Comparison of $A_\beta^{b-/-}$, H2-DM⁻, and CIITA⁻/⁻ in Second-Set Skin Allograft Rejection[1]

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Background. Responses against donor MHC antigens are the major contributor to allograft rejection. Currently, it is unclear whether both direct and indirect recognition pathways are necessary and/or sufficient for allograft rejection. Previously, we found donor MHC class II and H2-DM to have dramatic effects on cardiac allograft survival.

Methods. Here, we used H2-DM⁻ mice, which express CLIP–MHC class II complexes, and CIITA⁻/⁻ mice, which lack all class II proteins, to examine the role of direct and indirect recognition on skin allograft rejection. Recipients were primed with donor cultured keratinocytes and later tested for accelerated memory response by challenge with full-thickness tail skin grafts.

Results. As previously reported, $A_\beta^{b-/-}$ grafts survived longer than wild-type grafts, while H2-DM⁻ grafts were rejected as rapidly as wild-type grafts. Skin grafts deficient for both β_2m and H2-DM survived longer than grafts lacking only H2-DM, but not as long as $A_\beta^{b-/-}$ grafts. Additionally, CIITA⁻/⁻ grafts survived as long as $A_\beta^{b-/-}$ grafts.

Conclusions. The delayed rejection of $A_\beta^{b-/-}$ compared to H2-DM⁻ suggests that indirect recognition of surface-expressed donor MHC class II is sufficient to mediate rapid skin allograft rejection. The equivalent survival of CIITA⁻/⁻ and $A_\beta^{b-/-}$ grafts suggests that indirect presentation of donor class II molecules (Aα or Eβ) present in $A_\beta^{b-/-}$ but not CIITA⁻/⁻ mice does not contribute to graft rejection. These results reveal a modest role for surface-expressed donor class II in primed keratinocyte rejection, but also reveal a dramatic contrast to the cardiac allograft system and indicate tissue/organ-specific mechanisms of rejection. © 2001 Elsevier Science

Key Words: MHC class II; H2-DM; CIITA; CLIP; skin; graft; cultured keratinocyte; transplantation.

INTRODUCTION

Donor MHC antigens are the major target of anti-graft responses [1, 2]. Historically, T cells were thought to reject histoincompatible grafts by interacting directly with donor MHC antigens expressed by the graft. More recently, it has been appreciated that graft rejection may be mediated by the recognition of donor MHC-derived peptides presented by recipient antigen-presenting cells (APCs), a process known as indirect recognition [3]. Indeed, evidence which supports indirect recognition as an important mechanism of graft rejection is accumulating. For example, full-thickness skin grafts deficient for MHC class I, class II, or both are rejected as rapidly as wild-type grafts [4–6]. Consistent with this finding, a second skin allograft model has shown that MHC class II expression by donor cultured keratinocytes (CKs) will efficiently prime recipients and produce accelerated second-set rejection [7, 8]. However, it is unknown whether this accelerated second-set rejection is the result of activation of a direct or an indirect response.

We address this issue by taking advantage of the H2-DM⁻ mouse [9–11]. Despite their normal level of surface MHC class II expression, H2-DM⁻ mice have a reduced ability to stimulate direct responses against

MHC class II [9–13]. This is an important difference between the H2-DM$^-$ and the A$_\beta^{b-/-}$ models. The A$_\beta^b$ knockout does not thoroughly address the role of donor MHC class II in both direct and indirect recognition since eliminating its expression is likely to eliminate both pathways. By contrast, H2-DM$^-$ grafts can prime recipients for an indirect response against donor MHC class II because H2-DM$^-$ mice express a normal level of surface MHC class II. However, H2-DM$^-$ cells are deficient in the ability to stimulate donor MHC class II-specific allogeneic T cells through the direct recognition pathway. Previously, we used the H2-DM$^-$ mouse to show that rapid rejection of cardiac allografts is strongly influenced by direct recognition of donor MHC class II [13]. Here, we use H2-DM$^-$ mice to examine the role of direct and indirect recognition in primed skin allograft rejection.

