Chlamydia pneumoniae-induced memory CD4+ T cell activation in human peripheral blood correlates with distinct antibody response patterns

Running title: Chlamydia pneumoniae-induced memory CD4+ T cell responses

Sebastian Bunk1*, Hanne Schaffert1, Bianca Schmid1, Christoph Goletz1, Sabine Zeller1,
Marina Borisova1, Florian Kern2, Jan Rupp3 and Corinna Hermann1

1 Department of Biochemical Pharmacology, University of Konstanz, Konstanz, Germany
2 Division of Medicine, Brighton and Sussex Medical School, Brighton, United Kingdom
3 Institute of Medical Microbiology and Hygiene, University of Luebeck, Luebeck, Germany

*Corresponding author: Mailing address: Department of Biochemical Pharmacology, University of Konstanz, P.O. Box M668, 78457 Konstanz, Germany; Phone: 0049-7531-884527, E-mail address: sebastian.bunk@gmx.net

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Abstract

*Chlamydia pneumoniae* is a frequent pathogen of the respiratory tract and persistent infections with this obligate intracellular bacterium have been associated with different severe sequelae. Although T cell activation during acute *C. pneumoniae* infections has been described, little is known about the frequency and role of *C. pneumoniae*-specific memory T cells that reside in the human body after resolved infection. In the present study, *C. pneumoniae*-induced T cell responses in peripheral blood mononuclear cells of 56 healthy volunteers were analyzed and compared to the donor’s serum antibody reactivity towards whole *C. pneumoniae* as well as recombinant *C. pneumoniae* antigens. Following short-term stimulation with *C. pneumoniae*, dual IFN-γ- and IL-2-producing CD4+ T cell responses could be detected in 16 out of 56 healthy individuals. *C. pneumoniae*-activated CD4+ T cells expressed CD154, a marker for T cell receptor-dependent activation, and displayed a phenotype of central memory T cells showing dominant IL-2 but also IFN-γ production. Interestingly, compared to seropositive individuals with no or single cytokine-producing CD4+ T cell responses (IFN-γ or IL-2), individuals with dual IFN-γ- and IL-2-producing responses showed significantly decreased immunoglobulin G reactivity towards *C. pneumoniae* RpoA and DnaK, antigens known to be strongly upregulated during chlamydial persistence. Our results demonstrate that memory CD4+ T cells responding to *C. pneumoniae* stimulation can be detected in the circulation of healthy donors. Furthermore, among seropositive individuals the presence or absence of dual IFN-γ- and IL-2-producing T cell responses was associated with distinct patterns of antibody responses towards persistence-associated *C. pneumoniae* antigens.
**Introduction**

Beside primary respiratory infection, *Chlamydia pneumoniae* is thought to establish persistent infections when the bacteria are not eliminated by the host (15, 20). Unresolved *C. pneumoniae* infections may represent a risk factor for chronic inflammatory diseases (2, 6, 9, 21), but they are difficult to diagnose, since serologic tests are still unable to discriminate between past and persistent infections (1, 6, 15). We recently described novel *C. pneumoniae* antigens, of which some might prove useful serologic markers to determine persistent *C. pneumoniae* infections (8). Unlike for antibody responses, little is known about the frequency and role of *C. pneumoniae*-specific T cells in humans. Effector CD4\(^+\) T cells responding to *C. pneumoniae* could be isolated from *C. pneumoniae*-positive atherosclerotic plaque (4, 5) and by using MHC class I tetramers, *C. pneumoniae*-specific CD8\(^+\) T cells were detected in peripheral blood mononuclear cells (PBMC) of infected patients (10). Studies in mice revealed a protective role for CD4\(^+\) and CD8\(^+\) T cells during primary *C. pneumoniae* infection (33, 34, 39) and during reinfection (31, 32, 34), suggesting the development of specific memory T cells. Pathogen-specific memory T cells were found to have a key role in the immune control of persisting viruses, like CMV, EBV, Varicella zoster virus and HIV. Notably the cytokine profile of antiviral T cell responses was found to reflect the degree of efficiency of controlling the viral infection. Whereas CD4\(^+\) T cell responses with predominant IFN-\(\gamma\) production were found during uncontrolled viral infections with high virus titers, dual IFN-\(\gamma\) and IL-2-production of virus-specific CD4\(^+\) T cells reflected an efficient immune control of persistent viral infections associated with low or moderate virus titers (16, 17, 30).

