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Administration of anti-interleukin-6 monoclonal antibody prolongs cardiac allograft survival

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Summary

To investigate the effects of anti-IL-6 monoclonal antibody (anti-IL-6 mAb) on acute allograft rejection and the potential mechanisms in a mouse heart transplantation model. Heterotopical heart graft model was performed. The anti-IL-6 mAb was administered to recipient mice after cardiac grafting. Results were compared with administration of anti-IL-17 mAb or anti-IL-6 mAb + anti-IL-17 mAb (the 'double' treatment). The cardiac allograft survival was monitored by daily palpation in combination with histological evaluation. Quantitative polymerase chain reaction assay, mixed lymphocyte reaction, and flow cytometric analysis were employed to determine the mRNA expression of pro-inflammatory cytokines, allogeneic T-cell proliferation, and the proportion of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells in graft-infiltrating lymphocytes and splenocytes of recipients, respectively. The results showed that the cardiac allograft survival in anti-IL-6 mAb-treated mice was prolonged significantly when compared with that of the untreated or anti-IL-17 mAb-treated mice. Meanwhile, the 'double-treated' did not prolong graft survival significantly when compared with those treated with anti-IL-6 mAb. The increase of graft survival induced by anti-IL-6 mAb was associated with reduced transcript levels for IFN-γ and IL-17, accompanied by a dramatic reduction of T-cell proliferation capacity to alloantigen stimuli and a higher proportion of Treg cells. Thus, anti-IL-6 mAb may be protective against acute rejection after cardiac transplantation through suppressing the activation of effector T cells and promoting the induction of Treg cells.

Introduction

Acute allograft rejection is T-cell dependant, which is correlated with Th1 differentiation, IFN-γ expression and restimulation of peripheral T cells with alloantigen [1]. Recent studies demonstrated a novel pro-inflammatory subpopulation of IL-17-producing T helper cells, termed 'Th17', that are distinct from Th1 and Th2 [2]. These Th17 cells have been also implicated in allograft rejection of solid organs. IL-17 transcripts have been detected at the early stage of rat and human kidney allograft rejection [3,4], and IL-17-producing cells might regulate the rejection of rat lung [5]. Moreover, IL-17 plays a major role in cardiac allograft rejection in those T-bet (−) mice that have less IFN-γ production and Th1 cell differentiation, suggesting that Th17 cells are, at least partly, involved in acute rejection [6,7].

Similar to Th1 development, the cytokine environment plays a critical role in positive and negative regulation of Th17 development. As three individual groups demonstrated, the IL-6 and TGF-β induce the differentiation of Th17 cells in mice *in vitro* synergistically [8–10]. IL-1 and IL-6 induce the differentiation of human Th17 cells *in vitro* synergistically [11,12], indicating that IL-6 is essential in the induction of Th17 cells in both human and mice. IL-6 is a pleiotropic cytokine produced by multiple cell types that has well-described functions in both innate and adaptive immune responses [13–16]. The previous works indicated that IL-6 plays a key role as it inhibits TGF-β-induced Treg differentiation [8,17] and

induces RORγt (retinoid acid receptor-related orphan receptor), a transcription factor specifically required for Th17 development [9]. As a regulator of Th17 differentiation *in vitro*, IL-6 represents a potential target for the inhibition of Th17 development *in vivo*, which might relieve effector T cells from suppression by Tregs *in vivo*. Korn *et al.* reported that IL-6 (−) mice are resistant to the induction of experimental autoimmune encephalomyelitis (EAE). In the absence of IL-6, Th17 responses are impaired whereas Tregs responses are dominant, suggesting that IL-6 is a critical factor that shifts the immune response from Tregs response to pathogenic Th17 response [18]. Similarly, IL-6 has been included in other types of immunopathology [19–21]. However, the effect of IL-6 in the allograft rejection response on the reciprocal differentiation of Tregs and Th17 cells *in vivo* is unclear.

In this study, a mouse heart transplantation model was established and anti-IL-6 mAb was administered to evaluate its effects on acute rejection. As antagonism of IL-17 network (via expression of an IL-17R-immunoglobin fusion protein) can delay acute rejection in wild-type recipients of aortic [22] and cardiac [23] transplantation modestly, an additional aim of this study was to examine whether administration of anti-IL-17 mAb or anti-IL-6 mAb and anti-IL-17 mAb combined therapy (double-treated) could also delay acute rejection in recipient mice. Our results demonstrated that administration with anti-IL-6 mAb alone can delay acute cardiac allograft rejection and prolong graft survival significantly, which might be related with the reduced expression of pro-inflammatory cytokines and impaired Th1 as well as Th17 cell immunity in grafts.

Materials and methods

Mice

Inbred male BALB/c (H-2^d), C57Bl/6 (H-2^b) and C3H (H-2^k) mice were obtained from the Institute of Organ Transplantation, Tongji Hospital, Huazhong University of Science and Technology (Wuhan, China). Mice (age: 8–12 weeks; weight: 20–25 g) were selected for the study. The mice were housed in a specific pathogen-free facility (SPF) in microisolator cages supplied with autoclaved food and water. All of the studies were performed under the guidelines of Tongji Animal Use Regulations and approved by the Institutional Animal Care and Use Committee (IACUC) of the Tongji Medical College.

Antibodies and reagents

Antibodies were purchased from BD Pharmingen (San Diego, CA, USA) including APC-conjugated anti-CD3 (UCHT1), fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (GK1.5), FITC-conjugated anti-CD8α (53–6.7), phycoerythrin (PE)-conjugated anti-CD25 (PC61.5), PE-cy5-conjugated Foxp3 (FJK-16s), PE-conjugated anti-IL-17 (TC11-18H10), and PE-conjugated anti-IFN-γ (XMG1.2). Anti-IL-6 mAb (MAB406), anti-IL-17 mAb (MAB421) and isotype control immunoglobulin G1 (rat-IgG) mAb (MAB005) were purchased from R&D Systems (Minneapolis, MN, USA).

Heart transplantation model

For heterotopic cardiac transplantation, C57Bl/6 mice were used as recipients, whereas Balb/c mice were used as donors, and C3H mice as third-party control. Abdominal heterotopic cardiac transplantations were performed according to the protocol described by Corry *et al.* [24]. Briefly, the cardiac allograft was transplanted in the abdominal cavity by end-to-side anastomosis of aorta and pulmonary artery of the graft to the recipient's aorta and vena cava, respectively. The donors that did not beat immediately after reperfusion or stopped within 1 day after transplantation were excluded. Allograft survival was presented as the mean survival time (MST) ± SD. Graft function was assessed by daily abdominal palpation. The day of rejection was defined as the last day of a detectable heartbeat in the graft. Rejection was verified in selected cases by necropsy and pathological examination of hematoxylin/eosin (H&E)-stained graft sections.

