Unexpected role of TNF-α in graft versus host reaction (GVHR): donor-derived TNF-α suppresses GVHR via inhibition of IFN-γ-dependent donor type-1 immunity

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Abstract

Graft versus host disease (GVHD) is a major complication of allogeneic hematopoietic stem cell transplantation, leading to significant morbidity and mortality. Host-derived TNF-α play a role in the induction of allo-reactive donor T cell activation and the pathogenesis of GVHD. On the other hand, the precise role of donor-derived TNF-α in GVHD remains unclear. To elucidate this issue, we designed an acute GVHD model using (B6×D2) F1 recipient mice transferred with spleen cells derived from either wild-type or TNF-α±/± C57BL/6 mice. Surprisingly, we found that spleen cells from TNF-α±/± mice induce more severe graft versus host reaction (GVHR) than wild-type spleen cells upon transfer into B6D2F1 mice. Transplantation of TNF-α±/± mouse spleen cells was associated with enhanced anti-host CTL generation and augmented deletion of host cells. Moreover, mice receiving TNF-α±/± cells showed significantly higher levels of serum IFN-γ, which was mainly produced by donor CD8+ T cells. We also demonstrated that TNF-α deficiency in donor spleen cells caused a marked elevation of TNF-α producing capacity by LPS-stimulated host macrophages. Such enhanced GVHR was completely prevented by using TNF-α±/±IFN-γ±/± splenic cells. Our findings demonstrate, for the first time, that donor-derived TNF-α suppress GVHR by inhibiting IFN-γ-dependent donor type-1 immunity which is essential for host TNF-α elevation.

Introduction

Allogeneic hematopoietic stem cell transplantation (HST) has been an effective treatment of hematologic malignancies and genetic disorders (1). The success rate of HST has steadily increased in recent years, but graft versus host disease (GVHD) is still a major cause of post-transplantation mortality (2,3). The generation of a strong graft versus host reaction (GVHR) is induced by activated donor T cells which recognize major and/or minor histocompatibility Ag mismatches. Cytokine dysregulation and organ damage due to pre-transplantation conditioning regimens are also involved in the development of GVHR (4–7).

Tumor necrosis factor α (TNF-α) has been implicated in the pathogenesis of GVHD. TNF-α induces a direct toxicity to host tissues and enhances the expression of MHC (8) and adhesion molecules (9). Moreover TNF-α may act as an autocrine T cell growth factor (10) and thus augment donor T cell clonal expansion. Anti-TNF-α mAb can ameliorate the severity of GVHD (11–13). In a recent study, it was shown that TNF-αR p55 of the recipient controls early GVHD (14) and that TNF-αR p55 of the donor plays a critical role in allo-reactive T cell response (15). Therefore, recipient-derived TNF-α contributes to the activation of allo-reactive T cells and augments the severity of acute GVHD. In contrast, the role of donor-derived TNF-α in GVHD remains unclear.

In the present study, we have examined the role of donor-derived TNF-α in allo-reactive T cell responses in vivo using well-characterized mouse models of GVHD. We demonstrate that mice transferred with TNF-α-deficient mouse spleen cells...
exhibit augmented general parameters associated with GVHR, including early elevation of donor-derived IFN-γ, generation of anti-host CTL and producing host-derived TNF-α. These data document an unrecognized role of donor-derived TNF-α, which might suppress early GVHR through the control of IFN-γ-dependent donor type-1 immunity.

Methods

Mice

C57BL/6J (B6) (H-2b) and B6 × DBA/2 F1 (B6D2F1) mice were obtained from Charles River Japan (Yokohama, Japan), TNF-α+/− C57BL/6 mice were provided by Dr K. Sekikawa (Department of Immunology, National Institute of Animal Health, Tsukuba, Japan) and IFN-γ+/− C57BL/6 mice were provided by Dr Y. Iwakura (Institute of Medical Science, University of Tokyo, Tokyo, Japan). 6–10 week-old mice were used for all experiments.

Induction of GVHD

Single-cell suspensions from the spleens of B6 and B6D2F1 mice were prepared in RPMI 1640 medium (Gibco-BRL, Grand Island, NY). The cells were suspended in PBS. Acute GVHD was induced by injecting B6 spleen cells (5 × 10⁷) into B6D2F1 mice. Age-matched B6D2F1 mice transferred with syngeneic spleen cells (5 × 10⁷) were used as control mice.

