Transplant International

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

# The role of interleukin-17 in murine cytomegalovirus interstitial pneumonia in mice with skin transplants

Ji-Qiang Zhao,[1] Li-Zhong Chen,[1] Jiang Qiu,[1] Shi-Cong Yang,[2] Long-Shan Liu,[1] Guo-Dong Chen,[1] Wei Zhang[3] and De Qun Ni[3]

1 Department of Organ Transplantation, First Affiliated Hospital, Sun Yat-sen University, GuangZhou, China
2 Department of Pathobiology, First Affiliated Hospital, Sun Yat-sen University, GuangZhou, China
3 Department of Microbiology, ANHUI Medical University, HeFei, China

**Keywords**
interleukin-17, interstitial pneumonia, murine cytomegalovirus, skin transplantation.

**Correspondence**
Prof. Li-Zhong Chen, Department of Organ Transplantation, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou City, Guangdong Province, 510080, China. Tel.: +86 208 730 6082; fax: +86 208 730 6082; e-mail: clz@medmail.com.cn

**Conflicts of Interest**
The authors have declared no conflicts of interest.

Received: 27 January 2011
Revision requested: 23 February 2011
Accepted: 21 April 2011
Published online: 31 May 2011

doi:10.1111/j.1432-2277.2011.01273.x

## Summary

We hypothesized that the T helper (Th)17 response plays an important role in murine cytomegalovirus (MCMV) interstitial pneumonia. BALB/c mice with skin grafts from C57/BJ6 mice were intranasally inoculated with $1.0 \times 10^5$ PFU MCMV. Lung tissues and skin grafts were histologically evaluated and expression of interleukins (IL)-17, -6 and -8, monocyte chemotactic protein (MCP)-1 and interferon (IFN)-γ in serum and bronchoalveolar lavage (BAL) fluid, intracellular IL-4, -17, and IFN-γ, in spleen lymphocytes were analysed. The levels of IL-17 in the serum and BAL fluid were significantly higher in MCMV-infected mice versus not-infected mice ($P = 0.0286$ and $P = 0.007$, respectively) and the BAL levels of IL-17 peaked in 9 days ($P = 0.001$). The IL-17 level in the BAL was correlated with the grade of lung interstitial inflammation ($r = 0.554$, $P = 0.0144$). Serum IFN-γ levels were also higher after infection than that in the not-infected mice ($P = 0.0286$). IL-17 production increases locally and systemically during MCMV interstitial pneumonia. Neutralization of IL-17 significantly suppressed lung inflammation at day14 as assessed by histology. These findings suggest that IL-17 is important in the pathology of MCMV interstitial pneumonia.

## Introduction

Cytomegalovirus (CMV) is a ubiquitous herpesvirus that causes asymptomatic infection in up to 90% of the healthy adult population. Primary infection or reactivation in immunocompromised hosts can be associated with serious morbidity and mortality. It has been estimated that CMV causes symptomatic disease in 35% and death in 2%, of renal transplant recipients [1]. CMV infection induces an increase in plasma concentrations of interleukin (IL)-6, IL-8, tumour necrosis factor (TNF)-α, Interferon (IFN)-β, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in renal and liver transplant recipients [2,3]. During CMV disease, several cytokines, chemokines and adhesion molecules are released to recruit inflammatory cells for infection control [4]. In a murine model of ovalbumin (OVA)-induced allergic airway disease, murine cytomegalovirus (MCMV) infection altered T helper (Th)1/Th2 cytokine expression and decreased bronchoalveolar lavage (BAL) fluid eosinophilia. The decline in BAL fluid eosinophilia was associated with a change in lung Th1/Th2 cytokine profiles [5].

Interleukin-17 is a $CD4^+$ Th cell-derived cytokine that was identified originally as cytotoxic T lymphocyte (CTL)-associated antigen 8. It has a proinflammatory role and has been implicated in post-transplantation rejection and respiratory diseases [6–8], as it induces the expression of many mediators of inflammation [9]. Recent reports indicate that IL-17 is critical in the clearance of several pathogens, including certain viruses [10,11], bacteria [12,13] and fungi [14,15].

Cytomegalovirus-induced pneumonia is recognized as a major cause of mortality in immunocompromised patients and is a significant impediment to successful

organ transplantation. Although murine cytomegalovirus infection can induce a multifunctional $CD4^+$ T-cell response and a substantial percentage of the MCMV-specific CD4 T-cell population produces IL-17 upon peptide stimulation [16], very little is known about the role of this cytokine in the context of viral infection and transplantation. In this study, we report that the Th17 response and the dynamic evolution of IL-17 associated with pathological changes affect the replication of MCMV and the occurrence of interstitial pneumonia in allogeneic skin transplant BALB/c mice after intranasal inoculation of the virus.

## Materials and methods

### Animals

Six- to eight-week-old specific pathogen-free female BALB/c and C57Bl/6J mice (weighing 15–20 g) were purchased and kept in the pathogen-free mouse room in the experimental animal center of Sun Yat-sen University. The animal use protocol has been reviewed and approved by the Institutional Animal and Use Committee of Sun Yat-Sen University.

### Skin grafting

Using trunk skin from donor mice (C57Bl/6J), skin allografts were performed according to previous descriptions [17]. A square graft (approximately 10 mm × 10 mm) was placed on a graft bed prepared on the flank of a BALB/c recipient. The graft was covered with protective bandages for 8 days. Allograft recipients received intraperitoneal injections of cyclosporin A (CsA; Novartis Pharma, KG, Germany, 20 mg/kg/day) daily throughout the study. Rejection was considered to occur when grafts exhibited dark discoloration, scabbing and necrotic degeneration.

### Virus propagation and intranasal inoculation

The Smith strain of MCMV was routinely propagated on BALB/c mouse embryo fibroblasts (MEFs), maintained in Dulbecco's modified Eagles medium (DMEM, Gibco, Invitrogen Corporation, CA, USA) containing 10% fetal bovine serum (FBS) (Gibco, Invitrogen Corporation, CA, USA), 100 U/ml penicillin and 5 μg/ml streptomycin (Gibco, Invitrogen Corporation, CA, USA).

On the 5th day after grafting, the mice were weighed and lightly anesthetized with ether, and $1.0 \times 10^5$ PFU of virus were injected intranasally ($n = 17$, MCMV-infected group). Control animals were inoculated in parallel with an equal volume of virus-free medium ($n = 17$, not-infected group). Infected animals were housed in isolation apart from control animals.

### Neutralizing antibodies for IL-17

BALB/c mice with MCMV infection were treated with intraperitoneal injections of 100 μg of normal rat IgG ($n = 10$, eBioscience, CA, USA) or rat anti-mouse IL-17 ($n = 10$, eBioscience) at day 0 and day 7 after MCMV infection. Blood, lung tissues and spleens were collected at 9 and 14 days p.i from five infected mice. The values of body weight were recorded at the same time.