Experimental groups

Before the start of the transplant studies, the optimal dosage of anti-IL-6 mAb or anti-IL-17 mAb was determined based on our previous results. As a result, recipients with cardiac allografts were randomly allocated into seven groups: group I, untreated allogeneic control; group II, rat-IgG; group III, anti-IL-17 mAb 200 μg/day i.v. on postoperative day (POD) 0–6; group IV, anti-IL-6 mAb 300 μg/day i.v. on POD 0–6, followed by every other day until POD 10; group V, the 'double-treated' using the same regimens as group III and IV; group VI and VII, anti-CD25 mAb (250 μg/day) was administered to group IV and V on POD 1, 3, and 5, respectively, to investigate the role of Treg cells induced by IL-6 neutralization. Allograft and spleen were collected at desired PODs and divided into equal sections for real-time polymerase chain reaction (PCR) assay, histology, and flow cytometry.

Histology

The hearts were obtained and fixed in formaldehyde. Then, heart tissues were embedded in paraffin, cut into

4-μm sections, dehydrated with graded alcohol, transparentized with xylene, and stained with H&E.

Analysis of serum cytokines

The serum was collected from anti-IL-17 mAb treated mice (group III) or anti-IL-6 mAb-treated mice (group IV) at desired PODs. The serum level of IL-17 in group III and IL-6 in group IV were determined using enzyme-linked immunosorbent assay (ELISA), respectively. The assay was performed in triplicates for samples and in duplicates for standard, and experiments were repeated three times followed by averaging the data and subtracting the standard optical density of background. The calculation of cytokine titer was based on a regression analysis of the log of final optical density versus the log of standard dilutions, and data were presented as pg/ml.

Isolation of graft-infiltrating lymphocytes (GIL)

Cardiac grafts were rinsed *in situ* with Hank's Stock Solutions/1% heparin. Explanted hearts were minced through a sieve with a pore size of 50 μm into RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The tissue suspension was then digested with 1 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 60 min at 37 °C. After washing twice, viable lymphocytes were separated by Ficoll gradient centrifugation (Histopaque-1083; Sigma-Aldrich). Cells were washed thrice with RPMI 1640 medium supplemented with 10% FBS and used for the following experiments.

Expression analysis of cytokines

The allograft samples were removed at the desired PODs and total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was further treated with DNase I to remove genomic DNA. cDNA was synthesized with 1 μg of RNA using a single strand cDNA synthesis kit (MBI, Los Angeles, CA, USA). Real-time PCR was performed to detect the expression of IFN-γ, IL-17A and RORγt using an Icycler (BioRad, Hercules, CA, USA) with a SYBR Green qPCR kit (Invitrogen). The primer sequences were as follows: IFN-γ, 5′-ACGCTCTTCCTCATCGCTGTTT-3′ and 5′-GT CACCA TCCTTTTGCCAGTTC-3′; IL-17A, 5′-CCGCAAT GAAGACCCTGATAGA-3′ and 5′-CAGCATCTTCTCGA CCCTGAAA-3′; RORγt, 5′-ACCTCCACTGCCAGCTGT GTGCTGTC-3′ and 5′-T CATTTCTGCACTTCTGCATGT AGACTGTCC-3′; GAPDH, 5′-ACCACAGTCCATGCCA TCA C-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. The mRNA expression of each cytokine was normalized by the GAPDH followed by averaging. The relative quantification value, fold difference, was expressed as $2-\Delta\Delta Ct$.

For determination of intracellular cytokine levels, single-cell suspensions of erythrocyte-free splenocytes were prepared and incubated with 50 ng/ml Phorbol myristate acetate (Sigma-Aldrich), 500 ng/ml ionomycin (Bio-Vision, Palo Alto, CA, USA) and 1 μg/ml Golgi Plug (BD Biosciences, Mountain View, CA, USA) in a 96-well plate at 37 °C with 5% CO_2 for 4 h. Cells were then stained for surface marker, fixed in 4% formaldehyde in phosphate-buffered saline (PBS), and permeabilized with 0.5% saponin plus 1% bovine serum albumin PBS, followed by labeling with specific cytokine antibodies. Cells were then analyzed on a FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences) in accordance with the manufacturer's instructions.

Detection of $CD4^+$ $CD25^+$ Treg cells by flow cytometry

Splenocytes and GIL from C57Bl/6 recipients were isolated on POD 7 for flow cytometry analysis. The lymphocytes were prepared as a single-cell suspension. For Tregs analysis, APC-conjugated anti-CD3, FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 were added to the cell suspension simultaneously and incubated on ice for 30 min. After fixation and permeabilization, the cell suspensions were stained with PE-cy5-conjugated Foxp3 or isotype control. After washing twice, pellets were resuspended in 300 ml cold staining buffer and analyzed using flow cytometer.

Mixed lymphocyte reaction (MLR) and cell proliferation assay

Stimulator cells were prepared from splenocytes of BALB/c or C3H donors. The cells were irradiated and seeded into 96-well V-shaped plates. T cells of GIL isolated from each treatment recipients on POD 5 were used as responder cells and purified by the warm nylon wool technique. A total of 5×10^5 responder T cells and 5×10^4 stimulator cells were co-cultured at 37 °C in a humidified atmosphere with 95% air and 5% CO_2 for 5 days followed by pulsing with 1 μCi $[^3H]$ thymidine deoxyribose for 18 h. The cells were harvested with a semi-automated cell harvester and counted on a beta scintillation counter.

Statistics

Allograft survival curves were generated by the Kaplan and Meier method. The difference in allograft survival between multiple groups was determined using the

log-rank (Mantel-Cox) test. Difference in data from flow cytometry and RT-PCR assay was subjected to one-way analysis of variance. SPSS 11.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data were expressed as mean ± SD. A value of $P < 0.05$ was treated as statistically significant.

Results

Expression of cytokine in recipients' serum after mAb administration

The mAb was evaluated for its ability to reduce the proportion of cytokine in serum of recipients. The intention was to choose an optimal dose for the transplant model that had an adequate effect of neutralization for IL-6 or IL-17. Based on our previous results, different doses of anti-IL-6 mAb or anti-IL-17 mAb were administered to recipient mice at different time points, serum level of IL-6 or IL-17 was determined using ELISA, respectively. The results (Fig. 1a) indicated that with the addition of anti-IL-6 mAb (300 μg/day), serum level of IL-6 decreased completely from day 1 to day 20. Meanwhile, after administration of anti-IL-17 mAb (200 μg/day), serum level of IL-17 decreased completely from day 1 to day 12 (Fig. 1b). Therefore, the dose of anti-IL-6 mAb (300 μg/day) or anti-IL-17 mAb (200 μg/day) was chosen for the transplant model.