In the present study we investigated *C. pneumoniae*-induced T cell activation in PBMC of healthy donors and correlated the results with their serum immunoglobulin (Ig) A and G
reactivity towards whole *C. pneumoniae* and towards *C. pneumoniae* antigens, known to be upregulated during persistent chlamydial infection (3, 26, 28). For donors with dual IFN-γ- and IL-2-producing CD4⁺ T cell responses we further analyzed the cytokine profile and CD154 expression of activated T cells as well as their expression of CD45RA and CCR7 to discriminate between effector memory T cells (T<sub>EM</sub>) and central memory T cells (T<sub>CM</sub>). Our data demonstrate that after stimulation with *C. pneumoniae*, activated memory CD4⁺ T cells producing IFN-γ, IL-2 and CD154 can be detected in peripheral blood of healthy human donors. Furthermore in the case of seropositive individuals, the presence of *C. pneumoniae*-induced CD4⁺ T cell responses producing both IFN-γ and IL-2 was found to be associated with decreased serum IgG reactivity towards persistence-associated chlamydial antigens when compared to individuals with no or only single cytokine-producing responses.
Material and methods

Volunteers and PBMC preparation

Whole blood and sera were obtained from 57 healthy volunteers (University of Konstanz, Germany). The donors stated that they were healthy, had no chronic or severe respiratory illnesses during the last two years and had not taken immunosuppressive medication. To determine possible acute *C. pneumoniae* infections, as defined by a fourfold rise in specific IgG or IgA titers of consecutive serum samples, serum samples of each donor were taken at the start of the study as well as 2, 4 and 6 months after the initiation of the study and analyzed for specific antibodies. According to the criteria above one donor fulfilled the serologic parameters of an acute *C. pneumoniae* infection (fourfold rise in the IgG and IgA titer) and was excluded from the study. Acute infections with unrelated pathogens could further be excluded by differential blood cell count using ABX Pentra 60 (ABX, Montpellier, France). The age of the 56 donors without evidence for acute infection varied between 24 and 61 (mean 40.6) and the ratio of men to woman was 30 to 26. PBMC were isolated using BD Vacutainer® CPT™ Cell Preparation Tubes (BD Biosciences) according to the manufacturer’s instructions. The cells were washed and resuspended in RPMI-1640 supplemented with ultra-glutamine, 2.5 IE/ml liquemin (Hoffmann-La Roche) and 10% autologous serum at a concentration of 8x 10⁶ cells/ml. Then, 0.4 ml aliquots of PBMC were equilibrated in 15 ml polypropylene tubes at 37°C in a humidified 5% CO₂ atmosphere for 20 h before they were stimulated.

Stimulation of PBMC

Equilibrated PBMC aliquots of 0.4 ml (3.2x 10⁶ cells) were adjusted to 1 ml with RPMI-1640 containing ultra-glutamine, 2.5 IE/ml liquemin (Hoffmann-La Roche) and 1 µg of the
costimulatory antibody anti-CD28 (BD Biosciences). The cells were stimulated with $1 \times 10^9$ 
*C. pneumoniae* that was found to be the optimal dose for activation or with staphylococcal 
enterotoxin B (100 ng or 1 µg, Sigma-Aldrich) as a positive control for stimulation. PBMC 
samples of CMV-seropositive donors were also stimulated with a peptide pool (1 µg per 
peptide) specific for the CMV protein pp65 (22). Control stimulations with 1 µg and 10 µg 
LPS from *Salmonella abortus equi* were carried out under identical conditions to analyze the 
influence of TCR-independent T cell activation. After application of the stimuli the PBMC 
were incubated at 37°C in a humidified 5% CO$_2$ atmosphere for 6 h, the last 4 h in presence of 
10 µg/ml Brefeldin A (Sigma-Aldrich). Then, PBMC were incubated with 2 mM EDTA for 
15 min and vortexed roughly to detach adherent cells. The cells were fixed with 4.5 ml FACS 
Lysing Solution (BD Biosciences) for 10 min and stored at -70°C until flow cytometry 
analysis.

**Staining of surface markers and intracellular cytokines**

Fixed PBMC were thawed, transferred to 5 ml polystyrene tubes and centrifuged at 1100 g for 
10 min and cells were permeabilized for 10 min at room temperature using Permeabilizing 
Solution 2 (BD Biosciences). The cells were stained for surface markers with anti-human 
CD3-allophycocyanin (APC), CD4-phycoerythrin (PE) (both from BD Pharmingen) and 
CD8-peridinin-chlorophyll (PerCP) or CD4-PerCP (both from BD Bioscience), 
CD45RA-fluorescein-isothiocyanate (FITC) and CCR7-PE (both from BD Pharmingen) or 
CD4-PerCP alone. For the staining of intracellular cytokines FITC-conjugated antibodies 
against human IL-2 and/or IFN-γ (both from BD Biosciences) and anti-human IFN-γ-PE and
Flow cytometric analysis