### Determination of cytokine and chemokine levels

At various times after virus inoculation, four to five BALB/c mice were sacrificed by ophthalmectomy. The left lungs from each animal were lavaged *in situ* with 400 μl sterile saline and the returned fraction (approximately 200 μl) was immediately centrifuged at 500 rpm for 10 min at 4 °C. The supernatants were used for measurement of cytokines and chemokines using ELISA, according to the recommendations of the manufacturer. IL-21, IL-23, transforming growth factor beta (TGF-β), IL-6, IFN-γ, human monocyte-chemoattractant protein-1 (MCP-1), IL-8, and IL-17 ELISA kits were all purchased from R&D, MN, USA. The results are expressed as pg/ml of BAL and serum. In addition, blood was collected from skin transplant recipient mice via retro-orbital bleeding and serum was separated before and after MCMV inoculation.

### Histological and immunohistochemical analysis

The unmanipulated, noninflated, right lungs were removed from animals and dissected into several parts; some were fixed in 4% buffered paraformaldehyde solution and embedded in paraffin and the others were used for DNA and RNA extraction. Tissue sections were stained with H&E to assess general morphology by the Department of Pathology at the First Affiliated Hospital of Sun Yat-sen University. Each specimen was scored using the following criteria [18]: 0 = normal lung, 0.5 = 1 or 2 foci of 10–20 cells per section, or small areas with twofold thickened alveolar septa, 1.0 = 3–5 foci of 10–30 cells per section, or widespread areas with twofold thickened alveolar septa, $2.0 = 5^+$ foci of 10–50 cells per section, or two to three fold thickened alveolar septa throughout the lung, and $3.0 = 5^+$ foci of 10–100 cells per section, or three to four fold thickened alveolar septa throughout the lung.

The anti-murine (m)IL-8 and anti-mIL-6 antibodies were purchased from R&D systems (Minneapolis, MN, USA). Anti-mIL-17 monoclonal antibodies (mAb) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The sample collection and immunohistochemical staining were performed as described previously [7].

### PCR, RT-PCR

PCR was performed as previously described [19] using a genomic DNA Kit (Invitrogen, CA, USA) for DNA extraction, according to the manufacturer's instructions. For RNA extraction, fresh tissues were sonicated in Trizol reagent (Invitrogen) to disrupt the tissue and the RNA was purified according to the directions of the manufacturer. Reverse transcriptase PCR (RT-PCR) analysis for the MCMV IE1-RNA and DNA was performed according to the method described previously [20].

### Intracellular cytokine staining and flow cytometry

Splenocytes were harvested from mice and single cell suspensions were prepared followed by lysis of red blood cells. Intracellular cytokine staining was performed using the Th1/Th2/Th17 Phenotyping Kit (BD Pharmingen™, NJ, USA). In brief, lymphocyte suspensions from spleens of MCMV-infected mice were stimulated with 50 ng/ml of phorbol-12-myristate-13-acetat (Merck, NJ, USA) and 1 μg/ml of ionomycin (Merck) for 5 h at 37 °C in the presence of Protein Transport Inhibitor (BD GolgiStop™, NJ, USA). The lymphocytes were washed twice in phosphate buffered saline and adjusted to a density of $1 \times 10^6$/ml. The cells were fixed with 1 ml of fixation solution (BD Cytofix™, NJ, USA) and incubated in the dark at room temperature for 30 min. After washing with permeabilization buffer, the cells were resuspended and immunostained. Th1, Th2 and Th17 subsets were acquired and analysed using a FACSCalibur (Becton Dickinson, NJ, USA) with CellQuest Pro software (Becton Dickinson).

### Statistical analysis

The chi-square test or one-way ANOVA was used to analyse the statistical significance of the observed differences between two groups. In time course studies, one-way ANOVA was used, followed by LSD *post hoc* analysis. Pearson correlation analysis was used to study the correlation between two variables. The Kaplan–Meier method was used to compare survival curves of the studied groups. All data were expressed as the mean ± SEM. A $P$ value of 0.05 or less was considered significant. All analyses were performed using Graphpad Prism 5.0 software (GraphPad Software, Inc. San Diego, CA, USA).

## Results

### Establishing a protocol for MCMV interstitial pneumonia in mice with skin transplants

Some previous studies have reported that IL-17 levels are increased in an allograft transplant model [6,7,21]. The major goal of this study was to determine the change in IL-17 in mice with skin transplant that have MCMV interstitial pneumonia. Thus, the time course of IL-17 levels in skin transplant mice treated with CsA was measured using ELISA before MCMV infection. As shown in Fig. 3a, serum IL-17 persists at a low level for 2 weeks and has no significant difference over several days post-transplant ($P = 0.4159$). Based on these findings, mice were infected with MCMV 5 days after skin transplantation. Thus, the time course for collecting samples used in previous studies on MCMV interstitial pneumonia was followed [18,22].

### Histological evaluation of interstitial pneumonia and viral replication

To confirm that the mouse model was a useful model of MCMV interstitial pneumonia, we histologically analyzed the lungs of infected mice. Lung tissues from MCMV-infected mice were markedly impaired compared with control mice and displayed dense inflammatory foci. Nine days after infection, the inflammatory foci had diffused through the lung parenchyma. The alveolar walls had become thickened and caused focal, diffuse diminution or obliteration of alveolar space. Concurrent with these changes, a diffuse infiltration of monocytes had spread through the parenchyma. No lung abnormalities were noted in the not-infected mice. Histological scoring indicated that the lungs of infected mice had significant interstitial inflammation (Fig. 1c).

Our latest studies demonstrated that IL-17 contributed to MCMV interstitial pneumonia and the lungs on day 9 displayed significantly higher scores compared with days 5 and 21 (unpublished data). To further investigate the relationship between IL-17 and MCMV interstitial pneumonia, we studied whether neutralization of IL-17 would protect mice from MCMV-induced interstitial pneumonia. It was found that IL-17mAb alleviated the severity of interstitial pneumonia (Fig. 1a). Histological analysis of lung sections revealed that MCMV-infected mice developed severe interstitial pneumonia on day 9 with inflammation of lung tissue, whereas IL-17mAb led to a relief of lung inflammation on day 14 (Fig. 1c).

When allogeneic skin-transplanted mice were infected intranasally with $1.0 \times 10^5$ PFU MCMV, RT-PCR studies revealed that MCMV IE-1 DNA and MCMV IE-1 RNA had appeared in lung tissue as early as 5 days post-infection (Fig. 2). In addition, RT-PCR was performed to detect expression in the kidney, liver, skin graft, and salivary gland at each time point throughout the study, and the results were consistent with the results seen for the lungs. These data confirm that this mouse model can be used for analysis of interstitial pneumonia.