Anti-IL-6 mAb treatment delays acute cardiac allograft rejection

Mouse heart transplantation model was established by i.v. administration of anti-IL-6 mAb (group IV), anti-IL-17 mAb (group III) or 'double-treated' (group V) as described in Materials and Methods. The graft survival was observed and the effect of each treatment on allograft acute rejection was investigated. As shown in Fig. 2, all isografts survived for a long time. No obvious prolongation of allograft MST was observed between untreated (group I) and rat-IgG-treated mice (group II) (7.7 ± 0.8 and 8.1 ± 1.0 days, respectively), which was shorter than that of the anti-IL-17 mAb-treated mice (group III, 13 ± 1.8 days; log rank, $P < 0.05$). Interestingly, MST increased markedly (22.0 ± 2.3 days) in anti-IL-6 mAb-treated mice (group IV) compared with that in groups I, II, and III (log rank, $P < 0.01$). The MST of grafts in 'double-treated' mice (group V, 23.2 ± 2.1 days) was slightly longer than that of group IV, which showed no significant difference. Accordingly, histological analysis of cardiac allografts harvested on POD 7 in either group I or II revealed severe acute rejection characterized by rigorous infiltration of inflammatory cells and allograft destruction. Allografts with anti-IL-17 mAb therapy delayed acute rejection with a moderate decrease of inflammatory cell infiltration and less tissue damage on POD 7. By contrast, relatively intact myocardium and remarkable reduced infiltration of inflammatory cells were observed in allografts of group IV and V on POD 7 (Fig. 3, insets).

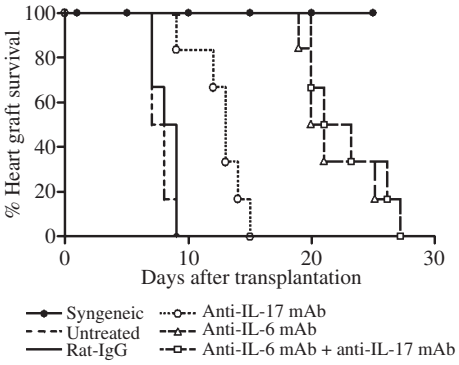

Figure 2 Survival of cardiac allografts. C57Bl/6 mice served as recipients and Balb/c mice as donors. After transplantation, the mice were treated with either anti-IL-6 mAb or anti-IL-17 mAb or 'double-treated' and cardiac allograft survival was examined over time ($n = 6$ mice/group).

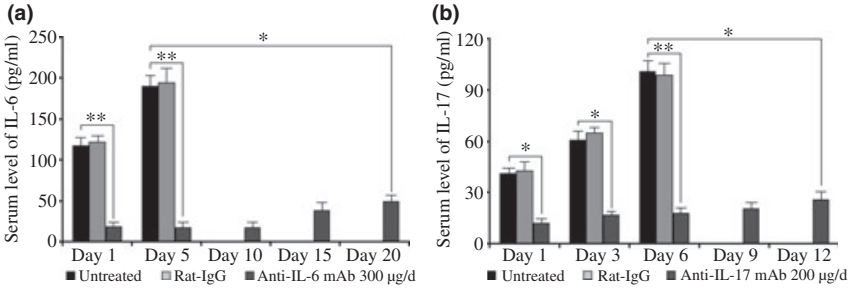

Figure 1 Serum level of IL-6 or IL-17 in mAb-treated mice. Recipient mice treated with optimal dosage of anti-IL-6 mAb (300 μg/day) (a) or anti-IL-17 mAb (200 μg/day) (b), and serum was collected serially at the indicated time points. Serum level of IL-6 and IL-17 was determined using ELISA, respectively ($n = 3$ at each time point, $^{**}P < 0.01$, $^{*}P < 0.05$, compared with those rat-IgG-treated or untreated mice). Data were presented as mean ± SD and experiments were performed in duplicates.

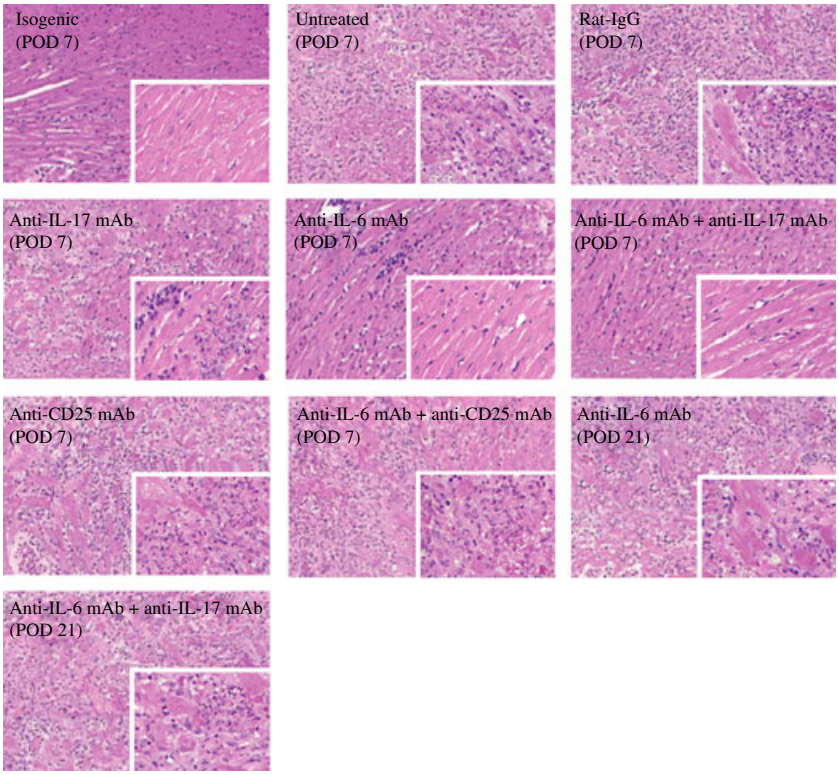

Figure 3 Histological analysis of cardiac allografts after transplantation. Representative histological findings of cardiac allografts harvested on postoperative day (POD) 7 or 21. On POD 7, relatively intact myocardium accompanied by markedly reduced infiltration of inflammatory cells was observed in allografts of recipients treated with anti-IL-6 mAb or anti-IL-6 mAb + anti-IL-17 mAb. By contrast, the anti-IL-6 mAb + anti-CD25 mAb group showed extensive leukocyte infiltration and developed severe rejection at this time point. Original magnification: 40× for main panels, 200× for insets; $n = 3$ animals/group.

Anti-IL-6 mAb treatment attenuates IFN-γ, IL-17 and RORγt expression

It has been demonstrated that Th1 cytokine response is predominant during acute allograft rejection. While Th17 cells have also been implicated in allograft acute rejection. We therefore checked the effect of each study groups on allograft expression of IFN-γ, IL-17 and RORγt (a transcription factor specifically required for Th17 development). The relative expression levels for IFN-γ, IL-17 and RORγt in each graft on POD 3, 7, 14, and 21 were determined with real-time PCR, respectively. Allograft expression for IFN-γ, IL-17 and RORγt increased significantly in untreated mice after transplantation, which was in accordance with the previous reports. Administration of anti-IL-17 mAb only modestly attenuated the mRNA expression of IFN-γ. No significant difference of IFN-γ expression was found between anti-IL-17 mAb-treated and untreated mice on POD 7. By contrast, anti-IL-6 mAb attenuated the mRNA expression of IL-17 and RORγt dramatically throughout the experimental time. Moreover, the IFN-γ expression in anti-IL-6 mAb-treated allografts was also reduced and the suppressive effects were sustained until POD 14. However, no significant difference of IFN-γ expression was observed between the grafts isolated from anti-IL-6 mAb-treated mice that failed for transplantation on POD 21, and untreated mice that failed for transplantation on POD 7. A similar down-regulation effect was also observed in the 'double-treated' group (Fig. 4a–c).

