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Disparate Primary and Secondary Allospecific CD8+ T Cell Cytolytic Effector Function in the Presence or Absence of Host CD4+ T Cells

Phillip H. Horne,† Mitchel A. Koester,* Kartika Jayashankar,* Keri E. Lunsford,† Heather L. Dziema,* and Ginny L. Bumgardner2*†

The role of CD4+ T cells in promoting CD8+ T cell effector activity in response to transplant Ags in vivo has not been reported. We used a hepatocellular allograft model known to initiate both CD4-dependent and CD4-independent rejection responses to investigate the contribution of CD4+ T cells to the development, function, and persistence of allospecific CD8+ T cell effectors in vivo. Complete MHC-mismatched hepatocellular allografts were transplanted into C57BL/6 (CD4-sufficient) or CD4 knockout hosts. The development of in vivo allospecific cytotoxicity was determined by clearance of CFSE-labeled target cells. CD8+ T cell cytotoxic effector activity was enhanced in response to allogeneic hepatocellular grafts with a greater magnitude of allogtocytotoxicity and a prolonged persistence of CTL effector activity in CD4-sufficient hosts compared with CD4-deficient hosts. Cytolytic activity was mediated by CD8+ T cells in both recipient groups. In response to a second hepatocyte transplant, rejection kinetics were enhanced in both CD4-sufficient and CD4-deficient hepatocyte recipients. However, only CD4-sufficient hosts developed recall CTL responses with an augmented magnitude and persistence of allogtocytotoxicity in comparison with primary CTL responses. These studies show important functional differences between alloreactive CD8+ T cell cytolytic effectors that mature in vivo in the presence or absence of CD4+ T cells. The Journal of Immunology, 2007, 179: 80–88.

Activation and maturation of CD8+ effector T cells are conventionally considered to require CD4+ T cell help. However, it is recognized that this requirement for CD4+ T cell help is conditional, such that CD8+ CTL maturation in the absence of CD4+ T cells occurs when APC activation is directly stimulated by adjuvants, infectious agents, or experimental agents (1–10). Furthermore, effective CD8+ T cell-mediated clearance of pathogens can occur in the absence of CD4+ T cells (11). More recently it has been recognized that the absence of CD4+ T cells significantly impairs CD8+ T cell memory or recall responses to Ag (11–15) (reviewed in Ref. 16). CD4+ T cells have been reported to contribute to the development of enhanced secondary or memory CD8+ CTL responses through epigenetic modification or programming (11, 17–19). Others have reported that CD4+ T cells are important for the maintenance (but not programming) of CD8+ memory T cells (13). In contrast to the critical influence of CD4+ T cells on development of efficient CD8+ T cell memory responses, the absence of CD4+ T cells during priming does not impair primary CD8+ T cell cytokine production, proliferation, or effector function in nontransplant models (17, 20).

In addition to the recognized roles of CD8+ T cells in clearance of infectious pathogens and tumor cells, CD8+ T cells are also known effectors of allograft rejection (21–25). Allograft rejection by conventional pathways is a CD4+ T cell-dependent process (reviewed in Ref. 26) that can be suppressed by targeting CD4+ T cells alone. This implies that allospecific CD8+ CTLs that develop concurrently with allospecific CD4+ T cells are CD4-dependent CD8+ CTLs. Several allograft tissues, including hepatocytes (26, 27), skin (28, 29), intestine (30, 31), and cardiac allografts in some models (32), have been reported to activate CD4-independent, CD8+ T cell rejection responses. These unconventional CD4+ T cell-independent immune pathways are associated with different costimulatory requirements (31, 33–35), skewed immune repertoires in the host (32), and resistance to immunotherapies that readily control CD4+ T cell-initiated rejection responses (27, 31, 32, 36–42). Furthermore, CD4-independent CD8+ T cell rejection responses contribute to costimulation blockade-resistant rejection and interfere with immune tolerance strategies (39, 41, 43, 44). Collectively, these studies underscore the importance of understanding the development and function of alloreactive CD8+ T cells that are activated independently of CD4+ T cells.

Few experimental transplantation studies have examined the in vivo development of alloreactive CD8+ CTL effector function in the presence or absence of CD4+ T cells. One study of cardiac allografts reported that host CD4+ T cells are necessary to license dendritic cells for CD8+ T cell activation, CTL function, and CD4-dependent graft rejection (45). However, the development of CTL effectors under CD4-independent conditions in response to allograft tissues such as skin, intestine, and hepatocytes has not been investigated, except for reports by our group showing that the rejection of allogeneic liver parenchymal cells in the absence of CD4+ T cells is accompanied by in vivo cytotoxic effector function (46, 47).

