EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 41, No. 10, 707-716, October 2009

Sequential evolution of IL-17 responses in the early period of allograft rejection

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DOI 10.3858/emm.2009.41.10.077

Accepted 26 May 2009

Abbreviations: CMN, coagulative myocyte necrosis; IACUC, institutional animal care and use committee; ISHLT, International Society for Heart and Lung Transplantation; T_h cell, helper T cell; T_{reg} cell, regulatory T cell

Abstract

In addition to $CD4^+CD25^+Foxp3^+$ regulatory T (T_{reg}) cells which protect against autoimmune tissue injury, IL-17-producing $CD4^+$ T (T_h17) cells have been recently described and shown to play a crucial role in autoimmune injury. It appears that there is a reciprocal developmental pathway between T_h17 and T_{reg} cells. Although IL-17 is known to be associated with allograft rejection, the cellular source of IL-17 and the nature of T_h17 in the context of allograft rejection remain unknown. In the current study, the dynamics of T_{reg} and IL-17-producing cells after syngeneic and allogeneic transplantation were examined using a wild-type murine cardiac transplantation model. $Ly6G^+$ cells were found to produce IL-17 during the early postoperative period and $CD8^+$ as well as $CD4^+$ T cells were also found to produce IL-17 during alloimmune response. Graft-infiltrating $Ly6G^+$, $CD4^+$, and even $CD8^+$ cells were found to express IL-17 highly compared to those in spleen. Although the frequencies of T_h17 and T_{reg} were found to gradually increase in both syngeneic and allogeneic recipients, T_h17/T_{reg} ratios were significantly higher in recipients with allograft rejection than in syngeneic recipients. In conclusion, IL-17 is produced by neutrophils during the early postoperative period and subsequently by T_h17 and $CD8^+$ T cells during allograft rejection. T_h17/T_{reg} imbalance is associated with the development of allograft rejection. This study would provide basic information on T_h17 biology for future investigation in the field of transplantation.

Keywords: graft rejection; interleukin-17; neutrophils; T-lymphocytes, regulatory

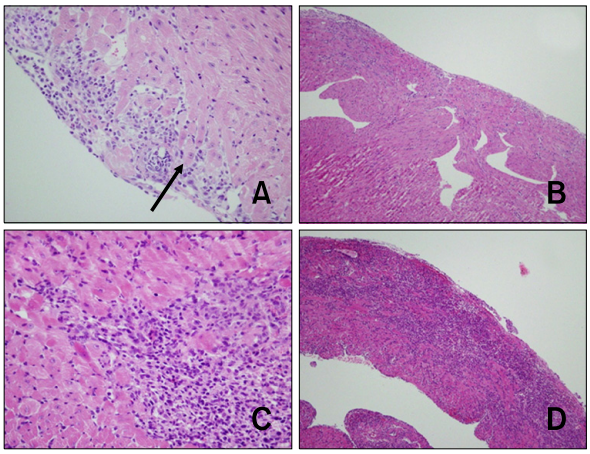
Introduction

$CD4^+$ helper T cells proliferate and differentiate into effector T cell subsets with specific cytokine phenotypes in response to antigen-specific activation. T helper (T_h) cells are traditionally classified as T_h1 and T_h2 cells based on their cytokine-expression profiles (Mosmann *et al*., 1989). In particular, T_h1 cells produce IFN-γ, which enhances cellular immunity and is required for the clearance of intracellular organisms. On the other hand, T_h2 cells produce IL-4, IL-5, IL-13 and IL-25, which enhance humoral immunity and are important for IgE production, eosinophilic inflammation, and the elimination of helminthic infections (Abbas *et al*., 1996). However, recent murine experiments have changed this T_h1/T_h2 paradigm by demonstrating that $CD4^+$ T_h cells can differentiate into T_h17 cells, which produce IL-17 and play a crucial role in inflammation and autoimmune diseases (Aggarwal *et al*., 2003; Cua *et al*., 2003; Langrish *et al*., 2005). In addition, $CD4^+CD25^+Foxp3^+$ regulatory T (T_{reg}) cells have also been described as a distinct subset from T_h1 and T_h2 cells. T_{reg} cells play an anti-inflammatory role and maintain tolerance to self-components by contact-dependent suppression or by releasing anti-inflammatory cytokines, such as, IL-10 and TGF-β (Ng *et al*., 2001; Dieckmann *et al*., 2002; Fontenot *et al*., 2003; Sakaguchi *et al*., 2006). Furthermore, the productions of T_h17 and T_{reg} cells appear to be mutually exclusive (Bettelli *et al*., 2006; Mucida *et al*., 2007) and therefore, the balance between T_{reg} and T_h17 cells may be important during the development of autoimmune diseases (Homey, 2006).

Recent studies have established that IL-17 re-

leased by T_h17 cells plays key roles during the pathogenesis of experimental autoimmune encephalitis (Langrish *et al*., 2005), rheumatoid arthritis (Kotake, 1999; Cho *et al*., 2008), inflammatory bowel disease (Fujino *et al*., 2003), psoriasis (Zheng *et al*., 2007) and uveitis (Amadi-Obi *et al*., 2007). However, the role of T_h17 and its archetypal cytokine, IL-17, is unclear in transplantation immunity. Some studies have shown that IL-17 is associated with acute allograft rejection. In a rat renal allograft transplantation model, IL-17 mRNA levels were found to peak on postoperative day 5, and then decline (Loong *et al*., 2002). Furthermore, the acute rejection of aortic allografts was prevented and cardiac allograft survival was prolonged by neutralizing IL-17 with an IL-17R:IgG-Fc fusion protein (Tang *et al*., 2001; Li *et al*., 2006). Although these data may suggest that T_h17 cells potentially impact acute allograft rejection, the cellular source of IL-17, and the nature of T_h17 cells during allograft rejection remain unknown.