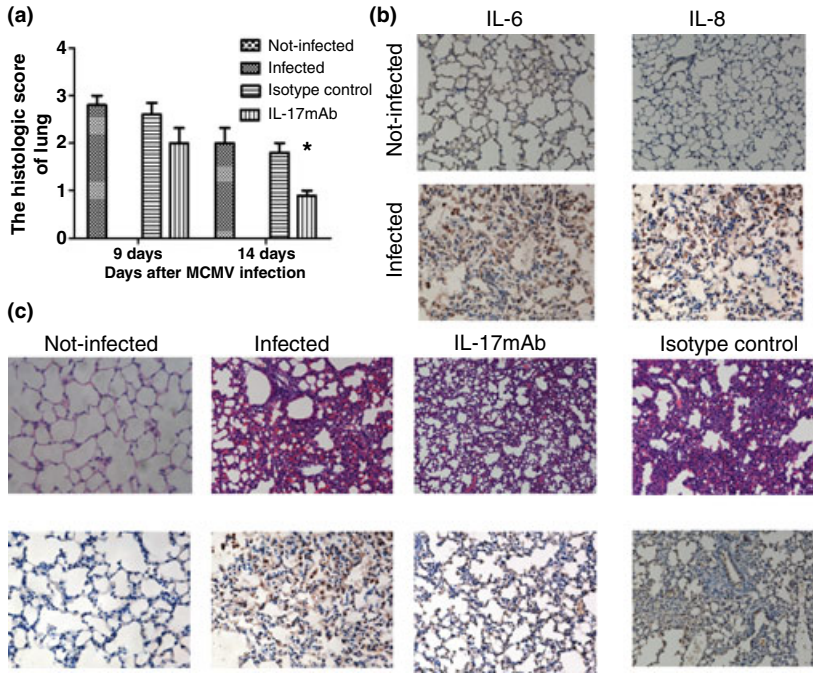


**Figure 1** IL-17mAb reduces disease severity in skin transplant mice infected with $1.0 \times 10^5$ PFU of MCMV. (a) The pathological score is lower in MCMV-infected mice treating with IL-17 mAb on day 14. (b) Immunohistochemical staining using anti-IL-6 and anti-IL-8 mAb, showing scattered expression of IL-6 and IL-8 in lung tissue from MCMV-infected mice compared with not-infected mice (magnification ×200). (c) The results of histopathology (magnification ×200) and immunohistochemistry (magnification ×200) in lung tissue show that IL-17 distributes in the lesions with inflammation and neutralization of IL-17 mAb makes the lesions restricted. $^*P < 0.05$ versus not-infected and Isotype control group. Values are means ± SEM of four to five animals per group at different stages. MCMV, murine cytomegalovirus; IL, interleukin; mAb, monoclonal antibody.


**Figure 2** Lung tissues of recipients were evaluated for MCMV-IE1 DNA and MCMV-IE1 RNA by RT-PCR 5 days after MCMV infection. Lung tissues from MCMV-infected recipients show definitive molecular MCMV replication. MCMV, murine cytomegalovirus; RT-PCR, reverse transcriptase PCR.

### Increased levels of serum cytokines and chemokines during MCMV interstitial pneumonia

Changes in serum IL-17 levels were examined over time (Fig. 3a). The serum IL-17 levels in mice with MCMV interstitial pneumonia were significantly higher than those of not-infected mice ($P = 0.0286$, Table 1). The IL-17 level on day 9 had increased to 53.90 pg/ml and was higher than that on other days, although this difference was not statistically significant ($F = 0.316$, $P = 0.858$).

As shown in Fig. 3b and c, the serum IL-8 and MCP-1 levels in infected mice were higher than in not-infected mice ($P = 0.0286$, $P = 0.0286$, respectively, Table 1). However, the difference in both IL-8 and MCP-1 between the indicated days after MCMV infection was not significant ($F = 0.225$, $P = 0.875$).

The serum IFN-γ levels in infected mice increased and peaked on day 9 after infection (Fig. 3d) and the difference was statistically significant between the groups ($P = 0.0286$). Moreover, the IFN-γ levels on both day 5 and day 9 were higher than that in not-infected mice, respectively ($144.43 \pm 14.21$ vs. $38.98 \pm 14.76$, $P = 0.001$; $243.51 \pm 103.34$ vs. $45.64 \pm 15.83$, $P = 0.024$, respectively).

Serum IL-6 levels were not different between infected mice and not-infected mice ($P = 0.809$).

