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Cytotoxic effector function of CD4-independent, CD8⁺ T cells is mediated by TNF- α /TNFR

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Conflict of Interest

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Abstract

Background—Liver parenchymal cell allografts initiate both CD4-dependent and CD4-independent, CD8⁺ T cell-mediated acute rejection pathways. The magnitude of allospecific CD8⁺ T cell *in vivo* cytotoxic effector function is maximal when primed in the presence of CD4⁺ T cells. The current studies were conducted to determine if and how CD4⁺ T cells might influence cytotoxic effector mechanisms.

Methods—Mice were transplanted with allogeneic hepatocytes. *In vivo* cytotoxicity assays and various gene-deficient recipient mice and target cells were utilized to determine the development of Fas-, TNF- α -, and perforin-dependent cytotoxic effectors mechanisms following transplantation.

Results—CD8⁺ T cells maturing in CD4-sufficient hepatocyte recipients develop multiple (Fas-, TNF- α -, and perforin-mediated) cytotoxic mechanisms. However, CD8⁺ T cells, maturing in the absence of CD4⁺ T cells, mediate cytotoxicity and transplant rejection that is exclusively TNF- α /TNFR-dependent. To determine the kinetics of CD4-mediated help, CD4⁺ T cells were adoptively transferred into CD4-deficient mice at various times posttransplant. The maximal influence of CD4⁺ T cells on the magnitude of CD8-mediated *in vivo* allocytotoxicity occurs within 48 hours.

Conclusion—The implication of these studies is that interference of CD4⁺ T cell function by disease or immunotherapy will have downstream consequences on both the magnitude of allocytotoxicity as well as the cytotoxic effector mechanisms employed by allospecific CD8⁺ cytolytic T cells.

Keywords

CD8⁺ T cells; FasL; TNF- α ; CD4⁺ T cells

Introduction

The prevailing dogma in transplant immunology is that CD8⁺ T cells mediate graft rejection through a perforin- and FasL-mediated cytotoxic mechanism (1–9). However, our previous results using a parenchymal cell transplant model suggest that this paradigm is likely too simplistic. We have previously reported that hepatocyte allografts initiate CD8-dependent rejection and *in vivo* allospecific cytotoxic effector function mediated by CD8⁺ T cells whose maturation is either CD4-dependent or CD4-independent (10, 11). While it is generally appreciated that CD8⁺ T cells require CD4⁺ T cell help for development of maximal effector function, CD8⁺ T cells can also be activated directly by antigen presenting cells without CD4⁺ T cell help when adjuvants or infectious agents are present (12–14). Although the contribution of CD4⁺ T cell help to CD8-mediated cytotoxic effector function is not clear, CD4⁺ T cells are known to contribute to CD8⁺ T cell expansion and, under some circumstances, facilitate trafficking to the site of inflammation (15, 16). In our model, allospecific CD8⁺ cytotoxic T lymphocytes (CTL) effector function is significantly enhanced in magnitude and persistence when primed in the presence of CD4⁺ T cells. However, our studies utilizing transgenic CD8⁺ T cells suggest that the enhanced cytotoxic effector activity generated in the presence of CD4⁺ T cells was not a result of enhanced proliferation, precursor frequency, or CD8⁺ T cell trafficking to the liver sinusoids (site of cellular transplantation) (17, 18). This led us to investigate the hypothesis that CD8⁺ T cell cytotoxic effector mechanisms which develop in CD4-replete conditions are fundamentally different, and perhaps more complex, from those which develop in CD4-deficient conditions. Additionally, we predicted that CD4⁺ T cell contribution to heightened *in vivo*

CD8-mediated cytotoxic effector function occurs early during transplant-initiated CD8⁺ T cell activation.

