Brief Definitive Report

$CD4^+$ but not $CD8^+$ Cells Are Essential for Allorejection

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Summary

The generation of knockout mice with targeted gene disruption has provided a valuable tool for studying the immune response. Here we describe the use of CD4 and CD8 knockout mice to examine the role of $CD4^+$ and $CD8^+$ cells in initiating allotransplantation rejection. Pretreatment with a brief course of depletive anti-CD4 monoclonal antibody therapy allowed permanent survival of heart, but not skin, allografts transplanted across a major histocompatibility barrier. However, skin as well as heart grafts were permanently accepted in the CD4 knockout mice. Transfer of $CD4^+$ cells into CD4 knockout recipient mice 1 d before skin engraftment reconstituted rejection, demonstrating that $CD4^+$ cells are necessary for initiating rejection of allogeneic transplants. Major histocompatibility complex disparate heart and skin allografts transplanted into CD8 knockout recipients were rejected within 10 d. This study demonstrates that $CD4^+$ but not $CD8^+$ T cells are absolutely required to initiate allograft rejection.

The relative importance of the $CD4^+$ and $CD8^+$ T cell subpopulations in mediating transplant rejection remains controversial. In certain settings, $CD8^+$ cells alone seem to be able to initiate allorejection (1). However, $CD4^+$ T cells have been shown to play a central role in transplantation rejection (2–6). Whether $CD4^+$ T cells are absolutely required to initiate allograft rejection has been the subject of a dispute. Naive $CD4^+$ and/or $CD8^+$ subpopulations have been adoptively transferred into immunoincompetent nude mice to assess their ability to reject skin allografts (7). This study indicated that skin allograft rejection was mediated by collaborations in vivo between T inducer and effector cells, and that two interacting T cell subsets can express different phenotypes as well as different antigen specificities. These experiments established the criterion that rejection requires two T cell subpopulations: one providing help, the other cytotoxic effector function. In certain mouse strains (e.g., B6 and B10), MHC class I–reactive $CD8^+$ cells can be activated in vitro independent of MHC class II–reactive $CD4^+$ cells (7–9). $CD8^+$ cells have also been shown to be the only subset effective in restoring rejection of MHC class I incompatible grafts (8, 10), and skin grafts from strains with isolated MHC class I mutations (7, 9). However, these investigators eliminated T cell subsets by treatment with specific mAbs in vivo to deplete either $CD4^+$ or $CD8^+$ T cells, and it was possible that reconstituted nude mice contained T cells derived either from the nude host animal or, more likely, from contaminating T cells in the reconstituting T cell population. It has recently been shown that, despite marked depletion of $CD8^+$ T cells after treatment with anti-CD8 mAbs in vivo, a unique subpopulation of $CD8^+$ cells remained which rejected MHC class I disparate skin grafts and generated allospecific CTL responses (11). Contamination of "purified" T cell subpopulations has also been shown to occur in an adoptive transfer study using mAbs to negatively select purified T cell subpopulations to determine the relative contributions of $CD4^+$ and $CD8^+$ cells from diabetic mice into NOD-scid mice, where purified donor $CD4^+$ populations revealed $<2.5\%$ contaminating $CD8^+$ T cells (12).

Targeting the CD4 or CD8 molecule with mAb to eliminate or inactivate $CD4^+$ or $CD8^+$ T cells has been a promising strategy for the induction of transplantation tolerance. Depleting regimens of anti-CD4 mAbs have been shown to induce long-term survival (tolerance) of pancreatic islet (13) and vascularized heart allografts (14–17), but only delay skin graft (18, 19) survival in rodents. A variety of mechanisms for anti-CD4–induced tolerance have been suggested (14, 20–23).

Anti-CD8 mAb therapy, on the other hand, has had variable results. Although nondepleting anti-CD8 therapy has been shown to induce permanent survival of vascularized heart allografts in mice (16, 24), mice treated with depleting anti-CD8 rejected their allografts (16). Anti-CD8 therapy also did not prolong heart or islet allograft survival in rats (25, 26), nor did it prolong skin graft survival in mice (19, 27). However, anti-CD8 combined with anti-CD4 treatment has been shown to prolong islet (28), bone marrow (19, 27), skin (27), and vascularized heart (16) graft survival.