Effect of anti-IL-6 mAb on intracellular cytokine expression of splenocyte on POD 7

To assess whether the inhibition of pro-inflammatory cytokines by anti-IL-6 mAb treatment was localized to the grafts or systematic, the amount of IFN-γ and IL-17 production by splenocytes of recipients from each study group on POD 7 was analyzed using flow cytometey. Anti-IL-6 mAb (group IV) treatment suppressed the production of IFN-γ in both $CD4^+$ and $CD8^+$ T cells (Fig. 5a). The proportion of $CD4^+$ IFN-γ^+ cells in the recipients of group IV decreased significantly compared with those mice treated with anti-IL-17 mAb or rat IgG. The population of T cells expressing IFN-γ reduced more markedly for $CD4^+$ T cells than for $CD8^+$ T cells (Table 1). Moreover, compared with untreated mice, the proportion of $CD4^+$ IL-17^+ cells (Th17) in group IV decreased dramatically, whereas IL-17-producing $CD8^+$ T cells in the spleen reduced slightly (Fig. 5b). Similar results were also found in anti-IL-6 mAb + anti-IL-17 mAb-treated mice.

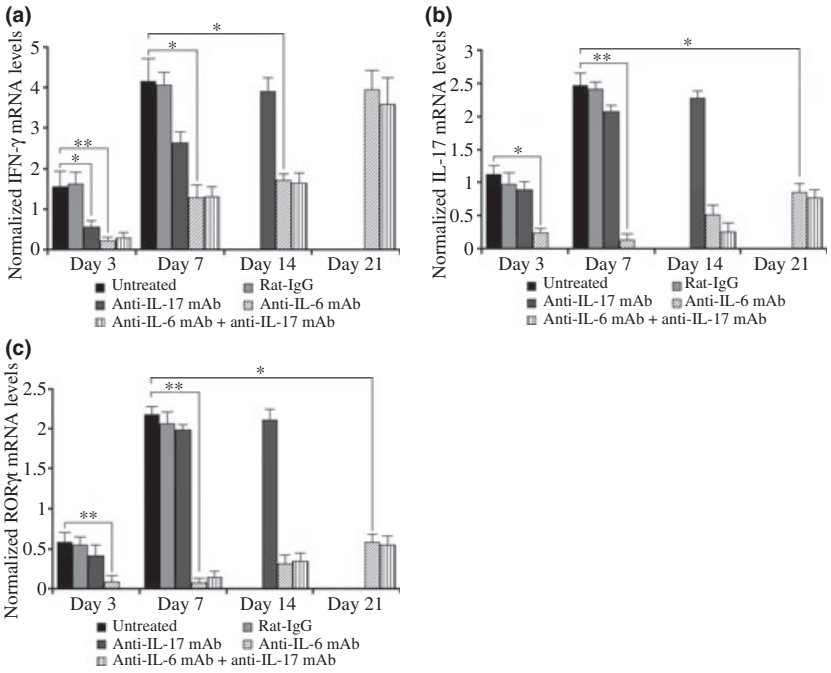

Figure 4 Anti-IL-6 mAb treatment attenuated IFN-γ, IL-17 and RORγt expression. Allograft of recipients from each treatment group was collected on postoperative day 3, 7, 14, and 21, and then subjected to expression analysis of IFN-γ, IL-17 and RORγt using real-time PCR. Administration of anti-IL-6 mAb not only inhibited allograft IL-17 and RORγt expression but also attenuated allograft IFN-γ expression. The mRNA expression of IFN-γ, IL-17 and RORγt was normalized by GAPDH ($n = 3$ in each time point, $^{**}P < 0.01$, $^{*}P < 0.05$, compared with those treated with rat-IgG or untreated). Data were presented as mean ± SD, and experiments were performed in duplicates.

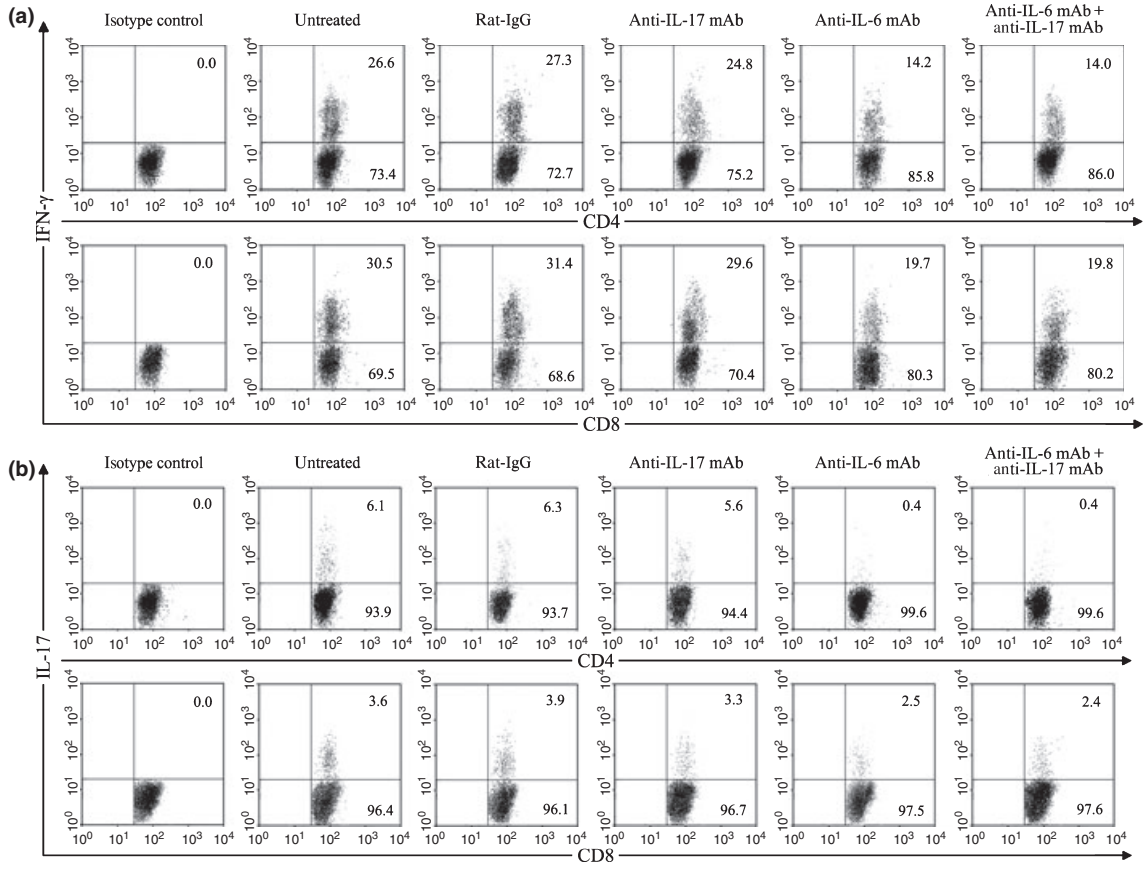

Figure 5 Anti-IL-6 mAb treatment affected splenic T-cell subpopulations. Splenic T cells were obtained from allograft recipients in each study group on postoperative day 7 and then subjected to intracellular IFN-γ (a) and IL-17 (b) staining. The percentages of $CD4^+$ and $CD8^+$ cells that were also positive for IFN-γ or IL-17 were determined using flow cytometric analysis. Data are representative of three independent results.

