Alpha-1-antitrypsin monotherapy reduces graft-versus-host disease after experimental allogeneic bone marrow transplantation

Isao Tawara*, Yaping Sun*, Eli C. Lewisb, Tomomi Toubai*, Rebecca Evers*, Evelyn Nieves*, Tania Azamc, Charles A. Dinarelloc1, and Pavan Reddy*a1

1Department of Internal Medicine, University of Michigan Comprehensive Cancer Center, Ann Arbor, MI 48109; 2Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel; and 3Department of Medicine, University of Colorado School of Medicine, Aurora, CO 80045

Contributed by Charles A. Dinarello, November 26, 2011 (sent for review August 8, 2011)

Acute graft-versus-host disease (GVHD) is a major complication that prevents successful outcomes after allogeneic bone marrow transplantation (BMT), an effective therapy for hematological malignancies. Several studies demonstrate that donor T cells and host antigen-presenting cells along with several proinflammatory cytokines are required for the induction of GVHD and contribute to its severity. Increasing evidence demonstrates that human serum-derived alpha-1-antitrypsin (AAT) reduces production of proinflammatory cytokines, induces anti-inflammatory cytokines, and interferes with maturation of dendritic cells. Using well-characterized mouse models of BMT, we have studied the effects of AAT on GVHD severity. Administration of AAT early after BMT decreased mortality in three models of GVHD and reduced serum levels of proinflammatory cytokines in the allogeneic recipients compared with vehicle (albumin) treated animals. AAT treatment reduced the expansion of alloreactive T effector cells but enhanced the recovery of T regulatory T cells, (Tregs) thus altering the ratio of donor T effector to T regulatory cells in favor of reducing the pathological process. However, despite altering the ratio in vivo, AAT had no direct effects on either the donor T effector cells or T regulatory cells Tregs in vitro. In contrast, AAT suppressed LPS-induced IL-1β-mediated vascular and liver disease, as well as reduced the size of infarct and the severity of heart failure in a mouse model of acute myocardial ischemia-reperfusion injury (32).

Given the role of proinflammatory cytokines in the pathogenesis of acute GVHD (2) and the tissue-protective and anti-inflammatory properties of AAT (22–27), we sought to investigate the effects of AAT administration in several well-characterized murine models of allogeneic BMT. We hypothesized that administration of AAT early in the time course of allogeneic BMT, during the intensification of the proinflammatory cytokine cascade (2, 4), would suppress cytokine production and reduce the severity of systemic GVHD.

Consistent with this hypothesis, we found that monotherapy with clinical-grade human AAT (hAAT) reduced circulating proinflammatory cytokines, diminished GVHD severity, and prolonged animal survival after experimental allogeneic BMT.

Results

Administration of hAAT Decreases Severity and Mortality from GVHD.

To determine whether administration of hAAT affects the severity of GVHD and subsequent mortality from the condition, three models for GVHD were examined. In the first model, hAAT was administered in a dose-dependent manner in a MHC disparate B6 (H-2b) → B6D2F1 (H-2b/a) mouse model, and the severity of GVHD was examined (33). Lethally irradiated B6D2F1 mice received bone marrow (BM) and splenic T cells from syngeneic (B6D2F1) or allogeneic (B6) donors. BMT recipient mice received i.p. with 1, 2, or 4 mg per mouse of either hAAT or human alpha-1-antitrypsin (α1-AT).


The authors declare no conflict of interest.

*To whom correspondence may be addressed. E-mail: cdinarello@mac.com or reddypr@umich.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.111765109/-/DCSupplemental.