Results

CD8⁺ T cells activated in the presence of CD4⁺ T cells develop FasL-, TNF- α /TNFR, and perforin-mediated *in vivo* cytotoxic effector function

To investigate mechanisms of CD8⁺ T cell cytotoxic effector function in response to hepatocyte transplant, an *in vivo* cytotoxicity assay was used, as previously described (17). Cytotoxicity observed in this model is CD8-mediated, as allocytotoxicity was significantly reduced or eliminated following CD8⁺ T cell depletion just prior to the *in vivo* cytotoxicity assay (Figure 1A, 1B; $p < 0.0009$ for both) (17, 19). To investigate the role of FasL and TNF- α , the *in vivo* cytotoxicity assay was performed with wild-type, Fas KO, and TNFR1/II KO allogeneic (H-2^b) splenocyte targets. As previously documented (17), we find a significant difference in the magnitude of allocytotoxicity which develops by day 7 in wild-type (52 $\pm$ 8%) versus CD4-deficient recipient mice (CD4-depleted; 22 $\pm$ 4%; $p = 0.032$; Figure 1A, 1B). Wild-type FVB/N (H-2^q) recipients demonstrated FasL/Fas-mediated and TNF- α /TNFR-mediated cytotoxicity since cytotoxicity against Fas-deficient (25 $\pm$ 7%) and TNFR1/II-deficient targets (21 $\pm$ 5%) was significantly reduced in comparison to the *in vivo* cytotoxicity occurring with wild-type targets (52 $\pm$ 8%; $p = 0.003$ and $p = 0.01$, respectively; Figure 1A). Treatment of wild-type recipient mice with anti-TNF- α mAb (400 μ g, i.p., day 5, 6) partially inhibited allospecific cytotoxicity against wild-type targets (from 52 $\pm$ 8% to 32 $\pm$ 4%; $p = 0.042$). Concurrent *in vivo* treatment with anti-TNF- α mAb to inhibit TNF- α /TNFR cytotoxicity and use of Fas-deficient targets to impair FasL/Fas-mediated cytotoxicity did not reduce allocytotoxicity beyond the results with use of Fas-deficient targets alone (28 $\pm$ 4% vs. 25 $\pm$ 7%, respectively; $p > 0.05$). This suggests that CD4-dependent, CD8⁺ allospecific CTLs use both FasL and TNF- α (and perhaps other) mechanisms of cytotoxicity. In the absence of methods to interfere with perforin-mediated cytotoxicity *in vivo*, we tested CD8⁺ T cells from wild-type recipients for perforin-mediated cytotoxicity using an *in vitro* cytotoxicity assay (Cr⁵¹ release). Perforin-dependent allospecific cytotoxicity was readily detected (Supplemental Figure 1B, C). Of note, wild-type mice exhibit serum alloantibody on day 7 that may contribute to residual cytotoxicity, following CD8-depletion or TNF- α inhibition (20). CD4⁺ T cells do not mediate *in vivo* cytotoxicity, as previously reported (17).

CD8⁺ T cells activated in the absence of CD4⁺ T cells only develop TNF- α /TNFR *in vivo* cytotoxic effector function

In CD4-depleted FVB/N recipients, *in vivo* cytotoxicity was completely abrogated when TNFR1/II KO targets were used (0 $\pm$ 0%; $p = 0.0007$) in comparison to wild-type target cells (22 $\pm$ 4%). Treatment of CD4-deficient recipient mice with anti-TNF- α mAb (400 μ g, i.p., day 5, 6) significantly inhibited allospecific cytotoxicity against wild-type targets (from 22 $\pm$ 4% to 12 $\pm$ 2%; $p = 0.027$) but the inhibition was not as complete as observed for the TNFR1/II mutant targets. A possibility exists that the anti-TNF- α mAb treatment dosing regimen was insufficient to inhibit *in vivo* TNF- α -dependent cytotoxicity. However, this is unlikely since the same regimen significantly inhibited *in vivo* cytotoxicity in both wild-type and CD4-deficient recipients. Furthermore, we have performed additional higher dosing regimens with anti-TNF- α mAb (800 μ g), without additional inhibition of cytotoxicity (data not shown). When Fas-deficient targets were used, *in vivo* cytotoxicity (32 $\pm$ 8%) was not significantly different from that detected with wild-type control targets (Figure 1B). These results demonstrate that alloreactive CD4-independent CD8⁺CTLs use only TNF- α /TNFR as a cytotoxic effector mechanism. Under these conditions, alloantibody does not contribute to cytotoxicity since CD4-deficient mice do not produce detectable serum alloantibody (20).