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The purpose of the current study was to examine CD8+ CTL maturation, magnitude, and productive memory development in response to allogeneic hepatocytes in the presence or absence of CD4+ T cells. Experimental advantages of the hepatocellular allograft model to study these immune parameters include the relative homogeneity of the alloantigen conferred by transplantation of a purified cell population and a standard cell number or graft size. Furthermore, CD4-independent, CD8+ T cell immune responses to allogeneic hepatocytes have been studied using three experimental models, including recipient CD4+ T cell depletion (anti-CD4 mAb, GK1.5), recipient genetic deficiency (CD4 knockout KO), or reconstitution of immunoincompetent SCID (or Rag1−/−) recipients with CD8+ T cells. All of these models produce comparable results and are available to explore the consequence of alloreactive CD8+ T cell maturation in the presence or absence of recipient CD4+ T cells (27, 36, 48). Our studies show that CD8+ CTL responses are generated by hepatocellular allografts both in the presence and absence of CD4+ T cells, but the kinetics, magnitude of effector responses, and second set responses are strikingly different.

Materials and Methods
Experimental animals
FVB/N (H-2b; Taconic Farms), C57BL/6 (H-2b; Taconic Farms), CD4 KO (H-2b, C57BL/6-Cd4−/−; The Jackson Laboratory), DesTCR (H-2b) transgenic mice with CD8+ T cells expressing TCR specific for H-2Kb; a gift from A. Mellor, Medical College of Georgia, Augusta, GA), and B10.BR (H-2b; The Jackson Laboratory) mouse strains were used in this study. Transgenic FVB/N and C57BL/6 mice expressing human α1-antitrypsin (hA1AT-FVB/N, H-2b, hA1AT-C57BL/6, H-2b) were the source of donor hepatocytes. These strains were created, bred, and maintained at the Biotechnology Center and Transgenic Animal Facility (Ohio State University) (27, 48). Mice that were 6–9 wk of age were used in experiments. All animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” (National Academy Press, revised 1996).

Hepatocyte isolation and purification
Hepatocyte isolation and purification were performed, as described previously (27, 48). Briefly, the liver was perfused with 0.09% EGTA-containing calcium-free salt solution, followed by a 0.05% collagenase solution (Sigma-Aldrich; type IV) in 1% albumin. Liver tissue was minced, filtered, and washed in RPMI 1640 with 10% FBS. Hepatocytes were purified on a collagenase (Sigma-Aldrich; type IV) in 1% albumin. Liver tissue was minced, filtered, and washed in RPMI 1640 with 10% FBS. Hepatocytes were purified on a 10% Percoll gradient (Pharmacia Biotech). Hepatocyte viability and purity were consistently >99%, respectively.

Hepatocyte transplantation and monitoring of hepatocyte graft function
Donor hepatocytes were retrieved from transgenic mice expressing hA1AT under control of the liver-specific hA1AT promoter and transplanted into recipients by intrasplenic injection with circulation of donor hepatocytes to the host liver, as previously described (48). C57BL/6 (H-2b) and CD4 KO (H-2b) recipients were transplanted with 2 × 106 purified allogeneic hA1AT-FVB/N (H-2b) hepatocytes. DesTCR CD8+ T cell-reconstituted B10.BR (DesB10BR) recipients (H-2b) were transplanted with 2 × 106 allogeneic hA1AT-C57BL/6 (H-2b) hepatocytes. Graft function was determined by detection of the secreted transgenic reporter product, hA1AT, in the host liver, as previously described (48). C57BL/6 (H-2b) and CD4 KO (H-2b) recipients by intrasplenic injection with circulation of donor hepatocytes to the host liver, as previously described (48).

Abs used for T cell subset depletion in hepatocyte recipients
Recipients were depleted of circulating CD4+, CD8+, or NK cells using mAbs. Anti-CD4 (GK1.5; American Type Culture Collection) and anti-CD8 (53-6.72; American Type Culture Collection) mAbs were obtained from Bioexpress Cell Culture Services. Anti-NK1.1 (PK136; American Type Culture Collection) mAb was purified from pristane-primed mice.

Washed hepatocytes suspended in 200 μl of DMEM into syngeneic B10.BR hosts (day 4 and 8) were retrieved (day 3 after hepatocyte transplant; day 4 after adoptive transfer of Des CD8+, T cells) for analysis of CFSE dilution in Des CD8+ T cells by flow cytometry.