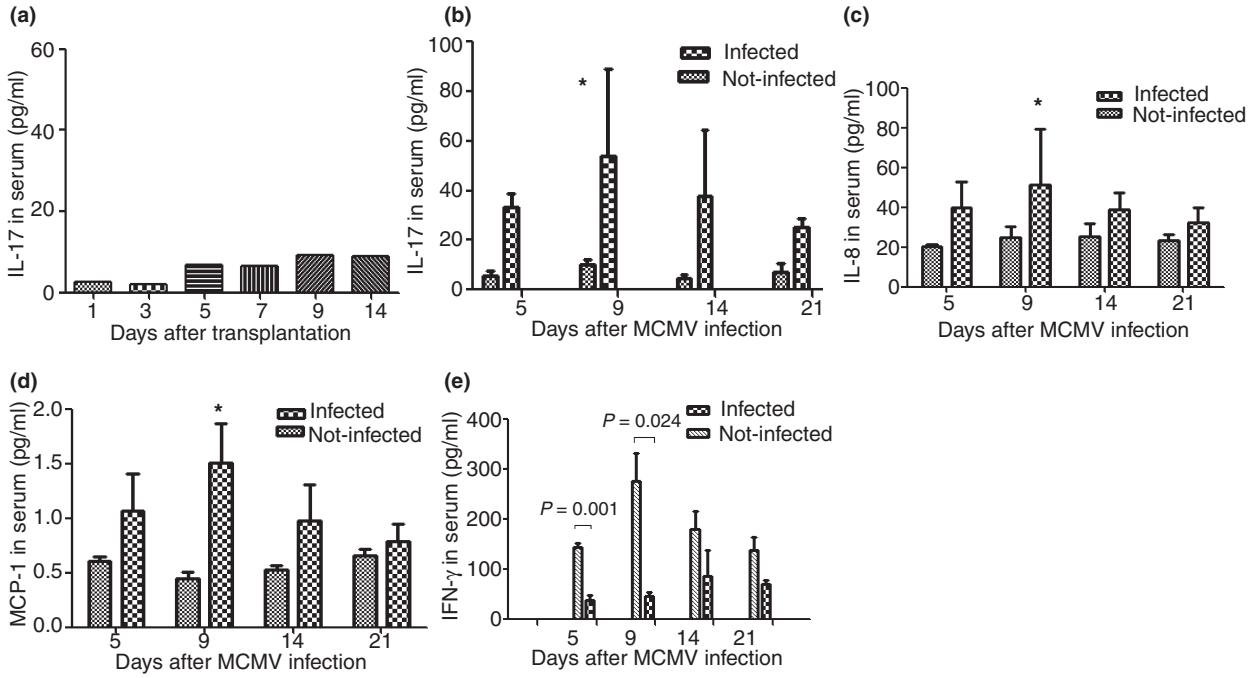


**Figure 3** Serum expression levels of cytokines and chemokines were altered in MCMV-infected mice. Serum was recovered from MCMV-infected and not-infected mice after 5, 9, 14 and 21 days of MCMV infection. The samples were assayed for IL-17 (a), post-transplantation (b), after MCMV infection, IL-8 (c), MCP-1 (d) and IFN-γ (e) by ELISA. The serum levels of IL-17, IL-8, IFN-γ and MCP-1 were increased in MCMV-infected mice compared with not-infected mice. However, the expression differences in infected mice between days were not significant. Serum IFN-γ levels in MCMV-infected mice on day 5 and 9 were higher than in not-infected mice. Results are expressed as mean ± SE of four to five animals per group at different stages. MCMV, murine cytomegalovirus; IL, interleukin; MCP-1, monocyte-chemoattractant protein-1; IFN, interferon.

**Table 1.** Serum and BAL fluid analysis of cytokines and chemokines from MCMV-infected mice.

| | Group | | | |
|---|---|---|---|---|
| | Not-infected | | MCMV-infected | |
| | Serum | BAL | Serum | BAL |
| IL-17 (pg/ml) | 6.94 ± 2.57 | 18.42 ± 17.63 | 37.52 ± 12.05* | 42.49 ± 17.50** |
| IL-8 (pg/ml) | 23.60 ± 2.21 | 18.20 ± 1.14 | 40.57 ± 7.87* | 39.65 ± 17.80* |
| IL-6 (pg/ml) | 6.08 ± 3.43 | 5.92 ± 2.80 | 6.12 ± 1.22 | 8.23 ± 5.73 |
| IFN-γ (pg/ml) | 60.09 ± 21.77 | | 184.7 ± 64.24* | |
| MCP-1 (pg/ml) | 0.56 ± 0.09 | 0.51 ± 0.19 | 1.08 ± 0.30* | 1.09 ± 0.45* |

Data are presented as mean ± SD. BAL, bronchoalveolar lavage; MCMV, murine cytomegalovirus; IL, interleukin; IFN, interferon; MCP, monocyte chemotactic protein.

*$P < 0.05$ for MCMV-infected mice versus not-infected mice; **$P < 0.01$ for MCMV-infected mice versus not-infected mice.

### Increased levels of cytokines and chemokines in BAL fluid during MCMV interstitial pneumonia

There was also an obvious trend towards higher IL-17 levels in the BAL of MCMV interstitial pneumonia mice than of not-infected mice, as shown in Fig. 4a ($P = 0.007$, Table 1). Moreover, the BAL fluid level of IL-17 on day 9 was higher than on days 5, 14 or 21 ($F = 47.651$, $P = 0.001$). The BAL fluid level of IL-17 in MCMV interstitial pneumonia mice was higher than the serum level of IL-17, but there was no statistical significance ($P = 0.2000$).

The levels of IL-8 in the BAL fluid of MCMV interstitial pneumonia mice were higher than those of not-infected mice ($P = 0.0286$, Table 1). More importantly, the BAL fluid levels of IL-8 were higher on day 9 than the expression levels on days 14 or 21 after MCMV infection (64.96 ± 30.00, 31.65 ± 12.32 and 24.08 ± 0.84; $P = 0.015$, $P = 0.004$, respectively; Fig. 4b).

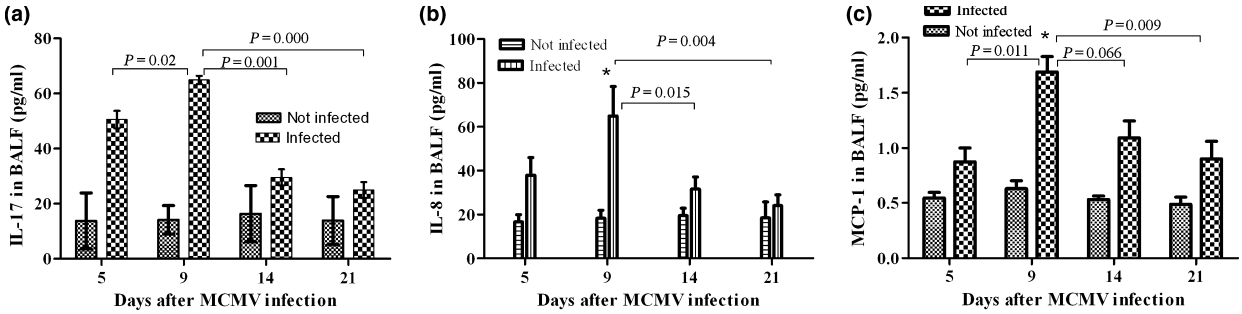


**Figure 4** Serial changes of IL-17 (a), IL-8 (b) and MCP-1 (c) were measured in BAL fluid from 5 to 21 days after MCMV infection. *represents the difference between the MCMV-infected mice and not-infected mice was statistically significant ($P < 0.05$). Results show mean ± SEM of four to five animals per group at different stages. IL, interleukin; MCP-1, monocyte-chemoattractant protein-1; BAL, bronchoalveolar lavage; MCMV, murine cytomegalovirus.

Levels of MCP-1 in BAL fluid were increased in MCMV-infected mice ($P = 0.022$, Fig. 4c). Furthermore, the BAL MCP-1 levels on day 9 were higher than on days 5 or 21 after MCMV infection ($P = 0.011$, $P = 0.009$, respectively).

Similarly to serum levels, the BAL fluid levels of IL-6 were not significantly different between the two groups.

### Correlation analysis for IL-17 with other cytokines in serum and BAL fluid

To investigate the correlation of IL-17 levels with those of other cytokines, we performed correlation analysis. The levels of IL-17 in BAL fluid were positively correlated with the levels of IL-8 and MCP-1 in BAL, respectively ($r = 0.6152$, $P = 0.033$, Fig. 5a; $r = 0.782$, $P = 0.022$, Fig. 5b).

