ORIGINAL ARTICLE

T helper 17 cells are involved in the local and systemic inflammatory response in community-acquired pneumonia

Marthe S Paats,1 Ingrid M Bergen,1 Wessel E J J Hanselaar,2 E Christine Groeninx van Zoelen,3 Henry A Verbrugh,4 Henk C Hoogsteden,1 Bernt van den Blink,1 Rudi W Hendriks,1 Menno M van der Eerden1

ABSTRACT

Background Recent findings in mouse models suggest that T helper (Th)17 cells, characterised by production of interleukin (IL)-17A and IL-22, are involved in the immunopathogenesis of pneumonia.

Objective In this study, we aimed to identify the involvement of Th17 cells in human community-acquired pneumonia (CAP).

Design Within 24 h of admission, T cells from peripheral blood (n=39) and bronchoalveolar lavage (BAL, n=20) of CAP patients and of 10 healthy individuals were analysed by intracellular flow cytometry for the production of various cytokines, including IL-17A and IL-22. Peripheral blood T cells were also analysed 7 and 30 days after admission. Th17 cytokine profiles were correlated with pneumonia severity index and microbial aetiology.

Results In the BAL of CAP patients, proportions of IL-17A and IL-22 double positive CD4 T cells were significantly increased compared with healthy individuals. Significantly increased proportions of IL-17A/IL-22 double positive CD4 T cells in BAL were found in non-severe and severe CAP patients, as well as in pneumococcal and non-pneumococcal CAP. In the peripheral blood of CAP patients upon admission, we found significantly increased proportions of IL-17A/IL-22 double positive CD4 T cells. One week after admission, the proportions of these double positive cells were still significantly increased in CAP patients compared with healthy individuals.

Conclusions These data indicate that Th17 cells are engaged in the local and systemic immune response in human pneumonia. Especially, IL-17A/IL-22 double positive Th17 cells may be involved in the immunopathogenesis of CAP.

INTRODUCTION

Community-acquired pneumonia (CAP) is a common and serious illness despite the use of pathogen-directed or empirical broad-spectrum antimicrobial therapy.1 Major gaps remain in the understanding of its pathogenesis; it is not clear why some individuals can easily control bacterial challenges and remain healthy, whereas others develop pneumonia.

The immunopathology of CAP is characterised by a strong local and systemic acute-phase response. Several components of the innate immune system, such as neutrophils and macrophages, have been identified as key mediators of bacterial clearance in the lungs.2 3 The role of T cells in the pathogenesis of pneumonia is, however, less evident. HIV patients with depleted CD4 T cells are at increased risk for developing lung infections, suggesting that these cells are involved in the immune defence against pneumonia.4

Recently, it has been demonstrated that the novel T helper (Th)17 subset expressing the signature cytokines interleukin (IL)-17A and IL-22, has a protective role in host defence against different respiratory pathogens in the mouse.5 IL-17A is thought to promote chemokine and proinflammatory cytokine production, and consequent recruitment and activation of neutrophils.6 IL-22 controls tissue responses during inflammation, for instance, by increasing lung epithelial proliferation and trans-epithelial resistance to injury.7 8 Several groups showed that IL-17 mediates pneumococcal immunity in mice,9 11 and that acquired immunity to pneumococcal colonisation is associated with the Th17 subset.9 11 Moreover, IL-17A increased pneumococcal killing by human neutrophils both in the absence and in the presence of antibodies.
and complement. In mouse models of *Klebsiella pneumoniae* infection, both IL-17A and IL-22 production were required for optimal bacterial clearance. Th17 responses are also involved in murine host defence against other bacterial pathogens including *Mycoplasma pneumoniae*, * Bordetella pertussis* and *Pseudomonas aeruginosa*, as well as respiratory viruses. Human studies on Th17 cells in pneumonia are currently lacking, except for the demonstration of a role for Th17 cells in clearing pulmonary infections in the hyper IgE-syndrome. In this disease, caused by mutations in STAT3, Th17, cells are lost and patients manifest chronic, recurrent and severe bacterial and fungal infections.