In vivo proliferation assay
Preparation of Des+ CD8+ T cells. Splenocytes from DesTCR mice were column purified for CD8+ T cells, according to manufacturer’s instructions (Mouse CD8 Subset Column Kit; R&D Systems), and phenotyped through immunolabeling with anti-CD45R0 (mouse anti-mouse Des) super-natant (100 μl neat), washing twice in cold PBS-FACS buffer (5% goat serum and 0.001% sodium azide), then staining with anti-CD8 FITC (53-6.7, 1:200; BD Pharmingen), anti-CD3 PE (145-2C11, 1:200; BD Pharmingen), and goat anti-mouse IgG Alexa Fluor 647-R-PE (1:200; Molecular Probes), in cold PBS-FACS buffer. The cells were washed twice and then analyzed by flow cytometry. Column-purified splenocytes were ~50% Des+ CD8+ CD3+.

Adaptive transfer of Des+ CD8+ T cells into B10.BR hepatocyte recipients. Des+ CD8+ T cells were stained with 5 μM CFSE in PBS at 37°C for 8 min, then washed in warm DME in 10% FBS (37°C) for 5 min. The 5.5–6 × 10⁶ colonized-splenocytes (~2–4 × 10⁵ Des+ CD8+ CD3+) were adoptively transferred through tail vein injection in 0.5 ml of DMEM into syngeneic B10.BR hosts (day −1), some of which were previously depleted of CD4+ T cells (anti-CD4 mAb, days −4 and −2). Transgenic hA1AT-C57BL/6 hepatocytes were transplanted on day 0 through intrasplenic injection in some of the Des+ CD8+ -reconstituted B10.BR (DesB10BR) hosts, and later host liver and spleen lymphocytes were retrieved (day 3 after hepatocyte transplant; day 4 after adaptive transfer of Des+ CD8+ T cells) for analysis of CFSE dilution in Des CD8+ T cells by flow cytometry.

Retention of graft-infiltrating cells (GICs) for phenotypic analysis
Hepatocytes transplanted by intrasplenic injection rapidly circulate to and engraft in the recipient liver (reviewed in Ref. 26) and stimulate the accumulation of GICs in the recipient liver (47). To retrieve GICs, recipient mice were euthanized for excision of the liver. The livers were digested in a solution containing 0.05% collagenase (Sigma-Aldrich; type IV) in 1% albumin for 10 min and cells were separated through mechanical disper-sion. Erythrocytes were lysed using hypotonic solution, and lymphocytes were enriched using 60% gradient (Histodenz; Sigma-Aldrich) density cen-trifugation. Lymphocytes were enumerated and phenotyped by immunolabelling with anti-CD8 FITC (53-6.7, 1:200; BD Pharmingen), anti-CD3 PE (1B11, 1:200; BD Pharmingen), anti-CD4 PE-Cy5 (IM7, 1:200; BD Pharmingen), and flow cytometry, gating on CD8+ T cells.
Allospecific target cell clearance was detected in both recipient groups, as seen by the increased clearance of allogeneic (FVB/N, H-2^q) CFSE^high relative survival time (MST) of 10 and 14 days, respectively (p < 0.05) (left) (p = NS). Allospecific target cell clearance was detected in both recipient groups, as seen by the increased clearance of allogeneic (FVB/N, H-2^q) CFSE^high relative to syngeneic (C57BL/6, H-2^b) CFSE^low^target cells; no allogeneic target cell clearance occurred in untransplanted controls. Greater allocytotoxicity was observed in C57BL/6 hosts (n = 5) in comparison with CD4 KO hosts (n = 5) (right) (p < 0.01) (a). Depletion of recipient CD8^+ T cells 48 h before the cytotoxicity assay on day 7 posttransplant in CD4^sufficient hosts (n = 5) and on day 5 posttransplant in CD4 KO hosts (n = 3) significantly decreased allocytotoxicity in both recipient groups (p < 0.001, p < 0.01, respectively), whereas depletion of host NK cells did not impair allocytotoxicity in CD4^sufficient or CD4 KO hosts (n = 4) at comparable times posttransplant (p = NS) (b). Untreated C57BL/6 (n = 3) and CD4 KO (n = 3) hepatocyte allograft recipients were assessed for specificity of in vivo cytotoxicity on day 10 (CD4^sufficient hosts) or day 5 (CD4^deficient hosts) posttransplant using third-party (B10.BR, H-2^b) CFSE^high^targets and syngeneic CFSE^low^targets. No clearance of third-party targets relative to syngeneic targets was observed in C57BL/6 or CD4 KO hepatocyte recipients (c).

