Analysis of the expressions of different interleukin-17 types in *Chlamydophila pneumoniae*-infected mice

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Abstract

Objective Investigation of the effects of different interleukin (IL)-17 cytokines in *Chlamydophila pneumoniae*-infected mice.

Methods BALB/c mice were infected with *C. pneumoniae* either once or 3 times at 4-week intervals and the gene expressions of the IL-17 cytokines were followed by quantitative reverse transcription PCR from day 1 to day 28 after infection and re-infection. After the treatment of mice with anti-IL-17A for the *in vivo* neutralization of IL-17A, ELISA was used to detect the differences in cytokine and chemokine production. The number and phenotype of the IL-17A-producing T cells were determined by ELISPOT.

Results C. pneumoniae induced high quantities of IL-17A and IL-17F at the mRNA level from the first day after infection, and their levels remained elevated on day 28. The expressions of IL-17C, IL-17D and IL-17E did not change significantly in response to a single infection. The *in vivo* neutralization of IL-17A resulted in a higher *C. pneumoniae* burden in the lungs of the mice, a decreased cell influx, and diminished chemokine levels. The phenotype of the IL-17A-producing T cells in the periphery was CD4$^+$. The re-infection of mice with *C. pneumoniae* led to an increased IL-17E expression.

Conclusion These results facilitate an understanding of the early inflammatory response after *C. pneumoniae* infection and suggest that *C. pneumoniae* re-infection induces the production of a high amount of IL-17E, which has an important role in the pathogenesis of allergic pulmonary diseases.

Keywords Animal models, *Chlamydophila pneumoniae,* IL-17

Introduction

The spectrum of illnesses caused by *Chlamydophila pneumoniae (C. pneumoniae)* ranges from severe community-acquired pneumonia to bronchitis, pharyngitis, laryngitis or sinusitis [1]. Chlamydial infections display high rates of recurrence [2], and a number of investigators have presented evidence suggesting a role of *C. pneumoniae* in chronic diseases such as atherosclerosis, asthma and reactive arthritis [3].

It has been demonstrated that *C. pneumoniae* infection induces the release of pro-inflammatory cytokines relevant to the exacerbation of chronic pulmonary diseases such as asthma and chronic obstructive pulmonary disease [4]. Moreover, in the host chlamydiae can achieve a state of latency in which they are viable, but dormant and do not multiply [5]. In this special state, the 60 kDa heat shock protein is expressed [6], a protein that is able to elicit a strong inflammatory response and appears to be involved in tissue injury and scarring processes.

Glucocorticoids are administered in the treatment of the inflammation that is present in almost all asthma patients. However, corticosteroids can negatively affect many aspects of cell-mediated immunity and favour the shift from a Th1-type response towards a Th2-type response. Corticosteroids are double-edged weapons: they can decrease the inflammation in the host, but they can also diminish the ability of the host to eradicate intracellular pathogens such as *C. pneumoniae*. Furthermore, they are able to reactivate dormant *C. pneumoniae* to an active growth phase [7] which, by enhancing the production of pro-inflammatory cytokines, can amplify the inflammatory processes in the respiratory tract of patients with asthma [8]. Previous studies have demonstrated that *C. pneumoniae* infections are characterized by lung neutrophilic inflammation and lung lymphocyte infiltration [9]. However, the mechanisms by which *C. pneumoniae* induces the influx of CD4$^+$ T cells and neutrophil granulocytes to the

lung tissues remain poorly understood.

The IL-17 cytokine family consists of six structurally related proteins (IL-17A, B, C, D, E and F). A well-characterized molecule of this family, IL-17A, is expressed primarily by Th17 cells (a subset of $CD4^+$ T cells) and signals through a receptor complex that contains IL-17RA and IL-17RC. The IL-17A signal increases matrix metalloproteinase and pro-inflammatory cytokine expressions. IL-17A also acts to recruit neutrophils to peripheral sites through the induction of chemokines and granulocyte-colony stimulating factor [10]. The expression of IL-17A is enhanced in several pulmonary diseases in which neutrophils are present, including severe asthma, chronic obstructive pulmonary disease and cystic fibrosis [11]. Administration of IL-17A into the airways induces significant increases in the levels of neutrophils associated with enhanced keratinocyte-derived chemokine (KC/CXCL1), macrophage inflammatory protein-2 (MIP-2/CXCL2) and lipopolysaccharide (LPS)-induced C-X-C chemokine (LIX) expression. In a model of LPS-driven airway inflammation, the neutralization of IL-17A significantly reduces the neutrophil numbers [12]. These data point to an important role of IL-17A in the regulation of airway inflammation and neutrophil recruitment.