Accordingly, we first analyzed the dynamic differentiations of T_{reg}, T_h17, and other IL-17$^+$ cells, including neutrophils and CD8$^+$ T cells, in the context of murine cardiac transplantations. We then evaluated the significances of T_h17/T_{reg} balance during allograft rejection.



E

Days after transplantation	Group (N)	CMN (Grade)	Rejection (Grade)
1	Syngeneic (5) Allogenic (5)	1 1	0 0
3	Syngeneic (6) Allogenic (5)	2 2	0 1R
5	Syngeneic (4) Allogenic (4)	1 1	0 2R

Figure 1. Histopathologic examination of syngeneic and allogeneic murine cardiac transplants. Tissue sections were stained with H&E. (A) Syngeneic graft in a B6 recipient showing predominant neutrophil infiltration and Grade 2 multifocal coagulative myocyte necrosis (CMN; arrow) on the 3rd postoperative day (×400). (B) Syngeneic graft in a B6 recipient showing an improved inflammatory response with little leukocyte infiltration on the 5th postoperative day (×100). (C) BALB/C allograft in B6 recipient showing characteristic mononuclear cell infiltration and Grade 1R acute cellular rejection (ACR) on the 3rd postoperative day (×400). (D) BALB/C allograft in B6 recipient showing Grade 2R rejection on the 5th postoperative day (×100). (E) Summary of histopathological features. Histopathology of these grafts (A-D) shows the grade of ACR deteriorated with time in allogeneic grafts and the extent of CMN was peaked on day 3 in both groups.

Results

Histopathological changes

The extent of coagulative myocyte necrosis (CMN), the most definitive characteristic of ischemic heart injury, was diagnosed in both groups of syngeneic and allogeneic recipients as grade 2 at 3 days and grade 1 at 5 days after transplantation (Figure 1A and 1B). Furthermore, regardless of transplantation group, at day 3, sections of cardiac grafts showed predominant neutrophil infiltration, regarded as archetypal cells of ischemic reperfusion injury. The grade of acute cellular rejection (ACR) deteriorated with time; grade 1R at day 3 and grade 2R at day 5 after transplantation (Figure 1C and 1D). Sections of allografts showed characteristic mononuclear cell infiltration during acute cellular rejection. Complete rejection of cardiac allografts occurred at 7.8 ± 0.12 days after transplantation. Histopathological features of cardiac graft recipients were summarized in Figure 1E.

Serial changes in serum levels of cytokines

Serum levels of IL-17 and IFN-γ were quantitated using Bio-Plex® kits in untransplanted control B6 mice (day 0), allogeneic (BALB/C → B6) recipients, and syngeneic (B6 → B6) recipients on days 1, 3, and 5 after transplantation (Figure 2). To determine whether increased production of IL-17 after cardiac transplantation is alloantigen-specific, we initially evaluated serial changes in the IL-17 serum levels after transplantation and compared the two study groups in this respect. In both allogeneic and syngeneic recipients, serum levels of IL-17 peaked on day 3, which corresponded to grade 2 CMN and grade 1R ACR (308.0 ± 74.9 pg/ml vs. 300.1 ± 63.1 pg/ml for allogeneic and syngeneic recipients, respectively), and these values were significantly greater than in naïve B6 mice ($P < 0.05$). However, on day 5, allogeneic recipients showed a sustained increase in IL-17 serum levels (285 ± 16.6 pg/ml), which contrasted significantly with that