***In vivo* CD8⁺ T cell cytotoxic effector function does not require perforin**

To determine the role of perforin-mediated cytotoxic mechanisms, FVB/N hepatocytes (H-2^q) were transplanted into wild-type (C57BL/6, H-2^b) and perforin KO (H-2^b) mice. A cohort of the recipients was CD4-depleted. Perforin KO recipients exhibited a similar degree of allocytotoxicity compared to wild-type mice (94±1% vs. 90±2%, respectively; Figure 2), suggesting that perforin is not critical to development or magnitude of *in vivo* CD8-mediated cytotoxicity. Of note, higher overall cytotoxicity is observed in C57BL/6 recipients (17) as compared to FVB/N recipients due to strain differences. Anti-TNF- α mAb reduces *in vivo* cytotoxicity in perforin KO recipients (84±2%; p=0.006). However, the overall magnitude of cytotoxicity remains high despite the absence of perforin and TNF- α , suggesting Fas/FasL-mediated cytotoxicity (and other mechanisms) are sufficient for CD8-mediated effector mechanism in wild-type recipients. In addition, in CD4-replete perforin KO recipients, alloantibody is detected and, in fact, serum levels are enhanced, as compared to alloantibody levels in wild-type recipients (21); this may account for some of the observed cytotoxicity. *In vivo* cytotoxicity in CD4-depleted wild-type (58±7%) and CD4-depleted perforin KO recipients (68±6%) were similar consistent with the interpretation that perforin is not critical to CD4-independent CD8-mediated cytotoxicity. In contrast, CD4-depleted perforin KO recipients treated with anti-TNF- α mAb have significantly reduced cytotoxicity (30±4%) as compared to control CD4-depleted perforin KO recipients (68±6%; p=0.01). These data, together with results in Figure 1B, demonstrate that *in vivo* CD8-mediated cytotoxic effector function in CD4-deficient recipients is critically dependent on TNF- α .

Hepatocellular allograft survival is enhanced following TNF- α inhibition in CD4-deficient recipient mice

We know from previous studies that hepatocyte rejection is T cell mediated either by CD4⁺ or CD8⁺ T cells (22). Hepatocyte rejection in CD4-deficient recipients is dependent on CD8⁺ T cells and transient CD8-depletion (100 μ g, day -4, -2) in CD4 KO recipients results in an enhanced hepatocyte allograft survival until repopulation of CD8⁺ T cells occurs (MST=35 days vs. 14 days, p=0.002). To investigate the correlation of CD8-dependent, TNF- α /TNFR-mediated *in vivo* allocytotoxicity with allograft rejection in CD4-deficient hosts, CD4 KO hepatocyte recipient mice were treated with anti-TNF- α mAb. We found that CD4 KO mice treated with anti-TNF- α mAb (400 μ g, daily i.p. injections days 5–20 posttransplant), demonstrated significant enhancement of hepatocyte survival as compared to untreated CD4 KO recipients (MST=24 vs. 14 days, p=0.002; Figure 3A). Anti-TNF- α mAb treatment (initiated 5 days posttransplant) was discontinued on day 20 and hepatocytes were rejected shortly afterwards consistent with the treatment targeting an effector mechanism. Anti-TNF- α mAb treated CD4-depleted wild-type mice exhibited similar enhanced allohepatocyte survival (data not shown) demonstrating that these results are not unique to the CD4 KO strain. An alternative interpretation for enhanced allograft survival by TNF- α -inhibition is that hepatocellular rejection is critically dependent on TNF- α -mediated cytotoxicity, both in the presence or absence of CD4⁺ T cells. To exclude this possibility we treated wild-type recipients with anti-TNF- α mAb (day 5–20); however, unlike results for CD4-deficient recipients, no enhancement of hepatocyte survival was observed (MST=10 days vs. 10 days in untreated wild-type recipients; Figure 3A). These data suggest that the beneficial effects of TNF- α blockade on allograft survival are unique to conditions where CD8⁺ T cells are alloactivated in the absence of CD4⁺ T cells.