In conclusion, whereas there is accumulating evidence for the involvement of Th17 cells in bacterial and viral respiratory infection in mice, in humans, Th17 cells are largely unexplored. In this study, we therefore aimed to investigate the presence of Th17 cells both locally and systemically in patients with CAP and to correlate these findings with disease severity and causative pathogen.

**RESULTS**

Venous blood samples were drawn from all 39 CAP patients included in the study; in 20 patients also a BAL was performed. No significant differences in clinical parameters were present between the total patient group and the subset of patients undergoing BAL procedure (table 1). Based on pneumonia severity index (PSI) scores, patients were allocated to the non-severe (PSI class I–III, n=22(56%)) or severe (PSI class IV–V, n=17(44%)) CAP group. In 10 patients (26%) *S pneumoniae* was isolated. Thirteen patients (33%) had a known non-pneumococcal bacterial (n=10) or viral (n=3) aetiology. In 16 patients (41%), no causative pathogen could be identified (table 1). Patients with a pneumococcal pneumonia were not more severely ill than patients with a bacterial non-pneumococcal aetiology (see online supplementary table S1). For the distribution of identified pathogens: see online supplementary table S2.

As a control group, we included 10 healthy individuals matched for age (54.8±5.7 years), gender (six males) and smoking status (four smokers).
Comparison of cytokine expression between CAP patients and healthy individuals

First, we quantified CD4, CD8 and γδ T cells in peripheral blood and BAL of all study subjects using flow cytometry. No significant differences were found in proportions of these lymphocyte subsets between CAP patients and healthy individuals (see online supplementary table S3). Absolute numbers of CD4 T cells in peripheral blood were similar in CAP patients upon admission compared with day 7 after admission (see online supplementary figure S1).

Next, we activated total peripheral blood mononuclear cell fractions and BAL cell suspensions and determined proportions of intracellular IL-17A and IL-22 single and double positive CD4 T cells (figure 1A), as well as proportions of IFNγ (Th1) and IL-4 (Th2) positive CD4 T cells in peripheral blood (data not shown).

Based on PSI scores upon admission, CAP patients were distributed over a non-severe (n=22) and a severe patient group (n=17). In peripheral blood, no differences in the proportions of IL-17A and IL-22 single positive CD4 T cells were found between CAP patients and healthy individuals. The proportions of IL-17A and IL-22 single and double positive CD4 T cells were significantly higher in BAL when compared with peripheral blood (all p<0.001; figure 1). However, no correlations were found between IL-17A and IL-22-positive CD4 T cells in blood and BAL of CAP patients (data not shown).

Moreover, we determined levels of IL-17 and IL-22 in serum and BAL fluid by ELISA. Levels of IL-17 were below detection in both compartments. IL-22 levels in serum, but not in BAL fluid, were significantly higher in CAP patients compared with healthy individuals (see online supplementary figure S3). No correlations were found between IL-17, IL-22 or IL17/IL-22 double positive CD4 T cells in blood or BAL and levels of IL-22 in serum or BAL fluid (data not shown).

We also correlated Th17 responses in blood and BAL with other clinical parameters. Proportions of IL-17A/IL-22 double positive CD4 T cells in peripheral blood upon admission of CAP patients admitted to the Intensive Care Unit (ICU) were significantly higher compared with the non-ICU patient group (p=0.03; see online supplementary figure S2). No other correlations were found between Th17 responses and parameters of disease severity including PSI score, need for mechanical ventilation, extent of pulmonary consolidations on chest x-ray, bacteremia, hypoxaemia, c-reactive protein (CRP) levels or mortality. The time of onset of symptoms before hospital admission, the use of antibiotics previous to hospital admission or the length of hospital admission also did not correlate with Th17 responses in blood or BAL of CAP patients (data not shown).

In addition to CD4 T cells, IL-17A and IL-22 can also be produced by other T-cell subsets, including γδ and CD8 T cells. The proportions of IL-17A+ or IL-22+ γδ T cells in peripheral blood and BAL were very low and did not differ between CAP patients and healthy individuals. Likewise, the proportions of cytokine positive CD8 T cells in peripheral blood were not significantly increased in patients compared with healthy individuals. In BAL, the proportions of IL-17A and IL-22 single and double positive CD8 T cells were significantly elevated in patients when compared with healthy individuals, but percentages were 5–10-fold lower than those in the BAL T cell compartment (data not shown).