Table 1. Intracellular expression of cytokine IFN-γ and IL-17 analyzed with flow cytometry in each group (n = 4 samples in each group) ($x \pm s$).

Groups	$CD4^+$IFN-$\gamma^+/CD4^+$ (%)	$CD8^+$IFN-$\gamma^+/CD8^+$ (%)	$CD4^+$IL-$17^+/CD4^+$ (%)	$CD8^+$IL-$17^+/CD8^+$ (%)
Untreated	27.2 ± 2.1	31.3 ± 1.7	6.1 ± 0.6	3.7 ± 0.6
Rat-IgG	28.1 ± 1.9	32.0 ± 2.1	6.5 ± 0.4	3.8 ± 0.9
Anti-IL-17 mAb	24.9 ± 1.4	29.8 ± 1.5	5.7 ± 0.4	3.2 ± 0.4
Anti-IL-6 mAb	14.0 ± 1.2*	20.5 ± 1.4	0.4 ± 0.3**	2.4 ± 0.5
Anti-IL-6 mAb + Anti-IL-17 mAb	14.2 ± 0.8*	20.2 ± 1.1	0.6 ± 0.2**	2.2 ± 0.3

**$P < 0.01$, *$P < 0.05$ compared with recipients treated with rat-IgG or untreated.

Enhancement of Treg cells in splenocytes and GIL by anti-IL-6 mAb treatment

It is well established that $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (Tregs) have a regulatory function and can modulate allograft survival [25], and Foxp3, a forkhead winged helixprotein transcription factor, controls the development of $CD4^+$ $CD25^+$ Tregs [26]. Therefore, we tested the hypothesis that prolonged graft survival in the group treated with anti-IL-6 mAb might be dependent on Tregs. As shown in Fig. 6b, $CD4^+$ $CD25^+$ T cells were detectable in the spleen and allografts of untreated mice on POD 7. The proportion of $CD4^+$ $CD25^+$ T cells increased in both spleen and allografts pretreated with anti-IL-6 mAb. These $CD4^+$ $CD25^+$ T cells expressed intercellular Foxp3 transcripts (>85%) throughout the experimental time (data not shown), indicating that the $CD4^+$ $CD25^+$ T cells detected after anti-IL-6 mAb administration expressed a Treg phenotype. The average percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ cells in the $CD3^+$ T-cell pool showed significant increase on POD 7 after anti-IL-6 mAb administration in the spleen and allografts compared with those recipients pretreated with rat-IgG or untreated (Fig. 6c), whereas the anti-IL-17 mAb-treated mice have no evident increase in the proportion of Tregs.

Depletion of $CD4^+$ $CD25^+$ T cells prevented against long-term acceptance of allografts mediated by anti-IL-6 mAb

To clarify the role of $CD4^+$ $CD25^+$ Tregs in long-term acceptance of cardiac allografts induction, we used anti-CD25 mAb to deplete $CD4^+$ $CD25^+$ T cells in recipient mice. Based on what has been reported in the literature [27,28], three doses of anti-CD25 mAb were administered to mice with anti-IL-6 mAb or 'double-treated'. Results showed that the percentage of $CD4^+$ $CD25^+$ T cells within the lymphocyte population derived from the spleen and allografts of anti-IL-6 mAb or 'double-treated' mice receiving anti-CD25 mAb decreased compared with those mice only treated with anti-IL-6 mAb (Fig. 7a). More than 86% of these $CD4^+$ $CD25^+$ Treg expressed Foxp3 (data not shown). The percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs in the $CD3^+$ T-cell pool were significantly reduced in the spleen and allografts of anti-IL-6 mAb or 'double-treated' mice receiving anti-CD25 mAb (Fig. 7b). These results indicated that the administration of anti-CD25 mAb to anti-IL-6 mAb-treated mice decreased the absolute number of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs.

Then, we observed whether administration of anti-CD25 mAb can prevent against long-term acceptance of cardiac allografts mediated by anti-IL-6 mAb or 'double-treated'. The MST of grafts in anti-IL-6 mAb + anti-CD25 mAb and anti-IL-6 mAb + anti-IL-17 mAb + anti-CD25 mAb treated mice was 10.6 ± 1.2 and 10.9 ± 1.4 days, respectively, which was slightly longer than that of the untreated mice (log rank $P > 0.05$, Fig. 7c). Moreover, anti-IL-6 mAb or 'double-treated' mice receiving anti-CD25 mAb restored the expression of IFN-γ when compared with those mice which were treated with anti-IL-6 mAb alone, as determined by real-time PCR (Fig. 7d). Accordingly, histological analysis of cardiac allografts in anti-IL-6 mAb + anti-CD25 mAb-treated mice revealed extensive leukocyte infiltration and developed rejection on POD 7 (Fig. 3).

Inhibition of MLR responses of GIL and splenocytes of recipients with anti-IL-6 mAb treatment

To analyze the effects of anti-IL-6 mAb on cellular alloimmune response *in vitro*, analysis of MLR responses with GIL was performed on POD 5. Proliferation responses were evaluated against donor BALB/c or third-party C3H splenocytes. In comparison with GIL from untreated mice, GIL from allograft of anti-IL-6 mAb or 'double-treated' mice showed a profound inhibition of proliferation in response to donor cells, but not to third-party cells (Fig. 8).