Four-colour flow cytometry was performed on a FACS Calibur™ flow cytometer. Data files were analyzed with the CellQuest™ software package (BD Biosciences). For the determination of IFN-γ- or IL-2-positive T cells, 80,000 to 100,000 CD3⁺ lymphocytes were analyzed and further characterized according to their expression of CD4 and CD8. The frequencies of IFN-γ⁺ or IL-2⁺ T cells were determined by quadrant statistics. To determine frequencies of specifically activated T cells, the portion of activated T cells of stimulated samples was subtracted by the respective values of unstimulated samples (level of noise, always between 0.00% and 0.02%). Frequencies of specifically activated T cells of ≥ 0.03% corresponding to 3 per 10,000 cells were considered as positive T cell response and frequencies below this threshold were considered as negative response. To analyze the expression of CD154 among activated CD4⁺ T cells, 100,000 CD4⁺ lymphocytes were assessed for the production of IFN-γ and/or IL-2 and further characterized for intracellular CD154. As before, the values of stimulated samples were corrected by subtraction of the proportion of cytokine- (maximal 0.02%) and CD154-positive cells of unstimulated samples. To determine the memory phenotype and the cytokine expression profile of CD154⁺CD4⁺ T cells responding to different stimuli, 100,000 to 200,000 CD4⁺ T cells were analyzed according to their expression of CCR7 and CD45RA as well as of intracellular IL-2 and IFN-γ, respectively.
Determination of *C. pneumoniae*- and CMV-specific antibodies

The sera of all donors were tested in a blinded fashion for the prevalence of total anti-*C. pneumoniae* IgG and IgA antibodies by microimmunofluorescence (MIF) assay and SeroCP™ Quant IgA ELISA (both from Savyon Diagnostics, St. Ashdod, Israel), respectively. Both assays are based on purified elementary bodies of *C. pneumoniae* TW-183. The MIF assay was also used to assess the prevalence of IgG antibodies against *C. trachomatis* and *C. psittaci* to exclude donors with previous infections with these pathogens. The CMV status of some donors was determined in a diagnostic laboratory (Labor Brunner, Konstanz, Germany) using Liaison IgG ELISA (DiaSorin, Saluggia, Italy) according to manufacturer’s instructions.

*C. pneumoniae* culture, preparation and quantification

*C. pneumoniae* TW-183 were propagated and harvested as described previously (8). In brief, semiconfluent monolayers of HEp-2 cells were infected with *C. pneumoniae* TW-183 (about 500 genomic equivalents per cell) and cultivated for 72 h. Infected cells were scraped off, disrupted by vortexing with glass beads and bacteria were purified using a discontinuous gradient containing 25/50% gastrografin (Schering). Purified *C. pneumoniae* were finally suspended in SPS buffer (10 mM Na(H)PO4, pH 7.4; 250 mM saccharose) at a concentration of ≥ 10¹¹ per ml, repeatedly passed through a syringe and stored at -70°C. The amount of *C. pneumoniae* genomic equivalents were determined by real-time PCR using primers specific for the 16S rRNA (forward: ATGTGGCTCTCAACCCCAT; backward: GGCGCCTCTCCTATAAATAGG) and calculated according to a standard curve of genomic DNA, which was prepared from a defined amount of *C. pneumoniae* elementary bodies as published previously (27).
Recombinant expression of *C. pneumoniae* antigens

The *C. pneumoniae* antigens RNA polymerase alpha, CpB0704 and polymorphic membrane protein 21 (Pmp21) m corresponding to the partial amino acid sequence 636-1142 of the full-length Pmp21 were cloned and expressed as previously described (8). In brief, the genomic sequences of the antigens were amplified by PCR using Pfu Polymerase (Fermentas) and primers flanked with *Bam*H1/*Sal*I restriction sites and ligated into pQE30 expression vector encoding a hexahistidine-tag (Qiagen). Protein expression was carried out in *Escherichia coli* M15 cells (Qiagen) for 6h at 30°C. Then, the cells were disrupted in buffer containing 8M urea and the proteins were purified using HisTrap HP columns (Amersham Biosciences). The same protocol was used to clone and express the full-length genomic sequence of *C. pneumoniae* molecular chaperone dnaK after amplification with gene specific primers (forward: CCGGATCCATGAGTGAACAAAAAATCAAG; backward: CCGTCGACTCGTCGTTATCAATAATTTTCTAC).

Immunoblot analysis

For immunoblotting, recombinant RNA polymerase alpha, molecular chaperone dnaK, polymorphic membrane protein 21 (middle part) and CpB0704 were separately applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12% polyacrylamide gels and 10 µg of protein per 7cm in width. After electrophoresis, the proteins were transferred to a Bio Trace NT nitrocellulose membrane (Pall) using a semi-dry blotting system (Bio-Rad). The membranes were blocked with TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) containing 5% nonfat dry milk for 2h at room temperature (RT). Then the membranes were cut into 4 mm strips and each strip was incubated with a serum sample of the 56 donors.
at a dilution of 1:1000 in blocking buffer overnight at 4°C. The strips were washed with TBST four times for 15 min and incubated with a peroxidase-conjugated rabbit anti-human IgG secondary antibody (1:2000 diluted; DakoCytomation) for 45 min at RT. After four subsequent washing steps, immunoreactive bands were visualized by ECL detection followed by 5-min exposure to a LAS-3000 imaging system (Fuji). The maximal signal intensity of immunoreactive bands was determined for each strip using the AIDA software package (Raytest/Fuji). An intensity threshold of 1000 counts above background level was used as cutoff.

Statistics
Statistical analysis was performed using GraphPad Prism 4 software. Significant differences of frequencies of activated T cells and mean antibody titers between different donor groups were tested by ANOVA and by Kruskal-Wallis test followed by Dunn’s post test, respectively.
Results

*C. pneumoniae*-induced CD4⁺ T cell responses can be detected among healthy donors

In this study we investigated whether, in addition to *C. pneumoniae*-specific antibodies, also memory T cells responding to *C. pneumoniae* could be detected among healthy individuals. Therefore, PBMC of 56 donors without evidence for ongoing infection were *ex vivo* stimulated with whole *C. pneumoniae* or control stimuli and the production of IFN-γ and IL-2 among CD4⁺ and CD8⁺ T cells was analyzed by flow cytometry (Fig. 1A and B). Following stimulation with *C. pneumoniae*, frequencies of activated CD4⁺ T cells producing either IFN-γ or IL-2 or both cytokines of ≥3 per 10,000 cells, here defined as positive response, could be detected in 26 out of 56 donors (Fig. 1A). For the remaining 30 donors no or only marginal activation with IFN-γ⁺ or IL-2⁺ CD4⁺ T cell frequencies below 3 per 10,000 was observed. The 56 donors were classified into three groups according to their individual *C. pneumoniae*-induced CD4⁺ T cell response: polyfunctional response with production of IFN-γ and IL-2 (n=16), monofunctional response with either IFN-γ or IL-2 production (n=10) and no-response (n=30), respectively (Table I). Among the three donor groups, no significant difference between sex ratio, mean age and smoking status was observed. The frequencies (mean ± SEM) of *C. pneumoniae*-activated IFN-γ⁺ and IL-2⁺ CD4⁺ T cells for polyfunctional responders were 5.4 ± 2.3 and 6.2 ± 3.1 per 10,000, respectively. For monofunctional responders showing only IFN-γ⁺ (n=5) or only IL-2-production (n=5) the frequencies were 3.7 ± 0.5 or 4.5 ± 0.5 per 10,000, respectively. In contrast to CD4⁺ T cells, *C. pneumoniae*-induced activation of CD8⁺ T cells could not be detected among the 56 donors (Fig. 1B). The ability of the donor’s CD8⁺ T cells to respond to exogenous antigens was confirmed by stimulating PBMC with staphylococcal enterotoxin B (SEB) as well as...
stimulation with a CMV-specific peptide mix (protein pp65) in case of CMV-seropositive donors (Fig. 1B).

Activation of CD4$^+$ T cells by *C. pneumoniae* depends on TCR-engagement

CD154 represents a sensitive marker for antigen-specifically activated CD4$^+$ T cells (11, 14), since its expression strictly depends on TCR engagement (24). To exclude TCR-independent activation through chlamydial components, such as *C. pneumoniae* LPS, that stimulate innate immune receptors, CD154 expression among *C. pneumoniae*-activated CD4$^+$ T cells was analyzed. Therefore, PBMC of donors showing polyfunctional CD4$^+$ T cell responses, i.e. production of IFN-$\gamma$ and IL-2, were stimulated with *C. pneumoniae* and assessed for CD154 expression. As shown for an exemplary donor in Fig. 2A, upon stimulation with both *C. pneumoniae* and SEB, a dominant population of IFN-$\gamma^+$ and/or IL-2$^+$ CD4$^+$ T cells coexpressed CD154. Compared to *C. pneumoniae* stimulation (mean 64%), the proportion of CD154 expressing IFN-$\gamma^+$ and/or IL-2$^+$ CD4$^+$ T cells was slightly higher after stimulation with SEB (mean 81%). Our findings indicate that upon stimulation with both *C. pneumoniae* and SEB, the majority of IFN-$\gamma^+$ and/or IL-2$^+$ CD4$^+$ T cells is activated in a TCR-dependent manner leading to CD154 expression. It is important to note, that CD4$^+$ T cell activation was independent from *C. pneumoniae* infection as demonstrated by a comparable activation of CD4$^+$ T cells after stimulation with a cell lysate containing corresponding amounts of sonicated *C. pneumoniae*, which were unable to infect HEp-2 host cells (data not shown). Furthermore, during the short incubation time of our assay we observed no TCR-independent T cell activation when the PBMC were stimulated with 10 µg LPS from *Salmonella abortus*.
equi, a potent Toll-like receptor stimulus (always below 2 IFN-γ+ or IL-2+ cells per 10,000 CD4+ T cells, data not shown).

To further characterize the cytokine profile of C. pneumoniae-activated CD154+CD4+ T cells, we analyzed the distribution of single IFN-γ+, single IL-2+ and dual IFN-γ+/IL-2+ cells. As shown for an exemplary donor in Fig. 3A, C. pneumoniae-activated CD154+CD4+ T cells were predominantly single IL-2+ or dual IFN-γ+/IL-2+ cells. For donors with polyfunctional responses C. pneumoniae-activated CD154+CD4+ T cells consisted of 8% single IFN-γ+, 48% single IL-2+ and 44% dual IFN-γ+/IL-2+ cells (Fig. 3B). Compared to SEB, stimulation with C. pneumoniae led to significant shift from single IL-2+ towards dual IFN-γ- and IL-2-producing CD154+CD4+ T cells arguing for a prominent production of effector cytokines among C. pneumoniae-activated CD4+ T cells.

Memory phenotype determination of C. pneumoniae-activated CD4+CD154+ T cells

The high proportion of single IL-2-producing CD154+CD4+ T cells observed in C. pneumoniae-stimulated PBMC indicate the presence of pathogen-specific memory CD4+ T cells. To determine the phenotype of these memory CD4+ T cells we investigated the expression of CCR7 and CD45RA to distinguish between CCR7-/CD45RA- T_EM and CCR7+/CD45RA+ T_CM (17, 18, 35). Beside the analysis of C. pneumoniae- and SEB-activated CD154+CD4+ T cells, we also determined CCR7 and CD45RA expression for total CD4+ T cells of unstimulated PBMC samples (Fig. 4A). Compared to total CD4+ T cells that consisted mainly of T_CM (mean T_CM 65%, T_EM 35%), increased frequencies of T_EM were observed among SEB-activated CD154+CD4+ T cells (mean T_CM 47%, T_EM 53%) (Fig. 4C). In contrast to this, C. pneumoniae-activated CD154+CD4+ T cells were found to be
predominantly T<sub>CM</sub> (mean T<sub>CM</sub> 70%, T<sub>EM</sub> 30%). To confirm these findings we further characterized the distribution of T<sub>EM</sub> and T<sub>CM</sub> among CMV-specific CD154<sup>+</sup>CD4<sup>+</sup> T cells of CMV-seropositive donors after stimulation with CMV-specific peptides (Fig. 4B). In line with published results (17, 37), the vast majority of CMV-specific CD154<sup>+</sup>CD4<sup>+</sup> T cells were found to be T<sub>EM</sub> (mean T<sub>CM</sub> 23%, T<sub>EM</sub> 77%) (Fig. 4C). We typically observed about 10-20% of activated CD154<sup>+</sup>CD4<sup>+</sup> T cells, which were positive for the expression of both CCR7 and CD45RA thus representing naïve CD4<sup>+</sup> T cells.

Association between C. pneumoniae-induced CD4<sup>+</sup> T cell response and serum antibody reactivity towards whole C. pneumoniae or recombinant antigens

The presence of C. pneumoniae-induced memory T cell responses in the peripheral blood of apparently healthy donors suggests a history of past infection. To address this, we analyzed IgG and IgA antibody reactivity towards whole C. pneumoniae in the sera of all donors using microimmunofluorescence (MIF) and ELISA, respectively seropositive individuals (Fig. 5A). Out of the 56 donors 31 (55%) had detectable anti-C. pneumoniae IgG antibodies in their serum (titer ≥64). In addition, the sera of 14 (25%) donors tested positive for IgA antibodies (titer ≥32), of which all were found to be IgG-positive. Interestingly, there was no significant difference between the number of seropositive samples for donors with undetectable CD4<sup>+</sup> T cell responses (IgG 57%, IgA 17%), donors with single IFN-γ- or IL-2-producing responses (IgG 40%, IgA 30%) and donors with dual IFN-γ- and IL-2-producing responses (IgG 63%, IgA 38%). For the latter group, however, the mean anti-C. pneumoniae antibody titer was highest. The absence of C. pneumoniae-induced CD4<sup>+</sup> T cell activation in 17 out of 31 seropositive donors was unexpected and we investigated whether this finding was
related to individual differences in the serological response pattern of these donors. To
compare the serological responses between donors with or without detectable CD4+ T cell
activation we analyzed the antibody reactivity towards the recombinant *C. pneumoniae*
antigens RpoA, DnaK and Pmp21m, which are upregulated during chlamydial persistence in
vitro (3, 26, 28). In addition we analyzed the antibody reactivity towards the control
*C. pneumoniae* antigen CpB0704 that lack sequence homology to proteins of other Chlamydia
species and represent a major target of antibodies contributing to the MIF reactivity (8). Using
quantitative strip immunoblot analysis (Fig. 5B), we found significantly increased IgG
reactivity towards RpoA and DnaK as well as higher mean reactivity towards Pmp21m among
donors with undetectable or single cytokine-producing CD4+ T cell responses compared to
donors showing dual IFN-γ- and IL-2-producing responses. In contrast, for subjects with
undetectable or single-cytokine producing responses, the IgG reactivity towards the control
antigen CpB0704 was significant lower compared to donors with dual IFN-γ- and
IL-2-producing CD4+ T cell responses (Fig. 5C) indicating distinct patterns of anti-
*C. pneumoniae* antibody responses among these donor groups.
Discussion

Antigen-specific T cells play an important role in the immune control of persisting pathogens, including viruses and bacteria. For *C. pneumoniae*, a pathogen that can establish persistent infections (15, 20) CD4+ T cell responses have not yet been studied. In our study *C. pneumoniae*-induced CD4+ T cell responses producing IFN-γ and/or IL-2 could be detected in PBMC of 26 out of 56 healthy human individuals without any evidence for acute respiratory infection. Following restimulation with *C. pneumoniae*, the majority of cytokine-producing CD4+ T cells expressed CD154, a marker for antigen-specifically activated CD4+ T cells (11, 14), indicating TCR-restricted activation of cells by *C. pneumoniae*. Among *C. pneumoniae*-activated CD154+CD4+ T cells we found two major populations of cells expressing either only IL-2 or both IFN-γ and IL-2, but also a small population of cells expressing only IFN-γ. The IL-2 production among the vast majority of *C. pneumoniae*-activated IFN-γ+CD4+ T cells suggests self-renewing populations of effector cells. Compared to *C. pneumoniae*-induced cytokine responses, the cytokine profile of SEB-activated CD154+CD4+ T cells showed a significant shift from both IFN-γ+ and IL-2+ cells towards single IL-2+ cells corresponding to reports demonstrating that SEB-activated CD154+CD4+ T cells produce IL-2 rather than IFN-γ (11, 14). Corresponding to their dominant IL-2 expression, the majority of *C. pneumoniae*-activated CD154+CD4+ T cells displayed the CCR7+/CD45RA- phenotype of TCM. In contrast, the vast majority of CMV-specific CD154+CD4+ T cells were CCR7+/CD45RA+ characteristic for TEM, which is in line with previous findings (17, 37). Interestingly, while having a predominant CCR7+ TCM phenotype more than 50% of *C. pneumoniae*-activated CD154+CD4+ T cells produced IFN-γ indicating the presence of CD4+ TCM cells producing the effector cytokine IFN-γ. Although
according to proposed models, CCR7+ TCM typically produce IL-2 and not IFN-γ (23, 35) our data are consistent with other reports showing that human antigen-specific TCM are able to produce effector cytokines, like IFN-γ (12, 13, 36, 38). It is also possible that C. pneumoniae-activated TCM represent a transitional population of cells in the differentiation process from TCM to TEM, which was described as to be the predominant population of memory CD4+ T cells during persistent viral infections (17). In our experiments C. pneumoniae-induced CD8+ T cell responses could not be detected. This was also observed in previous reports that demonstrated the inability of recombinant C. pneumoniae proteins or whole bacteria to activate CD8+ T cells (29, 39). We cannot exclude that stimulation of PBMC with whole C. pneumoniae was more effective in activating CD4+ T cells than CD8+ T cells, since stimulation of CD8+ T cell by exogenous antigens requires MHC class I cross-presentation of antigens by dendritic cells or macrophages (7, 19), which are rare in PBMC. Interestingly, studies in mice demonstrated an impaired development of Chlamydia-specific memory CD8+ T cells after primary and secondary C. trachomatis infection (25), a process that might also account for the lack of anti-C. pneumoniae CD8+ T cell responses observed in our study.

In our study, the proportion of C. pneumoniae IgG- and IgA-seropositive individuals was not significantly different between donors with and without T cell responses, although responders had higher mean antibody titers. Interestingly, compared to seropositive individuals with CD4+ T cell responses (IFN-γ- and IL-2-production) seropositive donors lacking these responses showed a different patterns of antibody reactivity towards recombinant C. pneumoniae antigens (8) associated with persistent chlamydial infection. We found an increased IgG reactivity towards RpoA, DnaK and Pmp21m known to be upregulated during
persistent chlamydial infection (3, 26, 28) in the sera of subjects with no or only single cytokine-producing CD4⁺ T cell responses compared to the donors showing dual IFN-γ- and IL-2-producing responses. There could be different explanations for the finding that a portion of seropositive donors lacked CD4⁺ T cell responses and displayed an altered antibody recognition pattern compared seropositive donors with detectable CD4⁺ T cell responses (IFN-γ- and IL-2-production). These findings could be related to (i) cross-reactive antibodies that bind to *C. pneumoniae* in the MIF assay but were gained during infection with unrelated pathogens. The presence of cross-reactive antibodies would also explain why seropositive individuals lacking CD4⁺ T cell responses showed only low reactivity towards *C. pneumoniae* CpB0704, the more specific antigen, but high reactivity towards RpoA and DnaK both representing rather conserved bacterial proteins. (ii) individual differences in the repertoire of *C. pneumoniae* antigens targeted by the host immune response or expressed by *C. pneumoniae* during infection (e.g. infections with different *C. pneumoniae* strains). In both cases, B and T cell responses of the donors might have targeted a set of *C. pneumoniae* antigens, which allow antibody binding and thus positive MIF results, but only inefficient restimulation of T cells in our assay (e.g. due to rare expression in cultivated *C. pneumoniae*) (iii) a direct influence of T cells on the course of infection. An impaired development of T cell responses during *C. pneumoniae* infection might favor the establishment of persistent infections, in which the production of antibodies with a preferential specificity for persistence-associated *C. pneumoniae* antigens, like RpoA and DnaK, might even take place in absence of strong T cell help due to the prolonged antigen exposure. Regarding this last possibility, our data demonstrate that the dual production of IFN-γ and IL-2 among *C. pneumoniae*-activated CD4⁺ T cells was associated with low reactivity to persistence-associated *C. pneumoniae*
antigens. Recent reports analyzing IFN-γ- and IL-2-production among antigen-specific CD4+ T cells during persistent viral infections, observed distinct functional T cell heterogeneity, which could be attributed to different levels of virus load (16, 30). Interestingly, the authors described that only polyfunctional CD4+ T cell responses, characterized by dual IFN-γ and IL-2-production, reflected an efficient immune control of viral infection. It could be speculated whether in our study the presence of polyfunctional C. pneumoniae-induced T cell responses also represents a signature of an effective immune response that is able to prevent the development or to improve the control of persistent C. pneumoniae infections. Unfortunately, we could not test this hypothesis due to the lack of reliable tools for diagnosing persisting C. pneumoniae.

In conclusion, this study demonstrates the presence of circulating memory CD4+ T cells in PBMC of healthy human blood donors that upon stimulation with C. pneumoniae produce IFN-γ, IL-2 and CD154. Furthermore, the presence or absence of dual IFN-γ- and IL-2-producing CD4+ T cell responses was found to be associated with distinct differences in the IgG reactivity pattern towards C. pneumoniae antigens that are upregulated during chlamydial persistence.
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**Figure 1** *C. pneumoniae*-induced T cell activation in healthy blood donors.

PBMC of 56 healthy volunteers were stimulated for 6 h with whole *C. pneumoniae* or control stimuli and assessed for intracellular IFN-γ and IL-2 using flow cytometry. For each donor 80,000 to 100,000 CD3⁺ lymphocytes were gated on CD4⁺ or CD8⁺ T cells and analyzed for cytokine-positive cells via quadrant analysis. (A) The frequency of IFN-γ- and IL-2-producing CD3⁺CD4⁺ T cells was determined in PBMC of an exemplary donor stimulated with *C. pneumoniae*, 100 ng SEB or left without stimulation. (B) The frequency of IFN-γ-producing CD3⁺CD8⁺ T cells in PBMC of an exemplary CMV-seropositive donor was determined after stimulation with *C. pneumoniae*, 100 ng SEB or CMV-specific peptides.

**Figure 2** CD154 expression among CD4⁺ T cells activated by *C. pneumoniae*.

In PBMC of donors with *C. pneumoniae*-induced CD4⁺ T cell responses producing IFN-γ and IL-2 the expression of CD154 among activated CD4⁺ T cells was determined after stimulation with *C. pneumoniae*. As controls, unstimulated PBMC or PBMC stimulated with 1 µg SEB were analyzed. For the analysis 100,000 CD3⁺CD4⁺ T cells were assessed for intracellular IFN-γ and/or IL-2 and further for the presence of intracellular CD154. (A) The coexpression of CD154 among IFN-γ⁺ and/or IL-2⁺ CD4⁺ T cells following stimulation with *C. pneumoniae* or SEB is shown for one exemplary donor. CD4⁺CD8⁺ T-cells were not analyzed. (B) Proportion of IFN-γ⁺ and/or IL-2⁺ CD4⁺ T cells coexpressing CD154 after stimulation with *C. pneumoniae* or SEB of 10 different donors. The horizontal line indicates the mean proportion of CD154 coexpression.
Figure 3  Cytokine profile of *C. pneumoniae*-activated CD154^+CD4^+ T cells

In PBMC of donors with *C. pneumoniae*-induced CD4^+ T cell responses producing IFN-γ and IL-2 the distribution of single IL-2^+, single IFN-γ^+ and dual IL-2^+/IFN-γ^+ CD154^+CD4^+ T cells was determined after stimulation with *C. pneumoniae* or SEB. For the analysis 200,000 CD4^+ T cells were measured. (A) The frequency of single IL-2^+, single IFN-γ^+ and dual IL-2^+/IFN-γ^+ CD154^+CD4^+ T cells after stimulation with *C. pneumoniae* or SEB is shown for one exemplary donor. The frequencies of the respective subpopulations were determined by quadrant analysis. (B) Mean frequencies (± SEM) of single IL-2^+, single IFN-γ^+ and dual IL-2^+/IFN-γ^+ CD154^+CD4^+ T cells of 7 different donors. For comparison, the frequencies of the three subpopulations were normalized to 100% in sum for each donor. The asterisk marks significant differences with p < 0.01 (tested by ANOVA).

Figure 4  Memory phenotype analysis of *C. pneumoniae*-activated CD154^+CD4^+ T cells

In PBMC of donors with *C. pneumoniae*-induced CD4^+ T cell responses producing IFN-γ and IL-2 the expression of CCR7 and CD45RA among total CD4^+ T cells and CD154^+CD4^+ T cells was analyzed after stimulation with *C. pneumoniae*, SEB or CMV-specific peptides. For the analysis 100,000 to 150,000 CD4^+ T cells were measured. The quadrants for analyzing CCR7^+/CD45RA^− (T_CM) and CCR7^−/CD45RA^+/+ (T_EM) were determined for each donor according to the expression of these markers among total CD4^+ T cells. (A+B) Proportions of T_CM and T_EM among CD154^+CD4^+ T cells determined after stimulation of PBMC with *C. pneumoniae* or SEB (A) or CMV-specific peptides (B) of one exemplary donor and another exemplary CMV-seropositive donor, respectively. (C) Mean frequencies (± SEM) of T_CM and T_EM among total CD4^+ T cells and CD154^+CD4^+ T cells for
12 donors and 4 CMV-seropositive donors stimulated with *C. pneumoniae*, SEB or CMV-specific peptides, respectively. For comparison the sum of the frequencies of $T_{CM}$ and $T_{EM}$ was normalized to 100% for each donor.

**Figure 5** Analysis of serum antibody reactivity towards whole *C. pneumoniae* and different antigens, which either are or are not associated with persistent infection

The sera of donors with IFN-$\gamma^{+}$ and IL-2$^{+}$ (▲), either IFN-$\gamma^{+}$ or IL-2$^{+}$ (□) or no *C. pneumoniae*-induced CD4$^{+}$ T cell responses (●) were analyzed for anti-*C. pneumoniae* antibodies. Significant differences are marked with *, ** and *** representing p-values of 0.05, 0.01 and 0.001, respectively (tested by Kruskal-Wallis). (A) IgG and IgA antibody titers specific for whole *C. pneumoniae* elementary bodies were determined for in the sera of 56 donors using MIF and ELISA, respectively. The horizontal line represents the mean titer of each donor group. (B) Strip immunoblots with sera of 15 different exemplary donors using the control *C. pneumoniae* antigen CpB0704. Each number represents an individual serum and the arrow indicates the location of CpB0704. (C) Quantitative comparison of chemiluminescence signals of the strip immunoblots of all 31 seropositive donors. The IgG reactivity towards the *C. pneumoniae* antigens RpoA, DnaK and Pmp21m as well as CpB0704 was determined.
### Table I C. pneumoniae-induced CD4+ T cell activation of 56 healthy donors

<table>
<thead>
<tr>
<th></th>
<th>Polyfunctional</th>
<th>Monofunctional</th>
<th>Non-responder</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ+IL-2 cells</td>
<td>16</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Mean (±SD)</td>
<td>5.4 (±2.3)</td>
<td>3.7 (±0.5)</td>
<td>n.a.</td>
</tr>
<tr>
<td>IL-2+ cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (±SD)</td>
<td>6.2 (±3.1)</td>
<td>4.5 (±0.5)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Male (n=30)</td>
<td>9</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Smokers (n=8)</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Mean age</td>
<td>40.9</td>
<td>40.3</td>
<td>40.4</td>
</tr>
</tbody>
</table>

*ab* Calculated for five donors with either IFN-γ+IL-2- (a) or IFN-γ-IL-2+ (b)

*n* not applicable
A

Unstimulated  C. pneumoniae  SEB

CD4/CD45RO

CD4 PerCP

Cytokine production

B

IL-2  IL-2 and IFN-γ  IFN-γ

% cytokine production

SEB  C. pneumoniae
A

Unstimulated

C. pneumoniae

SEB

80%

52%

10%

36%

CD45RA FITC

B

Unstimulated

CMV

12%

70%

CD45RA FITC

C

n=12

n=4

% memory CD4 T cells

CD4

SEB

C. pneumoniae

CD4

CMV

$T_{CM}$

$T_{EM}$