To further investigate the importance of specific CD8-effector mechanisms on susceptibility of transplanted hepatocytes to rejection, studies using donor hepatocytes from wild-type, Fas mutant, or TNFRI/II KO (all H-2^b) were performed. Wild-type (CD4-sufficient) FVB/N recipients rejected hepatocyte transplants by day 14, regardless of donor strain (data not

shown). CD4-depleted recipients rejected wild-type and Fas mutant hepatocytes with a median survival time of 14 days posttransplant. In contrast, CD4-depleted recipients (250 μ g, day -4, -2, 7, 14) transplanted with TNFRI/II KO donor hepatocytes exhibited significantly enhanced hepatocellular allograft survival (MST=35 days, $p=0.003$, Figure 3B). Collectively, these results are consistent with the critical role of CD8-dependent TNF- α /TNFR-mediated cytotoxicity on hepatocyte rejection in CD4-deficient recipients.

CD4⁺ T cell enhancement of CD8-mediated cytotoxic effector function occurs rapidly

To determine if CD8⁺ T cells in CD4 KO mice have the capacity to develop high magnitude cytotoxicity as observed in wild-type recipients, we performed CD4⁺ T cell reconstitution of CD4 KO mice. Allogeneic hepatocytes (FVB/N) were transplanted into CD4 KO mice on day 0. CD4⁺ T cells were adoptively transferred on day -1. We determined that 10×10^6 column purified CD4⁺ T cells from wild-type mice were sufficient to promote the development of higher magnitude allocytotoxicity as observed in wild-type recipients (95% cytotoxicity, Figure 4 and data not shown). In order to determine the kinetics of CD4⁺ T cell help for CD8⁺ T cell effector maturation, we varied the time of adoptive transfer of CD4⁺ T cells into CD4 KO mice relative to the time of hepatocyte transplant on day 0. 10×10^6 column purified CD4⁺ T cells were adoptively transferred into recipient groups on days -1, +1, +2, +3, +5, and +6 with respect to the hepatocyte transplant. Recipient mice were monitored for *in vivo* cytotoxicity on day 7 posttransplant by using allogeneic (FVB/N) and syngeneic (C57BL/6) target cells. *In vivo* cytotoxicity assays in mice adoptively transferred with CD4⁺ T cells on days -1, 1, 2, 3, and 5 all showed significantly higher mean values of cytotoxicity (95%, 84%, 76%, 82%, and 88%, respectively) than control CD4 KO mice (17%; $p<0.0002$) and equivalent mean values to control wild-type mice (Figure 4). The adoptive transfer of CD4⁺ T cells into CD4 KO mice on day +6 posttransplant also exhibited higher cytotoxicity (53%; $p=0.0001$) than CD4 KO recipients, however, significantly less cytotoxicity when compared to wild-type recipients ($p<0.0001$). Thus, CD4⁺ T cells require at least 48 hours to maximally enhance the magnitude of CD8-mediated *in vivo* allocytotoxicity.

Discussion

Activation of CD8⁺ T cells independently of CD4⁺ T cell help has been observed in many experimental models, such as tumor immunity (23), autoimmune diabetes (24), contact hypersensitivity (25), and allograft rejection (including intestinal, skin, cardiac, and hepatocellular transplantation (11, 26–35)). In fact, activation of alloreactive (CD4-independent) CD8-dependent immune responses has increasingly been identified as a major barrier to the establishment and maintenance of long-term allograft acceptance in both experimental and clinical settings (36–38). The activation of the CD8-dependent pathway of rejection is highly resistant to immunoregulation (26, 27, 29–33, 35, 39) and immunotherapeutic agents which readily suppress CD4-dependent rejection (27, 29, 31, 34, 40–42). For example, skin, cardiac, and intestinal allograft rejection have been observed to occur by CD8⁺ T cells activated despite interruption of CD28 and CD40L signaling, a powerful experimental “costimulatory blockade” treatment approach which effectively controls CD4⁺ dependent immune pathways (27, 29, 43–45).