Discussion

In this study, we demonstrated that neutralization of IL-6 exhibited both preventive and therapeutic effects on mouse acute allograft rejection. The results showed that graft recipients treated with anti-IL-6 mAb prolonged cardiac allograft survival significantly and significantly

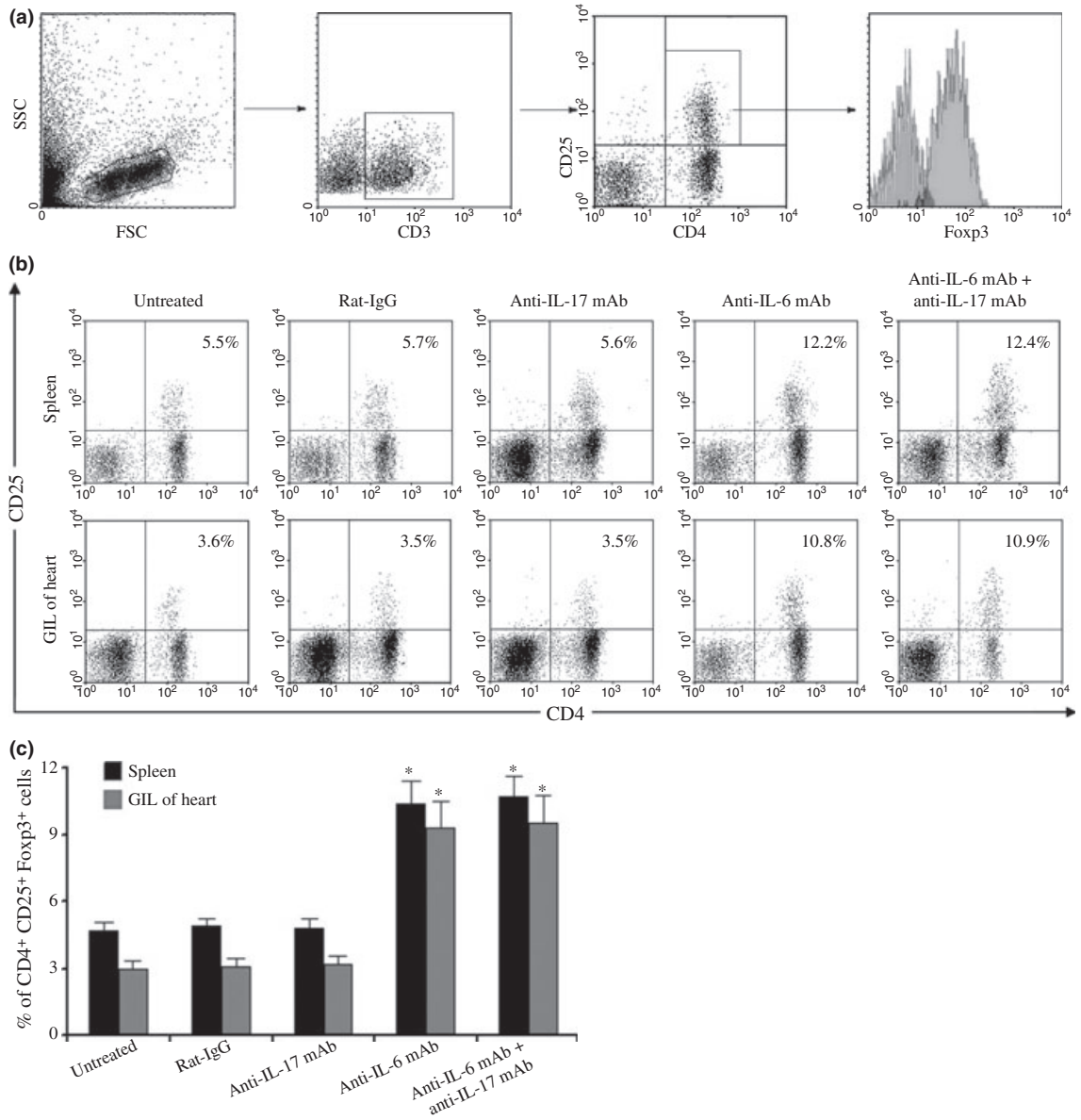

Figure 6 An increase of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells in splenocytes and graft-infiltrating lymphocyte (GIL) after anti-IL-6 mAb administration. Splenocytes and GIL from recipient mice were stained with APC-CD3, FITC-CD4, PE-CD25, and PE-Cy5-Foxp3, and analyzed using flow cytometry. Gating strategy: $CD4^+$ $CD25^+$ T cells were analyzed within the CD3 positive gate, and Foxp3 were analyzed in $CD4^+$ $CD25^+$ T cells (a). The percentages of $CD4^+$ $CD25^+$ T cells after administration with anti-IL-6 mAb, or anti-IL-17 mAb or 'double-treated' are shown in (b), and the results represented four separate experiments. The percentages of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells in the CD3-positive gate on postoperative day 7 in each study group are shown in (c), and the results represented four separate experiments. $^*P < 0.05$, compared with those treated with rat-IgG or untreated.

reduced the infiltration of mononuclear cells in allografts compared with that of the control groups.

Our results demonstrated that anti-IL-6 mAb treatment decreased the expression of IFN-γ mRNA in the allografts, and suppressed the induction of Th1 cells (Fig. 4a). The role of IL-6 in the regulation of Th1 cell differentiation was still unclear. Evidence showed that IL-6 could suppress the TGF-β-induced transformation of Tregs from naïve $CD4^+$ T cells [17]. A previous study in EAE showed that the generation and expansion of Tregs were observed in IL-6 (−) mice accompanied with suppression of Th1 as well as Th17 cell response, although $CD8^+$ T cells were not studied [18]. We proposed that the suppression on Th1 cells might be mediated by TGF-β-inducible Tregs,

