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Critical Role of Effector Macrophages in Mediating CD4-Dependent Alloimmune Injury of Transplanted Liver Parenchymal Cells

Phillip H. Horne,*† Jason M. Zimmerer,* Mason G. Fisher,* Keri E. Lunsford,† Gyongyi Nadasdy,‡ Tibor Nadasdy,‡ Nico van Rooijen,§ and Ginny L. Bumgardner2*

Despite the recognition that humoral rejection is an important cause of allograft injury, the mechanism of Ab-mediated injury to allograft parenchyma is not well understood. We used a well-characterized murine hepatocellular allograft model to determine the mechanism of Ab-mediated destruction of transplanted liver parenchymal cells. In this model, allogeneic hepatocytes are transplanted into CD8-deficient hosts to focus on CD4-dependent, alloantibody-mediated rejection. Host serum alloantibody levels correlated with in vivo allospecific cytotoxic activity in CD8 knockout hepatocyte rejector mice. Host macrophage depletion, but not CD4+ T cell, NK cell, neutrophil, or complement depletion, inhibited in vivo allocytotoxicity. Recipient macrophage deficiency delayed CD4-dependent hepatocyte rejection and inhibited in vivo allocytotoxicity without influencing alloantibody production. Furthermore, hepatocyte coinoculation with alloantibody and macrophages resulted in Ab-dependent hepatocellular cytotoxicity in vitro. These studies are consistent with a paradigm of acute humoral rejection in which CD4+ T cell-dependent alloantibody production results in the targeting of transplanted allogeneic parenchymal cells for macrophage-mediated cytotoxic immune damage. Consequently, strategies to eliminate recipient macrophages during CD4-dependent rejection pathway may prolong allograft survival. The Journal of Immunology, 2008, 181: 1224–1231.

Acute Ab-mediated allograft rejection is an important cause of solid organ graft dysfunction and failure. Complement-split product deposition at the graft site has become an important component of the pathologic diagnostic criteria for Ab-mediated graft damage (1). C4d deposition correlates with the occurrence of renal (2–5), lung (6, 7), liver (8–10), and cardiac (11–13) allograft loss, suggesting that complement is a key mediator of graft tissue destruction. The concurrent presence of innate and adaptive immune cellular infiltrates at the graft site reported in many studies in which complement deposition is observed and humoral immunity is operative (14–20), as well as known Ab-dependent cellular cytotoxicity (ADCC)3 mechanisms (21), bring into question the role of graft-infiltrating cells in alloantibody-mediated damage to the graft parenchyma.

We have previously reported that liver parenchymal cell (hepatocellular) allografts initiate a robust humoral immune response (especially when CD8+ T cell-mediated immunity is perturbed) which is sufficient to mediate hepatocyte rejection in a dose-dependent fashion. Acute rejection in CD8-deficient hosts is CD4+ T cell dependent, B cell dependent, and allospecific (22). The histology associated with alloantibody-mediated hepatocellular allograft rejection is remarkable for absence of injury to the native liver tissue. Hepatocellular allografts are distinguished from solid organ allografts by the absence of donor endothelium intervening between the vasculature and graft parenchymal cells. Consequently, the target of humoral alloimmunity after hepatocyte transplant is not donor endothelium, suggesting that alloantibody is able to target donor liver parenchymal cells specifically and clear them efficiently. A possible mechanism for this selective targeting and destruction of donor parenchymal cells include complement activation and resultant membrane attack complex-mediated lysis of the donor cells. Another mechanism, ADCC, involves other immune cell mediators, such as NK cells, macrophages, and neutrophils. These mechanisms are relevant to clinical cell (islet (23, 24) and bone marrow (25)) and solid organ allografts in which alloantibodies are a known barrier to early and long-term graft survival (14–20).

The current studies were performed to determine the in vivo mechanism of alloantibody-mediated damage of transplanted liver parenchymal cells. We found that host macrophages, and not complement, CD4+ T cells, NK cells, or neutrophils, were critical for in vivo-allospecific cytotoxic effector function generated during CD4-dependent Ab-mediated hepatocyte allograft rejection (CD8 KO recipients). The role of host macrophages as cellular effectors of Ab-mediated graft rejection was supported using three experimental approaches including the CD8-depleted macrophage “deficient” op/op host, macrophage depletion of a CD8 KO host, and an in vitro cytotoxicity assay in which hepatocellular cytotoxicity was determined in the presence of alloantibody, macrophages, or...
both alloantibody and macrophages. Therapies designed to limit or block interactions between alloantibody and host macrophages could prevent graft injury by humoral mechanisms which can occur despite effective control of T cell-mediated rejection responses.

