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High-mobility group box 1 accelerates early acute allograft rejection via enhancing IL-17$^+$ γδ T-cell response

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Keywords
acute allograft rejection, high-mobility group box 1, IL-17$^+$ γδ T cells, IL-23.

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The authors declare no conflict of interest.

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Summary

Th17 and γδ T cells are the dominant IL-17-producing cell. We previously reported that high-mobility group box 1 (HMGB1) is critical in inducing IL-17-producing alloreactive T cells during early stage of acute allograft rejection. However, the role of γδ T cells during this process and its implication in HMGB1–mediated allograft rejection are not fully understood. Here, we use a murine model of cardiac allograft transplantation to further study the role of HMGB1 and IL-17-producing γδ T cells in acute allograft rejection. It was found that the expression of HMGB1 was increased in allograft, while blockade of HMGB1 suppressed IL-17$^+$ γδ T-cell response and inhibited the gene transcription of IL-23 and IL-1β. Furthermore, *in vitro* HMGB1 indirectly promoted the development of IL-17$^+$ γδ T cells by stimulating dendritic cells to produce IL-23 and IL-1β, meanwhile depletion of γδ T cells *in vivo* prolonged allograft survival and reduced the level of IL-17 in serum. In conclusion, our findings inferred that increased HMGB1 expression could enhance IL-17$^+$ γδ T-cell response by promoting the secretion of IL-23 and IL-1β, while IL-17$^+$ γδ T cells contribute to the early stage of acute allograft rejection.

Introduction

Acute allograft rejection is one of the most common causes of graft failure after cardiac transplantation [1,2]. Helper T cells especially Th1 were considered as the dominant effector cells mediating acute allograft rejection [3]. Previously, we reported that high-mobility group box 1 (HMGB1) released during transplantation is essential for induction of IL-17-producing alloreactive T cell and induces early stage of allograft rejection [4,5]. Recent studies demonstrated that γδ T cells, in addition to CD4$^+$ T cells, are an important source of IL-17 [6,7]. For example, the majority of the IL-17-producing T cells in mycobacteria-infected mice are γδ T cells, rather than Th17 [8,9]. In addition, more and more studies in various experimental models demonstrated a critical role for IL-17$^+$ γδ T cells [10–12]. Some experts even speculated that IL-17$^+$ γδ T cells possess capabilities equivalent to Th17 cells, and IL-17$^+$ γδ T cells participate in protective immunity before the appearance of Th17 [13].

As an important component of IL-17 producing T cells, IL-17$^+$ γδ T cells share some common characteristic features with Th17 cells, such as expression of retinoid orphan receptor (ROR γt), chemokine receptor 6 (CCR6), aryl hydrocarbon receptor (AhR), and IL-23 receptor [14,15]. In contrast to Th17 cells, IL-17$^+$ γδ T cells can directly interact with certain pathogens via toll-like receptors (TLRs) [14]. When triggered by IL-23, γδ T cells produce a large amount of IL-17 and blockade of IL-23 leads to little IL-17 production [9,16]. Some γδ T cells even appear to have an inherent ability to produce IL-17 [7,17]. However, the precise role and exact regulatory mechanism of IL-17$^+$ γδ T cells in acute allograft rejection remain to be elucidated.

HMGB1 is a highly conserved nuclear protein that plays an important role in chromatin organization and transcriptional regulation [18]. It has been previously reported that HMGB1 could activate inflammatory pathways when released from ischemic cells [19]. By interacting with TLR2,

TLR4, TLR9, and RAGE, HMGB1 is able to activate many cell populations, such as macrophages and endothelial cells [20,21]. However, it is not clear whether HMGB1 could regulate IL-17 production in γδ T cells and is involved in acute allograft rejection.

In this study, we found that γδ T cells are another predominant source of IL-17 during early stage of acute allograft rejection and that HMGB1 could promote IL-17$^+$ γδ T cells by inducing IL-23 and IL-1β release from dendritic cells (DCs).

Materials and methods

Animals

C57Bl/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from the Institute of Experimental Animal, Chinese Academy of Medical Sciences (Beijing, China). Female mice (6–8 weeks) were used in all experiments. Mice were housed in the specific pathogen-free barrier facility at the Tongji Medical College for at least 1 week before transplantation. All of the studies were performed in accordance with the Tongji Medical College Animal Care and Use Committee guidelines.