Although using anti-CD4 or anti-CD8 mAbs is one strategy for studying the induction of tolerance, the interaction between the mAb and the target molecule could induce multiple immunological phenomena. Not all $CD4^+$

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or $CD8^+$ cells are depleted in studies using depleting anti-CD4 or anti-CD8 therapy. Thus, the use of depleting anti-CD4 or anti-CD8 mAbs does not exclude the possibility that signals generated as a result of the interaction between the antibody and the target molecule on residual $CD4^+$ or $CD8^+$ cells are involved in unresponsiveness (29). Additionally, nondepleting anti-CD4 or anti-CD8 mAbs may potentially affect $CD4^+$ or $CD8^+$ T cell function by direct blockade, by transmitting a negative signal, or by interfering with normal signal transduction mechanisms.

To avoid inherent questions regarding the efficacy versus mechanisms of anti-CD4 or anti-CD8 induced tolerance, we studied allotransplantation in CD4 and CD8 knockout mice that were generated using homologous recombination in pluripotent embryonic stem cells (30,31). Although it has previously been shown that skin allografts from mice lacking either class I (β2-microglobulin or TAP1 and β2-microglobulin), class II ($A\beta$3) or both class I and class II (β2-microglobulin and $A\beta$3) are rejected (32, 33), these mice contain a small number of $CD4^+$ and/or $CD8^+$ T cells. We expanded upon these studies by directly testing the hypothesis that the complete absence of $CD4^+$ (or $CD8^+$ cells) would block the initiation of allorejection and consequently allow the indefinite survival of allografts. Here we report that rejection can occur in the absence of $CD8^-$ cells, and that $CD4^+$ cells are required for allorejection.

Materials and Methods

Mice. Inbred male C57BL/6 (H-2b, B6), BALB/c (H-2d), and A/J (H-2a) mice were purchased from The Jackson Laboratory (Bar Harbor, ME); BALB/c CD4 knockout and BALB/c or PL/J(H2u) CD8 knockout mice (homologous for disrupted CD4 or CD8 gene as previously described [30, 31]) were the generous gift of Dr. Tak Mak (University of Toronto). The animals were housed and bred in pathogen-free conditions in the Stanford Department of Laboratory Animal Medicine (DLAM) according to the National Institutes of Health guidelines.

mAb and Immunosuppression. The mAb used in these studies, GK1.5 (CD4), is a rat immunoglobulin (IgG2b) directed against mouse L3T4 (34). GK1.5 was produced from ascites in nude mice primed with pristane (Sigma Chemical Co., St. Louis, MO) followed by intraperitoneal inoculation of GK1.5 hybridoma cells. The antibody was purified via passage over an affinity-gel protein A column. Antibody content was quantified by an optical density spectrometer (DU 640; Beckman Instruments, Inc., Fullerton, CA) and qualitated by FACS® analysis and depletion studies in vivo. The supernatant was passed through a 0.22-μm filter (Millipore Corp., Bedford, MA) before being stored at -20°C. 5 mg/kg of antibody was administered at -3, -2, -1, and 0 d relative to allograft transplantation.

Heterotopic Heart Transplantation. Vascularized heart grafts were transplanted using standard microsurgical techniques (35). Briefly, the harvested donor heart was placed in 4°C saline until transplantation. The mouse was anesthetized by intraperitoneal injection of Nembutal (50 mg/kg). The donor aorta was sutured to the recipient aorta and the donor pulmonary artery to the recipient inferior vena cava end to side using 10-0 suture. Transplant function was evaluated by daily abdominal palpation. Cessation of palpable heartbeat was used to determine the end point of rejection.

Table 1. *Anti-CD4 mAb (GK1.5) Allows Heart but not Skin Allograft Survival*

GK1.5	Allograft	Survival	MST ± SEM
		d	
+	Heart	60, 90, >100 × 6	93.8 ± 5.0*
−	Heart	6, 7 × 3, 8, 9	7.3 ± 0.4
+	Skin	8, 10, 11, 11, 11	10.2 ± 0.6
−	Skin	7, 8 × 5	7.8 ± 0.2

Pretreatment with a brief course of GK1.5 allowed long-term survival of A/J (H-2a) heart, but not skin allografts in C57BL/6 (H-2b, B6) recipients. 5 mg/kg of antibody GK1.5 was administered at -3, -2, -1, and 0 d relative to allograft transplantation.
* $P < 0.002$; Mann-Whitney U test.