Materials and Methods

Experimental animals

FVB/N (H-2b; Taconic Farms), CD8 KO (H-2b, C57BL/6Cdbtm1Mak, The Jackson Laboratory), and oopop osteopetrosis (B6C3Fe ala-Csf1op/Csf1op, H-2b, a gift from Dr. C. Marsh, Ohio State University) mouse strains were used in this study. Transgenic FVB/N mice expressing human α1-antitrypsin (hA1AT-FVB/N, H-2b) were the source of “donor” hepatocytes. This strain was created, bred, and maintained at the Biotechnology Center and Transgenic Animal Facility (Ohio State University) (26, 27). Mice were 6–9 wk of age were used in the experiments. All experiments were performed in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee of the Ohio State University (Protocol 2007A0071).

Hepatocyte isolation and purification

Hepatocyte isolation and purification was performed as described previously (26, 27). Briefly, the liver was perfused with 0.09% EGTA-containing calcium-free salt solution followed by a 0.05% collagenase solution (type IV; Sigma-Aldrich) in 1% albumin. Liver tissue was minced, filtered, and washed in RPMI 1640 with 10% FBS. Hepatocytes were purified on a 50% Percoll gradient (Pharmacia Biotech). Hepatocyte viability and purity were both consistently >99%.

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 (27). Graft function was determined by detection of the secreted transgenic reporter product hA1AT in serial recipient serum samples. Graft survival was reflected by stable and persistent serum hA1AT levels, whereas graft rejection was reflected by rapidly decreasing serum hA1AT (usually over 5–7 days) to undetectable levels (<0.5 μg/ml) (26, 27).

In vivo host treatment

Recipient mice were depleted of circulating CD4+ T cells, CD8+ T cells, NK cells (NK1.1-), or neutrophil (Ly6G+) cells using mAbs. Anti-CD4 (GK1.5), anti-CD8 (53.6.72), and anti-Ly6G (RB6-8C5) were obtained from Bioexpress Cell Culture Services. Anti-NK1.1 (PK136) mAb was purified from ascites produced in pristane-primed mice. Hepatocyte recipients (as well as nontransplanted control mice) were depleted of CD4+ T cells, NK cells, or neutrophils by i.p. treatment with 250 μg of anti-CD4, 300 μg of anti-NK1.1, or 100 μg of anti-Ly6G mAbs, respectively, 48 h before the in vivo cytotoxicity assay. Depletion was confirmed through flow cytometric analysis of recipient splenocytes. MCFSF−/− (oopop) and wild-type littermate recipient mice were depleted of CD8+ T cells using anti-CD8 mAb (300 μg i.p.) on days −4, −2, 7, and 14 relative to hepatocyte transplant. Depletion was confirmed through flow cytometric analysis of PBLs.

Recipient macrophages were depleted through i.p. injection of liposome-encapsulated clodronate. To determine the contribution of host macrophages to in vivo cytotoxic effector function, hepatocyte recipients were depleted of host macrophages (0.2 ml of liposome clodronate i.p.) 48 h before the in vivo cytotoxicity assay. To determine the role of host macrophages in the effector phase of hepatocyte rejection, CD8 KO hepatocyte recipients were depleted of host macrophages (0.2 ml of liposome clodronate i.p.) on days 5, 9, 13, 17, and 21 posttransplant while monitoring graft survival. Liposome clodronate and control liposomes containing only PBS were prepared as previously described (28). Clodronate was a gift from Roche Diagnostics. Depletion of macrophages was confirmed through flow cytometric analysis of F4/80+ (C1: A3-1, Caltag Laboratories) cells in recipient splenocytes.

Host complement was depleted through i.p. treatment of 25 μg of cobra venom factor (Naja melanoleuc: Venom Supplies). Host depletion of complement was confirmed through reduction in hemolysis of Ab-sensitized sheep erythrocytes in gelatin Veronal buffer according to the manufacturer’s instructions (Sigma-Aldrich).

