Published in final edited form as:

J Immunol. 2008 October 1; 181(7): 4580–4589.

Donor Deficiency of Decay-Accelerating Factor Accelerates Murine T Cell-Mediated Cardiac Allograft Rejection¹

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Abstract

Decay-accelerating factor (DAF) is a cell surface regulator that accelerates the dissociation of C3/C5 convertases and thereby prevents the amplification of complement activation on self cells. In the context of transplantation, DAF has been thought to primarily regulate antibody-mediated allograft injury, which is in part serum complement-dependent. Based on our previously delineated link between DAF and CD4 T cell responses, we evaluated the effects of donor Daf1 (the murine homolog of human DAF) deficiency on CD8 T cell-mediated cardiac allograft rejection. MHC-disparate *Daf1*^{−/−} allografts were rejected with accelerated kinetics compared with wild-type grafts. The accelerated rejection predominantly tracked with DAF's absence on bone marrow-derived cells in the graft and required allograft production of C3. Transplantation of *Daf1*^{−/−} hearts into wild-type allogeneic hosts augmented the strength of the anti-donor (direct pathway) T cell response, in part through complement-dependent proliferative and pro-survival effects on alloreactive CD8 T cells. The accelerated allograft rejection of *Daf1*^{−/−} hearts occurred in recipients lacking anti-donor Abs. The results reveal that donor DAF expression, by controlling local complement activation on interacting T cell APC partners, regulates the strength of the direct alloreactive CD8⁺ T cell response. The findings provide new insights into links between innate and adaptive immunity that could be exploited to limit T cell-mediated injury to an allograft following transplantation.

Decay-accelerating factor (DAF, CD55)⁴ is a 70-Kda gly-cophosphatidylinositol-anchored cell surface protein that regulates complement activation on self cell surfaces (1,2). In this capacity, following activation of the classical, lectin, or alternative pathways, DAF accelerates the dissociation of the bi- and trimolecular C3/C5 convertases, which are central amplification enzymes of the complement cascade (1). Rigorous studies have demonstrated that DAF regulates convertase activity only on the cell surface on which it is expressed (1).

Through this mechanism, DAF on donor tissue limits antibody-mediated transplant injury. Human anti-porcine xeno-antibodies, directed at the α 1,3-Gal residues expressed on porcine

¹This work was supported by the Roche Organ Transplant Research Fund (P.S.H.) and by National Institutes of Health Grants AI43578 (P.S.H.) and AI23598 (M.E.M.). P.N.L. received a fellowship grant from the Ohio Affiliate of the American Heart Association.

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Disclosures

The authors have no financial conflicts of interest.

⁴Abbreviations used in this paper: DAF, decay-accelerating factor; BM, bone marrow; MST, median survival time; WT, wild type.

endothelium elicit complement-dependent, hyperacute rejection, but their pathogenicity is diminished by transgenic over-expression of human DAF on cells in the xenograft (3). Consistent with this, Sykes and colleagues showed using a murine model that *Daf1^{-/-}* heart grafts are predisposed to accelerated antibody-mediated injury (4).

Recent studies by our joint group have delineated a novel role for DAF as a regulator of T cell immunity, a function that is also dependent upon the complement regulatory activity of DAF (5–8). During cognate T cell/APC interactions, both partners up-regulate production of alternative pathway complement components C3, factor B (fB), and factor D (fD) and down-regulate surface expression of DAF, with the latter alteration lifting restraint on local complement activation. T cell responses are stronger and more polarized toward type 1 immunity in the absence/blockade of DAF, and this enhanced response is abrogated if fD or C3 (both of which are required for alternative pathway activation) is absent from the APCs (5–8). Our joint group has shown that these effects of DAF on CD4 T cells are due to proliferative and pro-survival signals directly transmitted through locally produced C5a/C3a binding to the T cell-expressed C5aR/C3aR (5–9). These effects of DAF on T cell immunity have functional importance in vivo: T cell responses induced to model protein Ags, minor histocompatibility complex transplant Ags (e.g., HY), autoantigens, and viral Ags are stronger in DAF-deficient mice (5–7,10,11).

Herein we extend these observations to the study of graft-expressed DAF as a regulator of fully allogeneic heart transplant rejection, a pathogenic process that is predominantly mediated by CD8⁺ effector T cells. The results reveal that donor DAF deficiency, and specifically DAF deficiency of bone marrow (BM)-derived cells in the graft, significantly accelerates cardiac allograft rejection. The effect is dependent upon C3 production by graft cells, which regulates CD8 cell proliferation and apoptosis and occurs in the absence of alloantibody. The findings provide new insight into mechanisms regulating cellular alloimmunity and raise the concept that augmentation of DAF expression on allografts could be used to limit T cell-mediated, as well as antibody-mediated, graft rejection.

Materials and Methods

Mice

C57BL/6 (B6, Thy1.2, CD45.1), *RAG2^{-/-}*, Thy1.1, CD45.2, and B6.*C3^{-/-}* mice (all H-2^b) and BALB/c (H-2^d), C3H/HeJ (H-2^k), 129 (H-2^b), and C3H SCID (H-2^k) mice were purchased from The Jackson Laboratory. Mice deficient in the *Daf1* gene (*Daf1^{-/-}*) were produced as described (12) and backcrossed for >10 generations to B6 or 7 generations to C3H. B6 mice do not reject B6.*Daf1^{-/-}* skin (data not shown), confirming congenicity. The *Daf1^{-/-}* mouse was also crossed with B6.*C3^{-/-}* mice in our mouse facility to create *Daf1^{-/-}/C3^{-/-}*. All mice were housed in Mount Sinai School of Medicine Animal Resource Unit or the Cleveland Clinic Biological Resources Unit in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International.