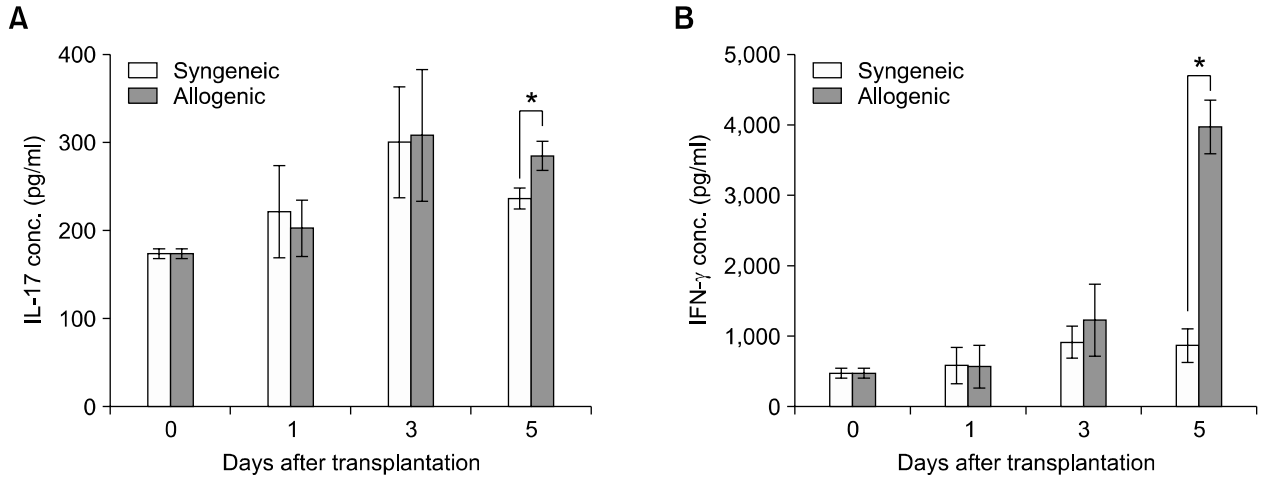


Figure 2. Changes in IL-17 and IFN-γ serum levels. Circulating levels of IL-17 and IFN-γ were quantified in mouse serum using BioPlex® Cytokine Assay kit. (A) IL-17 serum levels were peaked on the 3rd postoperative day and then decreased in both groups. However, allogeneic recipients had continuously higher IL-17 levels on the 5th postoperative day. (B) The serum levels of IFN-γ were elevated on the 5th postoperative day in allogeneic recipients. These changes in IL-17 serum levels suggest IL-17 production *in vivo* is associated with ischemic reperfusion injury during the early postoperative period and then accompanied by alloimmune responses during acute rejection. *$P < 0.05$ between the two groups on specific postoperative days.

observed in syngeneic recipients (236.1 ± 11.9 pg/ml) ($P < 0.05$). As illustrated in Figure 2B, serum levels of IFN-γ did not change significantly with time in syngeneic recipients (P = ns). On the other hand, in allogeneic recipients, serum levels of IFN-γ peaked on day 5 and showed significant elevations over other time points ($P < 0.05$). The results of this work led us to assume that IL-17 production *in vivo* is due to neutrophils contributing to ischemic reperfusion injury during the early postoperative period, which is then accompanied by alloimmune responses during acute rejection.

Serial changes in the frequencies of IL-17$^+$ cells and T_{reg} cells among splenocytes

To evaluate this hypothesis, concerning the sequential evolution of IL-17 response after transplantation, we examined the phenotype of IL-17$^+$ splenocytes using flow cytometry. In agreement with our hypothesis, the frequencies of Ly6G$^+$IL-17$^+$ cells (Ly6G$^+$IL-17$^+$/Ly6G$^+$ cells) peaked on day 3 (2.94 ± 1.50 and 4.0 ± 1.35%, respectively for allogeneic and syngeneic recipients), and thereafter decreased in both groups (Figure 3C, $P < 0.05$). The mean frequency of T_h17 (CD4$^+$IL-17$^+$/CD4$^+$ T cells) in naïve B6 mice was 0.32 ± 0.08% (Figure 3A). However, after cardiac transplantation, T_h17 frequencies peaked on day 5 in allogeneic recipients ($P < 0.05$). Allogeneic recipients showed high T_h17 frequencies on day 5 (1.70 ± 0.20%), which were significantly higher than T_h17 frequencies in the syngeneic group at this time (0.98 ± 0.17) ($P < 0.05$). Allogeneic recipients also showed significantly elevated CD8$^+$IL-17$^+$ T cell (CD8$^+$IL-17$^+$/CD8$^+$ T cells) frequencies on day 5 (Figure 3B, $P < 0.05$). Furthermore, allogeneic recipients had a higher mean frequency of CD8$^+$IL-17$^+$ T cells on day 5 (1.95 ± 0.3%) than syngeneic recipients (1.16 ± 0.2%) (P = 0.063).

T_{reg} cells constituted 4.58 ± 0.2% of CD4$^+$ T cells in naïve B6 mice. The frequencies of T_{reg} cells (CD4$^+$CD25$^+$Foxp3$^+$/CD4$^+$ T cells) increased progressively until day 5 in both syngeneic (6.52 ± 0.3%) and allogeneic (6.19 ± 0.6%) recipients (Figure 3D, $P < 0.05$). As shown in Figure 4, IL-17 producing CD4$^+$ T cells were predominantly CD4$^+$CD25$^+$ T cells. However, CD4$^+$CD25$^+$ T cells were mostly negative for IL-17, and CD4$^+$CD25$^+$Foxp3$^+$IL-17$^+$ T cells were virtually undetectable.

To ascertain whether the balance between pro-inflammatory T_h17 and anti-inflammatory T_{reg} is disrupted during acute rejection, the ratio of T_h17 to T_{reg} was evaluated. With progression of allograft rejection, allogeneic recipients had higher T_h17/T_{reg} ratios, i.e., allogeneic recipients had significantly higher T_h17/T_{reg} ratios on day 5 than on days 0 and 1 and also had significantly higher T_h17/T_{reg} ratios on day 5 than syngeneic recipients (Figure 5).

Expression of IL-17 in graft-infiltrating cells

To assess longitudinal changes in graft-infiltrating IL-17$^+$ cells after transplantation, we used double immunofluorescent staining and confocal analysis. It was found that neutrophils (Ly6G$^+$; Figure 6A-C), CD4$^+$ T cells (Figure 6D-F), and CD8$^+$ T cells