Cardiac allograft transplantation and HMGB1-neutralizing mAb or antimouse TCR γδ mAb administration

BALB/c (H-2^d) mice were used as donors, and C57Bl/6 (H-2^b) mice were used as recipients. C57Bl/6 (H-2^b) to C57Bl/6 (H-2^b) was used as the combination of syngeneic grafts transplantation. Heterotopic cardiac transplantation and assessment of allograft function were conducted as described previously [22]. Rejection was defined as complete cessation of cardiac contractility as determined by direct visualization. Two hundred microgram of antimouse HMGB1 mAb (Institute of Biophysics, Chinese Academy of Science, Beijing, China) was intraperitoneally injected daily into recipients from the day before transplantation till day 3 after transplantation. Recipients received the same amount of normal mouse IgG (Sigma, St. Louis, MO, USA) acted as controls [4]. For γδ T-cells depletion, 200 μg hamster antimouse TCR γδ mAb (UC7-13D5, Biolegend, San Diego, CA, USA) was given into recipients 2 days prior to transplantation, twice a week, as described previously [23]. Control mice received an intraperitoneal injection of isotype-matched Armenian hamster IgG (200 μg, Biolegend).

Graft histological analysis

Mice were killed by cervical dislocation, and cardiac allograft tissues were fixed in 4% paraformaldehyde. Serial sections were prepared using a microtome, and hematoxylin and eosin staining was used for the analysis of pathological changes.

Immunoblots

Total proteins were extracted from cardiac allograft and subjected to immunoblots assay as described previously [24]. The primary antibodies used in the study include rabbit anti-HMGB1 (Abcam, Cambridge, MA, USA) and rabbit anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubating with horseradish peroxidase conjugated antirabbit secondary antibody, the immunoreactivity was detected by an ECL system (Pierce, Rockford, IL, USA) and the blots were quantified by densitometry using an image analysis program [IMAGE J, National Institutes of Health (NIH), Bethesda, MD, USA].

SYBR green real-time PCR

Cardiac allografts were collected at indicated time points after transplantation and subjected to RNA isolation using the TRIzol (Invitrogen, Carlsbad, CA, USA) reagent according to the manufacturer's instruction. cDNA was synthesized from 5 μg RNA using a first-strand DNA synthesis kit (Fermentas Life Sciences, St Leon-Rot, Germany). The expression of IL-23, IL-1β, and IL-17 in the grafts was analyzed by real-time PCR using iCycler (Bio-Rad, Richmond, CA, USA). PCR mixture was prepared using SYBR super mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Primers for PCR amplification were as follows: IL-23p19:5′-GGGAACAAGATGCTGGATT-3′, 5′-CTTCACACTGGATACGGGG-3′; IL-1β: 5′-CAACCAACAAGTGATATTCTCCATG-3′, 5′-GATCCACACTCTCCAGCTGCA-3′; IL-17A: 5′-GACCAGGATCTCTTGCTGGA-3′, 5′-GGACTCTCCACCGCAATGA-3′; and GAPDH: 5′-TTCACCACCATGGAGAAGGC-3′, 5′-GGCATGGACTGTGGTCATGA-3′. Relative expression levels for these cytokines were normalized by GAPDH and calculated using the $2^{-\Delta\Delta C_t}$ method and expressed in arbitrary units. The expression of each cytokine in normal mice was used as calibrator.

Purification of splenic γδ T cells

γδ T cells were enriched from spleen using columns and a MACS kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction. In brief, spleen single-cell suspension was prepared and incubated with non-T-cell depletion cocktail for 15 min at 4–8 °C. Cell suspension was then passed through the LD column (Miltenyi Biotec) which is placed in the magnetic field of a suitable MACS separator (Miltenyi Biotec). Resulting cells were then incubated with antibiotin microbeads for 15 min

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at 4–8 °C and passed through the MS column (Miltenyi Biotec). Finally, the purity was tested by flow cytometry.

Generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were generated from bone marrow cells as described previously [4]. In brief, these cells were propagated from naïve C57Bl/6 mouse at 2×10^6 cells per well in 12-well flat-bottomed plates (Nunc, Roskilde, Denmark) supplement with 2 ml RPMI medium containing 10% fetal bovine serum (Invitrogen), penicillin (100 U/ml), and streptomycin (100 mg/ml) in the presence of rmGM-CSF (10 ng/ml) and rmIL-4 (2 ng/ml). Culture media were changed at day 3 and day 5, respectively. All recombinant cytokines used in *in vitro* experiments were obtained from PeproTech.

Measurement of IL-17A, IL-23, and IL-1β in serum or supernatant

After 7 days of induction, BMDCs were stimulated with HMGB1 (rHMGB1, 5 μg/ml) for 6 h or 24 h, respectively. Supernatant was then collected for IL-23 and IL-1β measurement by ELISA kits from Biolegend. Expression and purification of mouse recombinant HMGB1 (rHMGB1) were described previously [4]. For the measurement of IL-1β, ATP (10 mmol) together with HMGB1 was added into the culture. Spleen cells were stimulated with IL-23 (50 ng/ml, R&D) or IL-23 (50 ng/ml, R&D) combined with IL-1β (50 ng/ml, PeproTech, USA) for 48 h *in vitro*. Purified γδ T cells were treated with IL-23 (50 ng/ml, R&D) alone or IL-23 (50 ng/ml, R&D) in combination with IL-1β (50 ng/ml, PeproTech, USA) for 48 h, and the supernatants were harvested for the ELISA detection of IL-17A (eBioscience, San Diego, CA, USA). Sera collected from anti-γδ TCR antibody- or control IgG-treated recipients were used for the detection of IL-17A by ELISA kits from eBioscience (Sensitivity: 4 pg/mL).