Of equal interest is the influence the MHC-bound peptide has on direct recognition of allogeneic MHC. Three main theories have developed to address this question, each differing in the posited role of the MHC-bound peptide. First, the peptide-specific (or molecular mimicry) model of allorecognition suggests that allogeneic MHC complexed to self-antigen (Allo + Y) mimics the structure of self-MHC complexed to foreign antigen (Self + X) [14, 15]. In other words, this model suggests that "Allo + Y = Self + X." However, there is little crystallographic evidence for molecular mimicry in allorecognition [16–18]. In fact, the peptide specificity of alloreactive T cells is questionable considering that many alloreactive T cells respond to a range of different peptides [16, 18–23]. In general, these studies have been consistent with the peptide-dependent model of allorecognition. According to this second model, alloreactive T cells have little or no contact with the MHC-bound peptide, but instead are activated by differences in the polymorphic regions of the MHC molecule. The role of the peptide according to this model is to stabilize the MHC molecule and ensure that it assumes a natural conformation. Finally, the third model suggests that allorecognition can occur through interaction with polymorphic regions of the MHC even in the absence of bound peptide [24]. However, peptide-independent allorecognition has been detected in only some cases of MHC class I-directed allograft rejection [25–27].

H2-DM$^-$ mice are an ideal model to address this question because they are unable to load foreign peptides onto their MHC class II molecules, but still express a normal level of surface MHC class II. As a result, almost all the MHC class II molecules expressed by H2-DM$^-$ are bound to the invariant chain (Ii)-derived class II-associated invariant chain peptide (CLIP) [9–11]. However, some non-CLIP peptides are bound to H2-DM$^-$ MHC [28]. Because the H2-DM$^-$ mouse expresses a nearly uniform MHC class II-

peptide epitope, use of these mice in transplant models will help determine the importance of the MHC-bound peptide in allorecognition.

Finally, previous studies on the effect of MHC class II on allograft rejection have frequently relied upon the A$_\beta^{-/-}$ mouse. Some of these studies have suggested that indirect recognition of donor antigens is sufficient to mediate rapid skin allograft rejection. However, it is not known whether the source of these antigens is derived from donor MHC class I expression, minor antigens, or the expression of the remaining MHC class II genes. Although A$_\beta^{b-/-}$ mice lack surface MHC class II complexes, they still express the Aα and Eβ MHC class II proteins. These class II proteins are a potential source of donor MHC class II-derived peptides and could be presented by the indirect pathway. In order to determine the effect of inhibiting all MHC class II gene expression on graft rejection, we have used mice lacking expression of the master regulator of MHC class II gene expression, the class II transactivator (CIITA), as skin graft donors. CIITA is essential for transcription of all MHC class II genes and is required for the efficient expression of the Ii and DM genes [29, 30]. Mice lacking CIITA do not have detectable MHC class II protein expression and have reduced levels of Ii and DM [31–33]. Here, we use CIITA$^{-/-}$ mice to determine if inhibiting all MHC class II gene expression can alter the fate of a primed skin allograft.

MATERIALS AND METHODS

Animals. (BALB/c × DBA/2)F1 (a.k.a. CBYD2F1/J, H2^d, hereafter referred to as CBY), CBA (H2^k), C57BL/6 (B6, H2^b), and β_2-microglobulin$^{-/-}$ (β_2m$^{-/-}$, H2^b) mice on the B6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). H2-DM$\alpha^{-/-}$ mice and A$_\beta^{b-/-}$ mice were backcrossed onto the B6 (H2^b) background for nine and six generations, respectively. Mice lacking expression of both H2-DMα and β_2m (H2-DM$^-$ × β_2m$^{-/-}$) were described previously [13]. CIITA$^{-/-}$ mice were generated at the University of North Carolina at Chapel Hill, as described previously [33]. CIITA$^{-/-}$ mice were backcrossed to the B6 background for six generations. CIITA$^{-/-}$, H2-DM$\alpha^{-/-}$, A$_\beta^{b-/-}$, and H2-DM$^-$ × β_2m$^{-/-}$ mice were bred and maintained at the University of North Carolina under an Institutional Animal Care and Use Committee approved protocol.

Keratinocyte culture and flank grafting. Recipient mice were primed with cultured donor keratinocytes 3–5 weeks prior to challenge with a full-thickness tail skin graft. Keratinocytes were prepared and grafted essentially as described previously [7]. Briefly, full-thickness tail skin from euthanized donor animals was incubated in 1× dispase (Boehringer Mannheim, Indianapolis, IN) for 1 h at 37°C. The epidermal layer was then separated from the dermis and disrupted, and $4 × 10^6$ epidermal cells were plated on 100-mm^2 tissue culture plates (Costar–Corning) along with $2 × 10^6$ growth-inhibited 3T3's (4 μg/ml mitomycin-C). Cell culture medium was composed of 50% DMEM and 50% HAMS F12 and supplemented with 5% FCS, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, 0.01 μg/ml cholera enterotoxin, 10 ng/ml epidermal growth factor, and 5 μg/ml amphotericin. When the cultured keratinocytes reached confluence, they were detached from the plate as an intact sheet with dispase (Dispase, 1.2 U/ml; Boehringer Mannheim) and adhered to Vaseline gauze. The gauze was cut into 1- to
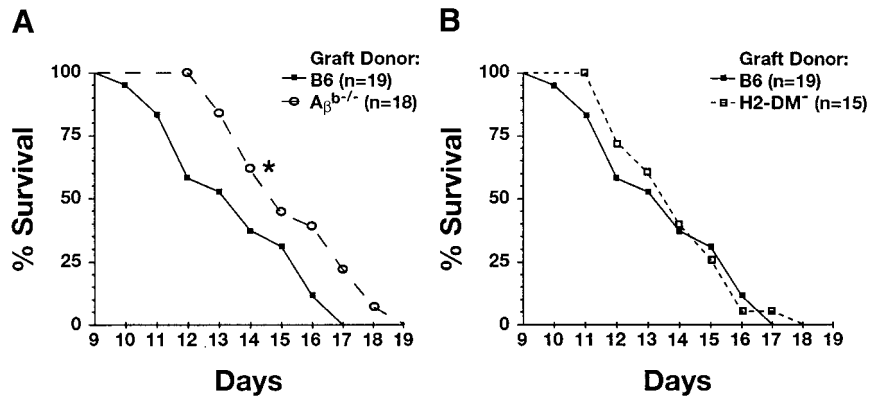
FIG. 1. Second-set survival of full-thickness skin allografts from H2-DM$^-$ and A$_\beta^{b-/-}$ donors on allogeneic recipients. (A) CBA recipients reject wild-type control B6 skin grafts rapidly. A$_\beta^{b-/-}$ allografts, by contrast, have a delayed rejection rate ($P < 0.05$). (B) Interestingly, H2-DM$^-$ allografts are rejected as rapidly as wild-type B6 grafts. Likewise, CBY recipients rapidly reject H2-DM$^-$ full-thickness allografts (data not shown). Statistical significance (compared to B6) is indicated by an asterisk (*) and was determined by Mann–Whitney U test and one-way t test.

2-cm^2 sections and placed keratinocyte-side down on a prepared graft bed on the recipient. The wound was covered with Vigilon (C. R. Bond, Inc., Berkeley Heights, NJ) and a bandage secured with skin staples. Bandages were removed 7 days after grafting. Keratinocyte grafts were not monitored for rejection.

Full-thickness tail skin grafting. Three-to-five weeks after priming, recipient mice were challenged with a full-thickness tail skin graft. Tail skin grafts were examined daily and scored on a scale from 1 to 4 for redness, dryness, scaliness, and the presence and quality of hair. Grafts were considered rejected when any one of the above characteristics received a score of 4. Statistical significance was determined using the Mann–Whitney U test and one-way t test, with statistical significance defined as $P < 0.05$.

Cytotoxic T lymphocyte assay. Cell-mediated cytotoxicity was assessed by the DNA fragmentation assay (JAM test) [34]. Spleen cells were recovered from skin graft recipients and stimulated *in vitro* for 6 days by coculture with irradiated (2500 rads) allogeneic spleen cells, from the same mouse strain as the donor CK and skin grafts. B6, CBA, and CBY spleen cells were stimulated with concanavalin A (Con A) for use as targets. Con A-stimulated blasts were labeled by incubating with [^{3}H]thymidine (5 μCi/ml) for 6–12 h. Washed effectors were incubated with labeled targets (10,000 per well) at the indicated effector/target ratios for 3–4 h before being harvested and counted. Percentage cytotoxicity was determined according to the formula [(S − E)/S] × 100, where E is the average experimental release of triplicate samples and S is the average spontaneous release of numerous samples.

RESULTS

Primed Recipients Reject H2-DM$^-$ Skin Grafts Rapidly

Previous reports have shown that priming with A$_\beta^{b-/-}$ as opposed to wild-type keratinocytes caused a modest delay in the rejection of the corresponding skin allograft. Unlike previous reports examining the role of donor MHC class II on skin graft rejection, which used MHC class II-deficient mice, the H2-DM$^-$ mouse expresses a normal level of MHC class II [9–11]. Despite abundant MHC class II expression, APCs from H2-DM$^-$ mice are unable to stimulate allogeneic T cells *in vitro* [9, 10, 13]. In fact, H2-DM$^-$ cells are as deficient at stimulating allogeneic T cells as are MHC class II null cells (A$_\beta^{b-/-}$). Thus, H2-DM$^-$ mice provide a unique system for examining the role of MHC class II in the absence of MHC class II antigen-presenting function mediated by donor tissue. Previously, we used the H2-DM$^-$ mouse model to examine the role of direct recognition in the rejection of a vascularized organ graft (heart) that was known to be influenced by MHC class II expression and found that eliminating H2-DM is as effective as eliminating A$_\beta$ in greatly prolonging allograft acceptance [13]. These experiments indicate a role for direct antigen presentation by donor MHC class II. Here, we extend upon these findings by examining the role of direct recognition in the rejection of skin grafts. Generally, wild-type and MHC class II null full-thickness primary skin grafts are rejected identically [4, 5]. However, by adopting a protocol that measures the second-set rejection of full-thickness tail skin grafts by CK-primed recipients, we were able to detect a modest but reproducible and statistically significant difference in the second-set survival of wild-type and MHC class II null grafts (Fig. 1) [7].

CBA (H2^k) recipients primed with wild-type B6 CKs reject B6 full-thickness tail skin grafts rapidly (Fig. 1A). Conversely, CBA recipients primed with A$_\beta^{b-/-}$ CKs reject A$_\beta^{b-/-}$ full-thickness skin grafts more slowly. Interestingly, CBA recipients primed with H2-DM$^-$ CKs rejected H2-DM$^-$ full-thickness grafts as rapidly as wild-type B6 grafts (Fig. 1B). While this model generates only a slight enhancement in graft survival, the result is reproducible and statistically significant with
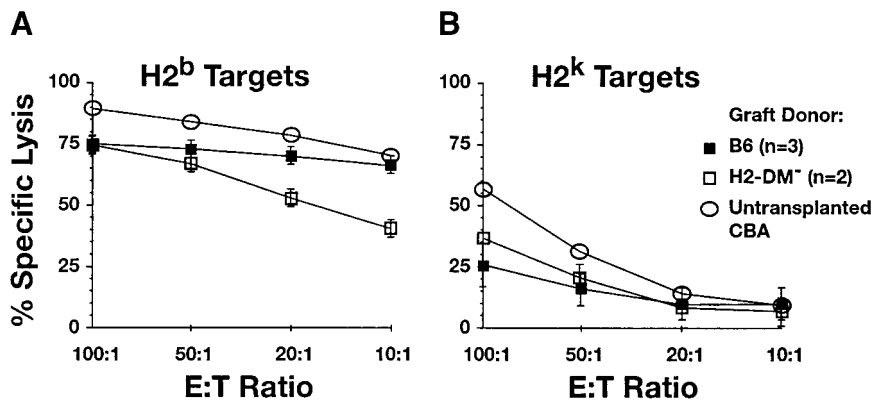
FIG. 2. Recipients of H2-DM$^-$ or wild-type skin grafts generate donor-specific CTL activity equally well. Splenocytes from recipients of H2-DM$^-$ or B6 grafts were harvested 14–21 days following transplant. Recipient splenocytes were stimulated for 6 days with irradiated spleen cells from the same donor strain used for the original graft. The restimulated recipient cells were harvested and assayed for the ability to lyse labeled targets. (A) CBA recipients of B6 (filled squares) or H2-DM$^-$ (open squares) skin grafts lyse H2^b targets equally well, but (B) react poorly with control H2^k targets. Similar results were obtained from CBY recipients (data not shown).

respect to wild-type and $A_\beta^{b-/-}$ donor grafts. Similar results were observed on a second inbred mouse strain, CBY (data not shown). These results are consistent with the interpretation that indirect recognition of donor MHC class II is responsible for the accelerated rejection of H2-DM$^-$ compared to $A_\beta^{b-/-}$ skin grafts.

H2-DM$^-$ and B6 Recipients Generate Donor-Specific Cytotoxic T Cell (CTL) Activity

We examined the level of CTL activity in our recipients in order to determine whether recipients of H2-DM$^-$ grafts were able to generate a normal level of CTL. CBA recipients of either B6 or H2-DM$^-$ donor grafts generated similar levels of CTL activity (Fig. 2). Similarly, CBY recipients were equally capable of generating CTL against B6 and H2-DM$^-$ skin grafts (data not shown). These results are consistent with the identical rates of rejection of B6 and H2-DM$^-$ grafts. They

also identify donor MHC class I as a potential target of the antigraft response. However, it is unlikely that donor class I-specific CTL are responsible for the accelerated rejection of H2-DM$^-$ compared to $A_\beta^{b-/-}$ skin grafts since recipients of $A_\beta^{b-/-}$ grafts also have a strong CTL response (Fig. 5).

H2-DM$^-$ × $\beta_2 m^{-/-}$ Mice Show the Importance of Indirect Recognition on Skin Allograft Rejection

To further explore the role of direct recognition of donor MHC class II on skin allograft rejection, we used mice deficient for both H2-DM and β_2m (H2-DM$^-$ × $\beta_2 m^{-/-}$) as donors. These donors allow us to reduce or eliminate anti-graft responses directed against donor MHC class I and focus on those responses directed against donor MHC class II. The survival of $\beta_2 m^{-/-}$ grafts is slightly longer than that of B6 grafts, but not as long as that of $A_\beta^{b-/-}$ grafts. There is no difference in
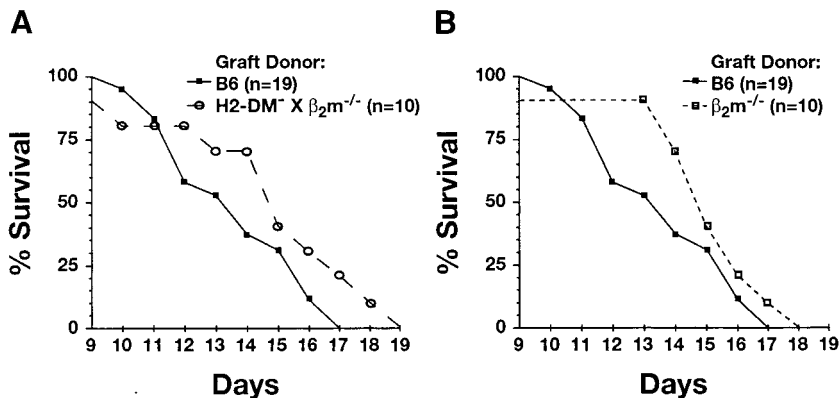

FIG. 3. Grafts deficient for both H2-DM and β_2m have a prolonged survival compared to wild-type grafts. Full-thickness tail skin allografts from (A) H2-DM$^-$ × $\beta_2 m^{-/-}$ donors have a modestly enhanced survival compared to control B6 and survive as along as (B) $\beta_2 m^{-/-}$ allografts on primed CBA recipients. Similar results were obtained with primed CBY recipients (data not shown).
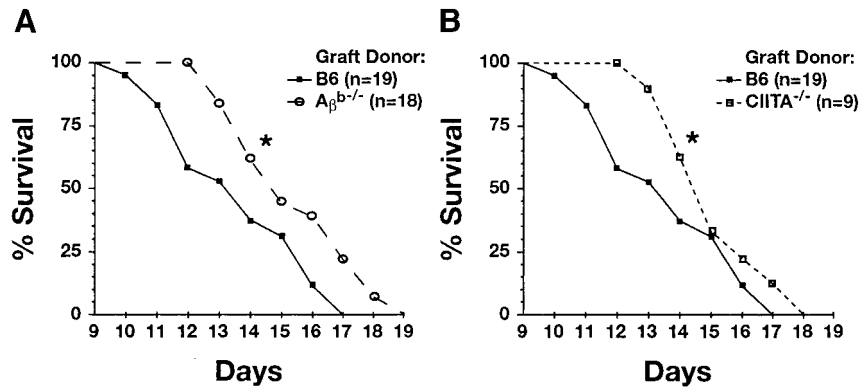
FIG. 4. Eliminating the expression of the $A\alpha$ and $E\beta$ MHC class II proteins does not significantly enhance CIITA$^{-/-}$ graft survival beyond that observed for $A_\beta^{b-/-}$ grafts. When grafted onto primed CBA recipients, (A) $A_\beta^{b-/-}$ allografts survive as long as (B) CIITA$^{-/-}$ allografts. Likewise, CIITA$^{-/-}$ allografts survive as long as $A_\beta^{b-/-}$ allografts on primed CBY recipients (data not shown). Statistical significance (compared to B6) is indicated by an asterisk (*) and was determined by Mann–Whitney U test and one-way t test.

the survival of $\beta_2m^{-/-}$ and H2-DM$^- \times \beta_2m^{-/-}$ grafts by CBA recipients (Figs. 3A and 3B), indicating that even in the absence of donor MHC class I, the elimination of H2-DM has no effect on skin graft survival. This is in contrast to the cardiac allograft model in which the effect of H2-DM is significant. Since the H2-DM$^{-/-}$ cells themselves cannot present peptides efficiently, these findings suggest that indirect recognition of donor MHC class II is a potent mediator of primed skin allograft rejection.

Elimination of CIITA Expression Enhances Skin Allograft Survival Similar to That Observed with the Traditional A_β^b Knockout Mouse

Until recently, the mouse model used to examine the effect of the absence of donor MHC class II on allograft rejection has been the A_β^b knockout mouse. However, this knockout mouse still expresses two ($A\alpha$ and $E\beta$) of the four MHC class II proteins. Peptides derived from the two expressed chains may find their way into the indirect pathway and contribute to allograft rejection. Therefore, the $A_\beta^{b-/-}$ mouse may represent an incomplete MHC class II knockout when used as allograft donor. We address this issue by using the CIITA knockout mouse. CIITA expression is essential for the transcription of all of the MHC class II genes, and CIITA$^{-/-}$ mice have no detectable MHC class II protein expression [31, 33]. In addition, CIITA$^{-/-}$ mice have substantially reduced expression of Ii and DM, which may further enhance allograft survival. CIITA$^{-/-}$ mice may, therefore, represent a more complete MHC class II knockout.

On primed CBA recipients, CIITA$^{-/-}$ skin allografts have an enhanced survival compared to wild-type grafts, but did not survive longer than $A_\beta^{b-/-}$ grafts (Figs. 4A and 4B). Similarly, CIITA$^{-/-}$ full-thickness skin allografts survive as long as $A_\beta^{b-/-}$ full-thickness

skin allografts on CBY recipients (data not shown). This result demonstrates that eliminating CIITA expression is an effective means of removing donor MHC class II expression and enhancing graft survival. However, in this transplant model, the inhibition of all MHC class II gene expression as well as a reduction in Ii and DM expression does not significantly enhance skin allograft survival beyond that observed with the A_β^b knockout. Further, these findings suggest that efficient activation of the indirect pathway by donor MHC class II requires donor class II expression at the cell surface or, alternatively, that donor A_β^b-derived peptides are the predominant donor class II antigens presented by the indirect pathway.

CIITA$^{-/-}$ and $A_\beta^{b-/-}$ Grafts Induce Similar Levels of CTL Activity

As observed with H2-DM$^-$ graft recipients, CBA recipients of CIITA$^{-/-}$ or $A_\beta^{b-/-}$ skin grafts generated a similar level of CTL activity. CTLs from CBA recipients reacted strongly against allogeneic H2^b targets, and poorly against syngeneic H2^k targets, as expected (Figs. 5A and 5B). Similarly, CBY recipients of CIITA$^{-/-}$ or $A_\beta^{b-/-}$ skin grafts displayed equivalent levels of CTL activity (data not shown). These results further suggest that CTLs specific for donor tissues may be contributing to the rejection of these skin allografts.

DISCUSSION

We have used a model of full-thickness skin graft rejection by CK-primed recipients to further define the role of direct and indirect recognition in skin allograft rejection and to determine the contribution of the non-surface-expressed $A\alpha$ and $E\beta$ MHC class II proteins in the rejection of A_β^b skin allografts. The failure of H2-
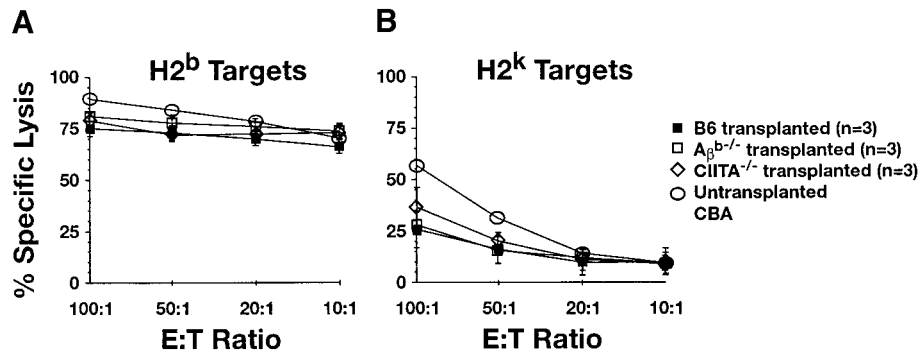
FIG. 5. Recipients of CIITA$^{-/-}$ and A$_\beta^{b-/-}$ skin grafts generate an equal level of CTL activity. Spleen cells were harvested from recipients of CIITA$^{-/-}$ or A$_\beta^{b-/-}$ full-thickness tail skin grafts and stimulated *in vitro* for 6 days. The stimulated recipient spleen cells were assessed for the ability to lyse donor targets. (A) CBA recipients of CIITA$^{-/-}$ or A$_\beta^{b-/-}$ skin grafts develop an identical level of H2^b donor-specific CTL activity, but (B) respond less well to H2^k control targets. Similar results were obtained with CBY recipients (data not shown).

DM$^-$ grafts to have any survival advantage at all suggests that (1) donor MHC class II antigen is presented by host cells through the indirect pathway and (2) MHC class II-bound peptide is not important for indirect recognition. Alternatively, H2-DM$^-$ skin grafts could be rejected by direct recognition of the CLIP–MHC class II complexes expressed by these mice. However, this seems unlikely since it has been difficult to detect or induce CLIP–MHC class II-specific responses in numerous systems [9–13]. Finally, our comparison of the A$_\beta^b$ and CIITA knockouts demonstrates that surface expression of MHC class II proteins is necessary to effectively stimulate an indirect response against donor MHC class II-derived peptides.

These results demonstrate that the elimination of MHC class I or MHC class II results in a small enhancement of skin allograft survival (data summarized

FIG. 6. Primed CBA recipients reject full-thickness skin allografts from H2-DM$^-$ mice almost as rapidly as wild-type control grafts. Graft survival was monitored daily. Each point represents an individual graft recipient, and the line in each group represents the mean survival. Statistical significance (compared to B6) is indicated by an asterisk (*) and was determined by Mann–Whitney U test and one-way t test.

in Fig. 6). Interestingly, CBA recipients rejected H2-DM$^-$ skin grafts as rapidly as wild-type grafts. The difference between the rejection of H2-DM$^-$ grafts and A$_\beta^{b-/-}$ or CIITA$^{-/-}$ grafts most likely represents the contribution of indirect recognition of class II MHC expressed by the former but not the latter two strains, although direct recognition cannot be completely ruled out. This result also suggests that the MHC class II-bound peptide is not important in the recognition and rejection of MHC disparate skin allografts through the indirect pathway. Rather, the more rapid rejection of H2-DM$^-$ grafts compared to MHC class II null grafts indicates that donor MHC class II-derived peptides can be presented by host APCs and contribute to rapid graft rejection.

Ultimately, indirect recognition of donor MHC and/or minor antigens is sufficient to mediate the rapid rejection of allogeneic skin grafts. Skin grafts are quite susceptible to rejection caused by minor histocompatibility antigen differences [3], as evidenced by the rapid rejection of MHC null skin grafts in numerous models [4–6]. This is in contrast with numerous other organ graft models (e.g., heart and kidney), which frequently demonstrate enhanced or indefinite survival of MHC null grafts [35–38]. This indicates that the mechanism of graft rejection is likely to be tissue/organ specific. The rapid rejection of skin grafts due to minor antigen differences illustrates the importance of indirect recognition in graft rejection, in general, and skin graft rejection in particular. However, these findings do not directly demonstrate whether allogeneic MHC participates in the rejection of skin grafts through a direct and/or indirect pathway. Our results can be reasonably interpreted to suggest that indirect recognition of donor MHC class II plays a clear role in the rejection of skin allografts by primed recipients. However, responses directed against MHC class I and minor histocompatibility antigens and direct responses against
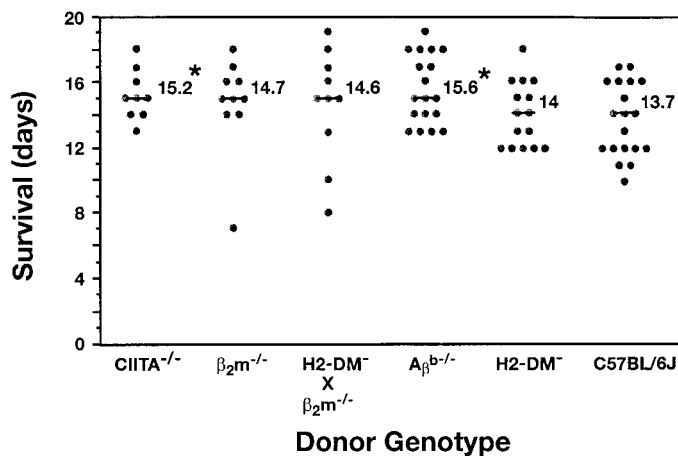
MHC class II may also contribute to rapid rejection of skin grafts.

The rapid rejection of H2-DM$^-$ grafts suggests an important role for indirect presentation of surface-expressed donor MHC class II proteins in skin allograft rejection. However, we also wanted to determine whether the presence of the non-surface-expressed Aα and Eβ MHC class II proteins contributed to the rejection A$_\beta^{b-/-}$ grafts through the indirect pathway. Because CIITA$^{-/-}$ mice lack expression of all MHC class II proteins they are ideally suited to address this question. The equivalent survival of A$_\beta^{b-/-}$ and CIITA$^{-/-}$ grafts demonstrates that indirect presentation is most efficient when the source of donor MHC class II-derived peptides is expressed on the cell surface.

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