IL-17E, also known as IL-25, has been implicated in Th2 cell-mediated immunity [13]. IL-17E binds to IL-17RB; the transcripts of this receptor are abundantly expressed in the liver and kidney, and at lower levels in the brain, testes and small intestine [14]. Mice deficient in IL-17E exhibited a reduced level of Th2 cytokine production, resulting in poor resistance to *Trichuris* infection [15]. Conversely, the overexpression of IL-17E or the administration of recombinant cytokine strongly induced Th2 cytokine expression and IgE production *in vivo* and in allergic pathological conditions [16].

The roles and the kinetics of different IL-17 types have not been investigated in an animal model after *C. pneumoniae* infection. Zhou et al. recently demonstrated that IL-17A plays a critical role in the regulation of the host susceptibility to *Chlamydia muridarum* infection in

mice; and the skewed IL-17/Th17 profile in C3H/HeN mice was predisposed by a higher basal level of IL-17RC expression and then further amplified by a higher inducible IL-17RA expression in the lungs [17]. Moreover, *C. muridarum* infection during sensitization enhances the subsequent neutrophilic inflammation and Th1/Th17 responses during allergen exposure [18].

In the study reported here, we investigated the kinetics and roles of IL-17 cytokines after *C. pneumoniae* infection in mice. We found that *in vivo* neutralization of IL-17A resulted in a higher *C. pneumoniae* burden and decreased chemokine levels in the lungs of mice. The phenotype of the IL-17A-producing cells in the spleen proved to be CD4^{+}. Re-infection of the mice induced an increased IL-17E expression.

Materials and methods

Inoculum preparation and culturing of *C. pneumoniae* from the lungs

C. pneumoniae CWL029 (ATCC) was propagated on HEp-2 cells, as described earlier [19]. The partially purified and concentrated elementary bodies (EBs) were aliquoted and stored at -80 °C until use. A mock preparation was prepared from an uninfected HEp-2 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 HEp-2 monolayers and, after a 48-h culture, cells were fixed with acetone and stained with monoclonal anti-*Chlamydia* LPS antibody (AbD Serotec, Oxford, UK) and FITC-labelled anti-mouse IgG (Sigma, Saint Louis, Missouri, USA). The number of *C. pneumoniae* inclusions was counted under a UV microscope, and the titre was expressed in inclusion forming units/ml (IFU/ml). Lung homogenates from each mouse were centrifuged

(10 min, 400g), serial dilutions of the supernatants were inoculated onto HEp-2 cell monolayers, and the titre of *C. pneumoniae* was determined as described previously for the titration of the EB preparation.

Mice and infection conditions

Eight-week-old female BALB/c mice were obtained from the Charles River Laboratory (Germany). The mice were fed a regular mouse chow diet *ad libitum* and housed under biosafety level 2 conditions. Before infection, the mice were mildly sedated with an intraperitoneal (i.p.) injection of 200 μl sodium pentobarbital (7.5 mg/ml); they were then infected intranasally with 5×10^5 IFU *C. pneumoniae* in 25 μl of sucrose-phosphate-glutamic acid (SPG) buffer. Uninfected mice were used as controls. After inoculation, mice were anaesthetized and sacrificed on days 1, 2, 4, 7, 14 and 28, 7 animals at each time point. Sera were taken by cardiac puncture. The lungs were removed and homogenized mechanically. One half of the homogenized lungs was processed for quantitative reverse transcription PCR (RT qPCR), while the other half was suspended in 1 ml of SPG buffer for the detection of viable *C. pneumoniae*, and for cytokine and chemokine measurements. Spleens were destroyed with a cell strainer, and the spleen cells were kept in foetal bovine serum medium containing dimethyl sulfoxide at -80 °C until use.

In a separate experiment, BALB/c mice were infected intranasally with *C. pneumoniae* 3 times at 4-week intervals. Groups of 7 mice were sacrificed at 2 or 4 weeks after each infection and the lungs were processed as mentioned above.

All experiments complied fully with the 'University of Szeged Guidelines for the Use of Laboratory Animals'.