Flow cytometry

Cells in allograft were prepared by digestion with 2 mg/ml collagenase IV (Invitrogen) and 10% fetal bovine serum in RPMI 1640 media for 1.5 h at room temperature. For the staining of cell surface markers, cells isolated from grafts or spleen were incubated with indicated fluorescence-conjugated Abs at 4 °C for 30 min. For intracellular staining, cells ($1–2 \times 10^6$ cells/ml) were first stimulated with cell stimulation cocktail (eBioscience) for 6 h at 37 °C and then stained with cells surface Abs (CD3, γδ TCR) for 30 min at 4 °C. Subsequently, cells were fixed by IC fixation buffer (eBioscience) for 15 min and permeabilized by 1× permeabilization buffer for 30 min at 4 °C and incubated with antimouse IL-17A. Abs used for flow cytometry are listed as follows: FITC-conjugated anti-CD3, PE-labeled anti-IL-17A, antigen-presenting cells (APC)-labeled γδ TCR and PerCP-cy5.5-labled IL-17A. Isotype-matched abs were used as control. All antibodies were purchased from eBioscience. Samples were harvested and analyzed using a BD LSRFortessa cytometer.

Statistical analysis

The data are presented as means ± SD. Statistical differences were determined by Student's *t*-test. Two-sided probability (P) values <0.05 were considered to be statistically significant.

Results

Kinetics of IL-17$^+$ γδ T cells in recipient mice during early stage of acute allograft rejection

Previously, we reported that HMGB1 induces IL-17-producing alloreactive T cells and mediates early stage allograft rejection, preceding the infiltration of IFN-γ-producing alloreactive Th1 cells [4]. Recent studies suggested that γδ T cells are another important source of IL-17 during acute allograft rejection [25].To investigate the role of IL-17$^+$ γδ T cells in acute allograft rejection, single-cell suspension were prepared from spleen and graft to examine the kinetics of IL-17-producing γδ T cells after allogeneic heart transplantation. As depicted in Fig. 1a, about 56.0% of IL-17$^+$ T cells in allograft are γδ T cells, suggesting that γδ T cells are an important source of IL-17 during the early stage of allograft rejection. Interestingly, there was a decrease of splenic IL-17$^+$ γδ T cells in mice with allogeneic grafts as compared to those with syngeneic grafts (Fig. 1b and c). In contrast, percentage of IL-17$^+$ γδ T cells in allograft was much higher than that of syngeneic graft 3 days after transplantation (Fig. 1d and e). Collectively, these data suggested that IL-17$^+$ γδ T cells are another important source of IL-17 and might contribute to acute rejection of allograft.

The levels of HMGB1 protein, IL-23, and IL-1β mRNA were increased during the early stage of acute allograft rejection

We have shown HMGB1 could promote IL-17 production during the early stage of acute allograft rejection [4]. To examine the involvement of HMGB1 in the induction of IL-17$^+$ γδ T cells and acute rejection, Western blot analysis was used to evaluate the level of HMGB1 in allograft and a time-dependent increase of HMGB1 was observed from day 1 through day 3 and day 7 after transplantation