The IL-17 level in BAL was correlated with the grade of lung interstitial inflammation (r = 0.662, $P = 0.0038$; Fig. 5c). The degree of lung inflammation strongly correlated with the IL-17 levels at 5 and 9 days after MCMV infection ($r = 0.972$, $P = 0.028$; $r = 0.977$, $P = 0.004$).

### Expression of cytokines in lung tissues from infected mice

We investigated the expression of IL-17, IL-6 and IL-8 in lungs from MCMV-infected mice by immunohistochemi-

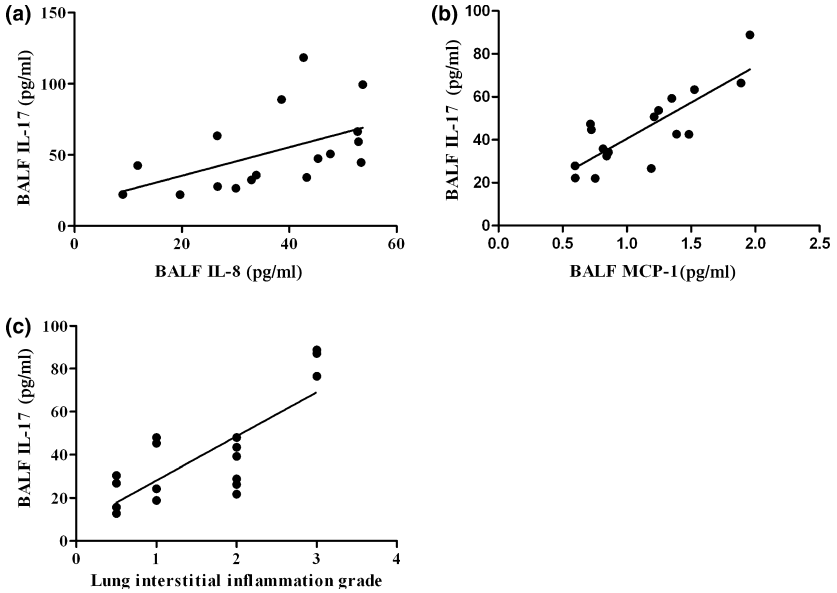


**Figure 5** IL-17 and IL-8 levels were measured in serum and BAL fluid after MCMV infection. Correlation between (a) IL-17 and IL-8 in BAL fluid ($r = 0.6152$, $P = 0.033$), (b) IL-17 and MCP-1 in BAL ($r = 0.782$, $P = 0.022$) and (c) IL-17 level in BAL and the grade of lung interstitial inflammation ($r = 0.5514$, $P = 0.0144$). Four to five mice were investigated per group at different stages. IL, interleukin; BAL, bronchoalveolar lavage; MCP-1, monocyte-chemoattractant protein-1.

cal staining. On day 9, there was no detectable IL-17 in lung tissues from not-infected mice (Fig. 1c). However, abnormal expression of IL-17 was detected in lung tissues from MCMV-infected mice. Consistent with IL-17 expression, both IL-6 and IL-8 were extensively expressed in lung tissues from MCMV-infected mice compared with not-infected mice (Fig. 1b).

### Cytokine expression profiles of CD4 T cells after MCMV infection

CD4 T cells play a very important role in MCMV infection. To determine levels of intracellular IL-17 and IFN-γ, we examined intracellular cytokines staining after MCMV infection by the re-stimulation of splenocytes (Fig. 6). We found that the percentage of IL-17$^+$ CD4 T cells in the spleens of mice with MCMV infection was higher than in the not-infected mice (0.71 ± 0.81% vs. 0.05 ± 0.06%, $P = 0.026$). IFN-γ$^+$ CD4 T cells were also significantly increased in spleens of mice over the course of infection compared with not-infected mice (5.34 ± 4.43% vs. 1.52 ± 1.68%, $P = 0.025$). The percentage of IL-17$^+$ lymphocytes in the spleens of mice on day 9 was higher than 5 days after MCMV infection (11.06 ± 1.32% vs. 1.59 ± 0.87%, $P = 0.000$). The expression of IL-4 in the two groups was not significantly different ($P > 0.05$). Treating these mice with IL-17mAb reduced the proliferation of IL-17$^+$ CD4 T cells in the presence of MCMV infection.

### Differentiation factors of Th17 cells after MCMV infection

In the study, we found that the difference of serum IL-6 level between the infected and not-infected group was not significant. Therefore, other differentiation factors expression levels, such as IL-21, IL-23, TGF-β, in mice serum were determined. As shown in Table 2, the IL-21 level was not different between the two groups, but the TGF-β level in MCMV-infected mice was lower than in not-infected mice ($P = 0.002$).The IL-23 level in MCMV-infected mice was higher than in not-infected mice ($P = 0.003$).

### Skin allograft survival time

Histological evaluations were performed by hematoxylin and eosin staining on paraformaldehyde-fixed, paraffin-embedded sections of skin grafts from MCMV-infected mice. The skin grafts of infected mice demonstrated dense inflammation at 9 days after MCMV infection. Immunohistochemical staining using an anti-IL-17 antibody detected scattered expression in skin grafts 9 days after MCMV infection (Fig. 7). In agreement with the expression seen in lung tissues with MCMV infection, IL-6 and IL-8 demonstrated scattered expression in skin grafts 9 days after MCMV infection. However, these cytokines are presented sporadically in skin allograft from not-infected mice (Fig. 7).

**Table 2.** Serum levels of differentiation factors after MCMV infection.

| | Group | |
|---|---|---|
| | Not-infected | MCMV-infected |
| IL-21 (pg/ml) | 205.04 ± 110.89 | 166.23 ± 46.89 |
| IL-23 (pg/ml) | 38.09 ± 34.84 | 89.45 ± 25.64* |
| TGF-β (pg/ml) | 544.16 ± 68.52 | 230.88 ± 98.51* |

Data are presented as mean ± SD. MCMV, murine cytomegalovirus; IL, interleukin; TGF-β, transforming growth factor beta.
*$P < 0.05$ for MCMV-infected mice versus Not-infected mice.