Flow cytometric analysis

The phenotypic characterization of spleen cells by flow cytometry was carried out using a FACSCalibur instrument and CELLQuest software (Becton Dickinson, San Jose, CA). mAbs used in these experiments [phycoerythrin (PE)-conjugated anti-CD4 mAb, PE-conjugated anti-CD8 mAb, PE-conjugated anti-B220 mAb and fluorescein isothiocyanate (FITC)-conjugated H-2Kd mAb] were purchased from PharMingen (San Diego, CA).

Intracellular cytokine expression

For the detection of cytoplasmic cytokine expression, cells stimulated with immobilized anti-CD3 mAb for 6 h in the presence of Brefeldin A were first stained with PerCP-conjugated anti-CD4 mAb or cyochrome-conjugated anti-CD8 mAb and FITC-conjugated anti-H-2Kd mAb, fixed with 4% paraformaldehyde and treated with permeabilizing solution (50 mM NaCl, 5 mM EDTA, 0.02% NaN₃ and 0.5% Triton X-100, pH 7.5). The fixed cells were then stained with PE-conjugated anti-IFN-γ mAb for 45 min on ice. The percentage of cells expressing cytoplasmic IFN-γ was determined by flow cytometry (FACSCalibur). PerCP-conjugated anti-CD4 mAb, cyochrome-conjugated anti-CD8 mAb and PE-conjugated anti-IFN-γ mAb were purchased from PharMingen.

Cytotoxicity assay

The cytotoxicity mediated by CTL was measured by 4 h ⁵¹Cr-release assay as described previously (16). H-2Kd-specific cytotoxicity was determined using DBA/2-derived P815 mastcytoma cells (H-2Kd) as target cells. As control, C57BL/6-derived MBL-2 T lymphocyte cells (H-2Kd) were used. The percentage cytotoxicity was calculated as described previously (16).

Generation of CTL in mixed lymphocyte culture (MLC)

Spleen cells (5 × 10⁶ cells) from GVHD mice and control mice were co-cultured with BDF1 mouse spleen cells (2.5 × 10⁶ cells) which were inactivated by pretreatment with mitomycin C (60 μg/ml; Kyowa Hakko Kogyo, Tokyo, Japan). Cells were co-cultured for 4 days in flat-bottomed 12-well plates. After culture, cells were harvested and their cytotoxicity was measured.

Measurement of cytokine levels by ELISA

IFN-γ levels in serum or culture supernatants were evaluated with commercial ELISA kit (Amersham International, Buckinghamshire, UK) according to the manufacturer’s instructions.

Measurement of serum TNF-α levels induced by LPS injection

LPS-induced TNF-α production was assayed in mice transferred with spleen cells from wild-type or TNF-α+/− mouse spleen cells. As a control, B6D2F1 mice transferred with syngeneic mouse spleen cells were used. Ten days after GVHR induction, the mice were treated with or without i.v. injection of LPS (10 μg) and their serum samples were harvested 90 min after LPS injection to determine serum TNF-α levels by ELISA (Amersham).

Statistical analysis

Difference between the means of experimental groups were analyzed using the Student’s t-test. Differences were considered significant where P < 0.05.

Results

TNF-α deficiency in donor cells accelerates GVHR in mice

B6D2F1 (H-2b,d) mice were treated with i.v. injection of wild-type or TNF-α+/− C57BL/6 (H-2b) spleen cells. As a control, mice were injected with syngeneic B6D2F1 spleen cells. After 14 days, mice were sacrificed to examine the frequency of host cell deletion, as detected with anti-H-2b mAbs and flow cytometry. As shown in Fig. 1, in mice transferred with wild-type C57BL/6 mouse spleen cells the percentage of host B cells decreased to 28.7%. Deletion of host B cells was further enhanced (84.3%) when TNF-α+/− mouse spleen cells were transferred into B6D2F1 mice. Host cell deletion was also demonstrated among CD4⁺ and CD8⁺ T cells (data not shown). Consistent with these findings, spleen cells obtained from B6D2F1 mice treated with TNF-α+/− splenocytes exhibited higher levels of anti-host CTL activity compared with spleen cells from mice transferred with control (B6D2F1) or wild-type C57BL/6 mouse splenocytes (Fig. 2).

TNF-α deficiency in donor cells accelerates the elevation of serum IFN-γ levels initiated by donor type-1 immunity during GVHD

As previously described (17,18), type-1 cytokines such as IL-12 and IFN-γ play a critical role in acute GVHD induction.
Fig. 1. Acceleration of host B cell deletion by GVH response in mice transferred with TNF-α−/− spleen cells. GVH response was induced in BDF1 mice by cell transfer with (A) syngeneic BDF1 (H-2b,d) spleen cells, (B) wild-type C57BL/6 (H-2b) spleen cells or (C) TNF-α−/− C57BL/6 (H-2b) spleen cells as described in Methods (n = 6). Fourteen days after GVH induction, host B cell deletion by GVHD response was determined by flow cytometry after staining with PE-labeled anti-B220 mAb and FITC-labeled anti-H-2Kd mAb. The numbers represent the percentage of cells in spleen cells. The total cell numbers of B cells in spleen is indicated in parentheses. Similar results were obtained in three different experiments.