The hepatocellular allograft model coupled with *in vivo* and *in vitro* cytotoxicity assays were used to investigate the CTL effector mechanisms for CD8⁺ T cells which are primed in a CD4-sufficient versus CD4-deficient environment. CD4-sufficient allograft recipients demonstrate CD8⁺CTL effector function mediated by FasL, TNF- α and perforin whereas CD4-deficient allograft recipients utilize only TNF- α -mediated CTL activity. The perforin/granzyme CTL lytic mechanism has been reported to mediate more efficient killing than FasL-mediated cytotoxicity (46, 47) and has been shown to require stronger activation

signals than are required to activate the FasL cytotoxicity pathway (48). In these studies, we further show that CD8⁺ T cells develop TNF- α /TNFR-mediated cytotoxicity in response to transplant even without CD4⁺ T cell help; however, CD4⁺ T cells can further promote the development of multiple cytotoxic effector mechanisms by alloreactive CD8⁺CTLs. Altogether these data suggest a hierarchy of *in vivo* CD8-dependent CTL mechanisms which range in complexity and which contribute to overall magnitude of allocytotoxicity.

These studies demonstrate the vulnerability of allogeneic hepatocytes to CD8-mediated TNFRI/II-dependent acute rejection. Prolongation of hepatocyte survival could be achieved in CD4-deficient recipients by treatment with anti-TNF- α mAb (Figure 3A). Similarly, TNFRI/II-deficient (unlike Fas-deficient) hepatocytes were protected from acute rejection in CD4-deficient recipients despite the absence of immunosuppression (Figure 3B). However, rejection eventually occurred by day 42 posttransplant in all recipients. Rejection in these recipients may have been due to non-TNFRI/II-dependent immune mechanisms associated with repopulating CD4⁺ T cells or to NK cells which have been reported to mediate cytotoxicity in CD4-independent conditions in the setting of anti-tumor immunity or co-stimulatory blockade resistant bone marrow rejection (49, 50). However, we suspect that repopulating CD4⁺ T cells are the dominant cells responsible for delayed rejection since we have previously reported that NK cell-depletion does not delay rejection or reduce *in vivo* cytotoxicity in the absence of CD4⁺ T cells or CD8⁺ T cells (33, 51). Interestingly, Langrehr *et al.* have also shown that anti-CD4 and anti-TNF- α mAb treatment leads to enhanced survival of allogeneic intestinal transplants (52). Clinical studies using infliximab (anti-TNF- α mAb) to suppress ongoing rejection have shown that blocking TNF- α has efficacy in steroid-resistant intestinal transplant recipients (53, 54). It is possible that the prolongation of intestinal allograft survival by TNF- α -inhibition was due to suppression of a CD4-independent, CD8-mediated response that we and others have shown to be resistant to therapies that readily control CD4-dependent rejection responses (31–33, 35, 39).

Although perforin is an important cytotoxic effector mechanism (46, 47), our studies show that perforin is not required for *in vivo* alloreactive CD8⁺ T cell mediated cytotoxicity (Figure 2) or hepatocyte rejection (data not shown) since both readily occur in perforin KO recipients. These findings regarding perforin are consistent with results in other transplant models (3, 55, 56). Our data shows that CD8 effector function (without CD4⁺ T cells) is TNF-dependent but perforin- and FasL-independent. CD8 effector function in CD4-replete recipients is FasL- and TNF-dependent (Figure 1A) and perforin independent (Figure 2). Our explanation for these data is based on alternate mechanisms known to mediate CD8-dependent cytotoxicity and on small amounts of alloantibody known to occur in wild-type recipients. For example, CD8⁺ T cell use of TRAIL, TWEAK, and/or TL1A (57) could account for the observation that cytotoxicity is not further suppressed by interference with both FasL/Fas and TNF- α /TNFR (Figure 1A) or both perforin and TNF- α /TNFR (Figure 2) cytotoxic mechanisms. In addition, cytotoxicity from alloantibody in CD4-replete wild-type recipients (no antibody in CD4-depleted recipients) could also account for detectable *in vivo* cytotoxicity. We don't think CD8⁺ memory cells contribute to *in vivo* cytotoxicity in these studies based on previous work showing that memory responses require a second transplant and that the magnitude and kinetics of *in vivo* cytotoxicity are quite different after a primary and secondary transplant in wild-type recipients (17). When testing for *in vitro* allocytotoxicity, we only detect perforin-mediated killing by CD8⁺ splenocytes from wild-type but not from CD4-deficient recipients (Supplemental Figure 1A & 1B). *In vitro* assays preferentially reflect perforin-mediated cytotoxic effector mechanisms as previously noted (58, 59) and accounts for the failure to detect *in vitro* cytotoxicity by CD8⁺ T cells from CD4-deficient recipients.