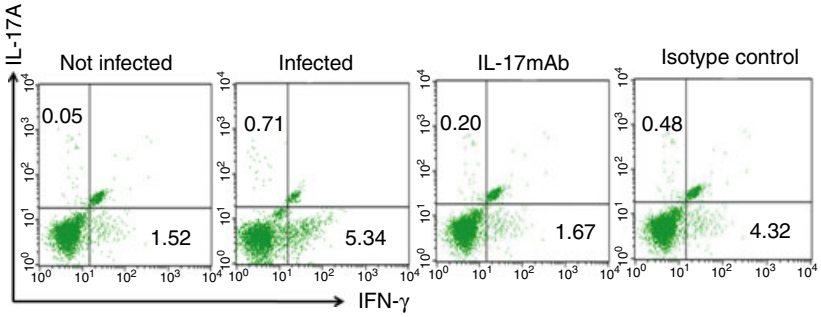


**Figure 6** Flow cytometry analysis of Th1/Th2/Th17 subsets in each group. The total population of splenocytes was gated using the forward scatter channel/side scatter channel profile and CD4 positive expression. The gated CD4$^+$ T-cell population was chosen to analyse cytokine expression. The positive populations of IFN-γ, IL-4 and IL-17 were gated, respectively, to calculate their percentage in CD4$^+$ T cells. Results of intracellular staining are representative of four or five mice per group from at least two separate experiments for per group at different stages. Numbers in quadrants indicate the per cent of IL-17$^+$ T cells and IFN-γ$^+$ T cells after cell division. IL, interleukin; IFN, interferon.

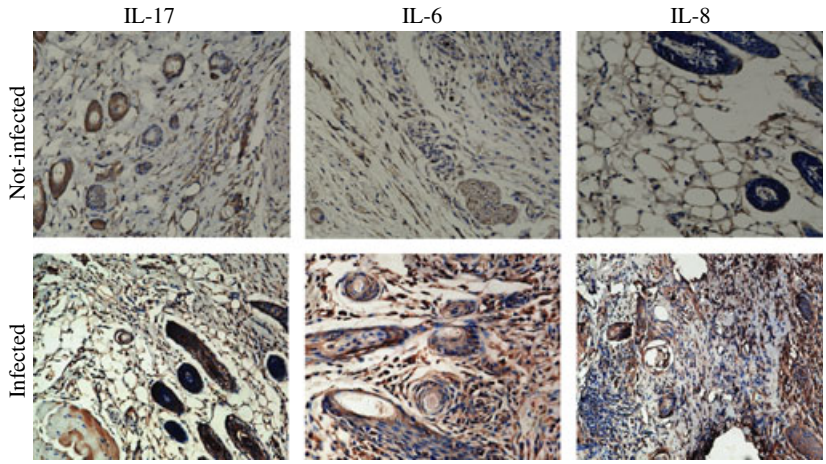


**Figure 7** IL-17, IL-6 and IL-8 are presented in skin allograft 9 days after MCMV infection. Immunohistochemical staining using an anti-IL-17, anti-IL-6 and anti-IL-8 monoclonal antibody showed scattered expression of IL-17, IL-6, and IL-8 in skin allograft from MCMV-infected mice. However, these cytokines are presented sporadically in skin allograft from not-infected mice (magnification ×200). IL, interleukin; MCMV, murine cytomegalovirus.

To investigate the influence of MCMV infection on skin allograft survival time, the skin allograft survival time of six mice in each group was recorded. The survival curve of skin allografts was calculated (Fig. 8). The median survival time of skin allograft in MCMV-infected mice was shorter than not-infected mice (14 days vs. 17 days, $P = 0.0038$). The survival curves were significantly different between the two groups ($\chi^2 = 8.427$, $P = 0.0037$, Hazard ratio = 0.1171). By 14 days after infection, most of the skin grafts had been rejected. In addition, the growth of MCMV-infected mice, as measured by body weight, was slower than that of not-infected mice ($F = 10.492$, $P = 0.002$).

## Discussion

The main result of the preliminary data described here demonstrates that MCMV infection results in the production of IL-17, which regulates the immune response as well as the development of lung lesions over the course of interstitial pneumonia. Moreover, IL-17 regulates the expression of inflammatory cytokines and chemokines in the lung. IL-17 might play an important role in the development of MCMV interstitial pneumonia after allogeneic transplantation.

A critical step to study the dynamic evolution of IL-17 is the selection of appropriate time points at which to collect samples. An important consideration is the pathological variation of the lung over time, as demonstrated by Price *et al.* [18]. In the current study, the authors identified a fixed time point for evaluation that represented the typical lung pathological characteristics of MCMV interstitial pneumonia. Given the influence of allogeneic transplantation on IL-17 [21], we chose to detect serum IL-17 levels in allogeneic skin transplant mice before MCMV infection. These results indicated that serum IL-17 levels were low and did not differ over various days after transplantation, allowing for statistically adequate comparison between MCMV-infected and control mice.

Interferon-γ produced by Th1 cells had been identified as responsible for a wide range of autoimmune and inflammatory diseases. In agreement with previous studies [23], we also found an increase in serum IFN-γ levels after MCMV infection in this model, although CsA can block lymphocyte activation by preventing the transcrip-

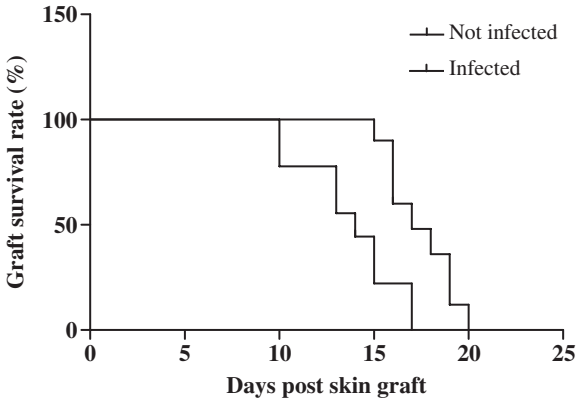


**Figure 8** MCMV infection shortened the skin allograft survival time compared with not-infected mice (14 days vs. 17 days, $P = 0.0037$). MCMV, murine cytomegalovirus.

tion of γ-interferon genes [24]. Strikingly, the IFN-γ levels on both days 5 and 9 after infection were higher than those of not-infected mice and then rapidly decreased. This trend in serum IFN-γ levels was compatible with previously reported IFN-γ expression in spleen lymphocytes after MCMV infection [16]. Recent studies have shown that IFN-γ can negatively affect IL-17 expression [25]. However, we did not observe this correlation between IL-17 and IFN-γ in this study. Interestingly, CsA alone or combined with corticosteroids was able to significantly inhibit the expression of IL-17 and IFN-γ *in vivo* and *in vitro*, which correlated with suppression of clinical disease activity [26–29]. Thus, how IL-17 regulates the expression of IFN-γ in MCMV interstitial pneumonia can only be speculated on at the moment.

