Mediators of Inflammation

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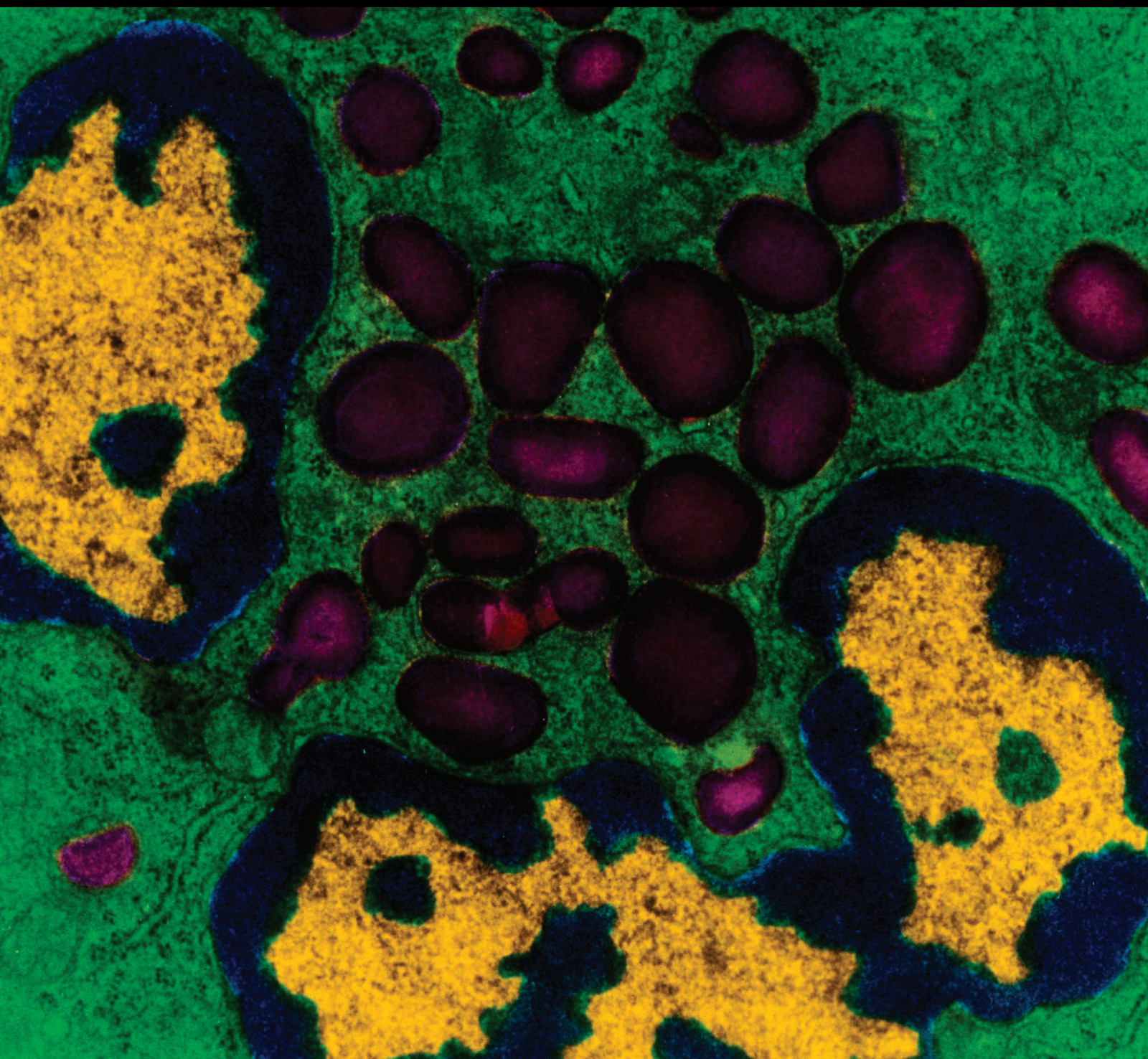
Guest Editors: Guansong Wang, Musheng Bao, Xiang Zhang, Juraj Majtan, and Kong Chen



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# Editorial Board

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*Editorial*

# Th17 Cytokines and Barrier Functions

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IL-17 functions in host defense against extracellular bacterial and fungal infections and contributes to the pathogenesis of various autoimmune inflammatory diseases. IL-17 producing CD4+ T helper cells (Th17) are a major source of IL-17 *in vivo* and play a central role in the pathogenesis of autoimmune diseases. Since the discovery of Th17 cells in 2005, there has been rapid progress in our understanding of their roles and plasticity in the immune system and their defects causing genetic diseases. The IL-17 pathway has been shown to be an attractive therapeutic target in many autoimmune and inflammatory disorders. Most Th17 cells were found to reside in the barrier tissues, including respiratory and intestinal tracts as well as skin. Cytokines produced by these Th17 and non-Th17 cells play critical roles in regulating tissue homeostasis and inflammation.

The pathogenic roles of Th17 in Multiple Sclerosis (MS) have been studied extensively in both human and animal models. E. Volpe et al. discuss recent advances in this field, with particular focus on the mechanisms conferring pathogenicity in MS and their potential modulation. G. R. D. Passos et al. reviewed the key findings supporting the relevance of the Th17 pathways in the pathogenesis of MS and Neuromyelitis Optica Spectrum disorders, as well as their potential role as therapeutic targets in the treatment of immune-mediated CNS disorders. The roles of Th17/IL-17 in other autoimmune diseases are also highlighted in this special issue. R. Kugyelka et al. summarized the recent advancements on the role of IL-17, particularly in the different rodent models of Rheumatoid Arthritis. Y. Li et al. summarized current knowledge about Th17 cells and gut microbiota involved in type 1 diabetes and proposed Th17 targeted therapy in children with islet autoimmunity to prevent progression to overt diabetes. After reviewing current literatures on Th17/IL-17 in asthma, X. Yang et al. proposed that Th17/IL-17 may be a key player in neutrophilic asthma. Recent findings on IL-17-driven mechanisms that promote breast cancer progression were thoroughly reviewed by T. Welte and X. H.-F. Zhang and the contradictory roles of IL-17 in cancer were also discussed. Owing to the increasing evidence for pathogenic roles of IL-17 in various diseases, Th17-targeted therapies are being actively investigated. H. Lin et al. thoroughly reviewed recent development and therapeutic potential of targeting Th17 cells with small molecules and small interference RNA. The research tools for interrogating the roles of IL-17 in human diseases are also of interest of many researchers in this field. Interestingly, F. Neves et al. studied IL17A from five lagomorphs and found that *IL17A* sequences of human and European rabbit are more closely related than the sequences of human and mouse, suggesting that European rabbit might be a more suitable animal model for studying human IL-17.

Several human studies in this special issue confirmed animal model findings. B. He et al. analyzed serum cytokine levels in patients with High-Altitude Deacclimatization Syndrome (HADAS ) and found that IL-17A levels correlate with disease incidence and severity, indicating that serum levels of IL-17A could serve as a novel predictive index of HADAS.

Y. Gong et al. examined a cohort of Ulcerative Colitis patients in a Chinese Han population and found that the levels of IL-17 and Th17 were significantly higher compared to healthy subjects while the frequency of Treg and the serum TGF-b1 were significantly reduced, suggesting that restoration of the Th17/Treg immune balance might have therapeutic potential in UC management. Using primary human nasal epithelial cells, M. Ramezanpour et al. provided data suggesting that Th17 cytokines may contribute to the development of chronic rhinosinusitis by promoting a leaky mucosal barrier. P. B. Linhartova et al. investigated the association of IL17 and IL17F gene polymorphisms with type 1 diabetes mellitus and chronic periodontitis and found the functional relevance of the *IL17A* polymorphism with higher IL-17 secretion in individuals with A allele. A. B. Christensen et al. observed increased *IL17A* mRNA expression in the intestinal epithelium of antiretroviral therapy (ART) suppressed HIV-1 infected individuals after treatment with histone deacetylase (HDAC) inhibitors (panobinostat), suggesting that panobinostat therapy may influence the restoration of mucosal barrier function in these patients.

Although Th17 cells seem to be mostly pathogenic in the setting of autoimmune diseases, the IL-17 pathway is instrumental in host defense against bacterial infections. Two papers in this special issue focus on the role of IL-17 in host defense: Z. Yan et al. summarize the recent advances in understanding the host-pathogen interaction of *A. baumannii* and propose a potential role of the IL-17 pathway in generating a protective immune response. Using a mouse model of septic peritonitis in mice induced by *E. coli*, Y. Ren et al. found a strong correlation of IL-17 production and surface TLR9 expression on neutrophils, suggesting that both molecules can be therapeutic targets for treating sepsis.

We hope that this special issue will not only be useful to the broad readership by providing insights into new and important aspects related to Th17 cytokines and barrier functions in the context of human diseases, but also stimulate novel research ideas and innovated therapeutic strategies in this field.

## Acknowledgments

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*Research Article*

# Association between Serum Interleukin-17A Level and High-Altitude Deacclimatization Syndrome

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High-altitude deacclimatization syndrome (HADAS) is emerging as a severe public health issue that threatens the quality of life of individuals who return to lower altitude from high altitude. In this study, we measured serum levels of SOD, MDA, IL-17A, IL-10, TNF-$\alpha$, and HADAS score in HADAS subjects at baseline and 50th and 100th days and to evaluate the relationship between interleukins, including IL-17A, and HADAS. Our data showed that and the serum IL-17A levels and HADAS score decreased over time in the HADAS group, and serum IL-17A levels were significantly higher in the HADAS group at baseline and 50th day compared with controls ($p < 0.05$). Furthermore, baseline serum levels of MDA and TNF-$\alpha$ were significantly higher, while SOD and IL-10 levels were lower in HADAS subjects compared with controls ($p < 0.05$). It is interesting that serum levels of IL-17A were clearly interrelated with HADAS incidence and severity ($p < 0.05$). ROC curve analysis showed that combined serum IL-17A and IL-10 levels were a better predictor of HADAS incidence than serum levels of IL-17A or IL-10 alone. These data suggest that serum levels of IL-17A are a novel predictive index of HADAS.

## 1. Introduction

When people originally from a lower altitude descend to sea level or lower altitude after high-altitude/hypoxia acclimatization, then they lose hypoxia tolerance and physiological adjustments. In addition, they experience changes in hemoglobin and hormone levels over time, and this is known as high-altitude deacclimatization (HADA) [1]. This physiological process has been documented in explorers [2], athletes [3], military personnel [4], and workers in high-altitude mines [5]. Following recent dramatic economic growth in plateau regions of China, such as Tibet, Qinghai, and Xinjiang, tens of millions of lower altitude individuals temporarily migrated to high-altitude regions for work, and then they returned to lower altitudes after they finished their work each year. When the workers returned to lower altitudes, most of them were in the process of HADA and suffered from physical discomfort and symptoms. A previous study has shown that individuals suffering from HADA experienced symptoms such as sleepiness, insomnia, unresponsiveness, memory loss, fidgetiness, headache, throat pain or discomfort, coughing, sputum, chest tightness, becoming flustered, increased appetite, decreased appetite, diarrhea, abdominal distention, abdominal pain, lumbago, and arthralgia [6]. These symptoms are characteristic and are usually referred to as HADA syndrome (HADAS), which affects the quality of life of these individuals [7, 8]. Our previous and other studies showed that HADAS subjects suffered a series of clinical symptoms, which could last for many years [6, 9–11]. Therefore, HADAS has been a public health issue in China and in other countries.

In our previous study [6], we found that subjects who suffered from HADAS experienced hypoxia/reoxygenation (H/R). The subjects lived in hypoxic conditions and then quickly returned to normoxic environments, and the levels of $PaO_2$ and $SO_2$ in HADAS subjects rapidly increased from

Table 1: Baseline characteristics of subjects at randomization according to the study group.

| | HADAS group ($n = 67$) | Control group ($n = 41$) | $p$ value |
|---|---|---|---|
| Demographic characteristics | | | |
| Age, years (SD, range) | 25.1 (7.6, 18–35) | 24.8 (8.1, 18–34) | >0.05 |
| Race, Han (%) | 67 (100%) | 41 (100%) | >0.05 |
| Sex, male (%) | 67 (100%) | 41 (100%) | >0.05 |
| Symptom score (SE, 95% CI) | 13.58 (0.41, 12.77–14.39) | | |
| Severity of HADAS | | | |
| Moderate reaction (%) | 15 (22.39%) | | |
| Mild reaction (%) | 52 (77.61%) | | |

The data indication (%) or the mean [(SD; range) or (SE, 95% CI)]. Symptom score = high-altitude deacclimatization syndrome (HADAS) scores.

81.58 hPa and 87.31% to 125.84 hPa and 96.78%, respectively. Evidence from studies showed that H/R induced oxidative stress and production of reactive oxygen species (ROS) [12, 13] and resulted in damage to tissue or cells [14]. Superoxide dismutase (SOD) plays an important role in removal of excess free radicals in humans experiencing oxidative stress [15, 16]. Malondialdehyde (MDA) is a product of lipid peroxidation, which occurs when unsaturated lipids are exposed to oxygen [15, 16]. Elevation of MDA levels leads to increased oxidative stress and oxidative-mediated damage [15, 16]. Zhou et al. [17] showed that serum SOD levels were elevated and MDA levels were decreased when subjects returned to lower altitudes upon short-term exposure to high altitudes. However, the roles of SOD and MDA in HADAS are unknown.

Research has shown that H/R could increase generation of proinflammatory mediators, such as tumor necrosis factor-$\alpha$ (TNF-$\alpha$) [18] and interleukin- (IL-) 17A [19], and suppress levels of anti-inflammatory factor IL-10 [20], and then it induced apoptosis and damage to cells and tissues [21, 22]. In our previous study, we showed that a systemic inflammatory response and myocardial injury were observed in HADAS subjects 3 d after returning to a lower altitude [6]. However, serum levels of IL-17A, IL-10, and TNF-$\alpha$ in HADAS subjects as well as correlations between these factors and occurrence rate and progression of HADAS are not clear.

In this study, we evaluated the HADAS score and measured serum levels of SOD, MDA, IL-17A, TNF-$\alpha$, and IL-10 in HADAS and control groups. We then analyzed the correlation of serum levels of SOD, MDA, IL-17A, TNF-$\alpha$, and IL-10 with HADAS occurrence and severity.

## 2. Methods

*2.1. Subjects and General Protocols.* Sixty-seven healthy male subjects (25.1 ± 7.6 years old) from Chongqing (180 m) had worked in Lhasa (3650 m) for about 8 months and then returned to Chongqing by airplane. After they returned, all subjects had been diagnosed with mild-to-moderate HADAS according to relevant diagnostic and scoring criteria, and they were evaluated for HADAS at 3 d (baseline), 50 d, and 100 d. They were considered the HADAS group. In addition, 41 healthy male subjects (24.8 ± 8.1 years old) who had always lived in Chongqing served as the control group. The 2 groups were not significantly different in age ($p > 0.05$) (Table 1). All subjects provided written informed consent. This study was approved by the Medical Ethical Committee of the Second Affiliated Hospital, Third Military Medical University.

*2.2. Diagnostic and Scoring Criteria for HADAS.* Subjects had been diagnosed with HADAS according to relevant diagnostic and scoring criteria [6]. Briefly, adult individuals who were less than 60 years old returned to a lower altitude from a higher altitude where they had worked for 4–12 months. They suffered from 3 or more of the following symptoms: fatigue, sleepiness, insomnia, unresponsiveness, memory loss, fidgetiness, headache, and throat pain or discomfort. The principal exclusion criteria included symptoms directly attributable to primary diseases affecting the cardiovascular, respiratory, nervous, urinary, and hematological systems, cancer or leukemia, and a recent history of influenza, upper respiratory tract infection, infectious diarrhea, or similar symptoms. HADAS symptom scores (HADAS scores) were evaluated according to the scoring criteria for HADAS. Scores from 6 to 15 indicated a mild reaction, and scores from 16 to 25 indicated a moderate reaction.

*2.3. Collection and Analysis of Blood Samples.* Morning fasting venous blood (3 mL) was collected, centrifuged at 4000 r/min for 10 min to separate serum, and stored at −80°C before assay. Human serum IL-17A, TNF-$\alpha$, and IL-10 ELISA kits were purchased from R&D Systems (Abingdon, UK). Serum IL-17A, TNF-$\alpha$, and IL-10 levels were detected according to the manufacturer's instructions. Optical density at 450 nm was measured with a spectrophotometer.

SOD and MDA assay kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Serum SOD levels were measured according to the manufacturer's instructions. Briefly, the serum of subjects was mixed with the reagents and incubated for 40 min at 37°C. After the reaction, absorbance at 560 nm was monitored using a spectrophotometer. The samples were then mixed with trichloroacetic acid and incubated for 40 min at 95°C. The absorbance of each sample was measured at 532 nm with a spectrophotometer. MDA concentration was calculated according to the formula provided in the protocol.

*2.4. Statistical Analysis.* SPSS 15.0 for Windows was used for statistical analysis. All data are presented as the arithmetic

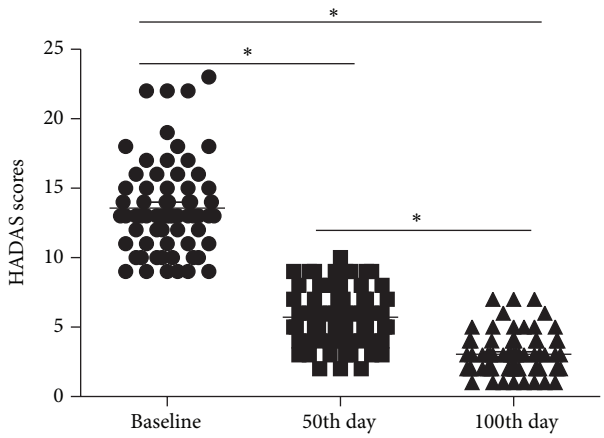


Figure 1: Scores of HADAS subjects. HADAS = high-altitude deacclimatization syndrome. The levels of HADAS scores of subjects tended to decrease as times went on. The dot plots show the levels of HADAS scores at baseline, 50th day, and 100th day. $^{*}p < 0.05$; the scores were significant different.

mean values (SD) or mean (SE, 95% CI). The changes in HADAS scores and levels of serum factors between HADAS and control group were examined across the study time course with a linear mixed effects modeling approach, and age and times were considered as covariates. Correlations of scores, SOD, MDA, IL-17A, IL-10, TNF-$\alpha$, and ages of HADAS subjects at baseline were analyzed using Pearson's correlation test. The effect of cytokine with HADAS occurrence and severity was analyzed via covariance adjusted logistic regression analysis, respectively, and age was considered as covariance. Based on logistic regression analysis, significant variables associated with HADAS occurrence were incorporated into a multivariate logistic regression model. Receiver operating characteristic (ROC) curve analysis was used to determine the value of serum IL-17A and IL-10 levels and in predicting HADAS. The prediction point was considered the point with the highest sensitivity and specificity. The level of significance was taken at $p < 0.05$.

## 3. Results

*3.1. Symptom Scores.* The HADAS scores were evaluated by physicians on the 3rd, 50th, and 100th days upon returning to low-altitude areas following 6 months of exposure to high altitudes and are shown in Figure 1.

The scores of subjects were 13.59 (0.41, 12.77–14.40) at baseline, 5.72 (0.25, 5.22–6.22) at the 50th day, and 3.06 (0.19, 2.69–3.43) at the 100th day, respectively. The differences in mean scores between baseline and the 50th day and 100th day were 7.87 (0.18, 7.41–8.32) ($p < 0.001$) and 10.52 (0.23, 9.54–11.09) ($p < 0.001$), respectively. The difference in mean scores between the 50th day and 100th day was 2.66 (0.10, 2.42–2.90) ($p < 0.001$). Thus, these data demonstrated that the HADAS scores decreased over time [6].

*3.2. Serum Levels of SOD, MDA, IL-17A, IL-10, and TNF-$\alpha$ between HADAS and Control Groups.* The serum concentrations of SOD, MDA, IL-17A, IL-10, and TNF-$\alpha$ were measured and compared between HADAS and control groups (Figures 2 and 3). At baseline, mean serum SOD and IL-10 levels were significantly lower in the HADAS group [serum SOD: 59.25 (0.52, 58.22–60.28) nu/mL versus 83.39 (0.67, 82.08–84.71) nu/mL, $p < 0.001$; serum IL-10: 42.29 (0.41, 41.47–43.10) pg/mL versus 56.55 (0.53, 55.51–57.59) pg/mL, $p < 0.001$]. Serum levels of SOD and IL-10 were not significantly different between HADAS and control groups at the 50th day and 100th day [serum SOD at the 50th day: 81.63 (0.52, 80.60–82.66) nu/mL versus 82.68 (0.67, 81.37–84.00) nu/mL, $p = 0.21$; serum IL-10 at the 50th day: 56.22 (0.41, 55.41–57.03) pg/mL versus 57.01 (0.53, 55.97–58.05) pg/mL, $p = 0.24$; serum SOD at the 100th day: 83.58 (0.52, 82.55–84.61) nu/mL versus 84.24 (0.67, 82.92–85.56) nu/mL, $p = 0.44$; serum IL-10 at the 100th day: 56.68 (0.41, 54.87–56.49) pg/mL versus 55.52 (0.53, 54.48–56.56) pg/mL, $p = 0.81$]. Moreover, serum SOD and IL-10 levels of HADAS subjects were lower at baseline than at the 50th and 100th days ($p < 0.001$).

Mean serum levels of MDA, IL-17A, and TNF-$\alpha$ were significantly higher in the HADAS group at baseline [serum MDA: 7.64 (0.03, 7.59–7.69) $\mu$mol/mL versus 4.89 (0.04, 4.82–4.96) $\mu$mol/mL, $p < 0.001$; serum IL-17A: 821.41 (9.52, 802.68–840.14) pg/mL versus 578.38 (12.17, 554.44–602.33) pg/mL, $p < 0.001$; serum TNF-$\alpha$: 259.03 (1.48, 256.16–261.89) pg/mL versus 231.11 (1.86, 227.46–233.57) pg/mL, $p < 0.001$]. Compared with the control group, serum IL-17A was higher at the 50th day [747.36 (9.52, 728.63–766.09) pg/mL versus 568.58 (12.17, 544.63–592.52) pg/mL, $p < 0.001$]. IL-17A concentrations were not significantly different between HADAS and control groups at the 100th day [566.90, 9.52 (548.17–585.64) pg/mL versus 573.12, 12.17 (549.17–597.06) pg/mL, $p = 0.69$]. Serum MDA and TNF-$\alpha$ levels were not significantly different between HADAS and control groups at the 50 and 100th days [serum MDA at the 50th day: 4.79 (0.03, 4.74–4.85) $\mu$mol/mL versus 4.819 (0.04, 4.75–4.89) $\mu$mol/mL, $p = 0.56$; serum TNF-$\alpha$ at the 50th day: 232.19 (1.46, 229.32–235.05) pg/mL versus 229.91 (1.86, 226.24–233.57) pg/mL, $p = 0.34$; serum MDA levels at the 100th day: 4.86 (0.03, 4.81–4.92) $\mu$mol/mL versus 4.85 (0.04, 4.78–4.92) $\mu$mol/mL, $p = 0.83$; TNF-$\alpha$ levels at the 100th day: 232.19 (1.46, 229.32–235.05) pg/mL versus 229.91 (1.86, 226.24–233.57) pg/mL, $p = 0.34$]. In addition, serum MDA, IL-17A, and TNF-$\alpha$ levels of HADAS subjects were higher at baseline than at the 50th and 100th days ($p < 0.001$), and the IL-17A concentration at the 50th day was higher than at the 100th day ($p < 0.001$).

*3.3. Correlation Analysis of Scores, SOD, MDA, IL-17A, IL-10, and TNF-$\alpha$ of HADAS Subjects at Baseline.* To understand correlations of scores, SOD, MDA, IL-17A, IL-10, and TNF-$\alpha$ of HADAS subjects at baseline, all baseline data from HADAS subjects were subjected to correlation analysis (Table 2).

HADAS score was positively correlated with serum levels of IL-17A and TNF-$\alpha$ ($r = 0.44$, $p < 0.001$; $r = 0.67$, $p < 0.001$, resp.) and negatively correlated with serum levels of

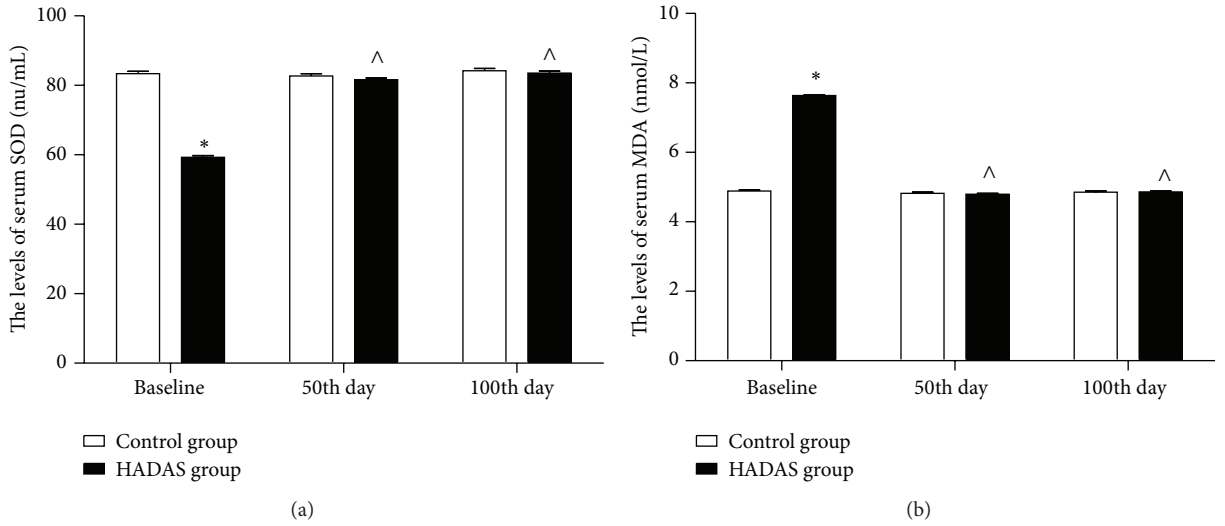


Figure 2: The serum SOD and MDA levels of subjects between HADAS and control groups. Data are presented as mean ± SE. SOD = superoxide dismutase; MDA = malondialdehyde. The serum SOD (a) and MDA (b) of subjects in both groups were assayed at baseline, 50th day, and 100 day. $^{*}p < 0.05$, relative to control group; $^{\wedge}p < 0.05$, relative to baseline.

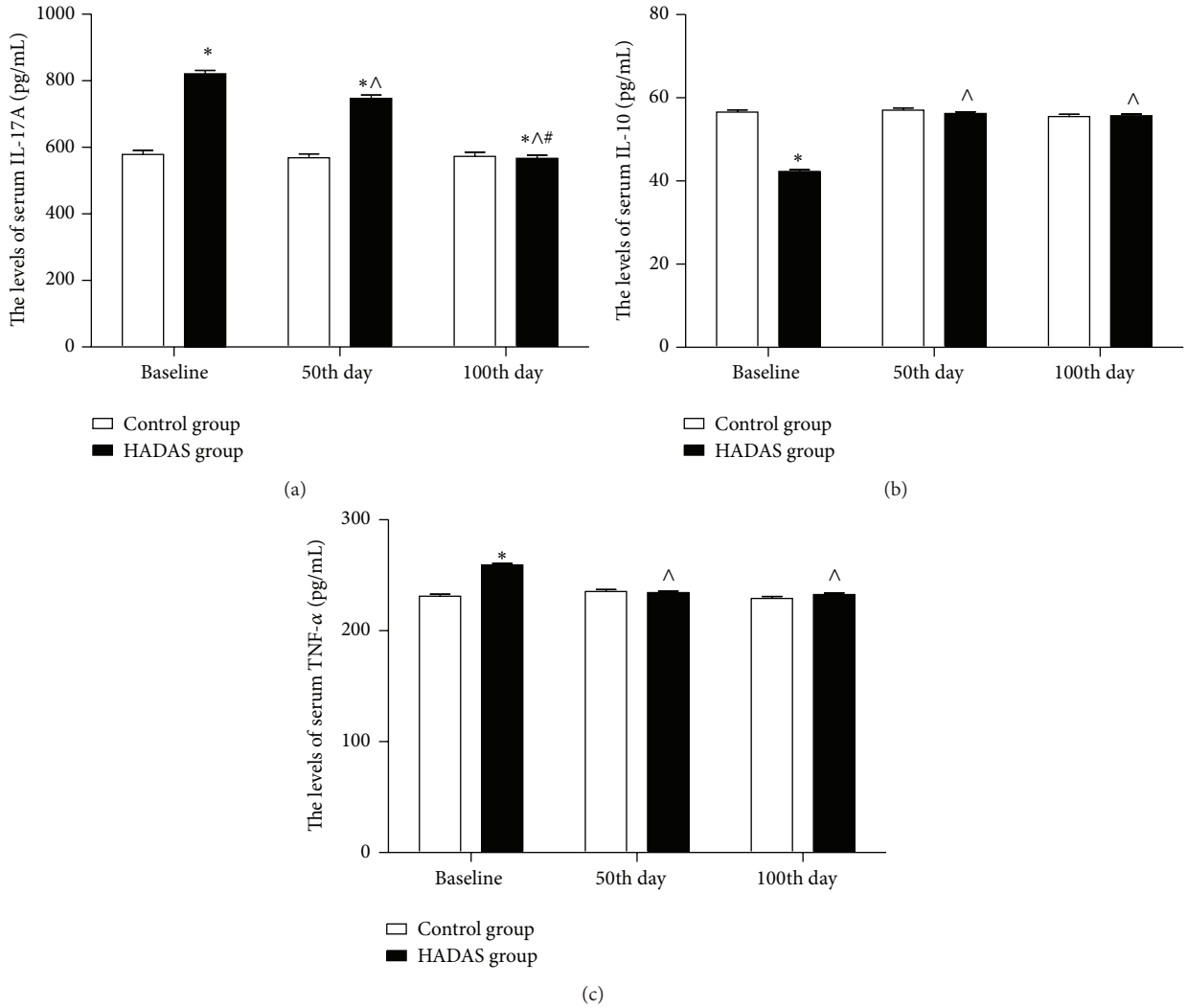


Figure 3: The serum IL-17A, IL-10, and TNF-$\alpha$ level of subjects between HADAS and control groups. Data are presented as mean ± SE. IL-17A = interleukin-17A, IL-10 = interleukin-10, and TNF-$\alpha$ = tumor necrosis factor-$\alpha$. The serum IL-17A (a), IL-10 (b), and MDA (c) of subjects in both groups were assayed at baseline, 50th day, and 100 day. $^{*}p < 0.05$, relative to control group, $^{\wedge}p < 0.05$, relative to baseline, and $^{\#}p < 0.05$, relative to the 50th day.

Table 2: Correlation analysis of scores, SOD, MDA, IL-17A, IL-10, and TNF-$\alpha$ of HADAS subjects on the baseline.

| Variables | Scores | | SOD | | MDA | | IL-17A | | IL-10 | | TNF-$\alpha$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $r$ | $p$ value | $r$ | $p$ value | $r$ | $p$ value | $r$ | $p$ value | $r$ | $p$ value | $r$ | $p$ value |
| Scores | — | — | −0.07 | 0.29 | 0.11 | 0.20 | 0.44 | 0.000* | −0.56 | 0.000* | 0.67 | 0.000* |
| SOD | −0.07 | 0.29 | — | — | 0.04 | 0.39 | −0.004 | 0.49 | −0.12 | 0.16 | 0.06 | 0.31 |
| MDA | 0.11 | 0.20 | 0.04 | 0.39 | — | — | 0.08 | 0.26 | −0.08 | 0.27 | 0.10 | 0.22 |
| IL-17A | 0.44 | 0.000* | −0.004 | 0.49 | 0.08 | 0.26 | — | — | −0.33 | 0.003* | −0.33 | 0.003* |
| IL-10 | −0.56 | 0.000* | −0.12 | 0.16 | −0.08 | 0.27 | −0.33 | 0.003* | — | — | −0.45 | 0.000* |
| TNF-$\alpha$ | 0.67 | 0.000* | 0.06 | 0.31 | 0.10 | 0.22 | −0.33 | 0.003* | −0.45 | 0.000* | — | — |
| Age | 0.08 | 0.27 | −0.14 | 0.13 | 0.25 | 0.02* | 0.03 | 0.40 | −0.24 | 0.03* | 0.11 | 0.18 |

* A significant correlation ($p < 0.05$).

Table 3: Logistic regression analysis of the serum SOD, MDA, IL-17A, TNF-$\alpha$, and IL-10 associated with HADAS severity.

| Variable | Univariate | | Multivariate | |
|---|---|---|---|---|
| | OR (95% CI) | $p$ value | OR (95% CI) | $p$ value |
| SOD | 0.951 (0.868–1.043) | 0.284 | 0.928 (0.819–1.053) | 0.247 |
| MDA | 2.789 (0.468–16.636) | 0.260 | 2.968 (0.251–35.144) | 0.388 |
| IL-17A | 1.064 (1.023–1.107) | 0.002* | 1.052 (1.012–1.093) | 0.010* |
| TNF-$\alpha$ | 1.078 (1.025–1.133) | 0.003* | 1.067 (0.989–1.150) | 0.095 |
| IL-10 | 0.702 (0.559–0.882) | 0.002* | 0.854 (0.622–1.172) | 0.328 |

* $p < 0.05$ is considered significant for statistical analyses.

Table 4: Logistic regression analysis of the serum IL-17A, TNF-$\alpha$, and IL-10 associated with HADAS occurrence.

| Variable | Univariate | | Multivariate | |
|---|---|---|---|---|
| | OR (95% CI) | $p$ value | OR (95% CI) | $p$ value |
| IL-17A | 1.033 (1.018–1.047) | 0.000* | 1.029 (1.003–1.056) | 0.030* |
| TNF-$\alpha$ | 1.081 (1.041–1.122) | 0.000* | 1.087 (0.989–1.195) | 0.083 |
| IL-10 | 0.594 (0.466–0.758) | 0.000* | 0.688 (0.505–0.938) | 0.018* |

* $p < 0.05$ is considered significant for statistical analyses.

IL-10 ($r = -0.56$, $p < 0.001$). However, HADAS score was not correlated with serum SOD or MDA or age of HADAS subjects ($p > 0.05$).

Serum IL-17A level was negatively correlated with IL-10 and TNF-$\alpha$ levels ($r = -0.33$, $p = 0.003$; $r = -0.33$, $p = 0.003$, resp.). However, serum IL-17A level was not correlated with serum SOD or MDA or age of HADAS subjects ($p > 0.05$). It is interesting that serum IL-10 level was negatively correlated with TNF-$\alpha$ level and subject ages ($r = -0.45$, $p < 0.001$; $r = -0.24$, $p = 0.03$, resp.). Moreover, there was no correlation between IL-10 and SOD and MDA ($p > 0.05$).

*3.4. Correlation Analysis of Scores, SOD, MDA, IL-17A, IL-10, and TNF-α Associated with HADAS Occurrence and Grading.* Correlations of IL-17A, TNF-$\alpha$, and IL-10 with HADAS occurrence and severity were analyzed via logistic regression. These data showed that serum IL-17A level was associated with HADAS severity ($p < 0.05$) (Table 3), and serum IL-17A, TNF-$\alpha$, and IL-10 levels were associated with HADAS occurrence ($p < 0.01$). In addition, SOD and MDA levels and subject ages were not significantly associated with HADAS occurrence ($p = 0.984$, $p = 0.994$, and $p = 0.984$, resp.). Multivariate logistic regression analysis showed that IL-17A level (OR = 1.025, $p = 0.044$) and IL-10 level (OR = 0.681, $p = 0.017$) were predictive of HADAS (Table 4).

To study further the role of serum levels of IL-17A and IL-10 in HADAS, ROC curve analysis was used (Figure 4). These data demonstrated that serum IL-17A predicted HADAS with sensitivity of 93.9% and specificity of 77.7% [AUC = 0.941 ± 0.025 (SE), 95% CI: 0.892–0.991, $p < 0.001$]. These data demonstrated that a serum IL-17A level > 726.41 pg/mL predicted HADAS with sensitivity of 83.8% and specificity of 93.9% [AUC = 0.941 ± 0.025 (SE), 95% CI: 0.892–0.991, $p < 0.001$]. A serum IL-10 level < 48.76 pg/mL predicted HADAS with specificity of 89.2% and sensitivity of 93.9% [AUC = 0.973±0.022 (SE), 95% CI: 0.000–1.000, $p < 0.001$]. It is interesting that combining IL-17A and IL-10 levels provided an additional benefit for predicting HADAS with specificity of 97.3% and sensitivity of 97.0% [AUC = 0.984 ± 0.016 (SE), 95% CI: 0.000–1.000, $p < 0.001$]. The findings indicated that the combination of serum IL-17A and IL-10 levels was a better diagnostic predictor of HADAS than serum IL-17A or IL-10 levels alone.

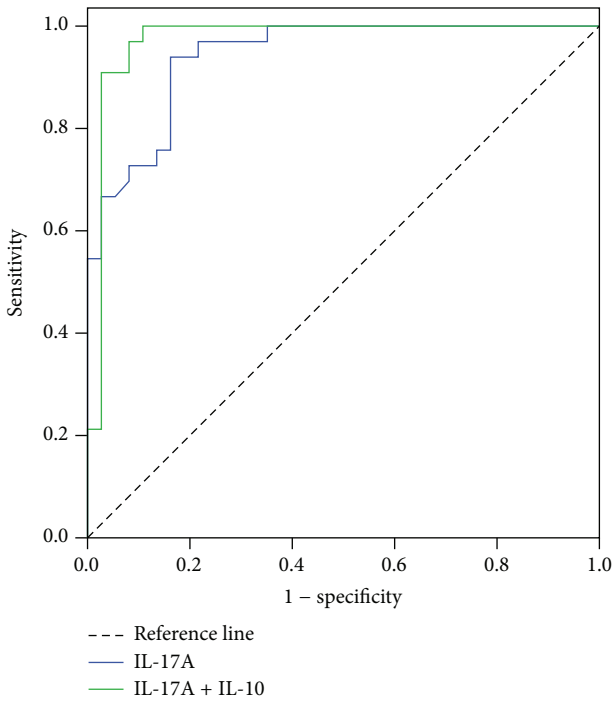


Figure 4: ROC curve analysis of the prediction of HADAS based on the serum IL-17A and combination of serum IL-17A and IL-10 levels. ROC curve analysis showing the performance of the biomarker IL-17A alone (blue) and a combination of IL-17A and IL-10 (green) in predicting the development of HADAS on the baseline. HADAS = high-altitude deacclimatization syndrome, IL-17A = interleukin-17A, and IL-10 = interleukin-10.

## 4. Discussion

Our data showed that the HADAS group had a significantly higher serum level of IL-17A compared with the control group at baseline and the 50th day and serum levels of IL-17A and HADAS score decreased over time in the HADAS group. Furthermore, serum levels of MDA and TNF-$\alpha$ were significantly higher and SOD and IL-10 levels were lower in the HADAS group than in the control group at baseline. Serum levels of IL-17A, IL-10, and TNF-$\alpha$ were significantly correlated with HADAS score. Serum IL-17A was correlated with HADAS severity, and serum levels of IL-17A and IL-10 were significantly correlated with HADAS occurrence rate. Thus, serum levels of IL-17A and IL-10 could be novel diagnostic predictors of HADAS.

H/R-mediated oxidative stress is involved in occurrence of HADAS. SOD is an antioxidant that reduces oxidative stress and protects tissues from damage induced by ROS [23]. Serum MDA is considered a marker of oxidative damage [24]. In our study, suppression of serum SOD and elevation of MDA levels was observed in HADAS subjects at baseline. Thus, these data indicate that the HADAS subjects had experienced oxidative stress. Serum SOD and MDA levels were not significantly different between the HADAS and control groups at the 50th and 100th days. This suggests that the levels of oxidative stress in HADAS subjects returned to normal levels. In addition, serum levels of SOD and MDA in HADAS subjects were not correlated with HADAS scores, severity, or occurrence rate at baseline. This could be because baseline serum levels of SOD and MDA reflected the very early stages of oxidative stress and antioxidant status in HADAS subjects, and they were indirectly involved in the appearance of symptoms.

H/R-mediated oxidative stress could induce release of mediators of inflammation and activate many signaling pathways, which result in cellular apoptosis and tissue damage [21, 25–29]. In our study, levels of proinflammatory mediators, TNF-$\alpha$ and IL-17A, were elevated, and levels of anti-inflammatory factor IL-10 were reduced at baseline in the HADAS group. This data suggests that an H/R-induced inflammatory response was involved in the occurrence of HADAS. Moreover, serum levels of IL-17A decreased over time in the HADAS group and were not different between HADAS and control groups at 100 d. Similar to serum levels of SOD and MDA, serum levels of IL-10 and TNF-$\alpha$ were not significantly different from the control group at the 50th and 100th days. These data suggest that the H/R-induced inflammatory response decreased with time.

Cytokine IL-17A is a member of the IL-17 family, and it is the hallmark cytokine of Th17 cells. Serum levels of IL-17A are elevated in several chronic inflammatory diseases [30–32], and IL-17A plays an important role in regulating inflammatory mediators and the inflammatory response. The mechanism of IL-17A in inflammation involves mediating recruitment of neutrophils to sites of inflammation and activating a number of proinflammatory chemokines and matrix metalloproteases [22]. Recently, the role of IL-17A in H/R or ischemia/reperfusion (I/R) has been explored. Barry and colleagues showed that elevation of IL-17A played a fundamental role in inflammation and apoptotic response in myocardial I/R injury [33]. Similarly, another study demonstrated that levels of serum IL-17A were increased by neutrophils and $CD4^+$ T cells produced following experimental I/R injury in mice [34]. Friedrich and colleagues [35] showed that IL-17A strongly induced TNF-$\alpha$ expression in inflammatory bowel disease. Xue and colleagues [36] showed that increased IL-17A not only enhanced production of proinflammatory cytokines but also impaired production of anti-inflammatory factors such as IL-10, resulting in renal tissue injury after I/R. Lee et al. [37] demonstrated that IL-17A played a critical role in intestinal, renal, and liver injury after I/R, and IL-17A knockout or inactivation significantly alleviated intestinal I/R injury and subsequent liver and kidney dysfunction. Based on the above research, we speculated that IL-17A may play a more important role than other inflammatory mediators, such as IL-10 and TNF-$\alpha$, in the occurrence of HADAS.

Exploring biomarkers for HADAS is very necessary. The diagnostic and scoring criteria of HADAS [6] included the essential diagnostic criteria, auxiliary diagnostic criteria, and symptom scores of HADAS. This was a complex diagnostic system, especially for HADAS symptom scores. Twenty-one symptoms had to be evaluated in scoring of HADAS symptoms by physicians and researchers. Evaluation of HADAS symptoms scores was a very heavy workload for physicians. Furthermore, symptom severity of HADAS subjects was based on subjective feelings, which resulted in some error for

HADAS symptom scores. The present findings showed that serum levels of IL-17A, TNF-$\alpha$, and IL-10 were significantly correlated with the HADAS score. It is interesting that IL-17A level was significantly associated not only with severity of HADAS, but also with HADAS occurrence based on multivariate logistical regression analysis. Serum level of IL-10 also correlated with occurrence of HADAS, and there was no correlation between IL-10 level and HADAS severity. This indicated that the serum level of IL-17A was a better independent predictor of HADAS than IL-10. In addition, ROC indicated that the combination of serum levels for IL-17A and IL-10 was a better predictor of HADAS occurrence than serum levels of IL-17A or IL-10 alone. These data suggest that a combination of serum levels for IL-17A and IL-10 may be a novel diagnostic predictor of HADAS.

Our study has some limitations. First, all of the subjects were male, and the majority of subjects were an average of 25 years old. Second, the spans between evaluation time points were too long to evaluate some parameters such as SOD, MDA, IL-10, and TNF-$\alpha$, which had already returned to normal levels in the HADAS group. All of these factors may have introduced bias into the results.

In conclusion, H/R-mediated oxidative stress and an inflammatory response are involved in occurrence of HADAS, and IL-17A level could be a novel predictive index of HADAS.

## Abbreviations

| | |
|---|---|
| HADAS: | High-altitude deacclimatization syndrome |
| H/R: | Hypoxia/reoxygenation |
| I/R: | Ischemia/reperfusion |
| IL-17A: | Interleukin-17A |
| IL-10: | Interleukin-10 |
| TNF-$\alpha$: | Tumor necrosis factor-$\alpha$ |
| SOD: | Superoxide dismutase |
| MDA: | Malondialdehyde |
| ROC: | Receiver operating characteristic. |

## Competing Interests

The authors declare that they have no competing interests.

## Authors' Contributions

Xiaolan Guo and Wei Yao designed the study and the experiments. Binfeng He, Mingdong Hu, Hongli Li, Weijie Dong, Zhenghua Wei, and Jin Li were responsible for data collection. Binfeng He and Hongli Li analyzed the data. Binfeng He, Hongli Li, and Mingdong Hu drafted the paper. Binfeng He, Wei Yao, and Xiaolan Guo revised and all authors approved the final paper. Hongli Li contributed equally to this work.

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*Research Article*

# Correlation of Surface Toll-Like Receptor 9 Expression with IL-17 Production in Neutrophils during Septic Peritonitis in Mice Induced by *E. coli*

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IL-17 is a proinflammatory cytokine produced by various immune cells. Polymorphonuclear neutrophils (PMNs) are the first line of defense in bacterial infection and express surface Toll-like receptor 9 (sTLR9). To study the relationship of sTLR9 and IL-17 in PMNs during bacterial infection, we infected mice with *E. coli* intraperitoneally to establish a septic peritonitis model for studying the PMNs response in peritoneal cavity. We found that PMNs and some of "giant cells" were massively accumulated in the peritoneal cavity of mice with fatal septic peritonitis induced by *E. coli*. Kinetically, the $CD11b^+$ PMNs were increased from 20–40% at 18 hours to >80% at 72 hours after infection. After *E. coli* infection, sTLR9 expression on $CD11b^+$ and $CD11b^-$ PMNs and macrophages in the PLCs were increased at early stage and deceased at late stage; IL-17 expression was also increased in $CD11b^+$ PMNs, $CD11b^-$ PMNs, macrophages, and $CD3^+$ T cells. Using experiments of *in vitro* blockage, qRT-PCR and cell sorting, we confirmed that PMNs in the PLCs did increase their IL-17 expression during *E. coli* infection. Interestingly, $sTLR9^-CD11b^+Ly6G^+$ PMNs, not $sTLR9^+CD11b^+Ly6G^+$ PMNs, were found to be able to increase their IL-17 expression. Together, the data may help understand novel roles of PMNs in septic peritonitis.

## 1. Introduction

Sepsis affects approximately 700,000 people annually and accounts for about 210,000 deaths per year in the US. Its incidence is rising at rates between 1.5% and 8% per year, despite continuous progress in the development of antimicrobial therapeutics and supportive cares [1]. Most of the sepsis is caused by bacteria and bacteria of abdominal origin contribute to the second major reason for sepsis. This type of sepsis is designated as abdominal sepsis or septic peritonitis. The septic peritonitis is the host's systemic inflammatory response to the bacteria, initiated by the pathogen associated molecule patterns (PAMPs) including lipopolysaccharide and lipid A from Gram-negative bacteria and lipoteichoic acid and peptidoglycan from Gram-positive bacteria [2]. The inflammatory initiation leads to the release of chemokines, such as interleukin-8 (IL-8), and proinflammatory cytokines, such as tumor necrosis factor-$\alpha$ (TNF-$\alpha$), IL-1, IL-6, and IL-12, in a massive amount. The cytokines, if not appropriately controlled, may severely impair the functions of vital organs or systems, resulting in death [2]. Data from the CIAOW study (complicated intra-abdominal infections worldwide observational study) showed that the overall mortality rate was 10.5% in the patients with septic peritonitis in a worldwide context [3].

Septic peritonitis is characterized by a massive infiltration of neutrophils into the peritoneum in response to bacterial invasion, where they are activated and act as a first line of

defense against the bacteria [4]. Morphologically, the mature neutrophils are unique among the other white blood cells by their lobulated nucleus, which inspired the renaming of them as polymorphonuclear neutrophils (PMNs) [5]. Based on cytokine production, macrophage activation, expression of Toll-like receptor (TLR), and surface antigen expression, murine PMNs have been classified into three types [6]: normal PMN (PMN-N), PMN-I, and PMN-II. PMN-I produces IL-12/CCL3, activates macrophages in a classical way, expresses TLR2/TLR4/TLR5/TLR8, and has a $CD49d^{+}CD11b^{-}$ phenotype. PMN-II produces IL-10/CCL2, activates macrophages in an alternative manner, expresses TLR2/TLR4/TLR7/TLR9, and possesses a $CD49d^{-}CD11b^{+}$ phenotype. PMN-N rarely produces cytokine/chemokine, displays no effect on macrophage activation, and expresses TLR2/TLR4/TLR9, with $CD49d^{-}CD11b^{-}$ phenotypes. The PMN-N may convert to PMN-I or PMN-II in response to bacterial infections [6]. Recently, macrophages have been reported to cooperate with neutrophils by promoting extravasation and activation of PMNs, even their clearance of bacteria [7].

Accumulated evidence revealed that Toll-like receptors (TLRs) could play a fundamental role in induction of hyperinflammation and tissue damage in sepsis. TLRs are pattern recognition receptors (PRRs) that sense microbial invasion and initiate innate immune responses. Positioned at the cell surface, TLR4 is essential for sensing lipopolysaccharide of Gram-negative bacteria and TLR2 is involved in the recognition of a large panel of microbial ligands, while TLR5 recognizes flagellin. Endosomal TLR3, TLR7, TLR8, and TLR9 are specialized in the sensing of nucleic acids produced notably during viral infections or during bacterial infections [8]. Conventionally, TLR9 is thought to sense microbial DNA in endolysosomes and not at the cell surface. Recently, it has been found that TLR9 is expressed on the surface of human and mouse PMNs, that the surface TLR9 (sTLR9) senses DNA in PMNs, and that sTLR9 expressing PMNs ($sTLR9^{+}$ PMNs) have roles in inducing rapid inflammation [9]. Human and mouse PMNs spontaneously express sTLR9, and the sTLR9 expression is upregulated in response to PMN stimulation [10]. In front of TLR9 ligands, $sTLR9^{+}$ PMNs could be activated, leading to cytokine secretion and CD11b upregulation [10]. Notably, TLR9 stimulation was demonstrated to be detrimental in mice with bacterial sepsis. $TLR9^{-/-}$ mice exhibited lower serum inflammatory cytokine levels, higher bacterial clearance, and greater survival after experimental peritonitis induced by cecal ligation and puncture (CLP). A single injection of TLR9 antagonist protected the wide type mice, even when administered as late as 12 hours after CLP [11]. It has been proposed that, during infection, $sTLR9^{+}$ PMNs could be involved in the detrimental effect by releasing massive proinflammatory cytokines, including IL-6 and TNF-$\alpha$, and possibly other cytokines in response to PAMPs [12, 13].

Interleukin-17 (IL-17) is a cytokine family that signatures T helper 17 (Th17) cell subset and contains six members (IL-17A to IL-17F), among which IL-17A is considered as one of the major proinflammatory cytokines mediating the innate and adaptive immune responses against bacterial infections [14]. In addition to Th17 cells, $\gamma\delta$ T cells, innate lymphoid cells (ILCs), mast cells, PMNs, and macrophages are also IL-17 producing cells [14]. Notably, innate immune cell-derived IL-17 constitutes a major element in the immune response against infectious agents by recruiting PMNs to the sites of infections and by inducing the production of antimicrobial peptides, CXC chemokines, and granulocyte colony stimulating factor (G-CSF) [15, 16]. In bacterial infection, PMNs act to produce increased IL-17, which in turn recruits more PMNs to join the fighting against invaded bacteria, resulting in increased production of innate immune cell-derived IL-17 [16, 17]. IL-17 was found to work in an IL-23-IL-17 axis which was critical for the survival of the host infected with bacteria [18]. In the axis, tissue-infiltrating PMNs could be the main source of IL-23 [19] which induces production of IL-17 from macrophages [20].

In the present study, we kinetically observed the infiltration of PMNs and expression of sTLR9 and IL-17 on/in the PMNs in the peritoneal lavage cells (PLCs) of mice intraperitoneally infected with various doses of *Escherichia coli* (*E. coli*), aiming to find the correlation of the expression of sTLR9 and IL-17 on/in the PMNs during the development of bacterial septic peritonitis. The achieved results could provide a basis for further investigation on the roles of sTLR9 expressing PMNs and IL-17 producing PMNs in bacterium caused septic peritonitis.

## 2. Materials and Methods

*2.1. Antibodies and Reagents.* The monoclonal antibodies of APC-conjugated rat anti-mouse CD45 antibody (561689), PE-conjugated rat anti-mouse CD14 antibody (561711), PE-conjugated rat anti-mouse CD11b antibody (561689), APC-Cy™ 7-cojugated rat anti-mouse CD3 antibody (560590), and APC-conjugated rat anti-mouse Ly-6G antibody (560599) were purchased from BD bioscience. The FITC-conjugated active anti-TLR9 monoclonal antibody (26C593.2) was from Abcam; FITC-conjugated anti-mouse IL-17A antibody (506908) and PerCP-Cy™ 5.5-conjugated rat anti-mouse IL-17A (3354955) antibody were from Biolegend. Trizol reagent (NC0301) was from Invitrogen. cDNA Synthesis Kit (Transgene biotech, I21021) and two-step SYBR green qPCR assays (Transgene biotech, G31227) were from Biotech.

*2.2. E. coli Strain and Mice.* *E. coli* strain of JM109 was recovered from lyophilized powder by being suspended into LB medium and then cultured on LB agar plate at 37°C overnight. The single colonies of *E. coli* on the plate were used as a group of original seed *E. coli*.

Female ICR mice were purchased from the Experimental Animal Center, Medical College of Norman Bethune, Jilin University, and maintained in microisolator cages under specific pathogen-free conditions. All the mice were used at 6 to 8 weeks of age. The experimental manipulation of mice was undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Science & Technology of Jilin Province.

*2.3. Preparation of E. coli Culture.* To prepare *E. coli* culture, each single colony of *E. coli* was picked up and cultured in 5 mL LB medium at 37°C with shaking at 200 rpm. When the OD value ($A_{600}$) of the culture reached 1.4, *E. coli* was harvested from the culture by centrifugation and mixed with 20% glycerol solution. The resultant glycerol *E. coli* was stored at −20°C as working *E. coli* seeds. The working seed *E. coli* were seeded into LB medium and cultured at 37°C till the OD value reached 0.7 followed by harvesting the *E. coli* pellet after centrifugation at 4000 g for 5 min. The *E. coli* pellet was diluted in a serial tenfold, plated on an eosin methylene blue agar plate, and then cultured at 37°C for 14 hours. The colony-forming units (CFUs) of the *E. coli* culture were calculated and determined as approximately $0.8 \times 10^8$ CFUs/mL. For animal experiment use, the *E. coli* pellet was washed twice using sterile 0.9% NaCl (saline) and then resuspended in 1.0 mL saline containing 0.4, 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, and was ready to use for infecting mice.

*2.4. Induction of Bacterial Septic Peritonitis and Preparation of PLCs.* For inducing bacterial septic peritonitis, mice were injected intraperitoneally (i.p.) with 1.0 mL of *E. coli* preparations containing 0.4, 0.8, 1.6, and $2.4 \times 10^8$ CFUs/mL in sterile saline, respectively. The saline injection was used as control. The survival of the mice was monitored every 4 hours for 4 days.

To harvest the peritoneal lavage cells (PLCs) from mice either infected with *E. coli* or injected with saline, the mice ($n \geq 3$ per group) were euthanized at defined time points with 50 mg/kg pentobarbital sodium followed by washing peritoneal cavities using 6 mL per mouse of ice-cold phosphate-buffered saline (PBS). The peritoneal lavage fluid was centrifuged at 750 g for 5 minutes at 4°C for harvesting the PLCs. The PLCs were resuspended in cold PBS for further use.

*2.5. Cell Culture and Treatment.* PLCs were harvested from the naïve mice and maintained in RPMI 1640 supplemented with 10% (V/V) fetal bovine serum (FBS) (GIBCO) and antibiotics (100 IU of penicillin/mL and 100 IU of streptomycin/mL). In experiments using viable bacteria, antibiotics were not added to the culture medium during the isolation, washing, or subsequent culturing period. No bacterial contamination was observed in PLCs cultured in the absence of antibiotics. Cells were counted and then plated in 24-well cell culture plates (Costar, Cambridge, MA) at an approximate density of $1 \times 10^6$ cells/well. The PLCs were cocultured with *E. coli* at $1 \times 10^5$ or $1 \times 10^6$ CFUs/well or saline as a vehicle control for 14 hours, and then they were cultured with BFA for another 4 hours, in a 5% $CO_2$ humidified incubator at 37°C. The PLCs were collected and stained with FITC-labeled anti-IL-17 mAb, followed by flow cytometry analysis to detect the expression of IL-17.

*2.6. Cell Counting.* To count the numbers of cells in each peritoneal lavage sample, the samples were spin down for harvesting the cell pellets. The cell pellets were smeared on slides and then stained using hematoxylin-eosin (HE) followed by counting the cell numbers on hemocytometer (Beckman Coulter, Fullerton, CA) and taking photos of the cells.

*2.7. Flow Cytometry.* For surface staining, the PLCs were stained with fluorescence-conjugated mAbs against CD45, CD11b, and sTLR9, respectively, for 30 minutes at room temperature in the dark followed by washing twice with PBS. For IL-17 intracellular staining, the PLCs were surface stained as described above and then fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin followed by staining with FITC-conjugated anti-IL17A monoclonal antibody. All stained cells were analyzed by flow cytometer FACSCalibur (BD) and CytoFLEX (Beckman Coulter). Live cells were carefully gated by forward and side scattering. Data were analyzed with FlowJo software (FlowJo 7.6.1).

*2.8. Cell Sorting.* The PLCs were harvested from mice ($n \geq 6$ per group) either infected with *E. coli* or injected with saline. The PLCs were stained with fluorescence-conjugated mAbs against CD14, CD11b, and CD3 for 30 minutes at room temperature in the dark, followed by washing twice with PBS. The stained cells were sorted using BD FACS Aria II by the methods described in Figure 5(g). The neutrophils were sorted by selection for $CD3^-CD14^-CD11b^+$ cells, and T cells were sorted by selection for $CD3^+$ cells, according to the manufacturer's recommendations. T cells (defined as $CD3^+$ cells with a purity >83% of living cells) and PMNs (defined as $CD3^-CD14^-CD11b^+$ cells with a purity >96.7% of living cells) were obtained.

*2.9. qRT-PCR.* Total RNA was isolated from the PLCs with Trizol reagent and reverse-transcribed using cDNA Synthesis Kit. Quantitative real-time PCR (qRT-PCR) was performed using two-step SYBR green qPCR assays and the target mRNAs were identified by the specific primers as follows: IL-17A, forward: 5′-AAGGCAGCAGCGATCATCCCT; reverse: 3′-TCTTCATTGCGGTGGAGAGTCC; GAPDH, forward: 5′-ATCACCATCTTCCAGGAGCGA; reverse: 3′-TCTCGTGGTTCACACCCATCA. The data were acquired using the Step One™ real-time PCR system (Applied Biosystems). The procedure of the target mRNA amplification was as follows: 1 cycle at 95°C (30 seconds) followed by 40 cycles at 95°C (5 seconds) and 64°C (31 seconds). Each assay plate included negative controls with no template. The relative amount of gene expression was calculated according to the formula $2^{-\Delta\Delta Ct}$, in which $\Delta Ct$ = [Ct(gene) − Ct(GAPDH)] and Ct is the crossing threshold value returned by the PCR instrument for every gene amplification.

*2.10. Statistical Analysis.* Comparisons between groups were conducted using analysis of Student's *t*-tests. Survival curves of mice were estimated using the Kaplan-Meier method and compared using the log-rank test. We considered the resulting *P* values of less than 0.05 (95% CI) to be statistically significant. Statistics were analyzed using GraphPad Prism 5.0 for Windows (San Diego, CA).