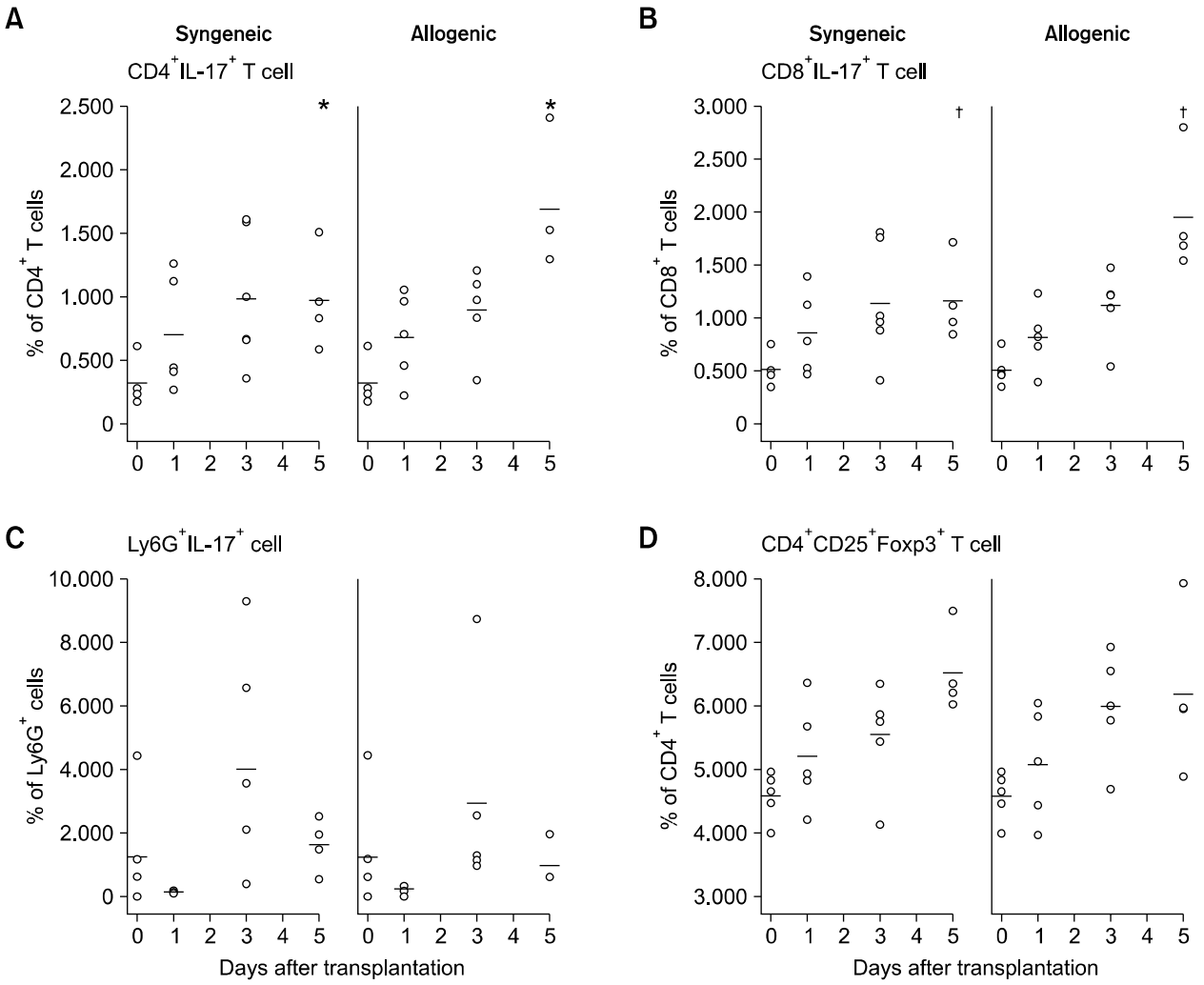


Figure 3. Flow cytometric analysis of splenocytes after syngeneic or allogeneic murine cardiac transplantations. The frequencies of $CD4^+IL\text{-}17^+$ T (T_h17) cells and $CD8^+IL\text{-}17^+$ T cells peaked on day 5 in allogeneic recipients (A, B) whereas $Ly6G^+IL\text{-}17^+$ cells peaked on day 3 in both syngeneic and allogeneic recipients (C). The frequencies of $CD4^+CD25^+Foxp3^+$ T (T_{reg}) cells increased gradually in both groups (D). $^*P < 0.05$ and $^\dagger P < 0.10$ between the two groups on specific days.

(Figure 6G-I) were IL-17-positive. In both syngeneic and allogeneic grafts, $Ly6G^+$ cells highly expressed IL-17 during the early postoperative period. In allogeneic grafts, on days 3 and 5, 13.1 ± 1.3% and 9.3 ± 1.2% of graft-infiltrating $Ly6G^+$ cells expressed IL-17, respectively. Likewise, in syngeneic grafts, 12.3 ± 1.1% and 5.9 ± 0.5% of graft-infiltrating $Ly6G^+$ cells expressed IL-17. Graft-infiltrating T_h17 and $CD8^+IL\text{-}17^+$ T cells were identified only in allografts. T_h17 cells (9.1 ± 1.3% of $CD4^+$ T cells) were demonstrated only on day 3, which corresponded to early acute rejection. In accord with our flow cytometry results, $CD8^+$ as well as $CD4^+$ T cells labeled for IL-17 during the acute rejection period. $CD8^+$ T cells expressed IL-17 on day 3 (13.3 ± 0.9%) and day 5 (11.7 ± 1.9%). Table 1 summarized the longitudinal changes in the absolute cell counts of $Ly6G^+$ cells, $CD4^+$ T cells and $CD8^+$ T cells and the percentages of $IL\text{-}17^+$ cells in each cell type.

Discussion

The role of T_h1 and T_h2 cells in acute allograft rejection has been widely investigated and the T_h1/T_h2 model has been used as a framework to explain the mechanism involved. Increased production of IFN-γ, a prototypic cytokine of T_h1 response, and mononuclear cell infiltration of the graft are commonly associated with acute allograft rejection. Thus, T_h1 cells are generally considered to be the main mediators of allograft rejection (Chan *et al*., 1995). On the other hand, T_h2 cells

Table 1. Absolute cell counts of $Ly6G^+$ cells, $CD4^+$ T cells and $CD8^+$ T cells and percentages of $IL\text{-}17^+$ cells in each cell type.