### Results

**Allospecific CD8^+ CTL effector function develops in CD4^sufficient (C57BL/6) and CD4^deficient (CD4 KO) hepatocyte recipients**

We have previously reported that allogeneic liver parenchymal cells initiate distinct rejection pathways in C57BL/6 (CD4^+ and CD8^-) T cell sufficient, H-2^b^and CD4 KO (CD4^deficient, CD8^- T cell sufficient, H-2^b^) hosts (27, 36). Rejection in C57BL/6 recipients is dependent on both CD4^+ and CD8^- T cells (36). Rejection in CD4 KO recipients is CD8^- T cell dependent (27). In the current study, C57BL/6 (H-2^b^) and CD4 KO (H-2^b^) mice were transplanted with hA1AT-FVB/N (H-2^b^) hepatocytes and had median survival time (MST) of 10 and 14 days, respectively (p = NS) (Fig. 1a). Hepatocyte recipients were assessed on day 7 posttransplant for in vivo allospecific cytotoxicity by measurement of CFSE-labeled allogeneic vs syngeneic target cell clearance. Both CD4^sufficient and CD4^deficient hepatocyte recipients demonstrated detectable in vivo allospecific cytotoxicity (Fig. 1a); however, the magnitude of allospecific cytotoxicity was greater in CD4^sufficient than in CD4^deficient recipient groups (p < 0.01). To determine whether CD8^- T cells were responsible for the observed allocytotoxicity, separate groups of C57BL/6 and CD4 KO recipients were depleted of CD8^- T cells (anti-CD8 mAb, 53-6.72) 2 days before the cytotoxicity assay. Depletion of CD8^- T cells abrogated allocytotoxicity in CD4^deficient recipients and significantly decreased allocytotoxicity in CD4^sufficient recipients (Fig. 1b). Furthermore, NK cells do not contribute to cytotoxicity in CD4^sufficient or CD4^deficient recipient groups, because depletion of NK cells (anti-NK1.1 mAb, PK136) 48 h before the in vivo cytotoxicity assay did not perturb cytotoxicity in comparison with NK replete hosts (Fig. 1b). The allospecificity of the CD8^- T cell-mediated cytotoxic effector function was assessed in separate groups of recipients that were transplanted, followed by assessment of in vivo cytotoxicity assay using syngeneic and third-party (B10.BR, H-2^b^) target cells. No enhanced clearance...
of third-party target cells was observed in hepatocyte allograft recipients (Fig. 1c). These studies demonstrate that allospecific CD8\(^+\) CTLs develop in both CD4-sufficient and CD4-deficient hepatocyte recipients.

**In vivo allospecific CD8\(^+\) T cell cytolytic effector function is greater in magnitude and persists longer in CD4-sufficient recipients than in CD4-deficient recipients**

To determine whether or not the difference in magnitude of in vivo alloctytotoxicity observed on day 7 posttransplant in CD4-sufficient compared with CD4-deficient recipients was sustained over time or was limited to that time point, we performed a kinetic analysis of CD8\(^+\) CTL maturation under these disparate conditions. Groups of CD4-sufficient (C57BL/6, H-2\(^b\)) and CD4-deficient (CD4 KO, H-2\(^b\)) recipients were transplanted with hepatocellular allografts (hA1AT-FVB/N, H-2\(^b\)) and assessed for allospecific in vivo cytotoxicity at serial time points posttransplant (Fig. 2). In CD4-sufficient recipients, CD8\(^{+}\) mediates cytotoxic effector function is first observed 3 days posttransplant and rises to its peak magnitude on day 7 posttransplant. CD8\(^{-}\)-mediated allospecific cytotoxic effector function decreases after rejection (day 10 posttransplant), and slowly returns to near baseline by day 21 following transplant.

The differences observed in the CD4-sufficient and CD4-deficient recipients raised the issue of whether CD4\(^+\) T cells might directly contribute to cytotoxic effector function in CD4-sufficient recipients. To investigate this possibility, C57BL/6 mice were transplanted with hA1AT-FVB/N hepatocytes and were then depleted of CD4\(^+\) T cells (anti-CD4 mAb, i.p., days 6 and 7 posthepatocyte transplant) 48 h before the assay for in vivo allospecific cytolytic activity (day 8 posttransplant). The depletion of CD4\(^+\) T cells did not significantly decrease in vivo alloctytotoxicity (allogeneic target cell clearance) relative to C57BL/6 control hosts (n = 5) (p = NS).
through enhanced proliferation, are not responsible for the in-
lation size, due to a higher frequency of alloreactive cells or
ident allograft rejection. To investigate the size of the CD8
effector population occurring in CD4-dependent vs CD4-indepen-

DesTCR (data not shown) were retrieved, enumerated, and immunolabeled for
B10.BR recipients, and 3 days posttransplant, host GICs and splenocytes
(data not shown) were retrieved, enumerated, and immunolabeled for
DesTCR and CD8. Allospecific DesTCR CD8+ T cells were assed
for in vivo proliferation by analysis of dilutional fluorescence and flow
cytometry. The percentages of DesTCR CD8+ T cells remaining un-
divided, dividing 1–3 times, 4–6 times, or 7+ times were compared between
CD4-sufficient (n = 4) and CD4-deficient (n = 4) Des+ TCR CD8+ T
cell-reconstituted (DesB10.BR) recipient groups. The data shown are rep-
resentative of four separate experiments.