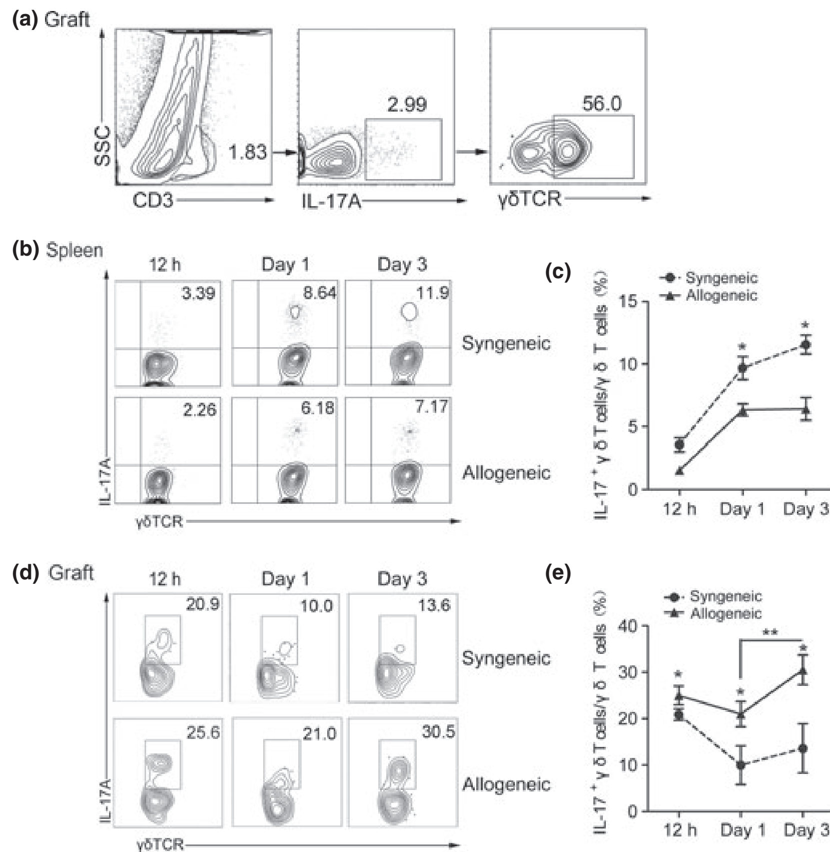

Figure 1 Kinetics of host-derived splenic IL-17$^+$ γδ T cells or IL-17$^+$ γδ T cells in cardiac grafts. Spleen and grafts were collected on 12 h, days 1 and day 3 after transplantation (n = 3–5/group/time point). (a) IL-17-producing T cells were gated in allograft and calculated for γδ TCR expression on day 3 after transplantation. To examine the production of IL-17 by splenic γδ T cells (b), (c) or γδ T cells in cardiac grafts (d), (e), spleen or graft single-cells suspension prepared from recipients was activated with Cell Stimulation Cocktail for 6 h, followed by flow cytometric analysis of intracellular IL-17 expression. In the sample gating, lymphocytes (SSC-A versus FSC-A) were gated for the analysis of CD3 and γδ TCR expression. CD3$^+$ γδ TCR$^+$ cells population was then gated for the analysis of IL-17. For cells in allograft, cells were gated on CD3$^+$ and then further analyzed for the expression of γδ TCR and IL-17A. Data are shown as mean ± SD. Three independent experiments were performed with at least three mice for each group at each time point. *$P < 0.05$; **$P < 0.01$.

(Fig. 2a). Furthermore, quantitative real-time PCR showed that the transcription of IL-23 and IL-1β was increased in allograft, and the highest mRNA level for IL-23 was noted at day 1 after transplantation (Fig. 2b).

HMGB1 augmented IL-17$^+$ γδ T-cell response during early acute allograft rejection

Previously, our laboratory demonstrated that blockade of HMGB1 prolong allograft survival [4]. Having found that IL-17$^+$ γδ T cells were another source of IL-17, we next tested whether HMGB1 has an effect on IL-17$^+$ γδ T-cell response in the context of transplantation. To this end, an antimouse HMGB1 mAb or control IgG was used in recipient mice of allogeneic transplantation. As depicted in Fig. 3a and b, blockade of HMGB1 reduced the proportion of IL-17$^+$ γδ T cells in both allograft and spleen. Consistently, blockade of HMGB1 inhibited the transcription of IL-23, IL-1β, and IL-17 in allograft on day 3 after transplantation (Fig. 3c). These results were further confirmed by histological examination (Fig. 3d). On day 3 postgrafting, allograft in anti-HMGB1 mAb-treated mice showed less inflammatory infiltration in comparison with IgG-treated group. These lines of evidence suggested that HMGB1 could be involved in the development of IL-17$^+$ γδ T cells, which depend on the expression of IL-23 and IL-1β.

Depletion of γδ T cells delayed cardiac allograft acute rejection

To further examine the impact of IL-17$^+$ γδ T cells on heart allograft survival, donor grafts from BALB/c mice were transplanted into antimouse TCR γδ mAb-treated recipient C57Bl/6 mice as described previously [23]. On day 3 after