Skin Grafts. Skin allografts taken from donor chest skin were grafted onto the flank of the recipients with a running 6-0 suture using the uncovered skin graft technique (36). Using this method, the skin graft was visible from the day of engraftment until rejection was complete, and mice were not burdened by circumferential body dressings. Skin graft changes of shrinkage and black coloration were defined as the time of rejection.

MINIMACS Purification of $CD4^+$ Cells. Single cell suspensions of freshly isolated spleen and LN cells from naive BALB/c mice were counted and incubated with anti-CD4 magnetic microbeads (Miltenyi Biotec, Auburn, CA) for 20 min on ice, washed, and purified by passage through magnetic flow columns. The eluent gave a population of 90% $CD4^+$ cells by FACS® analysis (data not shown). 5×10^7 $CD4^+$ cells were then inoculated intraperitoneally into each CD4 knockout mouse.

Results and Discussion

Skin but not Heart Allografts Are Rejected in anti-CD4 mAb–Treated Mice. Mice treated with anti-CD4 mAb accepted heart but not skin allografts (Table 1). B6 mice that received a brief course of anti-CD4 showed long-term survival of A/J heart allografts (mean survival time [MST], 94 d). Skin allograft survival was not prolonged in the anti-CD4 treated recipients compared with untreated controls (MST, 10 and 8 d, respectively). Other investigators have previously shown that heart but not skin allografts were permanently accepted in mice treated with a short course of anti-CD4 therapy (16, 17, 19).

Why are skin allografts rejected in the anti-CD4–treated mice? Generally, skin allografts induce stronger allospecific cellular immunity than heart allografts (1). It has been demonstrated in the anti-CD4–treated mice that "memory" T cells persist despite depletion of peripheral $CD4^+$ cells (23, 37). These residual $CD4^+$ T cells (resistant memory cells) may mediate the induction of graft rejection in response to highly immunogenic antigens present in skin grafts. Differ-

Table 2. *CD4 Knockout Mice Retain Skin and Heart Allografts Indefinitely*

Strain combination	Allograft	Survival	MST ± SEM
		d	
B6 to CD4 KO BALB/c	Heart	>100 × 8	100.0 ± 0.0*
B6 to BALB/c	Heart	7, 8, 8, 9, 10	8.4 ± 0.5
B6 to CD4 KO BALB/c	Skin	>100 × 8	100.0 ± 0.0*
B6 to BALB/c	Skin	8, 8, 9, 10, 10	9.0 ± 0.4

BALB/c CD4 knockout (KO) recipients permanently accept C57BL/6 (H-2b, B6) heart and skin allografts.
* $P < 0.01$, Mann-Whitney U test.

ences in graft immunogenicity of skin grafts may be due to either the number of class II passenger leukocytes (including Langerhans cells) or the MHC class I density in skin tissue. Recognition of minor or Qa differences or skin-specific alloantigens may be important as well (38). These differences may initiate a strong response that recruits the small number of residual $CD4^+$ cells in anti-CD4–treated recipients.

Mice Lacking $CD4^+$ Cells but with Functional $CD8^+$ Cells Permanently Accept Heart and Skin Allografts. C57BL/6 heart and skin allografts were permanently accepted in the BALB/c CD4 knockout recipients (MST >100 d) (Table 2). Why do CD4 knockout mice not reject allotransplants? Mice lacking $CD4^+$ cells through targeted gene disruption have previously been shown to have normal numbers of T and B cells, with peripheral expansion of the $CD8^+$ compartment (31). The CD4 knockout mice possess an expanded subpopulation of CD4-CD8-TCR-α/β^+ (double negative) T cells in the thymus and periphery that is not normally found in significant numbers in conventional mice (31). These mice have been shown to have intact Ig isotype class switch from IgM to IgG in response to sheep erythrocytes and vesicular stomatitis virus in vivo (30). It was also demonstrated (using depletive regimens of mAbs to various subpopulations of T cells in vivo) that the double negative cells were responsible for providing help in the antibody response of CD4 knockout mice to vesicular stomatitis virus infection (30). These cells were demonstrated to be class II MHC–restricted in responses against the T cell–dependent antigen KLH. CTLs were also shown to be generated against lymphocytic choriomeningitis and vaccinia virus, suggesting that $CD8^+$ cells in these CD4 knockout mice can mount an immune response in the absence of $CD4^-$ cells (30).