In vivo neutralization of IL-17A in mice

Groups of 7 female BALB/c mice were treated i.p. with 100 µg/mouse of either anti-IL-17A (MAB421, R&D Systems Europe, Ltd., Abingdon, UK) or an isotype control antibody (R&D Systems) 24 h before and 1 and 2 days after *C. pneumoniae* infection. The mice were sacrificed on day 1 or day 4 and processed in the same way as mentioned above.

Bronchoalveolar lavage (BAL) collection

The lungs were lavaged with 1 ml phosphate-buffered saline. 50 µl of a 5 x 10^5 cells/ml cell suspension was placed into a chamber which was attached to cytospin slides, and then centrifuged at 800 rpm for 3 min. The cells were examined morphologically and counted after staining with May-Grünwald-Giemsa solution.

ELISPOT assays

ELISPOT assays were performed to determine the number and phenotype of the IL-17A-producing spleen cells isolated from mice 2 weeks after *C. pneumoniae* infection, uninfected mice serving as controls. Splenocytes were re-stimulated *in vitro* with *C. pneumoniae* at a multiplicity of infection of 0.2, or with an equivalent amount of HEp-2 mock preparation. To determine the phenotype of the IL-17A-producing cells, the spleen cell suspensions were depleted of $CD4^+$ and $CD8^+$ cells, respectively, by using micro-beads coated with the respective antibody [CD4 (L3T4) or CD8a (Ly-2), Miltenyi Biotec, Bergisch Gladbach, Germany] and applying the magnetic cell sorting system of Miltenyi Biotec. The outcome of the procedure was controlled by flow cytometry after direct staining of the depleted cells with

α-CD4-TC and α-CD8-rPE antibodies (Caltag Laboratories, Burlingame, CA, USA). The IL-17A ELISpot kit (R&D Systems) was used during the experiments. Stimulated spleen cells (5×10^5) were distributed into each well (coated with anti-IL17A antibody) in triplicate, and incubated. After 24 h, the plates were washed, and biotinylated detection antibody, HRP-streptavidin, and substrate solution were added as recommended by the protocol. The mean number of spots counted in triplicate wells under a dissecting microscope was used to calculate the number of spot-forming cells (SFCs) per 1 million spleen cells.

Cytokine and chemokine measurements in the lungs

The supernatants of the lung homogenates and the sera were centrifuged (5 min, 12 000g) and assayed for the concentrations of IL-17A, KC, LIX and MIP-2 with different Quantikine$^®$ mouse chemokine/cytokine kits (R&D Systems). The sensitivities of the IL-17A, KC, LIX and MIP-2 measurements were in the ranges 10.9-700 pg/ml, 15.6-1000 pg/ml, 15.6-1000 pg/ml and 7.8-500 pg/ml, respectively. The clarified supernatants and sera 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 random hexamer primer 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 LightCycler® 2.0 Instrument (Roche Applied Science). Thermal cycling was initiated with a denaturation step of 10 min at 95 °C, followed by 40 cycles each of 5 sec at 95 °C, 20 sec at 60 °C and 25 sec 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', IL-17A antisense 5'- GGA ACG GTT GAG GTA GTC TGA G -3'; IL-17C sense 5'- TGC TGA GGA ATT ATC TCA CGG CCA-3'. IL-17C antisense 5'- ACT GTG TTC AGC TA GAG GTC CTT -3'; IL-17D sense 5'- CAA GCA CAT CAC ACA CAT CCC GTT -3', IL-17D antisense 5'- TTA GTA AGC TTG GGC CAC AGG AGA -3'; IL-17E (IL-25) sense 5'- CAG GTG TAC CAT CAC CTT GCC AAT -3', IL17-E (IL-25) antisense 5'- ACA ACA GCA TCC TCT AGC AGC ACA -3'; IL-17F sense 5'- AGC AAG AAA TCC TGG TCC TTC GGA -3', IL-17F antisense 5'- CTT GAC ACA GGT GCA GCC AAC TTT -3'. The primers used for IL-23 p19 cDNA amplification were: sense 5'- CCT GCT TGA CTC TGA CAT CTT C -3', IL-23 p19 antisense 5'- TGG GCA TCT GTT GGG TCT C -3', and for β-actin: sense 5'- TGG AAT CCT GTG GCA TCC ATG AAA C -3', β-actin 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. The lowest cycle number at which the various transcripts were detectable, referred to as C_t, was compared with that of β-actin, the difference being referred to as ΔC_t. 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.