Intriguingly, in agreement with CMV disease data from the solid organ transplantation and mouse bronchiolitis obliterans (BO) models [30,31], we did not observe an effect of MCMV infection on the concentrations of IL-6, a typical pro-Th17 cytokine, although it was detected in lung tissue by immunohistochemical staining. These results suggest that a specific local environment can support Th17 function, which might play a role in MCMV infection. However, IL-6 could be replaced by IL-21 (secreted by Th17 cells themselves) because an alternative IL-6-independent but still STAT3 and IL-21-dependent pathway of Th17 differentiation has also been described [32]. This last point underscores the importance of IL-21, which is considered an autocrine factor involved in Th17 generation, not only through the up-regulation of its own production but also by inhibiting IFN-γ production by Th1 cells and by inducing the expression of IL-23 receptor on Th17 cells [33,34]. In addition, TGF-β has also been identified as a key cytokine necessary for the induction of regulatory T cells (Tregs) from naive T cells and is produced by Tregs themselves [35]. More importantly, the presence of TGF-β producing Tregs in an inflammatory environment may favour the generation of proinflammatory Th17 cells, a situation that is potentially significant in the context of transplantation [36]. In this study, the level of IL23 was increased along with the reduction of circulating TGF-β level after MCMV infection. Thus, we conclude, on the basis of induced by alloantigen and MCMV infection, activated $CD4^+$ T cells differentiate into Th17 cells upon exposure to IL-23, which promotes the production of IL-17 by activated T cells [37].

Interleukin-8, the major neutrophilic chemoattractant, has been identified as an important trigger in the onset and progression of Bronchiolitis Obliterans Syndrome (BOS) [38]. Both *in vitro* and *in vivo*, IL-17 is capable of inducing neutrophil migration and activation, most likely through the release of IL-8 [39,40]. MCP-1, which recruits circulating monocytes to sites of inflammation, has been recognized as an inflammatory chemokine that is present during acute and chronic lung inflammation. In this study, similar to previously reported results in various acute or chronic lung diseases [6,41,42], serum MCP-1 and IL-8 expression was increased after MCMV infection; these levels were positively correlated with IL-17 levels. Moreover, the differences in IL-8 and MCP-1 expression in BAL fluid were more significant than in serum and both were positively correlated with IL-17 level. Although we did not specifically test for infiltration of inflammatory cells into the lungs, we suppose that local stimulation by IL-17-producing Th17 cells could directly or indirectly contribute to expression of these cytokines in lung tissues and to the progression of interstitial pneumonia through neutrophils and monocytes.

Several studies have shown that acute MCMV infection during the peri-transplant period can lead to allograft rejection [43], vasculopathy [44–46] and accelerated rejection [47,48]. In this study, the median survival of skin allografts in MCMV-infected mice was shorter than in control mice. Therefore, our results are consistent with those previously reported. Thus, acute MCMV infection after transplantation may accelerate skin allograft rejection, which may be induced by the Th17 response.

In summary, we have convincingly demonstrated that IL-17 is increased both in the BAL fluid and in the serum during post-transplant MCMV interstitial pneumonia and is correlated with the progression of pneumonia. IL-17 may play a major role in the development of MCMV interstitial pneumonia and merits further attention as to its role in disease progression. IL-17 may be an important signal of disease development and prognosis. However, the significance of IL-17 in MCMV interstitial pneumonia will require further investigation.

## Authorship

Z-JQ: designed and performed research, analyzed and interpreted data, wrote manuscript. QJ: helped in designing the study, performed research. Y-SC: analyzed histology and immunohistochemical staining of lungs and skin allograft. L-LS: helped in intracellular staining and analyzed flow cytometry analysis of Th1/Th2/Th17 subsets. ZW and N-DQ: extracted DNA and RNA of lung tissues and performed RT-PCR analysis. C-GD: analyzed data and wrote the manuscript. C-LZ: analyzed data, edited the manuscript.

## Funding

The authors have declared no funding.

## Acknowledgements

We gratefully acknowledge Prof. Wang Ming Li (AnHui Medical University) for the gift of MCMV and her assistance during viral infection. This work was supported by the Sun Yat-sen University 5010 project.

## References

1. Tornatore KM, Garey KW, Saigal N, *et al.* Ganciclovir pharmacokinetics and cytokine dynamics in renal transplant recipients with cytomegalovirus infection. *Clin Transplant* 2001; **15**: 297.
2. Farrugia E, Schwab TR. Management and prevention of cytomegalovirus infection after renal transplantation. *Mayo Clin Proc* 1992; **67**: 879.
3. Daniel V, Pasker S, Wiesel M, *et al.* Cytokine monitoring of infection and rejection in renal transplant recipients. *Transplant Proc* 1995; **27**: 884.
4. Nordøy I, Müller F, Nordal KP, *et al.* Chemokines and soluble adhesion molecules in renal transplant recipients with cytomegalovirus infection. *Clin Exp Immunol* 2000; **120**: 333.
5. Wu CA, Puddington L, Whiteley HE, *et al.* Murine cytomegalovirus infection alters Th1/Th2 cytokine expression, decreases airway eosinophilia, and enhances mucus production in allergic airway disease. *J Immunol* 2001; **167**: 2798.
6. Vanaudenaerde BM, Dupont LJ, Wuyts WA, *et al.* The role of interleukin-17 during acute rejection after lung transplantation. *Eur Respir J* 2006; **27**: 779.
7. Loong CC, Hsieh HG, Lui WY, *et al.* Evidence for the early involvement of interleukin17 in human and experimental renal allograft rejection. *J Pathol* 2002; **197**: 322.
8. Ye P, Rodriguez FH, Kanaly S, *et al.* Requirement of interleukin17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* 2001; **194**: 519.
9. Witowski J, Ksiazek K, Jörres A. Interleukin-17: a mediator of inflammatory responses. *Cell Mol Life Sci* 2004; **61**: 567.
10. Molesworth-Kenyon SJ, Yin R, Oakes JE, *et al.* IL-17 receptor signaling influences virus-induced corneal inflammation. *J Leukoc Biol* 2008; **83**: 401.
11. Hashimoto K, Durbin JE, Zhou W, *et al.* Respiratory syncytial virus infection in the absence of STAT 1 results in airway dysfunction, airway mucus, and augmented IL-17 levels. *J Allergy Clin Immunol* 2005; **116**: 550.
12. Dubin PJ, Kolls JK. IL-23 mediates inflammatory responses to mucoid *Pseudomonas aeruginosa* lung infection in mice. *Am J Physiol Lung Cell Mol Physiol* 2007; **292**: L519.
13. Mangan PR, Harrington LE, O'Quinn DB, *et al.* Transforming growth factor-β induces development of the T (H) 17 lineage. *Nature* 2006; **441**: 231.
14. Romani L, Fallarino F, De Luca A, *et al.* Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. *Nature* 2008; **451**: 211.
15. Palm NW, Medzhitov R. Antifungal defense turn Th17. *Nat Immunol* 2007; **8**: 549.
16. Arens R, Wang P, Sidney J, *et al.* Cutting edge: murine cytomegalovirus induces a polyfunctional CD4 T cell response. *J Immunol* 2008; **180**: 6472.
17. McFarland HI, Rosenberg AS. Skin allograft rejection. *Curr Protoc Immunol* 2009; **84**: 4.4.1.
18. Price P, Hopkins RM, Teo HK, *et al.* Modulation of immunocompetence by cyclosporin A, cyclophosphamide or protein malnutrition potentiates murine cytomegalovirus pneumonitis. *Pathol Res Pract* 1991; **187**: 993.
19. Hummel M, Zhang Z, Yan S, *et al.* Allergenic transplantation induces expression of cytomegalovirus immediate-early genes in vivo: a model for reactivation from latency. *J Virol* 2001; **75**: 4814.
20. Shanley JD, Pesanti EL, Nugent KM. The pathogenesis of pneumonitis due to murine cytomegalovirus. *J Infect Dis* 1982; **146**: 388.
21. Min SI, Ha J, Park CG, *et al.* Sequential evolution of IL-17 responses in the early period of allograft rejection. *Exp Mol Med* 2009; **41**: 707.
22. Yonemitsu J. Interleukin-4 induces mouse cytomegalovirus interstitial pneumonia in a latent infection model. *Kurume Med J* 2001; **48**: 49.
23. Orange JS, Biron CA. Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. *J Immunol* 1996; **156**: 4746.
24. Kwun J, Knechtle SJ, Hu H. Determination of the functional status of alloreactive T cells by interferon-[gamma] kinetics. *Transplantation* 2006; **81**: 590.
25. Harrington LE, Hatton RD, Mangan PR, *et al.* Interleukin 17-producing $CD4^+$ effector T cells develop via a lineage distinct from the T helper type 1and 2 lineages. *Nat Immunol* 2005; **6**: 1123.
26. Cho ML, Ju JH, Kim KW, *et al.* Cyclosporine A inhibits IL-15-induced IL-17 production in $CD4^+$ T cells via down-regulation of PI3K/Akt and NF-kappaB. *Immunol Lett* 2007; **108**: 88.
27. Zhang C, Zhang J, Yang B, *et al.* Cyclosporine A inhibits the production of IL-17 by memory Th17 cells from healthy individuals and patients with rheumatoid arthritis. *Cytokine* 2008; **42**: 345.
28. Liu X, Yang P, Lin X, *et al.* Inhibitory effect of Cyclosporin A and corticosteroids on the production of IFN-γ and IL-17 by T cells in Vogt–Koyanagi–Harada syndrome. *Clin Immunol.* 2009; **131**: 333.
29. Chi W, Yang P, Zhu X, *et al.* Production of interleukin-17 in Behcet's disease is inhibited by cyclosporin A. *Mol Vis* 2010; **16**: 880.
30. Cervera C, Filella X, Linares L, *et al.* Th1/Th2 cytokine release pattern during in vivo cytomegalovirus disease in