In vivo cytotoxicity assay

An in vivo cytotoxicity assay, initially designed to detect cytolytic T cell function in vivo through clearance of CFSE-stained allogeneic and syngeneic target cells, has been previously described (29). Syngeneic target splenocytes from C57BL/6 mice were stained with 0.2 μM CFSE (Molecular Probes). Allogeneic target splenocytes from FVB/N mice were stained with 2.0 μM CFSE (CFSE−/−). Equal numbers of CFSE-labeled syngeneic and allogeneic target splenocytes (20 × 106 each, mixed 1:1) were injected into the tail veins of allograft recipient and control untransplanted mice. Eighteen hours after CFSE-labeled target cell injection, splenocytes from hepatocyte recipients were retrieved and analyzed by flow cytometry, gating on CFSE-positive splenocytes. Percent allospecific cytotoxicity was calculated using the following formula where no. of CFSElow+ represents the number of allogeneic target cells and no. of CFSElow+<wbr/> Experimental represents the number of syngeneic target cells recovered from either untransplanted or experimental mice:

\[
\text{% Specific lysis} = \frac{\text{CFSElow naive} - \text{CFSElow experimental}}{\text{CFSElow naive} + \text{CFSElow experimental}} \times 100
\]

Donor-reactive Ab

Host serum was assayed for the presence of donor-specific IgG alloantibodies by incubating serum (diluted 1/10) obtained 14 days after transplant with FVB/N target splenocytes followed by incubation with FITC-conjugated goat F(ab’)2, anti-mouse IgG Fc (Organon Teknika) and analysis by FACS. Alloantibody level is represented as the percentage of target cells labeled by secondary fluorescent Ab as described previously (22).

Immunohistochemistry

Tissue was snap frozen in liquid nitrogen, fixed in cold acetone, quenched in hydrogen peroxide solution to block endogenous peroxidase activity, and endogenous biotin was blocked using a biotin-blocking system (DakoCytomation). For detection of donor hepatocytes engrafted within recipient liver parenchyma, the polyclonal rabbit anti-human α1-antitrypsin Ab was used (DakoCytomation). Biotinylated goat anti-rabbit IgG (H + L; Vector Laboratories BA-5000) secondary Ab (1/2000) was used before the Vectastain Elite ABC Kit (Vector Laboratories PK-7100) for detection. Liver macrophages (F4/80+ cells) were detected with rat anti-mouse F4/80 Ab (1/25; Serotec) and goat anti-rat IgG, HRP (mouse adsorbed, 1/25; Serotec). Both anti-hA1AT or anti-F4/80 Abs were visualized with liquid diaminobenzidine-positive substrate (DakoCytomation). Slides were counterstained with Richard-Allen hematoxylin and the histology was assessed by blinded analysis.

In vitro cytotoxicity assay

Purified mouse FVB/N allogeneic hepatocytes (H-2b) were incubated for 30 min (37°C) with serum from naive or repressor CD8 KO mice (H-2b; 14 days after hepatocyte transplantation). Aliquots of hepatocytes were then added to 12-well plates with 10% FBS medium. Macrophages were harvesteds from C57BL/6 mice (H-2b) as previously described (30). Briefly, 1 ml of 4% Brewer thioglycolate medium was injected into the peritoneal cavity of wild-type mice. Five days later, the peritoneal cavity was washed with 10 ml of PBS. Subsequently, the peritoneal exudate cells were plated for 1 h and the adherent macrophages were collected (85–95% purity by CD68 flow cytometry; data not shown). Macrophages were then added to the wells at various E:T ratios (10:1 or 40:1). Following the addition of

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effector function, such as CD4+ T cells or NK cells. FVB/N (H-2b) hepatocytes were transplanted into untreated CD8 KO (H-2b) mice. After graft rejection occurred (MST = 14 days), CD8 KO hosts were left untreated (no Rx), or were depleted of CD4+ T cells (CD8 KO anti-CD4), NK cells (CD8 KO anti-NK1.1), or both CD4+ T cells and NK cells (CD8 KO anti-CD4 anti-NK1.1) 48 h before the in vivo cytotoxicity assay. The bars represent average cytotoxicity ± SD for each group. Individual cytotoxicity values are represented by the circles. Alloreactivity in all groups was statistically similar (p, NS).

macrophages, hepatocytes were cultured for an additional 8 h at 37°C. Supernatants were then collected and analyzed for alanine transaminase (ALT) release (Department of Clinical Laboratories, Ohio State University).

Statistical analysis

Graft survival between experimental groups was compared using Kaplan-Meier survival curves and log-rank statistics (SPSS). Other statistical calculations were performed using Student’s t test to analyze differences between experimental groups. A value of p < 0.05 was considered significant.