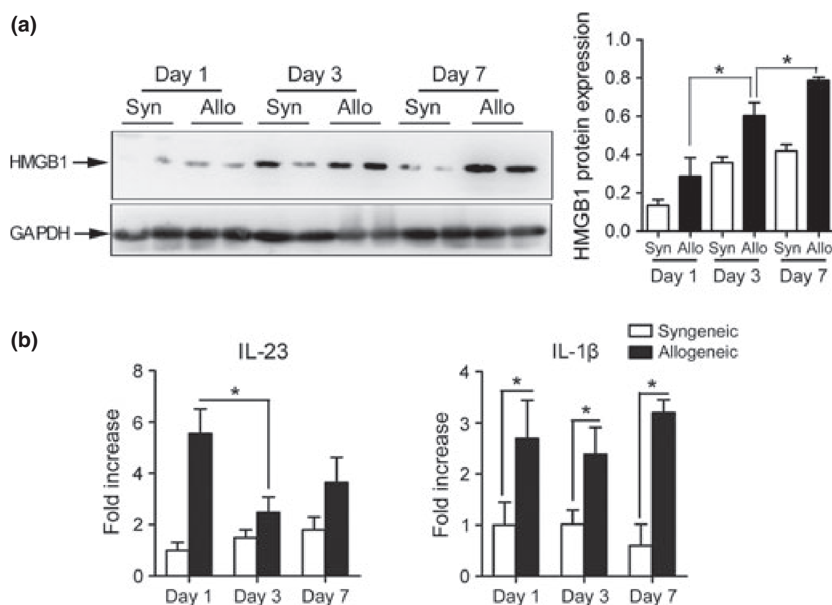

Figure 2 High-mobility group box 1 (HMGB1) protein, IL-23, and IL-1β mRNA level was increased in early stage of acute allograft rejection. Cardiac grafts samples were collected from mice on day 1, day 3, and day 7 after transplantation. (a) Expression of HMGB1 protein in total cardiac grafts tissue extracts was assessed by immunoblot. GAPDH was used as a loading control. Western blot assessment for HMGB1 in cardiac grafts was shown in the left panel, whereas the right panel showed densitometric analysis of the blots. (b) The mRNA expression of IL-23 and IL-1β was measured by qPCR in cardiac grafts. Data obtained from one representative of three independent experiments were shown (n = 3/group/time point). *$P < 0.05$.

transplantation, recipient mice were killed and splenic γδ cells were analyzed by flow cytometry. It was observed that TCR γδ almost disappeared in antimouse TCR γδ mAb-treated recipients, whereas this subpopulation of spleen cells was insusceptible at the same stage after intraperitoneal administration of control IgG (Fig. 4a). Importantly, treatment with anti-TCR γδ mAb modestly prolonged allograft survival compared with that of IgG-treated mice (MST, 11.0 ± 1.32 days vs. 7.28 ± 0.81 days, $P < 0.01$). No significant difference in allograft survival was observed between IgG-treated and untreated recipients (MST, 7.28 ± 0.81 days vs. 7.3 ± 0.75 days, $P > 0.05$; Fig. 4b). As shown in Fig. 4c and d, on day 3 postgrafting, lower serum level of IL-17 and less inflammatory infiltration in cardiac allograft were observed in anti-TCR γδ mAb-treated mice.

HMGB1 indirectly promoted dendritic cell activation and IL-23 and IL-1β secretion

It has been shown that HMGB1 induces DCs maturation and cytokine secretion [26]. To find out whether HMGB1 was able to augment IL-17$^+$ γδ T-cell response by stimulating DCs to secrete cytokines, BMDCs from recipient mice were stimulated with HMGB1 *in vitro*. LPS was used as a positive control. As shown in Fig. 5a, HMGB1 increased the secretion of IL-23 and IL-1β. Level of IL-23 was higher at 6 h compared with that of 24 h poststimulation, while similar levels of IL-1β were found at 6 and 24 h after stimulation. To further confirm whether γδ T cells produced IL-17 in response to the stimulation with IL-23 and IL-1β during acute allograft rejection, splenic cells were isolated from recipient mice and exposed to IL-23 in combination with IL-1β. It was found that IL-17$^+$ γδ T-cell response was enhanced by IL-23 combined with IL-1β *in vitro* (Fig. 5b). Finally, IL-17 produced by purified γδ T cells was further investigated by stimulation with IL-23 alone or IL-23 in combination with IL-1β. Figure 5c shows the purities of γδ T cells detected by flow cytometry (>87.2%). As a result, purified γδ T cells produced large amount of IL-17 in the presence of IL-23 or IL-23 in combination with IL-1β (Fig. 5d).

Discussion

Previously, we reported that blockade of HMGB1 is efficient on alleviating acute allograft rejection in mice [5]. Our subsequent findings suggested that HMGB1 is responsible for the augmented response of IL-17-producing cells during acute allograft rejection [4]. Both Th17 and γδ T cells are important sources of IL-17 production during a variety of pathologic conditions [6–9]. However, whether γδ T cells play a role in HMGB1-induced acute rejection is not clear. In the current study, we demonstrated that