Fig. 2. Augmentation of anti host CTL generation in mice transferred with TNF-α−/− spleen cells. BDF1 recipient mice were transferred with BDF1 mouse spleen cells (closed triangle), wild-type C57BL/6 (open circle) or TNF-α−/− C57BL/6 (closed circle) spleen cells. After 14 days, spleen cells were harvested from all mice and their CTL activity against host type P815 mastocytoma cells was measured by 4-h 51Cr-release assay. The data represent mean ± SE of three mice. Similar results were obtained in three different experiments.

Fig. 3. TNF-α−/− mice exhibited augmented producing ability of IFN-γ and TNF-α during GVHD. (A and B) Serum IFN-γ levels induced by allogeneic TNF-α−/− spleen cells during GVHD. BDF1 recipient mice were transferred with syngeneic BDF1, wild-type C57BL/6, TNF-α−/− C57BL/6 or IFN-γ−/− C57BL/6 spleen cells. (A). After 2, 6 or 10 days, serum IFN-γ levels of all mice were measured by ELISA. (B) Serum IFN-γ levels 6 days after donor cell transfer. (C and D) LPS-induced TNF-α production in recipient mice transferred with allogeneic TNF-α−/− spleen cells. BDF1 recipient mice were transferred with syngeneic BDF1, wild-type C57BL/6, TNF-α−/− C57BL/6 or IFN-γ−/− spleen cells. (C) After 2, 6 or 10 days, the recipient mice were treated with i.v. injection of LPS (10 μg/mouse) and their serum TNF-α level was determined by ELISA 90 min after LPS injection. (D) LPS-induced serum TNF-α elevation 10 days after donor cell transfer. The data represent mean ± SE of three mice. Similar results were obtained in three different experiments. *P < 0.05.
Therefore, we tested serum IFN-γ levels in recipient animals. Serum IFN-γ levels in recipient B6D2F1 mice became detectable at 4 days and reached a plateau at 6 days after transfer of wild-type or TNF-α−/− C57BL/6 spleen cells. The kinetics of serum IFN-γ production during GVHD is illustrated in Fig. 3(A). B6D2F1 mice transferred with TNF-α−/− spleen cells showed significantly higher levels of IFN-γ compared with mice transferred with wild-type C57BL/6 spleen cells. Of note, recipient mice treated with IFN-γ−/− C57BL/6 spleen cells did not show any increase in serum IFN-γ. This finding indicated that elevation of serum IFN-γ levels in recipient mice is dependent on the capacity of donor cells to produce IFN-γ.

**TNF-α deficiency in donor cells enhances TNF-α production by host cells during GVHD**

It has been reported that the capacity of host cells to produce TNF-α increases during the development of GVHR (19). Therefore, it was of great interest to determine how TNF-α deficiency in donor cells influences TNF-α production by host cells during GVHR. As shown in Fig. 3(C) and (D), we detected an increase of serum TNF-α levels upon injection of lipopolysaccharide (LPS) in B6D2F1 mice 10 days after treatment with wild-type C57BL/6 spleen cells but not B6D2F1 splenocytes. Recipient mice transferred with TNF-α−/− spleen cells, but not IFN-γ−/− spleen cells, showed robust elevation of TNF-α levels in response to LPS injection. Thus, these results demonstrate that defective TNF-α production in donor cells unexpectedly increases host TNF-α production. This phenomenon may contribute to the accelerated depletion of host cells observed in recipient animals that were treated with TNF-α−/− splenocytes.