Results

Recipient serum alloantibody levels correlate with the magnitude of in vivo cytotoxic effector function in CD8 KO hepatocyte rejector mice

We have previously reported that in the absence of host CD4+ T cells (CD8 KO, CD8+ T cell-depleted C57BL/6, and SCID hosts reconstituted with CD8-depleted splenocytes), rejection of hepatocellular allografts is CD4+ T cell dependent and mediated by alloantibody (22, 26). These studies prompted further analysis of the mechanism of Ab-mediated allogeneic parenchymal cell damage. Untreated CD8 KO (H-2b) recipients were transplanted with FVB/N (H-2b) hepatocellular allografts and monitored for graft rejection which occurred, as in previous studies, with median survival time (MST) of 14 days (26). Following rejection, the hosts were tested for in vivo cytotoxic effector function using an in vivo cytotoxicity assay by the adoptive transfer of syngeneic and allogeneic target splenocytes. This assay was originally designed to detect CD8+ T cell- or NK cell-mediated cytotoxicity activity in vivo (31, 32). Despite the absence of the CD8+ cytotoxic T cell subset, CD8 KO hepatocellular allograft rejector mice developed potent cytotoxic effector activity (n = 16; Fig. 1). All CD8 KO hepatocyte rejector mice generated detectable levels of alloantibody in recipient serum. To determine whether this cytotoxic activity was mediated by immune cells capable of cytotoxic effector function, such as CD4+ T cells or NK cells, CD8 KO hepatocyte hosts that had rejected hepatocyte allografts were depleted of CD4+ T cells (GK1.5) and/or NK cells (PK136) 48 h before the in vivo cytotoxicity assay. The high magnitude cytotoxic activity generated in CD8 KO transplant rejectors was not significantly decreased in the absence of host CD4+ T cells (n = 19), NK cells (n = 4), or both CD4+ T cells and NK cells (n = 5), in comparison to nondepleted rejectors (p, NS; Fig. 1). We noted a small decrease in cytotoxicity with CD4 depletion, although the effect was not statistically significant. Nevertheless, in subsequent cytotoxicity experiments, all hosts were depleted of CD4+ T cells to remove any potential cytotoxic contributions from CD4+ T cells and to clarify interpretation of data.

In previous studies, we reported that CD4-dependent rejection in CD8 KO recipients is accompanied by a variable level of alloantibody production (22). In the current studies, we also detected a variable level of alloantibody in CD8 KO hepatocyte rejectors (Fig. 1). To determine the correlation between recipient serum alloantibody and the magnitude of in vivo alloreactivity, we measured the serum alloantibody levels (day 14 posttransplant) and in vivo cytotoxic activity in CD8 KO hepatocyte rejectors (1 wk postrejection). A group of these CD8 KO rejector recipients were depleted of CD4+ T cells 48 h before the cytotoxicity assay. Fig. 2 shows that CD8 KO hepatocyte rejectors (with or without CD4+ T cell depletion) could be separated into groups with “high” (>60%, n = 10) or “low” (<60%, n = 6) serum alloantibody levels. Some hepatocyte rejectors with high (n = 8) or low (n = 4) alloantibody levels were also depleted of CD4+ T cells (anti-CD4) before the in vivo cytotoxicity assay to eliminate the potential contribution of CD4+ T cells to alloreactivity. Serum alloantibody levels in high compared with low alloantibody groups were statistically different (p < 0.0001) and are represented by the circles with the black lines indicating the averages. The average cytotoxicity ± SD for hepatocyte rejector mice with high or low serum alloantibody levels (with or without recipient CD4+ T cell depletion) is represented by the bars. Hosts with high levels of alloantibody demonstrated significantly higher levels of cytotoxicity than hosts with low Ab levels (p < 0.0001 for undepleted as well as CD4-depleted groups).
In vitro cytotoxic effector function in CD8 KO rejector mice does not require complement