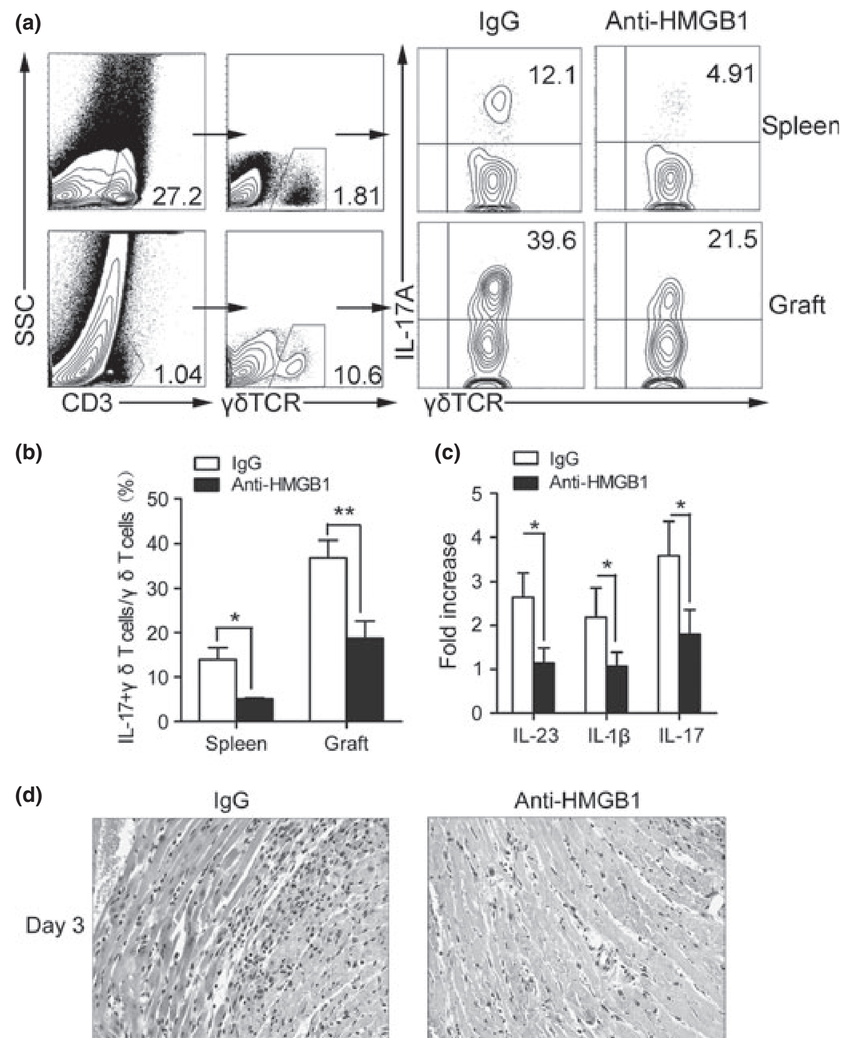

Figure 3 Blockade of high-mobility group box 1 (HMGB1) suppressed IL-17$^+$ $\gamma\delta$ T-cell response. Recipient mice (n = 3–5/group) were intraperitoneally injected with either anti-high-mobility group box 1 (HMGB1) mAb or mouse IgG, once per day, from the day before transplantation till day 3 after transplantation. Splenocytes and grafts were then collected. (a), (b) Spleen cells and cardiac allograft single-cell suspension from recipient mice were prepared on day 3 after transplantation and stimulated with Cell Stimulation Cocktail for 6 h and analyzed for intracellular IL-17 expression by flow cytometry. For cells in spleen or allograft, cells were gated on CD3$^+$ and then further analyzed for the expression of $\gamma\delta$ TCR and IL-17A. (c) Quantitative real-time PCR was performed to evaluate the effects of HMGB1 on IL-23, IL-1β, and IL-17 mRNA expression in cardiac allograft on day 3 after transplantation. (d) Representative H&E-stained sections of allograft harvested from anti-HMGB1 mAb-treated or IgG-treated recipient mice on day 3. Data are shown as mean ± SD. Three independent experiments (n = 3–5/group) were performed. *$P < 0.05$.

HMGB1 enhanced dendritic cell activation and release of IL-23 and IL-1β, which in turn promoted $\gamma\delta$ T-cell response and allograft rejection.

Although it remains unclear whether early acute allograft rejection is mediated by IL-17$^+$ $\gamma\delta$ T cells, there is now substantial evidence indicating that IL-17$^+$ $\gamma\delta$ T cells contribute to the pathogenesis of various autoimmune diseases [27], skin inflammation [15], ischemia-reperfusion injury [28], and host defense [29]. It was reported that IL-17$^+$ $\gamma\delta$ T cells are the predominant source of IL-17 [13,25]. Here, we presented evidence that IL-17$^+$ $\gamma\delta$ T cells participated in early stage of allograft rejection and acted as an important source of IL-17. In our study, $\gamma\delta$ T cells constituted a high proportion of IL-17$^+$ T cells. Furthermore, a larger amount of IL-17$^+$ $\gamma\delta$ T cells were observed in cardiac allograft, while splenic IL-17$^+$ $\gamma\delta$ T cells were decreased in allogeneic transplantation group compared to those with syngeneic grafts. This phenomenon might be related to the migration of IL-17$^+$ $\gamma\delta$ T cells from lymphoid organs to cardiac grafts, considering the fact that $\gamma\delta$ T cells expressed chemokine receptors CCR6, CXCR3, and CXCR4 [14,15,30]. However, the mechanisms underlying