Abs and reagents

All Abs for flow cytometry were purchased from BD Pharmingen except for anti-CD45.1-PECy-7 and anti-CD45.2-PE, which were purchased from eBioscience. CFSE was obtained from Molecular Probes (Invitrogen). Streptavidin was purchased from Sigma-Aldrich. Peptides Uty, Smcy, Dby, β -galactosidase peptide 96–103, and OVA_{323–339} were synthesized by Research Genetics at >90% purity. In selected experiments peptides were mixed in CFA and injected s.c. into the flanks of the animals (13).

Surgical procedures

Heterotopic heart transplantation and skin graft placement were performed as described previously (6,13,14). Heart graft function was monitored daily by palpation, and rejection was defined as the day on which a palpable heartbeat was no longer detectable. Skin graft rejection was defined as >90% visual necrosis. Following transplants into SCID recipients, purified syngeneic T cells were injected i.v. via the tail vein.

Cell isolation, labeling, and culture

T cells were isolated from spleen and lymph nodes of naive mice and isolated using a negative T cell selection kit (StemCell Technologies) according to the manufacturer's specification and purified to between 93 and 98% based on CD3 staining. Isolated cells were labeled with CFSE and used for subsequent experiments (5,6,13,14). In in vitro cultures, T cells were stimulated with peritoneal macrophages obtained by injecting 1 ml of thioglycollate and isolated 5 days later by peritoneal lavage. T cells were activated in 48-well plates in serum-free HL-1 media (Invitrogen) supplemented with 1% L-glutamine and 1% penicillin/streptomycin in a 2:1 T cell/APC ratio (5,6,13,14).

To isolate tissue-derived APCs, following euthanasia, the vascular system of animals was perfused with sterile PBS + 10 mM EDTA by intra-cardiac injection until the kidney and liver blanched (~10 ml). The heart tissue was harvested, minced, and washed three times with PBS/EDTA, and digested for 2.5 h at 37°C in 0.2 mg/ml collagenase type IV from *Clostridium histolyticum* (Sigma-Aldrich) diluted in RPMI 1640/10% FBS. The tissue was then dissociated through a 19-gauge needle and filtered through a 70-μm nylon mesh. Following three washes in RPMI 1640/10% FBS, the isolated single-cell suspension was stained for flow cytometric analysis.

BM chimeras

Male wild-type (WT) Thy1.1, WT Thy1.2⁺, and *Daf1*^{-/-} (Thy1.2) mice were irradiated (1200 Rads) and injected with 10×10^6 donor BM cells and treated with gentamicin for 10 days and used as recipients for cell transfers >21 days after reconstitution. Degree of donor BM chimerism was verified by flow cytometry using anti-Thy1.2 and anti-Thy1.1 mAb (BD Pharmingen) at the time of sacrifice.

Flow cytometry

Cells were stained for surface Ags by incubating with Abs in PBS, 0.1% BSA, and 0.01% sodium azide. Pilot studies revealed that CFSE staining did not alter IFN-γ production by TCR transgenic T cells stimulated with their cognate Ag (data not shown). Samples were collected using either a FACScan or FACSCanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

IFN-γ ELISPOT

IFN-γ ELISPOT assays were performed as previously described (5). Briefly, MultiScreen ELISPOT plates (Millipore) were coated overnight with the capture Abs. Following a blocking step, recipient spleen or T cells (0.2 to 1×10^6 per well) were plated and incubated with spleen cell stimulators (400,000 per well) or peptide Ag at 37°C, 5% CO₂ for 24 h. Donor spleen cell stimulators were treated with mitomycin C to limit proliferation and cytokine secretion (15). After washing, detection Abs were added overnight. After washing, alkaline phosphatase-conjugated anti-biotin Ab (Vector Laboratories) diluted 1/1000 in PBS with 0.17. Tween 20 was added for 2 h, the plates were developed, and the resulting spots were counted on an ImmunoSpot series 3 analyzer (Cellular Technology).

Cytotoxicity assays

In vitro CTL assays were performed using [³H]thymidine-labeled target cells as described previously (16).

Alloantibody detection

Serum alloantibody was assessed by flow cytometry using donor, self, or 3P thymocytes as targets (17,18).

Histological evaluation

Formalin-fixed paraffin sections of graft tissues were stained with H&E and for elastin as described previously (14,19). More than 14 individual sections were examined from each graft. Significant vasculopathy was defined as >50% occlusion of three or more large vessels on more than three different sections. C3d staining was performed on tissue frozen in OCT compound as described (18).

Statistical analysis

Graft survival was compared using log-rank survival statistics. Immunology assay results were compared using the Student's *t* test. A *p*-value of <0.05 was considered significant.