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. pneumoniae infection induces the expressions of IL-17A and IL-17F in the lungs of BALB/c mice

To investigate the production of different IL-17 types during _C. pneumoniae_ infection, BALB/c mice were inoculated intranasally with _C. pneumoniae_. On days 1, 2, 4, 7, 14 or 28 after infection, mice were sacrificed and their lungs were collected for the determination of _C. pneumoniae_ titres, the mRNA levels of the different IL-17 types and IL-23, and the IL-17A protein content in the individual lungs.

In previous studies, culturable _C. pneumoniae_ was demonstrated until 14 days after primary infection in BALB/c mouse lungs [20]. In our present experiment, the infectious bacterial titres were below the level of detectability at 24 h after infection, but had increased to 5.21×10^4 IFU/lung by day 2. The peak titre of _C. pneumoniae_ was observed on day 7, at 1.8×10^6 IFU/lung, while on day 14 after infection the titre had decreased to 6.8×10^3 IFU/lung (Fig. 1a).

The expression of IL-17A mRNA was increased as early as on day 1, but the highest level of expression (26.1-fold) was detected on day 7 after infection. It then decreased continuously, but the expression level was still rather high relative to the control on day 28 (8.57-fold) (Fig. 1b). The expression of IL-17F was highest on day 4 (16.25-fold) and then decreased rapidly

during the observed period. The expressions of IL-17C, IL-17D and IL-17E mRNA did not change during the course of the *C. pneumoniae* infection. The mRNA expression of IL-23, which is the main inducer of IL-17A production, was observed on the first day, and peaked on day 2 at 62-fold (Fig. 1c). The kinetics of IL-17A protein production correlated with the mRNA expression: it increased from day 1, with the highest concentration on day 7 (Fig. 1d).

The phenotype of the peripheral IL-17A-producing cells

In order to define the phenotype and the number of peripheral T cells which release IL-17A, the ELISPOT assay was carried out with the spleen cells of *C. pneumoniae*-infected mice after the depletion of CD4$^+$ or CD8$^+$ cells. Spleen cells of uninfected mice served as controls. The *C. pneumoniae* infection caused a significant increase in the number of IL-17A-producing T cells after *in vitro* re-stimulation of the splenocytes with *C. pneumoniae* for 24 h, i.e. 150 SFCs per million, as compared with the spleen cells of uninfected mice, where the number of IL-17A-producing cells after *in vitro C. pneumoniae* stimulation was only 2 SFCs per million (data not shown). The depletion of CD8$^+$ cells did not result in a significant reduction in the number of IL-17A-producing cells, whereas the depletion of CD4$^+$ cells resulted in a major reduction in the number of SFCs in *in vitro* re-stimulated spleen cells isolated from *C. pneumoniae*-infected mice (Fig. 2).

Effects of *in vivo* IL-17A neutralization

To investigate the role of IL-17A in acute *C. pneumoniae* infection, mice were pretreated 24 h before infection and at 24 and 48 h post-infection with anti-IL-17A monoclonal antibodies or with isotype control antibodies. Mice were sacrificed on day 1 or 4

after infection with *C. pneumoniae*. From the BAL of the mice, the numbers of lymphocytes and granulocytes were determined. From the homogenized lungs, *C. pneumoniae* was cultured and the levels of inflammatory chemokines were determined by sandwich ELISA.

The *in vivo* anti-IL-17A treatment led to a diminished IL-17A content in the BAL as compared with that in the isotype antibody-treated control mice (17.2 versus 64.8 pg/ml). The numbers of neutrophil cells in the anti-IL-17A-treated group on days 1 and 4 were 2.2×10^3 and 1.5×10^3, respectively, while those for the isotype-antibody-treated control were 2.12×10^5 and 4.51×10^5 cells (data not shown).

No *C. pneumoniae* was detected on day 1, because of the long life-cycle (48 h) of the pathogen. Surprisingly, the number of viable chlamydiae in the lung suspension of the anti-IL-17A-treated group on day 4 (2.06×10^5 IFU) was significantly higher than the *C. pneumoniae* content of the lungs of the isotype antibody-treated mice (6.6×10^4) (Fig. 3a).