Recipients, Des+CD8+ T cells underwent more rounds of cell di-
vision at the graft site (liver) than in the host spleen (data not
shown). Proliferation of allospecific CD8+ T cells was similar in
CD4-sufficient and CD4-deficient hepatic allograft recipients,
as reflected by comparison of the percentage of Des+ CD8+ T cells
remaining undivided, or which underwent 1–3 divisions, 4–6 di-
visions, or 7+ cell divisions (p = NS). This suggests that given an
equal number of alloreactive CD8+ T cells from the outset, these
cells proliferate to the same degree in response to allogeneic hep-
atoctyes in the presence or absence of CD4+ T cell help.

However, given the potential of TCR transgenic cells to bypass
the requirement for CD4+ T cell help due to high frequency or Ag
avidity (52, 53), we determined both the number of activated
CD8+ CTLs and the ultimate size of the nontransgenic CTL ef-

tector population occurring in CD4-dependent vs CD4-independ-
ent allograft rejection. To investigate the size of the CD8+ T cell
population in CD4-sufficient (C57BL/6) and CD4-deficient (C4 KO)
hepatic allograft recipients, activated CD8+ T cells were retrieved from the liver (CD8+ GICs)
and enumerated 1 wk posttransplant. The percentages and absolute numbers of CD8+ T cells with an activated phenotype (CD8+ CD62LlowCD44high) were equivalent between recipient groups
(p = NS). The number of recipient CD8+ GICs demonstrating a
cytotoxic effector phenotype (CD8+ CD43-highCD44-high) was also similar between CD4-sufficient and CD4-deficient recipient
groups (p = NS) (Fig. 5). Collectively, these data support the conclusion that differences in CD8+ effector T cell pop-
ulation size, due to a higher frequency of alloreactive cells or
through enhanced proliferation, are not responsible for the in-

creased magnitude of CD8+ cytolytic effector function ob-
served in CD4-sufficient recipients.

Potent secondary CD8-mediated CTL activity develops in
CD4-sufficient, but not CD4-deficient recipients

CD4+ T cell help in response to viral and other infectious stimuli
or nominal Ags has been reported to critically influence CD8+ T
cell memory and recall responses (3, 11–19). We hypothesized that
recipient CD4+ T cells also critically influence secondary allo-
reactive CD8+ T cell cytotoxic effector function after hepaticocyte
transplant. To test this hypothesis, CD4-sufficient (C57BL/6) and
CD4-deficient (CD4 KO) recipients were transplanted with allo-
genecic hepatocytes (hA1AT-FVB/N, H-2q). Following rejection of
the first hepaticocyte graft, recipients underwent a second donor-
matched hepaticocyte transplant (hA1AT-FVB/N, H-2q) on day
21–35 posttransplant (coinciding with return of in vivo CTL ac-
tivity to baseline levels). Hepatocellular allograft survival and CTL
activity were assessed in these primed recipients. Rejection of the
second graft was faster than rejection of the primary graft in both
CD4-sufficient (MST = 7 vs 10 days, p = 0.05) and CD4-deficient
(MST = 10 vs 14 days, p < 0.05) recipient groups, consistent with a
recall response. At 3 days following the second transplant, CD4-
sufficient hosts showed a higher magnitude of allocytotoxicity in
comparison with CTL activity at the comparable timepoint follow-
ing primary transplant (95 vs 33%, respectively, p < 0.001). In
cyte transplant (tence in CD4-sufficient recipients following secondary vs primary hepato-
cytotoxicity was significantly increased did not differ significantly in CD4-deficient recipients following primary or
respectively). In both recipient groups, this in vivo cytotoxicity was
clearance of the alloantigenic stimulus. In contrast, in CD4-defi-
cells could provide the minimum number of cells necessary to provide a suf-
cytes or receive help from other non-CD4
T cell alloimmunity (46, 47). To address the pos-
specific CD8**+** T cells was similar in the presence or absence of CD4**+**
which could circumvent the need for CD4**+** immune cells, or
or that a combination of these factors stimulates proliferation and
CD8**+** T cells in CD4-deficient condi-
tions (65). One caveat is that the in vivo proliferation of TCR
transgenic CD8**+** T cells may not reflect the proliferative require-
ments of wild-type CD8**+** T cells. This could occur because TCR
transgenic T cells could have decreased threshold for activation and
proliferation. It could also occur due to the relatively high
number of transgenic allospecific cells adoptively transferred (~2–
4 × 10^6), which could circumvent the need for CD4**+** T cell help
in proliferation (52). The number of Des**+** CD8**+** T cells transferred
in this study was determined from titration studies designed to
provide the minimum number of cells necessary to provide a suf-
cient yield of liver-infiltrating cells for flow cytometric analysis.
Despite this caveat, our results agree with published reports using
fewer CD8**+** TCR transgenic cells (1–2 × 10^6 OT-I cells) for
adoptive transfer and that also reported that proliferation of allo-
reactive CD8**+** T cells occurs independent of CD4**+** T cell help
(45). Our results demonstrate that although the presence of CD4**+**
T cells significantly influences the magnitude and longevity of in