## 3. Results

*3.1. The Establishment of E. coli Induced Septic Peritonitis in Mice.* To understand how neutrophils contribute to the development of sepsis, we first established a murine model of septic peritonitis induced by *Escherichia coli* (*E. coli*). The female ICR mice, 10 in each group, were intraperitoneally injected with $2.4 \times 10^8$, $1.6 \times 10^8$, $0.8 \times 10^8$, and $0.4 \times 10^8$ colony forming units (CFUs) of *E. coli*, respectively, and then monitored for their physical conditions and survival. Around 6 hours after the infection, all of the 10 mice received $2.4 \times 10^8$ CFUs of *E. coli*, 8 out of the 10 mice received $1.6 \times 10^8$ CFUs of *E. coli*, and 5 out of the 10 mice that received $0.8 \times 10^8$ CFUs of *E. coli* began to exhibit multiple neurological symptoms, including dispirited behavior, staggered gait, and trembling, but these phenomena were not present in all of the 10 mice that received $0.4 \times 10^8$ CFUs of *E. coli* or saline. Around 18 hours after the infection, the infected mice began to die. Around 48 hours, the infected mice underwent a massive death, especially in those that received high infectious dose of *E. coli*. By 72 hours after the infection, 80%, 60%, and 40% of the mice infected with 2.4, 1.6, and $0.8 \times 10^8$ CFUs of *E. coli*, respectively, died and yet 100% of the mice which were infected with $0.4 \times 10^8$ CFUs of *E. coli* or received saline survived (Figure 1). The results indicated that the infection with *E. coli* at $1.6 \times 10^8$ CFUs and $2.4 \times 10^8$ CFUs could induce septic peritonitis in ICR mice.

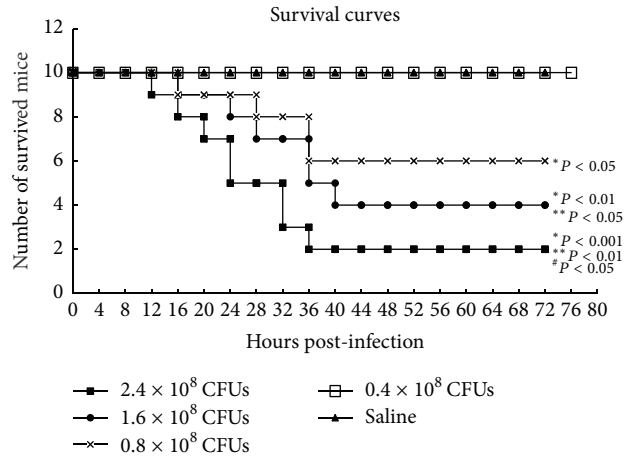
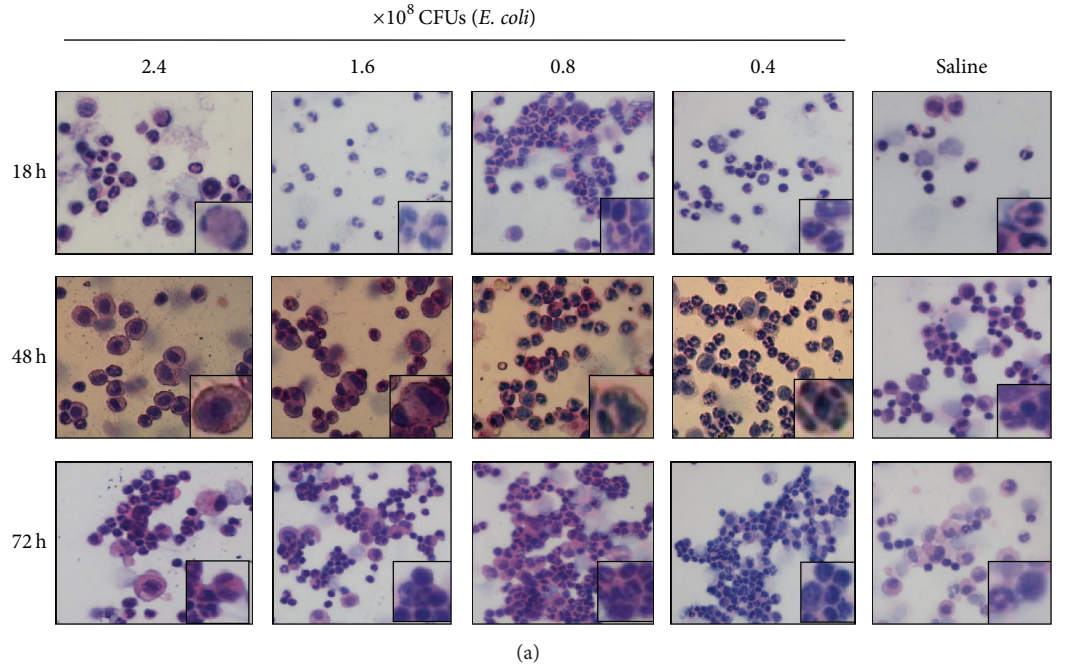


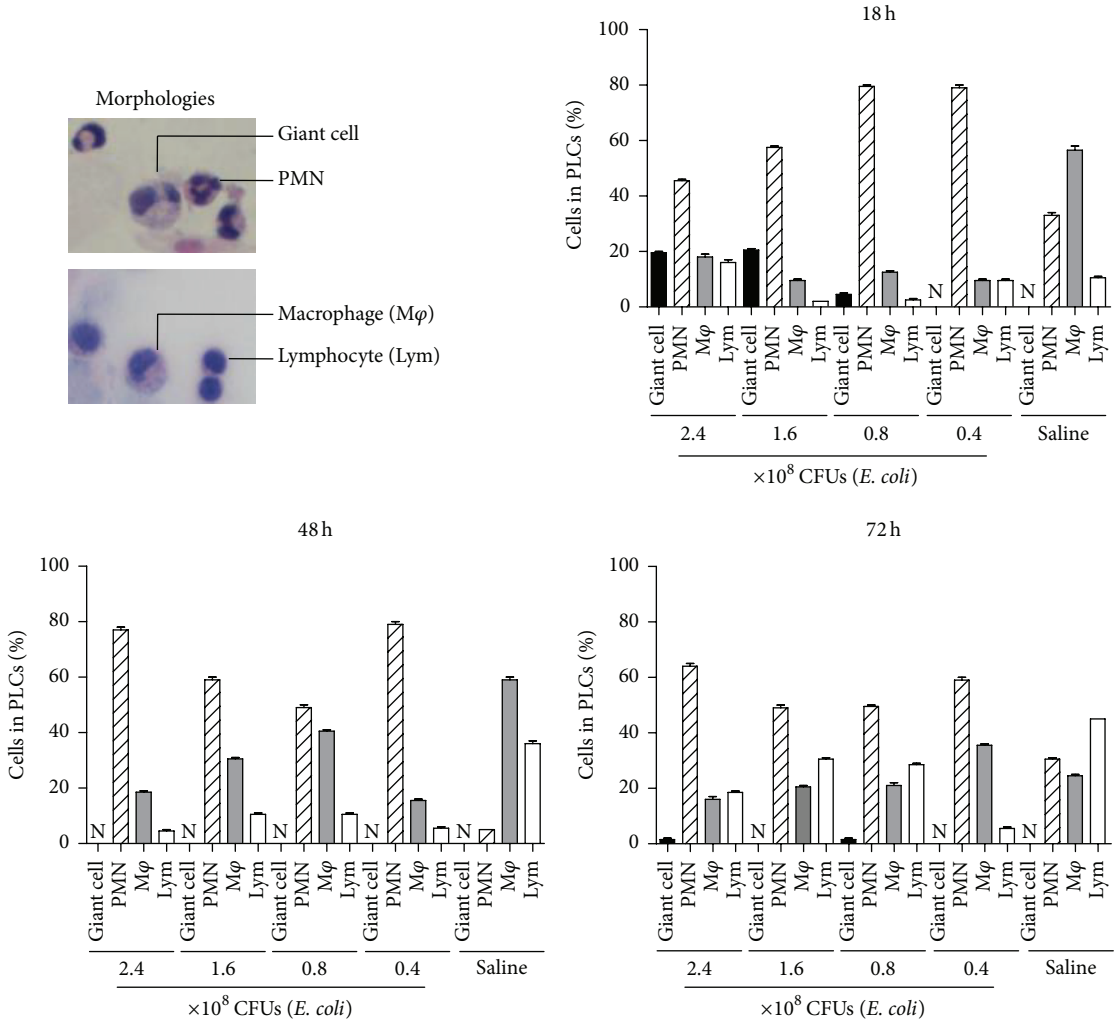

Figure 1: Survival curves of mice with septic peritonitis induced by *E. coli*. ICR mice were infected intraperitoneally with *E. coli* at different doses for inducing septic peritonitis. Saline-injected mice were as controls. The survival of the mice was counted. *Compared to control mice; **compared to mice infected with $0.4 \times 10^8$ CFUs of *E. coli*; #compared to mice infected with $1.6 \times 10^8$ CFUs of *E. coli*.

*3.2. The Infiltration and Morphology of Neutrophils in PLCs of Mice with Septic Peritonitis Induced by E. coli.* Since the polymorphonuclear cells (PMNs) are documented as the first innate immune cells recruited at inflammation sites and play a central role in host defense [21], we next collected the peritoneal lavage cells (PLCs) of the mice at 18, 48, and 72 hours after infection, respectively, for observing the infiltration and morphology of the PMNs in the PLCs. The collected PLCs were fixed on glass slides, stained with H&E dye, and photographed. A massive infiltration of PMNs was observed in the PLCs from the infected mice. At 18 hours after infection, in the PLCs of the mice infected with 1.6 and $2.4 \times 10^8$ CFUs of *E. coli*, the majority of the cells with the size of 0.2~0.4 micrometer were PMNs with a typical lobulated nucleus. Interestingly, there occurred a portion of giant cells, with the size of nearly 1.0~1.6 micrometer, characterized by lobulated nucleus that was squeezed to the marginal zone of the inner cell membrane. However, at 48 or 72 hours, the giant cells disappeared in the PLCs of the mice infected with 1.6 and $2.4 \times 10^8$ CFUs of *E. coli* (Figure 2(a)). Morphologically, there are four types of cells in the PLCs, including PMNs, macrophages, lymphocytes, and the giant cells (Figure 2(b)). When counting the cells, we found that at 18, 48, and 72 hours, PMNs constituted 50%–80% and macrophages constituted 20%–40% of the PLCs from the mice infected with 4 doses of *E. coli*, respectively, compared with nearly 30% for PMNs and 60% for macrophages in the PLCs from the mice that received saline. At 18 hours, interestingly, there were about 20% of the giant cells in the PLCs of the mice infected with 1.6 and $2.4 \times 10^8$ CFUs of *E. coli* (Figure 2(b), upper right). Noticeably, when the mice died of the infection, we collected the PLCs immediately and observed and found that plenty of bacteria coexisted with the PMNs in the PLCs of the mouse infected with $1.6 \times 10^8$ CFUs of *E. coli* for 31 hours (Figure 2(c)). These results indicate that neutrophils can move to infected peritoneal cavity by infiltration and change their morphologies after engulfing bacteria possibly.

*3.3. The Correlation of Neutrophil Numbers in PLCs with Severity of Septic Peritonitis in Mice Infected with E. coli.* During septic peritonitis, a large number of PMNs were recruited to the peritoneal cavity of the infected mice. To find the correlation of the numbers of PMNs at various time points with the severity of the disease in mice infected with *E. coli*, the PLCs of the mice were collected at 18, 48, and 72 hours after infection, counted by hemocytometer or flow cytometry after staining with fluorescence-labeled mAb of anti-CD45 and anti-CD11b. At 18 hours, the numbers of PLCs were nearly $1 \times 10^6$ cells/mL of peritoneal lavage fluid (PLF) in the mice received saline (control mice), significantly increased in the mice infected with 3 doses of *E. coli* in a dose dependent manner, and even reached nearly $3 \times 10^6$ cells/mL of PLF in the mice infected with $2.4 \times 10^8$ CFUs of *E. coli* ($P < 0.05$). At 48 hours, the numbers of PLCs were less than $1 \times 10^6$ cells/mL of PLF in control mice and significantly increased to over $3 \times 10^6$ cells/mL of PLF in the mice infected with three doses of *E. coli* and even to about $4 \times 10^6$ cells/mL of PLF in the mice infected with 1.6 and $2.4 \times 10^8$ CFUs of *E. coli* ($P < 0.05$). At 72 hours, the numbers of PLCs were decreased to $0.5 \times 10^6$ cells/mL of PLF in control mice and decreased to nearly $0.5 \times 10^6$ cells/mL of PLF in the mice infected with three doses of *E. coli* (Figure 3(a)). In the PLCs from the infected mice, most of the cells were $CD45^+$ nucleated leukocytes, representing PMNs, macrophages, and lymphocytes, respectively. At 18




(a)




(b)

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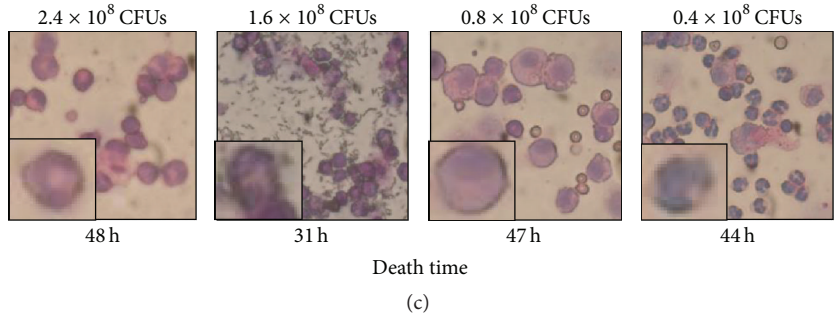
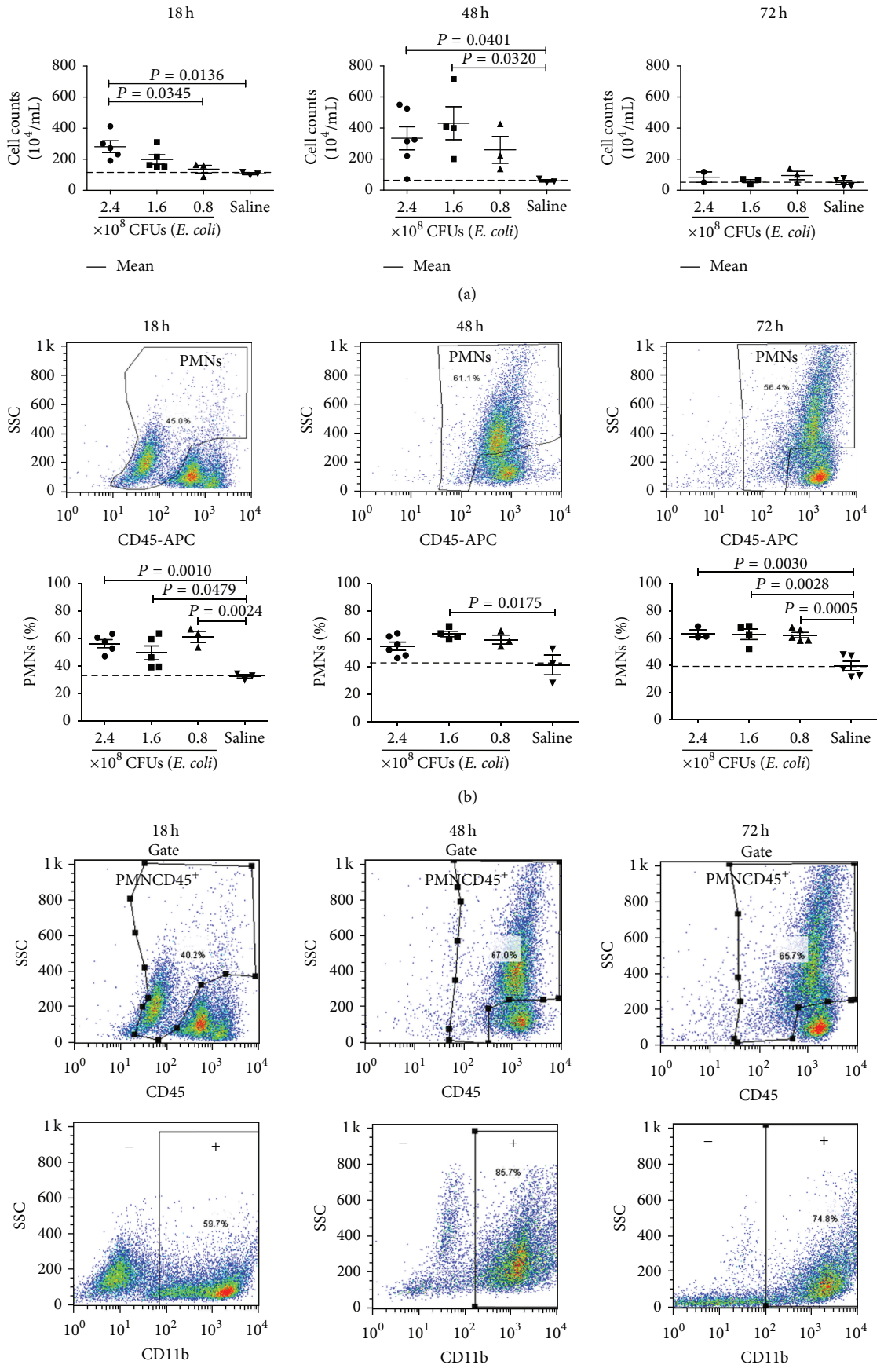



Figure 2: Morphologies of peritoneal lavage cells (PLCs) from mice infected intraperitoneally with *E. coli*. ICR mice were infected intraperitoneally with 3 doses of *E. coli* or received saline and then sacrificed at 18, 48, and 72 hours, respectively, for harvesting their PLCs. The PLCs were stained with H&E dye followed by taking photos and counting cell numbers under a microscope. (a) Photos of PLCs from killed mice. (b) The cell counts based on the PLC morphologies. (c) Photos of PLCs from dying mice. Each group is composed of 6 mice.

hours, the PMNs, macrophages, and lymphocytes were displayed on the left, middle, and right of the histogram, respectively, sequentially based on their nuclear structure and fluorescence intensity. At 48 and 72 hours, the PMNs were found to be shifted to the right upper quadrant, indicating that the PMNs were with more ovulated nuclei and expressed elevated CD45. As shown in Figure 3(b), at 18 hours after infection, the $CD45^+$ PMNs constituted 61.5%, 49.6%, and 56.4% of the PLCs in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, compared with 32.5% in the control mice. At 48 hours, the $CD45^+$ PMNs were increased to 59.5%, 63.7%, and 54.7% in the PLCs from the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, compared with 41.2% from the control mice. At 72 hours, the $CD45^+$ PMNs constituted 62.2%, 62.8%, and 63.5% of the PLCs in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, compared with 39.5% from the control mice. The PMNs were further detected as $CD11b^+CD45^+$ PMNs and $CD11b^-CD45^+$ PMNs, and their ratios in the PMNs were calculated. As shown in Figure 3(c), the $CD11b^+CD45^+$ PMNs constituted 49%, 36%, and 13% of the PMNs in the PLCs from the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli* for 18 hours, respectively. At 48 hours after the infection, the $CD11b^+CD45^+$ PMNs constituted 91%, 88%, and 86% in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively. At 72 hours after the infection, the $CD11b^+$ PMNs reached around 96% in all infected mice. Oppositely, at 18 hours, the $CD11b^-$ PMNs constituted 51%, 64%, and 87% of the PMNs in the PLCs from the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively. At 48 hours after the infection, the $CD11b^-$ PMNs constituted 9%, 12%, and 14% in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively. At 72 hours after the infection, the $CD11b^-$ PMNs only accounted for around 4% in all infected mice (Figure 3(c)). The results indicated that the peritoneal infection of *E. coli* induced massive infiltration of $CD45^+$ PMNs. Among the $CD45^+$ PMNs, $CD11b^+$ PMNs were increased with the progression of infection. In parallel, the $CD11b^-$ PMNs were decreased. At 18 and 48 hours after infection, the ratios of $CD11b^+$ PMNs or $CD11b^-$ PMNs were correlated with the doses of *E. coli* negatively or positively.

*3.4. The Expression of Surface TLR9 (sTLR9) on Neutrophils and Macrophages in PLCs with Development of Septic Peritonitis in Mice Infected with E. coli.* Since primary human and mouse blood neutrophils were reported to be able to express a functional sTLR9 [10], we tried to detect whether the expression of sTLR9 was different on $CD11b^+$ PMNs and $CD11b^-$ PMNs and correlates with development of septic peritonitis in mice. The PLCs collected from the mice which were infected with *E. coli* or received saline were gated for detecting the expression of sTLR9 on both $CD11b^+$ and $CD11b^-$ PMNs (Figure 4(a)). Results showed that, at 18 hours after infection, the ratios of $sTLR9^+CD11b^+$ PMNs were increased to 57%, 51%, and 70% of the $CD11b^+$ PMNs in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively ($P < 0.05$), compared to 12% in the mice that received saline control (control mice). At 48 hours, the ratios of $sTLR9^+CD11b^+$ PMNs were decreased to 2.3%, 2.3%, ($P < 0.05$) and 8% in the infected mice, respectively, and less than 13% as in control mice. At 72 hours, the ratios of $sTLR9^+CD11b^+$ PMNs were 7%, 5.5%, and 9% in the infected mice, respectively, compared with 18% in control mice (Figure 4(b)). The ratios of $sTLR9^+CD11b^-$ PMNs constituted 52%, 53%, and 68.5% of the $CD11b^-$ PMNs in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, for 18 hours ($P < 0.05$), compared with 3.5% in control mice, sharply decreased to 9%, 6%, and 9.5% in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, for 48 hours, compared with 8% in control mice, and reached 10%, 6%, and 10% in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, for 72 hours, compared with 8% in control mice (Figure 4(c)). These results indicated that *E. coli* could significantly induce the occurrence of both of the $sTLR9^+CD11b^+$ PMNs and $sTLR9^+CD11b^-$ PMNs in the peritoneal cavity at early stage of infection. Considering the fact that macrophages are the inflammatory cells which overlapped with the PMNs in the inflammatory sites, we also observed sTLR9 expression on macrophages in the PLCs. The results showed that the ratios of $sTLR9^+$ macrophages constituted 5%, 6%, and 6.7% ($P < 0.05$) of the macrophages in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, for 18 hours, compared with 3% in control mice, decreased to 0.9%

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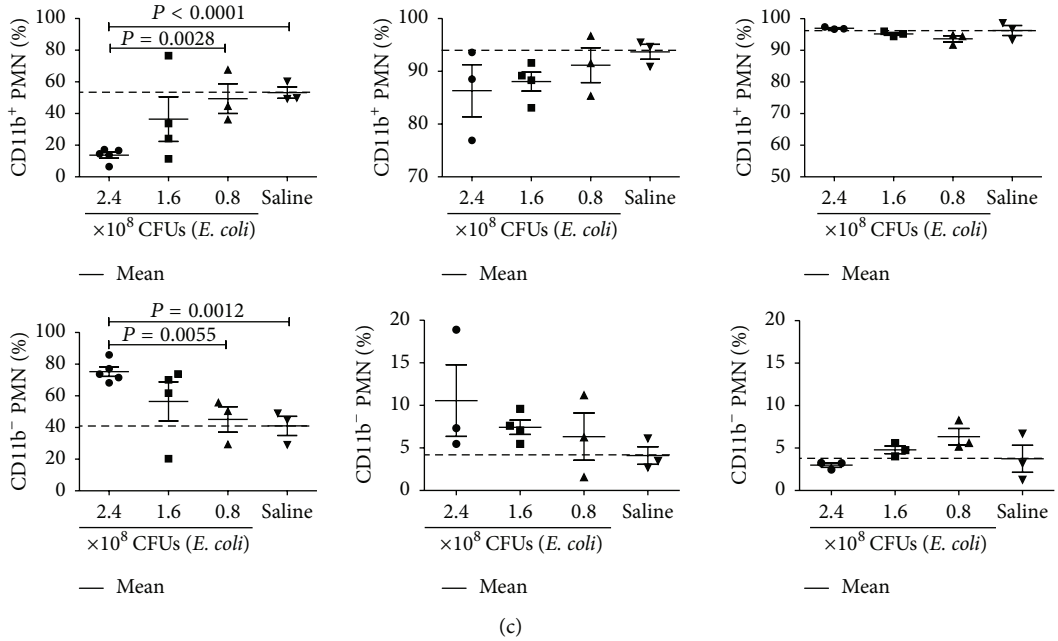
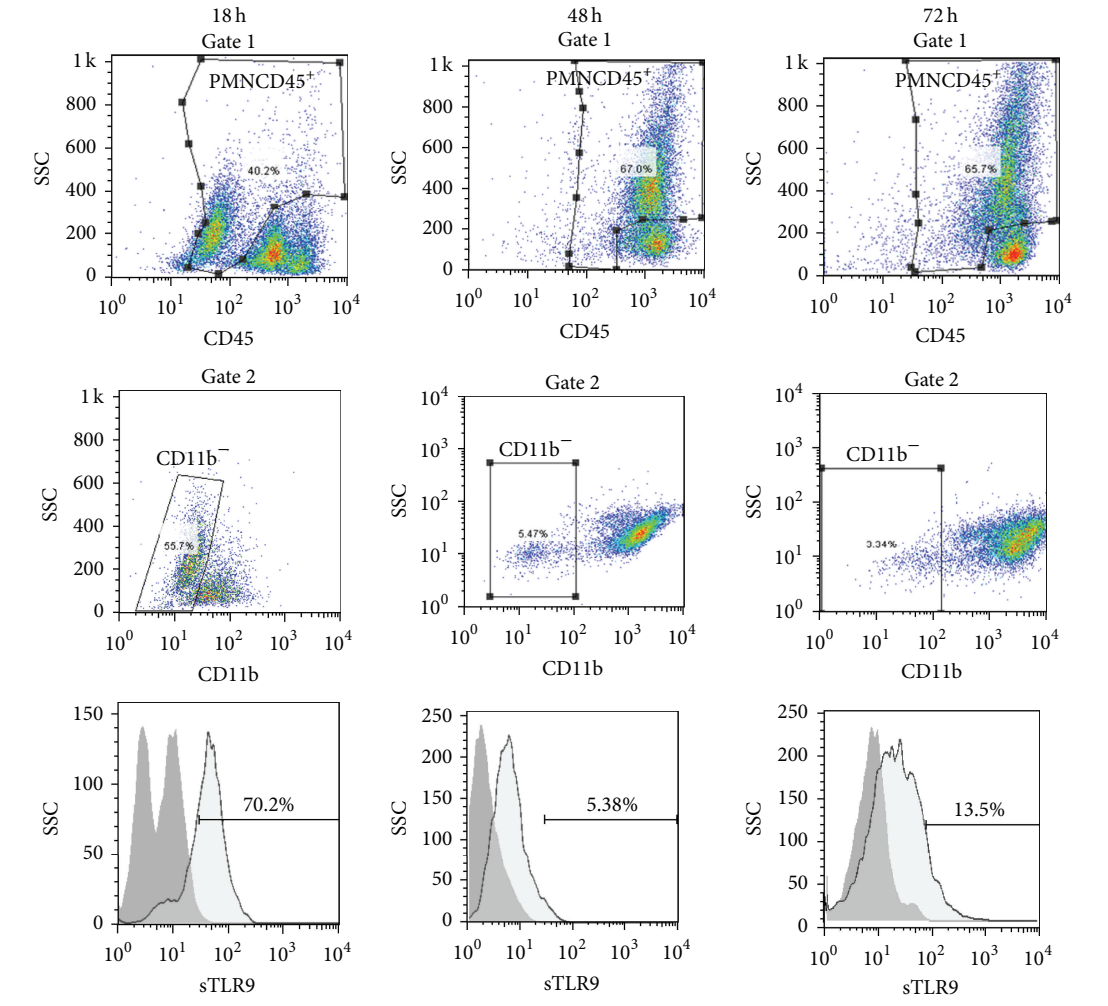



Figure 3: The numbers of PLCs and PMNs in mice infected intraperitoneally with *E. coli*. ICR mice were infected intraperitoneally with 3 doses of *E. coli* or received saline and sacrificed at 18, 48, and 72 hours to harvest their PLCs. The PLCs were counted immediately or stained with fluorescence-labeled mAbs of anti-CD45 and anti-CD11b followed by numerating with flow cytometry. (a) Numbers of PLCs. (b) Ratios of PMNs in PLCs. (c) Ratios of $CD11b^+$ PMNs and $CD11b^-$ PMNs in the PMNs. Each point represents the data from one mouse.

($P < 0.05$), 0.45% ($P < 0.05$), and 3% in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, for 48 hours, compared with 5% in control mice, and reached 4.8%, 2% ($P < 0.05$), and 2.8% in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, for 72 hours, compared with 5.5% in control mice (Figure 4(d)). These results indicated that, unlike PMNs, the macrophages barely express increased sTLR9 during septic peritonitis.

*3.5. Expression of Interleukin-17 (IL-17) in Neutrophils in PLCs from Mice with Septic Peritonitis Induced by E. coli.* In recent years, primary human and mouse neutrophils have been found to be able to display autocrine IL-17 activity that probably contributes to the etiology of microbial and inflammatory diseases [17]. To find whether the different subtype neutrophil derived IL-17 was involved in the development of septic peritonitis, we detected IL-17 expression in the $CD11b^+$ PMNs and $CD11b^-$ PMNs (Figure 5(a)) of the PLCs collected from the mice which were infected with *E. coli* or received saline (control mice). It was found that the ratios of $IL\text{-}17^+CD11b^+$ PMNs constituted 7%, 8.5%, and 10% of the $CD11b^+$ PMNs in PLCs of the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, for 18 hours ($P < 0.05$), compared with 25% in control mice, was 2% ($P < 0.05$), 7.5% ($P < 0.05$), and 25.5% in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, for 48 hours, compared with 25% in control mice, and reached 6%, 10%, and 8% in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, for 72 hours ($P < 0.05$), compared with 27% in control mice (Figure 5(b)). Noticeably, the saline injection seemed to stimulate neutrophils to express IL-17. To confirm this, the PLCs were collected from the mice injected with saline at 18, 48, or 72 hours after the injection, respectively, and stained with fluorescence-labeled mAb against CD45, CD11b, and IL-17, followed by detection using flow cytometry. The control mice in this experiment were not injected with saline. We found that saline stimulation tended to induce IL-17 expression (not statistically significant) with a big range of variation individually (data not shown). The ratios of the $IL\text{-}17^+CD11b^-$ PMNs were 5%, 4%, and 3% of the $CD11b^-$ PMNs in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, for 18 hours, compared with 7% in control mice; 3%, 11%, and 21.5% in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, for 48 hours, compared with 7% in control mice; and 7%, 8%, and 13% in the mice infected with 0.8, 1.6, and $2.4 \times 10^8$ CFUs of *E. coli*, respectively, for 72 hours, compared with 9% in control mice (Figure 5(c)). The results indicated that both of the $CD11b^+$ PMNs and the $CD11b^-$ PMNs could not increase IL-17 expression during the development of septic peritonitis in mice induced by *E. coli*. Also, as shown in Figure 5(d), macrophages could not increase the IL-17 expression in response to *E. coli*. Obviously, the results were in disagreement with the reports showing that PMNs could increase the production of IL-17 during bacterial infections. To validate the *in vivo* expression of IL17 in the PLCs, the PLCs pooled from the naïve mice were cocultured with *E. coli* at $1 \times 10^5$ CFUs, $1 \times 10^6$ CFUs or saline for 14 hours, and then with BFA for another 4 hours. The cultured cells were harvested, stained with FITC-labeled anti-IL-17 mAb, and detected by flow cytometry. As shown in Figure 5(e), *E. coli* could significantly increase $IL\text{-}17^+$ PLCs of the naïve mice, indicating that *E. coli* could stimulate

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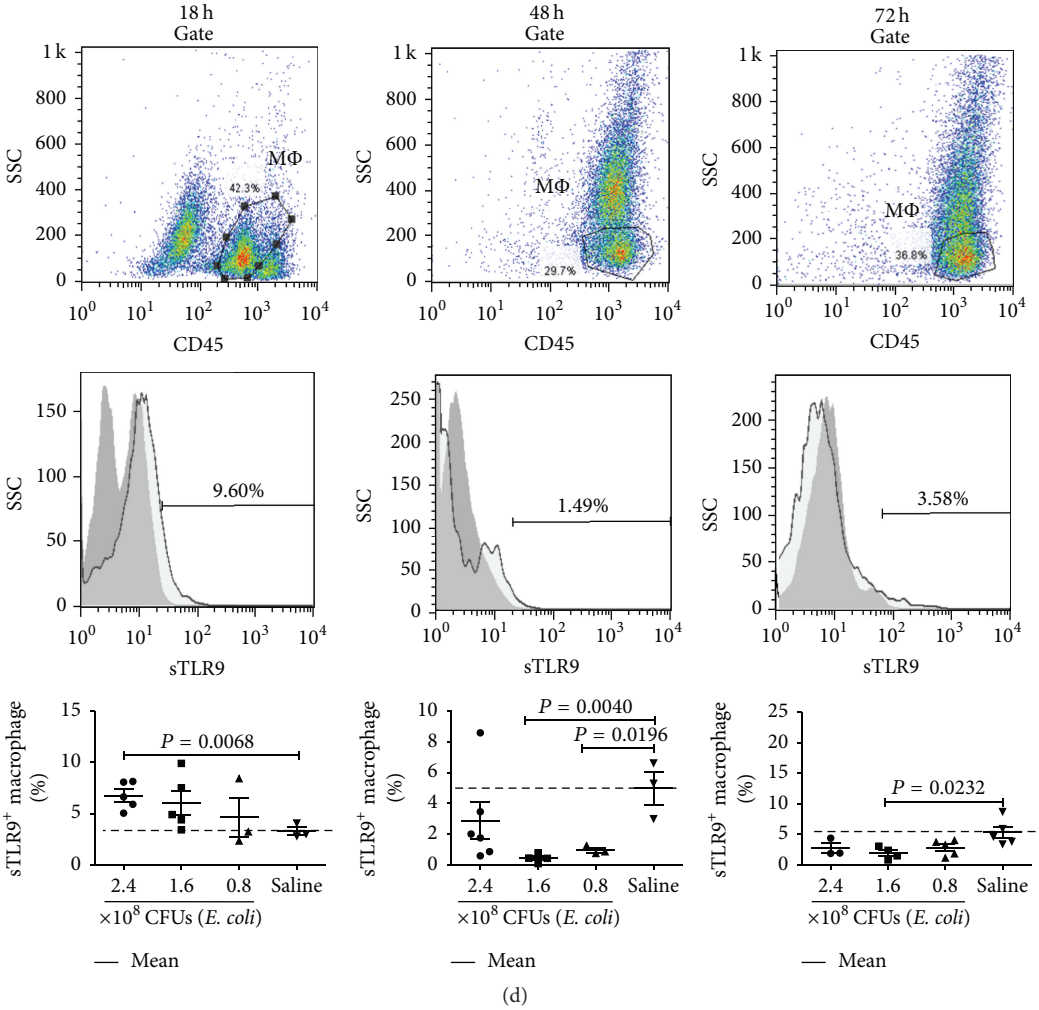
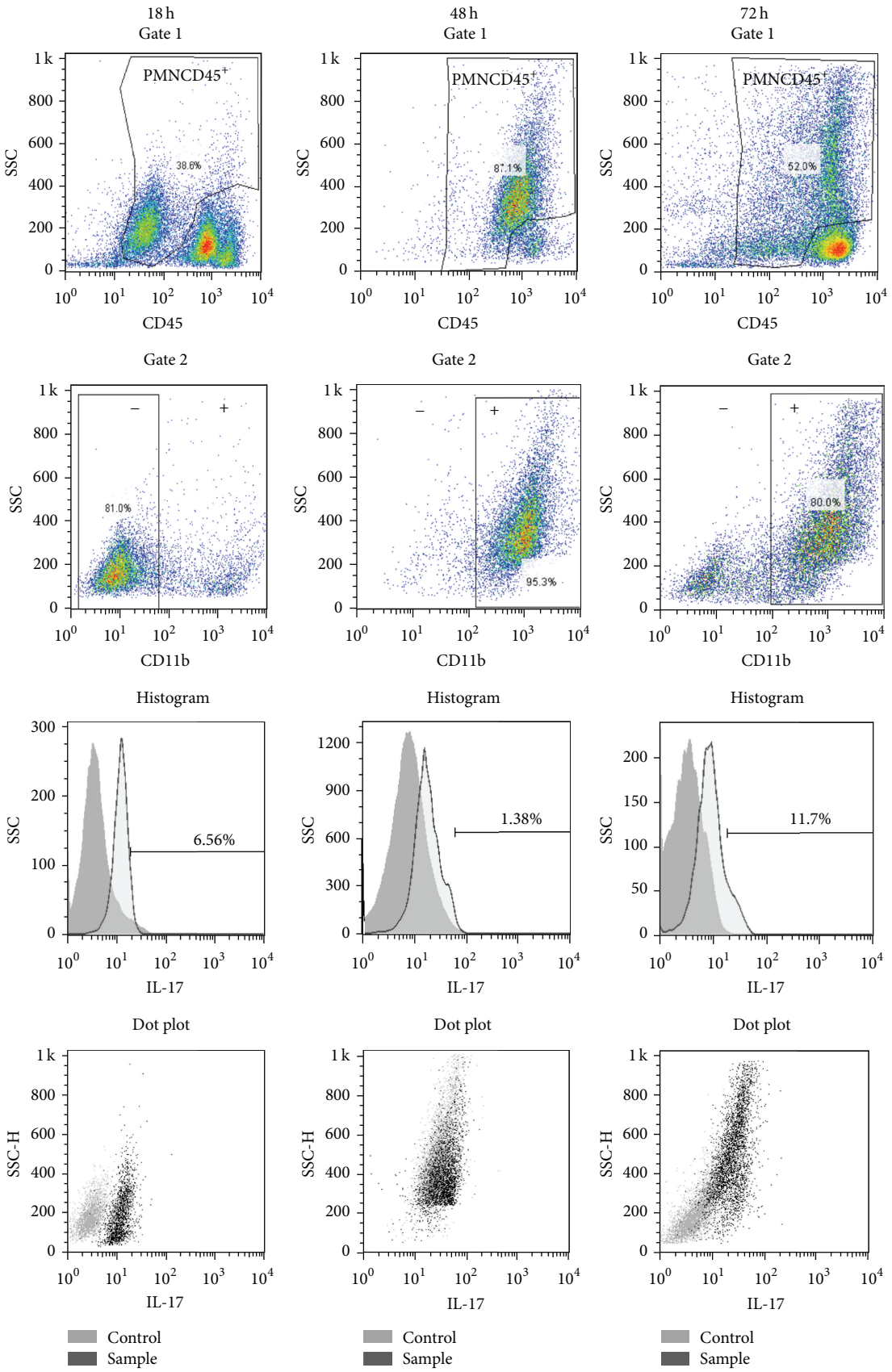



Figure 4: The ratios of sTLR9$^+$ PMNs and macrophages in PLCs from mice infected intraperitoneally with *E. coli*. ICR mice were infected intraperitoneally with 3 doses of *E. coli* or received saline and sacrificed at 18, 48, and 72 hours to harvest their PLCs. The PLCs were stained with fluorescence-labeled mAb of anti-CD45, anti-CD11b, and anti-TLR9 followed by detection with flow cytometry. (a) Gates of the PMNs from the CD45$^+$ PLCs followed by gating CD11b$^+$ PMNs or CD11b$^-$ PMNs from the PMNs. (b) Ratios of sTLR9$^+$ PMNs in CD11b$^+$ PMNs. (c) Ratios of sTLR9$^+$ PMNs in CD11b$^-$ PMNs. (d) Ratios of sTLR9$^+$ macrophages. Each point represents the data from one mouse.

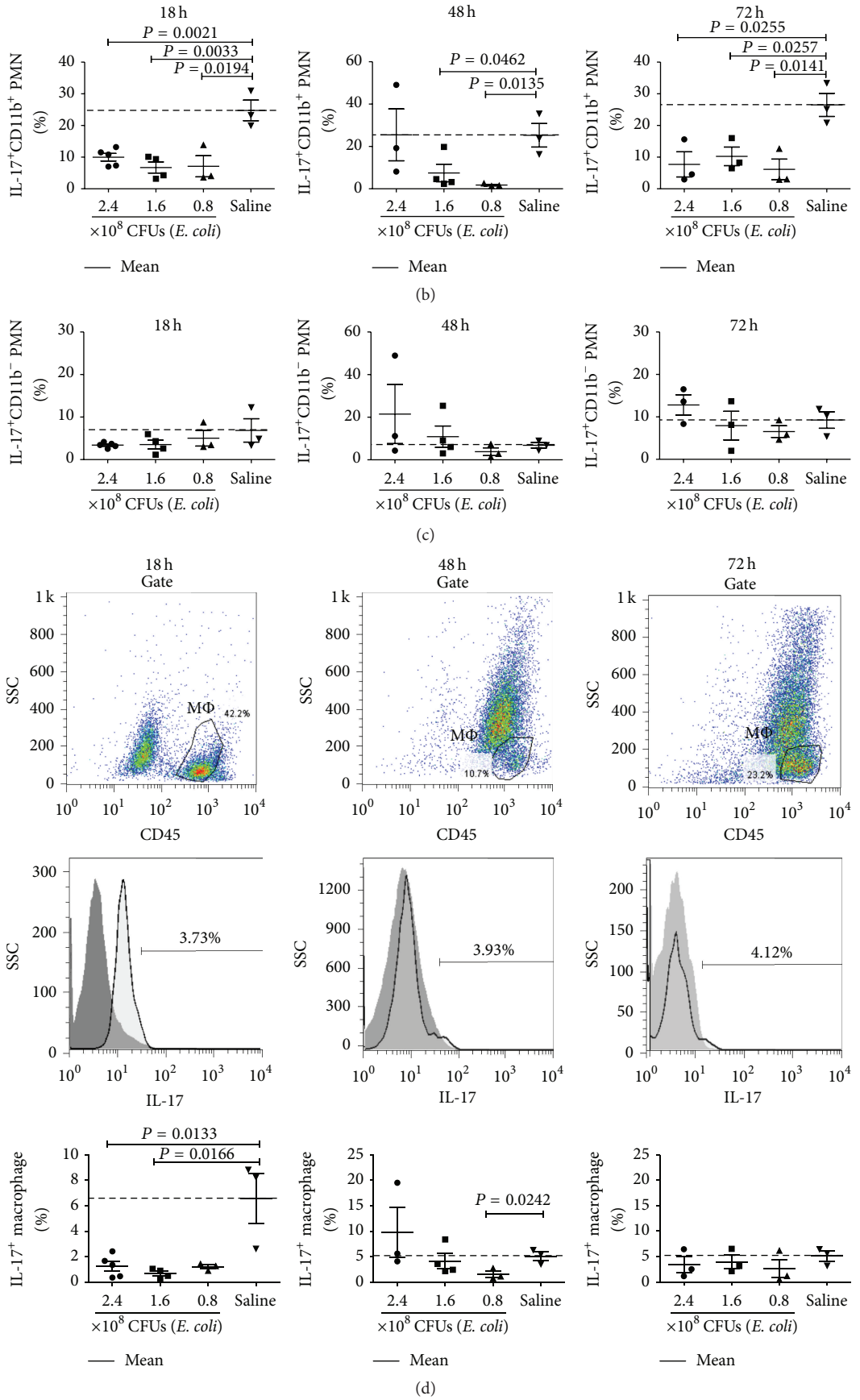
the PLCs to express IL-17. To clarify this, we set up an experiment to detect IL-17 mRNA expression in the PLCs from the mice infected with 0.8, 1.6, and 2.4 × $10^8$ CFUs of *E. coli* for 18 hours, respectively, or from the control mice and found that the IL-17 mRNA levels in the mice infected with 3 doses of *E. coli* were significantly elevated in a dose dependent manner (Figure 5(f)). Next we sorted CD3$^+$ cells and CD3$^-$CD14$^-$CD11b$^+$ cells from the PLCs of the mice which were infected with *E. coli* or received saline for 18 hours, detected their IL-17 mRNA expression by qRT-PCR, and found that IL-17 mRNA levels were significantly increased in both of the CD3$^+$ T cells ($P = 0.0006$) and the CD3$^-$CD14$^-$CD11b$^+$ cells ($P = 0.0434$) in the PLCs from the *E. coli* infected mice. Comparatively, the IL-17 mRNA levels in the CD3$^+$ T cells were much higher than those in the CD3$^-$CD14$^-$CD11b$^+$ neutrophils. The result indicated that both of T cells and PMNs were the IL-17 producers in the peritoneal cavity during the development of septic peritonitis (Figure 5(g)). The result suggests that the PLCs from the infected mice did express IL-17 and IL-17 in the PMNs might be secreted out during the development of septic peritonitis.

*3.6. Correlation of sTLR9 Expression with IL-17 Production in Neutrophils at Early Stage of E. coli Induced Septic Peritonitis in Mice.* To find the possible correlation between the production of IL-17 and the expression of sTLR9 in/on the PMNs and macrophages in peritoneal cavity of the mice during septic peritonitis, we harvested the PLCs from the mice infected with 0.8, 1.6, and 2.4 × $10^8$ CFUs of *E. coli*, respectively, at early stage of 8 hours after infection and detected their expression of IL-17 and sTLR9. The saline injected mice were as controls. The expression was represented by mean fluorescence intensity (MFI) to emphasize the increased expression of IL-17 and sTLR9 per cell. As shown in Figure 6, IL-17 expression




(a)

Figure 5: Continued.

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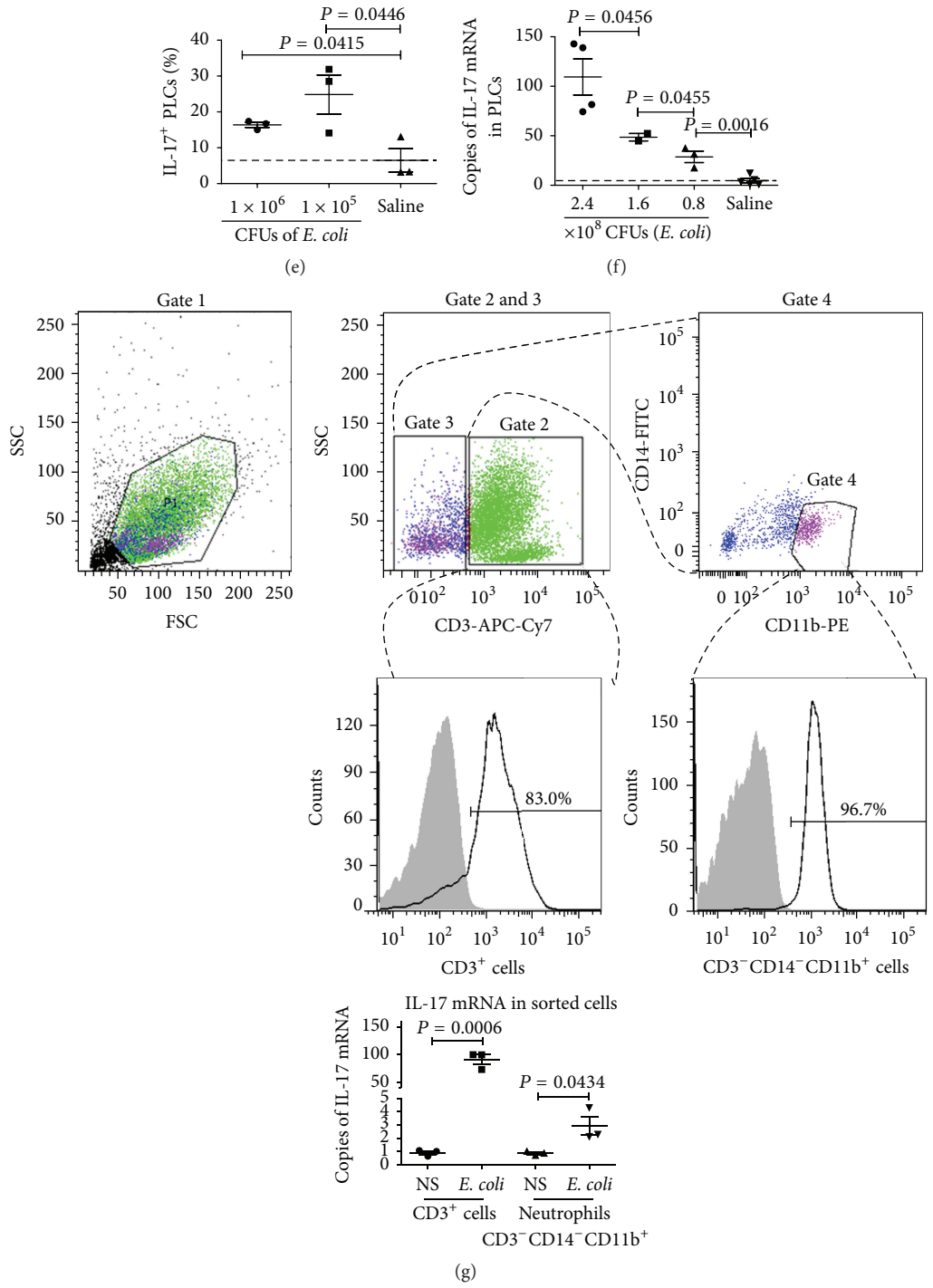


Figure 5: The ratios of IL-17$^+$ PMNs and macrophages in PLCs from mice infected with *E. coli* intraperitoneally. ICR mice were infected with *E. coli* at 3 doses or received saline and sacrificed at 18, 48, and 72 hours to harvest their PLCs. The PLCs were stained with fluorescence-labeled mAb of anti-CD45, anti-CD11b, and anti-IL-17, followed by detection with flow cytometry or lysed to isolate total RNA for amplifying IL-17 mRNA by qPCR. (a) PMNs gated from the CD45$^+$ PLCs (gate 1) and CD11b$^+$ PMNs or CD11b$^-$ PMNs from the gated PMNs (gate 2). (b) Ratios of IL-17$^+$ PMNs in CD11b$^+$ PMNs. (c) Ratios of IL-17$^+$ PMNs in CD11b$^-$ PMNs. (d) Ratios of IL-17$^+$ macrophages. (e) Percentages of IL17$^+$ PLCs in the PLCs cocultured with *E. coli*. (f) Copies of IL-17 mRNA in the PLCs. Each point represents the data from one mouse. (g) IL-17 mRNA expression in sorted CD3$^+$ cells or CD3$^-$CD14$^-$CD11b$^+$ neutrophils.

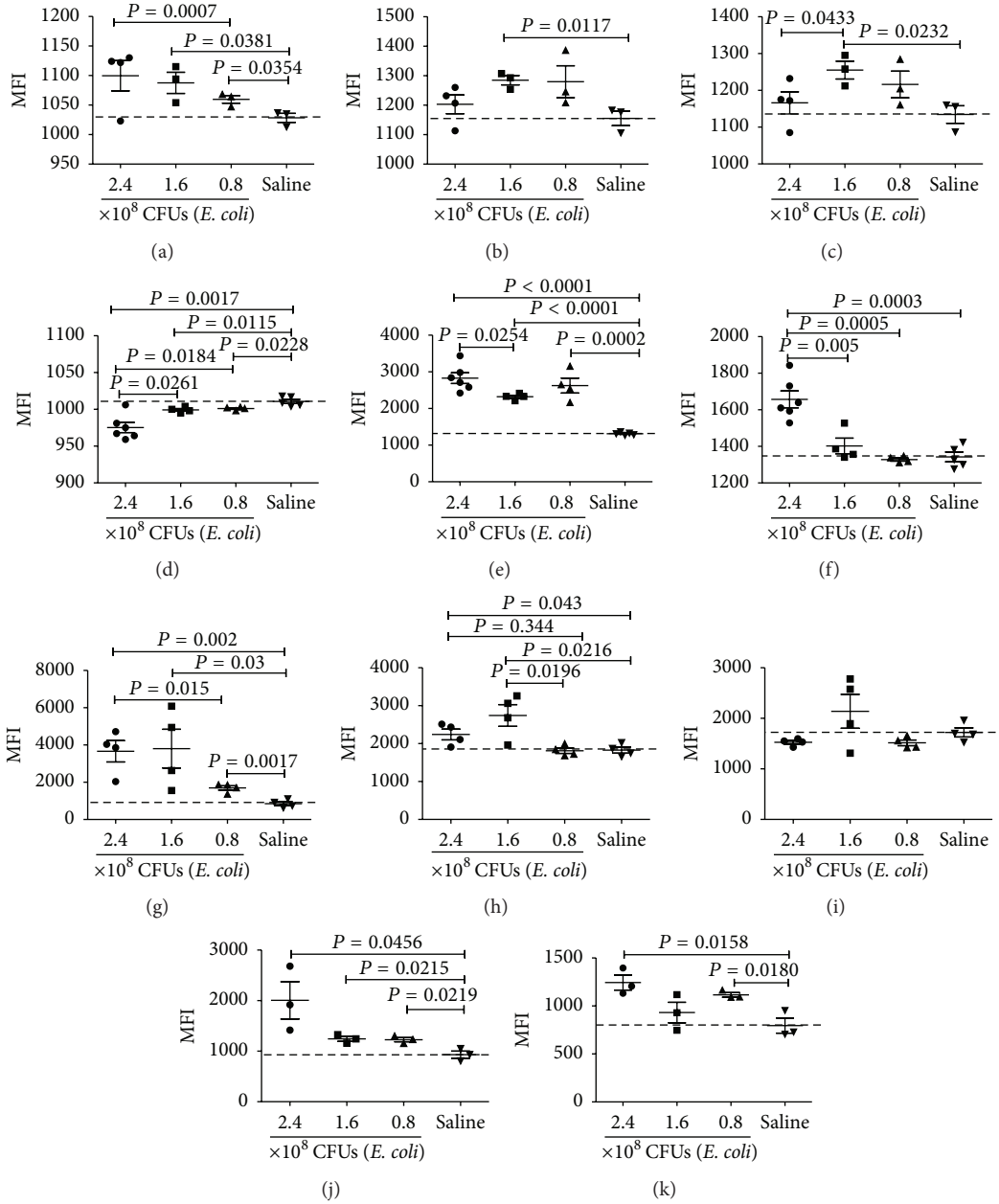


Figure 6: The expression of IL-17 and sTLR9 in/on PMNs, macrophages, and T cells in the PLCs from mice infected with *E. coli* intraperitoneally at early stage after infection. ICR mice which were infected with *E. coli* at 3 doses or received saline were sacrificed at 8 hours after infection for harvesting their PLCs. The PLCs were stained with fluorescence-labeled mAb of anti-CD45, anti-CD11b, anti-IL-17, anti-sTLR9, anti-CD3, and anti-Ly6G, followed by detection with flow cytometry. The expression levels of IL-17 and sTLR9 in various cells of the PLCs were indicated by mean fluorescence intensity (MFI). (a) IL-17 in $CD11b^-$ PMNs. (b) IL-17 in $CD11b^+$ PMNs. (c) IL-17 in macrophages. (d) sTLR9 on $CD11b^-$ PMNs. (e) sTLR9 on $CD11b^+$ PMNs. (f) sTLR9 on macrophages. (g) sTLR9 on $CD11b^+Ly6G^+$ PMNs. (h) IL-17 in $CD11b^+Ly6G^+$ PMNs. (i) IL-17 in $sTLR9^+CD11b^+Ly6G^+$ PMNs. (j) IL-17 in $CD3^+$ PLCs. (k) IL-17 in $CD3^-CD11b^+$ PMNs. Each point represents the data from one mouse.

was significantly upregulated in the $CD11b^-$ PMNs from the infected mice and their levels were positively correlated with the doses of *E. coli* (Figure 6(a)), while the upregulated IL-17 expression was only observed in the $CD11b^+$ PMNs or macrophages from the mice infected with $0.8 \times 10^8$ CFUs and $1.6 \times 10^8$ CFUs of *E. coli*. The highest dose of *E. coli* could not induce the highest levels of IL-17 in the $CD11b^+$ PMNs or macrophages after infection for 8 hours (Figures 6(b) and 6(c)). This result reveals that IL-17 may be mainly derived from $CD11b^-$ PMNs and secrete out earlier during bacterial infection in mice infected with high dose of *E. coli*. In parallel, we also tested the expression of sTLR9 on PMNs and macrophages and found that sTLR9 expression was significantly downregulated in the $CD11b^-$ PMNs from

the infected mice and the lowest downregulation happened in the $CD11b^-$ PMNs from the mice infected with $2.4 \times 10^8$ CFUs of *E. coli* (Figure 6(d)). In contrast, sTLR9 expression was significantly upregulated in the $CD11b^+$ PMNs from the infected mice (Figure 6(e)). Interestingly, the significantly upregulated expression of sTLR9 was observed also in the macrophages from the mice infected with $2.4 \times 10^8$ CFUs of *E. coli* (Figure 6(f)). To validate whether other types of cells in the PLCs also expressed IL-17 and sTLR9, the PLCs from mice infected with *E. coli* for 8 hours were stained with fluorescence-labeled mAbs against Ly6G, CD11b, IL-17, or TLR9 and then analyzed by flow cytometry. The results showed that *E. coli* infection significantly increased IL-17 expressing $CD11b^+Ly6G^+$ neutrophils (Figure 6(h)) as well as sTLR9 expressing $CD11b^+Ly6G^+$ neutrophils (Figure 6(g)) in a dose dependent manner but could not increase IL-17 expression in the $sTLR9^+CD11b^+Ly6G^+$ neutrophils (Figure 6(i)) at the time point. In parallel, the PLCs were stained with fluorescence-labeled mAbs against CD3, CD11b, and IL-17 and analyzed. It was found that both $CD3^+$ T cells (Figure 6(j)) in the PLCs and $CD3^-CD11b^+$ PMNs (Figure 6(k)) increased their IL-17 expression in response to the infection with *E. coli* for 8 hours. This result indicates that, at early stage, sTLR9 may be mainly expressed on $CD11b^+$ PMNs in PLCs of the mice infected with *E. coli*, and with the infection progression, macrophages may become successors to replace the $CD11b^+$ PMNs as the major $sTLR9^+$ cells.

## 4. Discussion

In this study, we tried to investigate the correlation of TLR9 expression with IL-17 production in PMNs during septic peritonitis and found that both sTLR9 and IL-7 could be expressed in the PMNs infiltrated into the peritoneal cavity of the mice infected with *E. coli*. At early stage of the infection, sTLR9 was increasingly expressed in the infiltrated $CD11b^+$ PMNs, and IL-17 was increasingly expressed in both of the $CD11b^-$ PMNs and $CD11b^+$ PMNs. IL-17 expression in $CD11b^-$ PMNs was positively correlated with the doses of *E. coli*. When infected with the highest dose of *E. coli* ($2.4 \times 10^8$ CFUs), IL-17 was increasingly expressed and sTLR9 was decreasingly expressed in/on the $CD11b^-$ PMN. When infected with the lowest dose of *E. coli* ($0.8 \times 10^8$ CFUs), both IL-17 and sTLR9 were increasingly expressed in $CD11b^+$ PMNs. Furthermore, we stained the PLCs with both CD11b mAb and Ly6G mAb and confirmed that the $CD11b^+Ly6G^+$ PMNs in the PLCs could increase their expression of both IL-17 and sTLR9 in response to *E. coli* infection. Taken together, with the fact that highest dose of *E. coli* ($2.4 \times 10^8$ CFUs) was the most deadly and the lowest dose of *E. coli* was the least deadly to the mice with septic peritonitis, we may deduce that increased expression of both sTLR9 and IL-17 in $CD11b^+$ PMNs might benefit the survival of the mice with septic peritonitis and that the decreased expression of sTLR9 and increased expression of IL-17 in $CD11b^-$ PMNs may be detrimental to the mice.