	Day 3		Day 5	
	Syngeneic	Allogeneic	Syngeneic	Allogeneic
$Ly6G^+$ cell No (mm^{-2})	754 ± 110	1,081 ± 220	480 ± 108	680 ± 34
$Ly6G^+IL\text{-}17^+$ (%)	12.3 ± 1.1	13.1 ± 1.3	5.9 ± 0.5	9.3 ± 1.2
$CD4^+$ cell No (mm^{-2})	375 ± 56	1,030 ± 117	128 ± 61	1,503 ± 372
$CD4^+IL\text{-}17^+$ (%)	-	9.1 ± 1.3	-	-
$CD8^+$ cell No (mm^{-2})	343 ± 74	1,194 ± 182	113 ± 43	1,900 ± 101
$CD8^+IL\text{-}17^+$ (%)	-	13.3 ± 0.9	-	11.7 ± 1.9

Longitudinal changes in graft-infiltrating cells after transplantation were assessed with double immunofluorescent staining and confocal analysis. With progression of rejection, allografts showed the relative predominance of $CD8^+$ T cells.

were initially predicted to promote unresponsiveness and to be associated with the induction of transplant tolerance. However, Th2-mediated rejection has been also reported to be involved in allograft rejection through graft infiltration by eosinophils, especially under conditions in which IFN-γ is depleted (Martinez *et al*., 1993; Bishop *et al*., 2001). However, this Th1/Th2 paradigm has been recently challenged by the finding that IL-17 is associated with allograft rejection, which suggests that Th17 may participate in transplant immunity (Van Kooten *et al*., 1998; Tang *et al*., 2001; Loong *et al*., 2002; Li *et al*., 2006). Furthermore, it has been reported that IL-17 is produced by Th17,

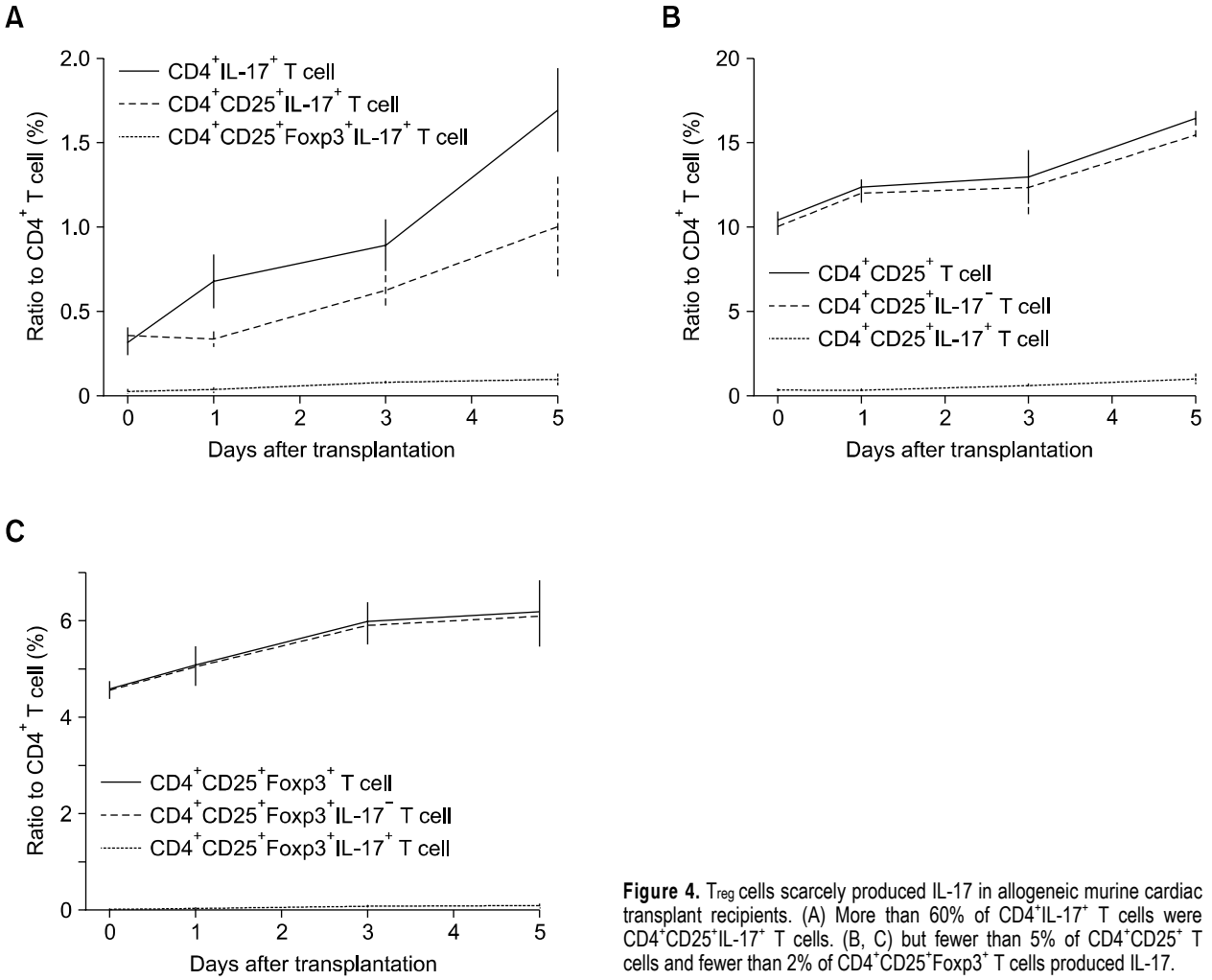


Figure 4. T_{reg} cells scarcely produced IL-17 in allogeneic murine cardiac transplant recipients. (A) More than 60% of $CD4^+IL\text{-}17^+$ T cells were $CD4^+CD25^+IL\text{-}17^+$ T cells. (B, C) but fewer than 5% of $CD4^+CD25^+$ T cells and fewer than 2% of $CD4^+CD25^+Foxp3^+$ T cells produced IL-17.