The quantity of LIX protein in the lung suspension of the IL-17A-neutralized mice on day 1 was significantly lower than the control level (Fig. 3b). The levels of KC and MIP-2 on day 4 were significantly lower than the chemokine levels in the lungs of the isotype antibody-treated control mice (Fig. 3c).

The effects of *C. pneumoniae* re-infection on the expressions of the different types of IL-17

It is known that re-infections with various infectious agents can play an important part in the pathogenesis of asthmatic disease [21]. It has also been described that IL-17E can promote the initiation of pro-allergic type 2 responses [22]. To study the expression of IL-

17E, BALB/c mice were infected with *C. pneumoniae* 3 times at 4-week intervals, and were sacrificed at 14 or 28 days after each inoculation.

After the first infection, the number of the recoverable *C. pneumoniae* was similar to that in our previous experiment. After the second and third inoculations, *C. pneumoniae* was not detected in the lungs of the mice at the examined time points (data not shown). The expression of IL-17E was not increased after a single inoculation (Fig. 1b), but after the second and third infections its expression increased dramatically. The level of expression of IL-17E mRNA 4 weeks after the third *C. pneumoniae* infection was still 400 times higher than the control. The expression of IL-17A mRNA was also increased, but not so markedly, in spite of the absence of viable *Chlamydia* in the lungs of the mice (Fig. 4).

Discussion

In contrast with the pathogenetic roles of IL-17 cytokines in autoimmune diseases, IL-17 cytokines protect the host from pathogens at the mucosal and epithelial tissues, including the lungs, intestines and skin [23]. We have demonstrated here that *C. pneumoniae* induced the expressions of IL-17A and IL-17F mRNA in mice, with peak levels on day 7 and day 4, respectively, whereas the mRNA expression of IL-17C, IL-17D and IL-17E did not change after a single infection with the pathogen. It was recently reported that *C. muridarum,* a close relative of the *Chlamydophila* genus, induces IL-17A production in mice, but the kinetics of IL-17A mRNA expression in that work differed from our findings [17]; moreover, a higher infectious dose and a different mouse strain were used in our experiments.

The involvement of IL-17A in protective immunity against intracellular pathogens such as *C. pneumoniae* is rather controversial. In the event of mycobacterial infection, IL-17A exerts an impact in inflammation and the formation of granulomas, but it is not required for overall

protection [24, 25]. Differences in the role of IL-17A have been observed for different *Salmonella* species: IL-17A is not required for the protection of *S. enterica*, but the depletion of Th17 cells in the intestines dramatically increases the frequency of bacteraemia in the case of *S. typhimurium* in monkeys infected with simian immunodeficiency virus [26, 27]. IL-17A is important in protective immunity at an early stage of listerial infection in the liver because IL-17A-deficient mice exhibited a reduced protective response [28].

As revealed by ELISPOT, the depletion of CD4^{+} spleen cells resulted in a major reduction in the number of SFCs, suggesting that the main source of the IL-17A after *C. pneumoniae* infection is the CD4^{+} cells. Although most of the recent studies focused on IL-17A produced by CD4^{+} T cells [29], γδ T cells are potent contributors to the immune responses following infections by intracellular pathogens such as *Mycobacterium tuberculosis*, *M. bovis* BCG and *Listeria monocytogenes* [28, 30, 31]. Other cell types, e.g. CD8^{+} T cells and natural killer cells [32], have been demonstrated to be IL-17A-producing cells, but in our experiment the depletion of CD8^{+} cells from the spleen did not influence the number of IL-17A-producing cells in the ELISPOT assay.

We found that the neutralization of IL-17A significantly reduced the number of neutrophil granulocytes in the BAL relative to that for the isotype antibody-treated animals. Moreover, the quantities of MIP, KC and LIX in the anti-IL-17A antibody-treated group were reduced 4 days after the infection with the pathogen. It was a somewhat surprising finding that the neutralization of IL-17A resulted in an increased pathogen burden at an early stage of infection; the amount of recoverable *C. pneumoniae* was increased 3-fold as compared with the isotype-treated controls, suggesting that IL-17A exerts an indirect antimicrobial effect. A similar antibacterial effect of IL-17A was seen in the case of extracellular *Klebsiella pneumoniae*. The over-expression of IL-17A after a challenge with *K. pneumoniae* resulted in the local induction of TNF-α, IL-1β and MIP-2, augmented polymorphonuclear leukocyte

recruitment, and enhanced bacterial clearance and survival [3333].

