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Alteration of Innate Immunity by Donor IL-6 Deficiency in a Presensitized Heart Transplant Model

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Abstract

Engraftment of IL-6 deficient donor into wild-type recipient could significantly improve allograft survival through T cell lineage particularly regulatory T cells (Tregs) in non-sensitized transplant host. However, its effect on innate immune responses remains uncertain. Our data revealed that donor IL-6 deficiency significantly increased infiltration of two subsets of MDSCs (CD11b+Gr1+ myeloid-derived suppressor cells), CD11b+Gr1low and CD11b+Gr1int with strong immunosuppression activity in the transplanted graft. It resulted in a dramatic increase of CD11b+Gr1int frequency and a significant decrease of the frequency of CD11b+Gr1hi and CD4-CD8-NK1.1+ cells in the recipient's spleen. Unexpectedly, donor IL-6 deficiency could not significantly reduce macrophage frequency irrespective of in the host's spleen or graft. Taken together, suppression of innate immune effector cells and enhanced activity of regulatory MDSCs contributed to tolerance induction by blockade of IL-6 signaling pathway. The unveiled novel mechanism of targeting IL-6 might shed light on clinical therapeutic application in preventing accelerated allograft rejection for those pre-sensitized transplant recipients.

Introduction

Interleukin-6 (IL-6) as a pleiotropic cytokine can be produced by various tissues particularly proinflammatory cell types and formed in local cytokine milieu. It is capable of orchestrating cellular differentiation, affecting early graft function recovery [1], and disrupting transplant tolerance induction [2,3]. IL-6 produced from injured allograft vessels could promote the magnitude of intimal expansion and allogeneic T cell infiltration through suppressing an increase of CD161+ regulatory T cells [4]. The multifaceted activities of IL-6 can be characterized by inhibition of Th1 response, impairment of suppressive function of regulatory T cells (Tregs), and promotion of Th2 and Th17 lineage development, directing against transplant tissue [5-7]. IL-6 amplifier and NF-kB-triggered positive feedback for IL-6 mediate in vitro hyperinduction of chemokine ligand 2 (CCL2) by IFN-γ in type 1 collagen cells, contributing to allogeneic responses and graft rejection [8]. Therefore, it was observed that donor graft-derived but not recipient source IL-6 becomes a systemic danger signal impairing constitutive immune suppression. Accumulating experimental and clinical evidences revealed that intragraft IL-6 gene expression level is closely associated with chronic [9] and acute graft rejection [3,7]. Elevation of urinary IL-6 concentration and serum IL-6R (IL-6 receptor) level was reported to be a suitable biomarker for predicting an early acute transplant rejection episode [10]. Conversely, donor IL-6 deficiency prolongs allograft survival via the presence of regulatory CD25+ T cells [3]. Neutralization of IL-6 can reduce T cell infiltration and decrease Th17 markers [4], rescuing early graft function [1] and significantly prolonging allograft survival [2,3,7].

Indeed, not only adaptive but innate immune cells are required for acute or chronic rejection in various transplant settings [11]. A recent study reported that TLR4/TRIF pathway contributes to allogeneic bone marrow cell rejection dependent on innate immune cells including F4/80+ (macrophages) or NK1.1+ cells (NK cells), causing a significant production of proinflammatory cytokine IL-6 and TRIF relevant chemokine
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MCP-1 [12]. During transplantation surgery the mechanical and ischemia-reperfusion injuries are inevitable, which causes microbial productions or endogenous pro-inflammatory ligands [11]. Thereafter, necrotic lysates released from graft can trigger higher inflammatory responses in dendritic cells (DCs) and natural killer cells, resulting in a remarkable production of inflammatory mediators including TNF-α and IL-6 in a MyD88-dependent manner, an insusceptibility of alloreactive T cells to suppression by CD4+CD25+ regulatory T cells (Tregs), and an initiation of T-cell immunoresponses against graft [2,11]. By contrast, absence of both IL-6 and TNF-α can result in permanent acceptance of allograft [2].

Although impact of IL-6 on adaptive immune responses was described in different transplant models [2,3,7], its specific contribution to the process of innate immune responses remains to be elucidated. To this end, we utilized a pre-sensitized transplant model for studying the effect of IL-6 on innate immune responses. A deeper understanding of the role of IL-6 during transplantation may pave the way to a more success in identifying novel transplant tolerance induction strategies [11].

Materials and Methods

Animals

Eight-twelve week male inbred mice were obtained from the Jackson Laboratories (JAX, Bar Harbor, ME) including wild-type C57BL/6 (B6; H-2b; Stock No. 000664), BALB/c (B/c; H-2b; Stock No. 00651), and B6:129S2-IL-6tm1Kopf/J (IL-6KO; H-2b; Stock No. 002650). The mice were housed under pathogen-free conditions at the Center for Life Science (Boston, MA). Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Beth Israel Deaconess Medical Center. Allogeneic heterotopic heart transplantations (HTx) were performed using BALB/c donors and C57BL/6 recipients.