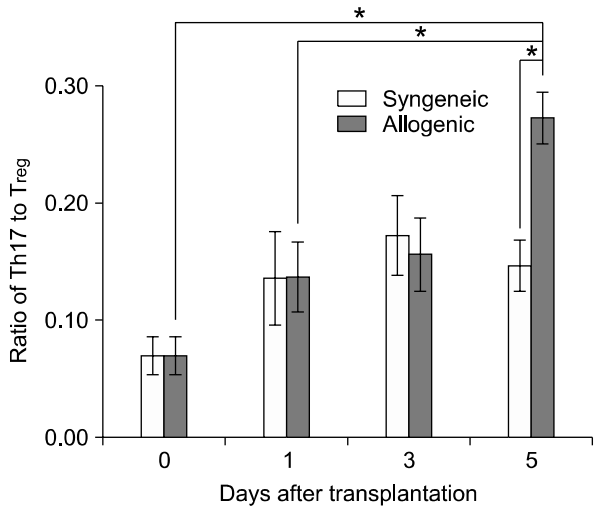


Figure 5. The T_h17/T_{reg} balance was disrupted with progression of allogeneic cardiac graft rejection. The ratio of T_h17 to T_{reg} cells were calculated using flow cytometric data of splenocytes in syngeneic and allogeneic murine cardiac transplant recipients. Allogeneic recipients showed higher T_h17/T_{reg} ratio on day 5 than on day 0 and day 1. On the 5th postoperative day, allogeneic recipients also had significantly higher T_h17/T_{reg} ratios than syngeneic recipients. $^*P < 0.05$.

natural killer, natural killer T, γδ T, and $CD8^+$ T cells, and even by neutrophils (Kryczek *et al*., 2007; Weaver *et al*., 2007; Hoshino *et al*., 2008). Yuan and colleagues reported that $CD4T_h17$ cells mediate accelerated vascular rejection and vasculopathy in T-$bet^{-/-}$ mice which exhibit profound deficiency in T_h1 response (Yuan *et al*., 2008). Thus, it would appear that the cellular source of IL-17 and the dynamics of IL-17-producing cells should be investigated in a wild-type transplantation model without pre-conditioning.

In the present study, we initially evaluated changes in IL-17 serum levels after cardiac transplantation in syngeneic and allogeneic recipients. In agreement with previous studies (Hsieh *et al*., 2001; Loong *et al*., 2002), we found that the serum level of IL-17 peaked on day 3 and declined thereafter in both recipient types (Figure 2A). However, on day 5, allogeneic recipients showed a continuously increased serum level of IL-17 whereas syngeneic recipients did not. This finding suggests that IL-17 is produced by innate immunity during the early postoperative period, and that IL-17 production is enhanced by allogeneic immunity during allograft rejection. To confirm this hypothesis, we analyzed the phenotype of IL-17^+ splenocytes by flow cytometry. On day 3, $Ly6G^+$ cells were found to express IL-17 highly in both syngeneic and allogeneic recipients. However, on day 5, in contrast to syngeneic recipients, allogeneic recipients showed a high proportion of T_h17 and $CD8^+$IL-17^+ T cells (Figure 3). Interestingly, IL-17 expression was comparable in $CD4^+$ and $CD8^+$ T cells in allogeneic recipients. Thus, our findings lead us to conclude that neutrophils that contribute to ischemic reperfusion injury produce mainly IL-17 during the early postoperative period and that $CD8^+$ as well as $CD4^+$ T cells secrete IL-17 during acute allograft rejection due to an adaptive immune response.

The second important finding of the present study arose from double-labeling confocal analysis. Specifically, IL-17^+ cells were found to contain a rather higher proportion of $Ly6G^+$, $CD4^+$ and $CD8^+$ T cells in grafts (up to 15%) than in spleen, which may have been due to the *in vivo* microenvironment. Similar results have been reported by Tzartos *et al*. (2008) in a study on multiple sclerosis (MS) in which IL-17^+ T cells were found to be associated with increased activity in MS lesion, and reported by Korn *et al*. (2007) in an experimental allergic encephalitis model, in which IL-17^+ cells were detected in the inflamed CNS, but not in the draining lymph nodes. In the present study, $CD8^+$IL-17^+ T cells were identified for up to 5 days in allografts after cardiac transplantation, whereas T_h17 cells were detected only on day 3 (Table 1). This contradicts an observation by Langrish *et al*. (2005) that IL-17^+ cells were mainly composed of $CD4^+$ T cells in an EAE model. This apparent difference may be due to the relative predominance of $CD8^+$ T cells during cardiac allograft rejection (Burrell *et al*., 2008).

It has been reported that there is a dichotomy in the generation of T_h17 that induces autoimmunity and T_{reg} that inhibits autoimmune tissue injury (Bettelli *et al*., 2006; Mucida *et al*., 2007). Thus, the balance between T_h17 and T_{reg} cells may be important in induction or regulation of tissue inflammation. This leads to the third major finding of the present study; we demonstrated for the first time that T_h17/T_{reg} balance is disrupted during allograft rejection. We observed that the frequencies of T_h17 (Figure 3A) and T_{reg} cells (Figure 3D) among $CD4^+$ T cells gradually and concomitantly increased after cardiac transplantation in both allogeneic and syngeneic recipients, but that the T_h17/T_{reg} ratio on day 5 was significantly higher in allogeneic than in syngeneic recipients (Figure 5). This suggests that the disruption of the balance between T_h17 and T_{reg} is associated with the development of allograft rejection, as well. Similarly, Cheng *et al.* (2008) also found that a T_h17/T_{reg} functional imbalance exists in patients with acute coronary syndrome (including unstable angina and acute myocardial infarction).