Our re-infection mouse model revealed that re-infection increased the expression of IL-17E (IL-25) relative to that in mice inoculated only once. The expression of IL-17A was also increased when viable *C. pneumoniae* was not present in the lungs, suggesting a role of IL-17A in the inflammatory process. This is an interesting finding in light of the reported putative role of respiratory pathogens such as *Chlamydia* and *Mycoplasma* in the activation of asthma. However the detailed pathomechanism has not been elucidated. In a recent publication, the transgenic overexpression of IL-17E by lung epithelial cells was shown to lead to increased mucus production and airway infiltration by macrophages and eosinophils, whereas the blockade of IL-17E reduced the airway inflammation and Th2 cytokine production in an allergen-induced asthma model [22]. The results of our experiments indicate that IL-17E induced by re-infection with *C. pneumoniae* may possibly influence allergic/asthmatic diseases.

In summary, a single infection with *C. pneumoniae* induced the production of IL-17A and IL-17F, and the *in vivo* neutralization of IL-17A resulted in a higher pathogen burden. CD4^{+} cells were identified as the main source of the IL-17A in response to *C. pneumoniae* infection. Re-infection with the pathogen led to a substantial expression of IL-17E.

Fig. 1 a *C. pneumoniae* IFU levels in the lungs of *C. pneumoniae*-infected mice. Lung homogenates were inoculated onto HEp-2 cell monolayers, and chlamydial inclusions were detected by indirect immunofluorescence, using LPS-specific monoclonal and FITC-labelled secondary antibodies. The data are means±SD of *C. pneumoniae* titres (IFU/lung) on the lung homogenates of 7 individual mice at each time point. **b** Expressions of IL-17A, IL-17C, IL-17D, IL-17E, IL-17F and **c** IL-23 mRNA in lung suspensions from infected mice. The total RNA extracted from the lungs was analysed by RT qPCR with the use of specific primers. Data are normalized for β-actin RNA content and plotted as fold change over the results for the control mice. **d** IL-17A protein production in the lungs of mice infected with *C. pneumoniae*. Lung homogenates were tested by using the IL-17A ELISA kit according to the manufacturer's instructions. Bars denote means±SD of the results on 7 mouse lungs.

Fig. 2 The number and phenotype of IL-17A-producing cells in mice infected with *C. pneumoniae*. Pooled spleen cells from 7 mice were depleted of CD4$^+$ or CD8$^+$ cells by using CD4- or CD8-specific antibodies and the Miltenyi magnetic cell sorting system. The depleted and non-depleted cells were tested after *in vitro* re-stimulation with *C. pneumoniae* antigen, or with a mock preparation in the ELISPOT assay, using the IL-17A ELISPOT kit. Bars indicate means±SD of SFCs per million spleen cells, counted in triplicate wells. The number of SFCs after re-stimulation with *C. pneumoniae* antigen was significantly lower in CD4-depleted cells than in non-depleted cells ($P<0.05$).

Fig. 3 a The *in vivo* neutralization of IL-17A. Mice were treated i.p. with anti-IL-17A or isotype control antibodies before *C. pneumoniae* infection and 24 and 48 h post-infection. Mice were sacrificed, their lungs were homogenized and the viable *C. pneumoniae* was quantified by culturing and indirect immunofluorescence tests. The number of viable chlamydiae in the lung suspension of the anti-IL-17A-treated group on day 4 was significantly higher than the *C. pneumoniae* content of the lungs of the isotype antibody-treated mice ($P<0.05$) **b** Cytokine secretions in lung tissue of mice on day 1 and **c** 4 after infection with *C. pneumoniae*. The quantities of KC, MIP-2 and LIX in the mouse lungs were determined by ELISA. Bars denote means±SD of the results on 7 mouse lungs ($P<0.05$).

Fig. 4 The effects of *C. pneumoniae* re-infection on the expressions of IL-17A and IL-17E. Mice were infected with *C. pneumoniae* 1-3 times at 4-week intervals. ↓ denotes re-infection. Animals were sacrificed 2 or 4 weeks after infection and the lungs were processed in the same way as mentioned in the legend to Fig. 1. The total RNA extracted from the lungs was analysed by RT qPCR with the use of specific primers. Data are normalized to β-actin RNA content and plotted as fold change over the results for the control mice.

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