Our findings that transplanted cells initiate multiple CD8⁺CTL cytotoxic effector mechanisms are consistent with results in other transplant as well as non-transplant experimental systems. For example, it has been reported that Fas/FasL (60–62), TNF- α /TNFR (60, 63), and perforin/granule release (61) are cytotoxic mechanisms that participate in CD8⁺ T cell-dependent hepatic viral clearance. These studies, however, did not investigate the influence of CD4⁺ T cells on the resultant CD8⁺ T cell *effector* mechanism. Borson *et al.* has reported that following skin transplantation, graft infiltrating cells express FasL, TNF- α , and perforin mRNA. Interestingly, they also found that following CD4-depletion, graft infiltrating cells express predominantly TNF- α mRNA compared to perforin and FasL mRNA (64). However, they did not determine what cells expressed these molecules. Our study is the first to show that CD8-mediated cytotoxic effector mechanisms which develop in the presence of CD4⁺ T cells are fundamentally different and more complex than those that develop in the absence of CD4⁺ T cell help. Immunotherapy to interfere with cytotoxic effector mechanisms mediating ongoing rejection will likely require a multifaceted and targeted approach.

In testing the kinetics of CD4-mediated help, we found that the adoptive transfer of CD4⁺ T cells into CD4 KO mice on day 0, at the time of transplant, resulted in the development of wild-type allocytotoxicity (which peaks on day 7 post-transplant). Surprisingly adoptive transfer of CD4⁺ T cells on day 5 after transplant was also sufficient to promote wild-type magnitude of cytotoxicity even when tested for cytotoxicity just 48 hours after CD4 transfer (on day 7 after transplant; Figure 4). Adoptive transfer of CD4⁺ T cells on day 6 after transplantation only partially restores CD8-mediated cytotoxicity suggesting that (while 48 hours is sufficient), 24 hours is insufficient duration for CD4⁺ T cell activation and delivery of help to CD8⁺ T cells. These studies indicate that CD4⁺ T cell contribution to CD8⁺ T cell allocytotoxicity occurs rapidly and is not necessarily required at the time of initial antigen exposure. Other recent studies which have investigated kinetics of CD4⁺ T cell interaction with CD8⁺ T cells *in vivo* have also concluded that these interactions occur rapidly (65–68).

Since CD8⁺ allo-CTLs cells exhibit distinct effector mechanisms in the presence or absence of CD4⁺ T cells, an interesting question is what precursors contribute to the development of these functionally disparate CTL subsets. Experiments to address this question, as well as specific mechanisms of CD4-help required for distinct CD8⁺ effector cells, are in progress. Finally, while the experimental models to compare maturation of alloreactive CD8⁺ T cells in wild-type and CD4-deficient hosts represent extreme differences in host immune repertoire, they are ultimately designed to reflect CD8-dependent immune outcomes in humans with CD4⁺ T cell function impaired by genetic deficiencies, acquired disease, and/or immunotherapies. Understanding the effector mechanisms activated under specific conditions could promote the development of novel immunotherapies that would avoid global perturbation of the immune system by targeting specific effector pathways.

Materials and Methods

Experimental animals

FVB/N (H-2^q MHC haplotype, Taconic), C57BL/6, CD4 KO, Fas mutant, TNFR I/II dKO, and Perforin KO (all others H-2^b, Jackson Laboratory) mouse strains (all 6–10 weeks of age) were used in this study. Transgenic FVB/N and C57BL/6 mice expressing human alpha-1 antitrypsin (hA1AT) were the source of “donor” hepatocytes, as previously described (33, 69). All experiments were performed in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee of The Ohio State University (Protocol 2008A0068).