**Suppression of GVHR by donor-derived TNF-α is associated with reduced donor type-1 immunity**

As shown in Fig. 3(B) and (D), donor-derived IFN-γ appears to be critical for initiation of early GVHR. To understand the precise role of donor-derived IFN-γ for enhanced IFN-γ and TNF-α production in B6D2F1 mice treated with TNF-α−/− mouse spleen cells, we evaluated TNF-α−/−IFN-γ−/− splenocyte for induction of GVHD in B6D2F1 mice. Consistent with previous results (Fig. 3), we observed a marked elevation of both serum IFN-γ and TNF-α in recipient mice transferred with TNF-α−/− spleen cells. However, no significant IFN-γ and TNF-α production was observed when animals received splenocytes from TNF-α−/−IFN-γ−/− mice (Fig. 4). These results suggest that defective TNF-α production by donor cells accelerates GVHR by activating donor IFN-γ-dependent type-1 immunity. To determine which subset of donor T cells is responsible for activating type 1 immunity, we examined the IFN-γ-producing capacity of CD4+ and CD8+ T cells from both donor (H-2b+, H-2d+) and recipient (H-2d+) mice by intracellular staining (Fig. 5). In keeping with the results of Fig. 3, B6D2F1 mice treated with TNF-α−/− spleen cells contained a higher frequency of IFN-γ-producing donor-derived CD8+ and CD4+ T cells. Donor-derived CD8+ Tc1 cells were particularly activated to produce IFN-γ (Fig. 5). In addition to the activation of donor Th1 and Tc1 cells, host Th1 and Tc1 cells were also activated to produce IFN-γ. However, when TNF-α−/−IFN-γ−/− spleen cells were used as donor cells, no significant increase in recipient-derived IFN-γ producing Th1 and Tc1 cells were induced (Fig. 5).

We further demonstrated that anti-host CTL generation, which is essential for the deletion of host cells, is greatly reduced in B6D2F1 mice transferred with TNF-α−/−IFN-γ−/− spleen cells, as compared with mice transferred with TNF-α−/− spleen cells (Fig. 6). In this experiment, CTL were induced from spleen cells of B6D2F1 mice transferred with donor cells by resensitized with B6D2F1 spleen cells because freshly isolated spleen cells from recipient mice 7 days after donor cell transfer exhibited low levels of cytotoxicity.

Thus, we concluded that in the absence of donor-derived TNF-α during the early phase of GVHR, donor-type-1 immunity, especially Tc1 activity, is activated in a manner that depends on IFN-γ production by donor cells. Subsequently, enhanced host type-1 immunity may induce TNF-α production by host macrophages, which in turn augments GVHR.

**Discussion**

In the present paper, we clarify the precise role of donor-derived TNF-α in acute GVHD using donor spleen cells from TNF-α−/− mice. We find that the defects in donor-derived TNF-α...
accelerate GVHR, including host IFN-γ and TNF-α production. These data suggest that donor-derived TNF-α may suppress GVHR via controlling donor IFN-γ-dependent type I immunity.

The critical role of proinflammatory cytokines, particularly TNF-α in acute GVHD, has been described in many experimental models and clinical experiments (20–22). The relationship between pre-transplant conditioning regimens, TNF-α production (22–25) and acute GVHD is particularly well-characterized. Chemotherapy and/or total body irradiation damages host tissues including the skin, intestine and liver. Subsequently, the damaged tissues themselves produce TNF-α and LPS, which leak into the systemic circulation and stimulate residual macrophages in the recipient to produce TNF-α. Using TNF-α receptor-deficient mice, it has been demonstrated that host-derived TNF-α plays a critical role in the early activation of allo-reactive donor T cells and increases morbidity and mortality of acute GVHD. In mouse models, anti-TNF-α mAb treatment of lethally irradiated recipients early in bone marrow transplantation reduces mortality and ameliorates pathology in skin and gut lesions. Thus, host-derived TNF-α has been considered to play a critical role in acute GVHD. In our present study we observed paradoxical effects indicating that donor-derived TNF-α suppresses early activation of allo-reactive donor T cells. We found that recipients transferred with TNF-α±/± spleen cells exhibit higher production of IFN-γ by donor Tc1 and Th1 cells, followed by enhanced IFN-γ secretion from residual recipient CD4+ and CD8+ T cells. Activated donor Tc1 and Th1 cells induce host tissue damage by activating host TNF-α-producing macrophages. Thus, TNF-α deficiency in donor cells accelerates the induction of anti-host CTL activity and host TNF-α-producing capacity, which induces severe GVHR. The augmented IFN-γ production is not derived from the different immunological condition between TNF-α-deficient and wild-type mice. TNF-α±/± mice possess the same percentage of immunoregulatory cells (CD4+ Th, CD8+ Tc and B cells) and exhibit the same levels of T cell responses induced by stimulation with anti-CD3 mAb or alloantigen (data not shown).