Activation of the classical complement cascade through cross-linking Abs on the surface of a target cell can result in lysis of the target cell through formation of the membrane attack complex. This potential mechanism in addition to evidence that complement is involved in Ab-mediated rejection in other experimental models (17, 33–39) prompted our investigation of whether complement is critical to the in vivo cytotoxic effector mechanism observed in CD8 KO hepatocyte rejector mice. Early and/or late phases of the classical complement cascade could contribute to target cell destruction through opsonization or direct lysis of allogeneic target cells; respectively, therefore, cobra venom factor was chosen as a reagent to physiologically exhaust functional complement in recipient hosts (reviewed in Ref. 40). Hepatocellular allografts were transplanted into CD8 KO hosts without immunosuppression. Alloantibody was assayed in CD8 KO rejector mice and only recipients with high serum alloantibody levels (>40% binding of target splenocytes) previously shown to correlate with maximal in vivo cytotoxic effector function (Fig. 2) were used for subsequent experimentation. Following graft rejection, hosts were depleted of CD4 + T cells alone (n = 10) or CD4 + T cells in addition to NK cells (n = 5), neutrophils (PMN, n = 6), complement (C; n = 6), or macrophages (Mac; n = 7). In vivo alloantibody was assessed within 48 h of host treatment. The average ± SD of alloantibody for each treatment group is shown by the bars. Serum alloantibody level for recipients within the treatment groups is represented by the circles. Alloantibody levels were similar between treatment groups (p, NS). Depletion of host macrophages significantly reduces in vivo alloantibody (p = 0.00001). Treatment of a separate cohort with PBS liposomes as a vehicle control for liposome clodronate did not interfere with cytotoxic activity (n = 2).

FIGURE 3. In vivo-allospecific cytotoxic effector function generated in CD8 KO rejector mice is mediated by macrophages. FVB/N (H-2b) hepatocytes were transplanted into untreated CD8 KO (H-2b) recipients. Following graft rejection, hosts were depleted of CD4 + T cells alone (n = 10) or CD4 + T cells in addition to NK cells (n = 5), neutrophils (PMN, n = 6), complement (C; n = 6), or macrophages (Mac; n = 7). In vivo cytotoxicity was assessed within 48 h of host treatment. The average ± SD of alloantibody for each treatment group is shown by the bars. Serum alloantibody level for recipients within the treatment groups is represented by the circles. Alloantibody levels were similar between treatment groups (p, NS). Depletion of host macrophages significantly reduces in vivo alloantibody in comparison to all other groups (p < 0.00001). Treatment of a separate cohort with PBS liposomes as a vehicle control for liposome clodronate did not interfere with cytotoxic activity (n = 2).

Whether ADCC by innate immune cells is involved in the alloantibody observed in CD8 KO rejector mice, CD8 KO recipients of FVB/N allogeneic hepatocytes were monitored for graft rejection and allograft survival. Following rejection, the hosts were depleted of CD4 + T cells alone (GK1.5) and neutrophils (RB6-8C5), NK cells (PK136), or macrophages (liposome-enclosed clodronate) 48 h before the in vivo cytotoxicity assay. As shown in Fig. 3, in vivo alloantibody was not influenced by the depletion of CD4 + T cells alone (n = 10) or CD4 + T cells in conjunction with neutrophils (n = 6) or NK cells (n = 5). In contrast, in the absence of macrophages (n = 7), all hosts showed significantly decreased alloantibody (p < 0.0001), despite similar levels of alloantibody (p, NS between groups). PBS-filled liposomes were used as a control for the liposome treatment and did not deplete host macrolayers or reduce the cytotoxic activity of CD8 KO rejector mice (p, NS; PBS liposome-treated hosts compared with hosts not treated with liposomes). Collectively, these data support the conclusion that humoral immune damage of transplanted liver parenchymal cells in CD8 KO hepatocyte rejector mice occurs by an ADCC mechanism mediated by recipient macrophages. We therefore hypothesized that CD8-deficient recipients with genetically impaired macrophage populations or which were macrophage-depleted would exhibit prolonged hepatocellular allograft survival.

Host macrophages are required to effect rapid CD4-dependent, allograft-mediated hepatic rejection

To determine whether macrophages are necessary for CD4-dependent, allograft-mediated allogeneic hepatocyte rejection, FVB/N hepatocytes (H-2b) were transplanted into CD8 KO recipient mice (H-2b), which are macrophage “deficient” due to the genetic absence of a macrophage growth and differentiation factor (M-CSF). This genetic defect is known to result in a general reduction of resident macrophages, including in the spleen and liver (42). These recipients were transiently depleted of CD8 + T cells to focus on the CD4-dependent, Ab-mediated rejection pathway. M-CSF +/+ littermates depleted of CD8 + T cells were used as controls and graft survival was compared between the groups (Fig. 4).
The CD8-depleted M-CSF<sup>+/+/</sup> hosts rejected hepatocyte allografts with a MST of 14 days (n = 4), which is equivalent to the rejection kinetics in untreated CD8 KO hosts (shown in Fig. 5 as “no Treatment”; p, NS). However, transiently CD8-depleted macrophage-deficient op/op (M-CSF<sup>+/−</sup>) hosts demonstrated delayed rejection of hepatocellular allografts with a MST of 35 days (n = 4; p = 0.007). Rejection at this time point coincided with reconstitution of CD8<sup>+</sup> T cells in the periphery (Fig. 4). The op/op hosts generated similar levels of alloantibody as the M-CSF<sup>+/+/</sup> control recipients (60 ± 12% in op/op vs 65 ± 36% in M-CSF<sup>+/−</sup>; p, NS), suggesting that host macrophage “deficiency” did not affect priming of alloantibody production after hepatocyte transplantation. Rather, host macrophages appear important to the efferent phase of CD4-dependent, alloantibody-mediated graft rejection.