In recent years, PMNs have been found to be able to express sTLR9 which engage PAMPs, such as bacterial DNA generated during infection, and damage-associated molecular patterns (DAMPs), such as mitochondrial DNA released from necrotic cells during sterile inflammation. The engagement, if intense, can prime PMNs to release massively produced cytokines [22], leading to sepsis or systemic inflammatory response syndrome (SIRS) [12]. In the present work, we found that the ratios of the $sTLR9^+CD11b^+$ PMNs and $sTLR9^+CD11b^-$ PMNs were significantly increased in the infiltrated $CD11b^+$ PMNs and $CD11b^-$ PMNs of the mice infected with *E. coli* for 18 hours, respectively. At this stage, we observed that a plenty of *E. coli* coexisted with the PMNs. When observed at 48 hours and 72 hours after infection, the ratios of both of the $sTLR9^+CD11b^+$ PMNs and $sTLR9^+CD11b^-$ PMNs were sharply decreased to the levels as those in saline control. Accompanying with the decrease, *E. coli* underwent disappearing. Possibly, the increased $TLR9^+CD11b^+$ PMNs and $sTLR9^+CD11b^-$ PMNs present a rapid innate immune response of the PMNs to bacterial invasion at the early stage of infection. PMNs armed with sTLR9 were demonstrated to be able to sense extracellular ligands and consequently initiate TLR9 mediated signaling in an intracellular TLR9 independent way. The response could offer a rescue mechanism for PMN activation when pathogen derived TLR9 ligands cannot reach the endosome in the early stage of infection [10]. Practically, the increased expression of sTLR9 on PMNs may be a proinflammatory activation marker [10] and $sTLR9^+$ PMN should benefit antibacterial defense during infection, evidenced by the following: (1) TLR9 agonists, if they exist, could activate $sTLR9^+$ PMN, not TLR9-deficient PMNs, to upregulate CD11b and secrete MIP-2 and IL-8 (CXCL8) [10]; (2) $sTLR9^+$ PMNs are involved in inducing the rapid inflammation which is needed in the initial phase of bacterial infection by secreting antimicrobial peptides and elastases [9]; (3) encountering microbial DNA, sTLR9 signaling is able to activate PMNs, and the activated PMN increases sTLR9 expression [9]. However, it is worthy to note that $sTLR9^+$ PMNs could be culprit for causing more severe pathological consequences. For instance, bacterial DNA or formulated peptides released following sepsis were reported to activate p38 MAP kinase through binding sTLR9 on PMNs, leading to acute lung injury which is characterized by protein-rich pulmonary oedema (swelling) and accumulation of large numbers of PMNs in the lungs [13].

It is well established that PMNs, as the firstly recruited inflammatory cells in infection sites, are IL-17 producing cells. IL-17 secreted from PMNs induces the release of proinflammatory factors from mesenchymal and myeloid cells, recruiting additional PMNs [15]. In addition to PMNs, macrophages located in the epithelial barriers are also important sources of IL-17. In this study, we initially found that the average ratios of the $IL17^+CD11b^+$ PMNs and $IL17^+$ macrophages were significantly decreased in the PLCs at 18, 48, and 72 hours in the mice infected with 3 doses of *E. coli*. Seemingly, the $CD11b^+$ PMNs and macrophages, in response to the peritoneal infection, could not produce increased IL-17. However, when checking mRNA expression, we found significantly upregulated IL-17 mRNA in the PLCs from the mice infected with *E. coli* for 18 hours (Figure 5(e)). The data imply that the infiltrated cells in peritoneal cavity of the mice infected with *E. coli*

could produce IL-17 and the produced IL-17 may promptly secrete out the cells, therefore resulting in the decreased ratios of $IL17^+CD11b^+$ PMNs and $IL17^+$ macrophages. To clarify this, we harvested the PLCs from the mice infected with *E. coli* at early stage of 8 hours after infection and detected their IL-17 levels which were expressed as mean fluorescence intensity (MFI). By doing this, we found that both of the $CD11b^-$ PMNs and $CD11b^+$ PMNs obviously produced IL-17 in the early stage of the peritoneal infection caused by *E. coli*. To confirm this, we set up another experiment in which the PLCs were harvested at 12 and 16 hours after the infection, cultured with BFA, a protein transport inhibitor capable of retaining the produced cytokines inside the cells, for additional 4 hours, and then detected their IL-17 expression. We found that the BFA *in vitro* treatment significantly increased the average ratios of the $IL17^+CD11b^+$ PMNs in $CD11b^+$ PMNs and $IL17^+$ macrophages in macrophages (data not shown). The results support the assumption that produced IL-17 in $CD11b^+$ PMNs and macrophages could be secreted out promptly in response to *E. coli* at the early stage of septic peritonitis in mice. Theoretically, deducing based on the published data, IL-17 derived from $CD11b^+$ PMNs and macrophages may initiate a response to induce chemokines which in turn recruit more PMNs [23, 24] to the site of infection [18, 25]. The new comer PMNs join the fighting against invaded bacteria, resulting in more IL-17 [16, 17]. In addition to the PMNs, macrophages could be recruited by IL-17 [20, 26], specifically by $CD11b^+$ PMNs derived IL-17. IL-17 could recruit more macrophages into peritoneal cavity during septic peritonitis. The increased macrophages could produce more IL-17 to join the $CD11b^+$ PMNs produced IL-17, playing a defense role in fighting bacterial infection and reducing mortality of mice with septic peritonitis [27]. In addition to the PMNs and macrophages, $\gamma\delta$ T cells were found as a major producer of IL-17 in the mice with experimental sepsis induced by CLP; $\gamma\delta$ T cell-derived IL-17 could promote production of proinflammatory mediators, resulting in enhanced lethality [28]. Compatibly, T cells, in this study, were confirmed to be involved in the development of septic peritonitis in mice by the evidence that (1) the IL-17 expression was increased in the $CD3^+$ cells of the PLCs from the mice infected with *E. coli* and (2) IL-17 mRNA levels were significantly increased in the sorted $CD3^+$ cells in the PLCs from the mice infected with *E. coli*. Furthermore, IL-17 expression was obviously increased in the sorted $CD3^-CD11b^+$ PMNs in the PLCs from the mice infected with *E. coli*. Together, these data suggest that the PMNs, like T cells, do express IL-17 as a response to bacterial infection during septic peritonitis. Although $CD3^+$ cells were one of the major producers of IL-17 during the development of septic peritonitis, probably, neutrophils might produce more IL-17 compared to $CD3^+$ cells because the neutrophils, as we found in this study, could constitute up to 70% of the PLCs (Figure 3(b)), whereas $CD3^+$ cells only constituted 10–15% of the PLCs (data not shown). Interestingly, $sTLR9^-CD11b^+Ly6G^+$ PMNs, not $sTLR9^+CD11b^+Ly6G^+$ PMNs, were found to be able to increase their IL-17 expression during septic peritonitis.

Interestingly, we found a type of giant cells with increasing numbers in the PLCs from the mice infected with *E. coli* up to $1.6 \times 10^8$ CFUs. Morphologically, the giant cells (Figure 2(a)) could be the PMNs with a changed lobulated nucleus located near to cell membrane, being at least 4 times larger than the regular PMNs. Obviously, the giant cells are the newly described cells and have several facets worthy of note. (1) Kinetically, the giant cells only appeared at the early stage of the infection and were not observed in the PLCs of the mice infected for 48 and 72 hours. (2) The giant cells only occurred in the PLCs of the severely infected mice, not in the PLCs of the mice infected with less amount ($0.4–0.8 \times 10^8$ CFUs) of *E. coli*. (3) The giant cells could constitute 20% of the PLCs. Possibly, the giant cells were developed from the regular PMNs after engulfing a large amount of bacterial pathogens. Biologically, the appearance of the giant cells could be used as a cell marker for signifying a real danger because the giant cells were intimately correlated with the severity of the infection and the death of the mice.

## Conflict of Interests

The authors declare that there is no conflict of interests.

## Acknowledgments

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*Research Article*

# The Th17/Treg Immune Imbalance in Ulcerative Colitis Disease in a Chinese Han Population

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*Objective*. To investigate the Th17/Treg immune balance in the ulcerative colitis (UC) patients in a Chinese Han population. *Methods*. Ninety UC patients and 30 healthy subjects were enrolled. The serum IL-17 and TGF-$\beta$1 levels of these participants were measured with ELISA; the percentage of Th17 and Treg cells in peripheral blood was determined with flow cytometry. *Results*. In UC patients, the levels of IL-17 and Th17 were significantly higher compared with healthy subjects; the percentage of Th17 and IL-17 level in moderate and severe subgroup was significantly higher than in mild subgroup; a positive correlation existed between these two indexes and clinical activity index and endoscopic evaluation. TGF-$\beta$1 level and Treg cells in UC patients were lower than healthy subjects. TGF-$\beta$1 level in moderate and severe subgroup was lower than in mild subgroup. There was a negative linear correlation between Treg cells and clinical activity index, endoscopic evaluation. A positive correlation was detected between Treg cells and TGF-$\beta$1 level. *Conclusions*. Th17/Treg immune imbalance might play a crucial role in the development of UC. To induce the production of Treg cells and TGF-$\beta$1, inhibit the level of Th17 and IL-17, and thus recover the Th17/Treg immune balance might imply new therapeutic targets in UC management.

## 1. Introduction

Ulcerative colitis (UC), as one of the major inflammatory bowel diseases (IBD), is a debilitating disorder of the gastrointestinal tract, characterized by a dysregulated immune response to unknown environmental triggers, including the inflammation of the intestine mucosa and submucosa, with a group of clinical symptoms of diarrhea, mucus purulent blood stool, and abdominal pain [1, 2]. Currently UC is incurable, yet the symptoms can be managed through anti-inflammatory steroids or immunosuppressants [3]. UC prevalence is currently highest in Europe (505 per 100,000 persons) and North America (249 per 100,000 persons) [4]. Now the global prevalence is rising, including Asian area [5], with rapid increases in incidence rates in younger people, placing an increased strain on healthcare resources; thus it represents a significant global health burden that is of growing concern [6].

Recent progresses in the immune mechanisms implicated in chronic inflammatory disorders have led to a more in-depth knowledge of the pathogenesis of UC; some common T cell mediated mechanisms for inflammation—Th17/Treg immune balance—are recognized as crucial mediators of the tissue damage causing mucosal ulceration of the colon in ulcerative colitis, yet the pathogenic role of this specific immune balance is still controversial [7].

Th17 and Treg cells are both originated from CD4+ T cells, circulating in peripheral blood or in the spleen. Th17 (CD4+ IL-17+) cells, as one of the inflammatory helper T cells and an important proinflammatory cells, promote inflammation progressing with specific secretion of IL-17 [8]. Treg (CD4+ CD25+ Foxp3+) cells mainly secrete some cytokines such as transforming growth factor-beta (transforming growth factor-beta, TGF-$\beta$) and act as suppressor cells with unique immune regulation [9]. The normality of Treg cells plays crucial role in the maintenance of the body's immune tolerance,

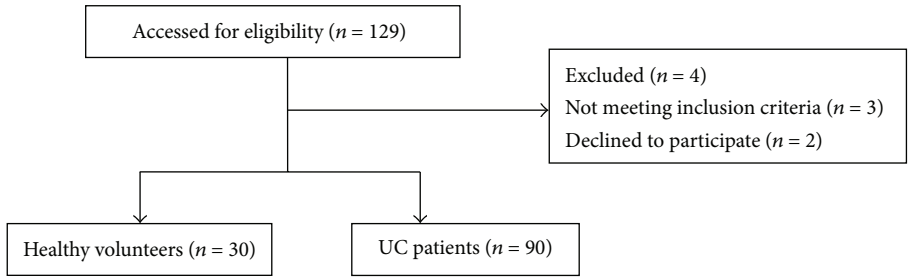


Figure 1: Study flow through study.

Treg cells accomplish their biofunctions via the inhibiting cytokines [10], which are important in the production and the proliferation of Treg cells and their mediation of immune tolerance [11].

UC is regarded as a kind of chronic inflammatory disease, under this condition, Th17 increases and Treg cell decreases. In our previous study, it was proved that the levels of IL-17 and Th17 in serum and colon of UC patients were significantly higher, yet the TGF-$\beta$1 and Treg cells were much lower compared with healthy control subjects [12]. Resveratrol in low dose (50 mg/kg·d) was reported to be able to regulate the Th17/Treg balance mainly through reducing the number of Th17 cells, and resveratrol in high dose (100 mg/kg·d) regulates this balance through both downregulating the number of Th17 and upregulating the number of Treg cells, thus to improve the inflammation in UC [13]. However, in some other studies, different conclusions were yielded; it was reported that only in moderate and severe UC patients, IL-17 could be determined, and the inflammation level was positively correlated with the level of IL-17 [14]. The Treg cells function was regarded as normal in the peripheral blood of UC patients; the number of Treg cells decreased during disease active stage, yet it increased during the disease remission period; the expression of Treg cells was higher in inflammation locations in mucous membrane of colon compared with noninflammation locations, yet still lower than control group [15].

Given these controversial studies, the variation of Th17/Treg immune balance in UC patients was still unclear. Therefore we conducted this clinical study to further investigate the change of Th17/Treg immune balance in a Chinese Han population with UC as well as the potential underlying mechanism.

## 2. Materials and Methods

*2.1. Patients.* Ninety Chinese Han patients with UC were recruited from the General Hospital of Shenyang Military Region from July, 2010, to February, 2014. Thirty healthy volunteers were enrolled from the neighbouring community. The diagnosis of UC was made based on clinical, laboratory, endoscopic, and histologic examinations with reference to the suggested guidelines for the diagnosis and treatment of IBD, which were approved in China in 2007 by the Chinese Medical Association Society of Digestive Disease Branch of inflammatory bowel disease Collaborative Group [16]. The study flow chart is shown in Figure 1.

All UC patients enrolled were aged between 18 and 70 years, diagnosed with UC, the initial onset, chronic persistent, or chronic relapsing type. Diagnostic facilities for high-quality endoscopy, radiology, and pathology should be available. Priority was given to the mild and moderate UC patients; cases with severe disease would also be enrolled if they did not need emergency therapy.

None of the enrolled subjects had any serious complications, such as local stricture, intestinal obstruction, intestinal perforation, rectum polyp, toxic colonic dilatation, colon or rectum cancer, anus diseases, or any severe primary diseases in cardiovascular, cerebrovascular, liver, kidney, or hematopoietic system, or mental diseases. Women who were pregnant or preparing to be pregnant or lactating were excluded from the study.

The study was approved by the Ethics Committee of Beijing Dongzhimen Hospital (Approved number ECPJ-DBY-2010-013), in accordance with the World Medical Association Declaration of Helsinki. All participants signed written, informed consent before participating.

*2.2. Sample Collection and Preparation.* Peripheral venous blood (8 milliliters in each subject) was collected from each participant. The subjects had to be fasted for at least 12 hours before blood sample collection. The blood sample was kept in the room temperature for 1 h and then centrifugated for 10 minutes at 4°C, 3000 r/min. Supernatant was then frozen at −80°C. Information on gender, age, height, body weight, marital status, disease course, complications, medication, clinical activity index, and endoscopic evaluation were collected from each patient. Clinical activity index and endoscopic evaluation are determined with reference to the Chinese Medical Association Society of Digestive Disease Branch of inflammatory bowel disease Collaborative Group [16].

*2.3. Enzyme-Linked Immunosorbent Assay (ELISA).* ELISA assay was performed according to the manufacturer's instructions of the ELISA kits (RapidBio, USA) for IL-17 and TGF-$\beta$1. The OD value at 540 nm was measured. The concentrations of IL-17 and TGF-$\beta$1 were calculated according to the standard curve. The Varioskan Flash was purchased from Thermo Scientific, USA.

*2.4. Flow Cytometric Analysis.* For detecting the percentage of Th17 cells, the PBMCs were stimulated with 20 ng/mL phorbol 12-myristate-13-acetate and 1 $\mu$g/mL ionomycin in the presence of 2 mmol/mL monensin (Sigma-Aldrich, USA) in 24-well plates. After being stimulated for 4 hours (37°C, 5% $CO_2$), the cells were collected and washed once with PBS. The cells were then incubated with APC-CD3 antibody and PE-Cy5-CD4 antibody at 4°C for 30 minutes. Next, the cells were fixed and permeabilized and stained with anti-human PE-IL-17 antibody at 37°C for 25 minutes. For detecting the percentage of Treg cells, the PBMCs were washed in PBS. Then the cells were stained with APC-CD3, PE-cy5-CD4, PE-Cy7-CD8, and FITC-CD25 antibodies at 4°C for 30 minutes. Then the cells were incubated with PE-Foxp3 antibody after fixation and permeabilization according to the manufacturer's instruction. All stained cells were analyzed by flow cytometry (FACSCalibur) and FlowJo software (Tristar, USA). The forward angle scattering light (FSC) and side scattering light (SSC) were adjusted to select the lymphocytes. Different cell subsets were detected by different cell labeling and gating. CellQuest software was used for data analysis and the percentage of positive cells was recorded.

*2.5. Statistical Analysis.* All data were analyzed by SPSS 17.0 software. All data except demographic data were expressed as mean ± SD. Measurement data in normal distribution were analyzed using Student's $t$ test; variables deviated from normality were analyzed by nonparametric tests Wilcoxon rank sum test. Numeration data were analyzed using Chi-square analysis. Pearson linear correlation analysis was adopted for variables in normal distribution; otherwise Spearman rank correlation analysis was used. A $P$ value of less than 0.05 was considered statistically significant.

## 3. Results

*3.1. Demographic Features and Clinical Characteristics.* Among 90 UC patients, 51 (56.7%) were male, the age ranged from 29 to 64 yr (37.19 ± 8.50); in 30 healthy control subjects, 17 (56.7%) were male and the age ranged from 28 to 61 (36.30 ± 9.12); there were no significant differences between the two groups in gender and age distribution ($P > 0.05$) as shown in Table 1.

In UC patients, 46 cases (51.1%) were diagnosed as mild type, 38 (42.2%) as middle type, and 6 (6.7%) as severe type. The clinical disease activity index was 6.80 ± 2.31; endoscopic index was 5.16 ± 1.99 (mean ± SD).

*3.2. Serum IL-17 and TGF-β1 Levels in UC Patients and Healthy Subjects.* In UC patients, the serum IL-17 level was significantly increased and the TGF-$\beta$1 level decreased compared with healthy subjects ($P < 0.05$) as shown in Table 1 and Figure 2.

The UC patients were further classified into 2 subgroups based on their disease severity, mild type and moderate and severe type (only 6 patients were diagnosed as severe type; thus they were combined with the moderate type). The serum IL-17 level in moderate and severe type of UC patients was significantly higher than mild type of patients ($P < 0.05$), yet TGF-$\beta$1 showed no difference between the two subgroups as shown in Table 2.

Table 1: Demographic details and clinical characteristics of UC patients and healthy subjects.

| | UC group ($n = 90$) | Control group ($n = 30$) | $P$ value |
|---|---|---|---|
| Male sex, number (%) | 51 (56.7) | 17 (56.7) | 1.000 |
| Age, year | 37.19 ± 8.50 | 36.30 ± 9.12 | 0.889 |
| Clinical activity index | 6.80 ± 2.31 | — | — |
| Endoscopic index | 5.16 ± 1.99 | — | — |
| IL-17 (pg/mL) | 24.86 ± 7.44 | 13.49 ± 1.87 | <0.001* |
| TGF-$\beta$1 (pg/mL) | 31.77 ± 9.27 | 37.01 ± 7.75 | 0.002* |
| Th17 (%) | 1.80 ± 0.82 | 0.23 ± 0.12 | <0.001* |
| Treg (%) | 7.25 ± 1.04 | 10.14 ± 0.59 | <0.001* |

The numbers are shown as mean ± SD.

Table 2: Demographic details and clinical characteristics in mild type and moderate and severe type of UC patients.

| | Mild type ($n = 44$) | Moderate and severe type ($n = 46$) | $P$ value |
|---|---|---|---|
| Male sex, number (%) | 29 (65.91%) | 22 (47.83%) | 0.064 |
| Age, year | 38.75 ± 9.76 | 35.6957 ± 6.87 | 0.207 |
| Clinical activity index | 5.18 ± 1.45 | 8.35 ± 1.88 | <0.001* |
| Endoscopic index | 4.14 ± 1.03 | 6.13 ± 2.21 | <0.001* |
| IL-17 (pg/mL) | 20.98 ± 6.88 | 28.58 ± 5.95 | <0.001* |
| TGF-$\beta$1 (pg/mL) | 31.49 ± 5.63 | 32.04 ± 8.45 | 0.678 |
| Th17 (%) | 1.32 ± 0.59 | 2.26 ± 0.76 | <0.001* |
| Treg (%) | 7.53 ± 0.89 | 6.98 ± 1.10 | 0.006* |

The numbers are shown as mean ± SD. * stands for $P < 0.05$.

*3.3. The Percentage of Th17 and Treg Cells in Peripheral Blood.* In peripheral blood of UC patients, the percentage of Th17 increased significantly and Treg cells decreased significantly compared with healthy subjects ($P < 0.05$) as shown in Table 1 and Figure 1.

In the moderate and severe subgroups, the percentage of Th17 was higher and the percentage of Treg cells was lower compared with mild subgroup ($P < 0.05$) as shown in Table 2 and Figure 3.

*3.4. Correlation Analysis.* In correlation analysis, it was detected that there was a positive linear correlation between serum IL-17 level and clinical activity index ($P < 0.05, r > 0$); between serum IL-17 level and endoscopic evaluation ($P < 0.05, r > 0$); between the percentage of Th17 in peripheral blood and clinical activity index ($P < 0.05, r > 0$); between the percentage of Th17 in peripheral blood and endoscopic evaluation ($P < 0.05, r > 0$); between the percentage of Th17 in peripheral blood and serum IL-17 level of UC patients($P < 0.05, r > 0$); between the percentage of Treg cells in peripheral blood and serum TGF-$\beta$1 level of UC patients ($P < 0.05, r > 0$).

Table 3: Correlation analysis between the indexes in UC patients.

| | IL-17 | TGF-$\beta$1 | Th17 | Treg |
|---|---|---|---|---|
| Clinical activity index | 0.498 (0.000)$^{\triangle}$ | −0.032 (0.762) | 0.653 (0.000)$^{\triangle}$ | −0.404 (0.000)$^{\triangle}$ |
| Endoscopic index | 0.448 (0.000)$^{\triangle}$ | −0.068 (0.379) | 0.488 (0.000)$^{\triangle}$ | −0.419 (0.000)$^{\triangle}$ |
| IL-17 | — | — | 0.562 (0.000)$^{\triangle}$ | |
| TGF-$\beta$1 | — | — | | 0.209 (0.004$^{*}$) |

The numbers are shown as $r$ ($P$). $^{*}P < 0.05$. The correlation coefficient $r > 0$ shows positive correlation; $r < 0$ stands for negative correlation. $^{\triangle}P < 0.05$ and $|r| > 0.4$ indicates the significant correlation between the two indexes.

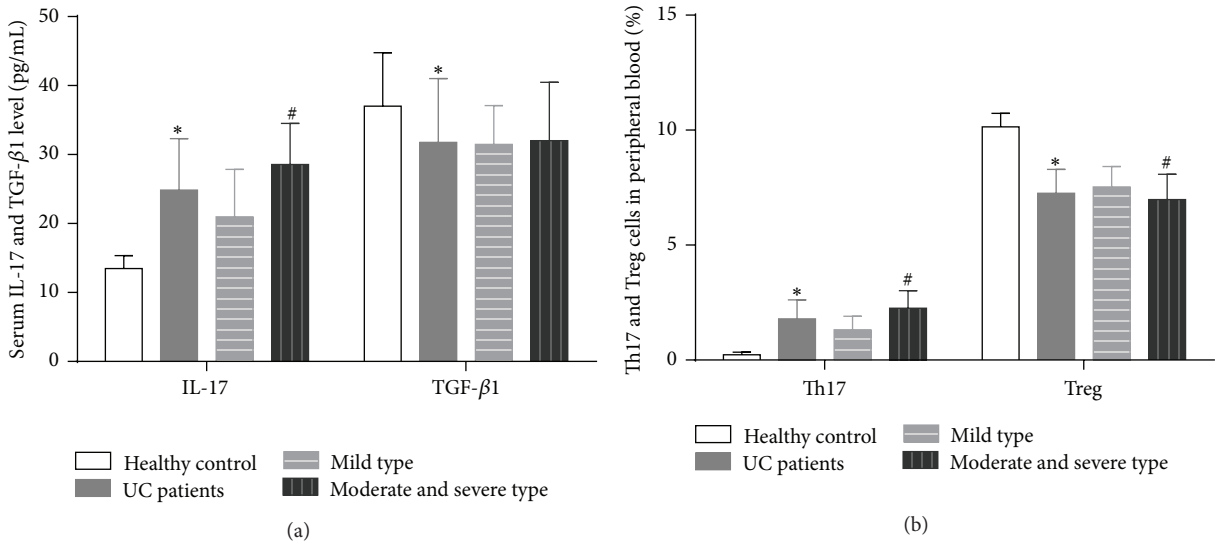


Figure 2: Serum IL-17 and TGF-$\beta$1 level (pg/mL) and percentage of Th17 and Treg cells in peripheral blood (%) in healthy control subject, UC patients, mild type, and moderate and severe subgroup. (a) Serum IL-17 and TGF-$\beta$1 level (pg/mL) in healthy control subject, UC patients, mild type, and moderate and severe subgroup. (b) Percentage of Th17 and Treg cells in peripheral blood (%) in healthy control subject, UC patients, mild type, and moderate and severe subgroup. $*$ presents $P < 0.05$ between UC patients and healthy control subject; # stands for $P < 0.05$ between mild type and moderate and severe type subgroups.

A negative linear correlation was detected between the percentage of Th17 in peripheral blood and clinical activity index ($P < 0.05$, $r < 0$) and between the percentage of Treg cells in peripheral blood and endoscopic evaluation ($P < 0.05$, $r < 0$). The correlation coefficient ($r$) and correlated $P$ value of the analysis were shown in Table 3.

## 4. Discussion

In this study, we verified the changing trends of serum Th17, Treg cells, IL-17, and TGF-$\beta$1 in UC patients, as well as their changes in patients with different disease severity and the correlation between the levels of these indexes and disease activity, thus to provide further evidence for the mechanism analysis in UC development.

UC is a chronic inflammatory bowel disorder with multifactorial pathogenesis factors. It has been proved that Th1/Th2 are involved in the development of UC [17, 18]; currently more and more evidence indicated that Th17/Treg balance is another key factor in UC.

Th17 and Treg cells are produced by CD4+ T cells and exist in spleen and peripheral blood in healthy individuals. Th17 and Treg cells are closely related in the process of differentiation and transformation, while they also can be independent or unified in the body's immune response which forms an immune balance as a switch of a variety of autoimmune diseases. In short, Th17 is one of the immune promoting cells, while Treg cell is a kind of immune suppressing cell; thus, the two kinds of cells and their balance are closely related to the immune function.

In UC patients, the increase of Th17 cell caused the higher level of serum IL-17, and the low Treg cell resulted in the decrease of serum TGF-$\beta$1; as a result, the autoreactive T cell was activated and inhibitive immune cytokines decreased, thus to aggravate the inflammation in the mucous membrane of colon. These results support that the Th17/Treg immune imbalance might play a crucial role in the development of UC. Thus to induce the production of Treg cells and TGF-$\beta$1, inhibit the level of Th17 and IL-17, and recover the Th17/Treg immune balance might imply new therapeutic targets in UC management.

Since Hovhannisyan et al. isolated Th17 and Treg cells from intestinal lamina propria of patients with IBD, the role of Th17 and Treg cells in pathogenesis of UC began to attract researcher's attention [19]. Then Ogino et al. proved that Treg cells were capable of suppressing colonic inflammation

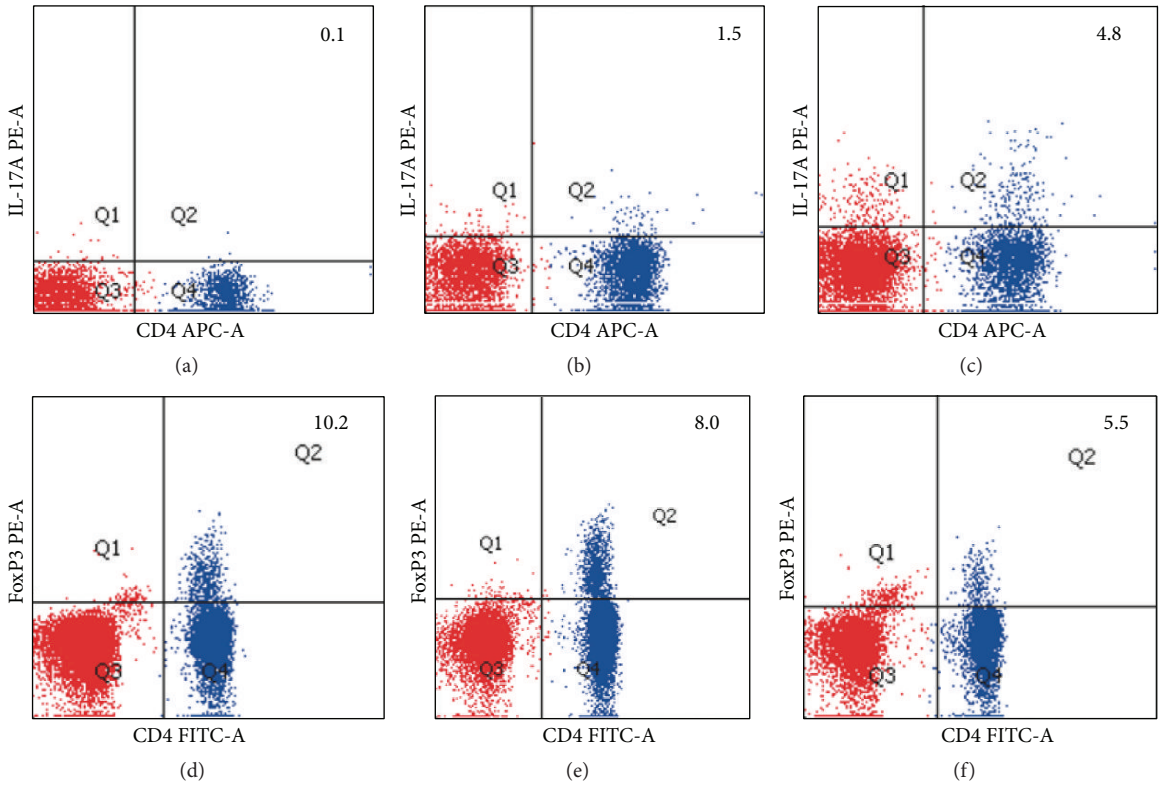


Figure 3: Flow cytometric analysis of the percentage of Th17 and Treg cells in peripheral blood. (a) Percentage of Th17 in peripheral blood in healthy control subjects. (b) Percentage of Th17 in peripheral blood in mild type subgroup of UC patients. (c) Percentage of Th17 in peripheral blood in moderate and severe type subgroup of UC patients. (d) Percentage of Treg cells in peripheral blood in healthy control subjects. (e) Percentage of Treg cells in peripheral blood in mild type subgroup of UC patients. (f) Percentage of Treg cells in peripheral blood in moderate and severe type subgroup of UC patients. In (a), (b), and (c), in the quadrant 1 (Q1), the IL-17A PE-A antibody is positive; the CD4 APC-A antibody is negative; in Q2, both the IL-17A PE-A and CD4 APC-A antibody are positive; in Q3, both the IL-17A PE-A and CD4 APC-A antibody are negative; in Q4, the IL-17A PE-A antibody is negative; the CD4 APC-A antibody is negative. In (d), (e), and (f), in Q1, the foxP3 PE-A antibody is positive; the CD4 FITC-A antibody is negative; in Q2, both the foxP3 PE-A and CD4 FITC-A antibody are positive; in Q3, both the foxP3 PE-A and CD4 FITC-A antibody are negative; in Q4, the foxP3 PE-A antibody is negative; the CD4 FITC-A antibody is negative.

by downregulating Th17 responses via TGF-$\beta$ by using UC mice model treated with Treg cells [20], which indicated the immune balance effect of Th17 and Treg cells. Yet, the pathogenesis of UC is still contradictive concerning some details, such as the changing trend of serum Th17, IL-17, Treg cells, and TGF-$\beta$ in UC patients and their correlation with disease activity and severity.

In this study, we indicated that the levels of serum IL-17 and percentage of Th17 in peripheral blood of UC patients increased significantly compared with healthy control; and these two indexes were positively correlated with clinical activity index and endoscopic index, which improved that serum IL-17 and percentage of Th17 in peripheral blood increased as disease condition aggravated. Percentage of Th17 was positively correlated with serum IL-17 level, which supports the point that Th17 and IL17, which is secreted by Th17 cells, can promote the inflammation during the development of UC and keep increasing as disease aggravates; thus they might be used as markers for judging the UC disease severity. These results are similar to most previous studies [12, 13, 21].

We also found out that the serum TGF-$\beta$ level and percentage of Treg cells in peripheral blood of UC patients were significantly lower than healthy individuals, and they showed negative correlation with clinical activity index and endoscopic index, which demonstrated that when UC disease aggravated, percentage of Treg cells in peripheral blood decreased, yet it is still unclear about the correlation between TGF-$\beta$ and disease severity. Percentage of Treg cells was positively correlated with serum TGF-$\beta$ level; Treg cells might be another important origination of TGF-$\beta$. Therefore, our study implied that Treg cells in the peripheral blood play the role of immunosuppression cells and should inhibit the inflammation in UC procedure. TGF-$\beta$, secreted by Treg cells, also possesses immunosuppression effect; thus, in the population who produce less Treg cells, there will be a higher incident rate of UC. Consequently, Treg cells and TGF-$\beta$ transfer therapy is expected to be efficacious for UC and inhibit the immunosuppression in UC patients.

There are still some limitation in this study. The sample size in the severe type subgroup of UC patients was small so

that we could only combine this subgroup with the moderate type subgroup in analysis. Thus more studies, especially clinical studies with larger sample size, are still warranted to explore the underlying mechanism of Th17/Treg immune balance in the development of UC.

## 5. Conclusions

In UC patients, the increase of Th17 cell caused the higher level of serum IL-17, and the low Treg cell resulted in the decrease of serum TGF-$\beta$1; as a result, the autoreactive T cell was activated and inhibitive immune cytokines decreased, thus to aggravate the inflammation in the mucous membrane of colon. These results support that the Th17/Treg immune imbalance might play a crucial role in the development of UC. Thus to induce the production of Treg cells and TGF-$\beta$1, inhibit the level of Th17 and IL-17, and recover the Th17/Treg immune balance might imply new therapeutic targets in UC management.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Yang Gong and Yifan Lin contributed equally to this work.

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*Review Article*

# *Acinetobacter baumannii* Infection and IL-17 Mediated Immunity

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*Acinetobacter baumannii* is a significant cause of severe hospital-acquired infections with a recent rise in multidrug-resistant infections involving traumatic wounds of military personnel. The interleukin-17 (IL-17) pathway is essential for neutrophil recruitment in response to a variety of pathogens, while the control of *A. baumannii* infection is known to be dependent on neutrophils. This suggests that IL-17 may play an important role in *A. baumannii* infection; however, this has yet to be studied. Here, we summarize the recent advances in understanding the host-pathogen interaction of *A. baumannii* and propose a potential role of the IL-17 pathway in generating a protective immune response.

## 1. Introduction of *A. baumannii*

*Acinetobacter baumannii* is microbiologically characterized as a rod-shaped, aerobic, pleomorphic, and nonmotile Gram-negative bacterium. *A. baumannii* can survive in natural environments such as soil and water for a prolonged period and mainly infect immunocompromised patients. This opportunistic organism is also known to be resistant to disinfectants and persist in hospital and health care facilities. As a consequence, *A. baumannii* has emerged as a major pathogen causing both community-acquired and nosocomial infections [1].

*A. baumannii* presents as an infection of the skin and soft tissue and causes pneumonia [2]. This pathogen has been strongly associated with wound infections of soldiers serving in Iraq and Afghanistan [3]. *A. baumannii* isolates were recovered from various tissues including respiratory tract, blood, flesh wounds, and urinary tract [4]. Many infections were caused by multidrug- and pandrug-resistant strains; this calls for the urgent need of new preventive and therapeutic options against this emerging threat.

Owing to drug resistance to all commonly used Gram-negative antibiotics, *A. baumannii* has generated an increase in research interest [5, 6]. Genomic analyses of multidrug-resistant isolates suggest that these drug resistance genes could be acquired from other Gram-negative species [7]. These studies have revealed that the major drug-resistant mechanisms are through several genes including $\beta$-lactamases, carbapenemases, DNA gyrase, dihydropteroate synthase/reductase, and efflux pumps [7]. This list is expected to become longer with the current abuse of antibiotics in clinical settings. Thus, the concept of therapeutic approaches that target host responses including the immune response has become more appealing.

## 2. Host Innate Immune Responses against *A. baumannii*

Although our understanding of the epidemiology, mechanisms of antibiotic resistance, and persistence of *A. baumannii* in various environments has advanced, the pathological characteristics are much less studied. Specifically, the immune pathways that are critical to host defense against *A. baumannii* are far from being well understood.

Since *A. baumannii* is a Gram-negative bacterium, it is not surprising that lipopolysaccharide (LPS), a highly immunostimulatory molecule on its surface, induces strong responses from mouse splenocytes and engineered human cells including the human monocytic cell line, THP-1 cells [8, 9]. LPS is recognized by TLR4 and activates NF-$\kappa$B, which leads to the secretion of MIP-2 and KC/IL-8 and subsequent neutrophil recruitment [9, 10]. TLR2 also seemed to contribute to the inflammatory response to *A. baumannii*, but the results are conflicting: in one report, TLR2-deficient mice exhibit accelerated neutrophil influx to the lungs, improved elimination of *A. baumannii*, and reduced proinflammatory responses. This suggests that TLR2 signaling may shift the immune response from protective to pathological [10]. Other studies have shown that purified TLR2 ligands from *A. baumannii* are immunostimulatory and result in the activation of NF-$\kappa$B and secretion of IL-8 [9, 11]. Variability in mouse versus human models could potentially explain the conflicting observations but could also be explained by contamination in the TLR ligands during purification.

Depletion of complement, macrophages, or neutrophils separately increased bacterial burden in an infection model with a hypovirulent strain but depletion of these compartments of immune system was insufficient to change lethality [12]. Although the mechanism is not clear, the loss of Fus1, a tumor suppressor protein, in mice significantly increased resistance to *A. baumannii* pneumonia [13]. However, depletion of neutrophils eliminates the enhanced antibacterial clearance of the $Fus1^{-/-}$ mice, further underscoring the importance of neutrophils in the host response to *A. baumannii* pneumonia. These data collectively suggest that neutrophils are an important cellular compartment that is involved in the controlling of *A. baumannii* infection. Based on the compelling evidence on the regulation of neutrophil recruitment by the IL-17 pathway, we hypothesize that the IL-17 producing T helper cells (Th17) play a role in mediating *A. baumannii* clearance.

## 3. IL-17 Pathway in Host Defense at Barrier Tissues

Effector CD4+ T cells differ in their phenotypes depending on differentiating conditions and can be categorized into various lineages [14]. Th1 cells make IFN-$\gamma$ as their signature cytokine, are potent IL-2 producers, and frequently coexpress TNF-$\alpha$. By contrast, Th2 cells do not produce IFN-$\gamma$ but are specialized in making cytokines IL-4, IL-5, and IL-13. The Th1/Th2 paradigm was a dominating theory in the field of T-cell immunology for more than 15 years until 2003, when a series of publications demonstrated a third distinctive effector lineage of CD4+ T cells, Th17 cells, discovered in mouse models of autoimmune encephalitis [15–20]. Most Th17 cells were found to reside in barrier tissues, including respiratory and intestinal tracts as well as the skin. Signature cytokines of Th17 cells include IL-17A, IL-17F, IL-22, and IL-26 (specific for humans) and these canonical cytokines produced by the classical Th17 (CD4+ IL-17 producing cells) and non-Th17 cells including $\gamma\delta$-T cells and innate lymphoid cells play critical roles in regulating tissue homeostasis and inflammation as well as antimicrobial responses upon infections caused by pathogens. Detailed roles and functions of IL-17 in host defense at three different mucosal sites including lung, digestive tract, and skin will be discussed below.

*3.1. Lung.* The pivotal roles of IL-17/IL-17 receptor signaling in the context of host defense against bacterial and fungal pathogens are very well recognized and appreciated even before the discovery of the Th17 lineage [21]. In pulmonary infection models, mice deficient in either IL-17 or IL-17RA have increased susceptibility to Gram-negative bacteria, such as *Klebsiella pneumoniae* [21] and *Mycoplasma pneumonia* [22]. During primary infection, IL-17 signals through the heterodimeric receptor IL-17RA/IL-17RC and promotes the production of CXC chemokines such as CXCL1, CXCL2, and CXCL5. These chemokines are critical for recruiting neutrophils which can ultimately clear the bacteria. IL-17 is also critical for the optimal production of G-CSF, an important cytokine that not only prolongs the survival of neutrophils but also improves the function of neutrophils. Th17 cells are also known for their ability to mediate serotype-independent protection in mouse models of *K. pneumoniae* [23], *Streptococcus pneumoniae* [24], and *Pseudomonas aeruginosa* [25]. In these models, Th17 cells have been shown to recognize antigens that are conserved among different bacterial species and provide broader protection upon secondary infection. It has been hypothesized that antigen-specific memory Th17 cells confer a host advantage by providing heterologous mucosal immunity through recognition of conserved antigens among different species of pathogens [23].

*3.2. Digestive Tract.* Th17 cytokines also play critical roles in the digestive system. The expression of IL-17 and IL-22 increases at other mucosal sites after infection with a number of pathogens including intestinal infections with *Citrobacter rodentium* [26–28] or *Salmonella* Typhimurium [29, 30]. The primary roles of IL-17 and/or IL-22 in these models are to control the infection within the mucosa and prevent the dissemination of these pathogens. In the *Citrobacter rodentium* infection model, which mimics infections by attaching and effacing (A/E) bacterial pathogens in humans, IL-22 is required for the colonic epithelial production of antimicrobial proteins, including RegIIIbeta and RegIIIgamma. The IL-22 dependent antimicrobial proteins are crucial in reducing intestinal epithelial damage and decreasing bacterial burden. Th17 cells and IL-17 receptor signaling are also essential for host defense against oral candidiasis caused by *Candida albicans* [31]. Upon oral *Candida* infection, Th17 signature genes including CXC chemokines and beta defensin-3 are strongly induced while IL-17RA deficient mice have more severe oropharyngeal candidiasis as compared to wild type mice.

*3.3. Skin.* In humans, skin is another important anatomical barrier from pathogens and *Staphylococcus aureus* is the most common cause of infection at skin. IL-17 has been shown to be critical in recruiting neutrophils in a skin infection model

Table 1: Genetic KO mice that are commercially available for studying *A. baumannii* infection.

| Gene products | Th1 pathway | Th2 pathway | Th17 pathway | Tfh pathway |
|---|---|---|---|---|
| Signature cytokines | Ifng$^{-/-}$ defective in IFN-$\gamma$ production | Il4$^{-/-}$ defective in IL-4 production | IL-17-GFP tracking IL-17A producing cells | |
| | Il12a$^{-/-}$ defective in Th1 differentiation | Il5$^{-/-}$ defective in IL-5 production | Il17aCre fate mapping IL-17A producing cells | |
| | Il12b$^{-/-}$ defective in Th1 and Th17 differentiation | Il13$^{-/-}$ defective in IL-13 production | Il22Cre fate mapping IL-22 producing cells | |
| | | | Il12b$^{-/-}$ defective in Th1 and Th17 differentiation | |
| Cytokine/chemokine receptors | Ifngr$^{-/-}$ defective in IFN-$\gamma$ signaling | Il4ra$^{-/-}$ defective in IL-4 and IL-13 signaling | | Cxcr5$^{-/-}$ defective in Tfh differentiation |
| Transcription factors | Tbx21$^{-/-}$ defective in Th1 differentiation | Stat6$^{-/-}$ defective in Th differentiation | Rorc$^{-/-}$ defective in Th17 differentiation | |
| | Stat4$^{-/-}$ defective in Th1 differentiation | | | |

although the primary cellular source appeared to be epidermal $\gamma\delta$-T cells [32]. In this mouse model of *S. aureus* skin infection, neutrophil recruitment to the infection sites was dependent on epidermal $\gamma\delta$-T-cell production of IL-17 and this IL-17 induction is controlled by signals from IL-1, TLR2, and IL-23 as IL-17 production upon *S. aureus* infection is diminished in the *Il1r1*$^{-/-}$, *Tlr2*$^{-/-}$, and *Il23a*$^{-/-}$ mice but not *Il12a*$^{-/-}$ mice.

## 4. Linking Th17 Responses to *A. baumannii*

Emerging evidence shows that the IL-17 pathway is critical in the host defense against various bacterial pathogens [23]; however, very little is known on the exact role of this pathway in *A. baumannii* infection. A recent study examined this in IL-17A KO mice as well as anti-IL-17 neutralization antibody and concluded that IL-17A is not required in primary infection [33]; however, this did not rule out the possibility of a compensatory role of IL-17F, an IL-17 family member that shares the most homology with IL-17A and signals through IL-17RA and IL-17RC complex. Thus, IL-17RA KO mice are required to adequately address this issue. Existing literature also suggested that Th17 cells play a role in vaccine-mediated immunity against *A. baumannii*. The rOmpA vaccine has shown efficacy in animal models through generating antibodies and inducing Th1, Th2, and Th17 responses [34]. However, the roles of each of these specific T helper lineages have not been thoroughly investigated. Furthermore, this study did not measure T follicular helper (Tfh) cell cytokines and markers that are critical in the formation and maintenance of B-cell germinal centers. Nonetheless, immune serum is capable of providing protection upon adoptive transfer, suggesting that a passive immunization strategy can be used in preventing and/or controlling *A. baumannii* infection. Future investigation focusing on the development of monoclonal antibodies against OmpA or other essential proteins from *A. baumannii* should be strongly considered for therapy. Studies using whole cell *A. baumannii* antigen for immunization demonstrated that elevated levels of Th17 polarizing cytokines such as IL-1$\beta$ and IL-6 were observed in nonimmunized mice [35], which correlates with elevated bacterial burden. It could be assumed that these cytokines are also induced by the immunization to promote Th17 responses. The role of such responses should be investigated using KO mice that are deficient in these pathways. A list of commercially available genetic KO mice for studying T helper responses in *A. baumannii* infection is summarized in Table 1. Antimicrobial peptides (AMPs) are another attractive solution in combating *A. baumannii* infection and several AMPs have been shown to have in vitro activities against *A. baumannii* including mastoparan [36] and LL37 [37]. Th17 cytokines including IL-17 and IL-22 are inducers of many AMPs such as $\beta$-defensin-2, lipocalin 2, and the S100 family proteins from barrier tissues such as gut, skin, and lung [38]. The activities of these AMPs on *A. baumannii* have not been carefully examined. However, Th17 cytokine inducible AMPs could potentially be used in treating antibiotic resistant infections. Indeed, Reg3$\gamma$, regulated by IL-22, has been shown to be highly effective in killing Methicillin-resistant *Staphylococcus aureus* [39]. The potential role of the IL-17 pathway in *A. baumannii* infections is summarized in Figure 1.

## 5. Conclusions and Perspectives

The increasing public threat posed by *A. baumannii* infections has greatly intensified clinical and research interest. Significant advances have been made towards understanding the mechanisms of its resistance to antibiotics and hospital hygiene procedures. Accumulating evidence on host-bacterial interactions and the host immune responses could impact available disease treatment. Th17 cells are a critical T-cell subset in controlling Gram-negative bacteria at mucosal barriers and could play a significant role in *A. baumannii* infection. Identifying exact host immune pathways induced

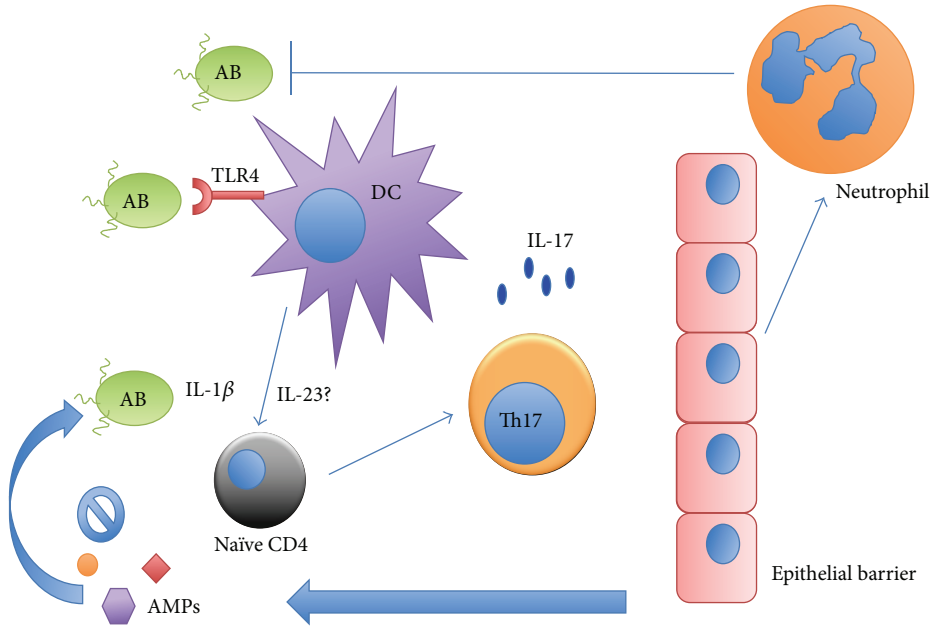


Figure 1: Potential role of Th17 cells in *A. baumannii* (AB) infection. As depicted above, TLR ligands of AB can activate TLRs on DCs and/or macrophages from the host and provoke the release of proinflammatory cytokines such as IL-1$\beta$ and possibly IL-23. Naïve CD4 T cells differentiate into effector Th17 cells and produce Th17 signature cytokines. These cytokines include IL-17 signal epithelial cells at barrier tissues and exert antimicrobial function through 2 possible mechanisms: recruiting neutrophils and antimicrobial peptides.

by *A. baumannii* infection or immunization will facilitate the discovery of new pharmacological and immunological drug targets to help combat this emerging public health crisis.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review Article*

# Th17 Cells Pathways in Multiple Sclerosis and Neuromyelitis Optica Spectrum Disorders: Pathophysiological and Therapeutic Implications

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Several animal and human studies have implicated CD4+ T helper 17 (Th17) cells and their downstream pathways in the pathogenesis of central nervous system (CNS) autoimmunity in multiple sclerosis (MS) and neuromyelitis optica spectrum disorders (NMOSD), challenging the traditional Th1-Th2 paradigm. Th17 cells can efficiently cross the blood-brain barrier using alternate ways from Th1 cells, promote its disruption, and induce the activation of other inflammatory cells in the CNS. A number of environmental factors modulate the activity of Th17 pathways, so changes in the diet, exposure to infections, and other environmental factors can potentially change the risk of development of autoimmunity. Currently, new drugs targeting specific points of the Th17 pathways are already being tested in clinical trials and provide basis for the development of biomarkers to monitor disease activity. Herein, we review the key findings supporting the relevance of the Th17 pathways in the pathogenesis of MS and NMOSD, as well as their potential role as therapeutic targets in the treatment of immune-mediated CNS disorders.

## 1. Introduction

Multiple sclerosis (MS) is a chronic immune-mediated demyelinating disease of the central nervous system (CNS) characterized by a relapsing-remitting (RR) or a progressive course with multifocal CNS dysfunctions [1]. Neuromyelitis optica spectrum disorders (NMOSD) include the entity previously known as neuromyelitis optica (NMO) and patients with limited forms (e.g., only myelitis or optic neuritis) and comprise a phenotypic continuum of primarily immune-mediated astrocyte injury, rather than a primary demyelinating disease, with preferential involvement of the optic nerves, brainstem, and the spinal cord [2, 3].

The nosology of NMO remained controversial for more than one century after its first description, by Devic, in 1894 [3]. It was speculated that it could represent a topographically restricted severe MS variant. A considerable advance in the understanding of those disorders was the identification of pathogenic autoantibodies against aquaporin-4 (anti-AQP4-IgG) in patients with NMO, which allowed for the establishment of NMO as a distinct nosological entity [3]. Despite the fact that both diseases have an inflammatory process restricted to the CNS and a relapsing course in the majority of patients, there are major differences in clinical definition and understanding of the two diseases. Astrocyte injury leading to secondary demyelination is the hallmark of NMO, at

least in those patients who are AQP4-IgG-seropositive, while primary demyelinating lesions with T cell and macrophage infiltration are seen in MS [2]. From the clinical and radiological standpoint, both disorders may present optic neuritis, transverse myelitis, and/or demyelinating brain lesions, but some features are specially suggestive of NMO, such as bilateral optic neuritis, involvement of the optic chiasm, or severe residual visual loss; a complete transverse myelitis, usually with longitudinally extensive lesions on the MRI; and an area postrema syndrome, characterized by intractable nausea, vomiting, and hiccups [3]. Besides that, it has been shown that several immunological therapies commonly used for MS fail to control or even increase disease activity in NMOSD [4], thus suggesting a distinct underlying pathophysiological process in each of those disorders and highlighting the need for a precise distinction between them in order to avoid the potentially harmful consequences of a misdiagnosis.

In both MS and NMOSD, T-B cell interaction has been pointed out as an important factor in the genesis of the disease process. In especially MS, increasing therapeutic options became available in recent years, and some of them involve control of autoreactive T cells, which highlights the importance of further understanding of the role of each of those cell types. Some knowledge about immune mechanisms involving autoreactive T cells comes from experimental autoimmune encephalomyelitis (EAE), the animal model of MS, and from animal models using passive human anti-AQP4-IgG transfer in NMO.

Initially, the group of CD4+ T lymphocytes known as helper T (Th) cells was believed to differentiate into two mutually exclusive phenotypes: type 1 ones (Th1), which are classically induced by interleukin- (IL-) 12 and produce interferon gamma (IFN-$\gamma$), and type 2 ones (Th2), which are stimulated and secrete IL-4 [5]. At that time, the Th1 pathway, regarded as proinflammatory, was considered to be the most important mediator of the pathogenesis of both EAE and MS, while the Th2 pathway would have an antagonist effect on Th1 cells and, consequently, a beneficial effect on the disease process [6]. However, subsequent studies provided consistent evidence that mice were still susceptible to EAE after genetic ablation of key cytokines of the Th1 pathway, such as IFN-$\gamma$, indicating that other unknown pathways were involved [7].

More recently, a new phenotype of Th cells was described, namely, Th17, whose signature cytokine is the IL-17 family. Th17 cells have been implicated in several autoimmune disorders and these cells seem to be relevant in the development of CNS autoimmunity. Here, we review the key findings from animal and human studies supporting the role of Th17 pathways in the MS and NMOSD pathogenesis and potential therapeutic targets under clinical investigation.

## 2. Brief Review of the Th17 Pathways

In 2005, it was demonstrated that näive Th cells would differentiate into a new lineage called Th17, due to its capacity to produce large amounts of IL-17 [8, 9]. Currently, the cytokine previously named as IL-17 is referred to as IL-17A, since it became clear that IL-17 actually represents a family of cytokines, which includes IL-17A to IL-17F.

The process of differentiation of näive Th cells into Th17 is dependent on IL-23 [10] and potently inhibited by IFN-$\gamma$ and IL-4 [8]. IL-23 knockout mice are resistant to EAE and lacked Th17 cells [11], suggesting that the Th17 pathway is implicated in the pathogenesis of EAE. However, the differentiation of T näive cells into Th17 cells may be induced not only by IL-23, but also by the combination of transforming growth factor beta 1 (TGF-$\beta$1) and IL-6. Moreover, IL-1$\beta$, in combination with IL-6 and IL-23 and independently of TGF-$\beta$1, may induce a different (and pathogenic) phenotype of Th17 cells, characterized by the coexpression of RAR-related orphan receptor gamma t (ROR$\gamma$t) and T-bet [12].

Indeed, Th17 cells can present different phenotypes, pathogenic or not, according to the modulating factors they are exposed to, such as IL-23, and the differential expression of some cytokines and chemokines, such as IL-17A, IL-17F, IL-21, and ROR$\gamma$t transcription factor [13]. Moreover, Th17 cells have impressive plasticity, that is, ability to transition between different phenotypes throughout their life span [7]. It has been demonstrated that a significant proportion of IL-17-producing (Th17) cells converts into IFN-$\gamma$-producing T cells, partially due to IL-23-mediated reprogramming [14]. There is also a subset of Th17 cells that, in addition to IL-17A, simultaneously express IFN-$\gamma$ and have chemokine receptors from both Th17 and Th1 cells [15].

Granulocyte macrophage colony stimulating factor (GM-CSF) is a growth factor that acts as a proinflammatory cytokine and is critically involved in Th17 and other cell-mediated immune responses. It is produced by several different cells, especially T cells, in response to IL-23 and IL-1$\beta$ [16] and induces the activation, maturation, and differentiation of macrophages and of dendritic cells (which secrete IL-23 and IL-6) [17]. Notably, there is a positive feedback loop between GM-CSF and IL-23, which plays a critical role in the expansion of pathogenic Th17 cells. Indeed, studies with EAE have shown that GM-CSF is essential for mediating Th17 cells-induced encephalitogenicity [16, 18, 19]. Recently, another study with EAE has suggested that the GM-CSF-producing T cells likely represent a distinct subset of T helper cells, designated as Th-GM [20].

Currently, after several studies indicating that the IL-17 family plays a crucial role in the development of EAE [21, 22], the pathogenic potential of Th17 pathways, in addition to that of Th1 pathways, in the development of EAE has been widely accepted, although not fully understood. Th1-induced EAE presents the classical phenotype, characterized by an ascending caudocranial paralysis, while Th17 polarization induces EAE with an atypical phenotype, characterized by ataxic gait [23].

Based on the previously mentioned findings, the classical Th1 paradigm was replaced by the Th1/Th17 paradigm in both EAE and MS. It is postulated that Th17 cells would play a more relevant role in the initial phases of EAE and MS, while Th1 cells would be more important in later stages of the inflammation in the CNS [6].

## 3. Modulation of Th17 Responses by Environmental Factors

Relevant modulation of Th17 responses occurs in mucosal tissues, especially those of the lungs [24] and gastrointestinal tract [25]. This modulation is dependent on the complex interaction of local immune elements with a multitude of pathogens, nutritional components, and other environmental factors. It is believed that T cell clones stimulated by these interactions in the periphery would induce activated T cells able to migrate through the blood-brain barrier (BBB) and induce damage in the CNS. Further studies are still required to better understand the role of environmental factors in MS and other autoimmune diseases, but this growing amount of evidence can provide new targets for therapeutic interventions.

One of the mechanisms of interaction between environmental factors and T cells is the aryl hydrocarbon receptor (AHR), which modulates the differentiation towards either Th17 or regulatory T (Treg) cells, increasing or decreasing, respectively, the severity of EAE, depending on its ligand [26]. Different compounds from the environment, including commensal microbiota and human pathogens, can act as a ligand for the AHR; moreover, such compounds may act only locally (i.e., in the mucosal tissues) or reach the circulation, causing changes in the immune system of different compartments [7].

Traditionally, systemic infections (especially by viral agents) have been believed to play a role in triggering or modulating the immune process that ultimately may lead to autoimmune CNS diseases. More recently, however, several studies have pointed out that, under special circumstances, normal intestinal microbiota may also activate previously quiescent autoreactive T cells in the gut-associated lymphoid tissues (GALT), thus precipitating autoimmunity [25]. There is some evidence implicating the small intestine as the major site for activation of effector Th1 and Th17 cells and segmented filamentous bacteria as the major inducers of IL-17-producing immune cells [27, 28]. As an example, cell cultures derived from NMO patients showed a higher Th17 responsiveness to *Escherichia coli*, associated with elevated IL-1$\beta$, IL-6, and IL-17 production and decreased IL-10 release, when compared to healthy controls [29]. Besides the complex regulation by cytokines and commensal or pathogenic microorganisms in T cell differentiation and function, Th cells are also regulated (and can be dynamically reprogrammed) by cellular metabolic pathways, including those related to glucose, amino acid, and lipid metabolism [30, 31]. Further studies are required to investigate the role of gut microbiota in the pathogenesis of MS and NMO.

Moreover, high concentrations of sodium chloride and high dietary salt intake have also been shown to enhance the differentiation of Th17 cells and lead to a more severe form of EAE [32]. That phenomenon seems to be mediated by serum glucocorticoid kinase 1 (SGK1), a downstream molecule of IL-23 signaling. SGK1 expression increases after elevation of salt concentration, promoting IL-23R expression and subsequently enhanced Th17 cell differentiation and autoimmunity development [33]. Interestingly, a recently published prospective study has found a positive correlation between higher sodium intake and increased disease activity, measured by both clinical and radiological parameters in patients with MS [34]. Further studies are required to evaluate if the large amounts of salt intake could increase the risk of developing MS.

Finally, substance P, a stress-related neuropeptide, was shown to influence T cell and cytokine profiles in cell cultures derived from patients with generalized anxiety disorder [35]. Complex T cell functional dysregulation (including Th1 and Th2 deficiency and Th17 hyperactivation) was further enhanced by substance P, thus suggesting rationale for the influence of chronic stress and anxiety disorders in individuals' susceptibility to autoimmune disease [35]. However, such influence of substance P in immune function has not been studied in MS or NMOSD yet.

## 4. Th17 as Pioneering Cells in the Breakdown of the BBB

After modulation and selection of T cells in peripheral tissues, pathogenic autoreactive T cells need to cross the BBB in order to cause inflammation into the CNS. Th17 cells have a large number of chemokines and chemokine receptors required to cross the BBB, which enables them to disrupt the BBB and access the CNS via several different pathways. *In vitro* and *in vivo* studies have shown that, through the action of IL-17A and IL-22, Th17 cells can efficiently disrupt BBB tight junctions, express high levels of the cytolytic enzyme granzyme B, and promote the recruitment of additional CD4+ lymphocytes from the systemic circulation into the CNS [36]. Th17 cells are also able to induce CXCL1 and CXCL2, chemokines that are potent attractants for polymorphonuclear cells and play an important role in the breakdown of the BBB in EAE [37]. Th17 cells can access the subarachnoid space via upregulation of the CCR6 receptor, expressed in the epithelium of the choroid plexus, in a process that is critical for the initiation of EAE [38]. Moreover, IL-17A is a key factor in the breakdown of the BBB by direct impairment of its integrity due to the formation of reactive oxygen species within the endothelial cells [39]. Evidences from EAE demonstrate that Th1 cells preferentially access the CNS by using the $\alpha 4\beta 1$ integrin, while Th17 cells do that by means of the $\alpha L\beta 2$ integrin (LFA-1) [40]. Melanoma cell adhesion molecule (MCAM) or CD146 is another adhesive molecule expressed by Th17 cells, but not by Th1 cells [41].

Some T cells can secrete both IL-17 and IFN-$\gamma$, being called Th1/Th17 cells. These cells can infiltrate the CNS early in the course of EAE and may be involved in microglial activation and thus may have an important role in the development of the disease [42]. In addition, increased proportions of Th1/Th17 cells were found in the blood and in the brain tissue of MS patients [15]. This amount of evidence suggests that Th17 cells act as pioneering cells in the induction phase of EAE and, presumably, in the early phases of MS.

## 5. Th17 in MS

Following studies that suggested the role of Th17 responses in EAE, several findings from different groups using different

Table 1: Hallmarks on the understanding of the role of the Th17 pathways in MS.

| Finding | Reference |
|---|---|
| Increased IL-17 found in the blood and CSF of RRMS patients, especially during relapse | [43] |
| IL-17-producing T cells identified in EAE | [10] |
| Increased Th17 cells and IL-17 found in the brain of MS patients | [48] |
| IL-17 production correlates with MRI activity | [53] |
| Secukinumab (anti-IL-17A monoclonal antibody) reduces MRI lesions in a phase II clinical trial | [114] |

techniques have provided substantial evidence that the Th17 pathways play a critical role in MS (a summary of the most important studies provided in Table 1).

An increased frequency of Th17 cells is detected in the peripheral blood and cerebrospinal fluid (CSF) of some RRMS and clinically isolated syndrome (CIS) patients, especially during the acute episode, when compared to patients with noninflammatory neurological diseases [43–47]. Increased proportion of Th17 cells, as well as increased levels of IL-17A (protein and messenger RNA [mRNA]), was observed in the brain tissue of MS patients, especially in acute and chronic active lesions, compared to healthy controls [48]. Th17 cells may also have a role in progressive forms of MS [49]. Finally, levels of GM-CSF, which is essential for Th17 responses as discussed earlier in this review, were also shown to be elevated in the CSF of MS patients [50, 51] and in the blood of MS (but not of NMO) patients [52].

Th17-related molecules were shown to correlate with parameters of disease activity in MS. *In vitro* studies demonstrated that the amount of IL-17 (and also IL-5) produced by mononuclear cell cultures from patients with MS after stimulation with human myelin basic protein correlates with the number of active lesions on magnetic resonance imaging (MRI) [53]. The proportion of Th17 cells, their subset effector memory Th17 cells (CD4+/CD45RO+/CCR7−), and the level of IL-17A correlated with disease severity as measured by the Expanded Disability Status Scale (EDSS), while the proportion of another subset, central memory Th17 cells (CD4+/CD45RO+/CCR7+), correlated with relapse frequency, in both MS and NMO [54]. Serum IL-17F (but not IL-17A) correlated with the number of MS relapses in two years [47].

## 6. Th17 in NMOSD and Its Animal Models

Much of the evidence regarding pathogenesis of NMOSD comes from studies on opticospinal MS (OSMS) and NMO. The former is no longer considered to be a variant of MS, since most of those patients actually had NMO [3]; hence OSMS is considered as an obsolete term.

Although the important role of B cell autoimmunity against aquaporin-4 (AQP4), by means of the anti-AQP4 immunoglobulin G (IgG), a T cell dependent immunoglobulin type (IgG1), in mediating CNS lesions is clearly established, many aspects of tissue damage in NMOSD remain poorly understood [55]. However, T cell-related mechanisms have been increasingly implicated in NMOSD [56–58]. AQP4-specific T cell responses were demonstrated to be amplified in NMO patients, whose T cells were shown to exhibit a Th17 polarization, partially mediated by increased production of IL-6 [59].

In some studies, IL-17 was increased in the CSF [60, 61] and in the blood [54] of NMO patients. Another study on cytokines in NMO did not find an increase in serum or CSF levels of IL-17 but did find an increased level of other Th17-related cytokines, notably IL-6 [52], which is a proinflammatory cytokine that increases the survival of plasmablasts capable of producing anti-AQP4-IgG and is also involved in the development of Th17 cells, which can also support B cell development and induce further tissue injury [62].

Th17-related markers have also been shown to correlate with parameters of disease activity and severity in NMOSD. The release of IL-6 and IL-21 by polyclonally activated CD4+ T cells derived from NMO patients was demonstrated to correlate directly with neurological disability [63] and *in vivo* and *in vitro* levels of IL-6 were higher among NMO patients who experienced relapse within a 2-year follow-up [64]. CSF levels of both IL-17A and the downstream cytokine IL-8 were found to have a positive correlation with spinal cord lesion length in NMO [60]. As previously mentioned in studies with MS, the proportion of effector memory Th17 cells and IL-17A levels correlated with EDSS, and the proportion of central memory Th17 cells correlated with relapse frequency in NMO [54].

The development of NMO-like disease models in animals has provided important insights into the pathogenesis of NMOSD. After the pathogenic potential of anti-AQP4-IgG has been demonstrated, it was shown that AQP4-specific T cells could also induce a NMO-like disease in rats, independent of anti-AQP4-IgG [65, 66]. Recently, another NMO-like model was developed in mice using AQP4-reactive T cells polarized into a Th17 phenotype (also independent of anti-AQP4-IgG), promoting lesions in the optic nerve and spinal cord [67].

A summary of the main studies supporting the involvement of Th17-related pathways in NMOSD is provided in Table 2.

## 7. Different Immunologic Profiles in MS and NMOSD

Several studies have already addressed the immunological differences between MS and NMO. However, a clear definition of immunologic profiles that differentiate MS and NMO remains controversial.

Investigators have assessed the levels of several cytokines and chemokines in the serum and/or CSF of patients with NMO and MS and compared them between both diseases and between each of them and a control group composed of patients with noninflammatory neurological disorders [52]. Both Th2- and Th17-related molecules were found to be

Table 2: Hallmarks on the understanding of the role of the Th17 pathways in NMOSD.

| Finding | Reference |
|---|---|
| Increased IL-17 found in the CSF of NMO patients | [60] |
| Increased Th17 cells found in the blood of NMO patients, especially during relapse | [69] |
| IL-17 and subsets of Th17 correlate with EDSS and relapse frequency in NMO | [54] |
| Pathogenicity of AQP4-specific T cells demonstrated in animal models | [65] |
| Pathogenicity of Th17-polarized AQP4-specific T cells demonstrated in animal models | [67] |

upregulated in NMO, except, interestingly, for the signature cytokine of each of those pathways, that is, IL-4 and IL-17 family [52]. Of note, among the Th17-related molecules elevated in NMO, IL-6 seemed to be the most relevant one [52]. In the same study, the Treg-related cytokine IL-10 was elevated in both NMO and MS, whereas Th1-related cytokines and molecules were upregulated only in MS [52].

Another study found increased levels of Th1-related markers in NMO when compared to MS, while the levels of Th17-related markers were similar between both diseases [68]. Investigators from the same group found increased proportion of Th17 cells and of IL-17-secreting T CD8+ cells, in both MS and NMO, especially during relapses [69]. The same study did find IL-17 levels, as well as those of IL-23, to be higher in NMO than in MS, leading the authors to speculate whether that would explain the more aggressive nature of NMO when compared to MS [69].

Another study compared NMO, RRMS, and PPMS and reported an increased expression of Th17- and Th1-related cytokines as being characteristic of NMO [70]. Further studies are required to clarify if cytokine levels are useful to indicate disease activity and if interference in the Th17 pathway can reduce inflammation in the CNS during relapses.

## 8. Effects of Current Therapy on the Th17 Axis

Since Th17 responses seem to be relevant in the pathogenesis of MS and NMOSD, great interest has been put in identifying possible effects of the currently available therapeutic agents on the Th17 pathways. A better understanding of those effects and of the different immunologic profiles of MS and NMOSD could potentially provide an explanation to why some NMOSD patients get worse when undergoing treatment with MS-targeted disease-modifying therapies, such as interferons. It could also provide valuable insights into potential mechanisms to be addressed by new or repositioned drugs.

Intravenous methylprednisolone (IVMP) is the most widely used treatment for acute relapses in both MS and NMOSD. A significant reduction in Th17 cell counts, IL-17A and IL-23R production, and RAR-related orphan receptor c (RORc) mRNA expression has been observed in MS patients after IVMP pulse therapy [54, 71]. A reduction in the same markers, except for IL-17A, was also seen in NMO patients after IVMP treatment [54]. Even though Th17 cells as a whole were decreased in both NMO and MS after IVMP therapy, a stratified assessment of their responses according to some of their subsets showed that central memory Th17 and effector memory Th17 cells were decreased only in NMO and not in MS [54]. These differences in the response to corticosteroids may explain the effectiveness of such drugs in NMO to reduce the risk of further attacks [54]. Progressive MS patients undergoing monthly IVMP pulse therapy presented no changes in the phenotype of Th17 cells [72].

Another therapeutic option for acute relapses in MS and NMOSD is intravenous immunoglobulin (IVIg). *In vitro* studies have demonstrated that IVIg inhibits the differentiation and amplification of Th17 cells and the production of IL-17A, IL-17F, IL-21, and CCL20 [73]. The inhibitory effect of IVIg on IL-17A seems to be mediated by the modulation of intracellular signaling pathways and not by passive neutralization by anti-IL-17 antibodies from the IVIg preparations [74].

Several drugs have been developed as disease-modifying therapies for MS, and many of them have been shown to modulate the Th17 axis. One of these immunomodulatory drugs is the recombinant IFN-$\beta$, which includes IFN-$\beta$ 1a and IFN-$\beta$ 1b. It was suggested that IFN-$\beta$ inhibits the differentiation of Th17 cells in mice and hence modulates the severity of EAE, by acting on the toll-IL-1 receptor domain-containing adaptor inducing IFN-$\beta$-dependent (TRIF-dependent) type I IFN induction pathway and its downstream signaling pathways, especially by means of an increased production of IL-27 [75]. In both mice with EAE and patients with MS, IFN-$\beta$ also decreased the ability of dendritic cells to stimulate the production of IL-17 by Th17 cells and increased the production of IL-27 by dendritic cells, shifting the proinflammatory response into an anti-inflammatory one [76]. In humans, IFN-$\beta$ therapy was shown to downregulate the expression of IL-1$\beta$, IL-23R, RORc, and IL-17A and upregulate the expression of IL-12, IL-27, and IL-10, suppressing the differentiation of näive T cells into Th17 cells. These effects may explain some of the IFN-$\beta$'s immunomodulatory effect in MS [77].

However, IFN-$\beta$ is not always effective in reducing CNS autoimmunity, and efforts have been done to identify factors that could predict the response to IFN-$\beta$ therapy. One study pointed out that mice with Th1-induced EAE did benefit from IFN-$\beta$ treatment, whereas deleterious effects were observed in mice with Th17-induced EAE [78]. Indeed, IFN-$\beta$ seems to be effective in diseases primarily driven by Th1, whereas it has proinflammatory effects in Th2-driven diseases [79]. In a study with RRMS patients, pretreatment levels of IL-17F and of endogenous IFN-$\beta$ were higher in nonresponders than in IFN-$\beta$ treatment responders [78]. A subsequent study, however, did not confirm the role of serum IL-17F in the prediction of poor response to IFN-$\beta$ therapy [80]. Interestingly, IFN-$\beta$ is not effective in reducing relapse rates or preventing disability in patients with NMOSD [81] and may even trigger severe exacerbations in those patients [82, 83]. It has been suggested that the poor response to IFN-$\beta$ in NMOSD may be related to elevated levels of IL-17 [4], which

corroborates some of the findings from earlier studies with EAE models and MS patients.

Glatiramer acetate (GA) is another traditional disease-modifying drug for MS. In EAE mice treated with GA, Th17 cells were drastically reduced, while Treg cells were increased [84]. However, further studies are required to clarify if the same effects are observed in MS patients treated with GA. Despite some anecdotal reports of patients with NMOSD who seemed to benefit from GA therapy [85, 86], as well as some speculations regarding the rationale for a potential benefit of GA in NMOSD [87], currently there is no evidence to support this indication, as well as no data regarding its effect on Th17 in NMOSD patients.

In patients with MS, fingolimod (FTY720) reduces Th17 central memory T cells in peripheral blood, presumably due to the retention of those cells in secondary lymphoid organs [88]. Another study pointed out that, after initiation of fingolimod therapy, half of the patients had a reduction in the proportion of circulating Th17 cells, whereas the other half (including the only one patient with relapses in that sample during the follow-up period) had an increase in the proportion of those cells, suggesting that a slower reduction in circulating Th17 cells after fingolimod initiation would predispose to relapses [89]. In NMOSD, however, treatment with fingolimod was reported to trigger extensive brain lesions [90] or a fulminant course [91]; thus it is not recommended for NMOSD. Since eosinophils have been implicated in NMOSD pathogenesis [92] and fingolimod may promote bone marrow egress of eosinophils [93] and other pathogenic proinflammatory cells, that would explain the severe disease activity in NMOSD patients exposed to fingolimod.

Natalizumab is another commonly used treatment for MS. It acts by interfering with lymphocyte migration across the BBB, which is mediated by the interaction between $\alpha 4\beta 1$ integrin (on the surface of lymphocytes) and vascular-cell adhesion molecule 1 (VCAM-1; an endothelial receptor in CNS vessels) [94]. Specifically, it binds to the integrins, thus preventing them from binding to their endothelial receptors [94]. Even though such process is also required for CNS inflammation to develop in NMOSD, natalizumab was reported to fail in controlling disease activity in patients with NMOSD [95] or even triggering catastrophic exacerbations [96, 97]. Like fingolimod, natalizumab may increase the number of peripheral eosinophils [94], which could account for the increased disease activity in NMOSD patients treated with natalizumab. The specific effects of natalizumab over the Th17 cells have not been described, and given the number of receptors present in such cells, it might be possible that pathogenic Th17 cells can use alternative pathways not requiring binding to $\alpha 4\beta 1$ integrin to access the CNS.

In MS patients, treatment with dimethyl fumarate inhibited the maturation of dendritic cells and thus the generation of IFN-$\gamma$-producing (Th1) and IL-17-producing (Th17) cells [98]. No evidence on the use of dimethyl fumarate in NMOSD is available, so the use of this drug is not recommended at this time point.

Finally, treatment of aggressive MS with chemoablation and hematopoietic stem cell transplantation seems to exert its effect by decreased Th17 and Th1/Th17 responses, rather than Th1 pathway responses [99]. Even though some studies reported cases of NMOSD treated with stem cell transplantation [100–103], with variable results, none of them reported its influence on Th17 cells.

Several cytotoxic, immunomodulatory, and B cell depleting therapies are available for prevention of attacks in NMOSD, including prophylactic corticosteroids, plasma exchange, and IVIg, as well as azathioprine, mycophenolate mofetil, methotrexate, mitoxantrone, cyclophosphamide, and rituximab [104]; however, their precise effects on Th17 cells in NMOSD are not known to date. Rituximab (an anti-CD20 monoclonal antibody) and IVIg in combination have been shown to modulate T cell subsets and humoral immune responses in NMOSD [105]. Some data regarding the specific effects of the aforementioned drugs on the Th17 pathways is available from studies on other autoimmune diseases. Rituximab decreases Th17 cell responses in rheumatoid arthritis [106] and the IL-17 production in Sjögren's syndrome [107]. Methotrexate, with or without corticosteroids, reduces Th17 cell frequency [108], normalizes the Th17/Treg balance [109], and suppresses IL-17 production [110] in rheumatoid arthritis.

## 9. Emerging Therapies Targeting the Th17 Pathways

A few monoclonal antibodies targeting different Th17-related cytokines have been tested in MS so far. The first one, ustekinumab, was an antibody against IL12 and IL-23, which are critical for the maintenance of Th17 cells. Nevertheless, in a phase II study, ustekinumab did not show efficacy in the reduction of new enhancing lesions on brain MRI, number of relapses, or change from baseline EDSS after 23 weeks [111].

Secukinumab (AIN457) is a recombinant, highly selective, fully human monoclonal antibody against IL-17A. *In vitro* treatment of human astrocytes with secukinumab was shown to upregulate the levels of IL-6 and to decrease the levels of proinflammatory molecules [112], thus making it suitable for phase II studies in MS. Clinical trials in some other autoimmune diseases yielded promising results, with no significant safety concerns [113], and so far, three phase II trials assessing secukinumab in MS have been started. The first one (ClinicalTrials.gov identifier: NCT01051817) was a randomized, multicenter, double-blind, proof-of-concept study to assess the effect of secukinumab versus placebo on MRI parameters of disease activity over a 24-week period in patients with RRMS [114]. The results have been presented in a conference, as follows: the primary outcomes, number of new gadolinium-enhancing lesions, and number of combined unique active lesions decreased by 67% ($P = 0.003$) and by 49% ($P = 0.087$), respectively, in the secukinumab group; as a secondary outcome, the annualized relapse rate decreased by 43% in the secukinumab group, which was not statistically significant, maybe because the study was not powered to assess that outcome [114]. However, due to methodological issues (especially small sample size and important demographic, clinical, and radiological differences between groups on baseline), caution must be taken in the

interpretation of those findings [115]. The second trial (ClinicalTrials.gov identifier: NCT01874340), which is an extension of the first one, has had no results published yet. The third trial (ClinicalTrials.gov identifier: NCT01874340), which is a larger phase II study, was terminated early "based upon development of another anti-IL-17 fully human monoclonal antibody with better potential for treating MS patients," according to information provided by the sponsor. Actually, the new compound is CJM112, a new fully human anti-IL17A monoclonal antibody, whose phase II trial was not yet registered on https://ClinicalTrials.gov (accessed on 19 September 2015).

Future perspectives include targeting genes and soluble factors that mediate Th17 cell expansion and have been shown to be differentially expressed in MS patients [116], as well as modulating metabolic pathways that are relevant for the regulation of Th17 responses [30, 31].

## 10. Conclusions

Even though not completely understood, the role of Th17 cells in the pathogenesis of both MS and NMOSD is very well established by several findings from studies in humans and animal models. Th17-related pathways seem to be modulated by many of the currently available therapies, and drugs targeting specific points on those pathways are already being tested on phase II studies, with promising results. Further studies focusing on the role of Th17 cells and their related molecules as biomarkers of diagnosis, disease activity, and response to specific therapies are warranted and may potentially lead to a more precise comprehension of MS and NMOSD, as well as more selective and effective therapies.

## Disclaimer

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*Review Article*

# Does IL-17 Respond to the Disordered Lung Microbiome and Contribute to the Neutrophilic Phenotype in Asthma?

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Th17/IL-17 plays an important role in host defense and hyperimmune responses against pathogenic bacteria accompanied by the recruitment of neutrophils. Th17-associated immune response is also involved in the pathogenesis of asthma, which is known as a noninfectious allergic airway disease and has been shown to be heterogeneous. Th17-associated inflammation usually contributes to the neutrophilic phenotype, which is often characterized by greater severity, airflow obstruction, and steroid resistance. Concurrently, advanced culture-independent molecular techniques have increased our understanding of the lung microbiome and demonstrated that disorders of the lung microbiome, including changes of the total burden, diversity, and community composition, may contribute to severe, treatment-resistant neutrophilic asthma, although the precise mechanism is still unclear. Because Th17/IL-17 plays a role in bacteria-mediated immune responses and is involved in neutrophilic asthma, there may be a link between them. We review the effects of Th17/IL-17 on bacteria and asthma, showing the possibility that Th17/IL-17 may be a key player in neutrophilic asthma which may be characterized as severe or treatment-resistant by responding to the disordered lung microbiome.

## 1. Introduction

Th17 cells are known as a distinct lineage of $CD4^+$ T cells, which are promoted by antigen-presenting cells (APCs) through IL-1$\beta$ (in humans)/TGF-$\beta$1 (in mice), IL-6, and IL-23 [1–4]. Th17 cells are one of the main sources of IL-17 (all referring to IL-17A in this review), which functions by recruiting neutrophils [5]. Neutrophils play an important role in preventing bacterial dissemination and increasing the clearance of pathogens [6] and promote inflammatory responses and mediate tissue injury [7, 8].

In addition to bacteria-mediated diseases, Th17/IL-17 has also been found to be elevated in asthma with levels that are positively correlated with disease severity [9–12]. Because it functions by recruiting neutrophils, Th17-associated inflammation contributes more towards neutrophilic asthma [13], one of the inflammatory phenotypes of asthma, which is often characterized by worse control levels, a greater need for inhaled corticosteroids, and treatment resistance compared to other phenotypes [14, 15].

The development of molecular technology has largely expanded our understanding of the lung microbiome. The lower airway is no longer thought of as sterile. Disorders of bacterial microbiota have been found in the airways of asthmatics [16], and the observed community composition has been shown to differ among different features and severity of asthma [17].

Because the disordered lung microbiome is involved in the pathogenesis of neutrophilic asthma and Th17/IL-17 plays a role in neutrophil recruitment in both bacteria-mediated diseases and asthma, there may be a link between Th17/IL-17-associated immune responses to some specific bacterial microbiota and the pathogenesis of severe or steroid-resistant neutrophilic asthma. In this review, we describe the possibility of the link based on recent discoveries.

## 2. IL-17 and Bacteria-Mediated Immune Responses

IL-17 mediates innate and acquired immunity to certain strains of bacterial infection to aid in host defense. In mice, IL-17 has been found to increase the clearance of bacteria by recruiting macrophages and neutrophils in the context

of *Streptococcus pneumoniae* infection [18]. This protection was defective in both IL-17 receptor knockout (KO) and neutrophil-depleted mice [19]. In acute *Pseudomonas aeruginosa* infection, Th17 cells and IL-17 levels also increased and induced the recruitment of neutrophils in the early period, which played protective roles [20]. In *Klebsiella pneumoniae* infection, IL-17 supported protection through the induction of granulocyte colony-stimulating factor (G-CSF) and neutrophil recruitment [21]. For intracellular pathogens, IL-17 also mediates bacterial killing and host responses by regulating IL-12-Th1 cell immunity [22]. In *Mycobacterium tuberculosis* resistance, IL-17 contributed to granuloma formation and CXCL13 expression [23], and, in IL-17 KO mice, granuloma formation was found to be impaired after infection with *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) [24]. Although our understanding of IL-17 in human host defense remains limited, studies with the aforementioned animal models have provided evidence for the role of IL-17 in mammalian immune responses against pathogens and the importance of neutrophil recruitment and activation is involved in the mechanisms.

Moreover, IL-17 also plays a complex role in bacteria-mediated hyperimmune response. Hypersensitivity pneumonitis (HP) is an inflammatory disease that can progress to lung fibrosis. Simonian et al. demonstrated that, in a *Bacillus subtilis*-induced HP model, IL-17 expressed by $\gamma\delta$ T cells play a role in bacterial clearance and downregulate inflammatory responses and lung fibrosis [25, 26], whereas in another *Saccharopolyspora rectivirgula*-induced HP model, IL-17 expressed by $CD4^+$ T cells, which acted as a proinflammatory cytokine, produced an unregulated inflammatory and fibrotic response and promoted rather than protect against hypersensitivity pneumonitis and lung fibrosis [7]. The two opposing effects suggest that the role of IL-17 in the pathogenesis of infectious and inflammatory diseases may differ depending on the microorganisms used in mouse models and cells that produce it.

## 3. IL-17 and Asthma

In addition to bacteria-mediated diseases, Th17/IL-17 has also been known to be involved in airway diseases, such as asthma [11], in which bacterial microbiota was traditionally thought to not play a role.

Asthma is a chronic inflammatory disease, which has been shown to be heterogeneous [27]. Indeed, less than 50% cases of asthma are attributed to atopy and present as typical Th2 inflammation [28, 29]. According to the proportions of neutrophil and eosinophil in induced sputum, asthma is categorized into 4 inflammatory subtypes: neutrophilic asthma, eosinophilic asthma, mixed granulocytic asthma, and paucigranulocytic asthma [30]. Whereas eosinophilic asthma is usually driven by Th2-associated inflammation, neutrophilic asthma correlates more strongly with Th17-associated inflammation [31].

IL-17 mRNA and protein levels have been found to be elevated in the lung tissue, induced sputum, and serum of asthmatics and to correlate with increased disease severity [9–12]. As IL-17 plays a role in recruiting neutrophils into the airways [11, 31, 32], some severe asthma patients exhibit airway neutrophilic inflammation, which is induced by Th17 cells and linked to elevated IL-17 [13, 14, 33]. These patients often appear to have difficulty in gaining improvements in symptoms and forced expiratory volume in one second (FEV1) [15]. This difficulty may partly be due to the resistance of Th17 cells to corticosteroid treatments [34]. Moreover, IL-17 induces human bronchial and murine epithelial cells to express Muc5ac and Muc5b [35], contributing to goblet cell hyperplasia, airway remodeling, steroid resistance, and the pathogenesis of severe or refractory asthma [36–38].

The precise mechanisms of Th17-associated neutrophilic inflammation and how it contributes to worsened clinical outcomes remain unclear. Because IL-17 plays an important role in host defense and hyperimmune responses against bacteria, are there any scenarios in which bacteria may be involved in the pathogenesis of Th17 inflammation in neutrophilic asthma?

## 4. Lung Microbiome

The lower airway is not sterile. Advanced culture-independent molecular techniques, including qPCR and 16S rDNA sequencing, have challenged our understanding of the lung microbiome [39].

Charlson et al. analyzed the bacterial abundance and composition throughout the respiratory tract by sampling at multiple sites in six healthy individuals, showing that bacteria were resident in both upper and lower airways of healthy people [40]. Compared with the upper airways, the lung microbiome displayed similar community composition but less biomass, suggesting possible aspiration of colonizing microorganisms from the upper airways [40]. This aspiration occurs commonly in healthy people [41]. It increases the risk of smoking-related lung diseases, for smoking significantly alters the species in the oro-/nasopharynx, although little direct influences have been found on the lung microbiome [42, 43]. When significant quantities of disordered supraglottic bacteria aspirate into the lungs, the normal relative abundance of the lung microbiota will change and subclinical lung inflammation increases [44]. This phenomenon may in part play a role in the development of COPD triggered by cigarette smoking.

Another study investigated the respiratory tract microbiome in 64 healthy individuals, and some specific bacteria, including Enterobacteriaceae, *Haemophilus*, *Methylobacterium*, and *Ralstonia*, were found to have much higher abundance in the lungs, indicating that not all bacteria in the lungs were derived from the oral cavity [43].

Alterations of the lung microbiome also contribute to pulmonary inflammation and participate in the pathogenesis of various airway inflammatory diseases. Rutebemberwa et al. showed that *Novosphingobium* spp. presented and played a role in more severe COPD [45]. In non-cystic fibrosis bronchiectasis, the loss of bacterial diversity in the lower airway is correlated with decreased FEV1 [46]. A significant association between idiopathic pulmonary fibrosis (IPF) disease progression and the relative abundance of *Streptococcus* and *Staphylococcus* genera has also been reported [47].

Moreover, exacerbations without acute infections during the aforementioned diseases have also been found to associate with increased bacterial burdens and decreased community diversity [48].

## 5. Lung Microbiome in Asthma

Although asthma is usually known as a noninfectious allergic disease, views challenging current concepts have emerged, which indicate an association between disordered bacterial microbiota and pathogenesis of asthma. Wood et al. reported that several potentially pathogenic bacteria with significant quantities were cultured from the sputum in 15% (17/115) of patients with stable asthma with increases of sputum total cell counts, the proportion and number of neutrophils, and IL-8 levels, suggesting the presence of lung microbiota and its effects on immunity in asthma [49]. With culture-independent 16S rDNA sequencing, Hilty et al. [50] found more bacterial microbiota in the airways of patients with asthma or COPD compared to healthy controls. All of the subjects enrolled were free of clinical infections and antibiotics in this study. The results showed significant increases in pathogenic Proteobacteria, particularly *Haemophilus* spp., in asthmatics and increases in Bacteroidetes, particularly *Prevotella* spp., in controls. However, glucocorticosteroids, the main treatment for asthma, promote the persistent colonization of *Haemophilus influenzae* in a mouse model of infection [51]. Thus, whether asthma or steroid treatment should be responsible for the disorder of the lung microbiome remains unclear. Marri et al. [16] collected the induced sputum samples from mild active asthmatics and nonasthmatics. Compared with those of nonasthmatics, sputum samples from patients contained higher proportions of Proteobacteria, which was consistent with the findings of Hilty et al., and possible lower proportions of Firmicutes and Actinobacteria. Because 80% of patients enrolled in this study were not using corticosteroids, the changes in the relative abundance of bacterial species should be attributed to the disease itself rather than the treatments. Another study which investigated the combined effects of colonization and allergic airway disease also showed a declined clearance rate of *Haemophilus influenzae* in OVA-sensitized mice, indicating the possibility that allergic airway diseases, such as asthma, contribute to chronic bacterial colonization and alterations of the lung microbiome [52]. Steroid treatment further promotes dysbiosis and exerts synergistic effects.

The lung microbiome also differs in patients with different asthma control levels and disease severity. In suboptimally controlled asthmatics, the burden and diversity of bronchial microbiota have been reported to be much higher, and an association between bacterial composition and degree of airway hyperresponsiveness was also investigated [53]. Moreover, compared to mild-moderate asthmatics, patients with severe asthma were significantly enriched in taxa belonging to Actinobacteria and had less abundant levels of several different families of Proteobacteria [17].

In contrast, however, one study compared the pathogenic microorganisms of the lungs, including bacteria, virus, and fungi, in predominantly allergic asthma, nonallergic asthma, mixed asthma, and unspecified asthma patients according to the latest edition of the ICD (J45.0–J45.9) from 2011 to 2012 with no limitations on the patients' state of exacerbation using methods including Gram staining, culture, and PCR and found no significant differences among the 4 groups [54]. Therefore, associations between asthma and microorganisms have not been firmly established.

The less sensitive methods used in this study may have been responsible for the negative result, but the inappropriate subject grouping may have had even more of an effect. In this study, subjects were grouped by whether they were allergic or not, but no direct associations between bacterial products and allergy have been found yet. In contrast, the dose-dependent effect of LPS on immune responses has been determined. In a mouse model using OVA challenges, very low doses of LPS induced Th2-dominant inflammation, whereas Th17-related inflammation was induced and promoted by increased doses of LPS. When the dose of LPS continued to increase and reached levels much higher than those found in the environment, large numbers of Treg cells accumulated [55]. Therefore, the major effect of the lung microbiome on asthma is likely to shape immunity and subsequently influence clinical manifestations and disease severity. Because of the importance of immune responses in the pathogenesis of asthma with disordered lung microbiomes, the fact that enrolled subjects comprised both attack and attack-free patients would aggravate the immune heterogeneity among the groups and veil any potential differences.

## 6. Bacterial Disorder in Asthma and Involvement of IL-17

The greater share of evidence indicates that disorders of the bacterial microbiota play a role in asthma development, corticosteroid response, and severity by regulating immunity with IL-17 involved in this regulation.

Birth cohort studies in Copenhagen revealed that neonates with bacterial colonization, especially *S. pneumoniae*, *H. influenzae*, or *Moraxella catarrhalis*, had a higher risk for asthma in their early lives [56]. These pathogenic bacteria may promote different types of immune responses. Følsgaard et al. reported that colonization by *M. catarrhalis* and *H. influenzae* induced a mixed Th1/Th2/Th17 response, whereas colonization by *S. aureus* induced a Th17 response with elevated IL-17 levels [57]. The colonization might be associated with acute wheezing episodes [58], but even if asymptomatic in infancy, children diagnosed with asthma at the age of 7 years still exhibited excessive immune responses with the aberrant production of IL-5, IL-13, IL-17, and IL-10 by their PBMCs when treated with those pathogens that had colonized them during their infancy [59]. These studies indicate that some specific pathogenic bacterial genera induced Th17-associated immune responses and may be responsible for increased risk of asthma. However, some other studies support the alternative hypothesis that exposure to diverse microbes in early life helps to protect against atopic diseases. Gollwitzer et al. found that the shifts of lung microbiome from predominant phyla of Gammaproteobacteria and

Firmicutes towards Bacteroidetes may be a key factor that induces Treg cells via PD-L1, promoting the tolerance to allergen during the maturation of the neonatal immune system and decreasing the responsiveness to allergen in adulthood [60]. Therefore, the actual effect on asthma development may be determined by the complex interactions among the burden, genera, microbe diversity, the types and routes of exposure, and host immunity [61].

A clinical trial investigating 42 preschool children with severe persistent wheezing and without symptoms of acute respiratory infections revealed that 81% of all subjects had neutrophilic inflammation and elevated bacteria counts presented in 59% among them. After treatment with antibiotics, 92% of the subjects obtained significant improvements, suggesting that bacterial colonization was responsible for the increased severity of symptoms and elevation of neutrophils [62]. To directly describe the inflammatory phenotype-specific alterations to the airway microbiome in asthma, Simpson et al. [63] grouped poorly controlled asthmatics according to their inflammatory phenotypes and analyzed their sputum microbiome, showing that neutrophilic asthma had expanded *Haemophilus influenzae* with reduced bacterial diversity and species richness, whereas eosinophilic asthma had greater percentages of *Tropheryma whipplei*. This study directly and strongly confirmed the disorder of lung microbiome in neutrophilic asthma.

As discussed previously, neutrophilic asthma is often characterized by a positive correlation with disease severity and steroid-resistant airway inflammation. Th17/IL-17-associated immune responses exhibit the same characteristics accompanied by recruiting neutrophils. Thus, Th17/IL-17 immune response likely acts as a bridge between the lung microbiome and neutrophilic asthma.

In steroid-resistant asthma, the dominance of *Moraxella catarrhalis* or *Haemophilus* or *Streptococcus* genera in the induced sputum is associated with a longer asthma disease course, worse postFEV1%predict (68.0% in average), and higher proportions and numbers of sputum neutrophils, which are also correlated with the total bacterial burden [64]. Another study that compared the lung microbiome composition in patients with corticosteroid-resistant (CR) and corticosteroid-sensitive (CS) asthma also showed the differences at genus level and that *Haemophilus* spp. are the most responsible for corticosteroid resistance [65]. In a murine model of allergic airway disease, *Haemophilus influenzae* colonization rather than active infection successfully induced steroid-resistant neutrophilic inflammation that was mediated by IL-17, suggesting the involvement of IL-17 in refractory neutrophilic asthma associated with disordered lung microbiome [52, 66].

In severe asthma, compared to mild-moderate asthma, taxa most belonging to Actinobacteria were found to be significantly enriched, whereas several different families in Proteobacteria were much less abundant [17]. More importantly, these researchers also analyzed the association of airway bacterial microbiota (sampled by bronchial brushings) and host epithelial gene expression, showing that several families in Proteobacteria positively correlated with Th17-associated gene expression [17]. However, interestingly, these families were different from those that belonged to Proteobacteria but correlated with FKBP5, a marker of steroid resistance. Proteobacteria levels were also negatively correlated with biopsy eosinophil numbers. Therefore, these results suggest that the increase of specific airway bacterial microbiota, such as some families in Proteobacteria, leads to Th17-associated inflammation and contributes to noneosinophilic asthma or neutrophilic asthma through the recruitment of neutrophils by IL-17. And this was independent of steroids effects. The involvement of IL-17 in neutrophilic asthma with a disordered microbiome has also been demonstrated in animal models. In a mouse model, *Streptococcus pneumonia* infection in infancy increased the risk of adulthood allergic airway diseases, with elevated Th17/IL-17 levels and accumulation of neutrophils, whereas the neutralization of IL-17 by monoclonal antibody significantly reduced neutrophil recruitment, alleviated airway inflammation, and decreased airway hyperresponsiveness, indicating an essential role of IL-17 in the pathogenesis of neutrophilic asthma [67].

## 7. Conclusion

The bronchial tree is not sterile. Lung microbiome disorders are involved in many chronic airway diseases, such as asthma. When some specific potentially pathogenic bacteria colonizing in the airways increase, the total burden and/or the community composition of the lung microbiome may change, which may subsequently induce Th17-associated inflammation. Large amounts of IL-17 are released and neutrophils are recruited into the airways, contributing to neutrophilic inflammation and promoting host defense or bacteria-mediated immune responses. Moreover, these responses are likely involved in the pathogenesis of severe, steroid-resistant neutrophilic asthma, which has been shown to be associated with the disordered lung microbiome. However, more studies on the precise mechanisms of how Th17/IL-17-associated immunity responds to the lung bacterial microbiota and determines the inflammatory and clinical phenotypes of asthma should be performed to help further understand the complex pathogenesis of asthma and generate more therapeutic options.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Research Article*

# *Interleukin-17A* Gene Variability in Patients with Type 1 Diabetes Mellitus and Chronic Periodontitis: Its Correlation with IL-17 Levels and the Occurrence of Periodontopathic Bacteria

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Interleukin-17 contributes to the pathogenesis of type 1 diabetes mellitus (T1DM) and chronic periodontitis (CP). We analyzed *IL-17A* −197A/G and *IL-17F* +7488C/T polymorphisms in T1DM and CP and determined their associations with IL-17 production and occurrence of periopathogens. Totally 154 controls, 125 T1DM, and 244 CP patients were genotyped using 5′ nuclease TaqMan® assays. Bacterial colonization was investigated by a DNA-microarray kit. Production of IL-17 after *in vitro* stimulation of mononuclear cells by mitogens and bacteria was examined by the Luminex system. Although no differences in the allele/genotype frequencies between patients with CP and T1DM + CP were found, the *IL-17A* −197 A allele increased the risk of T1DM ($P < 0.05$). Levels of HbA1c were significantly elevated in carriers of the A allele in T1DM patients ($P < 0.05$). Production of IL-17 by mononuclear cells of CP patients (unstimulated/stimulated by *Porphyromonas gingivalis*) was associated with *IL-17A* A allele ($P < 0.05$). *IL-17A* polymorphism increased the number of *Tannerella forsythia* and *Treponema denticola* in patients with CP and T1DM + CP, respectively ($P < 0.05$). *IL-17A* gene variability may influence control of T1DM and the "red complex" bacteria occurrence in patients with CP and T1DM + CP. Our findings demonstrated the functional relevance of the *IL-17A* polymorphism with higher IL-17 secretion in individuals with A allele.

## 1. Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease caused by T cell-mediated destruction of pancreatic $\beta$ cells resulting in the absence of insulin and uncontrolled hyperglycemia. A complex interplay between genetic and environmental factors participates in developing of T1DM and manifestation of its systemic and oral complications. Periodontitis has been identified as the sixth complication of diabetes; the other five complications are retinopathy (DR), nephropathy (DN), neuropathy (DPN), macrovascular disease, and poor wound healing [1]. Periodontitis is a chronic infection characterized by progressive inflammatory response to bacteria in dental plaque, which finally results in periodontal tissue destruction and tooth loss. The relationship between diabetes mellitus and periodontitis has been reported previously [2]. Chronic hyperglycemia induces a proinflammatory state in the gingival microcirculation characterized by an increased vascular permeability, and leukocyte and endothelial cell activation, which may contribute to periodontal tissue damage

in diabetes mellitus [3]. Chronic periodontitis (CP) is more frequent in T1DM patients and can worsen its metabolic control [4, 5]. Nevertheless, molecular mechanisms responsible for periodontal disease and its progression in T1DM patients remain unknown.

Although individuals at risk for T1DM are recognized by screening for HLA-associated risk genotypes and $\beta$ cell autoantibodies, recently the pathogenic role of IL-17-secreting T helper 17 (Th17) cells has been implicated in the development of T1DM [6–8]. The IL-17 cytokine family consists of six cytokine members designed from IL-17A (originally cloned and named CTLA-8), IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F, according to the order of their discoveries. IL-17A and IL-17F, the most studied members in the IL-17 family, are located close to each other on human chromosome 6, sharing the highest amino acid sequence identity (50%) and similar functions. They are primarily involved in autoimmune responses, tumor development, and host defense against bacterial and fungal infections by activating epithelial innate immune responses and production of antimicrobial peptides, cytokines (e.g., IL-6 or TNF-$\alpha$), chemokines (e.g., IL-8), and cartilage degrading metalloproteinases as well as cytokines promoting osteoclastogenesis that results in bone destruction [9–11].

Several lines of evidence suggest that IL-17 plays a role in human diabetes. Bradshaw et al. [12] observed that monocytes isolated from T1DM patients induced more IL-17 producing T cells compared with healthy controls. They also observed significantly increased IL-17 producing T cells in peripheral blood of patients with long standing T1DM [12]. Arif et al. [13] demonstrated that activation of IL-17 pathway accelerated pancreatic $\beta$ cell apoptosis and led to autoimmune diabetes. They observed significantly elevated IL-17A expression in pancreas from newly diagnosed T1DM patients. In addition, peripheral blood lymphocytes from T1DM patients had elevated IL-17A and IL-17F expression [14].

The emerging role of IL-17 in periodontal disease was also discussed in a recent study, based on evidence from human and animal models [15]. Upregulated *IL-17A* gene expression has been observed in patients with CP, suggesting that the net effect of IL-17 signaling promotes the disease development [16–22]. Additionally, IL-17RA deficient mice were found more susceptible to *Porphyromonas gingivalis* (*P.g.*), causal Gram-negative bacteria of the "red complex" [23, 24]. Based on the fact that increased IL-17 levels occur in the gingival tissue of patients with periodontal disease [25–27], Park et al. [28] hypothesized that *P.g.* lipopolysaccharide (LPS) might mediate IL-17 release from human periodontal ligament cells.

Despite the important role of IL-17 cytokine in T1DM and CP pathogenesis, no study has investigated *IL-17* gene variability in T1DM patients so far and only a few studies have reported a relationship between polymorphisms in the *IL-17* gene and periodontitis with contradictory results [29–33]. Therefore, in the present study, we aimed to investigate the association of *IL-17A* −197A/G (rs2275913) and *IL-17F* +7488C/T (His161Arg, rs763780) gene polymorphisms with T1DM and/or CP. In addition, *IL-17A* single nucleotide polymorphism (SNP) was examined in relation to the occurrence of selected periodontal bacteria in subgingival pockets and production of IL-17 by mononuclear cells in a subgroup of CP patients and healthy controls.

## 2. Material and Methods

The study was performed with the approval of the Committees for Ethics of the Medical Faculty, Masaryk University Brno and St. Anne's Faculty Hospital. Written informed consent was obtained from all participants in line with the Helsinki declaration before inclusion in the study.

*2.1. Study Population and Clinical Examinations.* In this case-control study, 523 unrelated adult subjects from the Czech Republic were included. One hundred and twenty-five patients with T1DM were followed in the outpatient unit of the Diabetology Clinics in South Moravia Region, Czech Republic. The diagnosis of T1DM was originally based on the presence of clinical symptoms (such as polyuria, polydipsia, and weight loss) and biochemical parameters (glycemia, ketoacidosis, and autoantibody status). All patients were receiving intensified insulin therapy or insulin pump and other medicaments according to the presence of diabetic complications, such as DN, DR, DPN, and other comorbidities as described on a part of our cohort previously [34]. Duration of diabetes was defined as the period from diabetes onset until the enrolment in this study. Levels of glycemia, glycated hemoglobin (HbA1c), total cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), body mass index (BMI), and further parameters were recorded. The periodontal status was evaluated in a subgroup of 38 diabetic patients, 154 healthy controls, and 244 CP subjects recruited from a patient pool of the Clinic of Stomatology, St. Anne's Faculty Hospital Brno, from 2005 to 2015. The controls were selected from subjects referred to the Clinic of Stomatology for reasons other than periodontal disease (such as dental caries, orthodontic consultations, and preventive dental checkups) during the same period as patients and matched for age and gender. Exclusion criteria were history of systemic diseases such as cardiovascular disorders (e.g., coronary artery diseases), diabetes mellitus, malignant diseases, immunodeficiency, and current pregnancy or lactation.

The diagnosis of periodontitis/nonperiodontitis was based on the detailed clinical examination, medical and dental history, tooth mobility, and radiographic assessment as described in our previous study [35].

*2.2. Genetic Analysis.* Isolation and storage of DNA and genotyping of samples were conducted in the laboratory of the Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.

Genotyping of two SNPs in *IL-17*, *IL-17A* −197A/G (rs2275913), and *IL-17F* +7488C/T (His161Arg, rs763780) was based on polymerase chain reaction using 5′ nuclease TaqMan assays (C_15879983_10, C_2234166_10, resp.). Reaction mixture and conditions were designed according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA) and fluorescence was measured using the ABI PRISM 7000 Sequence Detection System. SDS version 1.2.3

Table 1: Demographic data of the healthy controls and the studied subjects with CP, T1DM (and the T1DM + CP subgroup).

| Characteristics | Controls $N = 154$ | CP $N = 244$ | T1DM $N = 125$ | T1DM + CP $N = 38$ |
|---|---|---|---|---|
| Age (mean years ± SD) | 48.5 ± 10.7 | 52.5 ± 9.8 | 46.4 ± 13.8 | 49.9 ± 10.6 |
| Sex (males/females) | 75/79 | 112/132 | 67/58 | 16/22 |
| Smoking (no/yes, %) | 71.1/28.9 | 73.9/26.1 | 78.9/21.1[#] | 78.9/21.1 |
| BMI (mean ± SD) | 23.2 ± 4.6 | 26.4 ± 3.7 | 25.0 ± 4.9 | 25.3 ± 3.1 |
| Duration of DM (mean years ± SD) | — | — | 22.8 ± 10.3 | 24.0 ± 11.3 |
| HbA1c (mmol/mol, mean ± SD) | — | — | 76.5 ± 17.3 | 69.9 ± 11.4 |
| DN (no/yes, %) | — | — | 52.9/47.1 | 71.9/28.1 |
| DR (no/yes, %) | — | — | 30.4/69.6 | 42.4/57.6 |
| DPN (no/yes, %) | — | — | 39.3/60.7 | 47.1/52.9 |

BMI = body mass index, CP = chronic periodontitis, DN = diabetic nephropathy, DPN = diabetic peripheral neuropathy, DR = diabetic retinopathy, HbA1c = glycated hemoglobin, $N$ = number of subjects, SD = standard deviation, and T1DM = type 1 diabetes mellitus.
[#]Smoking status is known only in T1DM patients with CP.

software was used to analyze real-time and endpoint fluorescence data.

*2.3. DNA-Microarray Analyses of Oral Pathogens.* DNA-microarray analyses of oral pathogens based on a periodontal pathogen detection kit (Protean Ltd., Ceske Budejovice, Czech Republic) were previously used and described [35]. Bacterial colonization [*Aggregatibacter actinomycetemcomitans* (*A.a.*), *Tannerella forsythia* (*T.f.*), *P.g.*, *Prevotella intermedia* (*P.i.*), *Treponema denticola* (*T.d.*), *Peptostreptococcus micros* (*P.i.*), and *Fusobacterium nucleatum* (*F.n.*)] in subgingival sulci/pockets was investigated in a subgroup of controls ($N = 51$), CP patients ($N = 182$), and T1DM patients with CP ($N = 38$) before subgingival scaling. This test determined the individual pathogens semiquantitatively as follows: (−) undetected, which corresponds to the number of bacteria less than $10^3$, (+) slightly positive corresponding to the number of bacteria $10^3$ to $10^4$, (++) positive corresponding to the number of bacteria $10^4$ to $10^5$, and (+++) strongly positive, with the number of bacteria higher than $10^5$.

*2.4. Cultivation of Bacteria and Immunological Examination.* Cultivation of periodontopathic bacteria and immunological examination were performed in the laboratory of the Institute of Clinical and Experimental Dental Medicine, General University Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic.

IL-17 levels were measured in a subgroup of 15 healthy controls and 30 patients with CP. IL-17 levels were determined in mononuclear cells isolated from 20 mL of heparinized blood using the Luminex multiplex method (Luminex 100TM analyzer, R&D systems, USA). The isolation, cultivation, and stimulation of cells by selected bacteria (*A.a.*, *T.f.*, *P.g.*, and *P.i.*) and mitogens or Heat Shock Protein (HSP) 70 were described in detail in our previous article [36].

*2.5. Statistical Analysis.* Statistical analysis was performed using the statistical package Statistica v. 10 (StatSoft Inc., USA). Standard descriptive statistics were applied in the analysis: absolute and relative frequencies for categorical variables and mean with standard deviation (SD) or median with quartiles for quantitative variables. To compare independent groups, one-way analysis of variance (ANOVA) and Kruskal-Wallis (ANOVA) were performed to compare continuous variables. The allele frequencies were calculated from the observed numbers of genotypes. The differences in the allele frequencies were tested by the Fisher-exact test; Hardy-Weinberg equilibrium (HWE) and genotype frequencies were calculated by the chi-square test ($\chi^2$). The association was described by odds ratios (OR) with 95% confidence intervals (95% CI). Only the values of $P$ less than 0.05 were considered as statistically significant.

## 3. Results

*3.1. Case-Control Study.* The demographic data of the study population are shown in Table 1. The mean ages and BMI between the healthy controls and patient groups did not differ significantly ($P > 0.05$). There were also no significant differences between the subjects with T1DM or CP and the controls relating to males/females ratio or smoking status. All diabetic patients ($N = 38$) who were examined at the Periodontology Department were affected by periodontitis. Duration of DM and diabetic complications (DN, DR, and DPN) in the whole diabetic cohort versus subgroup of T1DM patients with CP were not statistically different.

*3.2. SNPs Analysis.* Both studied polymorphisms were in the HWE in the control group ($P > 0.05$). Considering the fact that, in the Czech population, SNP *IL-17F* +7488C/T (His161Arg, rs763780) TT genotype occurred in 93.7%, we analyzed this polymorphism only in the subgroup of subjects ($N = 190$, data not shown).

The allele and genotype frequencies of the *IL-17A* −197A/G (rs2275913) variant are shown in Table 2. While the genotype frequencies were not different between the controls and CP or T1DM patients, the A allele was marginally associated with an increased risk to T1DM ($P < 0.05$,

Table 2: *IL-17A* −197A/G (rs2275913) genotype and allele frequencies in healthy controls, patients with CP, T1DM (and the T1DM + CP subgroup).

| *IL-17A* −197 | Controls | CP | | | T1DM | | | T1DM + CP | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Genotype and allele | $N$ = 154 (%) | $N$ = 244 (%) | $P$ | OR (95% CI) | $N$ = 125 (%) | $P$ | OR (95% CI) | $N$ = 38 (%) | $P$ | OR (95% CI) |
| AA | 18 (11.7) | 32 (13.1) | 0.56 | 1.00 (0.52–1.93) | 23 (18.4) | 0.05 | 1.93 (0.93–4.00) | 6 (15.8) | 0.46 | 1.20 (0.42–3.48) |
| AG | 71 (46.1) | 97 (39.8) | 0.14 | 0.77 (0.50–1.19) | 59 (47.2) | 0.23 | 1.26 (0.75–2.11) | 14 (36.8) | 0.25 | 0.71 (0.33–1.55) |
| GG | 65 (42.2) | 115 (47.1) | | 1.00 | 43 (34.4) | | 1.00 | 18 (47.4) | | 1.00 |
| AA + AG versus GG | 89 versus 65 (57.8/42.2) | 129 versus 115 (52.9/47.1) | 0.20 | 0.82 (0.55–1.23) | 82 versus 43 (65.6/34.4) | 0.11 | 1.39 (0.85–2.27) | 20 versus 18 (52.6/47.4) | 0.35 | 0.81 (0.40–1.65) |
| A | 107 (34.7) | 161 (33.0) | | 0.92 (0.68–1.25) | 105 (42.0) | | 1.36 (0.96–1.92) | 26 (34.2) | | 0.98 (0.58–1.66) |
| G | 201 (65.3) | 327 (67.0) | 0.33 | 1.00 | 145 (58.0) | 0.05* | 1.00 | 50 (65.8) | 0.37 | 1.00 |

CI = confidential interval, CP = chronic periodontitis, $N$ = number of subjects, OR = odds ratio, and T1DM = diabetes mellitus type 1; * $P$ value < 0.05 (in comparison with healthy controls).

Table 3: Levels of HbA1c in correlation with *IL-17A* −197A/G (rs2275913) genotypes in T1DM#.

| *IL-17A* −197 | T1DM | | |
|---|---|---|---|
| Genotype | $N$ | HbA1c (mmol/mol) Mean ± SD | $P$ |
| AA + AG | 69 | 76.6 ± 16.5 | |
| GG | 35 | 69.8 ± 13.9 | 0.03* |

HbA1c = glycated hemoglobin, $N$ = number of subjects, SD = standard deviation, and T1DM = type 1 diabetes mellitus; * $P$ value < 0.05 (parametric test, ANOVA).
#Levels of HbA1c were available in 104 patients with T1DM.

OR = 1.36, 95% CI = 0.96–1.92). Moreover, mean levels of HbA1c were significantly elevated in carriers of the A allele (AA and AG genotypes in comparison to GG homozygotes) in a group of T1DM patients (76.6 mmol/mol versus 69.8 mmol/mol, $P < 0.05$, Table 3).

Although no significant differences in the *IL-17A* allele or genotype frequencies between patients with CP and healthy controls were found in the whole set (Table 2), stratification of subjects according to smoking status revealed the following differences: in healthy subjects, the A allele frequency was higher in smokers ($P < 0.05$, OR = 1.62, 95% CI = 0.97–2.70), whereas, in CP patients, this allele was found more frequently in nonsmokers ($P < 0.05$, OR = 0.64, 95% CI = 0.41–1.02). Although a similar distribution of alleles or genotypes between the groups of control nonsmokers versus CP nonsmokers was found, frequencies of the A allele ($P < 0.01$, OR = 2.14, 95% CI = 1.19–3.83), AA genotype ($P < 0.05$, OR = 3.17, 65% CI = 0.89–11.28), and AA + AG versus GG genotypes ($P < 0.05$, OR = 1.82, 95% CI = 0.81–4.09) were more frequent in the control smokers in comparison with the smokers with CP (Table 4). However, as the associations detected between the given subgroups were only of borderline significance and numbers of the individuals in the subgroups were small, the results obtained should be interpreted carefully. A subanalysis performed separately in the groups of females ($N = 269$) and males ($N = 254$) showed no significant difference in the *IL-17A* −197A/G (rs2275913) allele or genotype frequencies (data not shown). Due to the small number of T1DM patients with confirmed CP, this group was not analyzed according to gender or smoking status.

*3.3. Microbial Analysis.* Possible links between the *IL-17A* −197A/G (rs2275913) variant and the occurrence of seven selected periodontal bacteria in subgingival pockets were analyzed. The *IL-17A* −197 A allele carriers had an increased risk of *T.f.* occurrence in 182 CP patients (34.9% versus 19.6%, $P < 0.05$, OR = 2.20, 95% CI = 1.03–4.73) and a similar but nonsignificant trend was observed for the presence of *P.i.* (36.0% versus 28.7%, $P = 0.09$, OR = 1.40, 95% CI = 0.89–2.20). On the other hand, in patients with T1DM and CP carrying A allele of the *IL-17A* −197 polymorphism, *T.d.* in subgingival biofilm occurred less frequently than in subjects without this allele (26.9% versus 50.0%, $P < 0.05$, OR = 0.37, 95% CI = 0.13–1.01, Table 5). In subgroups of nonsmokers, the *IL-17A* −197 A allele carriers had an increased risk for the occurrence of *P.i.* in patients with CP only (39.6% versus 28.7%, $P < 0.05$, OR = 1.63, 95% CI = 0.96–2.76, Table 5). The *IL-17A* −197 SNP was not associated with the occurrence of any other bacteria (including *P.g.*), from those seven selected ones (data not shown).

*3.4. Immunological Analysis.* We analyzed IL-17 levels in relation to the *IL-17A* −197A/G (rs2275913) polymorphism in selected patients with CP and healthy controls. The IL-17 levels were measured after a 3-day *in vitro* cultivation of mononuclear cells, without or with stimulation by dental plaque bacteria, mitogens, or HSP70 in the CP patients ($N = 30$). Carriers of genotype with the A allele of *IL-17A* −197A/G (rs2275913) SNP had a higher production of IL-17 by unstimulated monocytes (0.98 pg/mL versus 0.27 pg/mL, $P < 0.05$) and also after stimulation with *P.g.* (1.51 pg/mL versus 0.10 pg/mL, $P < 0.05$) than GG homozygotes (Table 6).

Table 4: *IL-17A* −197A/G (rs2275913) genotype and allele frequencies in healthy controls and patients with CP stratified by smoking status.

| *IL-17A* −197 | Control nonsmokers | Control smokers | | CP nonsmokers | CP smokers | | | |
|---|---|---|---|---|---|---|---|---|
| Genotype and allele | $N = 108$ (%) | $N = 44$ (%) | $P$ | $N = 173$ (%) | $N = 61$ (%) | $P$ | $P^a$ | $P^b$ |
| AA | 10 (9.2) | 8 (18.2) | | 28 (16.2) | 4 (6.6) | | | |
| AG | 49 (45.4) | 22 (50.0) | | 67 (38.7) | 24 (39.3) | | | |
| GG | 49 (45.4) | 14 (31.8) | 0.16 | 78 (45.1) | 33 (54.1) | 0.15 | 0.22 | 0.04* |
| AA + AG versus GG | 59 versus 49 (54.6/45.4) | 30 versus 14 (68.2/31.8) | 0.09 | 95 versus 78 (54.9/45.1) | 28 versus 33 (45.9/54.1) | 0.14 | 0.10 | 0.02* |
| A | 69 (31.9) | 38 (43.2) | | 123 (35.5) | 32 (26.2) | | | |
| G | 147 (68.1) | 50 (56.8) | 0.04* | 223 (64.5) | 90 (73.8) | 0.04* | 0.22 | 0.01* |

CP = chronic periodontitis, $N$ = number of subjects, and * $P$ value < 0.05.
$P^a$ comparison of groups of nonsmokers.
$P^b$ comparison of groups of smokers.

Table 5: The presence of bacteria in correlation with *IL-17A* −197A/G (rs2275913) polymorphism in patients with CP and T1DM + CP#.

| *IL-17A* −197 | CP $N = 182$ (%) | | | CP $N = 182$ (%) | | | T1DM + CP $N = 38$ (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| Genotype and allele | *T.f.* neg | *T.f.* pos | $P$ | *P.i.* neg | *P.i.* pos | $P$ | *T.d.* neg | *T.d.* pos | $P$ |
| AA | 0 (0.0) | 24 (15.1) | 0.02* | 6 (8.0) | 18 (16.8) | 0.07 | 3 (25.0) | 3 (11.5) | 0.14 |
| AG | 9 (39.1) | 63 (39.6) | 0.33 | 31 (41.3) | 41 (38.3) | 0.51 | 6 (50.0) | 8 (30.8) | 0.11 |
| GG | 14 (60.9) | 72 (45.3) | | 38 (50.7) | 48 (44.9) | | 3 (25.0) | 15 (57.7) | |
| AA + AG versus GG | 9 versus 14 (39.1 versus 60.9) | 87 versus 72 (54.7 versus 45.3) | 0.12 | 37 versus 38 (49.3 versus 50.7) | 59 versus 48 (55.1 versus 44.9) | 0.27 | 9 versus 3 (75.0 versus 25.0) | 11 versus 15 (42.3 versus 57.7) | 0.06 |
| A | 9 (19.6) | 111 (34.9) | | 43 (28.7) | 77 (36.0) | | 12 (50.0) | 14 (26.9) | |
| G | 37 (80.4) | 207 (65.1) | 0.03* | 107 (71.3) | 137 (64.0) | 0.09 | 12 (50.0) | 38 (73.1) | 0.04* |
| *IL-17A* −197 | CP nonsmokers $N = 131$ (%) | | | CP nonsmokers $N = 131$ (%) | | | T1DM + CP nonsmokers $N = 30$ (%) | | |
| Genotype and allele | *T.f.* neg | *T.f.* pos | $P$ | *P.i.* neg | *P.i.* pos | $P$ | *T.d.* neg | *T.d.* pos | $P$ |
| AA | 0 (0.0) | 20 (17.5) | 0.06 | 4 (7.4) | 16 (20.8) | 0.04* | 2 (25.0) | 3 (13.6) | 0.27 |
| AG | 8 (47.1) | 44 (38.6) | 0.59 | 23 (42.6) | 29 (37.7) | 0.51 | 4 (50.0) | 7 (31.8) | 0.21 |
| GG | 9 (52.9) | 50 (43.9) | | 27 (50.0) | 32 (41.6) | | 2 (25.0) | 12 (54.5) | |
| AA + AG versus GG | 8 versus 9 (47.1 versus 52.9) | 64 versus 50 (56.1 versus 43.9) | 0.33 | 27 versus 27 (50.0 versus 50.0) | 45 versus 32 (58.5 versus 41.6) | 0.22 | 6 versus 2 (75.0 versus 25.0) | 10 versus 12 (45.4 versus 54.5) | 0.15 |
| A | 8 (23.5) | 84 (36.8) | | 31 (28.7) | 61 (39.6) | | 8 (50.0) | 13 (29.5) | |
| G | 26 (76.5) | 144 (63.2) | 0.09 | 77 (71.3) | 93 (60.4) | 0.05* | 8 (50.0) | 31 (70.5) | 0.12 |

CP = chronic periodontitis, $N$ = number of subjects, neg = negative, pos = positive, T1DM = type 1 diabetes mellitus, *P.i.* = *Prevotella intermedia*, *T.d.* = *Treponema denticola*, *T.f.* = *Tannerella forsythia*, and * $P$ value < 0.05.
# Of the seven periodontal pathogens analyzed, only those with significant differences are shown.

In the healthy controls ($N = 15$), unstimulated IL-17 levels were 0.10 (0.08–0.26) pg/mL (median; 25–75 quartiles) without any significant relationship with *IL-17A* polymorphism. After pooling both groups ($N = 45$), production of IL-17 was significantly associated with *IL-17A* polymorphism in the unstimulated mononuclear cells ($P < 0.05$), but not after stimulation by *P.g.* ($P = 0.06$, data not shown). *IL-17A* −197A/G SNP had no significant effect on IL-17 production after stimulation with other periodontal bacteria, HSP70, and/or mitogens (data not shown).

## 4. Discussion

Inflammation is a physiological immune response triggered during infection and injury in an attempt to prevent infection and promote regeneration. However, persistent and

Table 6: Levels of IL-17 in correlation with *IL-17A* −197A/G (rs2275913) genotypes in patients with CP[#].

| *IL-17A* −197 | IL-17 unstimulated (pg/mL); median (25–75 quartiles) | |
|---|---|---|
| Genotype | *N* | *P* |
| AA + AG | 19 | 0.98 (0.25–8.53) | |
| GG | 11 | 0.27 (0.00–0.54) | 0.04* |
| *IL-17A* −197 | IL-17 stimulated by *P.g.* (pg/mL); median (25–75 quartiles) | |
| Genotype | *N* | *P* |
| AA + AG | 19 | 1.51 (0.50–4.56) | |
| GG | 11 | 0.10 (0.00–1.51) | 0.02* |

CP = chronic periodontitis, *N* = number of subjects, * *P* value < 0.05 (Kruskal-Wallis test, ANOVA), and *P.g.* = *Porphyromonas gingivalis*.
Levels of IL-17 were available in 30 patients with CP.
[#]Only significant differences were shown.

unwarranted inflammation can result in host tissue damage [8]. Regulation of inflammation is a complex process, tightly controlled by signaling messengers of the immune system, such as cytokines. IL-17A and IL-17F, produced mainly by Th17 cells, have been found to be involved in the pathogenesis of autoimmune diseases including diabetes and chronic inflammation, such as periodontitis [12, 15, 37–41].

We evaluated *IL-17A* −197A/G (rs2275913) and *IL-17F* +7488C/T (His161Arg, rs763780) SNPs in a group of adults with and without T1DM and/or CP from Czech population. As the present study identified a low variability of *IL-17F* at position +7488C/T (similarly as other studies in other populations: http://www.snpedia.com/index.php/Rs763780), the TT genotype occurred in 93.7%, and no CC homozygote was detected, we investigated this polymorphism only in a subgroup of 190 subjects. Further, no associations of this polymorphism with aggressive periodontitis (AgP) or CP [31–33] have been previously found. In contrast, the *IL-17A* −197A/G SNP was analyzed in the whole set of our 523 subjects. The minor allele frequency (MAF) of this SNP was 0.36, which is in line with the allele frequency in other European population [42]. To this date, no study investigating an association of the *IL-17A* −197A/G or *IL-17F* +7488C/T variants with T1DM or periodontitis in European white population has been published. Although no significant differences were found in the genotype frequencies between the healthy subjects and T1DM or CP patients, the A allele was marginally associated with an increased risk of T1DM ($P < 0.05$). This allele displayed a higher affinity for the nuclear factor of activated T cells (NFAT), a critical transcription factor involved in the IL-17 regulation [43, 44]. Espinoza et al. [43] reported that healthy individuals possessing the A allele of *IL-17A* −197A/G (rs2275913) produced significantly more IL-17 after *in vitro* T cells stimulation than those without this allele. Shao et al. [45] described that uncontrolled expansion of Th17 cells was involved in T1DM pathology and might exert essential effects on its development.

In the studied diabetic population, significantly higher levels of HbA1c were present in T1DM carriers of the genotype with the A allele (AA + AG genotypes) versus the GG homozygotes (76.6 mmol/mol ± 16.5 mmol/mol versus 69.8 mmol/mol ± 13.9 mmol/mol). To our knowledge, no previous study has focused on this issue; however, polymorphisms in other genes, for example, *IL-6* [46] and *IL-18* separately [47] or in combination with the *IL-12B* gene [48], have been associated with higher concentration of HbA1c in T1DM populations.

In addition, we found no significant differences in the *IL-17A* −197A/G (rs2275913) allele or genotype frequencies between the healthy subjects and patients with CP, not even after stratification by sex (data not shown). No studies on this topic from European but only from Iranian and Brazilian populations have been reported. In the Iranian population, the CC genotype of another *IL-17A* variant (rs10484879) was associated with CP and peri-implantitis [29]. Three Brazilian studies examined variability in the *IL-17A* −197A/G (rs2275913) gene in relation to periodontal disease with controversial results. In the study by Saraiva et al. [32], the A allele was associated with the absence of periodontitis, but Corrêa et al. [31] and Zacarias et al. [33] found the AA genotype and the A allele as a risk factor for CP. Even in the separate subgroup of Czech patients with CP and T1DM, no relationship between *IL-17A* polymorphism and periodontal status (PD = probing pocket depth, CAL = clinical attachment loss, etc.) was found. However, Gürsoy et al. [49] recently described the association between PD and IL-17 levels in saliva of type 2 diabetic patients, but independently of glycemic status. Interestingly, the allele and genotype distributions of the *IL-17A* variant in the subgroup of T1DM patients with CP were closer to the values in nondiabetic CP than T1DM patients. The different findings in Czech population may be due to differences in European versus Brazilian populations.

In contrast to findings in the Brazilian cohort where the AA genotype was identified as a risk factor for CP in nonsmoker Caucasians [33], our results showed no differences in distribution of the *IL-17A* −197A/G alleles or genotypes between healthy and periodontitis nonsmokers. However, the G allele and the GG genotype were marginally significantly associated with an increased risk of periodontitis in smokers. Analysis of the allele frequencies in nonsmokers versus smokers showed borderline significant differences in both studied groups but in the opposite trend, in which 68.1% versus 56.8% for healthy controls and 64.5% versus 73.8% for patients with CP. Due to a relatively small number of subjects in the individual subgroups, number of comparisons

performed, and only marginally significant differences, our results should be interpreted carefully.

In the next step, according to the hypothesis about biological functions and regulation of IL-17 which plays a role in host defense [50], we assessed the *IL-17A* gene variability in relation to the presence of periodontopathic bacteria in subgingival pockets in patients with CP and T1DM + CP. In CP population, *IL-17A* −197 A allele carriers had an increased risk of *T.f.* occurrence and, in CP nonsmokers only, this allele increased risk of the occurrence of *P.i.*, but the same A allele was protective for the presence of *T.d.* in subgingival biofilm in T1DM patients with CP. In connection with periodontopathic bacteria, gene variability has been investigated in a few other interleukins to this date. The specific *IL-8* variants were associated with subgingival colonization with *A.a.* in AgP and *T.f.* in CP in the Czech population [35]. Finoti et al. [51] also found that *IL-8* haplotype influenced the presence of the "red complex" bacteria in gingival sulci. Several studies by Nibali et al. [52–56] focused on a correlation between the pathogenic bacterial colonization and variability in *IL-6* and *Fc γ receptor* genes. Additionally, SNPs in the *IL-1* gene cluster [57], *interferon γ*, and *IL-2* were associated with the presence of various periodontopathic bacteria, especially *A.a.* and the "red complex" bacteria. However, in our study, no other bacteria from those studied were associated with *IL-17A* −197 variant and there were no significant differences in the occurrence of these bacteria among groups.

Finally, in functional study, we associated IL-17 production *in vitro* by blood mononuclear cells with the *IL-17A* −197A/G (rs2275913) gene polymorphism in the subgroup of patients with CP. In Czech periodontitis patients, *IL-17A* −197 AA + AG carriers had higher IL-17 levels in unstimulated mononuclear cells than GG homozygotes. Our results are in accordance with the conclusion of Espinoza et al. [43], who connected the A allele with increased IL-17 levels *in vitro* after T cells stimulation. Even greater differences in IL-17 production were measured in CP carriers with the A allele in genotype after stimulation with *P.g.* It is in agreement with previous findings that *P.g.* LPS is a mediator of IL-17 release from human periodontal ligament cells [28]. Also Moutsopoulos et al. [58] proved that *P.g.* induced innate cell IL-17 production and promoted Th17 polarization. In a pooled group of CP and healthy subjects ($N = 45$), production of IL-17 was associated with the *IL-17A* polymorphism only in unstimulated mononuclear cells, but not after stimulation by *P.g.*, other periodontal bacteria, HSP70, and/or mitogens. In addition, recently Azman et al. [20] demonstrated that serum, saliva, and gingival crevicular fluid, IL-17A levels were higher in periodontitis patients and correlated positively with clinical parameters (PD, CAL, and BOP = bleeding on probing). Findings of the present study also demonstrated increased IL-17 levels in unstimulated monocytes in patients with periodontitis versus healthy controls (0.48 pg/mL versus 0.10 pg/mL; median). However, the comparison of absolute values of IL-17 levels in healthy subjects with results of other studies would require the use of the same method for the cytokine determination (including a kit from the same supplier), type and preparation of samples, and age of subjects [59, 60].

There are many possible limitations in this study. The present study is mainly limited by relatively low numbers of subjects and especially by the fact that from the group of 125 T1DM subjects, only 38 patients were evaluated for periodontal status. The negative findings of single marker analysis in this subgroup could be a result of a lack of statistical power to detect minor differences (small effect of genes/gene variants in multifactorial diseases is typical). In addition, most of the associations found were tightly below statistical significance ($P < 0.05$) without multitest corrections. Therefore, risk of relatively high false discovery rate means that our results should be interpreted with caution. Secondly, levels of IL-17 in plasma or in gingival tissue were not measured and the presence of periodontal pathogens was examined only in the 271 subjects. However, this study also has several strengths. This is the first study of the *IL-17* gene polymorphisms in T1DM population and in European patients with CP that was conducted in a relatively homogenous population of white Caucasians in Central Europe of the Czech origin. Secondly, the size of studied healthy subjects and CP patients is greater than in previous Iranian or Brazilian studies. Thirdly, we examined not only the polymorphisms alone but their relationship with clinical, bacterial, and biochemical parameters, which allowed a better biological assessment of the detected associations.

## 5. Conclusions

In conclusion, *IL-17A* gene variability may partially influence T1DM control and the "red complex" bacteria occurrence in patients with CP and diabetic patients with CP. Additionally, our findings confirmed the functional relevance of the *IL-17A* polymorphism with higher IL-17 secretion in the individuals with the A allele. However, the results of this study need to be proven in a larger independent cohort.

## Conflict of Interests

The authors declare no conflict of interests.

## Authors' Contribution

Petra Borilova Linhartova, Jirina Bartova, Katerina Kankova, and Lydie Izakovicova Holla designed the study and drafted the paper. Jirina Bartova, Hana Poskerova, Jan Vokurka, and Antonin Fassmann performed the clinical analysis and collected the blood samples. Jakub Kastovsky, Svetlana Lucanova, and Petra Borilova Linhartova carried out the molecular analysis, and Lydie Izakovicova Holla performed statistical analysis. All authors revised the final version of the paper.

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*Review Article*

# Enigma of IL-17 and Th17 Cells in Rheumatoid Arthritis and in Autoimmune Animal Models of Arthritis

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Rheumatoid arthritis (RA) is one of the most common autoimmune disorders characterized by the chronic and progressive inflammation of various organs, most notably the synovia of joints leading to joint destruction, a shorter life expectancy, and reduced quality of life. Although we have substantial information about the pathophysiology of the disease with various groups of immune cells and soluble mediators identified to participate in the pathogenesis, several aspects of the altered immune functions and regulation in RA remain controversial. Animal models are especially useful in such scenarios. Recently research focused on IL-17 and IL-17 producing cells in various inflammatory diseases such as in RA and in different rodent models of RA. These studies provided occasionally contradictory results with IL-17 being more prominent in some of the models than in others; the findings of such experimental setups were sometimes inconclusive compared to the human data. The aim of this review is to summarize briefly the recent advancements on the role of IL-17, particularly in the different rodent models of RA.

*The present scientific contribution is dedicated to the 650th anniversary of the foundation of the University of Pécs, Hungary.*

## 1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disorder characterized by chronic synovitis leading to the progressive destruction of joints accompanied by systemic inflammation and the production of autoantibodies [1]. RA affects 0.5–1 percent of the human population making it one of the most common autoimmune disorders. Since the first modern description of the disease in 1800 [2], our knowledge regarding the pathomechanisms of RA has expanded to such a degree that specific therapies targeting various modulators of the inflammatory phase could be introduced, truly revolutionizing the treatment of RA. Yet little is known about how and when the disease starts although the new therapeutic agents proved to be much more effective than the conventional drugs used earlier. However, these disease-modifying antirheumatic drugs (DMARDs) do not alter the autoimmunity per se; they do not lead to remission in all of the patients. The central role of TNF$\alpha$ in the inflammatory phase of RA has been described earlier, but with the description of Th17 cells and the involvement of IL-17 in RA some new potential treatment options became available only recently [3]. Our review will mainly focus on the role of IL-17 in RA, particularly the rodent models of the disease, which all contributed to the development of novel therapeutic agents.

## 2. Th17 Cells in RA

The synovitis in RA is characterized by massive cellular infiltration of the synovium consisting mainly of leukocytes such as T and B cells, macrophages, granulocytes, and dendritic cells together with the increased local production of proinflammatory cytokines and chemokines, eventually leading to the destruction of the joint and bone. T cells, especially $CD4^+$ T cells, play a major role in this process, also supported by

the effective use of Abatacept in the treatment of RA, an agent that selectively blocks T cell costimulation [4]. For a long time RA was considered to be a Th1-dependent disease. However, following the description and characterization of IL-17 and Th17 cells, more and more data indicated that these latter types of $CD4^+$ cells are key players in the development of RA and that anti-IL-17 therapies might have beneficial effects [3].

Th17 cells are a subgroup of helper T cells with the capability to produce high levels of IL-17 described as their main characteristic along with the expression of the chemokine receptor CCR6 and the transcription factor ROR$\gamma$t (RAR-related orphan receptor gamma t) [5]. In humans, Th17 commitment requires the production of mainly IL-1$\beta$, IL-6, IL-21, and IL-23, all of which are produced by tissue-resident activated macrophages and dendritic cells in an inflammatory environment [6, 7]. These members of the innate immune system are not only capable of inducing Th17 commitment but they also participate in the recruitment of Th17 cells to the site of inflammation through the production of chemokines that bind CCR6. CCR6 is expressed by a variety of cells such as immature dendritic cells, regulatory T cells ($T_{reg}$), and Th22 and Th17 cells. An increased proportion of $CCR6^+$ Th17 cells were described in the peripheral blood of patients with early untreated RA [8], and Th17 cells infiltrated the joints as these cells were detected both in the synovial fluids and in synovial membranes of RA patients [9, 10]. While the number of Th17 cells in the peripheral blood does not seem to be a reliable diagnostic tool to date, their increased presence in the synovial fluid correlates with increased disease activity in RA [11].

In addition to elevated IL-17 levels, an increased concentration of CCL20 was also detected in the synovial fluid of RA patients, and *in vitro* cultured synovial fibroblasts of RA patients were capable of producing CCL20 after treating them with IL-1 and TNF$\alpha$ [12, 13]. It was demonstrated that CCL20 acts as a chemoattractant of the CCR6 expressing Th17 cells in mice. Human peripheral blood Th17 also expressed CCR6 (see above); thus CCL20 might be a key element in the recruitment of Th17 cells to the inflamed joints of RA patients [13]. Therefore, CCL20 is the most significant ligand of CCR6, and the CCL20/CCR6 axis may serve as potential therapeutic target.

## 3. Discovery of IL-17

Interleukin-17 (IL-17), originally termed as CTLA-8, was first identified in 1993 as a transcript from a cDNA library derived from a T cell hybridoma generated by the fusion of murine cytotoxic T cells and rat T cell lymphoma cells [14]. The sequence showed 58% identity to the Herpesvirus Saimiri gene 13 (HSV13) and both the recombinant CTLA-8 and HSV13 stimulated the NF-$\kappa\beta$ pathway leading to increased IL-6 production in fibroblasts and also stimulated T cell proliferation acting similarly as the proinflammatory cytokines [15, 16]. Later, the CTLA-8 was renamed IL-17 [15] and in 1996 the originally identified rat CTLA-8 was confirmed as a homologue of the murine IL-17 [17].

In 1986, murine helper T cells were divided into Th1 and Th2 subtypes based on the cytokines they produced [18]. According to this hypothesis the naive $CD4^+$ T cells could differentiate either into IFN$\gamma$-producing Th1 or IL-4-producing Th2 cells, a process which is controlled mainly by the antigen presenting cells (APC) [19]. The discovery of IL-17 greatly challenged the former bipolar classification of helper T cells [20]. First, the amino acid sequence of IL-17 significantly differed from other cytokines previously described, and the structure of its receptor did not fit into those of other cytokine receptor families, making IL-17 a seemingly distinct signaling molecule. Subsequently, it was demonstrated that naive T cells primed with the lysate of *Borrelia burgdorferi* develop a phenotype characterized by markedly increased IL-17 production. However, IL-17 could not be classified as a Th1 or Th2 cytokine, which led to the discovery of Th17 cells as a distinct $CD4^+$ T cell population [21]. Since then, a large number of studies have been performed to reveal the physiological role of IL-17 and Th17 cells, as well as their participation in pathological conditions. One of the most targeted areas was the autoimmunity including RA, multiple sclerosis, and psoriasis. Regarding the detailed structure and signaling pathways of IL-17 and IL-17R we refer to some previous reviews on the subject [22, 23]. Briefly, the IL-17 cytokine family consists of six currently known members (IL-17A–F) and five receptors (IL-17RA–RE) in mammals. IL-17A is the most prominent member of the family and is simply referred to as IL-17 by many authors. IL-17A and IL-17F are closely related, both secreted by Th17 cells and having an amino acid sequence homology of 50%. IL-17A can form homodimers, or heterodimers with IL-17F; both forms are biologically active through the binding of IL-17-RA, although the IL-17A homodimer is more potent [24].

## 4. The Role of IL-17 in Rheumatoid Arthritis (RA)

The human IL-17A was first cloned in 1996 and, first time, it was found to be produced by activated $CD45(RO)^+$ memory helper T cells [25]. It was shown in the same study that IL-17A induces the production of IL-6, IL-8, $PGE_2$, and G-CSF in a dose-dependent manner in cultures of RA synovial fibroblasts. The IL-17A effect was blocked with anti-IL-17 antibodies [25]. Interestingly, TNF$\alpha$ had an additive effect on IL-17-induced secretion of IL-6. Soon after these observations it was found that synovial fluids of RA patients have high IL-17A levels compared to those with osteoarthritis. IL-4 or IL-13 completely inhibited the IL-17 production of *ex vivo* cultured RA synovium tissue [25], whereas exogenous IL-17 increased IL-6 production in synovial tissue cultures. These observations led to the conclusion that through the production of other proinflammatory cytokines, IL-17 has a significant, if not a central, role in the pathogenesis of RA [25].

## 5. IL-17 Regulates Bone Resorption in Human RA

Destruction of the articular cartilage accompanied by the juxta-articular bone resorption and marginal erosions in the bone are prominent histological features of RA [1].

Osteoclasts are large, multinucleated cells responsible for the degradation of bone [26]. It has been shown that certain cytokines play a major role in the differentiation of osteoclasts, although this field is complicated to study because many cytokines have both stimulatory and antagonistic effects on osteoclastogenesis, and their net effect is determined mainly by the specific bone microenvironment [27]. The cytokines promoting osteoclastogenesis act mostly via the RANKL expression, although some proinflammatory cytokines such as IL-1, IL-6, and TNF$\alpha$ might be able to induce osteoclastogenesis independently of RANKL. The introduction of TNF$\alpha$-inhibitors along with IL-1 and IL-6 antagonizing therapies further confirmed that these cytokines play a crucial role in bone and cartilage destruction, as these drugs proved to have a major protective effect on bone resorption [28].

IL-17, in particular, is also considered to be osteoclastogenic [29]. In an *in vitro* model of osteoclastogenesis, cocultured murine osteoblasts and hematopoietic cells were treated with IL-17 derived from the synovial fluids of RA patients resulting in an increased IL-17-dependent osteoclastogenesis [30]. Interestingly, this also induces an increased $PGE_2$ production in osteoblasts, and the addition of a COX2 inhibitor (e.g., indomethacin) had an inhibitory effect on osteoclast formation [30]. Later, it was shown that IL-17 is involved in increased bone resorption in human RA bone explant cultures and enhanced proteoglycan loss from mouse cartilage [31]. Another research group reported that IL-17 promoted osteoclastogenesis *in vitro* from human $CD14^+$ osteoclast precursors acquired from healthy donors through the upregulation of the receptor activator of NF-$\kappa\beta$ (RANK) [32].

Interestingly, some human $\gamma\delta$ T cells, capable of producing IL-17 under certain conditions, have been recently described in the peripheral blood [33], and it has been also confirmed earlier that the level of $\gamma\delta$ T cells in the synovial fluids of RA patients was elevated compared to healthy controls [34]. Even though IL-17 producing $\gamma\delta$ T cells are known to contribute to the pathogenesis of murine models of RA [35], activated $\gamma\delta$ T cells seemed to inhibit osteoclastogenesis in an IFN$\gamma$ dependent manner *in vitro* [36]. IL-17 producing ($IL\text{-}17^+$) $CD4^+$ cells infiltrated the synovium and these $CD4^+IL\text{-}17^+$ T cells were detected in close proximity of osteoclasts in the joints of RA patients [37]. The introduction of anti-IL-17 therapeutic antibodies is expected to refine our knowledge on the effects of IL-17 regarding altered bone homeostasis in RA.

## 6. IL-17 in Animal Models of RA

Much of our knowledge about IL-17 is derived from animal models of RA. Several mouse models exist that mimic some or more characteristics of the human disease. Herein, we mention only those few autoimmune models, either spontaneous or inducible, in which IL-17 has been proposed to play a role (summarized in Table 1). We will pay special attention to proteoglycan aggrecan-induced arthritis (PGIA) which is perhaps the most complex available immunological model of RA.

## 7. Th17 Cell Driven Arthritis in SKG Mouse

SKG mice spontaneously develop rheumatoid factor- (RF-) positive autoimmune arthritis, which resembles human RA [77]. This mouse strain harbours a point mutation in the gene encoding the Src homology 2 (SH2) domain of Zeta-chain-associated protein kinase 70 (ZAP-70), a key molecule in T cell receptor signaling [77]. The altered thymic selection resulted in highly self-reactive T cells, which spontaneously differentiated into Th17 cells. It had been shown that IL-17 and IL-6 were essential in the development of arthritis, while, unexpectedly, IFN-$\gamma$ had a protective role (Table 1) [38]. The role of IL-17 was further proven because the treatment with nondepleting anti-CD4 monoclonal antibodies prevented the onset of arthritis in SKG mice by altering $T_{reg}$/Th17 ratio in synovial tissue and draining lymph nodes (Table 1) [39]. Furthermore, treatment with neutralizing anti-IL-17A slightly inhibited the progression of arthritis in SKG mice (Table 1) [40].

## 8. IL-17 in K/BxN Mice and the Serum Transfer Model

K/BxN mice express the transgenic T cell receptor KRN against bovine RNase antigen in nonobese diabetic (NOD) background having the MHC class II allele $Ag^7$ (present in the NOD strain) spontaneously developing uniform, severe inflammatory arthritis by the age of 4 weeks. In addition to the spontaneous arthritis, the most evident pathological abnormality is the presence of anti-glucose-6-phosphate isomerase (GPI) antibody production in K/BxN mice [78]. Serum transfer from sick K/BxN mice causes a transient arthritis in a wide range of recipient mice [48, 49].

The importance of IL-17 in K/BxN was investigated by neutralization experiments using monoclonal anti-IL-17 antibodies (Table 1). When 25-day-old K/BxN mice housed in specific pathogen-free environment were treated with anti-IL-17 the onset of arthritis was delayed which was associated with a slower disease progression and reduced ankle thickening (Table 1). These anti-IL-17-treated mice also had lower serum levels of GPI autoantibodies (Table 1) [41]. Th17 cells play an important role in disease induction in K/BxN mice, which was supported by the fact that treatment of mice from birth with neomycin exacerbated arthritis, while treatment with vancomycin or ampicillin inhibited disease progression [41], most likely due to the fact that these latter two antibiotics are known to block Th17 T cell differentiation (Table 1). On the other hand, neomycin targets Gram-negative bacteria, which comprises the majority of gut microbiota, and, Th17 cells are known to mediate host defence against extracellular, especially Gram-negative bacteria (Table 1) [79].

Most recently it was investigated whether IL-17 or IL-17 producing cells play a role in the serum transfer model of arthritis (Table 1). Katayama and colleagues transferred K/BxN serum to $IL\text{-}17A^{-/-}$ mice and found that the disease was significantly less severe than when the K/BxN serum was transferred into wild-type mice or mice with severe combined immunodeficiency (SCID) (Table 1) [42]. Interestingly, high

Table 1: Summary of the most important data about IL-17 in animal models of RA.

| Model | Experiment | Effect | Reference |
|---|---|---|---|
| SKG | IL-17$^{-/-}$ mice | Inhibited arthritis | [38] |
| | IL-6$^{-/-}$ mice | Inhibited arthritis | [38] |
| | IFN-$\gamma^{-/-}$ | Exacerbated arthritis | [38] |
| | a-CD4 | Prevented arthritis (altering $T_{reg}$/Th17 ratio) | [39] |
| | a-IL-17A | Inhibited arthritis progression | [40] |
| K/BxN | a-IL-17 | Slower disease progression | [41] |
| | Neomycin | Exacerbated arthritis (Th17 differentiation) | [41] |
| K/BxN serum | IL-17A$^{-/-}$ mice | Less severe disease | [42] |
| | IL-17RA$^{-/-}$ mice | Reduced disease severity | [43] |
| | Neutrophils | Source of IL-17 in effector phase | [42] |
| CIA | Soluble IL-17R | Suppression of arthritis | [31] |
| | Ad-IL-17 | Exacerbation of arthritis | [31] |
| | | Angiogenesis | [44] |
| | IL-17$^{-/-}$ mice | Suppression of arthritis | [45] |
| | IL-17$^{-/-}$ BM transfer | Suppression of arthritis | [46] |
| | a-IL-17 serum | Suppression | [47] |
| | a-IL-1$\beta$ + a-IL-17A (sc, bs) | Suppression | [48] |
| | a-IL-1$\beta$ or a-IL-17A (c) | Suppression | [49] |
| | a-IL-1$\beta$ + a-IL-17A (bs, n) | Suppression | [50] |
| | a-IL-17 a-GM-CSF (c) | Suppression | [51] |
| | CD4$^{+}$ Th17 cells | Stimulation of osteoclastogenesis | [37, 52] |
| | V4$\gamma^{+}$V4$\delta^{+}$ $\gamma\delta$ T cells | IL-17 production | [53] |
| | IL-6 | Upregulated by IL-17 | [47] |
| | | Positive feedback loop triggered by IL-17A | [54] |
| | | Induces Th17 differentiation | [55] |
| | IL-1$\beta$ | Upregulated by IL-17 | [52] |
| | RANK, RANKL | Expression induced by Th17 cells | [56] |
| | Ad-IL-4 | IL-17 mRNA levels ↓ | |
| | | Prevention of osteoclastogenesis | [57] |
| | IL-12p35$^{-/-}$ mice | More severe arthritis | |
| | | IL-17 mRNA levels ↑ | [58] |
| | Ad-IL-27 | Suppressed arthritis | |
| | | IL-17 levels in joint and serum ↓ | |
| | | IL-1$\beta$, IL-6, and CCL2 expression ↓ | [59] |
| | Ad-IL-37 | Downregulates IL-17 | |
| | | Inhibits Th17 proliferation | [60] |
| | NK cell depletion | Exacerbation of arthritis | [61] |
| | $B_{reg}$ cells | Suppression of arthritis ($T_{reg}$ differentiation ↑) | [62] |
| | IL-10R Tg mice | Increased susceptibility of arthritis (IL-17 ↑) | |
| | | IL-17 producing $\gamma\delta$ T cells accumulate in joint | [63] |
| | IL-23p19$^{-/-}$ mice | Inhibition of arthritis (Th17 differentiation ↓) | [58] |
| | IL-23p19 vaccine | Suppressed arthritis | [64] |
| | IFN-$\gamma$R$^{-/-}$ mice | Exacerbated arthritis | [65] |
| | IFN-$\gamma$R$^{-/-}$ mice + a-IL-17 | Inhibited arthritis | [66] |
| PGIA | Initiation phase | Local and systemic IL-17 production | [67] |
| | IL-17$^{-/-}$ mice | Severity and onset similar to WT | [68] |
| | | IL-1$\beta$ ↑ IL-6 expression ↓ | |
| | IFN-$\gamma^{-/-}$ mice | Amelioration of arthritis | [69] |
| | | IL-17 levels ↑ | |
| | IFN$\gamma$/IL-17$^{-/-}$ mice | Suppressed arthritis | [69] |
| | | Reduced joint damage and cellular infiltration | |

sc: single-chain; bs: bispecific; n: neutralizing; c: combination therapy; Ad: adenoviral; Tg: transgenic; BM: bone marrow.

IL-17 serum levels were observed in transferred arthritic SCID mice, and it was revealed that neutrophils acted as source of IL-17 in the effector phase of arthritis (Table 1). Moreover, coinjection of wild-type neutrophils with K/BxN serum into IL-17A$^{-/-}$ mice resulted in the exacerbation of the disease (Table 1) [42]. K/BxN serum transfer studies into IL-17RA$^{-/-}$ animals resulted in similar findings: reduced clinical signs of arthritis and decreased expression of various chemokines, proinflammatory cytokines, and matrix metalloproteinases (Table 1) [43].

## 9. IL-17 in the Cytokine Network Regulation in Collagen-Induced Arthritis (CIA)

CIA is perhaps the most widely used inducible autoimmune (systemic) arthritis model, which can be induced by repeated intracutaneous immunization of DBA/1 mice with type II collagen (CII) emulsified with complete Freund's adjuvant.

The role of IL-17 in the development of CIA was first proved by the blockade of endogenous IL-17 with soluble IL-17 receptor protein in immunized DBA/1 mice [31], which resulted in the suppression of arthritis, accompanied by reduced joint damage. In contrast, either systemic or local (intra-articular) adenoviral gene transfer of IL-17 exacerbated CIA. These results were supported by Nakae and colleagues [80] using IL-17 deficient (IL-17$^{-/-}$) mice. Compared to wild-type animals both the incidence and severity of arthritis were markedly reduced and joint histology showed milder inflammation. Collagen-specific IgG2a levels were lower than that of wild-type mice. *In vitro* proliferative response to CII was reduced in IL-17$^{-/-}$ lymph node cell cultures, suggesting a crucial role for IL-17 in the induction of CIA. In another study, mice were immunized after allogeneic IL-17$^{-/-}$ bone marrow transplantation; type II collagen-immunized DBA/1 mice developed significantly less severe arthritis associated with reduced production of proinflammatory cytokines [46].

Both IL-17 producing CD4$^+$ Th17 cells [52] and $\gamma\delta$ T cells [53] were described in CIA. Th17 cells are thought to mediate their effects by stimulating osteoclastogenesis [37], but it remains to be evaluated how V4$\gamma^+$V4$\delta^+\gamma\delta$ T cells contribute to the development of CIA via their IL-17 production.

Over the years various IL-17 blocking antibodies were tested in CIA (summarized in Table 1). The severity of the disease was successfully reduced using polyclonal rabbit anti-murine IL-17 antibody [47], single-chain bispecific antibody (scBsAb1/17) against both human IL-1$\beta$ and human IL-17A [48], combination therapies with anti-IL-1$\beta$ and anti-IL-17A antibodies [49] or anti-IL-17 and anti-GM-CSF antibodies [51], or bispecific and neutralizing antibodies (BsAB-1, BsAB-2 and BsAB-3) against both human IL-1$\beta$ and human IL-17A [50].

IL-17 exerts its proinflammatory actions through various pathways (Table 1). IL-17 upregulates other proinflammatory cytokines, such as IL-6 and IL-1$\beta$ [44, 59]. IL-6 induces Th17 differentiation forming a positive feedback loop triggered by IL-17A [54]. Thus, the blockade of IL-6 in the early phase of CIA inhibits Th17 differentiation, hence suppressing disease progression [55]. Bone erosion in CIA is mediated by IL-17 and Th17 cells through the regulation of RANKL-mediated osteoclastogenesis [63, 64].

IL-17 was also found to contribute to angiogenesis, as local overexpression of IL-17 using adenoviral vectors resulted in increased endothelial staining in the ankles of CII-immunized mice compared to controls (Table 1) [44].

IL-17 production or differentiation of IL-17 producing cells is regulated by various cytokines and cells [3]. Intra-articular overexpression of IL-4 using adenoviral vectors resulted in reduced synovial IL-17 mRNA levels and prevented joint damage and bone erosion in CIA by suppressing osteoclastogenesis [57]. Sarkar and colleagues cocultured IL-4-transduced dendritic cells with splenic T cells from CII-immunized mice and found that IL-17 production by T cells was significantly reduced when T cells were harvested during the initiation phase of the disease, but that of T cells obtained during the end phase was not altered [81]. This result is particularly important and interesting because it shows that even during the progression of the disease there could be a shift in the cytokine balance and/or regulation, whose observation was further supported by results from the PGIA model (see Section 10) [82].

A number of cytokines regulate CIA in a network-like fashion (see also Table 1):

(i) IL-12 is an important cytokine in Th1 differentiation by promoting IFN$\gamma$ production. CIA is more severe in mice deficient of IL-12, accompanied by increased IL-17 mRNA levels, suggesting that IL-12 regulates IL-17 production in CIA [58].

(ii) Local expression of IL-27 in the ankles of mice ameliorated CIA by reducing IL-17 levels in the serum and joints of animals [59].

(iii) Monocyte and neutrophil recruitment as well as angiogenesis was inhibited in synovial tissue, and the expression of downstream targets of IL-17, such as IL-1$\beta$, IL-6, and CCL2, was reduced [59].

(iv) IL-37, a recently discovered member of IL-1 family, is expected to play an immunosuppressive role in CIA via the downregulation of IL-17 and Th17 cell proliferation [60].

(v) NK cell depletion exacerbated experimental arthritis, supporting a possible protective role of NK cells in CIA by inhibiting Th17 differentiation via their IFN$\gamma$ production [61].

(vi) Regulatory B cells control autoimmunity by their IL-10 production by means of promoting $T_{reg}$ differentiation over Th1/Th17 differentiation [62]. IL-10 signaling in T cells is important in ameliorating CIA, as blocking this pathway rendered mice highly susceptible to arthritis via increased IL-17 levels and accumulation of IL-17 producing $\gamma\delta$ T cells in the joints [63].

(vii) IL-23 proved to be essential in the development of CIA, supported by the fact that IL-23p19$^{-/-}$ mice were resistant to arthritis, with no signs of bone or joint destruction. Results indicated that IL-23

promoted differentiation of IL-17 producing $CD4^+$ T cells [58]. In contrast, immunization with peptide-based vaccines targeting the IL-23p19 subunit resulted in suppressed arthritis, but IL-17 mRNA level and T cell populations in the spleen were not altered [64].

(viii) IFN$\gamma$ receptor deficient mice develop exacerbated CIA [65]. Preventive treatment of these mice with anti-IL-17 inhibits CIA, with no signs of bone destruction, neutrophil infiltration, and granulopoiesis. It is supposed that besides inhibiting Th17 differentiation IFN$\gamma$ protects from autoimmunity by inhibiting effector functions of IL-17 [66].

## 10. PGIA at the Border of Th1/Th17 Disease

In genetically susceptible BALB/c mice repeated intraperitoneal immunizations with human cartilage proteoglycan (aggrecan) emulsified with a synthetic adjuvant lead to chronic joint inflammation [83], resembling human RA in both clinical (progressive irreversible cartilage destruction, bone erosion, and ankylosis) and immunological characteristics (T cell-dependent, autoantibody-driven disease) [84].

Originally, PGIA was thought to be completely a Th1-type disease with significant IFN$\gamma$ production [85]. Finnegan and colleagues demonstrated that in contrast to CIA, IFN$\gamma$ deficiency or treatment with anti-IFN$\gamma$ antibody resulted in the amelioration of arthritis. Similarly to CIA, PGIA is also controlled by a network of cytokines (Table 1): arthritis severity is regulated by IL-4 and IL-12 production as shown by testing PGIA in a number of knock-out animals [67].

Later, it was demonstrated that, during the immunization period of PGIA, that is, even before the onset of clinical signs, IL-17 is a prominent proinflammatory cytokine, produced in significant amounts both locally in the peritoneal cavity and systemically in the spleen or lymph nodes of mice (Table 1) [82]. This suggested that both Th1 and Th17 cytokines were involved in the development of PGIA. However, as implicated above (see Section 9), the cytokine balance could shift during the disease progression.

After repeated intraperitoneal immunizations with PG, resident B1 cells were replaced by T cells and conventional B cells in the peritoneum, which may trigger the effector phase of arthritis [82]. B1 cells shift the immune response towards Th1/Th17 direction, while conventional B cells favour $T_{reg}$ induction [75, 86]. Although the number of B1 cells significantly decreased in the peritoneal cavity upon immunization [82], the residual cells seemed to have sufficient capacity to maintain Th1/Th17 polarization in BALB/c mice or, alternatively, their presence is only necessary during the initiation of the disease.

The role of IL-17 in PGIA was further investigated in IL-17 deficient ($IL\text{-}17^{-/-}$) mice, in which, surprisingly, the onset and severity of arthritis were equivalent to wild-type animals. Although the expression of IL-1$\beta$ increased significantly in the inflamed joints, IL-6 expression was suppressed. Joint histology and PG-specific T and B cell responses were similar in $IL\text{-}17^{-/-}$ and wild-type mice, suggesting that IL-17 was not essential in PGIA (Table 1) [68].

There was a clear contradiction between the earlier mentioned study [82], where significant IL-17 production was detected in PG-immunized mice, and the latter results when $IL\text{-}17^{-/-}$ mice were used [68]. The discrepancy between the two observations was solved, when the relation of IL-17 and IFN$\gamma$ was assessed in the same model (PGIA) system (Table 1). In PG-immunized IFN$\gamma$-deficient mice IL-17 levels were significantly increased [69]. Double knockout ($IFN\gamma^{-/-}/IL\text{-}17^{-/-}$) mice were then immunized to investigate the relationship between IFN$\gamma$ and IL-17 in PGIA (Table 1). Compared to wild-type, $IFN\gamma^{-/-}$, or $IL\text{-}17^{-/-}$ mice, severity and onset of arthritis were significantly reduced in the double knockout mice, suggesting that PGIA "became" IL-17-dependent in the absence of IFN$\gamma$. Cellular infiltration in the synovium and joint destruction were diminished in double knockout $IFN\gamma^{-/-}/IL\text{-}17^{-/-}$ mice. The impaired migration of Th17 cells into the inflamed joints might be explained by the reduced synovial expression of CCL20, a ligand of chemokine receptor CCR6 on Th17 cells [69]. Results from $IFN\gamma^{-/-}/IL\text{-}17^{-/-}$ mice underline the network-like function of cytokines in PGIA: the absence of IL-17 was compensated by the overproduction of IFN$\gamma$. This may as well explain why $IL\text{-}17^{-/-}$ mice developed PGIA, and, in turn, the overproduction of IL-17 in IFN$\gamma$-deficient mice can be responsible for the residual disease activity.

## 11. Concluding Remarks about the Experimental Autoimmune Models of RA

There are many animal models of RA, which might represent the heterogeneity of the human disease itself. Above, we reviewed the involvement of IL-17 and IL-17 producing cells in some spontaneous (SKG and K/BxN) and inducible (K/BxN serum transfer, CIA, and PGIA) animal models of RA (summarized in Table 1). Based on the studies available, the autoimmune arthritis observed in SKG, K/BxN serum transfer, and CIA models might be clearly IL-17-dependent disease. In contrast, PGIA might represent a transition between Th1 and Th17 mediated forms of autoimmune arthritis [69].

These results clearly exemplify that in the development of experimental autoimmune arthritis various cytokines and immune cells [61, 62] act in close association, proving the network-like functioning of the immune system. Nevertheless, when working with animal models one must be aware of the characteristics of the chosen model and take into consideration that because of the complex interactions of cytokines genetic modifications might result in diverse compensatory mechanisms.

## 12. IL-17 as a Therapeutic Target in RA

Since IL-17 seems to play a major role in various autoimmune disorders characterized by chronic inflammation, several studies reached the conclusion that antagonizing IL-17 could be beneficial in these pathological conditions [87]. Various IL-17 blocking agents have been developed and are currently being tested in RA (summarized in Table 2), psoriasis,

Table 2: Summary of the most important therapeutical trials targeting IL-17 in RA.

| Name | Molecular target | Phase | Status | Patients | Weeks | Efficacy | Reference |
|---|---|---|---|---|---|---|---|
| Secukinumab | IL-17A | I | Completed | 52 | 6 | Supported | [70] |
| | | II | Completed | 237 | 16 | Supported | [71] |
| | | II | Completed | 174 | 52 | Supported | [72] |
| | | III | Ongoing | n/a | n/a | n/a | NCT01377012 |
| | | III | Ongoing | n/a | n/a | n/a | NCT01350804 |
| Ixekizumab | IL-17A | I | Completed | 97 | 10 | Supported | [73] |
| | | II | Completed | 448 | 12 | Supported | [74] |
| Brodalumab | IL-17RA | I | Completed | 40 | 48 | Not confirmed | [75] |
| | | II | Completed | 252 | 12 | Not confirmed | [76] |

n/a: no information available.

ankylosing spondylitis, and inflammatory bowel diseases, including Secukinumab, Ixekizumab, and Brodalumab [87].

Secukinumab, manufactured by Novartis Pharma AG under the trade name Cosentyx, formerly referred to as AIN457, is a human monoclonal IgG1 that targets IL-17A (Table 2). The safety and efficacy of the drug were first tested in a small number of patients with psoriasis, RA, and chronic noninfectious uveitis and produced clinically relevant response rates of different magnitude in each of the above-mentioned disorders [70]. The drug was approved by the United States Food and Drug Administration (FDA) for the treatment of psoriasis in January 2015 and is currently under investigation in RA (Table 2) and psoriatic arthritis [88].

The results of a phase II double-blind, randomized, placebo-controlled trial about the effectiveness of Secukinumab including 237 RA patients with incomplete responses to previous methotrexate therapy was published in 2013, in which different doses of Secukinumab (25, 75, 150, and 300 mg per month, resp.) were administered subcutaneously for a period of 16 weeks on a monthly basis [71]. Injections of 75, 150, and 300 mg Secukinumab reduced the levels of serum C-reactive protein (CRP) compared to placebo and also decreased the DAS28 (disease activity score 28) of patients, but the primary efficacy endpoint defined by the ACR20 (ACR: American College of Rheumatology) was not achieved (Table 2). The trial was extended to a period of 52 weeks to investigate the long-term effects with 174 of the original 237 patients [72]. The patients with improved CRP levels and DAS28 scores sustained their responses throughout the treatment, and the response of those patients who received 150 mg of Secukinumab was improved through week 52 (ACR50: week 16 = 45%, week 52 = 55%) (Table 2). Mostly mild or moderate adverse effects were found in 64.8% of the patients, and serious adverse effects were detected only in 8.9% of the patients. Based on these results, the authors concluded that 150 mg of monthly Secukinumab treatment can produce clinically relevant response in patients who previously failed to respond to cDMARD or bDMARD treatments [72]. The effectiveness and safety of Secukinumab are now being investigated in two phase III clinical trials (NCT01377012 and NCT01350804).

Ixekizumab is an anti-human IL-17A humanized IgG4 developed by Eli Lilly and Company under the name LY2439821 (Table 2). The first proof-of-concept study consisted of two phases. First, 20 RA patients received 1 intravenous dose of Ixekizumab or placebo and patients were regularly controlled by an 8-week period (Table 2). In the second study, 77 RA patients being already on stable doses of at least one DMARD received one intravenous dose of either Ixekizumab or placebo 5 times with two-week intervals (Table 2). Intravenous Ixekizumab added to oral DMARD therapy improved both DAS28 and the ACR20 scores greater than placebo [73]. The initial study was followed by a phase II clinical trial in which Ixekizumab was given subcutaneously to 260 bDMARD naive patients and to 188 patients with inadequate responses to anti-TNF$\alpha$ biologics combined with cDMARD therapies [74]. Using a logistic regression model, in the biologically naive group, a dose-related response rate was detected at week 12 by measuring ACR20 response rates. In patients with inadequate response rates to TNF$\alpha$-blocking agents, Ixekizumab also produced clinically improved ACR20 responses [74]. However, Ixekizumab has not been approved by the FDA to date, but there are ongoing phase III clinical trials in psoriasis (NCT01597245), ankylosing spondylitis (NCT01870284), and psoriatic arthritis (NCT02349295) (Table 2). Recently, no additional studies are performed with RA patients.

Brodalumab is also a novel therapeutic monoclonal antibody that targets the IL-17 system, although, unlike the previously listed agents, it directly binds to the IL-17RA. Brodalumab is a human IgG2 monoclonal antibody developed by Amgen Inc. under the name AMG 827 (Table 2), even though Brodalumab was found to be effective in psoriasis in phase II clinical studies [41, 79], and phase III trials are still ongoing in moderate to severe psoriasis (NCT01708590). The efficacy of Brodalumab in RA was not confirmed by clinical studies (Table 2). The results of a phase I study were published in 2013, in which 40 human subjects with methotrexate resistant moderate-to-severe RA were treated with Brodalumab [75]. Although the drug blocked the IL-17RA in circulating leukocytes, it did not have a clinically significant effect on the response rates as by day 85 (Table 2) [73]. A total of 252 RA patients with inadequate responses to methotrexate

were included in a subsequent phase II trial which also failed to find evidence on the clinical efficacy and, therefore, it was concluded that Brodalumab seems to be ineffective in RA (Table 2) [76].

The fact that the effectiveness of biological therapies targeting different proinflammatory cytokines differs from patient to patient seems to support the hypothesis of the heterogeneity of RA in terms of the possible underlying pathomechanisms and responsiveness to certain cytokines contributing to disease development in different RA patients. Moreover, while IL-17 is a relatively new therapeutic target in RA, IL-17, and IL-17R family members show a high variability in the expression in individual patients [10]. Therefore, it is not surprising that the blockade of IL-17A or its receptor with monoclonal antibodies did not lead to complete disease remission so far. As indicated in the above sections, discussing the animal models of RA, IL-17, and Th17 cells is part of a very complex immunopathological network, where targeting one single entity might not be sufficient in suppressing the autoimmune process. Thus, currently it is more likely that IL-17 targeting agents could be used to complement/augment current therapies.

However, it cannot be completely ruled out that, in the future, targeting the IL-17 axis in RA at different levels (Th17 differentiation, receptors, signaling, etc.) will not provide better therapeutical results than the currently available monoclonal antibodies. Moreover, we cannot neglect the complexity of the IL-17 system itself, which consists of 6 members with 5 known receptors, thus there is still place for developing new blocking/modifying agents, which might offer exciting new treatment forms in RA.

## 13. Concluding Remarks

Our knowledge has increased significantly about IL-17 and Th17 cells in the past 20 years. It became clear that this proinflammatory cytokine plays a key role in autoimmunity and more specifically in RA. We have detailed information about the immunological role of IL-17 based on different mouse models of arthritis complemented by some human data. However, as indicated above, care should be taken when analysing data derived from knock-out models and other genetically engineered mice, because the network-like function of the immune system might lead to unexpected compensatory mechanisms which could significantly alter the results. Nevertheless, our current understanding about IL-17 in RA (and new potential treatment directions) would not exist without the data from animal models. Therapeutic trials aiming to suppress IL-17 might provide some new treatments supplementing or replacing currently existing biological therapies in RA.

## Abbreviations

ACR: American College of Rheumatology
bDMARD: Biological disease-modifying antirheumatic drug
CII: Type II collagen
CCL20: Chemokine (C-C motif) ligand 20
CCR6: C-C chemokine receptor type 6
cDMARD: Conventional disease-modifying antirheumatic drug
CIA: Collagen-induced arthritis
COX2: Cyclooxygenase-2
CRP: C-reactive protein
CTLA-8: Cytotoxic T-lymphocyte-associated antigen 8
DAS28: Disease activity score 28
DMARD: Disease-modifying antirheumatic drug
FDA: United States Food and Drug Administration
G-CSF: Granulocyte colony-stimulating factor
GM-CSF: Granulocyte macrophage colony-stimulating factor
GPI: Glucose-6-phosphate isomerase
HSV13: Herpesvirus Saimiri 13
IFN$\gamma$: Interferon gamma
NF-$\kappa\beta$: Nuclear factor kappa-light-chain-enhancer of activated B cells
$PGE_2$: Prostaglandin E2
PGIA: Proteoglycan aggrecan-induced arthritis
RA: Rheumatoid arthritis
RANK: Receptor activator of nuclear factor kappa-B
RANKL: Receptor activator of nuclear factor kappa-B ligand
RF: Rheumatoid factor
ROR$\gamma$t: RAR-related orphan receptor gamma t
SCID: Severe combined immunodeficiency
SH2: Src Homology 2
TNF$\alpha$: Tumor necrosis factor alpha
ZAP-70: Zeta-chain-associated protein kinase 70.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

The first two authors Reka Kugyelka and Zoltan Kohl contributed equally to the work.

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*Research Article*

# Th17 Cytokines Disrupt the Airway Mucosal Barrier in Chronic Rhinosinusitis

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Cytokine mediated changes in paracellular permeability contribute to a multitude of pathological conditions including chronic rhinosinusitis (CRS). The purpose of this study was to investigate the effect of interferons and of Th1, Th2, and Th17 cytokines on respiratory epithelium barrier function. Cytokines and interferons were applied to the basolateral side of air-liquid interface (ALI) cultures of primary human nasal epithelial cells (HNECs) from CRS with nasal polyp patients. Transepithelial electrical resistance (TEER) and permeability of FITC-conjugated dextrans were measured over time. Additionally, the expression of the tight junction protein Zona Occludens-1 (ZO-1) was examined via immunofluorescence. Data was analysed using ANOVA, followed by Tukey HSD post hoc test. Our results showed that application of interferons and of Th1 or Th2 cytokines did not affect the mucosal barrier function. In contrast, the Th17 cytokines IL-17, IL-22, and IL-26 showed a significant disruption of the epithelial barrier, evidenced by a loss of TEER, increased paracellular permeability of FITC-dextrans, and discontinuous ZO-1 immunolocalisation. These results indicate that Th17 cytokines may contribute to the development of CRSwNP by promoting a leaky mucosal barrier.

## 1. Introduction

Chronic rhinosinusitis (CRS) is characterized by severe inflammation of the sinus mucosa leading to blockage of the nasal passageway and the accumulation of mucus and pathogens in the nose and paranasal sinuses [1, 2]. CRS affects around 1.9 million Australians [3] and puts a large financial burden on health care systems [4].

CRS is subdivided in CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP) based on the presence or absence of polyps in the sinonasal cavities [5]. CRSwNP patients typically display a T helper 2 (Th2) polarization, whereas patients without nasal polyps (CRSsNP) are often characterized by a Th1 polarization with high levels of Interferon-$\gamma$ [6].

Cytokines regulate innate and acquired immunity [7] and can disrupt mucosal barrier function by altering tight junction (TJ) composition and structure. This occurs through signalling pathways independent of cell death and the effect is cell type specific, pleiotropic, and time and dose-dependent [8]. Relatively few studies have demonstrated cytokine effects on nasal epithelial tissue or barrier function [5, 6, 9, 10]. Th1 cytokines such as interleukin-2 (IL-2), interferon-$\gamma$ (IFN-$\gamma$), and Tumour Necrosis Factor alpha (TNF-$\alpha$) are the primary source for proinflammatory Th1 responses [11], in which they are effective in controlling infection with intracellular pathogens and for perpetuating autoimmune responses [12]. In contrast, Th2 immune responses are characterized by the production of the interleukins IL-4, IL-5, and IL-13 [13] that are associated with the promotion of eosinophil recruitment and activation, and inhibition of several macrophage functions, thus providing phagocyte-independent protective responses [14]. Th17 cells are a subset of activated $CD4^+$ T cells and are characterized by the production of the interleukins IL-17A, IL-17F, IL-22, and IL-26 [15]. Th17 cells act as a bridge between adaptive and innate immunity where

they play crucial roles in the development of autoimmunity, inflammation, and allergic reactions [10]. Here, we tested the effect of interferon proteins and of Th1, Th2, and Th17 cytokines on the mucosal barrier structure and function of primary nasal epithelial cells harvested from nasal polyps of CRS patients.

## 2. Methods

*2.1. Harvesting and Culturing Human Nasal Epithelial Cells In Vitro.* Ethical approval for nasal brushing from CRS patients was granted from the Queen Elizabeth Hospital Human Ethics Committee and only consented patients were included in the study. Exclusion criteria included active smoking, age less than 18 years, and systemic disease. Primary human nasal epithelial cells (HNECs) were harvested from nasal polyps by gentle brushing in a method as described in [16]. Extracted cells were suspended in Bronchial Epithelial Growth Media (BEGM, CC-3170, Lonza, Walkersvill, MD, USA), supplemented with 2% Ultroser G (Pall Corporation, Port Washington, NY, USA). The cell suspension was depleted of monocytes using anti-CD68 (Dako, Glostrup, Denmark) coated culture dishes, and HNECs expanded in routine cell culture conditions of 37°C humidified air with 5% $CO_2$ in collagen coated flasks (Thermo Scientific, Walthman, MA, USA). HNECs were tested at passage two and confirmed to be of epithelial lineage via reactivity to pan-Cytokeratin and CD45 antibodies (both from Abcam, Cambridge, MA, USA), and a Diff-Quick staining method used in the assessment of cell morphology by professional cytologists (IMVS, The Queen Elizabeth Hospital, Woodville, Australia).

*2.2. Air Liquid Interface Culture.* HNEC were maintained at an Air Liquid Interface (ALI) medium, following the Lonza ALI culture method (Lonza, Walkersville, USA). Briefly, Transwells (BD Biosciences, San Jose, California, USA) were treated with collagen (Stemcell Technologies, Australia). 70,000 cells were seeded in a volume of 100 $\mu$L B-ALI medium into the apical chamber of the Transwell plate and 500 $\mu$L of B-ALI growth medium was added to the basal chamber in all wells containing the inserts. Cells were incubated at 37°C. On day 3 after seeding, B-ALI growth medium was removed from the apical and basal chambers and 500 $\mu$L B-ALI differentiation medium was added to the basal chamber only, exposing the apical cell surface to the atmosphere. Regular examinations were made to assess the integrity of the cell layer.

*2.3. Th1, Th2, and Th17 Cytokines Exposure.* Cytokines were added to the basal Transwell chamber at the following final concentrations: recombinant human Interferon-$\gamma$ (500 ng/mL, Sigma, Saint Louis, USA), interferon $\beta$ 1a (50 ng/mL, Sigma, Saint Louis, USA), interferon-$\alpha$ (500 ng/mL, Sigma, Saint Louis, USA), Tumour Necrosis Factor-$\alpha$ (500 ng/mL, Sigma, Saint Louis, USA), IL-1b (500 ng/mL, Sigma, Saint Louis, USA), IL-4 (50 ng/mL, Gibco, life Technology, USA), IL-5 (50 ng/mL, Gibco, Life Technology, USA), IL-13 (50 ng/mL, Gibco, Life Technology, USA), IL-17A (50 ng/mL, Gibco, Life Technology, USA), recombinant human IL-22 (50 ng/mL, Sigma, Saint Louis, USA), and recombinant human IL-26 (50 ng/mL, Abnova Taiwan Corp).

*2.4. Hematoxylin and Eosin (H&E) Staining and Histopathological Examination of Slides.* Paraffin-embedded tissue samples were cut in 4 $\mu$m thick sections on a microtome (Thermo Scientific HM 325 Rotary Microtome). Slides were then stained with routine hematoxylin and eosin (H&E) staining using Mayer's Hematoxylin and Eosin (Lillie's Modification, Dako, Thermo Fisher Scientific, Waltham, MA, USA). All slides were then scanned using digital whole-slide imaging technology (WSI) on the NanoZoomer Digital Pathology System (Hamamatsu Photonics, Hamamatsu City, Japan) under high resolution (40x objective magnification power). Eosinophil and neutrophil scoring was done according to a systematized methodology as detailed in [17].

*2.5. Transepithelial Electrical Resistance (TEER).* Transepithelial electrical resistance (TEER) was measured by using an EVOM volt-ohmmeter (World Precision Instruments, Sarasota, FL, USA). Briefly, 100 $\mu$L of B-ALI medium was added to the apical chamber of ALI cultures to form an electrical circuit across the cell monolayer and into the basal chamber. Cultures were maintained at 37°C during the measurement period using a heating platform. Only wells displaying baseline resistance readings greater than 500 $\Omega/cm^2$ were used for the experiments. Cytokines and control (B-ALI medium for the negative control and 2% Triton ×100 for the positive control) were added to the bottom chamber of each well, and TEER measurements were obtained at time 0, 4 h, and 24 h.

*2.6. Permeability Assay.* Paracellular permeability was studied by measuring the apical-to-basolateral flux of FITC dextran 4 kDa (Sigma, Saint Louis, USA). Briefly, after treating the cells for 24 h, the upper chambers were filled with 3 mg/mL of FITC-dextran and incubated for 2 h at 37°C. 40 $\mu$L samples were recovered from the bottom chamber and serially diluted on a 96-well plate (Corning Costar cell culture plates (96 wells)), and the fluorescence was measured with a microplate fluorometer (FLUOstar Optima, BMG Labtech, Ortenberg, Germany).

*2.7. Cytotoxicity Assay.* The amount of lactate dehydrogenase (LDH) in the medium was measured at 24 hours using the Cytotox Homogeneous Membrane Integrity Assay (Promega, Australia). Briefly, 50 mL of the media from each well was transferred to a new plate, and 50 mL of LDH reagent was added to the supernatant and incubated for 30 minutes in the dark at room temperature. The OD was measured at 490 nm on a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany).

*2.8. Trypan Blue Assay.* After cell incubation with the cytokines, the culture medium was discarded and washed

with PBS, and 100 $\mu$L of trypsin was added to each well. The plate was incubated for 5 minutes, and then 250 $\mu$L of supplemented culture medium was added. The contents of each well were aspirated, placed into labeled microtubes, and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, and the cells were suspended again in 100 $\mu$L of culture medium. An aliquot of 10 $\mu$L of cell suspension was removed and mixed with 10 $\mu$L of Trypan Blue. After homogenization, the live and dead cells were counted and the percentage of viable cells was calculated.

*2.9. Immunofluorescence Microscopy.* Cells were fixed with 2.5% formalin in phosphate-buffered saline (PBS) for 10 min, and then the cells were rinsed with tris-buffered saline-0.5% Tween (TBST) four times, permeabilized with 1% SDS in PBS, and blocked with serum free blocker (SFB; Dako, Glostrup, Denmark) for 60 minutes, at room temperature. Mouse monoclonal anti-human ZO-1 (Invitrogen, Carlsbad, CA, USA), diluted to 5 $\mu$g/mL in TBST-10% SFB, was added to the excised culture support membranes and allowed to incubate for 1 hour at room temperature. Excess primary antibody was removed with TBST, and 2 $\mu$g/mL anti-mouse Alexa-594 conjugated secondary antibody (Jackson ImmunoResearch Labs Inc., West Grove, PA, USA) was then added for 1 hour at room temperature. The filters were rinsed in TBST, and after the third wash 200 ng/mL of 4′,6-diamidino-2-phenylindole (DAPI; Sigma, Aldrich) was added to resolve nuclei. Membranes were rinsed once with ultrapure water, and 95% ice cold ethanol was added for 1 hour at 4°C. Membranes were transferred to a glass slide and a drop of anti-fade mounting medium (Dako, Glostrup, Denmark) was added before coverslipping. Samples were visualized by using a LSM700 confocal laser scanning microscope (Zeiss Microscopy, Germany).

*2.10. Statistical Analysis.* Data are presented as mean ± SEM. The TEER experiment was performed using three replicates from four CRSwNP patients with values normalised against the mean value from the patient at time 0. The dextran-FITC assay was performed using three replicates from four CRSwNP patients. Statistical analyses of all data were carried out using ANOVA, followed by Tukey HSD post hoc test. These tests were performed using SPSS software (version 18).

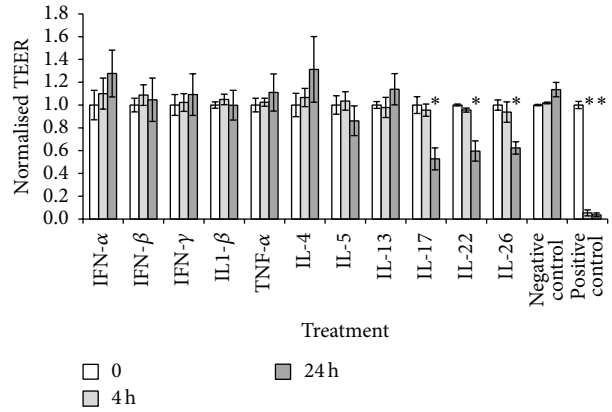


Figure 1: The Th17 cytokines IL-17, IL-22, and IL-26 cause a time-dependent reduction in TEER. Effect of Interferons $\alpha$, $\beta$, and $\gamma$ and of Th1, Th2, and Th17 cytokines on epithelial barrier function determined by measuring transepithelial electrical resistance (TEER) before the addition of cytokines ($t = 0$), and after 4 and 24 h. The values are shown as mean ± SEM for $n = 4$. Treatments significantly different from the untreated control at $p < 0.05$ are presented as $*$. ANOVA, followed by Tukey HSD post hoc test.

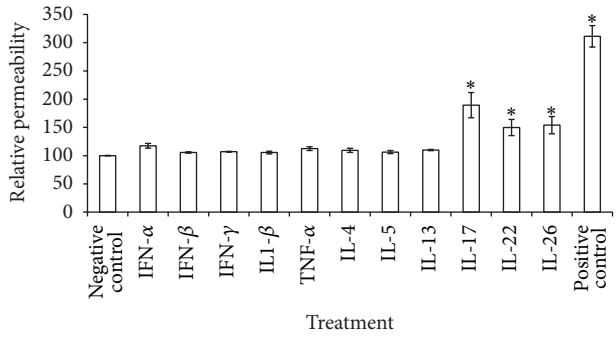


Figure 2: The Th17 cytokines cause an increase in paracellular permeability in CRS. Paracellular permeability of HNECs in ALI culture was determined by a Dextran-FITC assay after 24 h treatment of Th1, Th2, and Th17 cytokines. The values are shown as means ± SEM for $n = 4$. Treatments significantly different from the untreated control at $p < 0.05$ are presented as $*$. ANOVA, followed by Tukey HSD post hoc test.

## 3. Results

*3.1. Th17 Cytokines Disrupt TEER of HNECs.* ALI cultures were established from 4 independent CRS patient donors (2 males and 2 females, aged 45–60 years). Two patients were diagnosed with grass-pollen allergy, one had Aspirin-Exacerbated Respiratory Disease (AERD), and two were asthmatic. Eosinophil and neutrophil counts [11.1 (4.6–21.3) and 0.8 (0–2.4)] per High Power Field (HPF) were not different between the different patients ($p > 0.05$). The effect of interferons and of Th1, Th2, and Th17 cytokines was examined by measuring the TEER across HNEC monolayers from CRS patients at different time points. All Th17 cytokines tested (IL-17, IL-22 and IL-26) caused a significant reduction in TEER (average of 1.9 times; 1.7 times; 1.61 times for IL-17, IL-22, and IL-26 resp.) after 24 h of incubation. In contrast, Th1 and Th2 cytokines or interferon $\alpha$, $\beta$, or $\gamma$ did not show any significant effect on TEER (Figure 1).

*3.2. Th17 Cytokines Increase the Paracellular Permeability of HNECs.* All IL-17 family cytokines (IL-17, IL-22, and IL-26) led to a significant enhancement of paracellular permeability ($p < 0.05$) (Figure 2). IL-17 had the strongest effect, with 89.33% of the fluorescent dextran crossing the HNEC monolayer whereas IL-22 and IL-26 increased paracellular permeability with 49.85% and 53.92%, respectively. Th1 and Th2 cytokines and interferons $\alpha$, $\beta$, and $\gamma$ did not show any significant effect on the paracellular permeability in CRS patients (Figure 2).

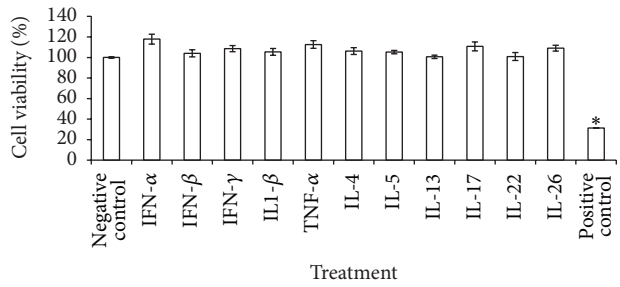


Figure 3: Th1, Th2, and Th17 cytokines and interferons do not have cytotoxic effects on HNECs. Relative viability as determined by the LDH assay after 24 h treatment of Th1, Th2, and Th17 on HNECs. Cell viability was calculated relative to the untreated cells (0 $\mu$g/mL) as negative control. The values are shown as means ± SEM, $n = 4$.

*3.3. Tight Junction Disruption Does Not Correlate with Cytotoxicity.* The effect of interferons and of Th1, Th2, and Th17 cytokines on cellular toxicity was examined by measuring LDH release from HNECs. There was no statistically significant increase in LDH release after 24 h incubation with any of the cytokines (Figure 3). In addition, the cell density estimated by the Trypan Blue assay did not show any significant differences in cell density or cell viability in cytokine treated cells compared to control cells (results not shown).

*3.4. Th17 Cytokines Cause a Profound Disruption of the Tight Junction Protein ZO-1.* The effect of interferon proteins and of Th1, Th2, and Th17 cytokines on the localization of Zona Occludens-1 (ZO-1) was examined by using immunofluorescence staining and confocal laser scanning microscopy, 24 hours after application of the cytokines. In untreated cells, ZO-1 was located at the periphery of the apical side of the monolayer, as expected. Similarly, interferons $\alpha$, $\beta$, and $\gamma$ and Th1 and Th2 cytokines, which had no effect on either TEER or paracellular permeability, led to no alterations in the localization of ZO-1. In contrast, application of Th17 cytokines, which significantly altered epithelial barrier function, resulted in profound disruption of ZO-1 immunolocalisation evidenced by faint or discontinuous regions of fluorescence (Figure 4).

## 4. Discussion

Cytokine mediated insult on mucosal membranes, causing disruption of tight junctions and increased paracellular permeability, contributes to a multitude of pathologic conditions in inflammatory diseases of the upper airways [18–20]. In this study, we compared the effect of interferons and of signature Th1, Th2, and Th17 cytokines on the barrier function of primary nasal epithelial cells harvested from CRS patients with nasal polyps. Immunolocalisation of the tight junction protein ZO-1 was used to analyse tight junction integrity to gain insights into mechanisms of cytokines dependent disruption of the airway epithelial barrier. Our study indicates that, in CRSwNP patients, IL-17 family cytokines (IL-17A, IL-22, and IL-26) can significantly disrupt epithelial barrier function in association with a disruption of tight junction integrity and without causing cellular toxicity. In contrast, Th1 and Th2 cytokines or interferons showed no significant difference on either TEER or paracellular permeability of HNECs.

It has been well established that different cytokines cause different, often opposing effects on epithelial barrier function depending on the cell type used and that any observed effect is dose and time-dependent (reviewed in [8]). Our results indicate that application of Th1 cytokines such as IFN-$\gamma$ and TNF-$\alpha$ does not have detrimental effects on epithelial barrier function. Rather, application of these cytokines appeared to slightly enhance the TEER of human nasal epithelial monolayers derived from some of the CRSwNP patients 24 hours after application. Whereas IFN-$\gamma$ and TNF-$\alpha$ generally decrease barrier function in different cell lines [21, 22], in airway epithelial cells, IFN-$\gamma$ has been reported to decrease barrier function by Soyka et al. [5] and promote epithelial barrier function by Ahdieh et al. [23]. These differences in the response to IFN-$\gamma$ could be attributed to many factors including interindividual variability in response to cytokines, different origin of cells (mucosa or polyps), and differences in experimental techniques. In the experiments by Soyka et al. [5], for example, TEER changes in response to IFN-$\gamma$ treatment from CRSwNP, CRSsNP and controls were pooled while in our studies, only cells from CRSwNP were used. Given the small number of samples used in most studies, further experiments using a larger number of donors will need to address the cause of these discrepancies. TNF-$\alpha$ can increase TEER in mammalian uterine cell monolayers in a dose-dependent manner [24]. Interestingly, a recent study revealed that patients had developed a recurrence of CRS after the start of TNF-$\alpha$ inhibitor administration with a remission of the disease only after cessation of TNF-$\alpha$ inhibitor treatment [25].

Moreover, we demonstrated no significant reduction of TEER by Th2 family cytokines (IL-4, IL-5, and IL-13) after 4 h and 24 h in our experiments. Using airway epithelial cells, Saatian et al. showed that IL-4 and IL-13 caused a reduction in TEER 72 h after challenge but not after 24 h [26]. Soyka et al. [5] also used airway epithelial cells from CRSwNP patients ($n = 2$) and controls ($n = 2$) and similarly showed significantly decreased TEER after IL-4 challenge; however, this effect was already evident after 12 and 24 hours. The reason for these discrepancies is not clear and can be dependent on numerous factors. While physiologically relevant, it is well known that experiments using primary cells have limitations due to inherent interindividual differences of age, genetic make-up and medical history [27]. This is particularly important in CRS, a multifactorial disease that can be associated with Th1 or Th2 responses. Also ethnicity may play a role as Caucasian CRSwNP patients are often characterised by a predominant Th2 type eosinophilic inflammation with high level of IL-5, whereas Asian CRSwNP patients preferentially have a Th1/Th17 polarization signature [28].

Th17 cells play a significant role in chronic allergic airway inflammation [29]. Th17 cytokines also affect the gut mucosal barrier function by promoting the amplification of the host response to secrete neutrophil chemoattractants and antimicrobial peptides such as lipocalin-2 and calprotectin. In addition, Th17 cells can expand within mucous layers in association with the presence of pathogens that are resistant

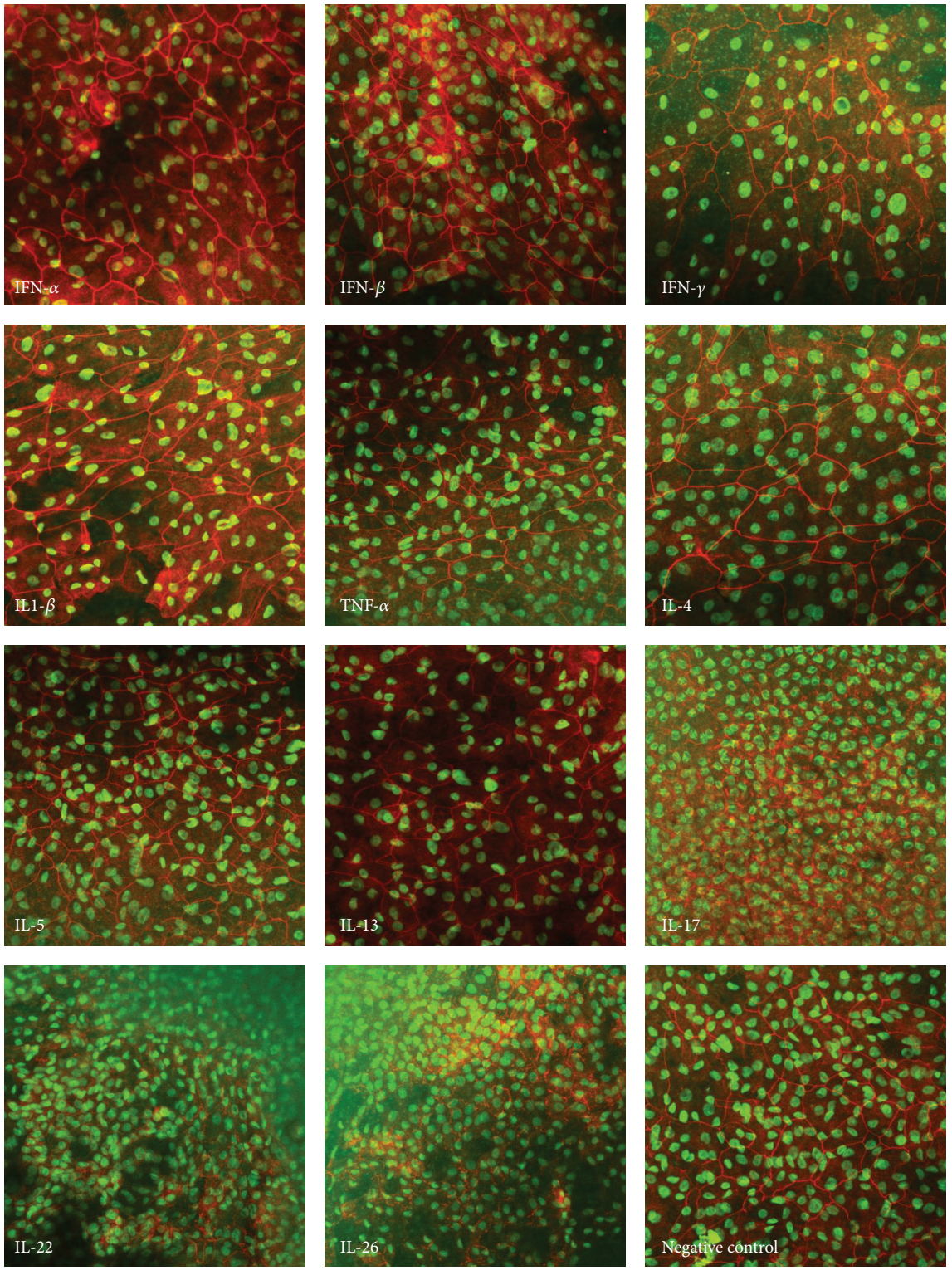


Figure 4: The Th17 cytokines cause a profound disruption of the tight junction in CRS. Effect of IFN-$\alpha$, IFN-$\beta$, IFN-$\gamma$, IL-1$\beta$, TNF-$\alpha$, IL-4, IL-5, IL-13, IL-17, IL-22, IL-26, and negative control on the localization of Zona Occludens-1 (ZO-1) (in red with DAPI stain in green) was imaged with 20x objective power using immunofluorescence confocal laser scanning microscopy 24 hours after application of the cytokines.

to some of the induced antimicrobial responses [30]. In allergic rhinitis, serum IL-17 levels were significantly related to clinical severity [31]. The expression level of IL-17 was also shown to be significantly higher in recalcitrant CRSwNP compared to controls [32]. In the present study, we found that IL-17 induced barrier dysfunction as assessed by reduction in TEER and enhanced macromolecular permeability, whereas Soyka et al. showed that IL-17 had no influence on TEER [5]. We also demonstrated significant reductions in TEER and enhanced macromolecular permeability of IL-22 and IL-26 which is the first such analysis using human nasal epithelial cells.

In tight junction formation, ZO-1 plays an essential role, by linking the transmembrane proteins occludin, claudin, and Junction Adhesion Molecule (JAM) cytoplasmic components of the tight junctions to the actin cytoskeleton [33]. Disruption of the actin-myosin structure has been understood to modulate paracellular permeability [8]. We observed a loss of normal ZO-1 immunolocalisation in HNEC monolayers of CRSwNP patients secondary to challenge with Th17 (IL-17, IL-22, and IL-26) cytokines, in association with disruption of barrier function. We also observed that IL-17, IL-22, and IL-26 treated cells appeared in higher cell densities than cells treated with other Th1 or Th2 family cytokines. It is known that TJs regulate epithelial proliferation by different molecular mechanisms, which generally suppress proliferation as cell density (and hence TJ assembly) increases (reviewed in [34]). Changes in expression of ZO-1 and ZO-1-associated nucleic acid binding protein (ZONAB), a Y-box transcription factor, affect cell proliferation; however, these effects take place at least 48 hours after changes in gene expression [35]. Given that the duration of exposure to the treatments in our experiments was only 24 hours and that cell counts did not show significant differences, we believe that TJ disruption secondary to IL-17, IL-22, and IL-26 exposure might render the pseudostratified layer of cells into a monolayer which might appear relatively overcrowded.

In summary, in patients with CRSwNP, Th1 and Th2 cytokines showed no significant effect on epithelial barrier structure and function. In contrast, the Th17 cytokines family (IL-17, IL-22, and IL-26) showed significant disruption of the epithelial barrier, leading to increased paracellular permeability associated with reduced tight junctionintegrity. In future studies, it will be important to determine the cellular mechanism of the effect of Th17 cytokines on the mucosal barrier in CRS patients to provide an opportunity for therapeutic modulation in inflammatory stress.

## Conflict of Interests

The authors have no conflicting financial interests that are relevant to this work.

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*Review Article*

# Th17 Cells in Type 1 Diabetes: Role in the Pathogenesis and Regulation by Gut Microbiome

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Type 1 diabetes (T1D) is an autoimmune disease which is characterized by progressive destruction of insulin producing pancreatic islet $\beta$ cells. The risk of developing T1D is determined by both genetic and environmental factors. A growing body of evidence supports an important role of T helper type 17 (Th17) cells along with impaired T regulatory (Treg) cells in the development of T1D in animal models and humans. Alteration of gut microbiota has been implicated to be responsible for the imbalance between Th17 and Treg cells. However, there is controversy concerning a pathogenic versus protective role of Th17 cells in murine models of diabetes in the context of influence of gut microbiota. In this review we will summarize current knowledge about Th17 cells and gut microbiota involved in T1D and propose Th17 targeted therapy in children with islet autoimmunity to prevent progression to overt diabetes.

## 1. Introduction

In 2005, T helper type 17 (Th17) cells were first identified as a distinct T helper cell lineage [1, 2]. The discovery of Th17 subset not only changes the classical Th1/Th2 paradigm in T cell immune responses, but also provides us with new insights into the pathophysiological process in several autoimmune diseases [3]. Type 1 diabetes (T1D), one of the most prevalent autoimmune diseases which were previously thought to be mediated by Th1 cells, is now establishing a connection with Th17 cells [4]. Exploration of Th17 cells in T1D pathogenesis has attracted more and more attention nowadays. Here, we briefly review the findings that led to the identification of Th17 cells, their differentiation and functions, and interaction between Th17 and T regulatory (Treg) cells and integrate current knowledge about the influence of microbiota on Th17 cells and Treg cells in T1D. Finally, several approaches are being explored for intervention to block interleukin- (IL-) 17 activity or suppress Th17 differentiation or restore balance of Treg and Th17 cells. Manipulation of gut microbiota is an attractive approach and has been investigated in animal models and humans. Small molecules which have been identified to block Th17 differentiation are also potential therapeutics in T1D. Monoclonal antibody based therapy targeting IL-17 has been well studied in other autoimmune diseases in humans. Two monoclonal antibodies targeting IL-17 or Th17 approved to treat psoriasis are potentially potent to protect prediabetic individuals from progression into diabetes.

## 2. Th17 Cells and Th17 Cytokines

In 2003, Cua and colleagues [5, 6] demonstrated that IL-23 was crucial for the induction of experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA). IL-23 stimulated IL-17-producing T cells could induce EAE in an adoptive transfer model. Furthermore, mice with a deleted p19 subunit of IL-23 showed significantly reduced number of IL-17-producing T cells and were protected from EAE. In contrast, p35 subunit of IL-12 deficient mice produced an increased number of IL-17-producing T cells and developed severe EAE and CIA. These findings indicated that IL-17-producing T cells driven by IL-23 rather than IFN-$\gamma$-producing Th1 cells driven by IL-12 are mediating these models of T cell mediated autoimmune diseases. In

2005, two independent groups [1, 2] formally proposed Th17 as a distinct T helper subset and further demonstrated the critical role of Th17 cells in autoimmune diseases. Cytokines required for Th17 cell differentiation and transcription factors governing Th17 cell development are unique and are different from Th1 and Th2 cells [7]. The central modulator of the Th17 lineage is retinoic acid related orphan nuclear receptor (ROR) $\gamma$t [8] which interacts with other transcription factors in a network to regulate Th17 cells [9, 10]. Th17 cells mainly produce signature cytokine IL-17A (commonly referred to as IL-17), hence its name. However, they also produce IL-17F, IL-21, IL-22, and granulocyte monocyte-colony stimulating factor (GM-CSF) and potentially produce tumor necrosis factor (TNF) and IL-6 [11]. From animal studies in various disease models, IL-17A and IL-17F have shown overlapping but differential functions [12]. The cytokines produced by Th17 cells have broad effects on many cell types and induce the production of proinflammatory cytokines and chemokines, whereas during the process of pathogen clearance, sometimes IL-17-driven inflammation is no longer protective but carries the risk of severe immunopathology and autoimmunity.

IL-17 family cytokines mediate their biological functions via correspondent receptors on the surface of target cells. The IL-17 receptors (IL-17R) constitute a distinct cytokine receptor family [13], which includes IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE. Functional receptors for IL-17 family cytokines with IL-17RA as a common subunit often exist in the form of heterodimers. The downstream pathways of IL-17 signaling involve NF$\kappa$B, MAPKs, and C/EBPs, Act1 being the common membrane proximal adaptor [13].

## 3. Th17 Cells in T1D

Several lines of evidence from studies of animal models of diabetes indicate involvement of Th17 pathway in the pathogenesis of T1D. In spontaneous autoimmune diabetes model in nonobese diabetic (NOD) mice, IL-17A and IL-17F expression in islet correlates with insulitis. Thus, young mice at prediabetic age do not but older diabetic mice do have increased expression of IL-17A or IL-7F in islet along with the development of insulitis [14]. Inhibition of Th17 cells significantly suppressed development of diabetes [15, 16]. IL-17 deficient NOD mice have delayed onset of diabetes with reduced insulitis [17]. In streptozotocin-induced diabetes, IL-23 promotes development of diabetes in subdiabetogenic doses of streptozotocin treatment by expansion of Th17 cells [18]. Moreover, IL-17A deficiency ameliorates streptozotocin-induced diabetes [19]. Purified islet antigen specific Th17 cells are able to transfer diabetes in immunodeficient mice [14, 20]. All these findings clearly demonstrate the critical role of Th17 cells in the development of diabetes. However, as seen in other models of autoimmune diseases, the role of Th17 cells in the pathogenesis and their relation with Th1 cells in mediating the disease in these diabetes models is not of a clear-cut picture. Th1 cells or IFN-$\gamma$ is often associated with increased expression of Th17 cells. Moreover, islet antigen specific Th17 cells need to convert into Th1-like cells to be able to induce diabetes in an adoptive transfer model [14, 20]. IL-17 and interferon- (IFN-) $\gamma$ receptor double knockout mice show significantly delayed onset of diabetes compared to IL-17 single knockout mice [17]. These data suggest that Th17 cells might cooperate with Th1 or IFN-$\gamma$ in mediating inflammation in diabetes. However, IFN-$\gamma$ induced by innocuous islet antigens shows therapeutic effect of diabetes in NOD mice through inhibition of IL-17 production [15]. A recent study provides a novel mechanism for Th17-mediated diabetes which is independent of IFN-$\gamma$ but dependent on TNF [21]. Nonetheless, data from most studies are in favor of an indispensable role of IL-17/Th17 cells in the development of T1D which is supported by the therapeutic effect of IL-17 blockade by anti-IL-17 antibody or IL-25 [16].

Human studies have also generated evidence to support the notion that Th17 cells are critical in the pathogenesis of T1D. Peripheral blood $CD4^+$ T cells from new onset T1D children produce higher levels of IL-17, IL-22 and increased *Rorc2* and *Foxp3* gene expression compared with those from healthy individuals upon polyclonal activation, while no increased IFN-$\gamma$ level or T-bet expression was detected in T1D patients. This observation clearly indicates a Th17 biased response in T1D patients. Interestingly, memory $CD4^+$ T cells from half of T1D patients show increased IL-17 and IL-22 secretion and *Rorc2* expression *ex vivo* indicating a Th17 response *in vivo* [22]. Similarly, in another study, increased number of IL-17-producing $CD4^+$ T cells was also readily detected in new onset T1D children [23]. More importantly, these circulating $CD4^+$ T cells in T1D patients produce IL-17 when they are activated by $\beta$-cell autoantigens including proinsulin, insulinoma-associated protein, and GAD65 peptides [24]. The increased levels of IL-17 in T1D may be attributed to the presence of proinflammatory cytokine milieu that drives toward Th17 differentiation. Indeed, monocytes from T1D patients spontaneously secrete substantially higher levels of IL-6 and IL-1$\beta$ which promote IL-17 production by memory $CD4^+$ T cells [25]. More compelling evidence for Th17 biased response in human T1D is provided by Ferraro and colleagues [26]. In response to polyclonal activation, $CD4^+$ T cells with memory phenotype from pancreatic-draining lymph nodes (PLN) of T1D patients produce higher levels of IL-17 but not IFN-$\gamma$ or IL-4. Moreover, these PLN memory $CD4^+$ T cells release increased levels of IL-17 in response to diabetes-related antigens, proinsulin, and GAD65 [26]. As seen in animal models and in rheumatoid arthritis, IL-17 and IFN-$\gamma$ dual producing cells have also been observed and are emerging preceding clinical diabetes suggesting their involvement in progression to full blown diabetes [27]. In a single case of patient who died 5 days after T1D diagnosis, increased mRNA expression of IL-17A, RORC, and IL-22 along with IFN-$\gamma$ was detected [24] suggesting the direct involvement of IL-17 in destruction of $\beta$ cells and this notion is supported by *in vitro* data where IL-17 has direct toxicity to human $\beta$ cells. Human islet cells express high levels of IL-17RA and IL-17RC. IL-17 alone and in synergy with IFN-$\gamma$ and IL-1$\beta$ increases expression of SOD2, NOS2A, and COX2 which are involved in the inflammatory response in islet cells. Furthermore, IL-17 inhibits BCL-2 gene expression leading to the enhanced proapoptotic effect of IL-1$\beta$/IFN-$\gamma$ in primary human islet cells [22, 24].

## 4. Th17 and Treg Cells in T1D

Th17 and Treg cells are two closely related $CD4^+$ T helper cells subsets in ontogeny. Both Th17 and Treg cells can be differentiated from the same naïve $CD4^+$ T cells depending on the presence of different amount of TGF-$\beta$ and absence or presence of proinflammatory cytokines. The homeostasis between Th17 and Treg is important in keeping autoimmunity in check. It is clear that in murine models dysfunction of Treg cells can lead to autoimmune diabetes with increased Th17 cells. Imbalance between Th17 and Treg cells has been noted in several autoimmune inflammatory conditions including T1D [26, 28, 29]. The imbalance is manifested by expansion of Th17 cells which is concomitant with decreased number or function of Treg cells. For example, Ferraro et al. found that expansion of Th17 cells and functional defects in Tregs are key features of the PLN in T1D patients [26]. Earlier studies in human T1D have reported conflicting results with those that T1D patients have decreased [30], increased [31], or equivalent [32–34] numbers of Treg cells compared with healthy individuals. This conflict may stem from poor understanding of the complexity of Foxp3 expressed Treg cells. It has been realized that some IL-17 secreting $CD4^+$ T cells also express Foxp3 but do not exert suppressive function. For example, Marwaha et al. demonstrate that new onset T1D children have an increased proportion of $CD45RA^-CD25^{int}Foxp3^{low}$ $CD4^+$ T cells secreting high levels of IL-17, which should be identified as effector Th17 cells [23].

The potential mechanisms of how Treg cells regulate Th17 cells response have been explored by several studies. Chaudhry et al. report that $CD4^+$ Treg cells control Th17 immune response in mice via Foxp3 binding to STAT3, a key factor in the initiation of Th17 differentiation [35]. Overexpression of Foxp3 results in a strong reduction of IL17A gene expression by inhibiting ROR$\gamma$t-mediated IL-17A mRNA transcription. This has been shown to be through direct interaction of Foxp3 with ROR$\gamma$t [36]. These observations support that impaired expression of Foxp3 may lead to defective control of Th17 cells. On the other hand, Th17 cells counteract the Treg cells to expand and allow the development of T1D. Emamaullee et al. found that NOD mice treated with anti-IL-17 could significantly increase the proportion of $Foxp3^+$Treg cells [16]. IL-17A and IL-21 induce Th17 and inhibit Tregs redifferentiation via Th17-associated signaling pathway in immune thrombocytopenia patients *in vitro* [37].

## 5. The Interplay between the Gut Microbiota and Th17/Treg Cells in T1D

Both genetic susceptibility and environmental factors are critical in T1D development. Gut microbiota is one of the important environmental factors in development of T1D. Microorganisms inhabiting humans have coevolved in a reciprocal manner with the host to form a status called symbiosis in health state. In particular, microorganisms residing in the mucosal surfaces such as the gut have a profound impact on the human immunity. The human immune system in turn has a great influence on the composition of gut microorganisms [38, 39]. The human gut is colonized with as many as 100 trillion bacteria [40] that are crucial for the development of the immune system, as well as lymphocyte development and their functions. It has been shown that alterations of the gut bacterial composition resulted in changes in T1D onset and progression in several animal models [41–48]. It was thought that studies with germ-free mice will generate definitive data about the role of gut microbiota in development of T1D. However, in contrast to observations in animal models of arthritis and multiple sclerosis, earlier studies reported that germ-free environment exacerbated autoimmune diabetes in NOD mice, but this was not reproduced in recent studies [42, 49]. Since differentiation of Th17 cells and generation of Treg cells are profoundly influenced by gut microbiota, several studies have investigated the effect on Th17 and Treg cells in diabetes models when gut microbiota is modulated. Ivanov et al. first demonstrated the critical role of gut commensal flora in Th17 cell differentiation in antibiotics treated mice [50]. In C57BL/6 mice, broad spectrum antibiotics or vancomycin treatment results in diminished differentiation of Th17 cells in small intestine lamina propria and germ-free environment essentially devoid of Th17 cells with an increased number of Treg cells [50]. These data suggest that composition of gut commensal microbiota regulate the balance of Th17 and Treg cells and may influence intestinal immunity. Later studies identified segmented filamentous bacterium (SFB) being the most important strain of bacteria to induce Th17 differentiation [37, 51], while other commensal bacteria and their metabolites such as short chain fatty acids promote Treg cells [52–54]. Surprisingly, germ-free NOD mice show a slight but significantly increased number of Th17 and Th1 cells in the colon, mesenteric and pancreatic-draining lymph nodes while the Treg cell number is decreased [42]. Consequently, these germ-free NOD mice develop accelerated insulitis although the incidence of diabetes is not changed compared with mice kept in specific pathogen-free (SPF) environment [42]. Whereas data presented above are in favor of a pathogenic role of IL-17/Th17 cells in T1D, recent studies in NOD mice suggested a protective effect of Th17 cells in T1D when gut microbiota is manipulated [55–57]. Biobreeding diabetes prone (BBDP) and biobreeding diabetes resistant (BBDR) rats which are classic models of T1D can provide novel evidence for gut microflora in the context of T1D [46, 58]. It was shown that oral transfer of *Lactobacillus johnsonii* strain N6.2 (LjN6.2) from BBDR to BBDP rats conferred T1D resistance in BBDP rats. The diabetes resistance in LjN6.2-fed BBDP rats was correlated to a Th17 cell bias within the mesenteric lymph nodes and it was concluded that this Th17 cell bias is most likely to contribute to the diabetes protection [56]. In an attempt to resolve the controversy in regard to pathogenic versus protective effects of SFB and Th17 cells on T1D, Kriegel et al. [57] conducted a detailed survey about incidence of diabetes in NOD mice and correlated the incidence with levels of SFB colonization and number of Th17 cells among NOD mice housed in different animal facilities including different facilities in authors' institution and commercial vendors, namely, Jackson and Taconic Farms. In female mice colonized with SFB, the incidence of diabetes is as low as 20%, while 80% of those without SFB colonized develop diabetes at 30 weeks of age. Interestingly, the incidence of diabetes in

males remains 10–15% regardless of their SFB colonization status. The number of Th17 cells in the SFB positive mice is correlated with SFB level in the feces. These studies clearly demonstrated correlation of Th17 cells with low incidence of diabetes but do not prove Th17 or IL-17 actually has the protective effect in T1D. The function of these "Th17" was not investigated. It is possible that these "Th17" cells are not the same effector Th17 cells. Indeed, $Foxp3^+$/$ROR\gamma t^+$IL-17-producing T regulatory cells in T1D in NOD mice have been reported. These regulatory cells migrate to the site of inflammation and protect NOD mice from diabetes [59]. In addition, how host cell signaling pathways are initiated by SFB is not clear. It is possible that SFB influence antimicrobial proteins and molecules expression in epithelium which participate in Th17 cell polarization. And SFB may also act directly on cells of the immune system (reviewed in [38]). Most recently, it is reported that *in vitro* generated IL-17-producing T regulatory cells for BDC2.5 TCR transgenic $CD4^+$ T regulatory cells (cultured with TGF-$\beta$ and IL-6) were able to inhibit transfer of diabetes [60]. However, it is not known whether these IL-17-producing $CD4^+$ T regulatory cells are induced by SFB. Obviously more studies are required to verify the role of Th17 cells in T1D and their relation to gut microbiome in NOD mice. For example, detailed analysis of cytokine profile of Th17 cells in those NOD mice with SFB is needed; recolonization of germ-free NOD mice with SFB and neutralization of IL-17 activity or deletion of Th17 cells will be required to further clarify this issue.

It must be noted that SFB is not present in humans. Many studies on gut microbiome of T1D children have revealed various results with several species of bacteria in abundance, but these have not been related to Th17 or Treg cell number or function (reviewed in [61]).

## 6. Therapeutic Implications

At present, the treatment of T1D mainly depends on insulin supplement. However the insulin treatment does not halt disease progression. So the treatment strategies directed on pathogenesis are needed imperiously. Emerging evidence has shown that therapeutic agents targeting the IL-17 or directly inhibiting Th17 cells regulate autoimmune diabetes in NOD mice. For instance, blocking IL-17 activity with anti-IL-17 antibody reduced peri-islet T cell infiltrates and prevented disease in NOD mice. Interestingly, inhibition of Th17 cell differentiation using recombinant IL-25 (IL-17E, a member of IL-17 family cytokine with an anti-inflammatory property and promoting Th2 cell differentiation) had more profound effect than that blocking a single Th17 cytokine, IL-17 using anti-IL-17 antibody in prevention of diabetes. The therapeutic superiority of Th17 cell blockade points out the importance of Th17 cells in the pathogenesis of diabetes and future therapeutic strategy should aim to target Th17 cells rather than targeting individual Th17 cytokines. In light of this view, it is of great interest to evaluate the therapeutic efficacy of recently discovered small molecules which act on $ROR\gamma t$. For instance, Solt et al. [62] administered SR1001, a selective $ROR\alpha/\gamma$ inverse agonist in NOD mice showing significantly reduced diabetes incidence and insulitis in the treated mice. Furthermore, SR1001 reduced proinflammatory cytokine expression, particularly Th17-mediated cytokines, reduced autoantibody production, and increased the frequency of $Foxp3^+$ $CD4^+$ Treg cells. These data suggest that use of ROR-specific synthetic ligands targeting this cell type may prove utility as a novel treatment for type 1 diabetes (Lin et al., this issue).

Moreover, it was reported that T cell vaccination (TCV) treatment inhibits autoimmune diabetes induced by multiple low-dose streptozotocin (MLD-STZ) in mice through the suppression of intrapancreatic Th17 cells through inhibition of STAT3-mediated RORgt activation [63]. Administration of B7-H4-immunoglobulin fusion protein (B7-H4.Ig), a newly identified T cell coinhibitory signaling molecule, blocks the onset of diabetes in NOD mice [64]. The reduction of diabetes is due to a transient increase of $Foxp3^+$ $CD4^+$Treg cells at one week posttreatment. Furthermore, the diabetes protection was associated with inhibiting the generation of Th17 cells which subsequently decreases IL-17 production and effectively inhibits the development of T1D in NOD mice [64]. Besides, Bertin-Maghit et al. reported that administration of tolerizing RelB(lo) dendritic cells in 4-week-old NOD mice showed significantly inhibited diabetes progression, which may depend on exacerbating the IL-1-dependent decline in Treg function and promoted Th17 conversion [65]. In addition, ONX 0914 is a selective inhibitor of the immunoproteasome subunit low molecular mass polypeptide (LMP) 7 ($\beta$5i) that attenuates disease progression in mouse models of diabetes. Immunoproteasome subunit LMP7 inhibition by ONX 0914 suppresses Th1 and Th17 but enhances Treg differentiation [66].

It has been reported that injection of adjuvants that contain mycobacterium such as bacillus Calmette-Guérin (BCG) or complete Freund's adjuvant (CFA) is effective in preventing from the onset of autoimmune diabetes in NOD mice [67]. Recently, Nikoopour et al. demonstrated in an adoptive model that $CD4^+$ T cells from CFA-immunized NOD mice which are stimulated with anti-CD3 in the presence of TGF-$\beta$ plus IL-6 or IL-23 can delay diabetes development in recipient mice, suggesting that CFA induces a regulatory Th17 subset [68]. These regulatory Th17 cells produce IL-17, IL-10, and IFN-$\gamma$. It is reasonable to speculate that the diabetic suppressive effect of these regulatory Th17 cells is mediated by IL-10 and IFN-$\gamma$. In addition, the above adjuvant treatment may be partially due to suppressing Th17 commitment [69].

Given the importance of gut microbiota in shaping Th17 and Treg cell balance, manipulation of composition of gut microbiota has been investigated for therapy of T1D. Markle et al. [47] report that transfer of adult male gut microbiota to immature female NOD mice shows a robust T1D protection. The diabetes transfer capacity of T cells from female recipients is tested in NOD-SCID mice. Onset of T1D in NOD-SCID mice that received T cells from female mice was significantly delayed. These results suggest that the capacity of T cells in female mice was downregulated by the manipulation of gut microbiota. Studies aiming to investigate whether composition alteration of gut microbiota will influence the balance between Th17 and Treg cells are required. Recently, Shi et al. [70] found that oral administration of *Cordyceps*

*sinensis*, a parasitic fungus used widely in traditional Chinese medicines, resulted in reduction in the overall incidence of diabetes in NOD mice, and this was due to an increase in the ratio of Treg to Th17 cells in the spleen and PLN. It is yet to be determined whether this effect was through alteration of gut microbiota. Diet can alter the composition of gut microbiota and potentially influence Th17 and Treg cells. Indeed, young NOD mice were fed with "antidiabetic" diet (ProSobee infant formula) that abolished inflammatory Th17 cells and IL-23 in the colon and significantly prevented diabetes [70]. This effect is presumably the result of changes in gut microbiota although they were not assessed. Future studies should assess whether strategies to modify gut microbiota will be able to halt or delay the onset of diabetes in high risk populations of healthy individuals.

Targeting IL-17 and Th17 pathway has been approved by FDA in treating psoriasis and psoriatic arthritis. For example, ustekinumab blocking the common subunit p40 of IL-12 and IL-23 has been shown to be highly effective in treating psoriasis. The therapeutic effect of this monoclonal antibody is considered by predominantly blocking IL-23 and subsequently Th17 cells [71, 72]. Secukinumab targeting IL-17A is also recently approved to treat psoriasis [73, 74]. Both antibodies appear to be safe and well tolerated. It would be worthwhile to investigate whether targeting IL-17 or Th17 will be islet protective in those children with islet autoimmunity but not yet diabetic by using these monoclonal antibodies.

## 7. Conclusion

T1D is a T cell mediated autoimmune disorder which targets and destroys insulin producing pancreatic $\beta$ cells. Accumulating evidence gained from animal models and humans closely connects IL-17/Th17 cells in the context of impaired Treg cells/function to the pathology of T1D. Dysbiosis of gut microbiota as one of the important environment factors in T1D has been considered. Several studies have highlighted the importance of gut flora in modulating the mucosal and systemic immune response involved in T1D, specifically in the disease onset and progression. The composition of intestinal microbiota regulates the Th17:Treg balance and may thus influence intestinal and systemic immunity involved in T1D. Studies on manipulation of gut microbiota with diet or medicine are required to assess the effect on Th17 and Treg cells and development and progression of diabetes in populations at high risk. Immunotherapy targeting IL-17/Th17 has achieved high efficacy in other Th17-mediated conditions. Clinical trials with these biologic drugs should be considered to prevent progression in prediabetic populations.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review Article*

# Targeting Th17 Cells with Small Molecules and Small Interference RNA

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T helper 17 (Th17) cells play a central role in inflammatory and autoimmune diseases via the production of proinflammatory cytokines interleukin- (IL-) 17, IL-17F, and IL-22. Anti-IL-17 monoclonal antibodies show potent efficacy in psoriasis but poor effect in rheumatoid arthritis (RA) and Crohn's disease. Alternative agents targeting Th17 cells may be a better way to inhibit the development and function of Th17 cells than antibodies of blocking a single effector cytokine. Retinoic acid-related orphan receptor gamma t (RORγt) which acts as the master transcription factor of Th17 differentiation has been an attractive pharmacologic target for the treatment of Th17-mediated autoimmune disease. Recent progress in technology of chemical screen and engineering nucleic acid enable two new classes of therapeutics targeting RORγt. Chemical screen technology identified several small molecule specific inhibitors of RORγt from a small molecule library. Systematic evolution of ligands by exponential enrichment (SELEX) technology enabled target specific aptamers to be isolated from a random sequence oligonucleotide library. In this review, we highlight the development and therapeutic potential of small molecules inhibiting Th17 cells by targeting RORγt and aptamer mediated $CD4^+$ T cell specific delivery of small interference RNA against RORγt gene expression to inhibit pathogenic effector functions of Th17 lineage.

## 1. Introduction

The differentiation of naïve $CD4^+$ T cells into effector T helper (Th) cells is induced by their T cell receptor and costimulatory molecules in the presence of other cytokines. It is that these cytokines and transcriptional factors ultimately determine the differentiation of $CD4^+$ Th cells into distinct subsets. Initially, $CD4^+$ Th cells were identified as having two subsets, Th1 and Th2 cells [1]. Th1 cells produce high levels of IFN-$\gamma$ and express the transcriptional factor T-bet, which protect the host against intracellular pathogens [2]. Th2 cells express GATA-3 and produce IL-4, IL-5, IL-9, and IL-13 which are mainly involved in protection against parasitic helminthes [3]. Recently, new subsets of effector Th cells that express different transcriptional factors and produce distinct cytokines have been discovered, including T regulatory (Treg) cells, Th17 cells, follicular helper T cell (Tfh), and Th9 cells [4, 5]. Treg cells are characterized by the production of IL-10 and TGF-$\beta$ as major cytokines and expression of forkhead box P3 (Foxp3) as transcriptional factor, which control immune response and maintain immune tolerance [6]. Th17 cells are characterized by the production of IL-17A (also known as IL-17), IL-17F, and IL-22 as signature cytokines and expression of retinoic acid-related orphan receptor gamma t (RORγt) as master transcriptional factor [7–9]. These cytokines play a critical role in host defense against extracellular pathogens such as bacteria and fungi [10] and many autoimmune diseases, including psoriasis, rheumatoid arthritis (RA), inflammatory bowel disease, uveitis, and multiple sclerosis [11–13]. Fully human monoclonal antibodies (mAbs) against IL-17 (ixekizumab and secukinumab) and IL-17 receptor A (IL-17RA) (brodalumab) have rapidly reduced clinical symptoms in patients with psoriasis [14–18]. However, in a Phase IB study on methotrexate-resistant RA patients, brodalumab did not improve disease symptoms [19]. In a Phase II study, secukinumab did not show clinical efficacy

in RA patient with inadequate response to methotrexate [20]. Furthermore, treatment of patients with Crohn's disease with secukinumab not only showed no good responses, but also worsened disease in some patients [21]. These data suggest that targeting IL-17 cannot completely alleviate Th17-mediated autoimmune diseases. Since Th17 cells also produce other cytokines such as IL-17F and IL-22 which are potent inflammatory mediators, targeting Th17 cells may provide a better efficacy in these clinical conditions [22].

Th17 differentiation requires the master transcriptional factor, RORγt, which is induced by activation of naïve $CD4^+$ T cells in the presence of inflammatory cytokines, such as IL-6, TGF-β, IL-21, IL-1β, and IL-23 [23]. Mice deficient in RORγt have reduced Th17 differentiation and are resistant to experimental autoimmune encephalomyelitis (EAE) [9]. Conversely, overexpression of RORγt promotes IL-17 production. The critical role of RORγt in the generation of Th17 cells provides a unique opportunity to develop novel therapeutics targeting Th17 cells. Given the fact that cytokines of IL-17 family are important in host defense and they are also produced by other immune cells other than Th17 cells, it is highly desirable to target the pathogenic Th17 cells. The disadvantage of mAbs targeting individual IL-17 cytokines is that it does not discriminate the cellular source of IL-17 and therefore poses potential adverse effects from blocking IL-17 activity produced for host defense. Moreover, the effector cytokines of Th17 cells include IL-17A, IL-17F, and IL-22 which are all to be blocked to abrogate Th17 cell activity which is a challenging task for individual mAbs.

Recently, small molecules targeting RORγt have been identified, which not only suppress Th17 differentiation and IL-17 production, but also reduce the severity of animal models of autoimmune diseases. In addition, recent advancement in technology of engineering nucleic acid enables a targeted delivery of small interference RNA (siRNA) or short hairpin RNA (shRNA) using aptamers which serve as vehicle to guide siRNA or shRNA to target cells. These two classes of agents, which are nonmonoclonal antibody or fusion protein based, are emerging to be useful in targeting Th17 cells rather than merely blocking individual cytokines. Small molecules directly interact with RORγt to block its activity while siRNA/shRNA specifically inhibits RORγt gene expression.

## 2. Identification and Differentiation of Th17 Cells

Before the identification of Th17 cells, T cell mediated autoimmunity was believed to be mediated by Th1 cells. Indeed, T-bet deficient mice were resistant to EAE, and polyclonal antibody targeting IL-12 was an effective therapy for EAE and CIA. However, the later studies provided contradictory results that IFN-γ and IFN-γ receptor deficient mice, as well as mice that lack IL-12p35, were not protected from EAE but developed rapidly progressing disease [24, 25]. Furthermore, IFN-γ knockout mice develop severe EAE and convert resistant strain of mice to be highly susceptible to collagen-induced arthritis (CIA) [26, 27]. Thus, the function of Th1 cells in T cell mediated autoimmunity was challenged. The discovery of IL-23, a cytokine which is composed of a unique p19 subunit and a p40 subunit which is shared with IL-12 [28], provided us with novel insights. It was IL-23, not IL-12, that was critical for the induction of EAE and CIA [29, 30]. Moreover, IL-23 failed to induce IFN-γ but instead expanded IL-17-producing T cells. When IL-17-producing T cells induced by IL-23 were adoptively transferred into naïve wild-type mice, EAE developed [30]. IL-23p19-deficient mice were resistant to EAE due to lack of IL-17-producing T cells [29, 30]. These studies led to IL-17-producing T cells to be described as a distinct Th cell subset, which was named Th17 cells [7, 8].

Differentiation of Th17 cells is induced by activation of naïve $CD4^+$ T cells in the presence of inflammatory cytokines. Transforming growth factor- (TGF-) β is a regulatory cytokine which has multiple effects on T cell development, homeostasis, and tolerance [31]. TGF-β not only induces naïve precursors into Foxp3-expressing inducible Treg (iTreg) [30], but also plays a crucial role in the generation of Th17 [31]. However, TGF-β alone is not capable of the induction of Th17 cells development. Unlike Th1, Th2, and iTreg cells, which only require a single cytokine for their generation, additional differentiation factors are required in Th17 cells development. Recent studies found that combination of IL-6 and TGF-β was the essential cytokine-mix of inducing naïve T cells to develop Th17 cells [32–34]. IL-6 is able to inhibit TGF-β-driven induction of Foxp3 in naïve T cells and instead leads to strong induction of IL-17 [33]. Furthermore, IL-21 together with TGF-β is also able to induce the differentiation of Th17 cells. During the initial Th17 differentiation, IL-6 induced IL-21 acting as a positive amplification loop to enforce Th17 differentiation [35, 36]. IL-21 was shown to be able to replace IL-6 at least *in vitro* [37]. In the absence of IL-6, IL-21 together with TGF-β was able to inhibit the development of iTreg and to promote the differentiation of Th17 cells [37]. *In vivo*, however, the role of IL-21 in the induction of Th17 cells remains controversial. It had been reported that the absence of IL-21 or IL-21R had no significant difference on the development of Th17 cells [38, 39]. Thus, IL-21 might be an alternative pathway in inducing and expanding Th17 cells [23]. IL-23 also plays an important role in regulation of Th17 cells indirectly. However, IL-23 receptors are absent on naïve T cells, so IL-23 is not involved in the initiation of Th17 cells, but expands an existing population of effector Th17 cells [40]. Without IL-23, activated $CD4^+$ T cells in the presence of IL-6 plus TGF-β were able to produce high amounts of IL-17 but did not fully develop into pathogenic Th17 cells [41]. The treatment with neutralizing IL-23p19 specific antibody not only inhibited the development of EAE but also ameliorated EAE after the onset of disease [42]. Ustekinumab, a mAb against IL-23/IL-12p40, has shown a marked efficacy in clinical studies involving psoriasis patients [43]. Ustekinumab also has shown increased clinical responses in patient with tumor necrosis factor- (TNF-) refractory Crohn's disease [44]. These studies indicate that IL-23 is an important cytokine in Th17-mediated autoimmune disease. In contrast to mice, combination of IL-6 and TGF-β is not capable of inducing human Th17 differentiation [45]. Instead of TGF-β, IL-1β

together with IL-6 or IL-23 was reported to upregulate RORγt and induce IL-17 production from $CD4^+$ T cells isolated from human peripheral blood, suggesting a fundamental difference in the biology of human and mouse Th17 cells [46].

## 3. Transcriptional Regulation of Th17 Cells

The differentiation of Th17 cells is initiated by the combined signals of activated TCR and cytokine receptors. These signals then induce specific transcription factors responsible for the expression of Th17 cell specific genes such as *Il17* and *Il17f*. Multiple transcription factors have been shown to be important for the development of Th17 cells, including RORγt, STAT3, IRF4, BATF, and RUNX1. RORγt is the master transcription factor that regulates the differentiation of Th17 cells [47]. RORγt belongs to the ROR subfamily. ROR is the member of retinoic acid nuclear receptor superfamily containing a ligand-binding domain (LBD). Usually, ligand binding to the LBD of ROR leads to conformational change and transcriptional activity. The ROR subfamily has three members in mammals: RORα, RORβ, and RORγ [48]. The RORγ has two different isoforms: RORγ and RORγt, which are encoded by the *Rorc* gene and have difference only at their N terminus [49]. RORγt is a splice variant of RORγ expressed in T cells [49]. Unlike RORγ, which is expressed in many tissue such as heart, kidney, liver, lung, brain, and muscle, RORγt is expressed exclusively in lymphoid cells [50]. RORγt is an important molecule to regulate gene expression during the development of T cells and the formation of secondary lymphoid organ [51–53]. *Rorc* gene knockout mice exhibited that $CD4^+CD8^+$ thymocytes showed early apoptosis, and lymph nodes, Peyer's patches, and lymphoid tissue inducer (LTi) cells failed to develop [52, 53]. *In vitro*, with the absence of *Rorc* in $CD4^+$ T cells, IL-17 expression was greatly decreased under Th17 polarizing conditions. Conversely, overexpression of RORγt in naïve $CD4^+$ T cells was sufficient to induce the expression of IL-17, IL-17F, and IL-22 [9]. RORγt is necessary for the expression of IL-17 as well as the differentiation of Th17 in mouse and human $CD4^+$ T cells [9, 54]. The number of Th17 cells was markedly reduced and the disease severity of EAE alleviated in *Rorc*-deficient mice. The role of RORγt is similar to transcription factors such as T-bet and GATA3 in Th1 and Th2 differentiation, respectively, and therefore RORγt has been considered to be a "master transcriptional factor" for Th17 differentiation [47]. RORγt promotes IL-17 expression by directly binding the promoter region of *Il17* gene at multiple sites [9, 55, 56].

Another related retinoic acid nuclear receptor, RORα, is also expressed in Th17 cells both *in vitro* and *in vivo*. In contrast to RORγt, RORα played minimal roles in mouse Th17 differentiation. However, mice deficiencies in *Rora* and *Rorc* markedly impaired Th17 generation and completely protected mice from EAE [57]. The coexpression of *Rora* and *Rorc* induced greater Th17 differentiation. It is demonstrated that RORα and RORγt acts as synergy in regulating Th17 cell gene expression.

Besides RORα and RORγt, other transcription factors are required in Th17 differentiation. The transcription factor signal transducer and activator of transcription 3 (STAT3), which is preferentially activated by IL-6, IL-21, and IL-23, is capable of inducing RORγt and regulating Th17 cells development [58, 59]. Deficiency of STAT3 in $CD4^+$ T cells impaired Th17 differentiation *in vivo*, and overexpression of a constitutively active STAT3 could increase IL-17 production [58, 60]. STAT3 might affect the production of IL-17 by increasing the expression of RORγt and RORα [57, 58]. Furthermore, STAT3 also binds directly to the *Il17* and *Il21* promoters and leads to the expression of IL-17 and IL-21 [61, 62]. Therefore, STAT3 and RORγt seem to cooperate to induce IL-17 production. Transcription factor interferon regulatory factor 4 (IRF4) also has a certain role in Th17 differentiation, which was previously associated with GATA-3 expression in Th2 differentiation [63]. Recently, it has been shown that IRF4 regulates IL-17 and IL-21 production [64]. IRF-4 deficient mice were shown to impair Th17 responses and were resistant to EAE [65]. IRF-4 deficient T cells failed to upregulate RORγt in response to IL-6 plus TGF-β and did not differentiate into Th17 cells [65], suggesting that IRF4 might also cooperate with RORγt to induce Th17 differentiation. In addition, BATF, a member of the AP-1 transcription factor family, and Runx1, a member of RUNX1 transcription factor, are also important for Th17 differentiation [66, 67].

As mentioned above, RORγt, STAT3, IRF4, BATF, and RUNX1-deficient mice show an impaired Th17 generation and an attenuated susceptibility to the induction of autoimmunity. Targeting these transcription factors might be a possible way to inhibit the development and function of Th17 cells. RORγt acts as the master transcription factor of Th17 differentiation, resulting in an attractive pharmacologic target for the treatment of Th17-mediated autoimmune disorders.

## 4. Small Molecules Target to Th17 Cells

*4.1. Digoxin.* By performing a chemical screen with an insect cell-based reporter assay, the cardiac glycoside digoxin was identified as a specific inhibitor of RORγt transcriptional activity. Digoxin suppressed murine Th17 cell differentiation without affecting differentiation of other T cell lineages. In addition, digoxin was effective in attenuating EAE in mice and in delaying the onset and reducing disease severity in a rat model of adjuvant-induced arthritis [68–70]. Digoxin was toxic for human cells at high doses, but its synthetic derivatives 20,22-dihydrodigoxin-21,23-diol and digoxin-21-salicylidene were nontoxic and specifically inhibited the induction of IL-17 in human $CD4^+$ T cells [68]. These data indicate that derivatives of digoxin might be used as chemical templates for the development of targeting RORγt therapeutic agents that attenuate inflammatory Th17 cells function and autoimmune disease.

*4.2. ML209/Compound 4n.* Using a cell-based gene RORγt and control reporter assay, a small molecule library comprising 300,000 compounds was screened at the NIH Chemical Genomics Center (NCGC), a series of diphenylpropanamide compounds as a selective RORγt inhibitor, including a highly potent compound ML209 (also known as compound 4n).

Huh and colleagues found that compound 4n inhibited transcriptional activity of RORγt, but not RORα, in cells. Like digoxin, compound 4n selectively inhibited murine Th17 differentiation without affecting the differentiation of naïve $CD4^+$ T cells into other lineages, including Th1 and regulatory T cells. Moreover, compound 4n suppressed RORγt-induced expression of IL-17 in human T cells [71]. This report demonstrates that compound 4n might serve as a valuable pharmacological agent to inhibit RORγt transcriptional activity and Th17 differentiation.

*4.3. SR1001 and SR2211.* Using the liver X receptor (LXR) agonist T0901317 [72] scaffold as a lead compound, Griffin and Burris developed a derivative, SR1001, which was devoid of all LXR activity yet retained its ability to suppress the transcriptional activity of RORα and RORγ [73]. SR1001 not only is high-affinity synthetic ligand that is specific to both RORα and RORγ, but also inhibits Th17 cell differentiation and function. SR1001 binds specifically to the LBD of RORα and RORγ, inducing a conformational change within the LBD, resulting in suppression of the receptors' transcriptional activity. By suppressing IL-17 gene expression and protein production, SR1001 inhibited the development of murine Th17 cells. Furthermore, SR1001 inhibited the expression of cytokines in murine or human Th17 cells and effectively reduced EAE severity in mice [73]. Therefore, SR1001 and its derivatives may represent a novel drug to treat not only Th17-mediated autoimmune diseases, but ROR-mediated metabolic diseases as well.

By modifying the SR1001 scaffold, SR2211 was developed. Unlike SR1001, SR2211 can specifically inhibit the transcriptional activity of RORγ, but not RORα. In cotransfection assays, SR2211 suppresses transcription activity in both GAL4-RORγ LBD and full-length RORγ contexts. Furthermore, SR2211 could result in suppression of gene expression and production of IL-17 in EL-4 cells [74]. These data strongly suggest that SR2211 is also a potent and efficacious RORγ mediator and represses its activity. Moreover, SR2211 suppressed inflammatory T cell function and Th17 cell differentiation and markedly reduced joint inflammation in mice with CIA [75]. It is shown that SR2211 has the potential utility for the treatment of Th17-mediated autoimmune disorders.

*4.4. Ursolic Acid.* Ursolic acid (UA), a small molecule present in herbal medicine, was identified by screening a small chemical library. In treatment with UA, the function of RORγt was inhibited selectively and effectively, and IL-17 expression was greatly decreased in developing and differentiated Th17 cells. In addition, UA ameliorated EAE in mice. The results thus indicate that UA might be a valuable drug candidate and can be used for developing treatments of Th17-mediated inflammatory diseases [76].

*4.5. TM920, TMP778, and GSK805.* Using a fluorescence resonance energy transfer (FRET) assay and two-cell line reporter assay (IL-17F promoter and RORγ-LBD promoter assays), a proprietary small-molecule library was screened and several compounds binding to RORγt were identified. TM920 and TM778 were identified as highly potent and selective RORγt inhibitors [77, 78]. *In vitro*, TM920 and TM778 suppressed Th17 development and inhibited IL-17 production from differentiated Th17 cells. Furthermore, TMP778 has increased potency and specificity for Th17 differentiation, resulting in blockade of nearly all Th17 signature gene expression [77]. Importantly, TMP778 displays no activity against any of the other 24 nuclear receptors tested, including RORα and RORβ, so TMP778 has very limited effects on the expression of other genes [78]. TMP778 potently impaired the IL-17 production not only by human $CD4^+$ Th17 cells, but also by human $CD8^+$ Tc17 cells, memory $CD4^+$ T cells, and PBMCs. TMP778 also blocked IL-17 production by skin mononuclear cells of psoriasis patients and significantly impaired expression of Th17 signature gene from psoriasis patients [78]. *In vivo,* TMP778 suppressed imiquimod-induced cutaneous inflammation and EAE [77, 78]. Although the specific RORγt inverse agonist, TM778, may have good RORγt target effects and low off-target effects, unexpected toxicity may occur in nonimmune cells and tissues (see below); in particular, it required a relatively higher dose of TN778 to exert its function. Another RORγt inhibitor, GSK805, is proved to be more potent than TM778 and can be orally administered. GSK805 could efficiently ameliorate the severity of EAE and strongly inhibited Th17 cell differentiation in the central nervous system [77]. It is interesting but unexpected that TMP778 and GSK805 were able to induce RORγt biding to GATA3 and led to an increase of GATA3 mRNA and protein expression. The apparent transactivation of GATA3 by RORγt may partially explain the inhibition of Th17 cell signature gene expression by TMP778 or GSK805 [77].

These compounds target RORγt, which inhibit the transcriptional activity of RORγt by binding to RORγt LBD [79], a domain present in both RORγ and RORγt. These compounds not only inhibit Th17 cell differentiation and IL-17 production, but also have shown variable levels of efficacy in EAE and CIA studies. Therefore, these compounds may serve as novel attractive drugs to treat Th17-mediated autoimmune disorders. However, we should note that RORγ is broadly expressed in many human tissues such as heart, kidney, liver, lung, brain, and muscle, so RORγ/RORγt inverse agonists might induce toxicity via inhabitation of RORγ in nonimmune tissue. Thus, in order to treat Th17-mediated autoimmune disorders, it is necessary to develop a specific strategy to only inhibit RORγ/RORγt transcriptional activity in immune cells, especially $CD4^+$ T cells.

## 5. Targeting Th17 Cells by CD4 Aptamer-RORγt shRNA Chimera

Recently, RNA interference (RNAi) technology provides a promise for studying basic T cell biology and for developing potential T cell targeted therapeutics. However, efficient delivery of small interference RNA (siRNA) into primary T cells remains a major hurdle of siRNA-based therapy [80]. Emergence of CD4 aptamers, which specifically bind $CD4^+$ T cells and efficiently deliver various biomolecules into these

cells, makes it possible to target RORγt and IL-17 production in $CD4^+$ Th17 cells with RNAi technology. Here we will discuss the advantage of aptamer-siRNA and contemplate whether CD4 aptamer-RORγt shRNA chimeras would be beneficial to inhibit Th17 differentiation in human T cells.

*5.1. Aptamers.* Aptamers, nucleic acid-based ligands, are small single-stranded DNA or RNA oligonucleotides that are produced *in vitro* via a process known as systematic evolution of ligands by exponential enrichment (SELEX) [81, 82]. In the SELEX process, aptamers are selected from a large pool ($>1 \times 10^{14}$) of single-stranded oligonucleotides with random sequences [83, 84]. After the incubation of the random aptamers pool with the target, followed by repeated cycles: the fixation of region containing binding, PCR or RT-PCR amplification, and modification of restriction endonuclease, aptamers with high affinity with their corresponding ligands are cloned [85]. With the technological improvement in the SELEX process, researchers can isolate aptamers from not only a protein target but also a complex mixture including cell-surface proteins and human plasma in the past decades. Recently, isolation of cell- and receptor-specific aptamers using living cells has been reported [86–88]. Therefore, the power of SELEX enables one to generate specific aptamers against a molecule, a protein, a cell-surface receptor, and even a cell [89, 90]. Notably, chemical modifications to aptamers, including sugar modifications (2′-O-Methyl, 2′-O-methoxyethyl, 2′-fluoro, or LNA), the phosphate backbone modifications (phosphorothioate, boranophosphate), or the nucleobase moiety modifications (4-thiouracil, 2-thiouracil, and diaminopurine), have been reported to greatly enhance the nuclease resistance of the aptamer probes [91, 92].

Similar to antibodies, aptamers, which are often regarded as nucleic acid "antibodies," gain entrance to target cells via receptor-mediated endocytosis upon binding to cell surface ligands [93, 94]. However, aptamers are generally nonimmunogenic or low-immunogenic [95, 96], whereas antibodies suffer from immunogenicity, resulting in immune responses in patients [97]. In addition, the cost of generation of aptamers *in vitro* is much less than the development process of antibodies [93, 98]. Importantly, aptamers can be generated through simple chemical approach in animals or cultured mammalian cells, making them easier to produce for large scale manufacturing that are necessary for clinical use [99]. The first therapeutic aptamer, antivascular endothelial growth factor (VEGF) aptamer, Macugen (pegaptanib) was approved by the US FDA for treatment of age related macular degeneration in 2005 [100]. Also, several aptamers are currently undergoing clinical trials [92, 101, 102]. Therefore, aptamers that target various proteins and cells are considered as ideal diagnostic and therapeutic approach for clinical disease, such as cancer, infection, and autoimmune disease [99, 103].

*5.2. Aptamer-siRNA.* RNAi offers a powerful approach to developing new therapeutics in human diseases [105]. siRNA, because of their ability to silence expression of sequence-specific gene [106, 107], has currently been developed as a new strategy in treatment of human disease. However, it is a big challenge to efficiently and safely deliver siRNA into "difficult-to-transfect" primary T cells by conventional transfection methods. For instance, electroporation and nucleofection cause excessive cell death and may require preactivation of T cells and electrical apparatus [108, 109]. Chemically modified synthetic siRNA with Acell agents can be transfected into primary T cells; however, they are needed to incubate with T cells for longer time and only a small portion of T cells are transfected [110]. The most disappointing defect of these methods is that it is difficult for them to be used *in vivo*. Retroviral vectors carrying shRNA cassette are able to effectively infect and enter T cells and make the shRNA to stably be expressed for the lifetime of the cells *in vitro* and *in vivo* [111, 112]. However, applying retroviral vectors *in vivo* gives rise to the danger about malignant transformation, which limits the viral vector transfection [80]. Nanoparticles are effective to deliver siRNA into T cells, but the delivery is not T cell specific [113]. Recently, a method that uses a fusion protein composed of a cell-target antibody fragment joined to a protamine peptide that binds nucleic acids has been reported for cell-specific siRNA transfection of immune cells [114, 115]. siRNAs mixed with the fusion protein can silence gene expression in cells, both *in vitro* and in tissues. Modifications of this approach effectively inhibit HIV infection in humanized mice [116]. However, antibody-based fusion proteins are expensive to manufacture, are potentially immunogenic, and are unsuitable for clinical use. Hence, an effective siRNA delivery system *in vivo* for targeting T cells has to be developed for treatment of T cells-mediated human disease. Because aptamers can enter target cells via endocytosis and maintain stability after endocytosis, aptamers have been developed as guiding moieties for both drug delivery and nucleic acid transport vehicles such as siRNA and shRNA [117]. Aptamer siRNA chimeras, composed of an siRNA/shRNA fused to an aptamer, provide an attractive alternative for *in vitro* and *in vivo* gene knockdown [118]. The aptamer portion of the chimeras binds to a cell-surface receptor such as prostate surface membrane Ag (PSMA), CD4, whereas the siRNA portion targets the overexpressed signaling molecules or regulatory nucleic acids, resulting in inhibition of cell proliferation and differentiation. Aptamer-siRNA chimeras (AsiCs) efficiently transfect and knock down gene expression in cells bearing the surface receptor recognized by the aptamer. The PSMA aptamer-siRNA chimeras targeting PSMA silenced target gene expression in prostate cancer mouse xenografts [96]. AsiCs containing an aptamer targeting HIV-gp120 inhibit HIV replication in already infected cells *in vitro* [119, 120] and *in vivo* [121]. CD4-AsiCs bearing siRNAs that recognized HIV *gag* and *vif* or host *CCR5* were specifically taken up by $CD4^+$ cells, knocked down genes expression, and inhibited HIV infection in primary $CD4^+$ T cells and in the female genital tract of humanized mice [122, 123] and at the same time do not activate lymphocytes or stimulate innate immunity [122, 123]. Moreover, the chimeras do not bind to or function in cells that do not express CD4, such as CD3, CD8, and CD45 [124]. Thus, aptamer-facilitated cell specific delivery of siRNA/shRNA represents an attractive novel approach

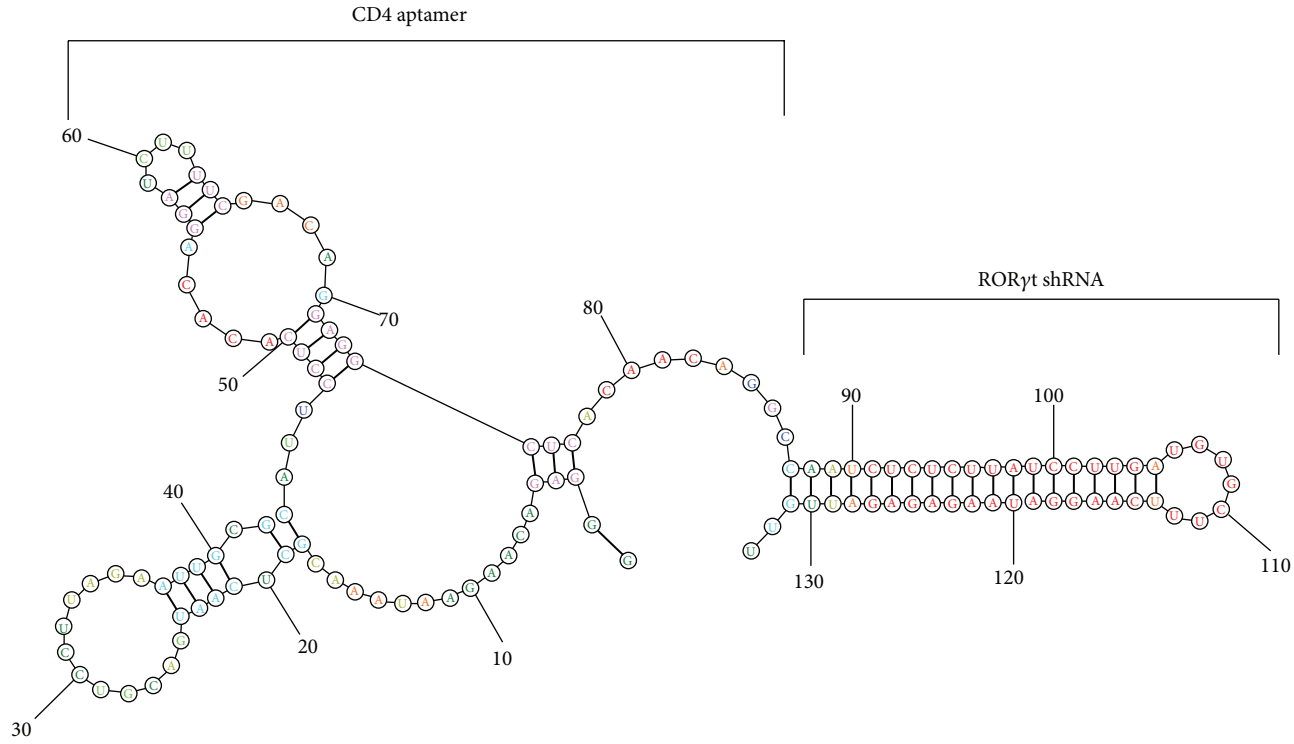


Figure 1: Predicted secondary structure of CD4-aptamer-RORγt shRNA chimera (modified from Song et al., BBRC, 2014, Figure 1(b) [104] with permission).

for efficient RNAi delivery. CD4-AsiCs overcome the hurdle of *in vivo* siRNA delivery to the immune cells and hold a promise to study immune responses and develop therapeutics in autoimmune diseases.

*5.3. CD4 Aptamer-RORγt shRNA Chimeras.* CD4 aptamers that bind surface CD4 can be applied as T helper cell-specific delivering vehicles. CD4-AsiCs bearing siRNAs or shRNA targeting RORγt might suppress Th17 differentiation and treat Th17-mediated autoimmune diseases. We selected CD4 RNA aptamers (86 nucleotides in length) that delivered RORγt-shRNA (Figure 1) into $CD4^+$ T cells and investigated its efficacy in suppressing Th17 cell differentiation and IL-17 production in human $CD4^+$ T cells *in vitro* [104]. Chemical modifications of nucleotides *2′-F-dCTP and 2′-F-dUTP* were done to enhance the nuclease resistance of the aptamer chimeras [104]. *In vitro* using fluorescent microscope and flow cytometric analysis, Cy3-labeled CD4 aptamer-RORγt shRNA chimeras (CD4-AshR-RORγt) (133 nucleotides in length) were shown to enter into human $CD4^+$ T cells but not Cy3-labeled mock CD4-AshR- RORγt. *In vitro* expression of RORγt is significantly and specifically diminished by CD4-AshR-RORγt in a concentration-dependent manner in human $CD4^+$ T cells compared with control CD4 aptamers [104]. Consistent with decreased RORγt, CD4-AshR-RORγt displayed a concentration-dependent inhibition of IL-17A release from $CD4^+$ T cells and intracellular IL-17A staining in $CD4^+$ T cells, while mock CD4-AshR-RORγt and CD4-AshR-scrambled control have no impacts [104]. This study indicates that intracellular delivery of CD4-AshR-RORγt could target RORγt and manipulate Th17 cell differentiation and IL-17 production in $CD4^+$ T cells. Additionally, CD4-AshR-RORγt does not significantly impact the expression of Th1 and Th2 lineage transcription factors T-bet and GATA-3 in PMBCs. Consistent with these, synthesis of IFN-$\gamma$ and IL-4 in PBMCs is not changed by CD4-AshR-RORγt.

These suggest that CD4-AshR-RORγt chimeras keep its specificity to target RORγt gene and Th17 cells. Thus, it is of interest to explore the use of CD4-AshR-RORγt chimeras in animal and clinical trials of autoimmune diseases.

## 6. Concluding Remarks

In conclusion, Th17 cells and their signature cytokines play crucial roles in the pathology of autoimmune and inflammatory diseases. Targeting IL-17 or IL-17R has shown clinical efficacy in psoriasis but not many other autoimmune disease such as RA and Crohn's disease. In contrast to blocking a single effector cytokine, targeting Th17 lineage provides promising therapeutic to impact multiple inflammatory cytokines. First attempts to target Th17 lineage are targeting RORγt, the master transcriptional factor of Th17 lineage, via small molecule inverse agonists. Several small molecules are shown to have potent suppressive effects on Th17 cells and their cytokines and have therapeutic efficacy in animal models of autoimmune diseases. Clinical studies are required to assess their usefulness for treating Th17-mediated human diseases. Aptamer mediated delivery of siRNA/shRNA specifically

against RORγt offers another strategy to target Th17 cells. By replacing the shRNA for targeted genes such as GATA3, T-bet, and STAT3; this CD4 aptamer may be used as a universal tool to introduce siRNA or shRNA into $CD4^+$ T cells to manipulate function of various Th cells. Further animal and clinical trials of CD4-AshR-RORγt chimeras are necessary to evaluate the beneficial outcomes in autoimmune diseases.

## Conflict of Interests

Cong-Qiu Chu has filed a patent application "Aptamer-RNAi Therapeutic Compositions." Other authors declare that there is no conflict of interests regarding the publication of this paper.

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*Research Article*

# Evolutionary Insights into IL17A in Lagomorphs

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In leporids, IL17A had been implicated in the host defense against extracellular pathogens, such as *Francisella tularensis* that infects hares and rabbits and causes the zoonotic disease tularemia. Here, we studied IL17A from five lagomorphs, European rabbit, pygmy rabbit, brush rabbit, European brown hare, and American pika. We observed that this protein is highly conserved between these species, with a similarity of 97–99% in leporids and ~88% between leporids and American pika. The exon/intron structure, N-glycosylation sites, and cysteine residues are conserved between lagomorphs. However, at codon 88, one of the interaction sites between IL17A and its receptor IL17RA, there is an Arg>Pro mutation that only occurs in European rabbit and European brown hare. This could induce critical alterations in the IL17A structure and conformation and consequently modify its function. The differences observed between leporids and humans or rodents might also represent important alterations in protein structure and function. In addition, as for other interleukins, IL17A sequences of human and European rabbit are more closely related than the sequences of human and mouse or European rabbit and mouse. This study gives further support to the hypothesis that European rabbit might be a more suitable animal model for studies on human IL17.

## 1. Introduction

Interleukin 17, first known as cytotoxic T lymphocyte associated antigen (CTLA) 8, is originated from a T-cell derived factor with cytokine-like activity [1, 2]. With a ubiquitous expression in different tissues, this protein, nowadays known as IL17A, has a sequence composition different from all the other cytokine families [1, 3]. IL17A, along with five functional homodimers (IL17B-F), one heterodimer (IL17A/F), and 5 receptors (IL17RA-RE), composes the IL17 family, which is important to adaptive immunity responses, namely, as mediator of chronic inflammation and autoimmune diseases [3–6]. There is a wide range of genes that are targeted by IL17, such as proinflammatory and hematopoietic cytokines, genes associated with acute phase response, and antimicrobial substances [3, 7]. This protein is also part of a subset of CD4 T helper (Th) cells known as Th17 which are able to establish a connection between innate and adaptive immune responses, being a complement to Th1 and Th2 defense mechanisms [8]. Furthermore, the production of IL17A is important for host defense against extracellular pathogens (fungi, viruses, bacteria, and parasites) assisting in neutrophils recruitment and activation and also promoting antimicrobial peptides [8–12]. Studies in mice [12–15] and humans [16–18] highlighted the importance of IL17 expressing cells for immunity against several diseases, and low expression levels of IL17 and IL17RA make organisms more susceptible to disease, including those caused by extracellular pathogens such as *Francisella tularensis*.

*F. tularensis* is highly pathogenic Gram negative intracellular bacteria included by the Center of Disease Control and Prevention (CDC) in category A of bioterrorism (http://emergency.cdc.gov/agent/agentlist.asp). Able to cause the zoonotic disease tularemia, this microorganism has several known hosts, from mammals to protozoans; however transmission to humans is normally associated with direct contact with lagomorphs, rodents, and some arthropods [15, 20–22]. In lagomorphs and rodents, *F. tularensis* has the ability to cause septicemia while in humans the outcome of infection is a multisystem organ failure [23]. There are several reports of *F. tularensis* infections in leporids, mainly in rabbits (European rabbit and cottontails) [24–26] and hares [24, 26, 27] and despite an apparent period of stasis (2006–2010) there were some recently documented outbreaks of tularemia in Europe [22, 28].

The order Lagomorpha includes two families, Leporidae (rabbits and hares) with eleven genera and Ochotonidae (pikas) with only one genus, *Ochotona* [29]. Together with rodents, lagomorphs form the clade Glires, a sister group of Euarchonta that includes primates [30, 31]. Along with mouse, the European rabbit had been used as a research model for several human diseases, development of therapeutics and vaccines [32]. Several studies have suggested that the European rabbit may be a better research model than mouse [33–37]. With the exception of humans and mouse, there is a big gap of information on IL17A in other mammalian groups, including leporids. Thus, considering the important biological role of the European rabbit immune response against several diseases, including tularemia, we performed a genetic characterization of IL17A in four leporid genera (*Oryctolagus*, *Brachylagus*, *Sylvilagus*, and *Lepus*).

## 2. Material and Methods

Samples of European rabbit (*Oryctolagus cuniculus cuniculus and Oryctolagus cuniculus algirus*), pygmy rabbit (*Brachylagus idahoensis*), brush rabbit (*Sylvilagus bachmani*), and European brown hare (*Lepus europaeus*) were provided by the CIBIO Lagomorpha tissue collection. Genomic DNA (gDNA) was extracted using the EasySpin Genomic DNA Minipreps Tissue Kit (Citomed, Torun, Poland) according to the manufacturer's instructions. Total RNA was extracted by using the RNeasy Mini Kit also according to the manufacturer's instructions (Qiagen, Hilden, Germany) from one specimen of European rabbit and one of European brown hares. Complementary DNA (cDNA) was synthesized using oligo(dT) as primers and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The European rabbit and American pika IL17A sequences were retrieved from public databases (accession numbers are given in bold in Figure 1). PCR amplification was performed with the Multiplex PCR Kit (Qiagen) by using two pairs of primers designed according to the retrieved sequences (for genomic DNA F1-CGTCCAACCTCAGTTGATC + R1-CACTGTACCATCTATCCTGC and F2-CCTTCATTTACTCCCATTCG + R2-CATCCATCACATGGCCTAA; for cDNA the combination of primers F1 + R2 was used). Sequencing was performed on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) and PCR products were sequenced in both directions. The sequences obtained were submitted to GenBank with the following accession numbers: KU163611–KU163619.

Haplotype phases of the sequences obtained were reconstructed with the program PHASE, built into the software DnaSP [38]. Multiple Sequence Comparison by Log-Expectation (MUSCLE; http://www.ebi.ac.uk/) [39] was used for sequence alignment. The putative N-glycosylation sites were predicted using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) [40].

The number of nucleotide differences per site between sequences was estimated in MEGA6 [41] with the following options: bootstrap method (1000 replicates), p-distance as model, and pairwise deletion for gaps/missing data treatment. A Maximum Likelihood approach was used to estimate the phylogenetic relationships between the IL17A nucleotide sequences by using MEGA6; the best-fit nucleotide substitution model was predicted by the same software and 1000 bootstrap replicates were used.

The secondary structure of IL17A was predicted using PsiPred (http://bioinf.cs.ucl.ac.uk/psipred/) [42, 43] and DiAminoacid Neural Network Application (DiANNA) (http://clavius.bc.edu/~clotelab/DiANNA/) [44]. Both methods predict protein cysteines that create disulfide bonds, but while PsiPred uses Position Specific Iterated-BLAST (PSI-BLAST) to obtain evolutionary information used to predict the secondary structure of the query protein, DiANNA is a neural network that recognizes cysteines in an oxidized state (sulfur covalently bonded) distinguishing them from those in a reduced state.

## 3. Results and Discussion

In this study we amplified and sequenced the IL17A gene for four leporids species (European rabbit, European brown hare, brush rabbit, and pygmy rabbit). For European rabbit (*O. c. cuniculus*) and European brown hare, both genomic and cDNA sequences were identical and only one of the sequences is presented; however both sequences have been assigned different accession numbers. These sequences were further compared to sequences of IL17A from another lagomorph, American pika (*Ochotona princeps*), and from representatives of the most relevant mammalian groups (e.g., Artiodactyla, carnivores, Chiroptera, Primates, rodents, etc.) available in online databases. In the European rabbit, IL17A is located in the forward strand of chromosome 12 and has a similar structure to other mammals with three coding exons. The IL17A cDNA sequence obtained in this work for *Lepus europaeus* showed a similar structure.

In humans, IL17A codes for a protein with 155 amino acids (aa) and has the ability to bind with high affinity to IL17RA and IL17RC [6, 45, 46]. The interaction between interleukins and their receptors is crucial for their function and signaling and any changes in the amino acid composition may induce alterations in the protein conformation. In humans and rodents these interactions sites are described [6, 45] and include Leu52, Ile54, Ser61, Ser70-Tyr72, Arg75, Arg84, Arg88-Val94, Trp96, Leu103, His114, His115, Asn117,

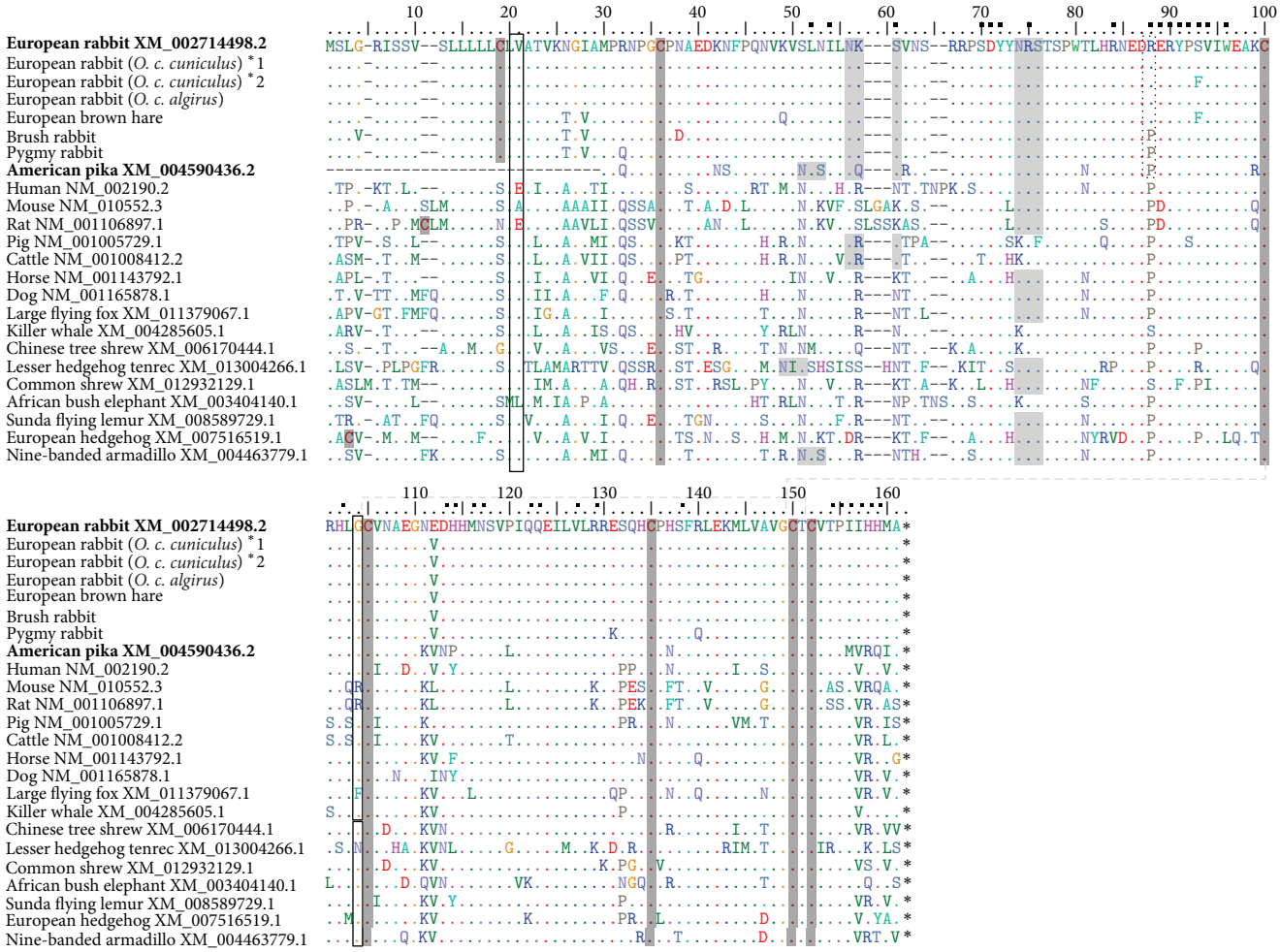


Figure 1: Alignment of IL17A for several mammalian species. GenBank and Ensembl accession numbers are indicated in bold for the retrieved sequences. Positively selected amino acids are boxed (according to [19]). N-glycosylation sites are shaded in light grey and cysteine residues are shaded in dark grey. A black dashed box represents the Agr>Pro mutation between leporids. ∗ represents stop codons; – represents indels; ▪ above the numbering represents the sites important for IL17A-IL17Ra interaction. *1 and *2 represent different alleles. Numbering is according to the European rabbit sequence (GenBank accession number XM_002714498.2) and the signal peptide and indels were included in the numbering. Disulfide bonds between side chain cysteines are represented by a light grey dashed line.

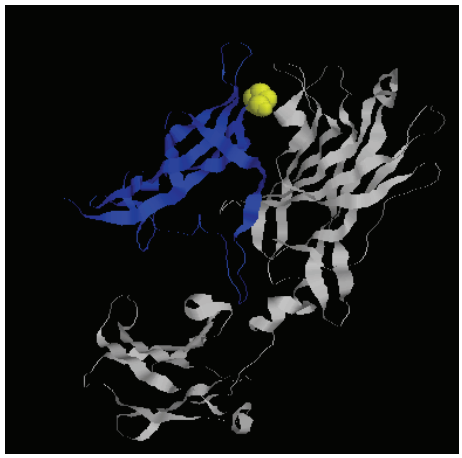

Figure 2: 3D structures of the IL17A-IL17RA complex. IL17A appears in blue while IL17RA appears in grey. Marked in yellow is the 88Arg>Pro mutation described for leporids.

Ser118, Gln122-Glu124, Leu128, Arg130, Phe139, and Pro155-Met160 (Figure 1). In leporids, IL17A codes for a protein with 153 aa and we observed that the sites that likely interact with the receptors are quite conserved. Indeed, from the thirty-three amino acids involved in the linkage between IL17A and IL17RA, eighteen are conserved: twelve are maintained between mammals and the other six, despite being different, do not alter the charge or the polarity. For the remaining fifteen amino acids, only three are differently charged, seven have distinct polarity, and five have both different charge and polarity (Table 1). Between leporids these sites are highly conserved, but a mutation was observed that is located in the external coil of the IL17A in a site where this protein interacts with IL17RA (Figure 2). This mutation, 88Arg>Pro, occurs in the European rabbit and in the European brown hare, while in brush rabbit and the American pika the amino acid present is a proline as in most mammals. Some studies showed that Arg>Pro mutations have crucial effects in the

Table 1: Characterization of the IL17A amino acids differences in the sites important for binding to IL17RA.

| Amino acid position | Amino acids | | | | |
|---|---|---|---|---|---|
| | Leporids | | | | Other mammals |
| | European rabbit | European brown hare | Brush rabbit | Pygmy rabbit | |
| 52 | | L$^{\#}$ | | | M$^{\#}$, S$^{*}$ |
| 54 | | I$^{\#}$ | | | V$^{\#}$, S$^{*}$, T$^{*}$ |
| 61 | | S$^{*}$ | | | N$^{*}$, K$^{*+}$ |
| 70 | | S$^{*}$ | | | T$^{*}$, L$^{\#}$ |
| 71 | | | D$^{*-}$ | | |
| 72 | | | Y$^{*}$ | | |
| 75 | | | R$^{*+}$ | | |
| 84 | | R$^{*+}$ | | | P$^{\#}$, V$^{\#}$ |
| 88 | R$^{*+}$ | | | P$^{\#}$ | P$^{\#}$, S$^{*}$ |
| 89 | | E$^{*-}$ | | | D$^{*-}$ |
| 90 | | | R$^{*+}$ | | |
| 91 | | Y$^{*}$ | | | F$^{\#}$ |
| 92 | | P$^{\#}$ | | | S$^{*}$ |
| 93 | S$^{*}$, F$^{\#}$ | F$^{\#}$ | | S$^{*}$ | F$^{\#}$, P$^{\#}$, R$^{*+}$ |
| 94 | | | V$^{\#}$ | | |
| 96 | | W$^{\#}$ | | | L$^{\#}$ |
| 103 | | L$^{\#}$ | | | Q$^{*}$, S$^{*}$, M$^{\#}$ |
| 114 | | H$^{*+}$ | | | P$^{\#}$, Y$^{*}$, F$^{\#}$, L$^{\#}$ |
| 115 | | | H$^{*+}$ | | |
| 117 | | | N$^{*}$ | | |
| 118 | | | S$^{*}$ | | |
| 122 | | Q$^{*}$ | | | K$^{*+}$ |
| 123 | | | Q$^{*}$ | | |
| 124 | | | E$^{*-}$ | | |
| 128 | | | L$^{\#}$ | | |
| 130 | | R$^{*+}$ | | | K$^{*+}$ |
| 139 | | | F$^{\#}$ | | |
| 155 | | P$^{\#}$ | | | S$^{*}$ |
| 156 | | I$^{\#}$ | | | M$^{\#}$ |
| 157 | | I$^{\#}$ | | | V$^{\#}$ |
| 158 | | H$^{*+}$ | | | S$^{*}$, R$^{*+}$, Q$^{*}$, K$^{*+}$ |
| 159 | | H$^{*+}$ | | | Q$^{*}$, Y$^{*}$, T$^{*}$ |
| 160 | | M$^{\#}$ | | | I$^{\#}$, V$^{\#}$, A, L$^{\#}$ |

The amino acid polarity ($^{*}$hydrophilic; $^{\#}$hydrophobic) and charge ($^{+}$positive; $^{-}$negative) are properly annotated. The amino acid present in the human IL17A sequence is underlined. Numbering is according to the European rabbit IL17A sequence.

protein function [47–49]. Indeed, the 332Arg>Pro mutation in human Trim5$\alpha$ restricts infection by HIV-1 (Human Immunodeficiency Virus-1) [49] while the 132Arg>Pro mutation in the helicase protein of coronavirus infectious bronchitis virus was lethal to infectivity *in vitro* [48]. Additionally, this mutation alters the physiochemical properties of the amino acid by changing from a basic polar and positively charged arginine to a nonpolar and neutral proline.

Disulfide bounds and N-glycosylation sites (Asn-X-Ser/Thr/Cys motifs where X can be any amino acid except proline) are important for the protein structure, stability, and function [50–52]. Disulphide bounds occur between cysteines side chains and these linkages are also important for protein protection [53]. In human and rodents, IL17A has a cysteine knot fold characterized by two sets of paired $\beta$-strands (1/2 and 3/4) interconnected by two disulfide bounds between strands 2 and 4 linked between four conserved cysteines (Cys100–Cys150 and Cys105–Cys152) [45, 54, 55]. In addition to these cysteines two other cysteines are common to all mammals, Cys36 and Cys135. For the European rabbit, the PsiPred predicted secondary structure and the DiANNA predicted disulfide bonds results are in agreement with those obtained and described for human and rodents [45, 54]. An extra linkage is also predicted between Cys31 and Cys135. When compared to other mammals, there is an extra cysteine (Cys19) in leporids located in the signal peptide. The rat and

Table 2: IL17A nucleotide distances (the lowest values are in bold and the highest values are underlined).

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| (1) European_rabbit (*O. c. cuniculus*) | — | | | | | | | | |
| (2) European_rabbit (*O. c. algirus*) | 0.002 | — | | | | | | | |
| (3) European brown hare | **0.011** | 0.013 | — | | | | | | |
| (4) Brush rabbit | **0.011** | 0.013 | 0.013 | — | | | | | |
| (5) Pygmy rabbit | 0.024 | 0.026 | 0.026 | 0.022 | — | | | | |
| (6) American pika XM_004590436.2 | 0.112 | 0.115 | 0.120 | 0.112 | 0.112 | — | | | |
| (7) Human NM_002190.2 | **0.169** | 0.171 | 0.175 | 0.171 | 0.173 | 0.159 | — | | |
| (8) Mouse NM_010552.3 | 0.251 | 0.251 | 0.249 | 0.249 | 0.245 | 0.240 | 0.236 | — | |
| (9) Rat NM_001106897.1 | 0.260 | 0.260 | 0.258 | 0.258 | 0.253 | 0.232 | 0.236 | 0.111 | — |

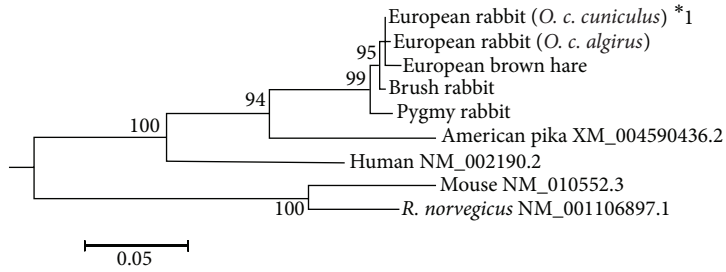


Figure 3: Maximum Likelihood (ML) tree of the IL17A nucleotide sequences. Only bootstrap values ≥ 94% are shown. In order to facilitate visualization, only one sequence/allele of each species was used.

the European hedgehog also have an extra cysteine located in different sites of the signal peptide (Cys11 and Cys3, resp.). Given that the signal peptide is cleaved in order for the protein to become active, this extra cysteine should not have an impact on the IL17A structure.

N-glycosylation is a crucial factor for the modulation of protein activity; therefore, alteration on these sites may interfere with recognition of targets, including receptors, and consequently affects the biological activity of the proteins and also their ability to diffuse through the organism [56, 57]. Human IL17A is N-glycosylated at Asn68. Detection of putative N-glycosylation sites indicated that this N-glycosylation site is present in the majority of mammals, including rodents and lagomorphs. Other putative N-glycosylation sites were detected and include Asn56 in lagomorphs, pig, and cattle, Asn51 in American pika and armadillo, and Asn49 in the lesser hedgehog tenrec. The killer whale and the African bush elephant have no putative N-glycosylation sites. The implications of the absence/presence of N-glycosylation sites in IL17A are unknown; however some studies indicate that presence/removal of glycans in some proteins do not alter their folding or function, although a decrease in the protein dynamics is observed [50, 52, 57].

Comparison of the nucleotide sequences (Table 2) indicated that, in leporids, the European rabbit and the European brown hare IL17A sequences are the least divergent (0.011) while the European rabbit and the pygmy rabbit IL17 sequences are the most divergent (0.026). Between the European rabbit and American pika, the genetic diversity obtained was 0.112–0.115. For the remaining mammals the highest divergence occurs for the lesser hedgehog tenrec (0.312) and the lowest divergence for the flying lemur (0.145). The comparison of the nucleotide diversity of several interleukins in the European rabbit suggested that it could represent a better animal model for research [34]. For IL17A, similar results were obtained, with the human sequence being more closely related to the European rabbit (0.169) than to mouse or rat IL17A sequences (0.236). This is further supported by a Maximum Likelihood tree inferred for IL17A mammalian sequences (Figure 3).

## 4. Conclusions

In the present study we sequenced and characterized IL17A for four leporids. Overall, the genomic organization, the location of the cysteine residues, and the presence of N-glycosylation sites are highly conserved in leporids. Nevertheless, a single mutation was detected within the interaction site with IL17RA which may induce crucial changes in IL17A structure, function, stability, signaling, and conformation. Further functional and structural studies should be performed to fully understand the impact of this specific mutation. The lowest divergence between the European rabbit and human IL17A sequences reinforces the hypothesis that the European rabbit might be a more suitable animal model for studies in the human innate immunity.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review Article*

# Interleukin-17 Could Promote Breast Cancer Progression at Several Stages of the Disease

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Metastatic disease accounts for more than 90% of deaths from breast cancer. Yet the factors that trigger metastasis, often years after primary tumor removal, are not understood well. Recently the proinflammatory cytokine interleukin- (IL-) 17 family has been associated with poor prognosis in breast cancer. Here we review current literature on the pathogenic mechanisms driven by IL-17 during breast cancer progression and connect these findings to metastasis. These include (1) direct effects of IL-17 on tumor cells promoting tumor cell survival and invasiveness, (2) regulation of tumor angiogenesis, and (3) interaction with myeloid derived suppressor cells (MDSCs) to inhibit antitumor immune response and collaborate at the distant metastatic site. Furthermore, IL-17 might also be a culprit in bone destruction caused by late stage bone metastasis. Interestingly, in addition to these potential prometastasis functions, there is also evidence for an opposite, antitumor role of IL-17 during cancer therapies. We hypothesize that these contradictory roles may be due to chronic, imbalanced versus acute transient nature of the immune reactions, as well as differences in the cells that interact with IL-17$^+$ cells under different circumstances.

## 1. Introduction

Although great progress has been made in breast cancer therapy, the treatments are often unsuccessful once metastases to vital organs occur. After removal of the primary breast tumor, the disease can be dormant over several years. As of today we are unable to predict reliably if or when the cancer will progress. Therefore it is important to elucidate pathogenic factors involved. IL-17 is a proinflammatory cytokine family with a documented association with poor prognosis in breast cancer. In our review, we discuss recent findings on IL-17-driven mechanisms that promote breast cancer progression. We also attempt to discern the context in which IL-17 has the opposite role and mediates antitumor reactions during cancer therapies.

## 2. IL-17 Family

The IL-17 family is comprised of 6 cytokines, including IL-17A to IL-17F. Among them, IL-17A and IL-17F share the highest sequence homology and have similar biological functions. Both bind to IL-17RA and IL-17RC chains [1]. IL-17 is primarily secreted by T helper (Th) 17 cells and innate lymphocytes ($\gamma\delta$ T cells, natural killer (NK) cells, and innate lymphoid cells) [2]. The prototypic IL-17A induces a signaling cascade in its target cells by binding to its receptor IL-17RA/RC. These receptor chains are broadly expressed in many cell types accounting for the pleiotropic effects of IL-17A on epithelial cells (including transformed tumor cells), endothelial cells, osteoblasts, fibroblasts, and various myeloid cells [3]. Downstream of the IL-17 receptor, NF$\kappa$B activator 1 (Act1) and TNF

receptor associated factor (TRAF) 4 are important adaptor proteins that transmit the intracellular signal cascade to activate transcription factors such as nuclear factor kappa B (NF$\kappa$B) and activator protein (AP) 1 in cell and tissue-specific manner. In turn these transcription factors regulate a wide array of target genes.

IL-17 induces expression of certain chemokines and vascular endothelial growth factor (VEGF) that lead to the recruitment of specific subsets of immune cells to the site of inflammation and the induction of angiogenesis, respectively. Loss of control in IL-17 signaling is a common pathogenic mechanism in chronic inflammatory diseases/autoimmune diseases such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease [4]. In rheumatoid arthritis, IL-17 induces the expression of receptor activator of nuclear factor kappa B ligand (RANKL) in osteoblasts which leads to the activation of osteoclasts. In addition among other IL-17 target genes, matrix metalloproteinases (MMPs) and IL-6 play important roles in bone resorption and pathogenesis of the disease [4]. Although IL-17A/F is typically associated with destructive tissue damage in autoimmune disease and pathogen infections [5–7], it is also involved in protective immunity. For example, IL-17A acts as a potent inducer of T cell mediated immune responses by activating and recruiting dendritic cells (DCs), monocytes, and neutrophils in various tissues [2]. The recruitment and modulation of neutrophils are critical in host defense [8, 9]. However, beyond certain pathogen infection, IL-17 has an almost ubiquitous role as coordinator of neutrophil-dominated inflammation. Furthermore, IL-17 stimulates dendritic cells (DCs) to produce IL-12, while inhibiting IL-10 to augment Th1 differentiation [10, 11].

## 3. Role of IL-17 in Breast Cancer

*3.1. Presence of IL-17 in Breast Cancer Patients.* Studies on tumor infiltrating lymphocytes (TILs) of breast cancer patients revealed the presence of Th17 cells and pointed out the role of tumor microenvironmental factors including chemokine (C-C motif) ligand 5 and monocyte chemoattractant protein 1 in generating and attracting these cells into the tumors [12]. IL-17$^+$ immune cells (either lymphocytes or macrophages) were also detected by Cochaud et al. in TILs of 8 of 40 breast cancer patients [13]. Furthermore, while analyzing 207 breast cancer specimens by IL-17 immunohistochemistry, Chen et al. found a correlation between large numbers of IL-17$^+$ cells and high histological grade of the tumors, triple negative molecular subtype, and shorter disease-free survival [14]. Another study showed that three single nucleotide polymorphisms (SNPs) in the IL-17A gene are associated with increased breast cancer risk in a Chinese patient population [15]. Studying invasive ductal carcinoma (IDC) of the breast, Benevides et al. determined an association between coordinately upregulated $T_{reg}$ and Th17 cells and aggressiveness of the disease [16, 17]. These and other findings discussed below in Sections 3.2 to 3.5 are in support of IL-17 as a marker of poor prognosis and risk in breast cancer. However, it was also recognized that the blood of metastatic breast cancer patients contains a higher frequency of $T_{reg}$ cells associated with risk. And in the HER2$^+$ subtype of breast cancer very low levels of Th17 cells were observed with an inverse relationship between $T_{reg}$ cells and Th17 cells. Anti-HER2 (trastuzumab) treatment increased Th17 cell numbers to restore a balance between $T_{reg}$ and Th17 cells [18]. Similarly, other IL-17 family members have been correlated to pro- and antibreast cancer activities [19–25]. Therefore it is necessary to investigate the detailed mechanisms to decipher IL-17's multiple roles in breast cancer.

*3.2. Direct Effects of IL-17 on Breast Cancer Cells.* Due to the high frequency of expression of IL-17 receptor chains on tumor cells, IL-17 family members can have direct effects on tumor cells. In a mouse breast cancer model, tumor-induced transforming growth factor beta (TGF-$\beta$) induced CD8 T cells to produce IL-17. *In vitro*, IL-17 in turn suppressed apoptosis of 4T1 mouse mammary carcinoma, CT26 mouse colon carcinoma, and MDA-MB231 human breast carcinoma cell lines. Furthermore, IL-17R knockdown in 4T1 breast cancer cells enhanced apoptosis and decreased tumor growth *in vivo* [26]. Treatment with endothelin-1 receptor dual antagonist decreased IL-17A levels and caused slower 4T1 tumor growth [27]. In another study, IL-17A via activation of tumor progression locus 2 (TPL2) induced mitogen activated protein kinase (MAPK), c-jun N-terminal kinase (JNK), and signal transducer and activator of transcription (STAT) 3 signaling and cellular transformation in JB6 CI41 mouse epidermal cells and promoted MCF7 (human breast cancer cell line) tumorigenicity [28]. MDA-MB231 cells express CD40 and interact with CD40L on activated T cells. This interaction increased TGF-$\beta$ production and consequently induced IL-17 expression, which enhanced MDA-MB231 proliferation through STAT3 activation [29]. Furthermore, IL-17A and IL-17E (IL-25) were involved in proliferation and survival of human breast cancer cell lines T47D and MCF7 as well as primary breast cancer biopsy cells IJG1731 and thereby promoted their resistance to the antimitotic chemotherapy agent docetaxel [19]. Interestingly, under some circumstances, IL-17E (IL-25) produced by nonmalignant mammary epithelial cells also displays antitumor function by targeting adjacent tumor cells, which express high levels of IL-25R and inducing apoptosis [20]. Similar to TNF-$\alpha$ receptor, a death domain (DD) portion in the IL-25R may be linked to apoptosis inducing adaptor molecules FAS-associated protein with death domain (FADD) and TNF-R1-associated death domain protein (TRADD) under certain conditions. These conclusions are also supported by [21]. IL-17 also plays a role in tumor invasion. For example, cells in the peritumoral area expressed IL-17 in 8 of 19 breast cancer patients studied. *In vitro*, the human breast cancer cell lines MDA-MB231 and MDA-MB435 were examined in a matrigel invasion assay by plating them on matrigel invasion chambers. Added IL-17 greatly induced invasion of these cells into matrigel [30].

*3.3. IL-17 in Angiogenesis.* It has been found that the tumor-promoting IL-17: tumor angiogenesis axis causes resistance to anti-VEGF therapy [31]. Tumor infiltrating Th17 cells and IL-17 induced the expression of granulocyte-colony stimulating

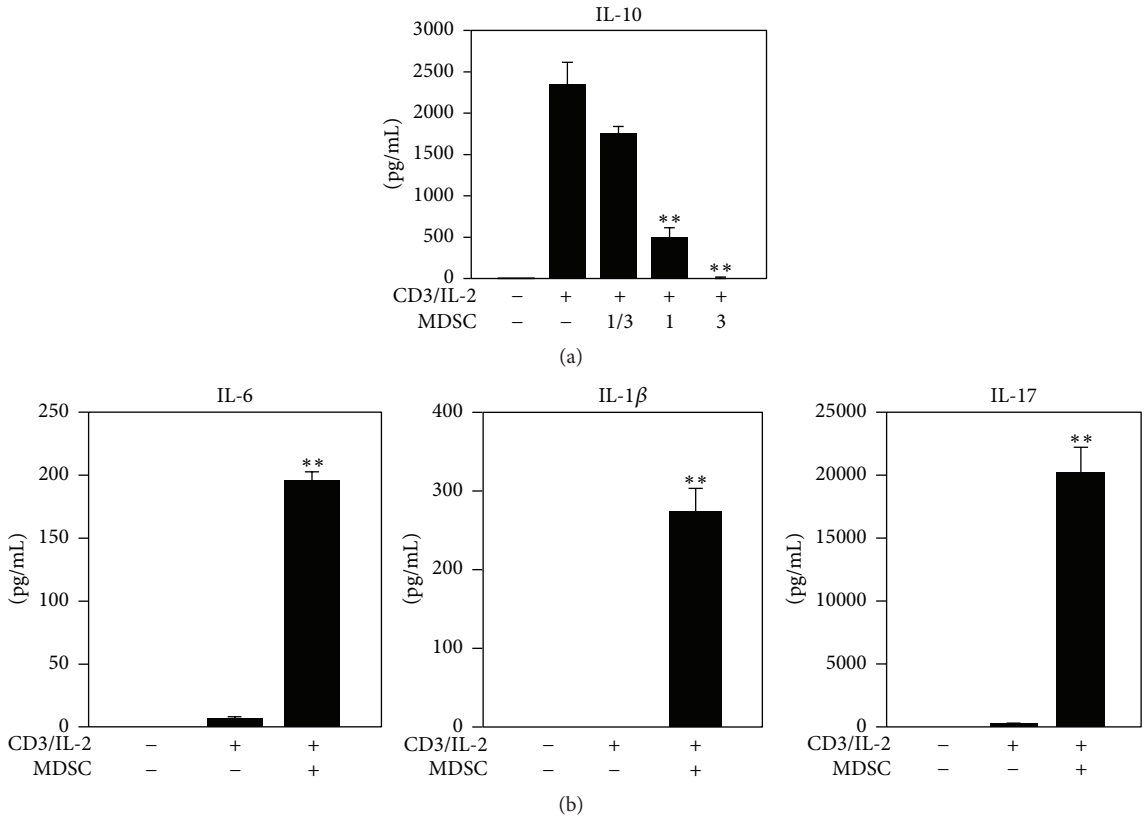


Figure 1: The effects of MDSCs on cytokine expression of T cells in coculture. Bio-Plex assays of indicated cytokines are shown. MDSCs and T cells were admixed at three ratios: 0.33 : 1, 1 : 1, and 3 : 1 (a) or 1 : 1 ratio (b) for five days. (a) Inhibited by MDSC coculture. (b) Cytokines of Th17 formation axis. $n = 4$. $^{**}p < 0.01$.

factor (G-CSF) leading to immature myeloid cell recruitment into the tumor microenvironment and anti-VEGF therapy resistance through the production of proangiogenic Bv8 by these myeloid cells [31]. In patients with invasive ductal carcinoma (IDC), tumor aggressiveness was reported to be enhanced by IL-17 via induction of angiogenic factors, such as chemokine CXCL8, MMP-2, MMP-9, and VEGF [16, 32]. Similarly, injection of recombinant IL-17 in the murine breast cancer model 4T1 was shown to increase microvascular density, a parameter for tumor angiogenesis [33].

*3.4. IL-17 and Tumor Escape from Host Immunosurveillance.* In an attempt to produce a specific antihuman cancer vaccine by combining one of the most specific cancer-associated structures, the Tn antigen (alpha-GalNAC-O-Ser/Thr) with mucin (MUC) 6, it was noted that Tn strongly diminished immunogenicity compared to MUC6 alone via a partial abrogation of Th1 response and promotion of IL-17 responses [34]. Other studies further revealed the underlying mechanism of IL-17's immunosuppressive function. In polyoma middle T- (PyMT-) induced breast cancer models, IL-17 induced the secretion of CXCL1 and CXCL5 from mammary carcinoma cells and increased the suppressive function of myeloid derived suppressor cells (MDSCs) on T cells by upregulating arginase (Arg), indoleamine 2,3-dioxygenase (IDO), and prostaglandin- endoperoxide synthase- (COX-) 2 expression [35]. The connection between breast cancer cells, IL-17, and MDSCs was further elucidated by Coffelt et al. [36] while studying a different model of breast cancer (tissue-specific cadherin (Cdh) 1/p53 double knockout). The tumor-derived IL-1$\beta$ induced IL-17 expression by $\gamma\delta$ T cells, which resulted in G-CSF-dependent expansion and polarization of neutrophils into cells with MDSC characteristics. These polarized neutrophils further inhibited cytotoxic CD8 T cells, which otherwise limited metastasis [36]. Thus, the collaboration of IL-17$^{+}\gamma\delta$ T cells with MDSCs is important for tumor escape from host immune reactions and metastasis formation in distant organs. In an attempt to understand the interactions of T cells and MDSCs in breast cancer models (Welte et al., submitted), we confirmed the immunosuppressive activities of breast tumor-induced MDSCs (Welte et al., submitted) in the *in vitro* coculture assay. MDSCs isolated from blood of breast tumor bearing mice were admixed with naïve splenic T cells. Cocultures were stimulated with combination of plate-bound anti-CD3 and IL-2 and T cell proliferation was assessed by the CFSE-labeling method. Furthermore, cytokine secretion was compared to that of T cells activated in the absence of MDSCs. It was noted that the regulatory cytokine IL-10 was diminished in the presence of MDSCs (Figure 1(a)), suggesting an IL-10- independent inhibition on T cell proliferation. Interestingly, T cells cocultured with large numbers of MDSCs produced higher levels of IL-6,

IL-1$\beta$, and IL-17, which indicates MDSCs promote Th17 cell differentiation (Figure 1(b)). In addition to Th17 cells, $\gamma\delta$ T cells serve as one main resource for IL-17 [36, 37]. In breast cancer MDSCs promoted the tumor-infiltration of V$\gamma$4$^+$CCR6$^+$ T cells (Welte et al., unpublished), subsets phenotypically related to IL-17 producers in previous studies [38]. Overall, these findings suggest a complex reciprocal interaction between MDSCs and IL-17 producing T cells.

*3.5. IL-17 and Breast Cancer Metastasis.* In addition to the enhancement of metastasis via suppression of antitumor immunity, IL-17 may also promote metastasis through its inflammatory activities. Eiró et al. found a link between intratumoral MMP-11$^+$ mononuclear inflammatory cells and metastasis [39, 40]. IL-17 was among the proinflammatory factors associated with MMP-11$^+$ cells, although its function in these cells still needs to be demonstrated. A very recent report analyzed the pathogenesis mechanism of metastasis in invasive ductal carcinoma (IDA) and found that IL-17A affected different steps of metastasis such as migration of tumorigenic neutrophils and tumor cells to distant metastatic sites and production of IL-6 and CCL20 in metastatic tumor cells [17]. A previous study also found that the metastasis promoting interaction of human bone marrow-derived stem cells with breast cancer tumor cells could be mediated by IL-17B/IL-17BR signaling [41]. The role of IL-17 in breast cancer metastasis to bone is also demonstrated by studies in Mukherjee's lab. High levels of IL-17A are associated with autoimmune arthritis. Mukherjee's group generated arthritic mouse models and found enhanced bone and lung metastasis of breast cancer line 4T1 and PyMT mouse breast cancer model upon arthritis induction. Combination therapy of anti-IL-17 and anti-inflammatory celecoxib abrogated metastasis [42–44]. A severe side effect of cancer treatment with aromatase inhibitor is arthralgia mediated by IL-17 [45–47]. Based on these findings, it is an attractive hypothesis that bone-destructive events in autoimmune arthritis and at late stage bone metastasis could both be caused by an IL-17-dependent mechanism.

## 4. Summary and Future Perspective

Since the discoveries that identified IL-17 as a central proinflammatory mediator, it has garnered a lot of attention and our knowledge on IL-17 is expanding quickly. In the biology of many tumors, IL-17 cytokines have a protumor role either directly on tumor cells or indirectly by having detrimental effects on the patient's antitumor response and by causing microenvironmental changes that worsen the disease towards more invasive and metastatic phenotype. Furthermore, IL-17-induced changes in rheumatoid arthritis resemble the bone destruction phenotypes observed at late stage bone metastasis. Therefore, treatment with anti-IL-17 holds a promise as anticancer therapy and in ameliorating the effects of bone metastasis. One concern in this treatment is that it could make patients more vulnerable to infections normally held in check by IL-17. Antitumor activity of interferon (IFN) $\gamma$ is well established. In comparison, it is less clear whether IL-17 could have beneficial effects in the immunotherapy of cancer. Recent studies by Zitvogel and colleagues described how certain chemotherapy drugs such as doxorubicin induce "immunogenic" tumor cell death [48, 49]. The immune response mounted to the dying tumor cells is critical for the efficacy of these chemotherapeutic drugs in preventing tumor progression. IL-17 expressed by $\gamma\delta$ T17 cells is important for initiating this immune reaction and eventually leads to activation of protective tumor-specific IFN$\gamma$ expressing CD8 T cells. The challenge in future studies will be to understand how to restrict IL-17's role to the promotion of CD8/IFN$\gamma$ antitumor activity without unleashing its other pathogenic protumor functions described above.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review Article*

# Advances in T Helper 17 Cell Biology: Pathogenic Role and Potential Therapy in Multiple Sclerosis

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The discovery of the T helper (Th) 17 lineage, involved in the protection against fungal and extracellular bacterial infections, has profoundly revolutionized our current understanding of T cell-mediated responses in autoimmune diseases, including multiple sclerosis (MS). Indeed, recent data demonstrate the pathogenic role of Th17 cells in autoimmune disorders. In particular, studies in MS and in its animal model (EAE, experimental autoimmune encephalomyelitis) have revealed a crucial role of Th17 cells in the pathogenesis of autoimmune demyelinating diseases in both mice and humans. Over the past years, several important aspects concerning Th17 cells have been elucidated, such as the factors which promote or inhibit their differentiation and the effector cytokines which mediate their responses. The identification of the features endowing Th17 cells with high pathogenicity in MS is of particular interest, and discoveries in Th17 cell biology and function could lead to the design of new strategies aimed at modulating the immune response in MS. Here, we will discuss recent advances in this field, with particular focus on the mechanisms conferring pathogenicity in MS and their potential modulation.

## 1. Introduction

Differentiation of naive $CD4^+$ T cells into T helper (Th) cells with diverse effector functions is crucial for the establishment of an adaptive immune response. Until recently, only two major cell subsets, Th1 and Th2, were used to describe the different adaptive immune responses established to eradicate pathogens [1–3]. Th1 cells induce cell mediated inflammatory responses against intracellular bacteria [4–7], while Th2 cells activate a protective response against helminth infection [8]. However, persistent or uncontrolled effector T cell responses are also associated with pathological states and tissue damage: an excessive Th2 cell response is responsible for atopic diseases, such as asthma [9], and an abnormal Th1 cell response can mediate chronic inflammation and is involved in several autoimmune diseases [10, 11]. In 1998 the discovery of $CD4^+$ T cells producing IL-17 [12] unveiled the presence of another subset of Th cells, the Th17 subset, distinct from Th1 and Th2 [13, 14], and its discovery has helped the understanding of immune responses unexplained by the Th1/Th2 paradigm, such as the response against fungi like *Candida albicans* [15] and extracellular bacteria such as *Pseudomonas aeruginosa* [16], *Klebsiella pneumoniae* [17], *Streptococcus pneumoniae* [18], and *Staphylococcus aureus* [19], and the development of autoimmune disorders, such as multiple sclerosis (MS), Crohn's disease, psoriasis, and rheumatoid arthritis. The pathogenic role of Th17 cells in autoimmune diseases is supported by both human studies and experiments performed in animal models. Indeed, IL-17A is highly expressed in the central nervous system (CNS) lesions and in the blood and cerebrospinal fluid (CSF) of patients with MS [20–24], in the colonic mucosa of patients with ulcerative colitis or Crohn's disease [25, 26], in the psoriatic skin [27, 28], and in the synovial tissues from rheumatoid arthritis patients [29]. Studies in murine models such as experimental autoimmune encephalomyelitis (EAE) [30], trinitrobenzene sulfuric acid- (TNBS-) induced colitis [31], and antigen or collagen-induced arthritis [32] reveal that the IL-17 pathway plays a pathogenic role in autoimmune disorders. Finally, the concept that Th17 cells are responsible for driving autoimmune inflammation was finally established when EAE, the mouse model of MS, was shown to be induced by passive transfer of IL-17-producing myelin reactive CD4 T cells [33].