Correlation of Th17 cells with microbiological aetiology

In the total CAP patient group, 10 (26%) patients had S pneumoniae isolated as the causative pathogen. Next, we compared patients with a pneumococcal pneumonia with patients with a known bacterial causative pathogen other than S pneumoniae (see online supplementary table S1). CAP patients with a pneumococcal aetiology were not sicker than patients presenting with CAP of another aetiology. Furthermore, cytokine profiles of peripheral blood CD4 T cells were not significantly different between pneumococcal pneumonia, bacterial non-pneumococcal pneumonia (NP), and healthy individuals (figure 2A). By contrast, both patients with pneumococcal pneumonia and patients with a bacterial non-pneumococcal pathogen isolated, had significantly increased proportions of IL-17A and IL-22 single and double positive CD4 T cells in BAL, compared with healthy individuals (figure 2B). Consistent with the heterogeneity of pathogens involved, non-pneumococcal patients showed a large range in the proportions of cytokine expressing CD4 T cells.

Table 1 Baseline characteristics of CAP patients

<table>
<thead>
<tr>
<th></th>
<th>PBMC (n=39)</th>
<th>BAL (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD) age (years)</td>
<td>55.6±18.5</td>
<td>60.6±19.0</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>25 (64)</td>
<td>13 (65)</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>15 (38)</td>
<td>8 (40)</td>
</tr>
<tr>
<td>Comorbidity, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COPD</td>
<td>10 (26)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Heart disease</td>
<td>8 (21)</td>
<td>6 (30)</td>
</tr>
<tr>
<td>Neurological disorder</td>
<td>6 (15)</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Chronic renal disease</td>
<td>1 (3)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>6 (15)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Pneumonia Severity Index, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low I</td>
<td>10 (26)</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Low II</td>
<td>7 (18)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Low III</td>
<td>5 (13)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Moderate IV</td>
<td>12 (31)</td>
<td>6 (30)</td>
</tr>
<tr>
<td>High V</td>
<td>5 (13)</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Mean ±SD CRP (mg/l)</td>
<td>207±151</td>
<td>219±169</td>
</tr>
<tr>
<td>Chest consolidation(s), n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unilobar, unilateral</td>
<td>32 (82)</td>
<td>16 (80)</td>
</tr>
<tr>
<td>Multilobar, unilateral</td>
<td>5 (13)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Bilateral</td>
<td>2 (5)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>ICU admission</td>
<td>8 (21)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Mechanical ventilation, n (%)</td>
<td>6 (15)</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Bacteraemia, n (%)</td>
<td>6 (15)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Hypoxaemia (sat&lt;90%), n (%)</td>
<td>13 (33)</td>
<td>7 (35)</td>
</tr>
<tr>
<td>Mean ±SD LOS (days)</td>
<td>10±14</td>
<td>13±18</td>
</tr>
<tr>
<td>Mortality, n (%)</td>
<td>2 (5)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Microbiological species, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>10 (26)</td>
<td>7 (35)</td>
</tr>
<tr>
<td>Other bacterial</td>
<td>10 (26)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Viral</td>
<td>3 (7.7)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Unknown</td>
<td>16 (41)</td>
<td>6 (30)</td>
</tr>
</tbody>
</table>

BAL, bronchoalveolar lavage; CAP, community-acquired pneumonia; COPD, chronic obstructive pulmonary disease; PBMC, peripheral blood mononuclear cells; LOS, length of hospital stay. For all clinical parameters p>0.35.

both in peripheral blood and in BAL. Up to 30% of CD4 T cells in BAL of CAP patients with a bacterial non-pneumococcal aetiology were IL-17 positive (figure 2B).