Vascularized heterotopic heart transplantation

Abdominal heterotopic heart transplantation was performed using standard microsurgical techniques as previously described [13,14]. Briefly, the mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg). The pulmonary artery and the ascending aorta of donor were anastomosed end-to-side to the recipient’s inferior vena cava (IVC) and the abdominal aorta. Graft function was daily monitored by observation of donor heartbeat palpation. The happening of rejection was judged by histology. The cardiac grafts that did not function at day 1 were engrafted with wild-type C57BL/6 hearts with PCI (7.0±0.5 hours, G5) or IL-6 deficient (IL-6KO) C57BL/6 hearts with PCI (6.5±0.8 hours, G6). This PCI time can partially cause cardiac graft injury rather than heart-beat failure (unpublished data).

Reagents and antibodies

Immunofluorescence analysis and mAbs. Harvested spleen and heart cells were directly labeled with fluorescently conjugated monoclonal antibodies (mAbs). All mAbs used for cell surface staining were purchased from BioLegend (San Diego, CA). For FACS staining, anti–mouse CD4 (Clone H129.19, Catalogue No. 130308) FITC-conjugated mAb, anti–mouse CD8a (Clone 53–6.7, Catalogue No. 100701) PE-conjugated mAb, Alexa Fluor® 700-conjugated anti–mouse Gr-1/Ly-6G (Clone RB6-8C5, Catalogue No. 108421) mAb, and the pacific blue-conjugated anti–mouse CD11b (clone M1/70, Catalogue No. 101223) mAb were used to label live T cells. All samples were acquired using an LSRII (BD Biosciences, Mountain View, CA). Data was analyzed using FlowJo 7.5 software (Tree Star, Ashland, OR), as we reported before [16]. The analysis gate was set on the side and forward scatters to eliminate debris and dead cells cell.

Isolation of graft-infiltrating leukocytes

The heart grafts were harvested, minced and then incubated in Roswell Park Memorial Institute (RPMI) 1640 medium for 30 minutes at 37ºC, followed by centrifugation at 1000 rpm for 5 minutes and re-suspension in RPMI 1640 medium.
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Results

Prolonged Survival of IL-6 Deficient Heart Allografts

Previously, various experimental studies demonstrated a critical role of donor IL-6, which mainly originated from the donor allograft tissue and could impair allograft survival [3]. This data was supported by our present studies, in which utilization of IL-6 deficient donor caused a prolonged allograft survival. The mean survival time (MST) of IL-6-/- heart grafts in the wild-type BALB/c recipients (IL-6KO B/c) was significantly increased (19.8±2.8 days) compared to that of allogeneic grafts (B6 B/c) (MST=8.0±1.2 days; Mantel-Cox Test, p=0.0012; Gehan-Breslow-Wilcoxon Test, p=0.0031) (Figure 2).

However, additional use of anti-CD25 mAb (PC61) to those IL-6-/- heart grafts (IL-6-/-BALB/c) pronouncedly shortened graft survival equivalent to allogeneic control group (B6 BALB/c) (Figure 2). An increase of frequency of peripheral CD4+CD25+Foxp3+ regulatory T cells (Figure 1) was caused owing to acute rejection event, accounting for a protective reactivity of recipient in the settings of strong effect of effector cells. It demonstrated that depletion of Tregs resulted in the poor transplant outcome despite lack of donor IL-6 cytokine, indicating that regulatory T cells were involved in the protective effect of donor IL-6 deficiency. Afterwards, pathological and immunohistochemical analysis were performed to further validate this findings. As a result, mild subepicardiac graft infiltrating leukocytes (GILs) particularly reduction of Gr1+ neutrophils were observed, while donor IL-6 deficiency combined with Tregs depletion caused a severe infiltration of leukocytes and Gr1+ neutrophils and cardiac structure destruction, accounting for acute rejection events on day 9 post-transplantation (Figure 3A and 3B).

Characterization of innate immune cells in PCI-mediated injury in the pre-sensitized transplant host engrafted with IL-6 deficient donor

To clearly identify the impact of PCI on allograft in the sensitized recipients, we dissected the process of accelerated transplantation and utilized syngeneic cardiac transplantation with PCI after recipients were pre-sensitized by fully MHC-mismatched skin. Based on the evident effect of IL-6 abrogation in the non-sensitized transplant model, we attempted to test whether lack of intragraft IL-6 cytokine could cause any significant alteration of innate immune response in the pre-sensitized transplant host's spleen. Based on aforementioned data on the critical role of Tregs in the transplantation, we firstly tested non-specific innate immunoregulatory cells, myeloid-derived suppressor cells (MDSCs), which have been thoroughly studied in our previous research. MDSCs can be sub-grouped into three different subsets, CD11b+Gr1- low, CD11b+Gr1- int, and CD11b+Gr1- high with distinct immunoregulatory effect. Therefore, we tried to...
characterize MDSCs subsets, macrophage, NK1.1+ cells in the spleen of wild-type donor + PCI (G5) and IL-6 deficient donor + PCI (G6) by flow cytometry analysis (Table 1). As a result, our studies revealed that donor IL-6 deficiency caused mild reduction of macrophage (F4/80+) frequency (G5 vs. G6: p=0.3), a dramatic increase of CD11b+Gr1low frequency (p=0.0021) and a significant decrease of CD11b+Gr1high frequency (p=0.0021) and a significant decrease of CD11b+Gr1high frequency.