Results

The absence of donor Daf1 accelerates graft rejection

In our previous studies, we showed that donor and recipient DAF deficiency accelerates minor Ag-disparate male skin graft rejection, a result associated with an enhanced frequency of HY-reactive T cells (5). To delineate the role of donor DAF in T cell-mediated rejection of a vascularized allograft, and more definitively to examine the CD8 cell response, we examined the survival of Daf1 deficient (*Daf1*^{-/-}) hearts following transplantation into fully allogeneic recipients. In C3H (H-2^k) recipients, B6 (H-2^b) *Daf1*^{-/-} allografts were rejected 9 days faster than WT hearts, results that were statistically and biologically significant (Fig. 1). Similar results were observed with BALB/c recipients (*Daf1*^{-/-} mean survival time (MST) of 10 days, WT MST of 14 days, *n* = 6/group, *p* < 0.05, data not shown). Microscopic examination of H&E-stained tissue sections obtained at cessation of heartbeats revealed diffuse mononuclear cell infiltration and perivascular inflammation in both groups, typical of acute cellular rejection (Fig. 1). Rare polymorphonuclear leukocytes were detected in blood vessels of the transplanted *Daf1*^{-/-} but not WT hearts (Fig. 1B, arrow).

Because DAF regulates complement activation (1) and because we previously showed that DAF deficiency augments T cell immunity via controlling local APC/T cell complement production (5), we tested whether the accelerated rejection in the absence of donor DAF required graft-derived C3. Hearts deficient in both C3 and DAF (backcrossed >12 generations to B6) survived significantly longer than did hearts deficient in DAF alone (MST of 22 days with one surviving >60 days, Fig. 1) and in fact survived longer than did WT hearts. *C3*^{-/-} hearts also exhibited prolonged survival (MST of 25 days with two surviving >60 days, Fig. 1), but unlike the indefinite survival of renal allografts deficient in C3 (20), 80% of the *C3*^{-/-} heart grafts were rejected by day 50. Histological examination of the *C3*^{-/-} and *C3*/*Daf1*^{-/-} hearts surviving to day 60 revealed mononuclear cell infiltration and fibrosis (not shown), indicating ongoing immune-mediated injury, not tolerance. In contrast to the marked effects of donor DAF deficiency on accelerating allograft rejection, WT B6 hearts rejected with similar kinetics in WT H-2^k vs *Daf1*^{-/-} H-2^k recipients (Fig. 1A).

To assess mechanisms underlying the accelerated rejection in the absence of donor DAF, we measured cellular and humoral alloresponses in additional groups of recipients. Confirming and extending previous work in which donor DAF deficiency augmented direct pathway alloimmunity (5), recall assays performed on day 3 posttransplantation revealed that spleens of recipients of B6 *DafI*^{-/-} allografts contained almost 4-fold more donor-reactive IFN- γ producers ($p < 0.05$ vs WT recipients). In contrast, the total number of anti-donor IFN- γ -producing spleen cells in C3H recipients of WT B6 allografts was low and was not significantly different from that detected in recipients of syngeneic C3H hearts (Fig. 1C). On day 8 posttransplantation, the number of IFN- γ producers was also significantly greater (~2-fold) in the recipients of B6 *DafI*^{-/-} vs WT hearts (Fig. 1C). Anti-donor immunity in recipients of C3/*DafI*^{-/-} hearts on day 8 was significantly weaker than in recipients of *DafI*^{-/-} hearts (and similar to that found in WT animals), confirming that the augmentation of the anti-donor immunity was dependent on C3 production by the donor graft. Control B6 *C3*^{-/-} hearts also induced weaker responses than did *DafI*^{-/-} hearts on day 8 (similar to the response induced by C3/*DafI*^{-/-} hearts, Fig. 1C). Direct anti-donor T cell responses in *DafI*^{-/-} H-2^k recipients of WT B6 hearts assessed at the time of rejection were not significantly different from those detected in WT H-2^k recipients (data not shown). Along with the graft survival data (Fig. 1A), these results show that donor DAF expression principally regulates the kinetics of graft rejection and the strength of the associated, direct anti-donor T cell response.

The titer of donor-reactive alloantibodies in the sera of the recipients on day 8 posttransplant was low and was not significantly different between recipients of WT and *DafI*^{-/-} heart grafts on day 8 posttransplantation (Fig. 2A). The low Ab titers were associated with focal vascular C3d staining in the WT and *DafI*^{-/-} heart grafts examined on day 8 posttransplant, without a discernable difference between the groups (Fig. 2B).

To precisely ascertain how DAF deficiency affects the recipient CD8⁺ T cell alloresponse to donor tissue, we evaluated immune responses in an allogeneic skin transplant model. The results shown in Fig. 3 reveal that the same pattern of alloreactive T cell immunity pertains in this system. Purified splenic CD8 T cells, the dominant effector cells in this skin rejection model (21,22), were isolated from WT BALB/c mice (H-2^d) transplanted with allogeneic (H-2^b) WT or *DafI*^{-/-} full-thickness trunk skin allografts on day 12 posttransplant and tested against donor strain WT stimulators in IFN- γ ELISPOT and in vitro cytotoxicity assays. The CD8 T cells obtained from mice primed with *DafI*^{-/-} skin produced donor-reactive IFN- γ ELISPOTs at ~2-fold greater frequency (Fig. 3A) and exhibited enhanced CTL activity compared with those isolated from recipients of WT allografts (Fig. 3B). The augmented frequency of recipient IFN- γ -producing CD8 T cells in response to *DafI*^{-/-} donor skin was abrogated by the absence of C3 as well as DAF from the donor graft. The responses measured in recipients of *DafI*^{-/-} /*C3*^{-/-} skin grafts were below those in recipients of WT skin grafts.

