive as that of normal CD8⁺ T cells. It is conceivable that high levels of antigen-induced TCR crosslinking are required to trigger anti-CD8 resistant effector cells, so that only antigens expressed in high quantities on graft cells are able to successfully trigger such cells.

The final requirement for in vivo generation of anti-CD8 resistant CD8⁺ effector cells is the recruitment and activation of additional CD4⁺ Th cells. In most cases, such CD4⁺ Th cells are not triggered by isolated MHC class I disparities expressed by skin grafts, but rather are triggered by the third party alloantigens expressed by the graft. In the present study, we have demonstrated that such third party alloantigens need not be linked to the MHC, and that any antigen capable of activating CD4⁺ Th cells should be capable of providing the necessary signals for anti-CD8 resistant CD8⁺ effector cells. We think that failure of anti-CD8 mAb treatment to block rejection of some MHC class I disparate skin grafts results from their expression of additional histocompatibility alloantigens which trigger CD4⁺ Th cells necessary for the activation of CD8⁺ anti-CD8 resistant effector cells in these mice. Indeed, production of alloantibody by CD8⁻ mice engrafted with MHC class I disparate skin (7) is presumptive evidence that the grafts did activate CD4⁺ Th cells, as such cells are essential for generation of H-2 specific humoral responses (25). The requirement for additional antigens to activate CD4⁺ Th cells in CD8⁻ mice dramatizes the failure of MHC class I H-2K alloantigen expressed on skin grafts to activate CD4⁺ Th cells in vivo (4). In contrast, MHC class I H-2K alloantigen on spleen APCs do activate CD4⁺ Th cells that recognize peptides of MHC class I alloantigen presented by APC Ia determinants (26). We think that failure of MHC class I H-2K alloantigen expressed on skin grafts to activate such CD4⁺ Th cells (4) results from defects in the ability of Langerhans cells, the predominant APC population in skin, to present class I MHC alloantigens in association with Ia determinants to allospecific CD4⁺ Th cells. Indeed, the markedly diminished expression of H-2K class

I MHC antigen on Langerhans cells (27) is consistent with a cellular defect in the expression and perhaps processing (28–30) of MHC class I H-2K antigens by these cells.

In summary, the present study demonstrates that mice depleted of $CD8^+$ T cells by in vivo administration of anti-CD8 mAb harbor a unique population of precursor cells able to give rise to anti-CD8 resistant, $CD8^+$ effector cells that mediate anti-MHC class I responses in vivo and in vitro but require help from third party Th cells to do so.

We thank D. Cohen, R. Gress, S. McCarthy, J. Sechler, J. Siegel, and S. Sharrow for critical readings of the manuscript. We further thank Atsushi Kosugi for teaching us the thymectomy procedure and Mr. Brian "Ivan" Simms for expert care of experimental animals.

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Received for publication 28 December 1990 and in revised form 21 February 1991.

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