PNAS | January 10, 2012 | vol. 109 | no. 2
564-569 | www.pnas.org/cgi/doi/10.1073/pnas.111765109

© 2012 National Academy of Sciences
All rights reserved.
AAT Alters Mature Donor T Effector-to-Treg Ratio After Allogeneic BMT. The expansion of mature donor cytopathic effector T cells (Teffs) and Tregs was determined, and the ratio of Teffs to Tregs (Teffs:Tregs) was calculated in allogeneic transplants treated with hAAT. Mice (B6) were irradiated and transplanted with BM and CD8+ T cells from the allogeneic C3H.SW donors. Because expansion of alloreactive T cells peaks at 3–4 wk in this model, splenocytes from recipient animals were isolated at 4 wk and analyzed for donor Teff and Treg cell expansion by donor-specific congenic markers.

To exclude the possibility of confounding effects of donor BM-derived Tregs in these analyses, and to analyze the kinetics of mature donor Teffs:Tregs, we used B6 GFP+ foxp3 knock-in mice as donors for Teffs (GFP+ and Ly5.1+) and mature Tregs (GFP+ and Ly5.1-). In a similar manner, B6 Ly5.2 (CD45.1) used as the source of donor BM is responsible for CD45.2+ mature Teff (GFP+) or Tregs (GFP-). These cells were infused into the MHC-matched but minor disparate C3H.SW animals, and the expansion of the mature Teffs and Tregs was determined.

Because both CD4+ and CD8+ T cells are responsible for mortality in this model, we analyzed the ratio of both mature donor CD4+ Teffs and mature CD8+ Teffs to the mature donor GFP+ Tregs on day 21 and day 28 after BMT (6). As shown in Fig. 3 A–D, the ratio of Teffs:Tregs was significantly altered for both CD4+ and CD8+ Teffs on both day 21 and day 28. The effect of hAAT was compared with rapamycin, an immunosuppressive agent that has been shown to enhance Tregs in these models (40). As shown in Fig. 3 C and D, alteration in the Teffs:Tregs ratio by hAAT produced effects similar to those induced by rapamycin on day 28.

Given the alteration in the expansion and the ratio of the donor Teffs and Tregs, we next determined whether hAAT had direct effects on donor Teff responses in vitro. The proliferation of Treg-depleted donor T cells (BALB/c) to allogeneic (B6) BM-derived dendritic cells (DC) was equivalent regardless of whether the donor T cells were pretreated with hAAT or vehicle (Fig. 4 A).

To determine the direct impact of hAAT on T cells without a confounding effect of hAAT on accessory cells, anti-CD3–induced T-cell activation was examined in the presence of hAAT. Consistent with previous reports (28), addition of AAT did not interfere with T-cell responses (Fig. 4). In addition, hAAT allowed uninterrupted T-cell lysis of host-type conA blast cells following priming [57% and 63% at 50:1 Effector:Target (E:T) ratio; P = not significant].

We next determined whether hAAT might directly enhance Treg functions, as determined by their ability to suppress T