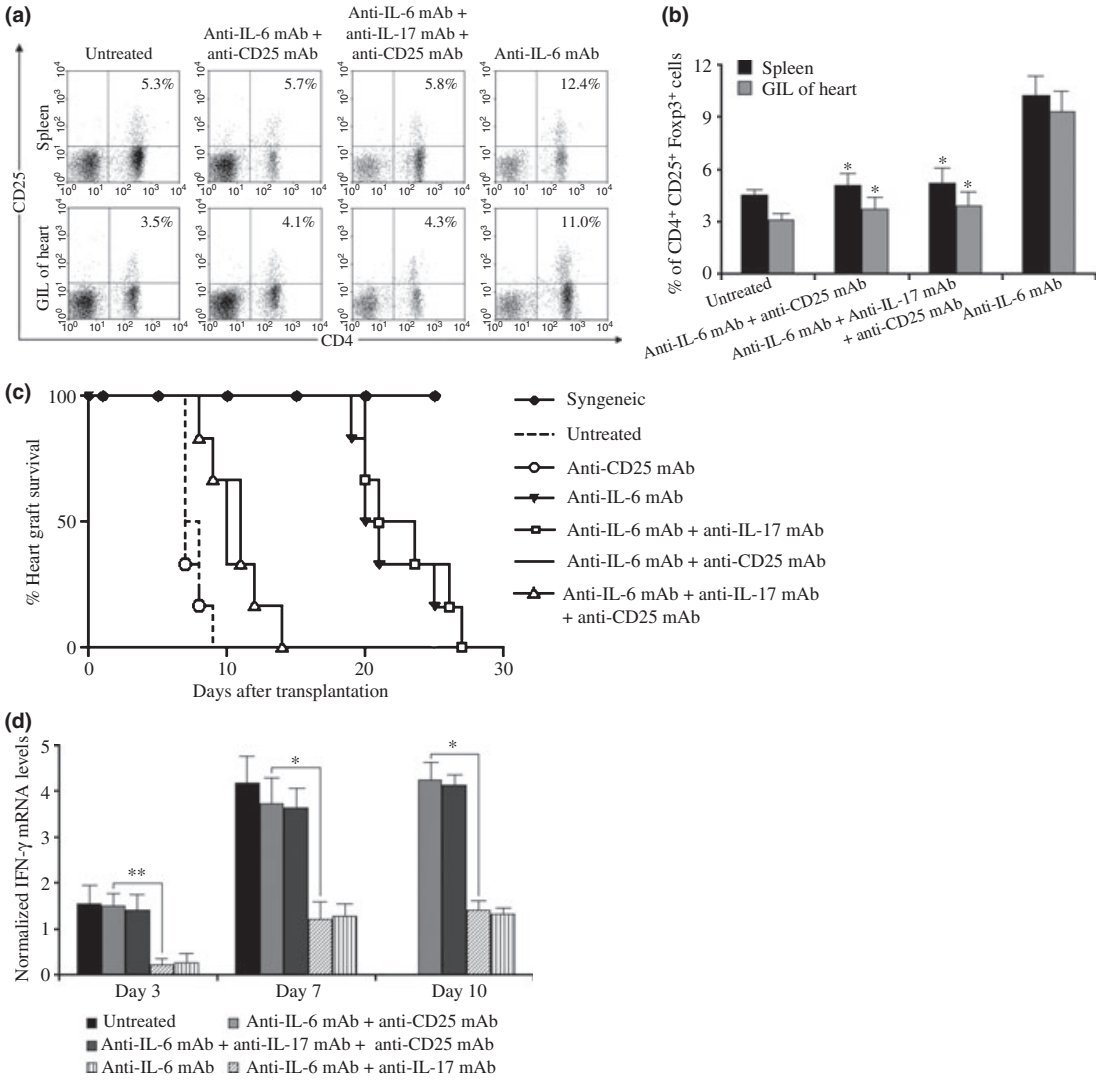

Figure 7 Administration of anti-CD25 mAb reduced $CD4^+$ $CD25^+$ $Foxp3^+$ T cells in the spleen and allografts of anti-IL-6 mAb or 'double-treated' mice and prevented against long-term acceptance of allografts. Recipient mice received three doses of anti-CD25 mAb on alternating days. Lymphocytes were isolated from the spleen and allografts of each treatment mice. These cells were analyzed using 4-color flow cytometry (as described in Fig. 6a). The percentage of $CD4^+$ $CD25^+$ T cells in anti-IL-6 mAb + anti-CD25 mAb treated mice was reduced in both spleen and allografts when compared with anti-IL-6 mAb treated mice (a). The percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs to total $CD3^+$ T cells in spleen and allografts of anti-IL-6 mAb + anti-CD25 mAb mice was reduced significantly (b). Depletion of $CD4^+$ $CD25^+$ T cells prevents the protection of heart allografts by anti-IL-6 mAb or 'double-treated' (c). Allograft of recipients were collected on postoperative day 3, 7 and 10, and then subjected to expression analysis of IFN-γ by real-time PCR. Anti-CD25 mAb administration increased the expression of IFN-γ mRNA in anti-IL-6 mAb or 'double-treated' mice (d). ($^{**}P < 0.01$, $^{*}P < 0.05$ compared with anti-IL-6 mAb treated mice). Data were from four independent experiments.

which was increased by IL-6 neutralization in allografts (Fig. 6). Our results supported this hypothesis that IL-6 neutralization prolonged graft survival because of the failure to activate T cells in the presence of immune suppression mediated by Treg cells. The depletion of $CD4^+$ $CD25^+$ T cells of anti-IL-6 mAb-treated mice by administration of anti-CD25 mAb significantly reduced the survival of a subsequently transplanted heart allograft (Fig. 7c). Moreover, no profound change in the population of Th1 cells was observed between anti-IL-6 mAb + anti-CD25 mAb-treated and untreated mice (Fig. 7d). Although, it has been suggested that anti-CD25 mAb treatment may inactivate but not preferentially deplete $CD4^+$ $CD25^+$ Treg [29]. There is strong evidence that PC61 does deplete a large fraction of $CD25^+$ Treg [27,30], as shown by our data.

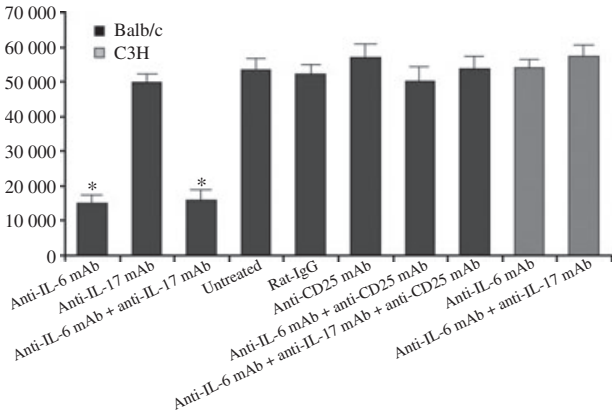

Figure 8 Inhibition of mixed lymphocyte reaction (MLR) responses in recipients with anti-IL-6 mAb treatment. Cells were harvested from grafts of each treatment group on postoperative day 5 and used for MLR assay. Cell response against first-party BALB/c or third-party C3H splenocytes, in the presence of IL-2, were analyzed after 5 days of co-culture. Results were expressed as the mean ± SD c.p.m. of one representative animal from six tested for graft-infiltrating lymphocyte (*$P < 0.05$ versus untreated or third party C3H mice). Data were from two independent experiments.

This study showed that the mRNA expression of IL-17 and RORγt (a specific transcription factor for Th17) was markedly up-regulated in allogeneic cardiac grafts, which was in accordance with the previous reports in which increased expression of IL-17 mRNA (in the renal allograft) and protein (in infiltrating mononuclear cells) was noted at as early as 2 days post-transplantation in acute rat rejection models [3,4]. Moreover, our results suggested that IL-6 neutralization significantly reduced the mRNA expression of IL-17 and RORγt in allografts throughout the experimental time, suggesting that IL-6 neutralization inhibited the induction of Th17 cells in heart grafts (Fig. 4b,c).

Furthermore, to assess whether the inhibition of pro-inflammatory cytokines by anti-IL-6 mAb treatment was confined to the heart grafts or systematic, intracellular cytokine staining was performed to detect the amount of IFN-γ and IL-17 in the splenocytes of recipients. As expected, anti-IL-6 mAb treatment dramatically reduced the production of IFN-γ in both $CD4^+$ and $CD8^+$ T cells on POD 7 (Fig. 5a). It is well established that Th1-type immune response was dominated in the acute allograft rejection, in which $CD8^+$ IFN-γ^+ cells (Tc1) were the main effector cells that destroyed the implanted allograft [31]. Moreover, $CD4^+$ IFN-γ^+ cells (Th1) played a detrimental role in the allograft rejection by recruiting and activating other effector cells including macrophages, neutrophils, and Tc1 cells [32,33]. Piccirillo *et al.* [34] reported that the proliferation of antigen-specific $CD8^+$ T cells was suppressed by Tregs. Therefore, the decreased population of $CD8^+$ IFN-γ^+ cells might be associated with TGF-β-inducible Treg cells. Meanwhile, intracellular cytokine staining of the splenocytes indicated that $CD4^+$ T cells exhibited a higher proportion of IL-17 secreting cells than $CD8^+$ T cells, the percentage of $CD4^+$ IL-17^+ (Th17) cells in the spleen of anti-IL-6 mAb-treated mice decreased markedly compared with that of rat-IgG-treated mice (Fig. 5b). As previously reported, the combination of IL-6 and TGF-β was essential for the differentiation of naïve $CD4^+$ T cells into Th17 cells *in vitro* [8–10]. Our results supported the importance of IL-6 in the differentiation of Th17 cells *in vivo*.