An additional experimental approach to focus on the role of macrophages during the effector arm of CD4-dependent Ab-mediated rejection was used. CD8 KO recipients were transplanted with allogeneic FVB/N hepatocytes and rejection permitted to ensue for 5 days posttransplant. Then host macrophages were depleted from untreated CD8 KO recipients during the effector immune phase with clodronate liposomes, a macrophage depletion treatment technology developed by Van Rooijen and Sanders (28), which is reported to be effective for ∼5–6 days per treatment. The effect of host macrophage depletion in CD8 KO recipients by treatment with clodronate liposomes was compared with CD8 KO recipients treated with control PBS liposomes. Serial host macrophage depletion beginning day 5 posttransplant did not affect the level of alloantibody production compared with control liposome-treated hosts or untreated hosts (alloantibody level: 68 ± 24% in liposome clodronate-treated hosts, n = 6; 62 ± 39% in PBS liposome-treated hosts, n = 4; 61 ± 26% in untreated CD8 KO hosts, n = 7). Hepatocyte allograft rejection was similar in untreated hosts (MST = 10 days; n = 7) and control PBS liposome treated hosts (MST = 14 days; n = 4; p, NS). In contrast, in hosts depleted of macrophages, hepatocellular allograft survival was prolonged with a MST of 25 days (n = 6; p < 0.001 compared with untreated hosts, p = 0.003 compared with liposome clodronate-treated hosts; Fig. 5).

Immunohistochemistry of recipient livers (days 8 and 9 after transplant) confirmed the depletion of host macrophages (F4/80<sup>+</sup>) cells in hepatic recipient treated with liposome clodronate (Fig. 6, A and B). Donor hepatocytes, detected by staining for the transgenic reporter product hA1AT, were present in the recipient liver in both macrophage-replete and macrophage-depleted hosts (Fig. 6, C and D). No evidence for C4d staining of recipient liver tissue was noted (data not shown). Cumulative histologic assessment of successive sections of the host livers on days 8 and after transplant showed, even at this early time point following macrophage depletion, more abundant donor hepatocytes in the macrophage-depleted hosts in comparison to the untreated hosts, concordant with the extended graft survival in macrophage-depleted hosts. In macrophage-replete (untreated) mouse liver sections, 44 ± 13.7 donor hepatocytes were detected (×20 high-power field) while significantly more donor (hA1AT-positive) hepatocytes were found in liver sections from macrophage-depleted mice (91 ± 13.2 hepatocytes; p = 0.0032).

**Alloantibody and macrophages together effect in vitro hepatocellular cytotoxicity**

To further explore the role of macrophages in humoral immune damage of allogeneic hepatocytes, hepatocytotoxicity was evaluated using an in vitro cellular cytotoxicity assay. Alloantibody in serum from CD8 KO (H-2<sup>d</sup>) mice was collected 14 days after allogeneic (FVB/N, H-2<sup>b</sup>) hepatocyte transplantation. Hepatocytes (FVB/N) were cultured with alloantibody, serum from untransplanted (H-2<sup>d</sup>) mice, or left untreated (37°C, 30 min). Following incubation with Ab, hepatocytes were plated into culture dishes and cultured alone or cocultured with macrophages for 8 h. Macrophages (H-2<sup>d</sup>) were added to the appropriate wells with hepatocytes at specific E:T ratios (E:T = 10:1 or 40:1). ALT release in the culture supernatant was measured as a reflection of hepatocellular injury. As a positive control, hepatocytes were mechanically disrupted in deionized H2O which resulted in a “maximum” ALT release.
Release of 202.0 ± 9.5 U/ml (data not shown). Hepatocytes that were cultured alone and hepatocytes cultured with serum-containing alloantibody showed similar baseline ALT release (50.7 ± 4.1 U/ml vs 44.4 ± 4.1 U/ml; Fig. 7). Untreated hepatocytes that were cocultured with macrophages (E:T = 10:1 and 40:1) also showed similar baseline ALT release (41.7 ± 5.9 and 44.7 ± 4.5 U/ml, respectively) when compared with the untreated control. However, hepatocytes that were treated with serum-containing alloantibody and cocultured with macrophages (E:T = 10:1 and 40:1) exhibited a significant increase in ALT release (p < 0.0001 for both E:T ratios; *p*, significance) compared with hepatocytes untreated or treated with serum from untransplanted (naive) mice and hepatocytes incubated with alloantibody or macrophages alone. Error bars denote the SDs of triplicate wells. The data are representative of three experiments.