Kaplan-Meier cardiac graft survival curve. Heterotopic abdominal heart transplantation was performed by using standard procedure. All transplanted mice were monitored every day until graft rejection, defined as the cessation of palpable cardiac activity. Although only three symbols of “triangle” are observed for group (WT B6B/c), two grafts survived for 7 days and two grafts survived for 8 days. It implies that each “triangle” on day 7 (60% remained to survive on day 7) and day 8 (20% remained to survive on day 8) represents two grafts. Graft survival of the allogeneic control group (C57BL/6BALB/c, n=5) is equivalent to IL-6 deficiency donor graft (IL-6KO/BALB/c) treated with anti-CD25 mAb (n=5) (Mantel-Cox Test, p=0.17; Gehan-Breslow-Wilcoxon Test, p=0.28), whereas IL-6 deficiency donor graft survival (IL-6KO/BALB/c) treated with iso-IgG (n=5) was significantly prolonged (Mantel-Cox Test, p=0.012; Gehan-Breslow-Wilcoxon Test, p=0.0031) in comparison to allogeneic control group.

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Graft histology and immunohistochemical analysis of grafts. (A) The heart transplanted recipients were sacrificed at day 9 after surgery. Allogeneic grafts were harvested, stained with H&E to assess inflammation and lymphocytes infiltration between different groups under microscope, as described in the Methods section. The figure shows that a mild leukocytes infiltration was observed in the IL-6 deficient donors (G1) (n=2), while a pronouncedly infiltration of leukocytes and preserved cardiac architecture were observed in the subepicardial area of the IL-6KO grafts treated by anti-CD25 mAb (PC61) (G2) (n=2) and wild-type allografts (G3) (n=2) without any treatment. (B) Grafts specimens harvested at day 9 post-transplant were snap-frozen into Tissue Tec and stained for Gr-1 protein as described in the Materials and Methods section. Immunohistochemical sections showed that depletion of Treg cells using anti-CD25 mAb (PC61) remarkably caused an infiltration of Gr-1+ cells in the IL-6 deficient grafts, which is similar to acutely rejected wild-type hearts. A mild subepicardial Gr-1+ cells infiltration was found within IL-6KO grafts without any treatment. Original magnification ✗40.

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Significant accumulation of CD11b+Gr1\textsuperscript{-}low and CD11b+Gr1\textsuperscript{-}int MDSCs within IL-6 deficient graft. To detect whether donor IL-6 deficiency affects two important subsets of MDSCs, CD11b+Gr1\textsuperscript{-}low and CD11b+Gr1\textsuperscript{-}int with strong immunosuppression activities, the pre-sensitized transplanted grafts with PCI including IL-6-deficiency and wild-type were harvested. The infiltrated leukocytes within graft were isolated and subject to cytometry analysis. The findings revealed that donor IL-6 deficiency did not remarkably suppress infiltration of macrophage within graft (p=0.3), while it caused a significant increase of intragraft CD11b\textsuperscript{-}Gr1\textsuperscript{-}low (p=0.00001) and CD11b\textsuperscript{-}Gr1\textsuperscript{-}int frequencies (p=0.032) in the early stage (Figure 5).
Discussion

The presensitized PCI-mediated transplant model was selected for two reasons. First, prior to empirical transplant surgery a considerable amount of transplant recipients have undergone previous failed transplants, multiple blood transfusions, ventricular assist device placement, or pregnancies, more likely to endanger hosts to alloantigen-sensitization situation. Our previous novel findings revealed that presensitized immune condition of recipient can exaggerate PCI-mediated injury of heart grafts. Second, owing to scarcity transplant organ much effort has been made to utilize remote donors, leading to a prolongation of cold-ischemic time during organ transportation.

Allogeneic immune responses directing allograft are the main obstacle to transplant tolerance induction [7]. Past studies on rejection process, blockade of IL-6 could delay the onset of acute rejection, prevent graft infiltration and reverse the ratio of Th1/Th2 in host, while in the setting of CD8+ cell-dominant rejection process, blockade of IL-6 could significantly prolong graft survival associated with reduced graft infiltration, altered Th1 responses, and inhibited production of serum alloantibody [7]. Nevertheless, the specific pathway of IL-6 toward triggering allogeneic innate immunoresponses remains unclear particularly the role of regulatory innate immune cells, MDSCs and further investigations are required to clarify the details of underlying molecular mechanisms.

Taken together, our present study revealed that blockade of IL-6 signaling pathway suppressed innate immune effector cells in large measure and enhanced activity of regulatory MDSCs. This unveiled novel mechanism of targeting IL-6 might shed light on clinical therapeutic application in preventing accelerated allograft rejection for those pre-sensitized transplant recipients.

Author Contributions

Conceived and designed the experiments: FG WG. Performed the experiments: ZS TH SY WG. Analyzed the data: LS ZS AH WG. Contributed reagents/materials/analysis tools: LS FG WG. Wrote the manuscript: FG WG.
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