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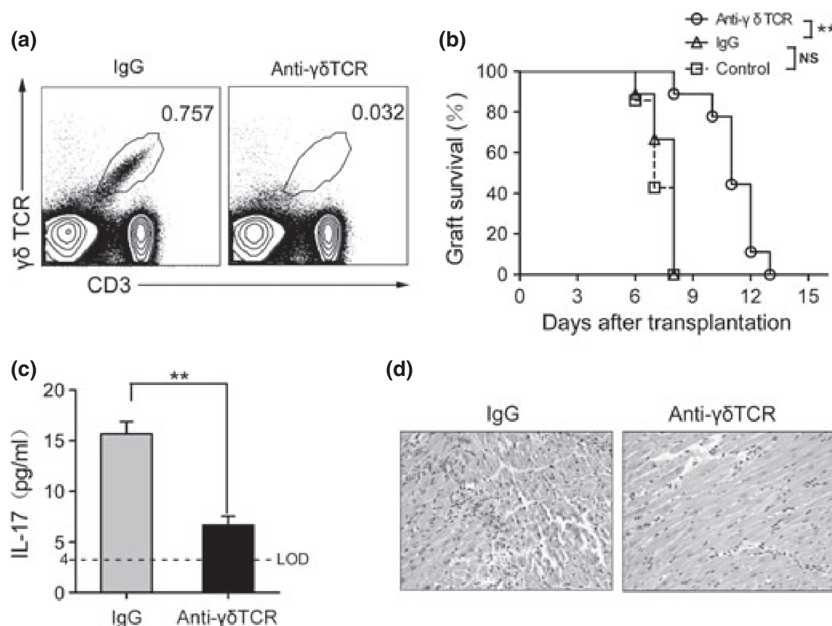

Figure 4 Depletion of γδ T cells modestly prolonged allograft survival. Recipient mice (n = 8/group) were intraperitoneally injected with either anti-mouse TCR γδ mAb or IgG 2 days before transplantation or untreated as controls. (a) Recipient splenic γδ T cells were detected by flow cytometry on day 3 after transplantation. (b) Graft survival was prolonged in antimouse TCR γδ mAb-treated recipients (11.0 ± 1.32, $P < 0.01$ vs. IgG) compared with IgG treated (7.28 ± 0.75 days, $P > 0.05$ vs. control) or controls (7.3 ± 0.81 days). (c) Serum levels of IL-17. Serum samples were collected on day 3 after transplantation, and IL-17 were detected by ELISA (the lower limit of detection is 4 pg/ml). (d) Histological images (HE, ×400).Grafts from antimouse TCR γδ mAb-treated and IgG-treated recipient mice were harvested on day 3. Cardiac allograft tissues were then sectioned for H&E staining. Data are shown as the mean ± SD (n = 6–8/group) and are representative of three independent experiments. **$P < 0.01$; LOD means limit of detection.

the recruitment of γδ T cells are not completely understood, and additional work is required to demonstrate the detailed mechanism.

Consistent with our current results, some investigators indicated that IL-17$^+$ γδ T cells are identified in allograft after transplantation [25]. Unexpectedly, in our present study, γδ T cells rapidly produced IL-17 even in the first 12 h, which indicated that IL-17$^+$ γδ T cells probably develop differently from Th17 cells and that these γδ T cells already seem to exist as effector memory cells, ready to produce IL-17 quickly [7]. These findings in our study were consistent with previous report that IL-17$^+$ γδ T cells possess the same capabilities as Th17 cells and IL-17$^+$ γδ T cells participate in protective immunity before the appearance of Th17 [13].

Although it is widely accepted that γδ T cells act as an important potential source of IL-17, little is known about the role they play during acute allograft rejection. We previously observed an increase in IL-17 mRNA, which reach a peak on day 3 after transplantation [4]. Interestingly, in the present study, the expression of IL-23, a cytokine closely related to the production of IL-17 [9,14], was also increased in cardiac allograft and preceded the increase in IL-17 (reached its highest level on day 1). Based on this current knowledge, here arises a question whether IL-17$^+$ γδ T-cell response was enhanced by the early up-regulated IL-23 expression. This mystery was further solved by the *in vitro* results that γδ T cells produced large amount of IL-17 when triggered by IL-23. These lines of evidence suggested that IL-17 produced by γδ T cells during acute allograft rejection might be related to IL-23.

IL-23 and L-1β are produced by APCs and regulated by several 'danger' signals through TLRs or other pattern recognition receptors [31]. We therefore hypothesized that HMGB1 activated γδ T cells by indirectly stimulating DCs to produce IL-23 and IL-1β. As it was noted that HMGB1 induced the production of IL-23 and IL-1β by BMDCs quickly, even in the first 6 h, which then forced recipient γδ T cells to produce sufficient IL-17 *in vitro*. These results were also agreed with previous report about the temporal expression of IL-23 [32]. However, up to now, the mechanisms involved in the development of IL-17$^+$ γδ T cells are not yet disclosed.