Hepatocyte transplantation

Hepatocyte isolation and purification were performed, as described previously (33, 69). Hepatocyte viability and purity has been determined to be consistently >95%. Recipients (non-transgenic) were transplanted with 2×10^6 purified allogeneic hA1AT⁺ hepatocytes and graft survival was monitored as previously described (33, 69).

Antibodies used for *in vivo* T cell subset depletion

CD4⁺ T cells were depleted by intraperitoneal injections of anti-CD4 mAb (250 µg, day -4,-2; clone GK1.5; Bioexpress, West Lebanon, NH). CD8⁺ T cell were depleted by intraperitoneal injections of anti-CD8 mAb (100 µg, day -1,-2; clone 53.6.72; Bioexpress). Depletion was confirmed through flow cytometric analysis of recipient peripheral blood lymphocytes.

In vivo cytotoxicity assay

Detection of *in vivo* cytolytic T cell function through clearance of Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE; Invitrogen, Carlsbad, CA) stained allogeneic (CFSE^{high}) and syngeneic (CFSE^{low}) target cells was performed as previously described (17, 51). Cohorts of these recipients were also treated with anti-TNF-α mAb (clone MP6-XT2.2-11, 400µg, i.p.; National Cell Culture Center, Minneapolis, Minnesota) just prior to the *in vivo* cytotoxicity assay (day -1 and 0 with respect to the cytotoxicity assay). Syngeneic and allogeneic target cells (20×10^6 each) were injected into allograft and control mice. Spleens were harvested eighteen hours after injection, analyzed by flow cytometry for CFSE⁺ splenocytes, and percent allospecific cytotoxicity was calculated, as described (17).

CD4⁺ T cell isolation and purification

Isolation and purification of CD4⁺ T cells was performed via negative selection column as per the manufacturer's recommendations (R&D Systems, Minneapolis, MN). Briefly, splenocytes were treated with an antibody cocktail, bound to a column, and the non-antibody bound cells were eluted. The purity of the recovered CD4⁺ T cells ranged from 90% to 93%.

Statistical analysis

Graft survival between experimental groups was compared using Kaplan Meier survival curves and log-rank statistics (Predictive Analytics SoftWare, SSPS Inc.). Other statistical calculations were performed using Student's t test to analyze differences between experimental groups. $P < 0.05$ was considered significant. To demonstrate the distribution of the data, results are listed as the mean plus or minus the standard error.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations

hA1AT human alpha-1 antitrypsin

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Figure 1. *In vivo* CD8-dependent cytotoxicity is mediated by FasL and TNF- α in wild-type recipients but only TNF- α in CD4-deficient recipients
 C57BL/6 hepatocytes (H-2^b) were transplanted into FVB/N (H-2^q) recipients that were untreated (CD4-dependent CD8-mediated allograft toxicity) or CD4-depleted (CD4-independent CD8-mediated allograft toxicity) prior to transplantation. *In vivo* cytotoxicity

was performed with different allogeneic targets: wild-type (susceptible to all mechanisms of cytotoxicity, wt); Fas mutant (resistant to FasL-mediated killing); or TNFR I/II KO (resistant to TNF- α -mediated killing) (all H-2^b). **A)** In wild-type hosts, cytotoxic activity was significantly decreased against both Fas mutant ($25\pm 7\%$, $n=5$; $p=0.003$) and TNFR I/II KO targets ($21\pm 5\%$, $n=5$; $p=0.01$) relative to wild-type ($52\pm 8\%$, $n=7$) targets. **B)** In contrast, CD4-deficient hosts demonstrated similar killing of Fas mutant ($32\pm 8\%$, $n=3$; $p>0.05$) as compared to wild-type targets ($22\pm 4\%$, $n=4$), but significantly reduced killing of TNFR I/II KO targets ($0\pm 0\%$, $n=5$; $p=0.0007$). Similar results were observed in wild-type and CD4-deficient recipients when utilizing anti-TNF- α mAb treatment ($400\mu\text{g}$, i.p., day 5, 6) to block this effector mechanism ($n=4$). Further downregulation of cytotoxicity was not observed in wild-type recipients when both FasL and TNF- α mechanisms were blocked ($28\pm 4\%$, $n=4$; Fas mutant targets and recipients treated with anti-TNF- α mAb) in comparison to control conditions of Fas mutant targets alone or TNF- α inhibition alone. Cytotoxicity was reduced or eliminated when CD8⁺ T cells were depleted ($100\mu\text{g}$, day 5, 6) prior to the *in vivo* cytotoxicity assay in both untreated and CD4-depleted recipients, respectively ($p<0.0009$ for both). Significance is depicted by a “*” ($p<0.05$).