solid organ transplantation. *Transplant Proc* 2007; **39**: 2233.
31. Nakagiri T, Inoue M, Morii E, *et al.* Local IL-17 production and a decrease in peripheral blood regulatory T Cells in an animal model of bronchiolitis obliterans. *Transplantation* 2010; **89**: 1312.
32. Weaver CT, Harrington LE, Mangan PR, *et al.* Th17: an effector CD4T cell lineage with regulatory T cell ties. *Immunity* 2006; **24**: 677.
33. Nurieva R, Yang XO, Martinez G, *et al.* Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 2007; **448**: 480.
34. Chen Z, Laurence A, O'Shea JJ. Signal transduction pathways and transcriptional regulation in the control of Th17 differentiation. *Semin Immunol* 2007; **19**: 400.
35. Chen W, Jin W, Hardegen N, *et al.* Conversion of peripheral $CD4^+CD25^-$ naive T cells to $CD4^+CD25^+$ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003; **198**: 1875.
36. Atalar K, Afzali B, Lord G, *et al.* Relative roles of Th1 and Th17 effector cells in allograft rejection. *Curr Opin Organ Transplant* 2009; **14**: 23.
37. Aggarwal S, Ghilardi N, Xie MH, *et al.* Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem* 2003; **278**: 1910.
38. Reynaud-Gaubert M, Marin V, Thirion X, *et al.* Upregulation of chemokines in bronchoalveolar lavage fluid as apredictive marker of post-transplant airway obliteration. *J Heart Lung Transplant* 2002; **21**: 721.
39. Hoshino H, Lötvall J, Skoogh BE, *et al.* Neutrophil recruitment by interleukin-17 into rat airways in vivo. Role of tachykinins. *Am J Respir Crit Care Med* 1999; **159**: 1423.
40. Lindén A, Hoshino H, Laan M. Airway neutrophils and interleukin-17. *Eur Respir J* 2000; **15**: 973.
41. Nakano Y, Kasahara T, Mukaida N, *et al.* Protection against lethal bacterial infection in mice by monocyte-chemotactic and-activating factor. *Infect Immun* 1994; **62**: 377.
42. Winter C, Herbold W, Maus R, *et al.* Important role for CC chemokine ligand 2-dependent lung mononuclear phagocyte recruitment to inhibit sepsis in mice infected with Streptococcus pneumonia. *J Immunol* 2009; **182**: 4931.
43. Lemström K, Koskinen P, Krogerus L, *et al.* Cytomegalovirus antigen expression, endothelial cell proliferation, and intimal thickening in rat cardiac allografts after cytomegalovirus infection. *Circulation* 1995; **92**: 2594.
44. Lemström K, Sihvola R, Bruggeman C, *et al.* Cytomegalovirus infection-enhanced cardiac allograft vasculopathy is abolished by DHPG prophylaxis in the rat. *Circulation* 1997; **95**: 2614.
45. Orloff SL, Streblow DN, Soderberg-Naucler C, *et al.* Elimination of donor-specific alloreactivity prevents cytomegalovirus-accelerated chronic rejection in rat small bowel and heart transplants. *Transplantation* 2002; **73**: 679.
46. Streblow DN, Kreklywich C, Yin Q, *et al.* Cytomegalovirus-mediated upregulation of chemokine expression correlates with the acceleration of chronic rejection in rat heart transplants. *J Virol* 2003; **77**: 2182.
47. Carlquist JF, Shelby J, Shao YL, *et al.* Accelerated rejection of murine cardiac allografts by murine cytomegalovirus-infected recipients. Lack of heliotype specificity. *J Clin Invest* 1993; **91**: 2602.
48. Shao YL, Shelby J, Hisatake G, *et al.* Accelerated cardiac allograft rejection in murine cytomegalovirus infected C3H recipients. *Transplant Proc* 1991; **23**: 129.