**Figure 1**  Cytokine expression by CD4 T cells in peripheral blood and BAL of CAP patients and healthy individuals. CAP patients were under divided as non-severe CAP (PSI class I-III) or as severe CAP (PSI class IV-V). (A) Flow cytometric characterisation of CD4 T cells. CD4 T cells were analysed for IL-17A expression (left panel), IL-22 expression (middle panel) and double expression of IL-17A and IL-22 (right panel). PBMC were gated from live CD56− cells, BAL cells from live cells. Results are shown as dot plots. (B). Proportions of total CD4 T cells positive for the indicated cytokines in PBMC from the total group of CAP patients (n=39), non-severe (n=22) and severe (n=17) CAP patients, versus healthy individuals (n=10). (C) Proportions of total CD4 T cells positive for the indicated cytokines in BAL from the total group of CAP patients (n=20), severe (n=10) and non-severe (n=10) CAP patients versus healthy individuals (n=10). Data are shown as box and whisker plots with 10th and 90th percentiles. Bold lines represent median values. Differences between CAP groups and HC were tested with the Mann–Whitney U test. *: p<0.05; **: p<0.01; ***: p<0.001. CAP, community-acquired pneumonia; HC, healthy control; PBMC, peripheral blood mononuclear cells; BAL, bronchoalveolar lavage.

**Systemic Th17 cytokine responses over time in CAP**

Peripheral blood samples were drawn from CAP patients upon admission and at days 7 and 30 after admission. To investigate systemic Th17 responses over time, we focused on proportions of IL-17A"IL-22" CD4 T cells, which upon admission were significantly higher in CAP patients than in healthy controls (figure 1B). At day 7, these proportions were still significantly higher than those found in healthy individuals, and showed a small but not significant increase compared with values upon admission (figure 3A). At day 30, proportions of IL-17A"IL-22" CD4
T cells in CAP patients were similar to those in healthy individuals (figure 3A).

Similar to the total group of CAP patients, also the group of severe CAP patients had significantly elevated proportions of IL-17A+IL-22+ CD4 T cells upon admission and at day 7, when compared with healthy individuals (figure 3B). In non-severe CAP patients, the proportions of these cells were not significantly different from values in healthy individuals at any time point (figure 3B).

When comparing pneumococcal and bacterial non-pneumococcal CAP patients with healthy individuals at different time points, we found that patients with pneumococcal pneumonia only had significantly higher proportions of IL-17A+IL-22+ CD4 T cells in their peripheral blood on day 7 after admission (figure 3C).

DISCUSSION
In this study, we aimed to identify the involvement of Th17 cells in the immunopathogenesis of CAP. The most important findings are that, in the BAL of CAP patients, proportions of IL-17A and IL-22 single and double positive CD4 T cells were significantly increased compared with healthy individuals. Significantly increased proportions of IL-17A+IL-22+ CD4 T cells in BAL were found in non-severe and severe CAP patients, as well as in pneumococcal and non-pneumococcal CAP. Upon admission, we found no differences in the proportions of IL-17A or IL-22 single positive CD4 T cells in peripheral blood between CAP patients and healthy individuals. The proportions of IL-17A+IL-22+ CD4 T cells were, however, significantly elevated in the blood of CAP patients, and were still significantly increased 1 week after admission. Moreover, upon admission, proportions of IL-17A/IL-22 double positive CD4 T cells in peripheral blood of CAP patients admitted to the ICU were significantly higher than those in the non-ICU patient group.

While Th17 responses to pneumonia are well established in mice,7–18 this is the first study demonstrating in vivo Th17 involvement as a distinct feature of CAP in humans. Our findings in peripheral blood point to an isolated Th17 response since we did not find increased proportions of circulating IFNγ+ (Th1) or IL-4+ (Th2) T helper cells. An important difference between our study and the other studies on T cell cytokine responses in human pneumonia is the fact that we studied patients with a heterogeneous microbial aetiology. This could explain that, contradictory to our findings, previous studies showed Th1 or Th2 responses against certain respiratory pathogens.10–17 24–26 Kemp et al24 showed a Th1 response at the onset of pneumococcal infection. However, their study population also included patients without pneumonia. Furthermore, in...
another study, human monocytes were in vitro infected with pneumococci, which resulted in both Th1 and Th17 responses. Patients with a pneumococcal aetiology had higher proportions of IL-17 and IL-22 single and double positive CD4 T cells in the BAL compared with healthy individuals. CAP patients with a bacterial non-pneumococcal aetiology, however, showed even higher proportions compared with the pneumococcal patient group, suggesting that the extent of Th17 involvement is pathogen-dependent. In pandemic 2009 H1N1 influenza, elevated plasma levels of both Th17 and Th1 related cytokines were observed, but the cellular source of these cytokines was not identified. The three patients in our study in whom a viral pathogen was isolated showed a wide range in cytokine expression, but IL-17A and IL-22 single and double positive CD4 T cells were, in all cases, above the median values of healthy individuals. A Th2 response was reported in Mycoplasma pneumonia. However, since we included only two patients with M pneumonia, we were not able to confirm these results.