High-mobility group box 1 (HMGB1) is an innate alarmin implicated in the initiation of inflammatory response following acute, local organ injury [33]. We previously demonstrated that HMGB1 can be passively released by damaged cells of allograft and actively secreted by allograft infiltrated immune cells such as DCs and macrophages [5] and extracellular HMGB1 functions as an innate alarmin

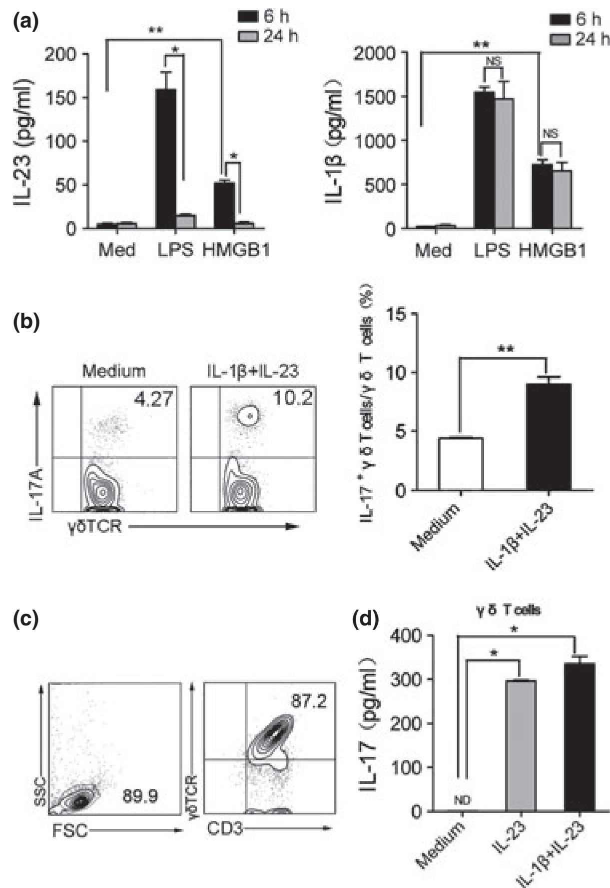

Figure 5 High-mobility group box 1 (HMGB1) indirectly promoted the development of IL-17$^+$ γδ T cells via activating dendritic cells (DCs) to secrete IL-23 and IL-1β. (a) Bone marrow-derived dendritic cells (BMDCs) were exposed to LPS (1 μg/ml), HMGB1 (rHMGB1, 5 μg/ml), or control vehicle for 6 or 24 h, respectively. For the measurement of IL-1β, ATP (10 mmol) was added into the culture. Supernatants were then harvested for the measurement of IL-23 and IL-1β by ELISA. (b) Splenic cells from recipient mice were cultured in the presence of IL-23 combined with IL-1β for 48 h. Splenic cells were harvested and IL-17$^+$ γδ T cells were analyzed by flow cytometry. Cells were gated on CD3$^+$ population. (c) Splenic γδ T cells isolated from recipient mice were used for the detection of purity. (d) Splenic γδ T cells were stimulated with IL-23 alone or in combination with IL-1β for 48 h. IL-17 production in the supernatants was measured using ELISA. Results shown are averages from three independent experiments (±SD). $*P < 0.05$; $**P < 0.01$; ND means not detected.

implicated in acute rejection of cardiac allograft [34]. In parallel, we detected a time-dependent expression of HMGB1 at protein level. To confirm whether HMGB1 have an effect on IL-17$^+$ γδ T cells during early stage of acute allograft rejection, blockade of HMGB1 suppressed IL-17$^+$ γδ T-cell response *in vivo* and reduced inflammatory infiltrates in allograft. Furthermore, blockade of HMGB1 also inhibited the mRNA level of IL-23, IL-1β, and IL-17. Combined with the experiments conducted *in vitro*, these lines of findings indicated that HMGB1 acts as a regulatory cytokine in IL-17$^+$ γδ T-cell response by promoting the secretion of IL-23 and IL-1β.

As a potent pro-inflammatory cytokine, previous investigators have demonstrated that IL-17 derived from γδ T cells also plays a critical role in neutrophil recruitment in infectious disease [29,35]. In our present study, depletion of γδ T cells prolonged allograft survival. Thus, we inferred that the absence of IL-17$^+$ γδ T cells in recipients reduced the production of IL-17, which may result in less neutrophil recruitment and prolong allograft survival. However, we cannot rule out the possibility that some other effector cells also participate in early acute allograft rejection as the effects of depletion of γδ T cells were modest.

In summary, the present results allowed us to reach the following conclusions. First, IL-17$^+$ γδ T cells contribute to the early stage of acute allograft rejection. Second, HMGB1-regulated IL-17$^+$ γδ T-cell response may be related to the secretion of IL-23 and IL-1β by DCs.

Authorship

MF and FG: participated in research design. QX, LD and LS: participated in the performance of the study. FZ: participated in data analysis. QX and MF: participated in the writing of the paper.

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