Finally, Benghiat *et al.* (2008) reported that T_h17 cells could be part of $CD25^+$ memory T cells and

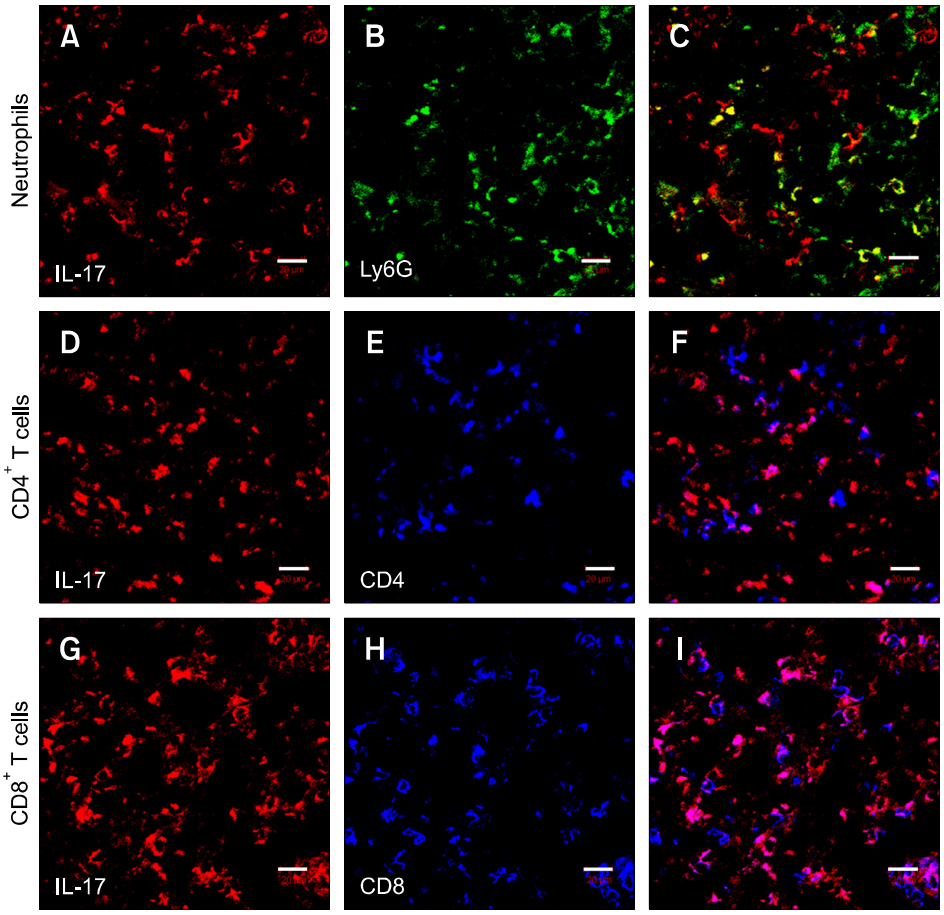


Figure 6. IL-17 production during murine cardiac transplant rejection. Double-immunofluorescence staining confocal microscopy images. Micrographs of staining for IL-17 (A, D and G; red), Ly6G (B; neutrophils, green), CD4 (E; blue) and CD8 (H; digitally converted to blue) in active inflammatory areas of a allogeneic murine cardiac transplant on the 3rd postoperative day. Overlays demonstrate IL-17 expression in neutrophils (C; yellow), $CD4^+$ T cells (F; pink), and $CD8^+$ T cells (I; pink). $Ly6G^+IL\text{-}17^+$ cells were observed at 3 and 5 days postoperatively in both syngeneic and allogeneic cardiac grafts. $CD4^+IL\text{-}17^+$ T (T_h17) cells were present only at 3 day postoperatively in cardiac allografts and $CD8^+IL\text{-}17^+$ T cells were observed on days 3 and 5 in allogeneic cardiac grafts. Scale bar = 20 μm.

that isolation of $nT_{reg}s$ solely on CD25 expression may lead to undesired selection of them. Likewise, in the present study, a considerable amount of T_h17 cells were found to be positive for CD25, which is also a marker for activated T cells, whereas $Foxp3^+$ $T_{reg}s$ were found to scarcely express IL-17 (Figure 4). However, as $CD4^+CD25^+Foxp3^+$ regulatory T cells can induce $CD4^+CD25\text{-}Foxp3^-$ T cells to become T_h17 cells and can differentiate into T_h17 cells (Xu *et al*., 2007), close attention should be paid to the selection of $T_{reg}s$ and also to the development of the T_h17 responses during $T_{reg}s$-based immunotherapy.

Altogether, the present study demonstrates the dynamic differentiation of IL-17-producing cells, including $Ly6G^+$ (neutrophils), $CD4^+$ and $CD8^+$ T cells in the context of allograft rejection, in a wild-type murine cardiac transplant model. We found that neutrophils produce IL-17 during the early postoperative period and that $CD8^+$ as well as $CD4^+$ T cells produce IL-17 during allograft rejection. In addition, our findings also demonstrate that $IL\text{-}17^+$ cells contain higher proportions of $Ly6G^+$ cells, $CD4^+$ and $CD8^+$ T cells in cardiac grafts than in spleens. Finally, T_h17/T_{reg} ratios were found to be significantly higher during allograft rejection, which suggests that T_h17 to T_{reg} imbalance plays a role in the development of allograft rejection. To draw firm conclusion, however, it needs an allograft transplantation model where only some of the recipients accept the graft. The T_h17/T_{reg} balances between allogeneic acceptor and rejector groups would be compared to test whether the balance is disrupted in the rejector group.