It is conceivable that Th17 cytokines contribute to the vigorous recruitment and activation of neutrophils, which is one of the most important components of the innate immune response against bacterial infection. Particularly, since IL-17A has the ability to induce the expression of various proinflammatory mediators by epithelial and stromal cells, including IL-1, IL-6, TNF-α, CXCL8, G-CSF and GM-CSF, all of which support recruitment or activation of neutrophils. In agreement with this notion, in a subgroup of patients for which peripheral blood neutrophil counts were available, we observed a positive correlation between the absolute numbers of circulating...
neutrophils and the proportions of IL-17+CD4 T cells in BAL (p=0.016, r=0.73; M.P. et al, unpublished).

In vitro differentiation of Th17 cells leads to cell populations with IL-17A or IL-22 single or double expression, a phenomenon also observed in vivo in the skin and lung.\(^8\) \(^28\) \(^29\) Coexpression of IL-17A and IL-22 is particularly of interest, as we observed high proportions of IL-17A/IL-22 double-producing T helper cells in both BAL and peripheral blood of CAP patients. IL-17A and IL-22 can act synergistically to augment the expression of genes involved in defence against microbial pathogens, such as β-defensin-2 in keratinocytes.\(^28\)

Additionally, in bleomycin-induced lung inflammation, IL-17A can regulate the expression, proinflammatory properties and tissue-protective functions of IL-22, thereby determining the functional consequences of IL-22 expression in the lung.\(^29\) Because of the protective effects of IL-17A and IL-22 in mouse models of pneumonia,\(^3\) \(^10\) our finding of increased proportions of IL-17A+IL-22+ CD4 T cells in BAL supports the hypothesis that Th17 cells are necessary for a vital beneficial host response in human pneumonia.

Our results show increased IL-17A and IL-22 single positive CD4 T cells in BAL, but not in peripheral blood of CAP patients. Furthermore, CAP patients had higher proportions of IL-17A and, to a lesser extent, of IL-22 CD4 T cell in BAL than in peripheral blood, indicating that these cells preferentially home to, or are generated at the site of infection, for instance, the lungs.

Several factors have to be considered for the interpretation of our results. First of all, the number of patients included in this study is small, although comparable with other studies investigating local inflammatory responses.\(^1\) \(^1\) \(^2\) \(^3\) \(^4\) \(^5\) \(^6\) \(^7\) \(^8\) \(^9\) \(^10\) Furthermore, like in other observational patient studies, there are uncontrolled factors present before patients enter the study. We cannot completely exclude the possibility that, for instance, differences in time of onset of symptoms and hospital admission causes modulation of inflammatory responses, for instance, cytokine responses from γδ T cells could have been missed due to their early response in infection, and may also play an important role in the host defence. Likewise, from mouse models, respiratory inflammatory responses depend on the causative pathogen. We included CAP patients with different microbial etiology. It would therefore be interesting to analyse larger cohorts of patients with viral or NP to investigate if Th17 responses are completely excluded the possibility that, for instance, differences in other observational patient studies, there are uncontrolled factors present before patients enter the study. We cannot completely exclude the possibility that, for instance, differences in time of onset of symptoms and hospital admission causes modulation of inflammatory responses, for instance, cytokine responses from γδ T cells could have been missed due to their early response in infection, and may also play an important role in the host defence. Further, from mouse models, respiratory inflammatory responses depend on the causative pathogen. We included CAP patients with different microbial etiology. It would therefore be interesting to analyse larger cohorts of patients with viral or NP to investigate if Th17 responses are

In conclusion, our data indicate that CD4 T cells with a Th17 profile are engaged in local and systemic immune responses in CAP. Especially, our novel finding of significantly increased IL-17A+IL-22+ Th17 cells in BAL and peripheral blood supports their role in host defence in CAP.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES


