in vivo *27*: 787-792 (2013)

IL-17E Production Is Elevated in the Lungs of Balb/c Mice in the Later Stages of *Chlamydia muridarum* Infection and Re-infection

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Abstract. *Background: Pathogens can influence allergic respiratory diseases. We previously found that multiple infections with Chlamydophila pneumoniae induce the production of interleukin-17A (IL-17A) and IL-17E, which have roles in the pathogenesis of asthma. The present work was designed to investigate our hypothesis that infections with another pathogen can induce the production of IL-17A and IL-17E. Materials and Methods: At an internal of 28 days, mice were infected twice with Chlamydia muridarum; the kinetics of IL-17A and IL-17E expression was subsequently determined at the mRNA and protein levels. The amounts of IL-17 cytokines produced by the stimulated spleen cells were determined by enzyme-linked immunosorbent assay (ELISA). The presence of IL-17E in the lungs was revealed by an indirect immunofluorescence test. Results: The infection with C. muridarum induced the production of IL-17A at the early stages of infection. The quantity of IL-17E was highest on days 28 and 56 after the first infection (28 days after the second infection). In the later stages of infection, IL-17E was produced by epithelial cells. The re-stimulated peripheral spleen cells produced IL-17A. Conclusion: Multiple infection with C. muridarum induces the production of a high amount of IL-17E, which plays an important part in the pathogenesis of allergic pulmonary diseases.*

Chlamydiae are medically important bacteria responsible for a wide range of human infections and diseases. These obligate intracellular pathogens have developed a unique developmental cycle involving a conversion between two distinct morphological forms. The infectious process begins with the attachment of the elementary bodies (EBs) to the host cells, inside which they develop into metabolically-active reticulate bodies, which replicate by binary fission (1). There are two important genera among the Chlamydiae: the Chlamydia and the Chlamydophila (2). *Chlamydia trachomatis* infects the mucosal epithelium of the eyes and the genital tract. *Chlamydia muridarum* is a mouse pathogenic strain commonly used to investigate human chlamydial infections (3). *Chlamydophila pneumoniae* belongs to the other genus. It causes approximately 10% of the cases of community-acquired pneumonia and 5% of those of bronchitis and sinusitis (4). *C. pneumoniae* has been identified in 5-25% of children with asthma exacerbation (5).

The discovery of T-helper 17 (Th17) cells constitutes one of the most important advances in T-cell immunology since the discovery of Th1 and Th2 cells (6). Th17 cells preferentially produce interleukin-17A (IL-17A), IL-17F, IL-21 and IL-22 (7), whereas Th1 and Th2 cells mainly produce interferon-γ (IFN-γ) and IL-4, respectively. Recent progress in studies of IL-17A and Th17 cells revealed important roles for IL-17A in the development of allergic and autoimmune diseases, and in protective mechanisms against bacterial and fungal infections, functions previously believed to be mediated by Th1 or Th2 cells (8).

Murine IL-17A is a 21-kDa glycoprotein of 147 amino acid residues that shares 63% amino acid identity with human IL-17A (155 amino acids). Five additional structurally-related cytokines were recently identified: IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25) and IL-17F, to form the IL-17 family (9, 10). IL-17A and IL-17F are highly homologous and bind to the same receptor. These two molecules are therefore likely to exhibit similar biological activities (11, 12). Indeed, both IL-17A and IL-17F are involved in the development of inflammation and the host defence against infection by inducing the expression of genes encoding pro-inflammatory cytokines (tumor necrosis factor, IL-1, IL-6, granulocyte colony-stimulating factor (G-CSF) and

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Key Words: Animal models, *Chlamydia muridarum*, IL-17A, IL-17E, allergic pulmonary diseases.

granulocyte macrophage colony-stimulating factor, chemokines [chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL5, IL-8, chemokine (C-C motif) ligand 2 (CCL2) and CCL7], antimicrobial peptides (defensins and S100 proteins) and matrix metalloproteinases (MMP1, MMP3 and MMP13) from fibroblasts, endothelial cells and epithelial cells. IL-17A also promotes CSF- and G-CSF-mediated granulopoiesis and recruits neutrophils to the inflammatory sites (8).

IL-17E enhances Th2 cell immune responses by inducing Th2 cell cytokines such as IL-4, IL-5 and IL-13 in auxiliary cells and induces IgE production and eosinophilia, contributing to the host defence against nematodes and allergic disorders (8). The heterodimer composed of IL-17RA and IL-17RB serves as the receptor for IL-17E (13). IL-17E is produced by Th2 cells, caecal patch $CD4^+$ and $CD8^+$ T-cells, mast cells and eosinophils (12). Alveolar macrophages and endothelial and epithelial cells may also produce IL-17E in rodents (8). The important role, played by IL-17E production in the pathogenesis of chronic inflammatory diseases is clearly different from those of IL-17A and IL-17F, which are involved in airway neutrophilia. In contrast with IL-17A, IL-17E activates macrophages and airway epithelial cells to secrete Th2 cell cytokines, resulting in airway eosinophilia (14).

In the present work, we demonstrated that *C. muridarum* infection and re-infection induce the expression of IL-17E at the mRNA and protein levels in the lungs of Balb/c mice in the later stages of infection. The production of IL-17E is compartmentalized, occurring in the epithelial cells around the bronchi.

Materials and Methods

Inoculum preparation. C. muridarum strain Nigg EBs (a kind gift from H.D. Caldwell, Hamilton, MT, USA) were purified from infected McCoy cells (ECAC, London, UK) by density-gradient centrifugation, as described previously (15), aliquoted and stored at –80˚C. A mock preparation was prepared from an uninfected McCoy cell monolayer, processed in the same way as the infected cells. The titre of the infectious EBs was determined by indirect immunofluorescence assay. Serial dilutions of the EB preparation were inoculated onto McCoy cells and, after 24-h culture, the cells were fixed with acetone and stained with monoclonal anti-Chlamydia lipopolysaccharide (LPS) antibody (AbD Serotec, Oxford, UK) and fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG (Sigma, St Louis, MO, USA). The number of *C. muridarum* inclusions was counted under a UV microscope, and the titre was expressed in inclusion forming unit/ml (IFU/ml).

Mice and infection conditions. Specific pathogen-free 6 to 8-week-old female Balb/c mice obtained from INNOVO Kft. (Budapest, Hungary) were maintained under standard husbandry conditions at the animal facility of the Department of Medical Microbiology and Immunobiology, University of Szeged, and were provided with food and water *ad libitum*. Before infection, the mice were mildly sedated with an intraperitoneal injection of 200 μl of sodium pentobarbital (7.5 mg/ml); they were then infected intranasally with 1×10^3 IFU of *C. muridarum* in sucrose–phosphate–glutamic acid (SPG) buffer; half of the mice were re-infected 28 days after the first infection. Seven mice at each time point were anaesthetized and sacrificed on each of days 1, 7, 14, 28, 29, 35, 42 and 56 after the first infection. Sera were taken by cardiac puncture. The lungs were removed and homogenized with acid-purified sea sand (Fluka Chemie AG, Buchs, Switzerland). One-half of the homogenized lungs was processed for quantitative reverse transcription polymerase chain reaction (RT qPCR), while the other half was suspended in 1 ml of SPG for the detection of viable *C. muridarum*. Lungs of three mice from each group were removed, frozen and kept at –80˚C for immunofluorescent staining. Spleens were dissected and homogenized by pressing through a nylon mesh into complete growth medium containing RPMI-1640 (Sigma), 10% foetal calf serum, 10 mM HEPES (Sigma), L-glutamine (0.3 mg/ml; Sigma), gentamycin (60 μg/ml, Sanofi Aventis, Budapest, Hungary) and 50 μM 2-mercaptoethanol (Sigma) for *in vitro* cytokine production testing. All experiments fully complied with the University of Szeged Guidelines for the Use of Laboratory Animals (III./42/2012).

Culturing of C. muridarum from the lungs. Lung homogenates from individual mice were centrifuged (10 min, 400 ×*g*), serial dilutions of the supernatants were inoculated onto McCoy cell monolayers and, after 24-h culture, the cells were fixed with acetone and stained with monoclonal anti-Chlamydia LPS antibody (AbD Serotec) and FITC-labelled anti-mouse IgG (Sigma). The number of *C. muridarum* inclusions was counted under a UV microscope.

Lymphocyte proliferation assay. Single-cell suspensions from spleens of seven infected, re-infected or uninfected mice were pooled and resuspended in the complete growth medium used during the homogenization of the spleen cells. The proliferative responses of 5×10^5 splenocytes in three parallel wells to purified heat-inactivated *C. muridarum* EBs, viable *C. muridarum* or the similarly treated mock preparation were examined after incubation for two days. Supernatants of the spleen cells were taken and frozen for further analysis.

Cytokine and chemokine measurements in the lungs and supernatants of the re-stimulated spleen cells. The supernatants of the lung homogenates and the supernatants of the re-stimulated spleen cells were centrifuged (5 min, 12,000 ×*g*) and assayed for the concentrations of IL-17A and IL-17E with different Ready-SET-Go! kits (eBioscience Inc., San Diego, CA, USA). The sensitivities of the IL-17A and IL-17E measurements were in the ranges 10.9-700 pg/ml and 31.2-2000 pg/ml, respectively. The clarified supernatants were tested in duplicate in accordance with the manufacturer's instructions.

mRNA extraction and RT qPCR. Total RNA was extracted from the lung suspensions by using the TRI Reagent (Sigma). During purification, all samples were treated with DNase 1, Amplification Grade (Sigma) to remove genomic DNA contamination. The RNA was quantified by spectrophotometric analysis, and the RNA integrity was confirmed by agarose gel electrophoresis. First-strand cDNA was synthesized by using 2 μg of total RNA with Superscript III (Invitrogen, Carlsbad, CA, USA) and 20 pmol of random hexamer primers in 20 μl of reaction buffer. The cDNA product was diluted 1/30, and the qPCR was conducted with the diluted cDNA, primers (10 pmol/μl) and SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma) in a total volume of 20 μl, with a Roche

LightCycler® 2.0 Instrument. Thermal cycling was initiated with a denaturation step of 10 min at 95˚C, followed by 40 cycles each of 5 s at 95˚C, 20 s at 60˚C and 25 s at 72˚C. Dissociation curves were recorded after each run to ensure primer specificity. The different mouse IL-17-specific primers were as follows: *IL-17A* sense 5'-AAG GCA GCA GCG ATC ATC C-3', antisense 5'-GGA ACG GTT GAG GTA GTC TGA G-3'; *IL-17E* (IL-25) sense 5'-CAG GTG TAC CAT CAC CTT GCC AAT -3', antisense 5'-ACA ACA GCA TCC TCT AGC AGC ACA-3'; and for β-actin: sense 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3', antisense 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'. All primers were synthesized by Integrated DNA Technologies Inc. (Montreal, Quebec, Canada). Cycle threshold (C_t) values were determined by automated threshold analysis with Roche Molecular Biochemicals LightCycler Software version 3.5 (Roche Applied Science, Penzberg, Germany). The lowest cycle number at which the transcripts were detectable, referred to as C_t, was compared with that of β-actin, the difference being referred to as ΔCt. The relative expression level was given as $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta C_t = \Delta C_t$ for the experimental sample minus ΔC_t for the control sample. We defined a threshold value, *i.e.* increases greater than 2-fold in the amount of transcript relative to the control samples were considered significant. Uninfected mice served as controls.

Lung histopathology. Four weeks after infection mice were anaesthetized and then sacrificed by exsanguination through cardiac puncture. The lungs were removed *in toto* and immersed in frozen tissue matrix, OCT (Sakura Finetek Europe, Alphen aan den Rijn, the Netherlands). For the detection of IL-17E antigen by immunofluorescence test, lungs were cut into 5-μm sections. The sections were stained with IL-17E antibody (Acris Antibodies GmbH, Herford, Germany) as primary antibody for 45 min at room temperature, followed by staining for 30 min with FITC-labelled anti-mouse IgG antibody (Sigma). Uninfected mice served as controls.

Statistical analysis. Statistical analysis of the data was carried out with SigmaPlot for Windows Version 11.0 software, using the two-tailed unpaired Student's *t*-test. Differences were considered statistically significant at $p<0.05$.

Results

C. muridarum infection and re-infection induce the expression of IL-17A and IL-17E mRNA in the lungs of mice. To investigate the production of IL-17A and IL-17E cytokines during *C. muridarum* infection, Balb/c mice were infected intranasally with *C. muridarum*, and subsequently re-infected on day 28 after the first infection. On days 1, 7, 14, 28, 29, 35, 42 and 56 after the first infection, groups of seven mice were sacrificed and their lungs were collected for the determination of *C. muridarum* titres, mRNA levels and protein contents of the IL-17A and IL-17E cytokines in individual lungs. The recoverable *C. muridarum* was determined by indirect immunofluorescence assay. The infectious *C. muridarum* titre in the mice increased to 8.25×10^3 IFU/lung by day 1, peaked at 5.23×10^5 IFU/lung on day 7, and then decreased to 3×10^1 IFU/lung by day 28 after infection. On day 29, one day after the re-infection the bacterial titre was 5.25×10^3 IFU/lung but at later time points, viable *C. muridarum* was not detected in the lungs of the re-infected mice (Figure 1).

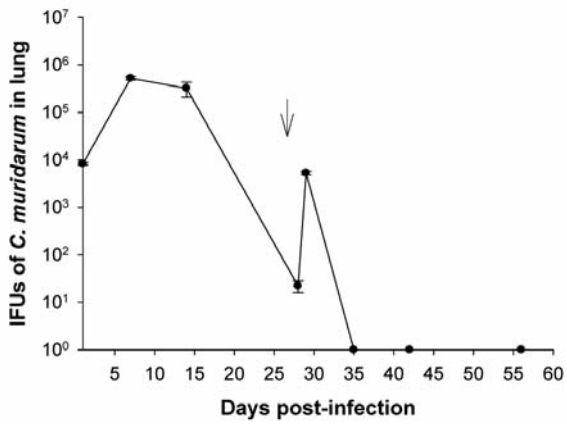

Figure 1. *Chlamydia muridarum inclusion forming unit (IFU) levels in the lungs of C. muridarum-infected and re-infected mice. Lung homogenates were inoculated onto McCoy cell monolayers, and chlamydial inclusions were detected by indirect immunofluorescence, with the use of lipopolysaccharide (LPS)-specific monoclonal and fluorescein isothiocyanate (FITC)-labelled secondary antibodies. The data are means±SD of C. muridarum titres (IFU/lung) of the lung homogenates of seven individual mice at each time point. ↓ Denotes the time of re-infection.*

The expression of *IL-17A* mRNA was highest (20-fold) on day 7, and then decreased continuously. The expression of *IL-17A* mRNA after re-infection displayed similar kinetics as after the primary infection, but the fold increases in transcripts were higher: 30-, 90- and 40-fold on day 29, 35 and 42, respectively (Figure 2a). The kinetics of IL-17A protein production correlated with the mRNA expression: the production increased from day 1, with the highest concentration observed on day 7 (Figure 2b). Unlike that of *IL-17A* mRNA, the expression of *IL-17E* mRNA did not demonstrate a parallel with the bacterial burden in the lungs of the mice. The expression started to increase on day 7, and the highest level (860-fold) was detected on day 28 after the first infection. On day 29 (one day after re-infection), the expression of *IL-17E* mRNA decreased dramatically, but after that it increased again and was highest (1600-fold) 28 days after re-infection, when the experiment was terminated (Figure 2a). The kinetics of IL-17E protein production was similar to that of the expression of *IL-17E* mRNA in the lungs of the infected and the re-infected mice (Figure 2b).

The production of IL-17E is compartmentalized. IL-17E is known to be produced by different cell types, *e.g.* T-lymphocytes and epithelial cells (16). However, it was not clear which cells are responsible for the production of IL-17E after *C. muridarum* infection. To investigate whether there is

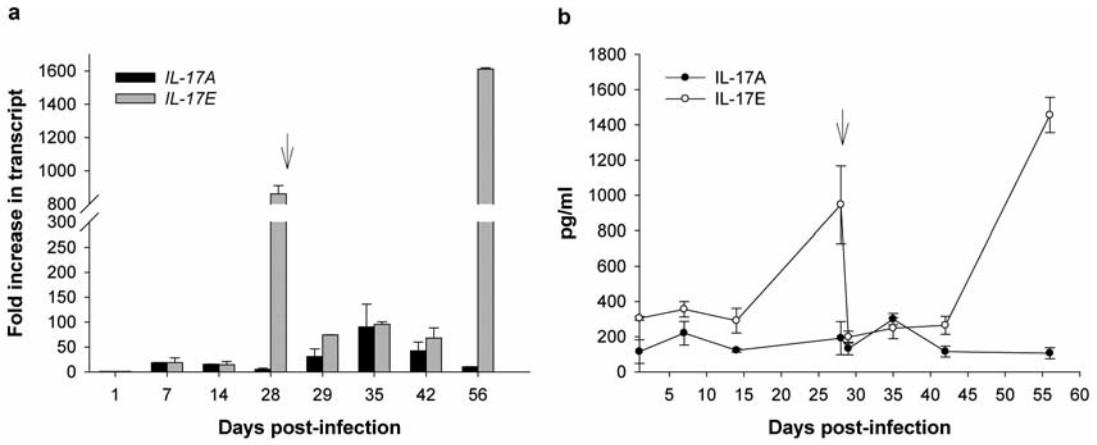

Figure 2. *a: Expression of interleukin-17A (IL-17A) and IL-17E mRNA in lung suspensions from Chlamydia muridarum-infected and re-infected mice. The total RNA extracted from the lungs was analyzed by quantitative reverse transcription polymerase chain reaction (RT qPCR) with the use of specific primers. Data are normalized for β-actin mRNA content and plotted as fold change of the results for the control mice. Uninfected mice served as controls. Bars denote means±SD of the results of seven mouse lungs. ↓ Denotes the time of re-infection. b: IL-17A/E protein production in the lungs of mice infected and re-infected with C. muridarum. Lung homogenates were tested by using an IL-17A and IL-17E ELISA kit according to the manufacturer's instructions. The data are means±SD of the results of seven mouse lungs. ↓ Denotes the time of re-infection.*

an increase in IL-17E level at the periphery, a lymphocyte proliferation assay was carried out. Spleen cells from mice which had been infected once or twice and killed two or four weeks after infection and re-infection, were re-stimulated with a mock preparation, or with live or heat-inactivated *C. muridarum*, or left unstimulated. Spleen cells from uninfected mice served as controls. The IL-17A and IL-17E cytokines were measured by ELISA.

There was no detectable IL-17E production in the supernatants of the re-stimulated or unstimulated spleen cells collected from *C. muridarum*-infected and uninfected mice. The quantity of IL-17A was also not increased in the supernatants of the spleen cells isolated from uninfected mice or of the spleen cells left unstimulated (data not shown). In contrast, the spleen cells from infected or re-infected mice produced IL-17A after *in vitro* re-stimulation with viable or heat-inactivated *C. muridarum*. There were no significant differences in IL-17A production between the spleen cells re-stimulated with viable or heat-inactivated *C. muridarum*. The quantity of the IL-17A cytokine produced by the spleen cells was lower in the mice which had been infected *in vivo* with *C. muridarum* only once, independently of the stimulation conditions (Figure 3).

To investigate IL-17E-producing cells further, the lungs of infected, re-infected and uninfected mice were sectioned and stained with monoclonal antibody against IL-17E as primary, and FITC-labelled anti-mouse IgG as a secondary antibody. No fluorescence was seen in the lung sections of the uninfected mice (Figure 4a). The production of IL-17E was observed in the lungs of the infected and re-infected mice four weeks after infection. The IL-17E-positive cells were situated especially among the epithelial cells of the bronchi,

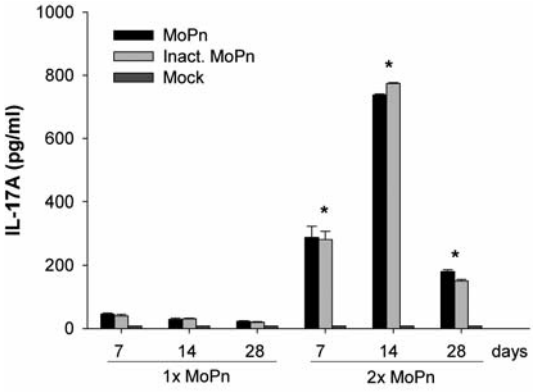

Figure 3. *The interleukin-17A (IL-17A) production of the spleen cells isolated from infected (1× MoPn) and re-infected (2× MoPn) mice. The IL-17A production of pooled spleen cells from seven mice was tested after in vitro re-stimulation with viable C. muridarum (MoPn), heat-inactivated C. muridarum (inact. MoPn) or a mock preparation by ELISA. Bars indicate means±SD of the results on the spleen cells of seven mice. *p<0.05 compared with the spleen cells isolated from once-infected mice killed at similar time points.*

and only a few positive cells were found in the interstitium of the lungs (Figure 4b and c).

Discussion

IL-17 cytokines can aggravate the pathogenesis of autoimmune diseases, but they have a beneficial role during infection caused by different pathogens (11). The role of IL-17E in respiratory diseases, and particularly in re-infections,

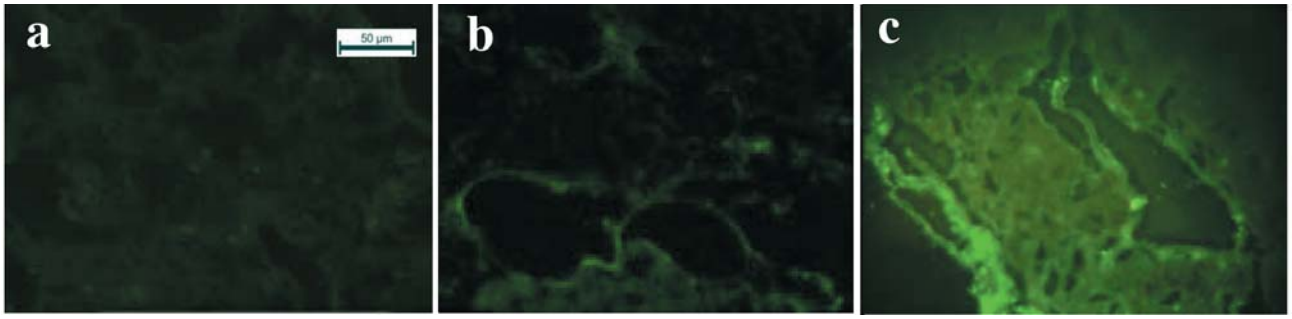

Figure 4. *The production of interleukin 17E (IL-17E) in the lungs of uninfected (a), Chlamydia muridarum-infected (b) and re-infected mice (c). The lung sections were stained with IL-17E-specific monoclonal antibody and with fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG.*

has not been elucidated. The role of pathogens in the development and reactivations of asthma is an exciting field, but the exact pathomechanism is not known.

We recently reported that the re-infection of mice with *C. pneumoniae* can induce a high level of IL-17E four weeks after infection, which indicates that IL-17E induced by re-infection with *C. pneumoniae* possibly influences allergic/asthmatic diseases (17).