In spite of an observational study, this study would provide basic information on T_h17 biology in transplant immunity, which needs further investigation.

Methods

Mice

Female C57BL/6 (B6, H-2b) and BALB/C (H-2d) mice were purchased from Japan SLC (Kotoh-cho, Japan), and housed in a specific pathogen-free animal facility. Mice were routinely used at about 8 weeks of age. This study was approved by Institutional Animal Care and Use

Committee (IACUC) of the Clinical Research Institute at Seoul National University Hospital. This facility is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Heterotopic cardiac transplantation

For allogeneic transplantations, BALB/C and B6 mice were used as donors and recipients (the allogeneic group), respectively, and for syngeneic transplantations group, B6 mice were used as donors and recipients (the syngeneic group). Heterotopic cardiac transplantations were performed with microsurgical technique in a similar manner as previously described (Corry *et al*., 1973; Niimi, 2001). Briefly, donor hearts were harvested after cool perfusion with heparinized lactated Ringer's solution and then placed into cooled lactated Ringer's solution while the recipients were prepared for transplantation. The graft aorta was anastomosed to the recipient abdominal aorta and the graft pulmonary artery was anastomosed to the recipient inferior vena cava. Effective ventricular contraction usually occurred within 30 s after reperfusion.

Histology

Cardiac grafts were recovered at several time points after transplantation, fixed in formalin, paraffin embedded, serially sectioned at 4 μm, and stained with H&E by routine technique. At least two separate slides were examined to assess myocyte viability and acute rejection. The extent of coagulative myocyte necrosis (CMN), which is the most definitive manifestation of ischemic myocyte injury, was graded semiquantitatively (Fyfe *et al*., 1996; Tan *et al*., 2007): grade 0, no evidence of CMN; grade 1, mild (focal) CMN; grade 2, moderate (multifocal) CMN; and grade 3, severe (confluent) CMN. Acute cellular rejection (ACR) was graded according to the criteria proposed by the International Society for Heart and Lung Transplantation (ISHLT) (Stewart *et al*., 2005), as follows: grade 1R, mild ACR; grade 2R, moderate ACR; and grade 3R, severe ACR, regardless of the presence or absence of CMN on slides. All the slides were examined by single pathologist.

Cytokine levels

Circulating levels of IL-17 and IFN-γ were quantified in mouse serum using the Bio-Plex® Cytokine Assay 18-Plex kit (BioRad Laboratories, Hercules, CA) and the Bio-Plex 200 platform, according to the manufacturer's instructions.

Flow cytometry analysis

Spleens were retrieved, mashed, and then erythrocytes were lysed using an RBC lysing buffer. Splenocytes were first stained extracellularly with anti-mouse CD3 APC, anti-mouse CD4 PE, anti-mouse CD8 PECy7, anti-mouse CD25 PECy7, anti-mouse CD45 APC, anti-mouse Ly6G PE, and appropriate isotype control mAb (BD Pharmingen, San Jose, CA), as per manufacturer's instructions. After surface staining, cells were fixed and permeabilized with Fixation/Permeabilization solution (BD Pharmingen), and then stained intracellularly with anti-mouse IL-17 FITC and anti-mouse Foxp3 APC (eBioscience, San Diego, CA), according to the manufacturer's protocol. Cell analysis was performed using a FACScan cytometer equipped with CellQuest software (BD Bioscience).

Immunofluorescent staining and confocal analysis

For immunostaining, cardiac grafts were recovered at several time points after transplantation and embedded in OCT compound (Sakura Finetechnical Co., Tokyo, Japan), and then snap-frozen in liquid nitrogen. Frozen sections (4 μm) were fixed in acetone for 10 min and air-dried. Slides were stained with anti-mouse Ly6G Alexa 647 (BioLegend, San Diego, CA), anti-mouse CD4 Pacific Blue, anti-mouse CD8 Alexa 488 (BD Pharmingen), and anti-mouse IL-17 PE (eBioscience) for direct immunofluorescent staining, and then analyzed with a LSM 5 Image Browser using an LSM 510 META confocal laser scanning microscope (Zeiss, Jena, Germany). Areas which showed the most extensive infiltration of inflammatory cells were selected and images were acquired at high-power (×400) using a confocal microscope. The $CD4^+$, $CD8^+$, $Ly6G^+$, and $IL\text{-}17^+$ cells were counted in each image using ImageJ software (National Institutes of Health, Bethesda, MD). The percentages of $IL\text{-}17^+$ cells with respect to $CD4^+$, $CD8^+$, and $Ly6G^+$ cells were calculated using double immunofluorescent data. A second 'blind' observer re-determined percentages using the same images.

Statistical analysis

Statistical analysis was performed using SPSS software version 15.0 for Windows (SPSS, IL). Data are presented as mean ± SEM and analyzed using the Mann-Whitney and Kruskal-Wallis tests. A P value < 0.05 was considered to be statistically significant.

Acknowledgements

We would like to thank Si Hwa Kim and Yu Jin Jung for their excellent microsurgical assistance and technical support. This work was supported by a research grant from the Clinical Research Institute (CRI), Seoul National University Hospital (#0620082780).

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