BM cell expression of DAF in the donor heart influences the kinetics of rejection

Our published results revealed that DAF regulates T cell immunity during cognate T cell/APC interactions through controlling activation of complement locally produced by both partners (5). This finding raised the possibility that the absence of DAF expression on BM-derived cells (APCs), as opposed to parenchymal cells, in the graft may drive the accelerated rejection. We tested this hypothesis in our transplant system by making BM chimeric mice to use as cardiac allograft donors. Thy1.2⁺ *DafI*^{-/-} BM was transplanted into lethally irradiated Thy1.1⁺ WT recipients to produce chimeras in which DAF was expressed on parenchymal (non-BM-derived) but not BM-derived cells (*DafI*^{-/-} BM $\rightarrow$ WT). Conversely, WT Thy 1.1⁺ BM was transplanted into lethally irradiated Thy1.2⁺ *DafI*^{-/-} recipients to produce chimeras with DAF on BM-derived but not parenchymal cells (WT BM $\rightarrow$ *DafI*^{-/-}). WT BM $\rightarrow$ WT and *DafI*^{-/-} BM $\rightarrow$ *DafI*^{-/-} chimeras were made as control heart graft donors. Six to 8 wk after

preparing the chimeras, we verified that the peripheral blood BM phenotype was >95% donor-derived (Fig. 4A). In a separate analysis, we made BM chimeras using CD45.1/2 polymorphisms (expressed on dendritic cells and macrophages) and analyzed the degree of chimerism 7 wk later. Fig. 4B shows that the CD11c⁺ and CD11b⁺ APCs within hearts of the chimeric animals were donor BM-derived and that the ratio of donor-to-recipient cells detected in the peripheral blood matches that in cells isolated from the heart tissue. Similar numbers of APCs were detected in each organ regardless of the source of BM used for reconstitution (WT vs *DafI*^{-/-}). We then transplanted the chimeric H-2^b hearts into allogeneic WT C3H recipients.

Hearts from *DafI*^{-/-} BM → WT donors (BM cells deficient in DAF, non-BM cells DAF⁺) rejected in C3H recipients significantly faster (MST of 10 days) than did hearts from WT BM → WT controls (MST of 14 days, Fig. 4C). Chimeric hearts deficient in DAF on both BM and parenchymal cells (*DafI*^{-/-} BM → *DafI*^{-/-}) were similarly rejected with accelerated kinetics (MST of 7.5 days, *p* = NS vs *DafI*^{-/-} BM → WT donors). Allografts from WT BM → *DafI*^{-/-} chimeras (MST of 13 days) were rejected with similar kinetics to the hearts from WT BM → WT controls (Fig. 4C).

Consistent with the above rejection rates, the frequency of IFN- γ -producing spleen cells reactive to donor alloantigens (Fig. 4D) was significantly higher in recipients of hearts from chimeras with *DafI*^{-/-}, BM-derived cells (*DafI*^{-/-} BM → WT and *DafI*^{-/-} BM → *DafI*^{-/-}) compared with recipients of hearts from chimeras with BM cells that expressed *DafI* (WT BM → *DafI*^{-/-} and WT BM → WT controls).

APC DAF deficiency enhances proliferation of alloreactive CD8 T cells

Our previously published work showed that locally produced complement regulates CD4 T cell proliferation and cell survival (5,6,8,9). To verify that APC DAF deficiency (which decreased regulation of APC complement activation) also affects proliferation/ survival of alloreactive CD8 T cells, we performed in vitro proliferation assays, co-staining with annexin V to assess apoptosis. For these analyses we stimulated purified CFSE-labeled BALB/c CD8 T cells from naive mice with allogeneic H-2^b peritoneal macrophages (WT, *DafI*^{-/-}, *C3*^{-/-}) in serum-free medium. Flow cytometric analysis revealed that at 72 h the BALB/c T cells proliferated in response to allogeneic B6 but not to syngeneic BALB/c APCs (Fig. 5A). The assays showed both that the BALB/c CD8 T cells proliferated to each of the allogeneic APCs at this early time point, but a significantly higher proportion of the dividing cells stained with annexin V in response to *C3*^{-/-} APCs compared with either B6 *DafI*^{-/-} or WT APCs. At 120 h (Fig. 5B), the CD8 cells proliferated more in response to B6 *DafI*^{-/-} vs WT APCs (10-fold more cells underwent more than seven divisions). Fewer proliferating CD8 T cells were annexin V⁺ following stimulation with B6 *DafI*^{-/-} APCs than with the controls. Despite initiating the cultures with the same number of BALB/c CD8 T cells, in one representative experiment, there were ~6-fold more CD8 T cells at 120 h in cultures with *DafI*^{-/-} APCs (~490,000 CD8 T cells per well) vs WT APCs (~85,000 CD8 T cells per well, data not shown). Conversely, stimulation with *C3*-deficient APCs resulted in less proliferation and more cell death at 120 h (Fig. 5B), with an associated ~4-fold fewer live cells compared with WT controls (~19,000 CD8 T cells, data not shown). Together with our previous work (5,8,9), the data reveal that APC-derived complement regulates CD8 T cell proliferation and apoptosis.

Because primed alloreactive T cells reencounter their target Ags on graft cells to mediate their effector functions (e.g., cytokine release and CTL activity), we next assessed whether target cell-expressed DAF also influences the cytotoxicity of alloreactive CD8 effector T cells. We primed BALB/c mice with B6 skin grafts and 14 days later isolated splenic CD8 effector T cells (>95% CD8⁺, CD44^{high}, CD62L^{low}, data not shown) for in vitro analysis. IFN- γ production and CTL activity were similar when the primed T cells were challenged with WT

or *Daf1*^{-/-} targets (Fig. 6, A and B), indicating that DAF expression does not influence these “immediate” effector functions.

Because the above experiments showed that APC DAF expression modulates the proliferation and survival of CD8 T cells from naive mice through regulating local complement, we questioned whether target cell DAF/C3 also regulates secondary expansion of primed CD8 T cells. We isolated CD8 T cells from BALB/c mice bearing WT B6 skin grafts (day 12 posttransplant), labeled them with CFSE, and assayed CFSE dilution following incubation with allogeneic B6 APCs. As found for naive CD8 T cells, the primed CD8 T cells proliferated more and underwent less apoptosis in response to *Daf1*^{-/-} APCs than did WT APCs (Fig. 6C). These effects required C3 produced by the APC.

To determine whether the effect of target cell DAF applies in vivo, we primed female B6 mice with the immune-dominant class I-restricted male antigenic determinant Uty and the subdominant class I-restricted epitope Smcy mixed together in CFA. Two weeks later, we divided the immunized mice into three groups (Fig. 7A). One group was not given an additional stimulus. The other two groups were given i.p. injections of WT or *Daf1*^{-/-} B6 male spleen cells (18 million per mouse) to induce secondary expansion of the primed CD8 T cells. We performed recall assays by ELISPOT 7 days later (21 days after the initial immunization). As shown in Fig. 7B, the initial immunization induced Uty-specific and Smcy-specific IFN- γ producers in all animals. Spleens from animals boosted with WT or *Daf1*^{-/-} male cells (secondary i.p. immunization) contained more IFN- γ producers than did those given a primary immunization alone, consistent with induction of a secondary T cell expansion. Significantly more Uty-specific and Smcy-specific IFN- γ producers were detected in mice boosted with *Daf1*^{-/-} vs WT APCs, documenting that APC DAF exerts control over secondary expansion in vivo.

To assess whether enhanced secondary CD8 T cell expansion induced by the absence of DAF increases graft injury, 7 days after Uty/Smcy immunization of B6 females and boosting with male B6 WT or *Daf1*^{-/-} cells i.p., we transplanted these mice with B6 WT male hearts. We showed previously that female B6 mice do not acutely reject male hearts, but augmenting the number of effector CD8 T cells can induce graft vasculopathy and can precipitate late graft loss (13,14). One of five male hearts transplanted into recipients boosted with *Daf1*^{-/-} male cells was rejected on day 40, and all (4/4) of the surviving grafts exhibited significant vasculopathy (at least three vessels with >50% occlusion on three or more sections) upon examination on day 90 (Fig. 7C). In contrast, none of the four hearts in the control animals (boosted with WT male cells) was rejected, and only one graft exhibited a single large vessel with partial occlusion (<50%) at day 90 posttransplantation (Fig. 7C). Taken together, the data reveal that the absence of *Daf1* on target cells augments secondary T cell expansion, which, in turn, confers augmented pathogenicity.

Donor DAF deficiency results in accelerated graft rejection independent of antibody

The observation that donor DAF deficiency augments graft injury and anti-donor T cell immunity in mH-disparate male heart grafts transplanted into syngeneic females (Fig. 7), in which anti-donor Abs are not known to participate, suggests the possibility that the effects of DAF on transplant injury might be antibody-independent. We performed additional experiments to test this because in MHC-disparate transplant models greater local complement activation induced by donor DAF deficiency could amplify antibody-initiated, complement-mediated injury in addition to augmenting T cell responses. We transplanted WT or *Daf1*^{-/-} B6 hearts into allogeneic H-2^k *SCID* recipients. Ten days later we administered 5×10^6 T cells from naive C3H mice (>96% CD3⁺, <1% CD19⁺) i.v. through the tail vein and followed graft survival by palpation. The adoptively transferred T cells caused acute rejection of the *Daf1*^{-/-} hearts by day 9, while WT hearts exhibited significantly prolonged survival (Fig.

8A) despite transfer of the same number of WT T cells. Histologic examination of the *Daf1*^{-/-} grafts confirmed massive mononuclear cell infiltration consistent with rejection (Fig. 8B). Mononuclear cell infiltrates were also found in the WT grafts, but the extent of infiltration was modest compared with the *Daf1*^{-/-} hearts that had ceased beating by day 9 (Fig. 8B). As anticipated in SCID mice, no anti-donor alloantibodies were detectable in the sera of any of the recipients (data not shown). While we detected donor-reactive IFN- γ -producing T cells in the spleens of recipients with WT hearts surviving >19 days (confirming that the adoptively transferred cells responded to the graft), >2-fold more were present in recipients of *Daf1*^{-/-} hearts at rejection (day 9) than in recipients of WT grafts on day 19 (101,000 vs 36,300, $p < 0.05$, data not shown). WT and *Daf1*^{-/-} grafts transplanted into unmanipulated SCID recipients survived indefinitely (not shown), confirming that innate immunity and/or ischemia-reperfusion injury is insufficient to cause graft loss regardless of donor DAF expression. As a positive control to confirm that the recipients can reject a WT allograft, we repeated the adoptive transfer experiments using >15 million T cells obtained from naive WT syngeneic mice. Under these conditions, all grafts in both groups were rejected by day 7 ($n = 3/\text{group}$, not shown).

As an additional test for Ab independence in the accelerated rejection of *Daf1*^{-/-} hearts, we transplanted B6 hearts into mH-disparate 129 (H-2^b) recipients, a strain combination that results in acute rejection but does not induce alloantibodies (both strains express the same MHC haplotype). Median survival of B6 *Daf1*^{-/-} hearts was 8 days ($n = 4$), shorter than WT hearts (MST of 10 days, $n = 7$, $p < 0.05$), a result associated with ~2-fold more IFN- γ -producing anti-donor T cells in the spleens (WT, 86 IFN- γ producers per 10^5 spleen cells; *Daf1*^{-/-}, 184 IFN- γ producers per 10^5 spleen cells, $p < 0.05$; no difference in total number of spleen cells per animal, not shown). No anti-donor Abs were detected in the sera of animals from both groups (not shown). Conversely, *C3*^{-/-} hearts transplanted into 129 recipients exhibited statistically longer survival than did WT hearts (MST of 15 days, $n = 3$).

Discussion

DAF accelerates the decay dissociation of C3/C5 convertases on cell surfaces on which it is expressed, and it thereby blocks local amplification of the complement cascade (1). Because alloantibodies initiate complement activation through the classical pathway, it has been largely assumed that effects of DAF on transplant rejection are mediated through its ability to inhibit systemic C3/C5 convertases assembled from systemic complement components and thereby block complement-dependent, antibody-initiated graft injury. The data presented herein reveal that this interpretation is incorrect since donor DAF regulates the strength of the transplant-induced direct pathway alloreactive T cell response.

The results of this study provide new insights into the link between DAF, local complement, and T cell-mediated transplant rejection. We showed that *Daf1*^{-/-} allografts were rejected with accelerated kinetics compared with WT control hearts, and this accelerated rejection compared with control grafts was not a consequence of enhanced antibody-mediated injury (Fig. 2). Accelerated rejection was observed in the absence of DAF from BM-derived cells within the graft (Fig. 3) despite the presence of DAF on graft parenchymal cells, in accordance with the effect being related to T cell/APC interactions. Consistent with this interpretation, transplantation of *Daf1*^{-/-} allografts, including those in which *Daf1* was absent from BM-derived cells in the graft, induced a significantly higher number of IFN- γ -producing, donor-reactive T cells responding through the direct pathway (Figs. 1 and 3).

To provide further mechanistic insight into these observations, we evaluated effects of DAF deficiency on primary and secondary CD8 T cell immune responses. We found that for both naive and alloantigen-activated CD8 T cells, stimulation with DAF-deficient APCs (which

permit unregulated local complement activation) induced more CD8 T cell proliferation and allowed less apoptosis. The expansion occurred as a result of the primary interaction with a DAF-deficient APC and was compounded during the secondary interaction with DAF-deficient cells in the graft (Figs. 5–7). The data imply that these two effects together contribute to markedly enhancing CD8 T cell expansion, which accelerates graft injury. It is notable that despite these remarkable effects on primary and secondary CD8 T cell expansion, target cell DAF expression does not modulate immediate effector functions of the expanded and activated T cells (i.e., secretion of IFN- γ or cytolytic function, Fig. 6).

Because DAF also regulates antibody-initiated amplification of the complement cascade, we performed several experiments to formally verify that DAF deficiency accelerates transplant injury primarily through regulating effector T cells. In the male to female mH-disparate model in which injury is T cell-dependent (anti-donor Abs are not known to participate), graft injury was more severe in the absence of donor DAF (Fig. 7). We also showed that rejection of *Daf1*^{-/-} grafts was accelerated in T cell-reconstituted, immunodeficient SCID mice (Fig. 8). The adoptive transfer experiment does not permit us to differentiate whether the accelerated rejection of *Daf1*^{-/-} hearts is a result of primary and or secondary expansion because T cells undergo homeostatic proliferation in immunodeficient hosts, a process that can induce an effector/memory phenotype (23). Nonetheless, the accelerated rejection of *Daf1*^{-/-} hearts in the absence of anti-donor Abs in two CD8 T cell-dominated graft rejection models proves that donor DAF regulates T cell-mediated allograft rejection independent of alloantibody.

These findings, together with previously published results by our joint group using other models (5–8), are consistent with a central role for DAF in regulating activation of complement produced during alloreactive T cell/APC interactions. In the absence of APC DAF, activation of the locally produced complement is enhanced, augmenting T cell alloimmunity. Our previously reported data implicate the anaphylatoxins C3a and C5a in augmenting the strength of the induced T cell immune response through multiple interrelated mechanisms (5–8). C3a and C5a can bind to the seven transmembrane-spanning, G protein-coupled C3a receptor (C3aR) and C5a receptor (C5aR) expressed on APCs, and thereby up-regulate IL-12 production, guiding the developing immune response toward a pathogenic type 1 cytokine-secreting phenotype. Additionally, others (24) and we have evidence that T cells express C3aR and C5aR, and our published data indicate that C3a/C3aR and C5a/C5aR interactions function as costimulatory signals to directly enhance T cell proliferation (7,8) but also limit death of activated CD4 T cells, the latter via down-regulating T cell-expressed Fas and upregulating Bcl-2 (9). The current set of data extends these concepts, demonstrating that they also apply to alloreactive CD8 T cells. As a result, the local T cell/APC-derived complement activation loop, regulated by cell surface DAF, influences the strength and cytokine profile of the alloreactive T cell response, which, as we have shown herein, can have important consequences for allograft injury.

While the absence of donor DAF expression augments the strength of the direct pathway T cell alloresponse, anti-donor effector T cells are activated even though APCs in WT grafts express DAF (Fig. 1). One explanation for this observation derives from our previous work in which we showed that in normal mice, DAF expression is physiologically down-regulated on APCs during T cell/APC interactions, permitting local complement activation that enhances T cell expansion and differentiation (5). Thus, while WT heart cells express DAF, T cell/allo-APC interactions result in DAF down-regulation on T cell/APC partners, which in turn permits activation/expansion of the pathogenic T cell response.

It is also likely that because of the strength, heterogeneity, and polyclonality of the alloreactive T cell repertoire, a proportion of the donor-reactive T cells (potentially the cells with strongest avidity) may respond in a C3-independent fashion. This latter possibility is supported by the

observation that T cell immunity, and in particular CD8 T cell immunity, is partially but not fully abrogated in mice deficient in C3 (11,25–27). Graft-derived C3 and graft-expressed DAF can also influence transplant injury through mechanisms that do not involve direct effects on T cells, including modulation of ischemia-reperfusion injury induced by natural Ab or mannose-binding lectin, and potentially contributing to antibody-mediated injury (28,29). The abrogation of such mechanisms may in part account for the prolonged survival of C3-deficient allografts despite the similar numbers of anti-donor T cells in detected recipients of $C3^{-/-}$ and WT control hearts on day 8 posttransplantation (Fig. 1).

While our studies focused on the role of donor DAF expression in direct allorecognition pathway T cell responses, we and others have shown that $DafI^{-/-}$ T cells respond stronger than do WT T cells (5,10). Nonetheless, our current data (Fig. 1) reveal that recipient DAF expression does not affect rejection as significantly as does donor DAF expression. The dominant effects of donor DAF on acute rejection can be explained by 1) the principal role of direct alloreactivity as a mediator of acute rejection; 2) our previous observations that APC-DAF, not T cell-DAF, is the key regulator of T cell immune responsiveness (5); and 3) the fact that APCs produce 1000-fold more complement than T cells (8).

Donor and/or recipient DAF expression could also influence the strength of the indirect alloresponse, but detailed evaluation of the effects of DAF deficiency on indirect Ag processing, presentation, and T cell activation will require further study.

In addition to providing mechanistic insight, our results have clinically relevant implications for approaches aimed at prolonging transplant survival. The data support the concept that regulating complement activation through overexpression of DAF (or other complement regulatory molecules) on donor organs, through genetic, pharmacologic, and or protein chemistry approaches, could be useful. Interestingly, other groups have shown that in vitro perfusion of donor organs with a lipid-tailed complement regulator CR1 is feasible, and while effects on T cell immunity were not tested, the approach limited ischemia-reperfusion injury in animals (30). Our data imply that it will be important to additionally test how this manipulation influences anti-donor T cell immunity. Blocking Abs specific for individual complement components (31,32) and small molecule inhibitors of complement receptors have already been developed for human use, and it will be important to assess whether and how these agents influence alloreactive T cells responding to a transplant.

In conclusion, these results extend previous work by showing that local complement activation, regulated by donor DAF expression, exerts control over the strength of the direct pathway ($CD8^{+}$) alloimmune repertoire, enhancing its pathogenicity. The findings have implications for the design and interpretation of complement-based therapies aimed at prolonging allograft survival.

Acknowledgements

The authors thank Robert Fairchild and Austin Schenk (Cleveland Clinic, Cleveland OH) for scientific criticisms and technical assistance, and Feng Lin (Case Western Reserve University, Cleveland OH) for the $DafI^{-/-}$ mice.

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FIGURE 1.

Donor DAF deficiency accelerates kinetics of graft rejection associated with augmentation of anti-donor T cells. **A**, Survival of B6 (H-2^b) WT, *Daf1*^{-/-}, *C3*^{-/-}, and *Daf1/C3*^{-/-} hearts in C3H (H-2^k) recipients and WT B6 into C3H *Daf1*^{-/-} recipients ($n = 6 - 8/\text{group}$). *, $p < 0.05$ for MST vs WT donors. There was no significant difference in survival between *C3*^{-/-} and *Daf1/C3*^{-/-} hearts. **B**, Representative H&E-stained sections of B6 WT or *Daf1*^{-/-} cardiac allografts obtained on the day of rejection. White arrowhead marks a focal infiltrate of polymorphonuclear leukocytes. Magnification, $\times 400$. **C**, Spleen cells were obtained from groups of transplanted animals on day 3 (*left*) or day 8 (*right*) and tested in IFN- γ ELISPOT assays against WT B6 stimulator cells. Total number of responding cells was calculated by multiplying the frequency times the number of spleen cells per mouse. Mean values $\pm$ SE are shown for $n = 3 - 5/\text{group}$. *, $p < 0.05$ vs control WT (Student's t test).

FIGURE 2.

Donor DAF deficiency does not alter alloantibody production. **A**, Serial dilutions of sera obtained from recipients ($n = 3/\text{group}$) were tested for reactivity to donor WT B6 thymocytes by flow cytometry. Alloantibody titers detected in recipients of WT vs *Daf1*^{-/-} hearts were not different on day 7 but were both significantly greater than that detected in naive serum. Sera from recipients of WT hearts obtained on day 18 (rejection) contained more alloantibody than did day 7 samples (*, $p < 0.05$). *Daf1*^{-/-} hearts were rejected by day 9, and thus day 18 samples from this group were not collected/ tested. **B**, C3d stained frozen sections of WT or *Daf1*^{-/-} heart grafts obtained on day 8 posttransplant (while still beating). Representative of at least three sections obtained from three individual animals per group.