Discussion

Both clinical and experimental studies highlight the barrier that acute Ab-mediated allograft rejection (AAMR) poses to successful allograft survival (reviewed in Refs. 1, 25, and 43–46). Acute Ab-mediated rejection occurs despite the use of maintenance immunosuppression to prevent rejection and is associated with worse graft outcome than T cell-mediated rejection (47). The detection of C4d complement-split product in graft tissue is an integral component of the recently established standards used to diagnose AAMR in kidney and cardiac allograft recipients (48). Complement deposition is usually detected perivascularly, supporting the inference that Ab and complement primarily target graft endothelial cells with subsequent ischemic graft damage (reviewed in Refs. 1, 21, 44, and 48). The current studies which focus on humoral immune damage of transplanted allogeneic liver parenchymal cells add an additional dimension to existing studies which focus on donor endothelial cell damage.

Our studies used a well-characterized hepatocellular allograft model that is known to initiate brisk alloantibody production and CD4-dependent rejection in the absence of CD8+ T cells (26). Alloantibody is sufficient to mediate acute rejection of hepatocellular allografts engraved in the liver in the absence of host T or B cells and without nonspecific tissue or vascular injury to the native liver (22). In the current studies, CD4-dependent (CD8-independent) rejection of allogeneic liver parenchymal cells in CD8 KO recipients was accompanied by alloantibody production and the development of high-magnitude in vivo cytoxic effector activity which is not mediated by CD4+ T cells or NK cells. In vivo cytotoxic effector activity is highly correlated with graft rejection in this transplant model since rejection is always accompanied by the detection of in vivo alloantibody and in vivo alloantibody is always absent with graft acceptance (induced by effective immunotherapy) (29). Since in vivo alloantibody in CD8 KO rejector mice is not mediated by CD4+ T cells, it is likely that the allospecificity of the observed cytotoxic effector function is attributable to serum alloantibody. The observation that recipient serum alloantibody levels correlated with the magnitude of in vivo-allospecific cytotoxic activity is consistent with this conclusion. Furthermore, we have previously reported that alloantibodies in rejector mice bind to allogeneic but not third-party or syngeneic hepatocytes by flow cytometric analysis (22). Additionally, the observed in vivo cytotoxicity is allospecific since differential target cell clearance is only observed when allogeneic and syngeneic targets are injected into recipient mice. Rejctor mice do not differentially clear third-party targets when cojected with syngeneic targets (32).

Different mechanisms of Ab-mediated graft endothelial cell injury have been described. These include both complement-dependent (membrane attack complex lysis, recruitment of innate inflammatory cells, and opsonization by complement proteins with subsequent phagocytosis) and complement-independent cytotoxic mechanisms (ADCC) (21). Our studies did not support a complement-dependent cytotoxic mechanism of allogeneic parenchymal cell damage since complement-depleted hosts retained high cytotoxic effector function. Rather, our studies are consistent with a paradigm of acute humoral rejection by an ADCC mechanism in which CD4+ T cell-dependent alloantibody production results in targeting of transplanted allogeneic parenchymal cells for macrophage-mediated cytotoxic immune damage (Fig. 8). This paradigm is supported by the observation that only depletion of host macrophages (and not NK cells or neutrophils) in CD8 KO rejector mice markedly reduces in vivo cytotoxic effector function. The remaining low-level in vivo alloantibody could be attributed to incomplete macrophage depletion or to the activity of CD4+ CD8− TCR+ killer cells and/or eosinophils in CD8 KO mice. Furthermore, genetic deficiency of recipient macrophages in op/op hosts, or transient depletion of host macrophages with liposome clodronate following the onset of CD4-dependent allograft rejection in CD8 KO recipients, resulted in significant prolongation of allograft survival in comparison to macrophage-replete controls. Prolongation of graft survival in macrophage-deficient recipients could not be attributed to differences in serum alloantibody since alloantibody levels were equivalent in both macrophage-deficient and macrophage-replete hosts. The data also demonstrate that host macrophages were critical during the effector phase of rejection in CD8 KO hosts since transient macrophage depletion after the onset of rejection effectively delayed rejection. Furthermore, macrophages are sufficient to effect alloantibody-dependent hepatocyte cytotoxicity in vitro. Resident liver Kupffer cells are presumably a primary macrophage population involved in ADCC-associated humoral rejection in this model since hepatocytes are transplanted to the liver. Human Kupffer cells have been shown to express FcγRI in areas of liver inflammation, as well as FcγRII and FcγRIII, all...
of which participate in ADCC (49, 50). The donor-directed Ab developing in response to hepatocellular allografts is polyclonal, developing high titters of IgG1, IgG2a, IgG2b, and IgG3 isotypes (P. H. Horne, unpublished observation); consequently, all Fc receptor types may be relevant. However, if further experimentation revealed that one receptor was used predominantly, therapy targeting the individual receptor could be developed to avoid systemic macrophage depletion.

The role of ADCC in allograft rejection in humans has been proposed, but limited clinical data are available to support this as an effector mechanism during AAMR (reviewed in Refs. 21 and 44). Many clinical and experimental studies of AAMR after cardiac (14, 15, 17), renal (18, 51), and liver (8, 52) transplantation have been notable for increased macrophage infiltration in concert with C4d staining in the graft tissue. However, in these studies, the role of macrophages as effectors of acute humoral rejection was not examined. To our knowledge, the current report is the first to demonstrate a role of macrophages as effectors of CD4/alloantibody-mediated in vivo cytotoxicity and acute rejection of allogeneic parenchymal cells. Results of the current studies have direct implications for humoral immune damage of clinical cellular allografts, such as hepatocyte, bone marrow, or islet allografts; alloantibody in sensitized bone marrow or islet allograft human recipients is a known barrier for successful cellular engraftment (23, 25). These results also suggest that the macrophage infiltration noted in solid organ allografts during Ab-mediated rejection may be active participants in the humoral rejection process.

Although the current studies did not show a role for complement for in vivo cytotoxic effector function or evidence of C3d staining during hepatocyte allograft rejection, allograft rejection is a complex process requiring recruitment of inflammatory cells to the graft site, recognition of target cells by host immune cells, and local activation of effector mechanisms. Complement can contribute to macrophage-dependent infiltration of the graft tissue, as has been reported in clinical (reviewed in Refs. 11, 43, and 53) and experimental studies (34, 54). Experimental studies have also demonstrated a role for complement in the pathogenicity of alloantibody for acute rejection of cardiac allografts (55). Another study demonstrated that recipient macrophages provide an important source of terminal complement protein C6, which is necessary for membrane attack complex formation and humoral rejection of cardiac allografts (56). Thus, complement may be important for recruitment of macrophages, alloantibody pathogenicity, and/or have a role as a macrophage effector product. Perhaps in solid organ allografts, which possess donor-type vasculature, complement activation on donor endothelial cells with membrane attack complex formation results in damage to the graft blood vessels with subsequent inflammatory cell and alloantibody influx into the parenchyma. Once the vascular bed is compromised, macrophages can more readily access the parenchyma and along with native tissue macrophages exert complement-independent humoral immune injury (ADCC) to graft parenchymal cells. In allograft tissues such as the liver, which possesses fenestrated endothelial barriers, damage to the graft endothelium may not be necessary to allow Ab access to graft parenchyma. In this situation, immune damage to engrafted allogeneic parenchymal cells could occur in the absence of endothelial damage and complement activity.

In conclusion, acute humoral rejection of organ allografts is marked in severe cases by vasculitis, thrombosis, and hemorrhage. In the absence of these advanced hallmark histologic characteristics, the presence of C4d in concert with relevant clinical indicators is diagnostic for Ab-mediated rejection. The current studies support a novel paradigm of humoral immune injury to allogeneic parenchymal cells, which occurs through a macrophage-mediated ADCC mechanism. Depletion or impairment of recipient macrophages significantly reduced in vivo allogycotoxicity and enhanced hepatocellular allograft survival in two models, suggesting a promising therapeutic target for CD4-dependent, Ab-mediated rejection of graft parenchymal cells. Targeting macrophage-mediated mechanisms of graft parenchymal destruction could afford time to attempt more permanent therapies to inhibit alloantibody production.

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Disclosures
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