In conclusion, IL-6 plays an important role in the allografts in controlling the migration of Th17 and Th1 cells into grafts. Although administration of anti-IL-6 mAb delayed acute rejection, it ultimately failed to prevent it. It has been suggested that graft acceptance or rejection was determined by a balance between activation of effector T cells versus suppression by Tregs. Anti-IL-6 mAb therapy might control allograft rejection, and the effect of which is mediated by the inhibition of allogenic T-cell proliferation, particularly Th17 cells and the enhanced activity of regulatory T cells. Thus, anti-IL-6 mAb treatment might be a promising strategy in preventing allograft rejection.

Authorship

JL: designed research, performed research, collected data, analyzed data, wrote the paper. FH: designed research, performed research, collected data, analyzed data. MW and XZ: performed research, collected data. XC and ZC: designed research, analyzed data.

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References

1. Koga S, Auerbach MB, Engeman TM, Novick AC, Toma H, Fairchild RL. T cell infiltration into class II MHC-disparate allografts and acute rejection is dependent on the IFN-gamma-induced chemokine Mig. *J Immunol* 1999; **163**: 4878.
2. Chen Y, Wood KJ. Interleukin-23 and TH17 cells in transplantation immunity: does 23 + 17 equal rejection? *Transplantation* 2007; **84**: 1071.
3. Loong CC, Hsieh HG, Lui WY, Chen A, Lin CY. Evidence for the early involvement of interleukin 17 in human and experimental renal allograft rejection. *J Pathol* 2002; **197**: 322.

4. Hsieh HG, Loong CC, Lui WY, Chen A, Lin CY. IL-17 expression as a possible predictive parameter for subclinical renal allograft rejection. *Transpl Int* 2001; **14**: 287.
5. Yoshida S, Haque A, Mizobuchi T, *et al.* Anti-type V collagen lymphocytes that express IL-17 and IL-23 induce rejection pathology in fresh and well-healed lung transplants. *Am J Transplant* 2006; **6**: 724.
6. Burrell BE, Csencsits K, Lu G, Grabauskiene S, Bishop DK. CD8+ Th17 mediate costimulation blockade-resistant allograft rejection in T-bet-deficient mice. *J Immunol* 2008; **181**: 3906.
7. Yuan YX, Paez CJ, Schmitt KI, *et al.* A novel role of CD4 Th17 cells in mediating cardiac allograft rejection and vasculopathy. *J Exp Med* 2008; **205**: 3133.
8. Bettelli E, Carrier Y, Gao W, *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006; **441**: 235.
9. Mangan PR, Harrington LE, O'Quinn DB, *et al.* Transforming growth factor-β induces development of the T_H17 lineage. *Nature* 2006; **441**: 231.
10. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006; **24**: 179.
11. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 2007; **8**: 942.
12. Wilson NJ, Boniface K, Chan JR, *et al.* Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 2007; **8**: 950.
13. Coletta I, Soldo L, Polentarutti N, *et al.* Selective induction of MCP-1 in human mesangial cells by the IL-6/sIL- 6R complex. *Exp Nephrol* 2000; **8**: 37.
14. Ford HR, Hoffman RA, Tweardy DJ, Kispert P, Wang S, Simmons RL. Evidence that production of interleukin 6 within the rejecting allograft coincides with cytotoxic T lymphocyte development. *Transplantation* 1991; **51**: 656.
15. Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. *Biochem J* 1990; **265**: 621.
16. Kopf M, Baumann H, Freer G, *et al.* Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature* 1994; **368**: 339.
17. Pasare C, Medzhitov R. Toll-dependent control mechanisms of CD4 T cell activation. *Immunity* 2004; **21**: 733.
18. Korn T, Bettelli E, Gao W, *et al.* IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 2007; **448**: 484.
19. Möller B, Villiger PM. Inhibition of IL-1, IL-6, and TNF-alpha in immune-mediated inflammatory diseases. *Springer Semin Immunopathol* 2006; **27**: 391.
20. Serada S, Fujimoto M, Mihara M, *et al.* IL-6 blockade inhibits the induction of myelin antigen-specific Th17 cells and Th1 cells in experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA* 2008; **105**: 9041.
21. Korn T, Mitsdoerffer M, Croxford AL, *et al.* IL-6 controls Th17 immunity *in vivo* by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells. *Proc Natl Acad Sci USA* 2008; **105**: 18460.
22. Tang JL, Subbotin VM, Antonysamy MA, Troutt AB, Rao AS, Thomson AW. Interleukin-17 antagonism inhibits acute but not chronic vascular rejection. *Transplantation* 2001; **72**: 348.
23. Antonysamy MA, Fanslow WC, Fu F, *et al.* Evidence for a role of IL-17 in organ allograft rejection: IL-17 promotes the functional differentiation of dendritic cell progenitors. *J Immunol* 1999; **162**: 577.
24. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation* 1973; **16**: 343.
25. Karim M, Bushell AR, Wood KJ. Regulatory T cells in transplantation. *Curr Opin Immunol* 2002; **14**: 584.
26. Sakaguchi S. Regulatory T cells key controllers of immunologic self-tolerance. *Cell* 2000; **101**: 455.
27. Li W, Kuhr CS, Zheng XX, *et al.* New insights into mechanisms of spontaneous liver transplant tolerance: the role of Foxp3-expressing CD25+CD4+ regulatory T cells. *Am J Transplant* 2008; **8**: 1639.
28. Li W, Carper K, Liang Y. Anti-CD25 mAb administration prevents spontaneous liver transplant tolerance. *Transplant Proc* 2006; **38**: 3207.
29. Kohm AP, McMahon JS, Podojil JR, *et al.* Cutting Edge: anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells. *J Immunol* 2006; **176**: 3301.
30. Jiang X, Morita M, Sugioka A, *et al.* The importance of CD25+ CD4+ regulatory T cells in mouse hepatic allograft tolerance. *Liver Transpl* 2006; **12**: 1112.
31. Dai Z, Li Q, Wang Y, *et al.* CD4+CD25+ regulatory T cells suppress allograft rejection mediated by memory CD8+ T cells via a CD30-dependent mechanism. *J Clin Invest* 2004; **113**: 310.
32. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol* 1996; **17**: 138.
33. Szabo SJ, Sullivan BM, Stemmann C, Satoskar AR, Sleckman BP, Glimcher LH. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science* 2002; **295**: 338.
34. Piccirillo CA, Shevach EM. Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. *J Immunol* 2001; **167**: 1137.