FIGURE 3.

DAF-deficient skin allografts prime stronger anti-donor CD8 T cell responses. Groups of BALB/c recipients mice were transplanted with full-thickness trunk skin allografts from WT or congenic knockout donors ($n = 4 - 8/\text{group}$). Animals were sacrificed on days 10–12, and pooled splenic CD8 T cells were isolated ($>92\%$ CD8⁺) and tested in IFN- γ ELISPOT (A) or CTL assays (B) against WT B6 target cells. Each point represents the mean $\pm$ SE of triplicate wells. *, $p < 0.5$ vs control WT. Representative of at least two independent experiments per group. Note that in this fully allogeneic skin transplant model all grafts are rejected by day 14 regardless of donor origin (no significant difference among donor groups).

FIGURE 4.

BM cell expression of DAF in the donor heart drives the kinetics of graft rejection. **A**, Flow cytometry demonstrating spleen cells in chimeras are >92% BM donor-derived. Representative plots of splenocytes from control Thy1.1⁺ WT and Thy1.2 *Daf1*^{-/-} are shown in the top two panels and spleens from Thy1.2 *Daf1*^{-/-} BM $\rightarrow$ Thy1.1 WT chimeras or Thy1.1 WT BM $\rightarrow$ Thy1.2 *Daf1*^{-/-} chimeras in the bottom two panels. **B**, Representative plots of heart-derived cells (*top*) or spleen cells (*bottom*) from CD45.2 *Daf1*^{-/-} BM $\rightarrow$ CD45.1 WT chimeras (*left*) or CD45.1 WT BM $\rightarrow$ CD45.2 *Daf1*^{-/-} chimeras (*right*). Plots show CD45.1 vs CD45.2 gated on CD11b/CD11c⁺ cells, which were all >95% donor BM-derived. **C**, Median survival time of chimeric hearts transplanted in C3H recipients ($n = 4 - 6$ /group). *, $p < 0.05$ by log-rank test vs WT $\rightarrow$ WT controls. **D**, Recall anti-B6 IFN- γ ELISPOT assays performed at the time of rejection. Bars represent mean values of triplicate wells for $n = 4 - 6$ /group. *, $p < 0.05$ by Student's t test vs WT $\rightarrow$ WT controls.

FIGURE 5.

Enhanced primary in vitro proliferation of alloreactive CD8 T cells in response to *Daf1*^{-/-} APCs. Representative 72 h (A) or 120 h (B) two-color, annexin V/CFSE flow cytometry plots of CFSE-labeled BALB/c splenic T cells obtained from naive mice stimulated with peritoneal macrophages from syngeneic BALB/c or allogeneic B6 WT, *Daf1*^{-/-}, or *C3*^{-/-} mice. Plots are gated on CD8⁺ cells (anti-CD8-APC mAb). Numbers represent the percentage of cells within each quadrant. Dashed boxes show the percentage of CD8 T cells that underwent more than 7 divisions, which was significantly greater in response to *Daf1*^{-/-} compared with WT APCs at each time point. Percentage proliferating cells was greater to all of the allostimulators than to syngeneic controls. The response to *C3*^{-/-} APCs was significantly less than to WT APCs at both time points. The experiment was repeated with similar results.

FIGURE 6.

Target cell expression of DAF enhances proliferation but does not influence IFN- γ secretion or CTL activity of primed alloreactive CD8 T cells. Splenic CD8 effector T cells (CD44^{high}, CD62L^{low}) were isolated from B6-skin graft-primed BALB/c mice and tested in IFN- γ ELISPOT assays (A) and in CTL assays (B) using congenic WT B6 and *Daf1*^{-/-} target/stimulator spleen cells. There was no significant difference between groups. Representative of three independent experiments. C, Representative annexin V-PE/CFSE two-color plots of CFSE-labeled splenic CD8 effector T cells (CD44^{high}, CD62L^{low}) isolated from B6-skin graft-primed BALB/c mice assayed on day 3 or 6 after in vitro stimulation with macrophages obtained from WT, *Daf1*^{-/-}, *Daf1/C3*^{-/-}, or *C3*^{-/-} mice. The responses to *Daf1*^{-/-} stimulators were significantly greater than those to all other stimulators at all time points. Representative of at least two individual experiments assayed at each time point.

FIGURE 7.

Target cell DAF deficiency induces enhanced secondary expansion of HY-reactive CD8 T cells in vivo. *A*, Schematic of experimental design. *B*, IFN- γ ELISPOT assays performed on day 21 ($n = 3/\text{group}$). *, $p < 0.05$. *C*, Representative photomicrographs of elastin-stained hearts obtained on day 90 posttransplant in Uty/Smcy-immunized mice boosted with WT male spleen cells (*left*) or *Daf1*^{-/-} spleen cells (*right*). Significant vasculopathy ($\rightarrow$) was noted in all animals boosted with *Daf1*^{-/-} spleen cells and in none of the four animals boosted with WT spleen cells. Magnification, $\times 100$.

FIGURE 8.

Donor DAF deficiency accelerates T cell-mediated allograft rejection. **A**, Survival of WT or *Daf1*^{-/-} hearts transplanted into C3H SCID recipients following adoptive transfer of purified C3H T cells ($n = 3/\text{group}$). *, $p < 0.05$. **B**, Representative H&E sections of a rejected *Daf1*^{-/-} heart (harvested on day 8) and a beating WT heart (harvested on day 19). Magnification, $\times 100$.