Figure 2. *In vivo* CD8⁺ T cell cytotoxic effector function does not require perforin

Wild-type (C57BL/6, H-2^b) and perforin KO (H-2^b) mice were transplanted with FVB/N (H-2^q) hepatocytes. Cohorts of the recipients were depleted of CD4⁺ T cells prior to transplant (days -4 and -2). *In vivo* cytotoxicity was performed with wild-type (FVB/N) allogeneic targets. Perforin KO recipients (94±1%, n=3) exhibit similar cytotoxicity to wild-type mice (90±2%, n=10). Anti-TNF- α mAb reduces cytotoxicity in perforin KO recipients (84±2%, n=3; p=0.006). CD4-depleted wild-type (58±7%; n=4) and CD4-depleted perforin KO recipients (68±6%, n=3) exhibit similar cytotoxicity. However, CD4-depleted perforin KO recipients treated with anti-TNF- α mAb have significantly reduced cytotoxicity (30±4%, n=3) as compared to CD4-depleted perforin KO recipients (68±6%; p=0.01, as denoted by “*”).

Figure 3. Hepatocellular allograft survival is enhanced following TNF- α inhibition in CD4-deficient recipient mice

A) FVB/N hepatocyte allografts were transplanted into wild-type and CD4 KO (H-2^b) hosts. On day 5 posttransplant, a cohort of recipients were treated with anti-TNF- α mAb (n=4). Some recipients were left untreated for comparison. Wild-type recipients rejected hepatocytes with a mean survival time of 10 days posttransplantation in both untreated (n=15) or anti-TNF- α mAb treated (n=4) recipients. In CD4 KO recipients, treatment with anti-TNF- α mAb (discontinued on day 20) resulted in significant enhancement of hepatocyte survival (MST=24 days; n=4) as compared to untreated mice (MST=14;

p=0.002; n=10). **B)** To further determine the role of TNF- α /TNFR interactions, additional studies were performed with wild-type, Fas mutant, or TNFRI/II KO hepatocytes. Hepatocyte survival in untreated or CD4-depleted FVB/N recipients were monitored over time. CD4-sufficient recipients rejected wild-type, Fas mutant and TNFRI/II KO hepatocytes with median survival time of 10, 14, and 10 days, respectively (not shown). CD4-depleted recipients rejected wild-type and Fas mutant hepatocytes with a median survival time of 14 days posttransplant (n=3 for both). In contrast CD4-depleted recipients demonstrate prolonged survival of TNFRI/II KO donor hepatocytes (MST=35 days, n=3; p=0.003).

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Figure 4. CD4⁺ T cells are not required at the time of transplant to develop maximal magnitude of CD8-mediated cytotoxic effector function

Allogeneic hepatocytes from FVBN mice were transplanted into either wild-type or CD4 KO mice on day 0. 10×10^6 CD4⁺ T cells were adoptively transferred on day -1, +1, +2, +3, +5, or +6 ($n = 3$ for all time points) into CD4 KO mice. The *in vivo* cytotoxicity assay was performed on day 7. The allogeneity detected in all the CD4 KO recipient mice receiving adoptive transfer of CD4⁺ T cells was significantly higher than that of CD4 KO mice without adoptive transfer (collectively, $p < 0.0002$, as denoted by “*”; “dots” represent individual samples). However, recipient CD4 KO mice receiving adoptive transfer of CD4⁺ T cells on day +6 posttransplant exhibited significantly less cytotoxicity than control wild-type recipients ($p < 0.0001$, as denoted by “†”).