Fig. 1. AAT reduces mortality from GvHD. (A) B6D2F1 mice were irradiated with 1,000 centigray (cGy) of total-body irradiation on day −1 and transplanted with 5 × 10^6 T-cell-depleted BM cells and 2 × 10^6 CD90+T cells from either syngeneic F1 or allogeneic B6 donors. Each allo-recipient was injected i.p. with either 4 mg hAAT (n = 9) or human albumin (n = 9) for 6 d from day −2 to day +13. Data shown are combined from two similar experiments. Percentage survival after BMT is shown. For ▲ vs. ▼, P = 0.02. (B) B6 mice were given 1,000 cGy of total-body irradiation on day −1 and transplanted with 5 × 10^6 T-cell-depleted BM cells and 2 × 10^6 CD90+T cells from either syngeneic B6 or allogeneic C3H.SW donors. Each allo-recipient was injected i.p. with either 2 mg hAAT (n = 17) or human albumin (n = 16) for 6 d from day −2 to day +13 and was monitored for GvHD survival. Data shown are combined from three similar experiments. Percentage survival after BMT is shown. For ▲ vs. ▼, P = 0.029. (C) C3H.SW mice were irradiated as above and transplanted with 4 × 10^6 T-cell-depleted BM cells and 1 × 10^6 CD90+T cells from either syngeneic C3H.SW or allogeneic B6 donors. The allo-recipient were injected i.p. with either 2 mg hAAT (n = 12) or human albumin (n = 15) and were monitored for GvHD survival as above. Data shown are combined from two similar experiments. Percentage survival after BMT is shown. For ▲ vs. ▼, P = 0.019.
enhance the expansion of Teffs while mitigating the responses of T cells and that the in vivo alterations in the ratios of donor T cells suppress BALB/c CD25+/FoxP3- T cells were then used at varying ratios to analyze their ability to harvest, sorted, and pretreated with hAAT or vehicle. The effect of hAAT on host APC. The effect of hAAT on dendritic cells and GvHD severity might be the consequence of an inflammatory cytokine, IL-10, has been shown to play a critical role in determining the severity of GvHD (2, 9, 10). Therefore, we reasoned that the alteration of the Teffs: Tregs ratio and the protection from GvHD might be the consequence of an inflammatory cytokine secretion by hAAT. Mice (B6D2F1) were irradiated and transplanted with BM and T cells and were injected with either hAAT or albumin as before. Serum samples were analyzed on day +7 from transplantation for TNF-α, IL-1β, and IL-6. Administration of hAAT significantly reduced serum levels of all three proinflammatory cytokines compared with control allo-recipients (Fig. 5).

Given the lack of direct effect of hAAT monotherapy on donor T cells, we next reasoned that the reduction in proinflammatory cytokines and GvHD severity might be the consequence of an effect of hAAT on host APC. The effect of hAAT on dendritic cells was thus examined. BM-derived DC from B6 mice were incubated overnight with hAAT before stimulation with 100 ng/mL LPS for 8 h. The secretion of proinflammatory cytokines from hAAT-treated DCs was significantly reduced (Fig. 6). In contrast, the secretion of an anti-inflammatory cytokine, IL-10, was significantly enhanced by hAAT compared with albumin-treated controls. The changes in IL-6 and IL-10 were also observed under similar conditions in host F4/80+ macrophages (Fig. S1).

AAT Inhibits LPS-induced NF-κB Translocation in DCs. To examine a possible mechanism for the reduction of LPS-induced proinflammatory cytokine secretion by hAAT in BM DCs, NF-κB translocation into the nucleus was determined. AAT or human albumin were added to BM-derived DCs and then stimulated with LPS. NF-κB translocation was analyzed by electrophoretic mobility shift assay (EMSA) in the nuclear fraction of cell lysates. Treatment with hAAT significantly reduced LPS-induced translocation of NF-κB into the nucleus compared with control DCs (Fig. 7). However, consistent with previous observations (43), the levels of expression of MHC class II CD80 and CD86 were high following stimulation of DCs with LPS. NF-κB translocation to the nucleus was determined by expression of the above costimulatory molecules (Fig. S2). However, despite the lack of effect on phenotype, AAT attenuated the functional responses of DCs, the secretion of proinflammatory cytokines, and NF-κB translocation.

**Fig. 2.** hAAT reduces Teff expansion but enhances Treg expansion. B6 mice were irradiated and transplanted with either allogeneic C3H.SW donor. The recipient animals received either hAAT or albumin as above. Expansion of C3H.SW donor (CD229.1+) CD8+ and CD4+FoxP3+ T cells was analyzed using absolute numbers of C3H.SW donor-derived (CD229.1+) (A) CD8+ and (B) CD4+FoxP3+ T cells are shown. (C) The ratio of CD8+ to CD4+FoxP3+ T-cell absolute numbers is shown. Each point represents one individual mouse (n = 4–5/group). *P < 0.05 for A and P < 0.04 for B and C.

**Fig. 3.** hAAT alters the ratio of mature donor Teff to Treg cells. C3H.SW mice were irradiated and transplanted with either allogeneic B6GFP+Foxp3 knock-in donor. The recipient animals received either hAAT or rapamycin or the control vehicle as above. Expansion of mature donor (CD45.2+CD45.1+CD229.1+GFP+) CD4 and CD8+ effectors and the mature donor (CD45.1+CD45.2+CD229.1+GFP+) Tregs was analyzed in the peripheral blood on day +21 and day +28 for the ratio of CD8+ to CD4+FoxP3+ T-cell absolute numbers of mature donor-derived (CD45.2+CD45.1+CD229.1+). Each point represents one individual mouse (n = 2–5/group). (A) CD4+Treg ratio on day +21, vehicle vs. hAAT; P = 0.037. (B) CD8+:Treg ratio on day +21, vehicle vs. hAAT, P = 0.022. (C) CD4+Treg ratio on day +28, vehicle vs. hAAT, P = 0.0223. (D) CD8+:Treg ratio on day +28, vehicle vs. hAAT; P = 0.0127.

**AAT Inhibits Proinflammatory Cytokine Release After Allogeneic BMT.** The presence of proinflammatory cytokines has been shown to enhance the expansion of Teffs while mitigating the responses of Tregs (41, 42). In particular, IL-1β, TNF-α, and IL-6 have been shown to play a critical role in determining the severity of GvHD (2, 9, 10). Therefore, we reasoned that the alteration of the Teffs: Tregs ratio and the protection from GvHD might be the consequence of the suppression of proinflammatory cytokine secretion by hAAT. Mice (B6D2F1) were irradiated and transplanted with BM and T cells and were injected with either hAAT or albumin as before. Serum samples were analyzed on day +7 from transplantation for TNF-α, IL-1β, and IL-6. Administration of hAAT significantly reduced serum levels of all three proinflammatory cytokines compared with control allo-recipients (Fig. 5).

Given the lack of direct effect of hAAT monotherapy on donor T cells, we next reasoned that the reduction in proinflammatory cytokines and GvHD severity might be the consequence of an effect of hAAT on host APC. The effect of hAAT on dendritic cells was thus examined. BM-derived DC from B6 mice were incubated overnight with hAAT before stimulation with 100 ng/mL LPS for 8 h. The secretion of proinflammatory cytokines from AAT-treated DCs was significantly reduced (Fig. 6). In contrast, the secretion of an anti-inflammatory cytokine, IL-10, was significantly enhanced by hAAT compared with albumin-treated controls. The changes in IL-6 and IL-10 were also observed under similar conditions in host F4/80+ macrophages (Fig. S1).
Discussion

The induction of acute GvHD is a consequence of donor T-cell responses to host alloantigens and the dysregulation of proinflammatory cytokine cascades (2, 4, 9, 10). Inflammatory cytokines play a key role in the pathogenesis of GvHD (2, 9, 10). In the current study, monotherapy with hAAT reduced several proinflammatory cytokines and GvHD mortality in multiple models.

The effect of AAT in regulating inflammatory processes is increasingly appreciated (44) in several experimental models (26–29, 45, 46). The concentrations and doses used in the current study are derived and further extended from these reports, according to which 2 mg hAAT per mouse is sufficient to allow islet allograft acceptance (a dose that is comparable to that used routinely in humans who are deficient in AAT) (27), and 0.5 mg/mL hAAT protects various cell types in vitro from multiple injuries. Thus, we explored in vivo doses of 1, 2, and 4 mg per mouse and also tested in vitro concentrations up to 4 mg/mL. The present data demonstrate that exogenous administration of hAAT after allogeneic BMT suppresses proinflammatory cytokines; alters the ratio of T effector cells to T regulatory cells; and, more importantly, reduces GvHD severity and related mortality.

In examining the cellular targets that can mediate such profound changes in animal model outcomes, we found that the presence of AAT did not directly alter the activity of T effector or T regulatory cells to host antigens in vitro, suggesting that the in vivo effects on donor T-cell subset expansion is likely an indirect effect, perhaps as a consequence of reduced inflammation. AAT appears to promote an environment that facilitates expansion of T regulatory cells and IL-10 (29, 30) while mitigating the expansion of donor T effectors by suppressing the proinflammatory milieu early after allogeneic BMT. Both rapamycin and AAT increase the ratio of Tregs after allogeneic BMT. Recent data have shown that rapamycin can directly modulate Tregs and that its effects can be further augmented by IL-2 (40, 47). In contrast to rapamycin, the mechanism of AAT in enhancing the ratio of Tregs might be more indirect, perhaps a consequence of increased secretion of IL-10, mitigation of the proinflammatory milieu (such as IL-6 that has been shown to affect the generation and stability of Tregs), and lack of impact on AAT on IL-2 secretion (27, 28, 48).

The anti-inflammatory effects of exogenous AAT observed in our study are consistent with several recent reports of this activity in solid organ allograft and autoimmune models (29–32). Monotherapy with AAT was reported to reduce cytokine-mediated islet damage and promote tolerance toward islet cell allografts (29, 30). Similarly, treatment with AAT dampened inflammation without

Fig. 4. AAT does not affect T-cell responses in vitro. (A) BALB/c splenic CD4+25+ T cells were treated overnight with hAAT or albumin at either 2 or 4 mg/mL and were then used as responder in an MLR with B6 BM DCs as stimulators. Freshly isolated BALB/c CD4+25+ Tregs were added at the indicated ratios to the above cultures, and T-cell proliferation was determined by 3H-thymidine incorporation at 96 h. Data are mean ± SEM of quadruplicate cultures. P = not significant, control vs. hAAT-treated CD4+ T cells. Data shown are from one of two similar experiments. (B) Treg treatment with hAAT. BALB/c splenic CD4+25+Tregs were treated overnight with hAAT or albumin at either 2 or 4 mg/mL and were then used as suppressors of freshly isolated BALB/c CD4+25+ at the indicated ratios in an MLR with B6 BM DCs as stimulators. T-cell proliferation was determined by 3H-thymidine incorporation at 96 h. Data are mean ± SEM of quadruplicate cultures. P = not significant, control vs. hAAT-treated CD4+25+ Tregs. Data shown are from one of two similar experiments.

Fig. 5. Injection of hAAT inhibits in vivo proinflammatory cytokine production after BMT. B6D2F1 mice were exposed to 1,000 cGy of total-body irradiation and transplanted with 5 × 10⁶ T-cell-depleted BM cells and 2 × 10⁶ T cells from either allogeneic (B6) or syngeneic (B6D2F1) donors. Each F1 recipient of the allogeneic cells was injected i.p. with 4 mg hAAT or human albumin on days −2, +1, and +4. Sera from the recipient animals (n = 4–5/group) were obtained on day 7 after BMT and analyzed for TNF-α, IL-1β, and IL-6. Albumin-treated allogeneic controls (solid bars) vs. hAAT allogeneic recipients (open and gray bars) for TNF-α, *P < 0.04, IL-1β, All, **P < 0.03; IL-6, *P < 0.04.
direct inhibition of T-cell activation and restored euglycemia and immune tolerance to β-cells in NOD mice with recent-onset type 1 diabetes (31, 46). Our data are also consistent with recent observations that AAT inhibits IL-32 and results in attenuation of GVHD (49). However, in contrast to those observations, in the present study, the allo-responses were not completely abrogated and tolerance was not induced after allo-BMT, as demonstrated by the persistence of some GvHD despite the reduction in mortality. Our observations nonetheless confirm the in vivo anti-inflammatory effects of AAT in clinically relevant models of GvHD. We further extend the observations by demonstrating that the beneficial effects of hAAT are, at least in part, because of direct modulation of DC responses. The suppression of proinflammatory cytokine secretion responses of DCs by AAT is consistent with other studies (26, 30). Furthermore, similar suppression of proinflammatory cytokines from human PBMCs has been observed, supporting the applicability of these observations to the human context (25–28, 50, 51). The role of AAT in suppressing inflammation in humans is also supported by the observation that Staphylococcus epidermis-stimulated blood collected from AAT-deficient individuals demonstrated significantly greater IL-8, IL-6, and IL-1Ra production compared with blood from healthy donors (26). Our data show that AAT directly reduced LPS-stimulated NF-κB nuclear translocation and indicate that this might be a crucial mechanism by which AAT reduces DC responses. These data are consistent with other observations in other cell types that demonstrate inhibition of NF-κB activation by AAT (52, 53).

The role of AAT in the context of clinical acute GvHD has not been well explored. A negative correlation between fecal AAT concentrations and serum albumin levels in patients with severe gastrointestinal GvHD has been reported (54). The monocyte-macrophage contribution to serum levels of AAT after BMT has also been reported (18). It is possible that the levels of AAT might be elevated in acute GVHD similarly to the elevated levels of other acute-phase reactant proteins, such as CRP or ferritin (55). The increased production of AAT following many inflammatory conditions may be a protective regulatory response to any aggressive systemic inflammation, and, as such, greater-than-normal concentrations might be needed to further dampen the ongoing inflammation. Although these issues are yet to be resolved, our data demonstrate a potent role for AAT in reducing the early proinflammatory responses contributing to GvHD without having a direct effect on donor T cells. Because AAT replacement has already been safely used in several patients with little toxicity and is FDA approved, AAT may be examined in human allogeneic BMT recipients as an adjunct in the prevention or treatment of GvHD.

Materials and Methods

Human Alpha-1-Antitrypsin and Albumin. hAAT (Aralast) and human albumin were obtained from Baxter. Rapamycin and LPS were purchased from LC Laboratories and Sigma, respectively.

Mice. Female C57BL/6 (B6, H-2b, CD45.2) and C3H.SW (H-2b, CD45.2+) mice were purchased from the Jackson Laboratories. B6-Ly5.2 (H-2b, CD45.1+) and B6D2F1 (B6, H-2b/d, CD45.2+) mice were purchased from the Charles River Laboratories. GFP-FoxP3 knock-in mice were provided by Alexander Rudensky (University of Washington, Seattle, WA) and then bred at the University of Michigan animal facility.

Bone Marrow Transplantation. Bone marrow transplantation was performed as described in detail in SI Materials and Methods. Briefly, recipient mice
were irradiated (³²P) source with 10 Gy total-body irradiation and injected with either syngeneic or allogeneic T cells along with T-cell-depleted bone marrow cells. Animal studies were approved by the University of Michigan Committee on the Use and Care of Animals.

Bone Marrow Dendritic Cell Generation. DCs were generated from BM in the presence of 20 ng/mL recombinant murine GM-CSF (Peprotech) for 7 d and isolated using CD11c microbeads (Milteny). Flow Cytometric Analysis. Flow cytometric analysis was performed using FITC, phycocyanin (PE), PerCP-Cy5.5, or APC-conjugated monoclonal antibodies to mouse CD4 (clone RM4-4), CD229.1 (3C7), CD8a (53-6.7), CD25 (PC6.15), CD45.1 (A20), and CD45.2 (B220) and analyzed on a FACSCalibur (Becton Dickinson) or C6 cytometer (Accuri Cytometers) (see Materials and Methods for details).

EMSA. CD11c⁺ DCs from B6 mice were treated with human albumin (hALB) or hAAT for 4 h and then treated with LPS or diluent for another 3 h after which EMSA was performed (see Materials and Methods for details).

ELISA. ELISAs for TNF-α, IL-1β, IL-6, and IL-10 (BD Pharmingen) were performed according to the manufacturer’s protocol.

In Vitro Suppression Assay. CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were isolated, and suppression assay was performed as described (6, 10, 34, 36).

Statistical Analysis. Survival curves were plotted and compared by log-rank analysis; P < 0.05 was considered statistically significant. A paired t test was used to evaluate significant differences between groups in in vitro experiments.

Tawara et al.