To explain our finding, several possible mechanisms are considered as follows: (1) TNF-α±/± mice produce higher levels of IFN-γ because they produce less amounts of soluble TNF-α receptor (TNF-αR), which is a blockade for TNF-α, in comparison with wild-type mice; and (2) TNF-α produced by activated T cells or APC has a capability of suppressing hyperactivity of T cells. In terms of soluble TNF-αR production, it was demonstrated TNF-α±/± mice produced the same levels of soluble TNF-αR2 as wild-type mice when they were injected with LPS (data not shown). This result is consistent with previous report that demonstrated the shedding of TNF-α R was induced independently on TNF-α levels (26). Therefore,
it appears to be unlikely that augmented GVHD is derived from the defect of TNF-α R production in TNF-α−/− mice. TNF-α−/− mice exhibit the same levels of T cell responses to anti-CD3 mAb and alloantigen as wild-type mice (data not shown). This observation suggests that TNF-α produced in wild-type mice does not suppress naive T-cell response to antigen. However, recently, we found that TNF-α−/− mice exhibited greatly enhanced T-cell responses in secondary allogeneic responses compared with wild-type mice, if pre-immunized mouse spleen cells were used as responder cells of MLC (data not shown). These findings strongly suggest that TNF-α may act as a negative feedback factor for T-cell hyperactivity as IL-27 does (27).

As classical risk factors of acute GVHD, HLA mismatches between donor and recipient are well-documented (28,29). Many studies have examined risk factors by studying the production of inflammatory cytokines. Holler et al. (30) have demonstrated that host-related secretion of TNF-α during pre-transplant conditioning correlates with subsequent GVHD and mortality after transplantation. Analysis of clinical risk factors for enhanced TNF-α response suggested a role for endogenous endotoxin and immunogenetic factors of cytokine activation. In human, the gene encoding TNF-α is located within the MHC locus on chromosome 6 (31), and the inducibility of TNF-α has been associated with certain HLA-class II genotypes (32). A close association between cytokine gene polymorphisms and cytokine inducibility has also been identified. There is a single base polymorphism in the TNF-α gene at position −308(G/A) (33). The rare allele TNF2(A) is closely associated with HLA A1, B8 and DR3 (34). When allelic distribution of this polymorphism was analyzed in 72 BMT recipients, a clear association of the TNF2(A) allele with enhanced in vitro TNF-α production in response to LPS and subsequent development of acute GVHD was noted (35). Another study has reported that the d3 homozygous allele of the TNF-α microsatellite is preferentially associated with grade III/IV GVHD (36). While these data indicate a role for TNF-α production by the recipient in predicting the development of GVHD, the relationship between donor TNF-α production and GVHD severity has never been elucidated. In a mouse model, Cooke et al. (37) have reported that LPS responsiveness of donor accessory cells correlates with GVHD severity. These investigators suggested that TNF-α production by LPS-stimulated donor cells may be a risk factor for the development of donor T cell responses to host antigens. In contrast, our data indicate that donor-derived TNF-α has a suppressive effect on the initiation of GVHR. The discrepancy in these results may be due to different cellular sources for TNF-α production. Cooke et al. (37) investigated TNF-α producing capacity of LPS-reactive TLR4-bearing APC populations using C3H/HeJ TLR4-deficient mice, while we assessed TNF-α producing ability of donor T cells in addition to APC. We found that accelerated GVHR induced by transfer with TNF-α−/− spleen cells was abrogated when either CD4+ T cells or CD8+ T cells were eliminated from donor spleen cells (data not shown). This result indicates a critical role of T cells in the initiation of GVHR, which might be triggered independently of donor APC populations. Our finding that (i) donor TNF-α−/−IFN-γ−/− cells do not accelerate GVHD (Figs 4–6) and (ii) donor-derived CD8+ T cells are major IFN-γ producing cells during the early stage of GVHR (Fig. 6), strongly suggests that memory-type IFN-γ producing CD8+ T cells play a critical role in GVHR induction. We are currently investigating the detailed cellular mechanisms by which donor-derived TNF-α suppressed donor type-1 immunity.

Donor lymphocyte infusion (DLI) has been frequently utilized for treatment of recurrent hematologic malignancies after allo-HST (38). This procedure re-induces complete remission in many patients, but the risk of lethal GVHD is still hard to predict. Because DLI is performed after primary allo-HST, TNF-α production by damaged tissues in the host after conditioning therapy is not as high as compared with donor-derived TNF-α. At present, it may be of great importance to investigate whether severity of GVHD can be predicted by examining donor TNF-α producing capacity, especially by T cells. We are currently investigating this issue.

In conclusion, we have shown, for the first time, that TNF-α−/− donor cells accelerate GVHR early after allogeneic transplantation. Therefore, donor-derived TNF-α may suppress GVHD morbidity. These findings provide novel approaches to detect patients undergoing allo-HST with increased risk for GVHD. Further studies will be required to determine the role of donor-derived TNF-α in a clinical transplantation setting.

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Abbreviations

DLI donor lymphocyte infusion
GVHD graft versus host disease
GVHR graft versus host reaction
HST hematopoietic stem cell transplantation

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