contrast, there was no increase in CTL activity following the sec-
ond transplant in CD4-deficient hosts (Fig. 6). Additionally, 2 wk
after rejection of the second hepatocyte transplant, CD4-sufficient
recipient groups still manifested heightened CTL effector function,
whereas CTL activity in CD4-deficient recipients had returned to
baseline (Fig. 6). These results support the conclusion that recip-
cient CD4**+** T cells facilitate development of an early augmented
and sustained allospecific CD8**+** CTL memory response beyond
clearance of the alloantigenic stimulus. In contrast, in CD4-defi-
cient recipients, secondary CTL responses are no different from the
primary CTL response and are significantly decreased in magni-
tude and extinguished more rapidly than in CD4-sufficient recipi-
ents (Fig. 6).

Discussion
The role of CD4**+** T cell help on primary and secondary CD8**+** T
cell cytolytic responses has been extensively investigated in vitro
(54–59). The requirement for, and consequences of CD4**+** T cell
help on maturation of Ag-specific CD8**+** CTLs in vivo have been
examined primarily in response to infectious pathogens (viral and
other microbial stimuli) and in response to immunization with
nominal Ags (reviewed in Refs. 16, 18, 19, and 60). To our know-
ledge, this is the first study to investigate the development of pri-
mary and secondary in vivo alloreactive CD8**+** CTL activity in
CD4-sufficient and CD4-deficient transplant recipients.
We found that allogeneic hepatocytes stimulate the development of
in vivo alloreactive cytotoxicity in both CD4-sufficient
(C57BL/6) and CD4-deficient (CD4 KO) hepatocyte recipients
(Fig. 1). In both recipient groups, this in vivo cytotoxicity was
mediated by CD8**+** CTL effectors (Fig. 1b) and was allospecific
(Fig. 1c). Although NK cells have been reported to initiate and
affect cytolytic activity in CD4-independent conditions in the
setting of antitumor immunity (61) and costimulation blockade-
resistant bone marrow allograft rejection (62), we found that
NK cells do not participate in CTL effector function in hepa-
tocyte rejectors (Fig. 1b). These studies expand upon our pre-
vious observations that CD8**+** GICs in hepatocyte rejectors ex-
press a CTL phenotype (CD8**+**CD43+) (47) and that the
detection of in vivo allocytotoxicity correlates with graft sur-
vival status (46, 47).

Experimental models that investigate CD8**+** CTL responses to
viral infection or to nominal Ag have reported that CD4**+** T cell
help does not significantly influence primary CD8**+** T cell re-
sponses, but critically influences CD8**+** T cell recall and memory
responses (11–20, 63). Of note, in these nontransplant models,
CD8**+** T cell cytokine production, proliferation, and CTL effector
function during primary Ag-specific activation were determined to
be comparable whether or not recipient CD4**+** T cells were present
(17, 20). In contrast, we found that CD4**+** T cells significantly
influence the development of alloreactive CD8**+** CTLs during
primary alloantigen exposure as reflected by the differences in kinet-
ics and magnitude of in vivo allospecific cytotoxicity in CD4-
sufficient vs CD4-deficient hepatocyte recipients (Fig. 2). These
results agree with studies in which CD4-sufficient hosts developed
higher CTL activity in response to allogeneic tumor cells than
CD4-deficient hosts (64).

Heightened CTL activity occurring in the presence of CD4**+** T
cells has several possible explanations. First, CD4**+** T cells could
directly mediate in vivo allospecific cytotoxicity; however, it was
observed that CD4**+** T cell depletion before the in vivo cytotox-
icty assay in CD4-sufficient hepatocyte recipients did not signifi-
cantly inhibit clearance of allogeneic target cells (Fig. 3). Second,
CD4**+** T cell signals could drive enhanced CD8**+** T cell effector
proliferation. We have noted in other studies that a small percent-
age of CD8**+** GICs in CD4 KO hepatocyte rejectors expresses the
IL-2Ralpha (CD25), implicating a role for local IL-2 in (CD4-inde-
dependent) CD8**+** T cell alloimmunity (46, 47). To address the pos-
sibility of enhanced CD8**+** T cell proliferation in the presence of
CD4**+** T cells, we analyzed in vivo proliferation of allospecific
TCR transgenic Des**+** CD8**+** T cells in CD4-sufficient and CD4-
deficient conditions. As shown in Fig. 4, proliferation of allospe-
cific CD8**+** T cells was similar in the presence or absence of CD4**+**
T cells. This suggests that either alloreactive CD8**+** T cells provide
self help through autocrine production of IL-2 and perhaps other
cytokines or receive help from other non-CD4**+** immune cells, or
that a combination of these factors stimulates proliferation and
maturaton of alloreactive CD8**+** T cells in CD4-deficient condi-
tions (65). One caveat is that the in vivo proliferation of TCR
transgenic CD8**+** T cells may not reflect the proliferative require-
ments of wild-type CD8**+** T cells. This could occur because TCR
transgenic T cells could have decreased threshold for activation and
proliferation. It could also occur due to the relatively high
number of transgenic allospecific cells adoptively transferred (~2–
4 × 10^6), which could circumvent the need for CD4**+** T cell help
in proliferation (52). The number of Des**+** CD8**+** T cells transferred
in this study was determined from titration studies designed to
provide the minimum number of cells necessary to provide a suf-
cient yield of liver-infiltrating cells for flow cytometric analysis.

During the second week following rejection of the initial
hepatocyte transplant, a second allogeneic FVB/N (H-2q) hepa-
tocyte transplant was performed (~21–35 days following the initial hepa-
tocyte transplant). The presence of in vivo allospecific cytotoxic activity was assessed in both
CD4-sufficient and CD4-deficient recipient groups on days 3 (n = 5, n = 6, respectively) and 21 (n = 5, n = 6, respectively) following the second
hepatocyte transplant. Allospecific cytotoxicity was significantly increased
in magnitude (p < 0.001 for day 3) and demonstrated prolonged persis-
tence in CD4-sufficient recipients following secondary vs primary hepa-
tocyte transplant (p < 0.001 for day 21). In contrast, allospecific cytotoxicity
did not differ significantly in CD4-deficient recipients following primary or
secondary hepatocyte transplant (p = NS for days 3 and 21 posttransplant, respectively).

![FIGURE 6. CD4-sufficient (but not CD4-deficient) hepatocyte transplant recipients develop recall responses with enhanced magnitude and persistence of allospecific cytotoxicity.](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/)
vivo allocytotoxicity, this does not appear to occur due to enhanced proliferation of CD8⁺ CTLs.

A third possibility is that more alloreactive CD8⁺ T cell precursors are present in C57BL/6 vs CD4 KO hosts, resulting in a greater number of CD8⁺ CTL effectors. To analyze this, we measured the number of activated and cytotoxic CD8⁺ T cells arising in both immune conditions following hepatocyte transplantation. We observed that CD4-sufficient and CD4-deficient recipients had similarly sized effector CD8⁺ T cell populations; enumeration of CD8⁺ T cells from the graft site (liver) and in the spleen showed similar numbers of activated (CD62LlowCD44high) and cytotoxic (CD43highCD44highb) CD8⁺ T cells (Fig. 5). These results are consistent with the in vivo proliferation experiments using TCR transgenic Des⁺ CD8⁺ T cells, demonstrating equivalent proliferation of allospecific CD8⁺ T cells in response to allogeneic hepatocytes in the presence or absence of host CD4⁺ T cells. Therefore, regardless of any potential differences in the number of alloreactive CD8⁺ T cell precursors or their in vivo proliferation in C57BL/6 vs CD4 KO hosts, we find that there is no significant difference in the number of activated or cytotoxic CD8⁺ effector cells.

A fourth possibility is that alloreactive CD8⁺ T cells develop along a continuum in response to local activation signals such that in the presence of CD4⁺ T cell help, CD8⁺ allo-CTLs reach their maximum differentiation state and cytolytic effector potential. In contrast, in CD4-deficient conditions, CD8⁺ CTLs may only reach intermediate differentiation and cytolytic effector function. In this scenario, it is possible that the increased allocytotoxicity observed in CD4-sufficient hepatocyte recipients could result from the development of more efficient cytolytic mechanisms in the presence of CD4⁺ T cell help. The perforin/granzyme CTL lytic mechanism has been reported to mediate more efficient killing than Fas ligand (FasL)-mediated cytotoxicity (66, 67) and has been shown to require stronger activation signals than are required to activate the FasL cytotoxicity pathway (68). Consistent with this idea, CD8⁺ CTL activation in response to hepatocellular allografts in CD4-sufficient conditions could develop both efficient perforin/granzyme- and FasL-killing mechanisms, whereas CD8⁺ CTLs that mature in CD4-deficient conditions may only develop the less efficient FasL cytotoxic mechanism. Studies to define the mechanisms of CD8⁺-mediated cytotoxicity in the presence or absence of CD4⁺ T cells are ongoing.

Alloreactive CD8⁺ T cells maturing in a CD4-sufficient immune environment exhibit peak cytolytic activity on day 7 posttransplant, which declines to near-baseline levels on day 21 posttransplant (1–2 wk after hepatocyte rejection). In contrast, alloreactive (CD4-independent) CD8⁺ CTLs exhibit a distinct effector phase profile in which peak cytolytic activity occurs early posttransplant (day 3) and rapidly declines to baseline by the time of hepatocyte rejection (day 10–14) (Fig. 2). This kinetic profile of a rapid peak effector response that resolves rapidly is reminiscent of the kinetics of innate immune cell responses. It is intriguing to consider the possibility that CD4-independent, CD8⁺ allo-CTLs are novel, intermediate (less vigorous) cytotoxic effectors bridging innate and adaptive immune pathways.

Whether or not CD4-independent CD8⁺ allo-CTLs represent differential maturation of the same precursor CD8⁺ T cells or whether they represent maturation of a unique subset of CD8⁺ T cells remains to be determined in future studies. One approach to this question would be to determine the functional fate and gene expression profile of a labeled population of in vivo activated CD8⁺ T cells (retrieved from CD4 KO hepatocyte recipients) that are subsequently adoptively transferred into CD4-sufficient vs control CD4-deficient hosts. If CD8⁺ allo-CTLs develop along a continuum in response to local stimuli including signals from CD4⁺ T cells, one would expect that CD8⁺CD43highCD44high T cells transferred into CD4-sufficient (C57BL/6) hepatocyte recipients would acquire the characteristics of CD8⁺ T cells developing de novo in CD4-sufficient hosts. In contrast, if CD4-independent CD8⁺CD43highCD44high T cells are terminally differentiated, there should be no difference in intracellular cytokine expression, expression of FasL vs perforin/granzyme molecules, gene expression profile, or in vivo CTL effector function between the populations when transferred into CD4-sufficient vs CD4-deficient hepatocyte recipients.

In nontransplant experimental models, CD4⁺ T cells clearly contribute to the development of enhanced secondary or memory CD8⁺ CTL and cytokine effector responses, although the mechanisms by which this occurs may vary (11–20, 63, 69, 70). In the current study, we found that the absence of CD4⁺ T cells significantly impairs the magnitude and persistence of alloreactive CD8⁺ CTL recall responses, consistent with observations made in other experimental systems (Fig. 6). CD4-sufficient secondary hepatocyte transplant recipients demonstrated an enhanced magnitude and persistence of CTL activity, whereas CD4-deficient recipients displayed secondary in vivo cytolytic effector responses that were similar in magnitude and kinetics to the primary CD8⁺-mediated CTL response. Despite the impaired development of enhanced secondary CTL responses in CD4-deficient recipients, both CD4-sufficient and CD4-deficient recipients demonstrated enhanced rejection kinetics in response to a second hepatocyte transplant. The accelerated kinetics of rejection, but not CTL activity, during the secondary response in CD4-deficient recipients could be explained by a reduced threshold for CTL activation without any enhancement in cytolytic differentiation, effector pool expansion, or CTL survival.

These studies used a hepatocellular allograft model to assess the influence of CD4⁺ T cells on the development of allospecific CD8⁺ CTL responses in vivo. The results support the concept that CD4-independent activation of alloreactive CD8⁺ T cells produces unique cytotoxic functional activity that is efficient in responding to and clearing donor Ag, but is not as potent in primary or memory in vivo cytolytic effector function when compared with CD8⁺ allo-CTLs, which mature in the presence of CD4⁺ T cells. A better understanding of alloreactive CD8⁺ T cells maturing in CD4-sufficient or CD4-deficient conditions is important for prevention of allograft rejection by these pathways as well as for design of immune tolerance strategies.

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