Double-negative T cells have been previously shown to have suppressive properties (39). It is possible that these CD4-CD8-TCR-α/β^+ T cells are not only unable to initiate but may actively suppress a response against the allograft. The activity of these double-negative cells in CD4 and CD8 knockout mice, and in CD4-CD8- double knockout mice, however, has been variable. In CD4 knockout mice, CD4-CD8-TCR-α/β^+ cells have been shown to provide MHC class II–restricted help in vitro as stated above (30). Although naive CD8 knockout mice have normal numbers of CD4-CD8-TCR-α/β^+ cells, double-negative cells are increased in CD8 knockout mice engrafted with an MHC class I–disparate skin graft (40). Double-negative cells also significantly increase when thymocytes from these CD8 knockout mice are transferred to nude mice who receive and subsequently reject MHC class II–deficient skin grafts. CD4 depletion with anti-CD4 mAbs in CD8 knockout mice has no effect on rejection of MHC class I–disparate skin allografts; thus residual nondepleted $CD4^+$ or CD4-CD8-TCR-α/β^- cells may play a role in this rejection. In CD4-CD8- knockout mice, CD4-CD8-TCR-α/β^+ cells have been shown to generate alloreactive cytolytic T cells, and recognize MHC class I antigens in vitro (41). These CD4-CD8- double-knockout mice have been shown to reject skin grafts with major H-2 histocompatibility disparities, but accepted grafts with only minor antigen differences (41). To address the possibility that CD4-CD8-TCR-α/β^+ cells in CD4 knockout recipients serve as "suppressor cells," $3.0–4.0 \times 10^7$ spleen cells from CD4 knockout mice bearing B6 hearts for over 100 d were transferred into irradiated (200 rads) BALB/c hosts along with a fresh donor-matched B6 heart allograft. Tolerance was not adoptively transferred to these naive recipients; all B6 heart allografts were rejected within 18 d, similar to irradiated controls (Table 3). These data suggest that unresponsiveness in the CD4 knockout recipients was not due to the presence of suppressor CD4-CD8-TCR-α/β^+ T cells. That double-negative cells do not actively suppress allorejection is consistent with previous results in both CD8 and CD4-CD8- knockout mice that demonstrated that double-negative cells tend to play a role in skin allograft rejection rather than suppression.

It is therefore most likely that the mechanism of unresponsiveness to allografts in the CD4 knockout mice was due to the complete absence of $CD4^+$ T cells which would

Table 3. *Adoptive Transfer of Spleen Cells from "Tolerant" CD4 Knockout Mice Does Not Prolong Allograft Survival*

Strain combination	$3–4 \times 10^7$ Splenocytes	200 rads	Survival	MST ± SEM
			d	
B6 to BALB/c	Tolerant CD4 KO	+	17, 18 × 3	17.8 ± 0.4
B6 to BALB/c	None	+	16, 18 × 3	17.5 ± 0.8

Transfer of $3–4 \times 10^7$ spleen cells from CD4 KO mice bearing allografted hearts for over 100 d into irradiated (200 rads) syngeneic hosts did not prevent allorejection of fresh B6 heart allografts in the naive recipients of adoptive transfer.

Table 4. *$CD4^+$ Cells Reconstitute Allorejection*

Strain combination	Transferred cells	Mice with rejected grafts	Survival
			d
B6 to CD4 KO	None	0/5	100 × 8
B6 to CD4 KO	$CD4^+$ cells	5/7	13, 14, 15 × 2, 16, >30 × 2
B6 to CD4 KO	CD8 KO cells	3/3	15, 16, 17

CD4 KO BALB/c mice were given 5×10^7 purified $CD4^+$ cells from conventional naive BALB/c mice. 1 d after this adoptive transfer, the CD4 KO BALB/c mice received a B6 skin graft

suggest that $CD4^-$ cells are required for initiation of allograft rejection. To address this possibility, we reconstituted CD4 knockout mice with naive $CD4^+$ cells just before engraftment to see whether the addition of $CD4^+$ cells would allow graft rejection. 1 d after adoptive transfer of 5×10^7 $CD4^+$ cells from conventional naive BALB/c mice obtained by MINIMACS purification, the CD4-reconstituted CD4 knockout BALB/c mice received a B6 skin graft. Control CD4 knockout mice received identical B6 skin grafts but did not receive CD4 cells before engraftment. 5 of 7 mice reconstituted with $CD4^+$ cells rejected their grafts (Table 4). This experiment was then modified to avoid the possibility that the $CD4^+$ cells isolated by MINIMACS purification were "activated." CD4 knockout mice were reconstituted with cells from CD8 knockout mice which have functionally intact $CD4^+$ cells (31). Three of three CD4 knockout recipients reconstituted with "CD4 cells" from CD8 knockout mice rejected their skin grafts (Table 4).

CD8 Knockout Mice Reject Heart and Skin Allografts. Although we have demonstrated that $CD4^+$ cells are essential for allorejection, what is the role of the $CD8^+$ cell? In certain settings, $CD8^+$ cells seem capable of initiating rejection in concert with MHC class I disparity (1). However, as demonstrated in the current study, $CD8^+$ cells alone, although present in the CD4 knockout mice, could not initiate allorejection. It is possible that the $CD8^+$ cells which may normally play a role in graft rejection were unable to respond to alloantigens in the complete absence of $CD4^+$ cell-mediated help. This question has been previously addressed in CD8 knockout mice, which lack $CD8^+$ cells but have functional $CD4^+$ cells (31). It has been previously shown that CD8 knockout mice reject MHC class I–or MHC class II–disparate skin grafts without delay compared with wild-type mice, suggesting that $CD8^+$ cells are not necessary for allorejection of either MHC class I or class II grafts (40). More recent studies have demonstrated that adoptive transfer of naive or sensitized $CD4^+$ cells from these CD8 knockout mice into nude mice that had been grafted with allogeneic skin from mice deficient in MHC class I or class II (MHC class II or MHC class I allogeneic, respectively) reconstituted rejection, suggesting that $CD4^+$ cells were sufficient to mediate rejection (42). Although MHC class I skin allografts were rejected, $CD4^+$ cells did not display alloantigen-specific cytotoxic activity, though they proliferated in vitro in response to allogeneic targets. We also studied CD8 knockout mice as recipients of MHC disparate allografts. C57BL/6 skin allografts transplanted into BALB/c CD8 knockout mice were rejected in 8.7 ± 0.3 d (compared to 9.0 ± 0.4 d for BALB/c controls), which concurs with the results of Dalloul et al. (42). C57BL/6 heart and skin allografts transplanted into PL/J CD8 knockout mice were rejected within 10 d for each graft separately ($n = 14$, data not shown). Collectivity these results suggest that elimination of cells bearing the CD8 molecule does not prevent allorejection. These data also demonstrate that $CD4^+$ cells can initiate rejection. Thus, our results demonstrate that the initiation of allorejection requires $CD4^+$ and not $CD8^+$ cells.

In these experiments we have explored the role of CD4 and CD8 cells in transplant allorejection using knockout mice as recipients of MHC disparate allografts. The results demonstrate that heart and skin allografts are permanently accepted in CD4 knockout mice, but are rejected in CD8 knockout mice. Thus, lack of $CD4^+$ cells allows permanent survival of heart and skin allografts in mice, whereas lack of $CD8^+$ cells does not prevent allorejection. These results demonstrate that $CD4^+$ cells, not $CD8^-$ T cells, are absolutely required in initiating allorejection. Our results also demonstrate that allorejection does not require both $CD4^+$ and $CD8^+$ T cell subpopulations.

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