In the present article, we wished to demonstrate that infection with another pathogen can also induce the production of IL-17E, which plays an important part in allergic asthma by promoting the release of Th2 cytokines such as IL-4, IL-5 and IL-13. We examined another member of the Chlamydiaceae family, *C. muridarum*, which belongs not in the Chlamydophila, but in the Chlamydia genus. The kinetics of *IL-17A* mRNA expression in our experiment was similar to that observed earlier after a single infection with *C. muridarum* (18).

Besides their roles in inflammatory lung diseases, IL-17A-producing $CD4^+$ T-cells are believed to be necessary for protective immunity against airborne pathogens (19). For instance, IL-17A-producing cells play a critical role in protective immune responses to mucocutaneous candidiasis, mainly through the recruitment of IFN-γ-producing $CD4^+$ T-cells (20). γδ T-cells have been reported to be the initial source of IL-17A during infection with *Mycobacterium tuberculosis* or *Mycobacterium bovis* (21). IL-17A induction of neutrophil activation and migration is important in the defence against a variety of microorganisms that infect the lung (22). Both IL-23 p19-deficient and IL-17R-deficient mice were unable to develop a full neutrophil response to *Klebsiella pneumoniae* infection in the airways, and hence were more susceptible to lung infection compared to their normal counterparts (23). Taken together, these findings suggest that IL-17A-producing cells are critical for normal immunity against microbial infections of the airways. Moreover, the significance of IL-17A in the immune response is demonstrated by the production of a higher quantity of IL-17A by peripheral cells of mice infected twice with pathogens, which suggest that re-infection with the same pathogen results in a higher number of IL-17A-producing cells in the spleen (Figure 3).

Concordant with our earlier results, the quantity of IL-17E increased four weeks after both the first and second infections with *C. muridarum* at the mRNA and protein levels. This is very interesting because by that time after the second infection there were extremely few pathogens if any at all in the lungs. Our earlier results had revealed that viable pathogen is needed for the expression of *IL-17E* mRNA, because there was no increase in *IL-17E* mRNA expression after treatment or re-treatment with heat-inactivated *C. pneumoniae* (17).

It is well-characterized that Chlamydia infections are followed by significant pathogenic changes, mediated by the cellular and soluble component of the inflammation. Re-infections with *C. trachomatis* elicit some degree of protective immunity in mice, but the limited growth of Chlamydia induces severe inflammation, which may lead to irreversible tissue damage (24). Furthermore, a primary *C. pneumoniae* infection proved to confer a partial resistance to re-infection in a mouse model, but provided no protection against inflammatory changes (25). In accordance with these findings, our results suggest that the synthesis and release of chlamydial antigens from mucosal epithelial cells or alveolar macrophages repeatedly infected with Chlamydia may provide prolonged antigenic stimulation, which strongly amplifies chronic inflammation. It is noteworthy that the re-infection of mice with *C. muridarum* resulted in acutely decreased levels of expression and production of IL-17E (Figure 2a and b). We speculate that the strong Th1 cytokine IFN-γ can inhibit the expression of IL-17E during the early stages of *C. muridarum* infection.

We found that the peripheral cells of the infected mice are not able to produce IL-17E, in contrast to IL-17A, which means that IL-17E production is compartmentalized and the IL-17E-producing cells must be in the lung. Use of the adoptive transfer of IL-17E-deficient cells clearly showed that

the production of IL-17E by T-cells, mast cells and other haematopoietic immune cells of stem cell origin, was not essential for the development of Th2-type/eosinophilic airway inflammation, suggesting that the IL-17E produced by non-immune cells such as airway epithelial cells, is crucial for its development (16).

In summary, re-infection with *C. muridarum* led to a substantial expression of *IL-17E* mRNA in the later stages of the infection. After infection and re-infection with *C. muridarum*, the epithelial cells of the lung are responsible for the production of IL-17E.

Acknowledgements

We thank Müllerné Deák Györgyi for excellent technical support. This work was supported by OTKA National Research Fund Grant PD 100442, and Grants TÁMOP-4.2.2.A-11/1/-2012-0035 and TÁMOP-4.2.2.B-10/1-2010-12.

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Received July 26, 2013
Revised October 11, 2013
Accepted October 14, 2013