In this review we discuss our current understanding of the Th17 lineage, focusing on the factors regulating their differentiation, their typical features, their pathological roles in MS, and the potential modulation of their response for therapeutic approaches.

## 2. Cytokine Production by Th17 Cells

IL-17 is the cytokine produced specifically by Th17 cells. IL-17A (commonly referred to as IL-17) is part of a cytokine family including IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F [34]. All members of the family show some conserved regions: IL-17A and IL-17F (the only cytokines of this family produced by Th17 cells) are the most similar to a 55% homology and exert similar functions [35]; IL-25 has the sequence with lowest similarity to IL-17A (only 16%) and plays distinct roles in immunity, mainly regulating the Th2 response against helminthic parasites and allergic inflammation [36–38]. IL-17B, IL-17C, and IL-17D have been shown to induce the production of proinflammatory cytokines, but their biological function is largely unknown [39–42]. Recent studies by three different groups have highlighted the function of IL-17C in mucosal immunity and in autoimmune responses [43–45].

Within the IL-17 family of cytokines, the biological function and regulation of IL-17A and IL-17F are the best understood. Both are produced by Th17 cells and can also act as heterodimers [46]. The effective signalling of IL-17A and IL-17F requires the IL-17 receptor (IL-17R), a heteromeric complex consisting of IL-17RA and IL-17RC [47]. Although both receptors are extensively expressed in different tissues and cell types [48–50] functional studies have focused mainly on epithelial cells. Both IL-17A and IL-17F induce epithelial cells to produce granulopoietic colony stimulating factor (G-CSF), stem cell factors that regulate granulopoiesis, and CXC chemokines (CXCL1, CXCL2, CXCL5, and CXCL8) responsible for neutrophil recruitment [51–53]. IL-17A increases the expression of mucins such as MUC5AC and MUC5B in primary human bronchial epithelial cells *in vitro* [54]. In addition, IL-17A also induces the expression of human beta defensin-2 [55] and CCL20 in lung epithelial cells [56]. This cooperative induction of neutrophil recruitment and antimicrobial-peptide production improves epithelial-barrier integrity and may be critical for mucosal host defense against extracellular bacteria and fungi.

Moreover, several studies have documented the role of IL-17 in regulating antibody generation by plasma cells and germinal center and ectopic inducible bronchus-associated lymphoid tissue (iBALT) formation [57–59].

Altogether, these functions of IL-17 make Th17 cells potentially relevant for vaccine development: in experimental models, Th17 cells are effective in providing vaccination-induced immunity against a range of pathogens, and the identification of Th17-specific antigens for common prevalent pathogens could help formulate a serotype-independent effective vaccination strategy [60].

However, the Th17 cytokine profile is not restricted to IL-17 production. In fact, Th17 cells are potentially producers of a broad array of cytokines, including IL-21, IL-22, IL-26, IL-6, TNF, and in certain conditions also GM-CSF, IL-9, IL-10, and IFN-$\gamma$ [61–70]. Because each of these cytokines has different functions, they collectively affect the global outcome of the Th17 response and generate different Th17 responses (Figure 1).

## 3. Stimuli Required for Th17 Cell Differentiation

Human *in vitro* studies reveal that dendritic cells (DCs) exposed to bacteria or fungi, but not viruses, elicit strong Th17 responses [62, 80]. Among pathogen-associated molecular patterns (PAMPs), peptidoglycan (TLR2 agonist) [62, 80] and its product muramyl dipeptide (NOD2 agonist) [80] are the most potent stimuli for the production of IL-17. In mice, an alternative pattern-recognition pathway activated by fungal infection has also been described, involving the engagement of a C-type lectin receptor, dectin-1, by fungal $\beta$-glucans components of zymosan [81]. Moreover, DCs stimulated with the pure $\beta$-glucan curdlan (dectin-1 agonist) prime T cells for a much higher IL-17 production than does IFN-$\gamma$ [82].

Stimulation of DC by pathogen-derived structures induces the production of cytokines which ultimately drive Th17 cell differentiation [83, 84]; these cytokines have been identified by using antigen presenting cell- (APC-) free models of T cell polarization. In mice, several reports have shown that transforming growth factor-$\beta$ (TGF-$\beta$) and IL-6 [85–87] have a critical role in inducing Th17 differentiation. Although not necessary, other proinflammatory cytokines such as IL-1$\beta$, TNF, and IL-21 can enhance this differentiation [87, 88]. Finally, IL-23 induces expansion of murine Th17 cells both *in vitro* [87] and *in vivo* [89] (Figure 1).

Studies on human Th17 cell differentiation have reported a critical role for IL-1$\beta$, IL-6, IL-23, and TGF-$\beta$ [65, 66]. TGF-$\beta$ was also shown to act in synergy with the inflammatory mediator IL-21 in driving Th17 cell differentiation [90]. Moreover, in humans it has been demonstrated that supernatants from DCs stimulated with zymosan or $\beta$-glucan induce the development of Th17 cells with requirements for TGF-$\beta$, IL-1$\beta$, and IL-6 [91]. Collectively, these studies reveal that similar pathways regulate both human and mouse Th17 cell differentiation [92] (Figure 1).

## 4. Transcription Factors Required for Th17 Cell Differentiation

The key transcription factor involved in the differentiation program of Th17 cells is the retinoic acid-related orphan receptor (ROR) $\gamma$t [93, 94], a member of the ROR family of nuclear receptors encoded by the *RORC* gene. Studies in mice [93] and humans [65, 66] revealed that ROR$\gamma$t controls the expression of IL-17A and IL-17F by Th17 cells. However, ROR$\gamma$t does not regulate all genes related to the Th17 lineage, suggesting that other transcription factors contribute to the expression of genes involved in its functional differentiation.

Although all cytokine pathways involved in Th17 cell differentiation result in the upregulation of ROR$\gamma$t expression, IL-6, IL-21, and IL-23 signalling pathways additionally

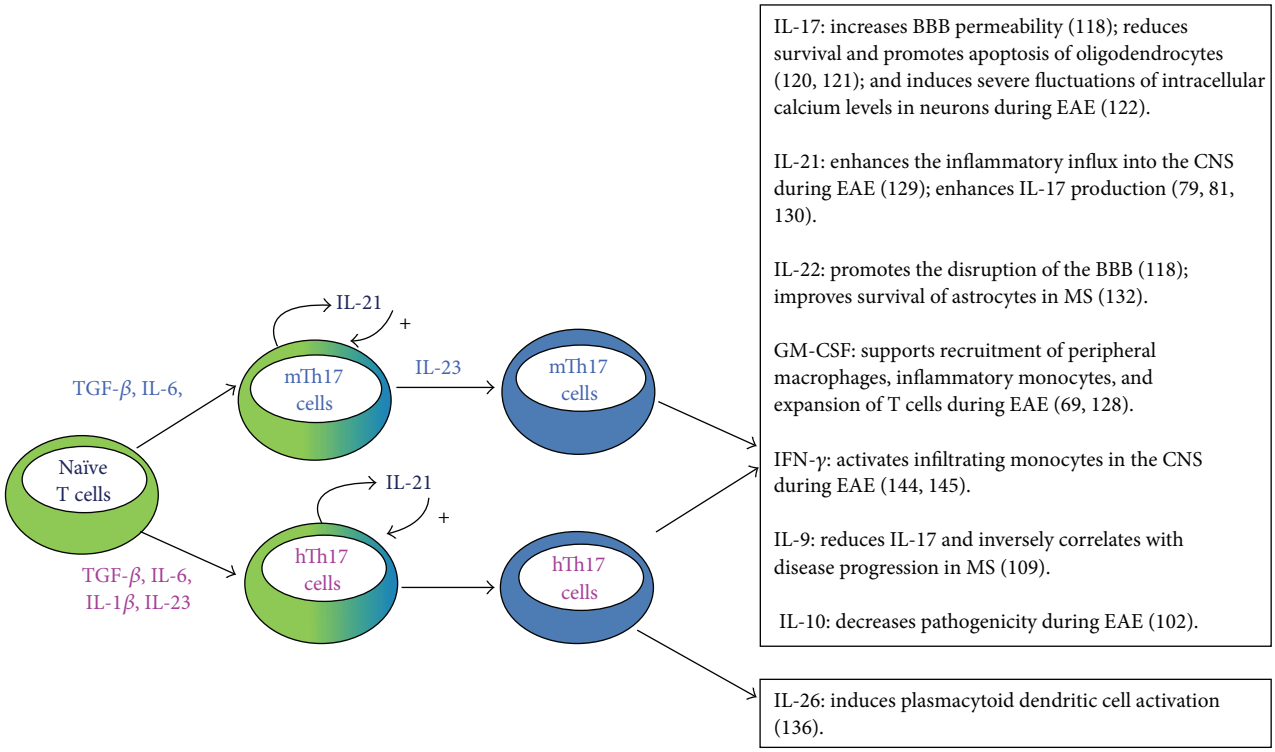


Figure 1: Differentiation of Th17 cells and their potential functions in MS. The T helper (Th) 17 cell differentiation program in mice (blue labels) and humans (purple labels) shares significant similarities. In both organisms, IL-6, TGF-$\beta$, IL-23, and IL-21 are involved in complete Th17 differentiation. Human Th17 cell differentiation requires also IL-1$\beta$. Fully differentiated Th17 cells produce specific sets of cytokines. Both murine and human Th17 cells produce IL-17, IL-21, IL-22, GM-CSF, IFN-$\gamma$, IL-9, and IL-10, with potentially relevant functions in MS pathogenesis. Human Th17 cells also produce IL-26.

activate STAT3, which directly binds the IL-17 and IL-21 promoters [95, 96]. Cells transduced with both RORγt and an active form of STAT3 (STAT3C) produce more IL-17 per cell, suggesting a cooperation between RORγt and STAT3 at transcriptional target sites [63]. Patients with autosomal-dominant hyper-IgE syndrome associated with dominant-negative mutations in *STAT3* lack Th17 cells, revealing the importance of STAT3-dependent signals in the differentiation and/or expansion of human IL-17-producing cells [97].

IRF4 has also been reported to be essential for Th17 cell differentiation [98]. IRF4-deficient mice have an impaired RORγt induction, since IRF4 is located upstream of RORγt in the Th17 cell differentiation process [98].

Another transcription factor known to cooperate with RORγt is the basic leucine zipper transcription factor ATF like (BATF), which is upregulated in Th cells following T cell receptor (TCR) activation [99, 100] and which is essential for the maintenance of RORγt expression induced by TGF-$\beta$ and IL-6 [100]. Recent studies have identified IRF4 and BATF as "pioneer factors" that bind and govern the accessibility of chromatin, enabling RORγt recruitment and binding to Th17 signature genes [101, 102].

Another member of the ROR family, RORα, also contributes to mouse Th17 cell development, and coexpression of RORα and RORγt causes synergistic increases in IL-17A, IL-17F, and IL-23R expression [103]. RORα and RORγt bind to the same retinoid response-like elements individually or as heterodimers and may be functionally redundant [104].

Retinoic acid receptors can be regulated by the ligand-dependent transcription factor aryl hydrocarbon receptor (AHR) [105, 106], whose binding to environmental pollutants such as dioxin influence Th17 cell differentiation [107, 108]. Different cellular contexts might provide distinct transcriptional partners for AHR and determine diverse outcomes of the immune response.

Another transcription factor associated with environmental conditions is the hypoxia-inducible factor 1α (HIF1α), which is a key sensor of hypoxia. HIF1α directly binds and drives transcription of RORC [109], and lack of HIF1α results in diminished Th17 development [110].

Therefore, combinatorial interactions of multiple transcription factors, including RORγt, RORα, activated STAT3, IRF4, BATF, AHR, HIF1α, and other unidentified factors regulate the genes that define the Th17 lineage. Decoding this transcriptional network will provide a better understanding of pathways involved in the differentiation of Th17 cells and may facilitate the development of strategies to manipulate the immune responses associated with these cells.

## 5. Immunomodulation of Th17 Responses

The conjunct action of multiple soluble factors fine-tunes and regulates the outcome of immune responses. For instance,

IL-10 has an important immunomodulatory role during the Th17 response. In fact, IL-10 production by restimulated mouse Th17 cells in the presence of TGF-$\beta$ and IL-6 is able to regulate Th17 cell immunopathology and reduces EAE disease severity [111]. In contrast, restimulation in the presence of IL-23 does not induce IL-10 production, conferring pathogenic functions to Th17 cells [111]. Thus, IL-10 production in Th17-polarizing conditions may be a mechanism of self-regulation of the potentially dangerous Th17 cell response. In humans, the regulation of IL-10 production in Th17 conditions is dependent on IL-1$\beta$ [65, 112]. Human Th17 cell differentiation in the presence of IL-1$\beta$ results in an enhancement of IL-17 and in a decrease of IL-10 production suggesting that, during the resolution of inflammation, a decrease in or a lack of IL-1$\beta$ may simultaneously decrease the production of IL-17 and enhance the production of IL-10 [65, 112]. Moreover, several studies suggest that IL-10 inhibits differentiation of Th17 cells in a direct manner [113, 114]. Given the role of IL-10 as a potent negative regulator of inflammation [115, 116], its presence could be an important mechanism for controlling the Th17 response.

The cytokine IL-2 has a similar inhibitory effect on Th17 cell differentiation. In fact, addition of exogenous IL-2 reduces the proportion of murine Th17 cells differentiated from naive T cells in the presence of TGF-$\beta$ and IL-6, whereas inversely blocking autocrine IL-2 by the addition of neutralizing antibodies enhances Th17 differentiation. Furthermore, IL-2-deficient mice contain a substantially greater fraction of Th17 T cells, and *in vitro* stimulation of T cells from these mice results in higher proportions of IL-17-producing cells [117]. In contrast, IL-2 seems to have a positive effect on IL-17 expression by human Th17 cells, and the addition of an IL-2-blocking antibody during differentiation prevents cell proliferation and IL-17 production [66].

We recently demonstrated that IL-9 has an inhibitory effect on IL-17 production by human Th17-polarized cells. Importantly, the interaction between IL-17 and IL-9 reveals a decisive mechanism regulating the pathogenic inflammation generated by Th17 cells in MS [118]. In fact, we found that IL-9 level in the CSF of MS patients inversely correlates with the progression of MS and with the levels of IL-17 observed in the CSF, indicating that inhibition of IL-17 via IL-9 could be protective in MS [118], despite the controversial results on the role of IL-9 in EAE [68, 119].

Th17 responses can also be suppressed by antigen presenting cells producing IL-27: this cytokine inhibits IL-17 production by human and mouse Th17 cells [120]. Interestingly, plasma levels of IL-27 negatively correlate with the percentage of circulating Th17 or with plasma IL-17 concentration in patients with progressive MS, suggesting that IL-27 might be involved in this disease [121].

Thus, the establishment, progression, and outcome of chronic inflammation, which underlies the pathogenesis of MS, are highly dependent on the nature of the complex network of cytokines which modulate Th17 cells and which are produced during the immune response.

## 6. Pathogenic Role of Th17 Cells in Multiple Sclerosis

MS is a heterogenous disease characterized by a wide variety of neurological symptoms and signs attributed to discrete areas of inflammation, demyelination, and axonal loss in the CNS [122]. Two main courses of MS exist: relapsing-remitting (RR) and primary or secondary progressive (PP and SP). RR-MS is characterized by recurrent neurologic symptoms interspersed by periods of stability, with full or partial recovery; the progressive form is characterized by gradual neurological dysfunction with or without exacerbations [123, 124].

Immunological mechanisms such as myelin destruction by specific CD8 T cells, activated microglia, invading macrophages, natural killer cells, and autoantibodies produced by B cells contribute to demyelination and axonal loss. Importantly, Th17 cells are mainly responsible for the persistent inflammation that characterizes both forms of MS [122].

Among the typical features of Th17 cells that could confer pathogenicity to MS, their abilities to enter the encephalic compartment, to penetrate the blood brain barrier (BBB), and to recruit inflammatory cells have been documented. In particular, Th17 cells express high levels of the C-C chemokine receptor 6 (CCR6) on the cell surface [62, 125] that binds the C-C chemokine ligand 20 (CCL20) constitutively expressed by the vascular endothelium of the blood-cerebrospinal barrier, thus enabling the entry of Th17 cells into the encephalic compartment through the choroid plexus [125]. Once in the brain parenchyma, Th17 cells release several proinflammatory mediators including IL-17A, responsible for the downregulation of tight junction proteins of the BBB, increasing BBB permeability, and favoring migration of both soluble inflammatory molecules and other circulating immune cells into the CNS [126]. Consistent with murine studies, IL-17A levels in the CSF of MS patients are associated with neutrophil expansion and blood brain barrier disruption, indicating that IL-17 may have similar pathogenic roles in EAE and MS [127].

It has been proposed that IL-17 also interferes with remyelinating processes, reducing survival [128], and promoting apoptosis of oligodendrocytes, the myelin-forming cells [129]. Moreover, a potential neurotoxic effect of Th17 cells has also been reported, by the induction of severe fluctuations of intracellular calcium levels in neurons during EAE [130] or through the release of granzyme B in human fetal neurons [126].

However, murine studies suggest that not only production of IL-17, but also other features of Th17 cells collectively confer encephalitogenic potential to these cells in MS. In fact, the course of EAE is unperturbed or only lightly ameliorated in IL-17 knockout mice and in wild-type mice treated with neutralizing antibodies specific for IL-17A [131, 132].

Among the proinflammatory mediators produced by Th17 cells, GM-CSF is gaining much attention in MS pathogenesis. Recent studies have suggested that this cytokine plays a fundamental role in the pathogenicity of Th17 cells in EAE [69, 70]. In fact, GM-CSF-deficient mice are resistant to EAE

[69, 133] and GM-CSF production by Th17 cells is crucial for their capacity to induce EAE [70, 134], likely supporting recruitment of peripheral macrophages, inflammatory monocytes, and expansion of T cells [69, 135, 136].

Another cytokine produced by Th17 cells is IL-21, whose administration before induction of EAE enhances the inflammatory influx into the CNS as well as the severity of the disease [137], likely due to the role of IL-21 in the induction and expansion of Th17 cells [88, 90, 138]. Interestingly, IL-21 and its receptor have been detected in lymphocytes infiltrating acute and chronic active white matter MS lesions [139], underlining the role for this cytokine in CNS inflammation.

The role of IL-22 in MS is still unclear. High levels of IL-22 and of IL-22 producing cells have been detected in the serum, CSF, and peripheral blood of MS patients [140–142], and it has been demonstrated that IL-22 promotes the disruption of BBB *in vitro* and *in vivo* [126]. However, in the brain tissue IL-22 improves survival of astrocytes that express its receptor [140], thus suggesting a protective role of this cytokine in MS.

Among the cytokines produced by Th17 cells, IL-26 has never been studied in the context of brain inflammation, due to the fact that only human Th17 cells produce it, thus making the EAE model inappropriate. However, the identification of a risk locus containing *IL26* and single-nucleotide polymorphisms within the *IL26* gene region associated with MS [143] encourages the study of its role in MS. Interestingly, IL-26 forms complexes with bacterial DNA and self-DNA released by dying cells, thus inducing plasmacytoid dendritic cells to produce IFN-$\alpha$ [144], that could have important implications in the modulation of the autoimmune response.

Moreover, the potential pathogenic role of Th17 cells in MS and in other autoimmune disorders could be related to their enhanced capability to survive, self-renew, generate effector progeny, and enter the memory pool with an efficiency superior to that of Th1 cells [145], and to their resistance to activation induced cell death [146–148]. In particular, we have recently demonstrated that human Th17 cells derived from healthy donors and MS patients express lower levels of FASL compared to Th1 cells, with a consequent lower sensitivity to cell death [148]. This mechanism could explain the persistence of IL-17-producing cells in autoimmune diseases, such as MS, contributing to the chronic inflammation typical of the disease.

Interestingly, Th17 cells could play a pathogenic role in MS also by converting their phenotype into a proinflammatory Th1 profile, as demonstrated by IL-17F reporter mice, where committed Th17 cells give rise to a progeny that shifts toward enhanced IFN-$\gamma$ expression, contingent upon limited or absent TGF-$\beta$ [149].

Similarly, in humans there are evidences indicating that, in the presence of IL-12, Th17 cells produce also IFN-$\gamma$. These cells which produce both IL-17 and IFN-$\gamma$, called Th1/17 cells [26], together with "nonclassical Th1 cells" might contribute to disease pathogenesis through properties shared by both the Th1 and Th17 subsets [150]. Moreover, Th17 cells producing IFN-$\gamma$ are enriched in myelin oligodendrocyte glycoprotein-specific T cells [151]. IFN-$\gamma$ produced by these cells could strongly activate macrophages [152] whose infiltration in the CNS correlates with EAE severity [153].

## 7. Therapeutic Approaches Targeting Th17 Cells in MS

In recent years several therapies directed against Th17-related cytokines, including IL-17, IL-23, and GM-CSF, have been developed, and some are currently being tested in ongoing clinical trials (Table 1). The fully humanized antibody neutralizing IL-17A called AIN457 or Secukinumab (NCT01708603 Clinicaltrial.gov) is already approved for the first-line systemic treatment of moderate to severe plaque psoriasis [154, 155]; in MS patients, although it showed a reduction by 63% of new magnetic resonance imaging (MRI) lesions compared to placebo-treated patients, the reduction of annualized relapse rate (ARR) was not statistically significant [71]. Moreover, Secukinumab was ineffective and resulted in higher rates of adverse events, mainly infections, compared with placebo in patients with Crohn's disease [156], and the study in MS terminated early based upon development of another anti-IL-17 monoclonal antibody with better potential for treating MS patients, Ixekizumab (NCT02387801 Clinicaltrial.gov).

Another Th17-related cytokine that has recently gained attention, as a promising molecular target, is GM-CSF, and MOR103 (NCT01517282 Clinicaltrial.gov) a fully human monoclonal antibody against human GM-CSF was tested in clinical trials. However, although performed on a limited number of patients, the first clinical trial did not show efficacy in MS [73].

Selective targeting of single Th17 cytokines, such as IL-17 or GM-CSF, with monoclonal antibodies has not shown efficacy in MS and in other autoimmune diseases such as Crohn's disease and rheumatoid arthritis, although the involvement of Th17 cells in their pathogenesis has been widely documented. This suggests that Th17 cells' pathogenicity relies also on other factors, and thus targeting this T cell subset, inhibiting the Th17 differentiation program as a whole, may be more effective in limiting inflammation.

In this context, Ustekinumab, an antibody neutralizing the p40 subunit common to IL-12 and IL-23, was developed but the clinical trial has been completed and did not show efficacy in reducing inflammation in MS (NCT00207727 Clinicaltrial.gov) [74–76]. However, by blocking both IL-12 and IL-23 which sit upstream of the Th1 and Th17 differentiation program, respectively, this monoclonal antibody suppressed both of these responses and this could explain its low efficacy in MS, considering that interference with Th1 responses determines increased susceptibility rather than protection from EAE [157–159]. Specific inhibitors of the p19 subunit of IL-23, including Tildrakizumab, Guselkumab, AMG 139, BI 655066, and LY3074828, that have been developed and are being currently tested in other autoimmune diseases [72] could give encouraging results in MS treatment.

The ROR$\gamma$t transcription factor represents the ideal target for the manipulation of Th17 cell responses. Digoxin, a small molecule which binds ROR$\gamma$t and interferes with transcription, has been shown to inhibit Th17 cell differentiation in the mouse and to reduce EAE severity [77], and another natural product, ursolic acid, has similar effects [78]. Chemical modification of other small molecules shown to bind the ligand-binding domains of ROR$\alpha$ and ROR$\gamma$t has

Table 1: Therapeutic approaches targeting Th17 cells in MS.

| Agent | Functional role | Clinical trial identifier in MS | Clinical stage | Reference |
|---|---|---|---|---|
| Secukinumab (AIN457) | Neutralizes IL-17A | NCT01708603 | 63% reduction of new MRI lesions compared to placebo; ARR reduction not statistically significant | [71] |
| Ixekizumab | Neutralizes IL-17 | | Currently tested in psoriasis | [72] |
| MOR103 | Neutralizes GM-CSF | NCT01517282 | Did not show efficacy in MS | [73] |
| Ustekinumab | Neutralizes the p40 subunit common to IL-12 and IL-23 | NCT00207727 | Did not show efficacy in MS | [74–76] |
| Tildrakizumab | Neutralizes the p19 subunit specific of IL-23 | | Currently tested in other autoimmune diseases | [72] |
| Guselkumab | Neutralizes the p19 subunit specific of IL-23 | | Currently tested in other autoimmune diseases | [72] |
| AMG 139 | Neutralizes the p19 subunit specific of IL-23 | | Currently tested in other autoimmune diseases | [72] |
| BI 655066 | Neutralizes the p19 subunit specific of IL-23 | | Currently tested in other autoimmune diseases | [72] |
| LY3074828 | Neutralizes the p19 subunit specific of IL-23 | | Currently tested in other autoimmune diseases | [72] |
| Digoxin | Interferes with ROR$\gamma$t | | Preclinical phase | [77] |
| Ursolic acid | Interferes with ROR$\gamma$t | | Preclinical phase | [78] |
| SR1001 | Interferes with ROR$\alpha$ and ROR$\gamma$t | | Preclinical phase | [79] |

led to the development of SR1001, which reduces human and murine Th17 cell differentiation, and suppresses the clinical severity of autoimmune disease in mice [79]. Although still in preclinical phase, the results of these studies indicate that this novel class of compounds has potential utility in the treatment of autoimmune diseases.

## 8. Conclusions

Over the past few years, remarkable advances in the understanding of Th responses have been reported. The discovery of the Th17 subset, of the cytokines and transcriptional factors regulating its differentiation, and of the biological functions of its effector cytokines has advanced our understanding of the role of $CD4^+$ T cells in adaptive immunity. However, many issues remain to be addressed, especially concerning the balance between pathological and protective roles during autoimmune and infectious diseases. Advances in these points are critical for the future development of new therapeutic strategies able to modulate Th17 pathways for the treatment of MS and of diseases where Th17 cells play a pathogenic role.

## Conflict of Interests

The authors declare that they have no financial conflict of interests.

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*Clinical Study*

# Administration of Panobinostat Is Associated with Increased IL-17A mRNA in the Intestinal Epithelium of HIV-1 Patients

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Intestinal $CD4^+$ T cell depletion is rapid and profound during early HIV-1 infection. This leads to a compromised mucosal barrier that prompts chronic systemic inflammation. The preferential loss of intestinal T helper 17 (Th17) cells in HIV-1 disease is a driver of the damage within the mucosal barrier and of disease progression. Thus, understanding the effects of new therapeutic strategies in the intestines has high priority. Histone deacetylase (HDAC) inhibitors (e.g., panobinostat) are actively under investigation as potential latency reversing agents in HIV eradication studies. These drugs have broad effects that go beyond reactivating virus, including modulation of immune pathways. We examined colonic biopsies from ART suppressed HIV-1 infected individuals (clinicaltrials.gov: NCT01680094) for the effects of panobinostat on intestinal T cell activation and on inflammatory cytokine production. We compared biopsy samples that were collected before and during oral panobinostat treatment and observed that panobinostat had a clear biological impact in this anatomical compartment. Specifically, we observed a decrease in $CD69^+$ intestinal lamina propria T cell frequency and increased IL-17A mRNA expression in the intestinal epithelium. These results suggest that panobinostat therapy may influence the restoration of mucosal barrier function in these patients.

## 1. Introduction

Intestines play a major role in maintaining our health. Beyond nutrient and liquid absorption, the intestines serve as a barrier between us, our intestinal microbiome, and foreign organisms/toxins. It is therefore expected that significant morbidity and mortality result from intestinal disorders like those associated with abnormal immune function (e.g., inflammatory bowel disease) and infectious diseases (e.g., various foodborne illnesses) [1, 2]. HIV-1 is an infectious disease that has a major negative impact on the intestines. Viral infiltration and replication in early HIV-1 infection result in rapid and profound intestinal $CD4^+$ T cell depletion [3–10]. This leads to a compromised mucosal barrier and then to chronic systemic inflammation [11]. The preferential loss of intestinal T helper 17 (Th17) cells in HIV disease is a driver of the damage within the mucosal barrier and this damage is not reversed during antiretroviral therapy (ART) [12]. A key effector molecule produced by Th17 cells (as well as $\gamma\delta$ T cells and a subset of innate lymphoid cells) is IL-17A. IL-17A is

a multifunctional cytokine with proinflammatory properties (e.g., neutrophil recruitment) along with a role in orchestrating mucosal barrier functions [13–15]. Given the pathogenesis of HIV-1 in the intestines, understanding the effects of new therapeutic strategies within this organ is a priority.

Recently, we conducted a single-arm, phase I/II clinical trial designed to evaluate the therapeutic effect of the HDAC inhibitor panobinostat on HIV-1 persistence despite successful ART [16]. Panobinostat is a potent hydroxamic acid HDAC inhibitor with inhibitory effects in the low nanomolar range against class I HDAC. It was approved in 2015 by the US Food and Drug Administration for the treatment of multiple myeloma [17]. HDAC inhibitors, like panobinostat, are actively under investigation as potential latency reversing agents because HIV-1 proviruses that are integrated into deacetylated, condensed chromatin lead to virus recrudescence when ART is interrupted. Accordingly, HDAC inhibitors have been extensively studied *in vitro* [18–20] and *in vivo* [16, 21–23] for their latency reversing potential and the results have been very promising. Furthermore, many HDAC inhibitors have robust anti-inflammatory properties [24]. These effects were observed in our trial cohort where panobinostat treatment was associated with reduced levels of peripheral blood (PB) inflammatory biomarkers (e.g., high-sensitivity C-reactive protein, interleukin-6, matrix metalloproteinase 9, E-selectin, and soluble CD40 ligand) as well as reduced expression of genes related to inflammation [25]. Because of the importance of understanding the varied intestinal effects of HDAC inhibitors as HIV-1 therapeutics, the study design included the collection of intestinal biopsies from consenting participants. These paired biopsies are the source material for this study to quantitate the biological impact of panobinostat in the intestines of individuals during suppressive ART.

## 2. Methods

*2.1. Study Design and Participants.* Between September 2012 and February 2014 we conducted an investigator-initiated, single-arm, phase I/II clinical trial as previously described (clinicaltrials.gov ID number NCT01680094) [16]. In accordance with the principles of the Helsinki Declaration, the Regional Ethics Committee for Region Midtjylland and the Danish Data Protection Agency approved the study design prior to patient enrollment and each patient provided written informed consent before any study procedures. Fifteen HIV-1 infected adults were enrolled in the study. These individuals exhibited virological suppression (<50 copies per mL, at least two measurements per year) for at least 2 years and $CD4^+$ T cell counts above 500 cells per $\mu$L. Each patient received oral panobinostat 20 mg three times per week every other week for eight weeks while maintaining ART (Figure 1). Study exclusion criteria included coinfection with hepatitis B or C viruses, clinically significant cardiac disease (including QTc prolongation), and current use of a protease inhibitor (because of potential drug interactions). Of the 15 patients, 9 individuals (all infected with Clade B virus) consented to participate in an endoscopic substudy with collection of mucosal biopsies from the sigmoid colon in the week prior to panobinostat dosing and during the fourth dosing week (Figure 1). The biopsies from the fourth week of panobinostat treatment period were collected between 22 and 24 hours after the most recent oral panobinostat dose.

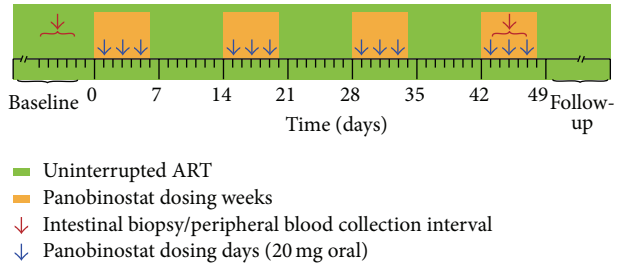


Figure 1: Schematic representation of the trial design. The timing of panobinostat dosing and the collection of samples utilized in these analyses are illustrated.

*2.2. Sigmoid Biopsies and Isolation of Lamina Propria Mononuclear Cells (LPMCs).* Sigmoidoscopy was performed by the same experienced endoscopists at baseline and during the fourth week of panobinostat treatment. An Olympus Exera II CLV-180 with an Olympus GIF-H180 scope (Olympus, Tokyo, Japan) was used to perform the procedures. The sigmoidoscopies were done without prior bowel cleaning. During all endoscopies mucosal tissue samples were taken from a standardized location in the sigmoid colon (approximately 35 cm from the anal verge). Endoscopic biopsies were collected for multiple analyses from macroscopically normal intestinal mucosa. Two biopsies were randomly selected to be fixed in 4% PFA (6–12 hours at room temperature) and embedded in paraffin blocks for gross histopathology. The remaining biopsies were collected in ice-cold Dulbecco's phosphate buffered saline (PBS) and immediately placed on ice. These biopsies were then processed to obtain lamina propria mononuclear cells (LPMCs). First, epithelial cells were removed by three 15 min incubations at 37°C in CMF HBSS-EDTA (calcium-magnesium free Hank's buffered salt solution supplemented with 2% human AB serum, 1.5 mM HEPES (Gibco Life Technologies, Auckland, New Zealand), and 2 mM EDTA (Thermo Fisher Scientific/Ambion, Waltham, Massachusetts)). After each incubation period, the epithelial cell-containing CMF HBSS-EDTA was removed and discarded. Following the third CMF HBSS-EDTA incubation the tissue samples were washed in RPMI 1640 supplemented with 10% AB serum and 1.5 mM HEPES. LPMCs were then isolated via a 90 min incubation at 37°C in 5 mL digestion media (RPMI 1640 supplemented with 10% human AB serum, 1.5 mM HEPES, 0.1 mg of collagenase D (Sigma-Aldrich, St. Louis, Missouri), and 50 U/mL DNase I (Sigma-Aldrich)). Following digestion, LPMC-containing supernatants were collected via filtration through a 70 $\mu$m nylon mesh (BD Biosciences, San Jose, California). Viable cells were counted and then aliquoted for quantitative PCR and flow cytometry analyses.

*2.3. Quantification of Viral DNA.* For four patients (identified as ▲, ●, ■, and ◆ in figures), LPMCs were used to isolate

$CD4^+$ T cells using a $CD4^+$ T cell isolation kit (Miltenyi Biotec, #130-096-533) and magnetic-activated cell sorting (MACS) columns (purity > 95%). The goal was to gain more specific insights into these potential viral reservoir cells. However, the total cell yields from these four isolations were low and therefore $CD4^+$ T cell enrichment was not performed with biopsies from the remaining five patients (identified as △, ○, □, ×, and ★ in figures). To compare anatomical compartments, PB $CD4^+$ T cells collected on the nearest date to the time of biopsy were analyzed for viral DNA load by the same method used for the LPMCs. Isolated LPMCs, LP $CD4^+$ T cells, and PB $CD4^+$ T cells to be used for nucleic acid quantification were lysed in RLT Plus Buffer immediately following isolation and the lysates were stored at −80°C until DNA was extracted (Allprep isolation kit, Qiagen #80204). Just prior to extraction, the lysates were subjected to shredding (Qiashredder, Qiagen #79656) and then nucleic acids were extracted according to manufacturer's instructions with each nucleic acid sample being eluted with three repeated applications of the same 50 $\mu$L elution buffer aliquot to their respective columns.

HIV-1 DNA was quantified in samples obtained from both the intestinal biopsies and PB cells at baseline and on panobinostat essentially as described [26, 27]. Briefly, extracted DNA was used directly for HIV-1 DNA quantifications using the QX100 Droplet Digital PCR system (BioRad) (~100 ng total DNA per PCR replicate) to determine the absolute levels of total HIV-1 DNA per $10^6$ LPMCs, LP $CD4^+$ T cells, or PB $CD4^+$ T cells. The PCR reaction mixture was loaded into the BioRad QX100 emulsification device fractionating each sample into ~20,000 nanoliter-sized droplets according to the manufacturer's instructions. After cycling, droplet data were collected using QX100 droplet reader (BioRad) and then analyzed with the QuantaSoft analysis software (BioRad). No panobinostat-associated cohort-wide changes in HIV-1 DNA levels were observed in intestinal cells ($p = 0.91$) (similar to [23]) or in time-matched PB $CD4^+$ T cells ($p = 0.57$) (consistent with our previous report [16]).

*2.4. Cell Surface and Intracellular Cytokine Flow Cytometry Analyses.* LPMCs surface expression of the T cell activation markers was measured essentially as described [28]. Briefly, within one hour of the isolation, cells were incubated with heat-inactivated mouse serum (Invitrogen, cat. number 10410) for 10 min and then stained with CD3 PerCP (BD Biosciences, cat. number 345766), CD4 PE (BD Pharmingen, cat. number 555347), CD8 PE-Cy7 (BD Pharmingen, cat. number 557746), CD69 APC (Biolegend, cat. number 310910), and HLA-DR APC-Cy7 (Biolegend, cat. number 307618). Following staining, the cells were fixed in 250 $\mu$L PBS containing 1% formaldehyde.

Intracellular cytokine detection in LPMCs was performed essentially as described [28]. Briefly, freshly isolated cells were incubated unstimulated overnight at 37°C in flat-bottomed wells (Nunc, Denmark, cat. number 140675) at a concentration of $2 \times 10^6$ LPMCs/mL in 2 mL of culture medium (RPMI 1640 with 10% pooled heat-inactivated human AB serum, 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin). Following this incubation, the cells were stimulated for 4 hours at 37°C with 1 $\mu$g/mL ionomycin (Sigma-Aldrich, Denmark, cat. number I0634) and 50 $\mu$g/mL phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich, Denmark, cat. number P1585) in the presence of 10 $\mu$g/mL brefeldin A (Sigma-Aldrich, Denmark, cat. number B7651). Next, cells were incubated with antibodies to detect surface expression of CD3 FITC (BD Biosciences, cat. number 345764) and CD8 PE-Cy7 (BD Pharmingen, cat. number 557746). Following surface staining, the cells were fixed with 1.5 mL BD FACS Lysing Solution (BD Biosciences, cat. number 349202) and then the cells were permeabilized with 0.5 mL FACS Permeabilizing Solution 2 (BD Biosciences, cat. number 340973). Blocking was performed with heat-inactivated mouse serum (Invitrogen, cat. number 10410) prior to staining with IFN-$\gamma$ PE (eBioscience, cat. number 12-7319) and anti-IL-17A APC (eBioscience, cat. number 17-7179-42). Following intracellular staining, the cells were fixed in 250 $\mu$L PBS containing 1% formaldehyde.

For all samples, data were collected using a FACSCanto analyzer (BD Biosciences) within 24 hours of staining. A total of $10^5$ events in the forward-side scatter lymphocyte gate were recorded. Single cells (according to forward-scatter-height and forward-scatter-area) were gated for CD3 expression. $CD3^+$ cells were next gated for $CD4^+$, $CD8^+$, or $CD8^{neg}$, as indicated in the figures. The cell populations were then assessed for expression of cellular activation markers and intracellular cytokines. Gates for CD69-, HLA-DR-, IFN-$\gamma$-, and IL-17A-positive events were based upon isotype or fluorescence-minus-one controls. Data from each patient from both time points were batch analyzed using FlowJo v.10 (Treestar).

*2.5. In Situ Hybridization (ISH).* *In situ* hybridization for IFN-$\gamma$, IL-1$\beta$, IL-8, and IL-17A mRNA as well as for HIV-1 RNA was performed in formalin-fixed paraffin-embedded (FFPE) tissues using the RNAScope 2.0 RED assay (cat. number 310036) according to the manufacturer's instructions (Advanced Cell Diagnostics, Inc., Hayward, CA, USA). First, 4 $\mu$m sections were cut from FFPE blocks and mounted on Superfrost Plus microscope slides. Consecutive sections were mounted and a minimum separation distance of 12 $\mu$m was ensured for the sections hybridized with a given probe (2 sections per probe). Slides were placed at 60°C for 1 hr in a dry oven. Mounted sections were deparaffinized in xylene, dehydrated in 100% ethanol, and then treated serially with ACD Pretreatment 1 (endogenous hydrogen peroxidase block) for 10 minutes at room temperature; ACD Pretreatment 2 (boiling in citrate buffer) for 8–12 minutes at 98–100°C; and ACD Pretreatment 3 (protease digestion) for 12–30 minutes at 40°C. Note that Pretreatment 2 and 3 conditions for each paraffin block were optimized to maximize the signal-to-noise ratio using positive (Hs-PPIB cat. number 313901) and negative (DapB cat. number 310043) control RNA probes. Washes with deionized water were performed after each pretreatment step. Next, hybridization between the target RNA and the selected ACD probe set (Hs-IL-17A cat. number 310931; Hs-IFN-$\gamma$ cat. number 310501; Hs-IL-8 cat. number 310381; Hs-IL1$\beta$ cat. number 310361; V-HIV1-CladeB cat. number 416111) took place at 40°C during a 2-hour incubation

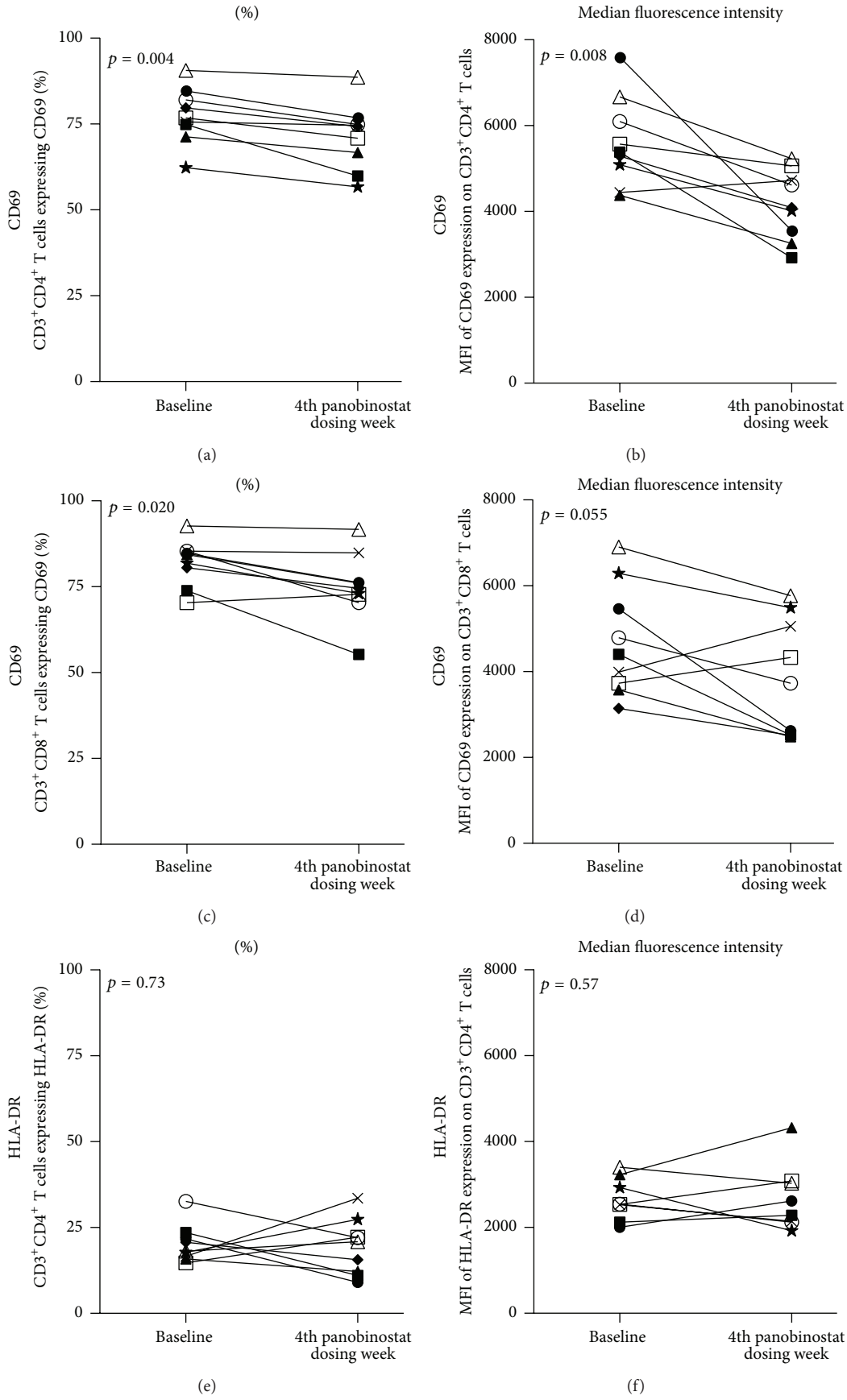


Figure 2: Continued.

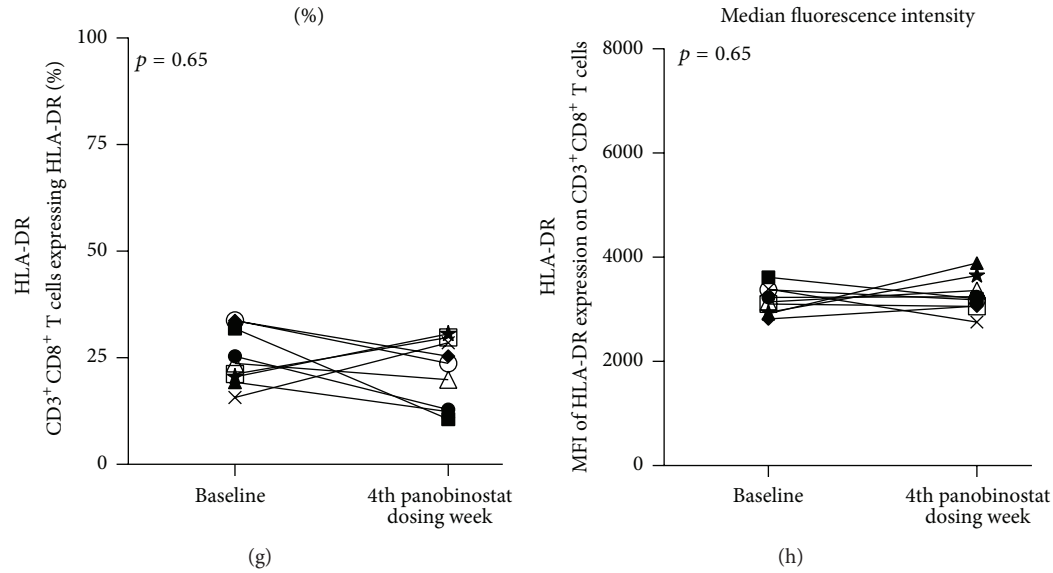


Figure 2: Panobinostat treatment was associated with reductions in the proportions of $CD69^+$ intestinal T cells. (a–h) Flow cytometric analyses were performed on freshly isolated LPMCs. Intestinal $CD4^+$ T cells (a-b; e-f) and $CD8^+$ T cells (c-d; g-h) were assessed for CD69 (a–d) or HLA-DR (e–h) expression. The proportion of cells expressing the respective activation marker is graphed in the left column while the levels of marker expression are represented in the right column. Each patient participant is represented in all figures by the same distinct symbol. Wilcoxon matched-pairs signed rank tests were used to generate the reported $p$ values.

in a HybEZ Oven (Advanced Cell Diagnostics, Hayward, CA). Two 2 min washes with ACD wash buffer were followed by signal amplification via the serial application of Amplifications 1–6 with two 2 min wash buffer washes after each step. Chromogenic detection of the target RNA was performed using Fast Red (10 min) followed by counterstaining with hematoxylin and mounting with Ecomount.

*2.6. Quantification of ISH Signals.* All of the sections were blinded before quantification and all counts were performed by the same individual via visual inspection with compound light microscopy with a robotic stage that randomly selected areas for quantification (Visiopharm NewCast, version 5.0.3.1247). In a minimum of 35 systematic random selected, nonoverlaying 25.600 $\mu m^2$ (160 $\mu m \times 160\ \mu m$) counting frames the number of positive cell profiles was counted. Test points were used to determine the area of examined tissue and the nature of the tissues within the counting frame (e.g., epithelial region, lamina propria) was recorded to allow for stratification of the data by anatomical region. In accordance with a recommendation from ACD, a cell profile was defined as RNA positive if it had at least one red spot visible at 20x magnification. Ratios were calculated for each of the probes and the background was subtracted. ISH data are expressed as positive cell profiles per $mm^2$. Validation of the ISH quantification strategy was performed according to standard methods. Briefly, all sections were counted prior to unblinding. After all sections were counted, 25 sections that covered all probe sets were assigned for recount prior to unblinding of the individual performing the quantification. These two count values for the same section (C1 and C2) were first plotted in a C1/C2 plot and a linear regression was performed ($r^2 = 0.36$). Next, the C1 and C2 values were compared using an unpaired $t$-test ($p = 0.31$). Finally, a Bland-Altman plot was generated using the average of the two counts versus the difference between the two counts (bias = 19.1; SD = 59.5). Together these values demonstrate that consistency in performing the quantification was maintained throughout the entire counting process. Note that the area measurements reported will not be the same as the true parameters present *in vivo* given that paraffin embedding and preparation caused an unavoidable, 30–50% shrinkage of tissue [29, 30]. One goal in this project was to quantify the number of cell profiles exhibiting productive HIV-1 infection via ISH in the intestinal tissues collected from these patients. Unfortunately, the low number of cell profiles with productive infection identified in the examined tissue sections precluded drawing conclusions regarding the ability of panobinostat treatment to induce viral RNA production in the intestines.

*2.7. Statistics.* All statistical tests were performed using an alpha level of 0.05. Wilcoxon matched-pairs signed rank tests were utilized to assess whether there was a significant difference between the baseline and on-treatment measures.

## 3. Results and Discussion

*3.1. Panobinostat Treatment Reduced Intestinal T Cell Activation In Vivo and Augmented IFN-γ Production by Intestinal T Cells Stimulated Ex Vivo.* Given our previous findings regarding the general anti-inflammatory activity of panobinostat in blood obtained from these patients [25, 31], we examined the activation status of freshly isolated LPMCs at baseline and during panobinostat treatment for surface

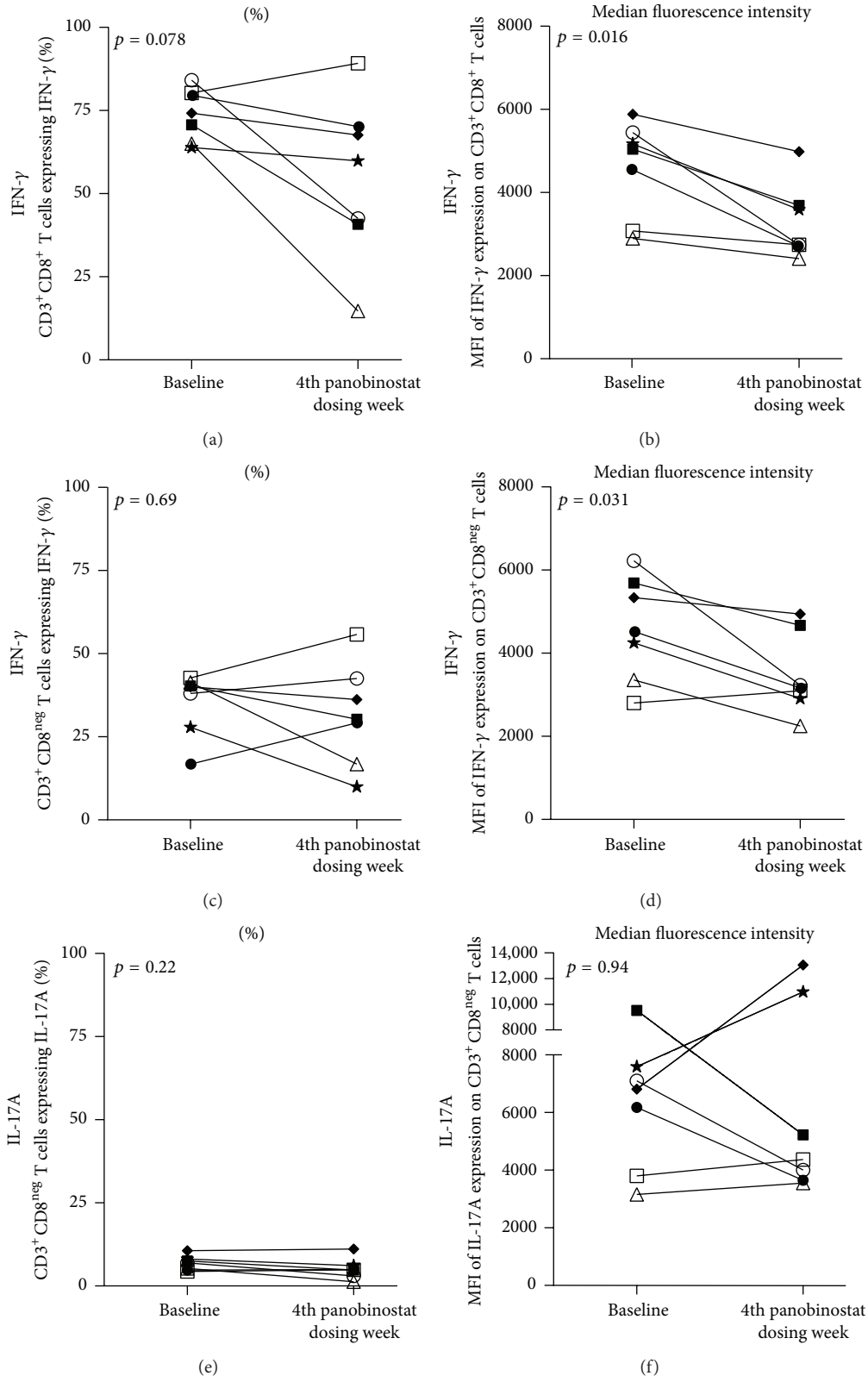


Figure 3: Intestinal T cells exhibit reduced intracellular IFN-$\gamma$ expression following *ex vivo* stimulation. (a–f) LPMCs were stimulated *ex vivo* with PMA/ionomycin and then assessed for intracellular cytokine expression. Intestinal $CD8^+$ T cells (a and b) and $CD8^{neg}$ T cells (c–f) were assessed for INF-$\gamma$ (a–d) or IL-17A (e and f) expression. The proportion of cells expressing the respective cytokine is graphed in the left column while the levels of cytokine expression are represented in the right column. Patient × and Patient ▲ were excluded from the intracellular cytokine expression analyses due low cell yields that precluded performance of the *ex vivo* stimulation either at the "baseline" or "during panobinostat" time point, respectively. Each patient participant is represented in all figures by the same distinct symbol. Wilcoxon matched-pairs signed rank tests were used to generate the reported $p$ values.

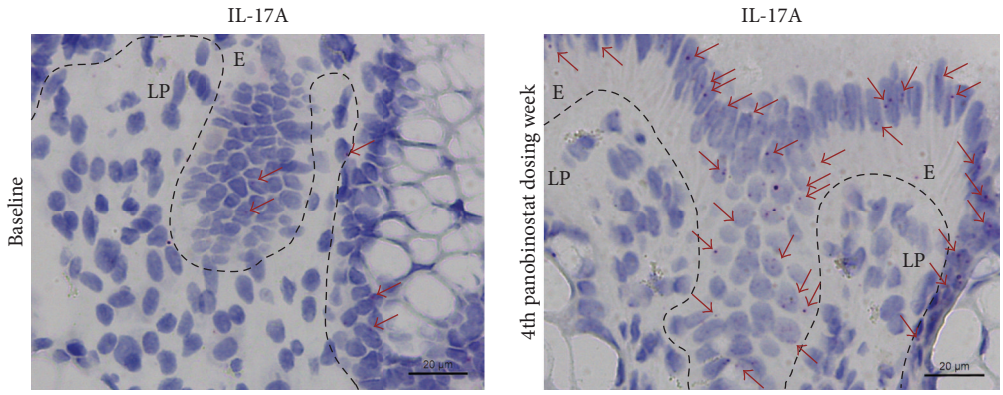


FIGURE 4: Intestinal IL-17A mRNA expression increased with panobinostat treatment. Representative images for IL-17A RNAScope ISH from Patient ★ are presented. Red arrowheads indicate a sampling of mRNA positive cell profiles in each image. Dashed lines demarcate lamina propria (LP) and epithelial (E) regions.

expression of early (i.e., CD69) and late (i.e., HLA-DR) T cell activation molecules [32]. We observed a decrease in the proportion of $CD69^+$ intestinal $CD4^+$ and $CD8^+$ T cells ($p = 0.004$, Figure 2(a); $p = 0.020$, Figure 2(c), resp.). Although we were unable to definitely determine whether this change resulted from decreased activation of resident cells versus an influx of naive cells, we did observe a significant decrease in the expression levels of CD69 on the intestinal $CD4^+$ T cells ($p = 0.008$, Figure 2(b)) and a trend towards reduced CD69 expression on intestinal $CD8^+$ T cells ($p = 0.055$, Figure 2(d)). In contrast to our findings regarding CD69 expression on intestinal T cells, we did not observe changes with panobinostat dosing in the proportions of HLA-DR expressing intestinal T cells or in the expression levels of HLA-DR on intestinal T cells (Figures 2(e) and 2(f)). It should be noted that these results from intestinal cells differ from PB outcomes from these patients [31]. Notable distinctions are that the baseline proportion of $CD69^+$ T cells in the intestines is considerably higher than in the PB, as expected [33], and that the greatest increase in the proportion of activated $CD4^+$ T cells was observed in PB during the first dosing week, while the observations made in the intestines are from the last dosing week. Thus, the differences between the observations in the intestines and PB regarding T cell activation may reflect differential stimulatory environments between these two anatomical compartments and the differential in the timing of the observations relative to the course of panobinostat treatment.

Next, we performed an *ex vivo* mitogenic stimulation of LPMCs that were rested overnight following isolation. We examined $CD8^+$ and $CD8^{neg}$ T cells for intracellular expression of two proinflammatory cytokines (i.e., IFN-$\gamma$ and IL-17A) [34]. We did not observe changes in the proportions of IFN-$\gamma$-producing intestinal T cells (Figures 3(a) and 3(c)). However, we did observe decreases in the expression levels of IFN-$\gamma$ in both intestinal $CD8^+$ and $CD8^{neg}$ T cells ($p = 0.016$, Figure 3(b); $p = 0.031$, Figure 3(d), resp.). Overexpression of IFN-$\gamma$ and other type 1 interferons in T lymphocytes has been observed both in experimental animal models of colitis and in patients with ulcerative colitis [35]. Thus, the detected reduction in IFN-$\gamma$ expression could reflect a general pleiotropic anti-inflammatory effect of panobinostat on lamina propria-resident T lymphocytes. This reduction in expression does not readily conform to our observation that panobinostat treatment generally did not affect IFN-$\gamma$ secretion by PB $CD8^+$ T cell memory subsets from this patient cohort [36]. However, direct comparisons between these PB and intestinal cell analyses are challenging. The main reasons are the difference in timing of sample selection as noted above and the fact that the *ex vivo* stimulations were performed with staphylococcal enterotoxin B (SEB) antigen for the PB cells and PMA/ionomycin for the intestinal cells. Further, the blood analyses were stratified by memory subset providing different assay resolution. IL-17A production was not examined in the PB cells, but it was characterized for the intestinal $CD8^{neg}$ T cells in response to mitogen stimulation. In this analysis, we did not observe cohort-wide changes in the proportion of cells expressing IL-17A or in the per cell expression ($p = 0.22$, Figure 3(e); $p = 0.94$, Figure 3(f), resp.). In accordance with the anticipated anti-inflammatory activity of panobinostat, these analyses demonstrate that panobinostat dosing generally reduced expression of the activation marker CD69 on intestinal T cells and reduced the capacity of intestinal T cells to produce IFN-$\gamma$ in response to *ex vivo* stimulation.

*3.2. The Number of IL-17A mRNA Producing Intestinal Cell Profiles Increased in the Epithelial Region with Panobinostat Dosing.* To gain insights into changes in the numbers of individual cells producing cytokine mRNA in the intestine during panobinostat therapy, we performed quantitative RNA *in situ* hybridization using limited amounts of biopsy material with the assumptions that these tissue samples were representative for the whole intestine and that two-dimensional profile counts correlate with total cell counts. Analyses were stratified according to the anatomical region harboring the RNA expressing cells (i.e., lamina propria and epithelial regions). We found that panobinostat treatment was associated with a significant increase in the number of cell profiles ($p = 0.04$) exhibiting IL-17A production within the epithelial region but not the lamina propria ($p = 0.13$) (Figures 4, 5(a), and 5(b)). In contrast, we did not observe cohort-wide differences in the number of cell profiles exhibiting INF-$\gamma$, IL-8, or IL1$\beta$

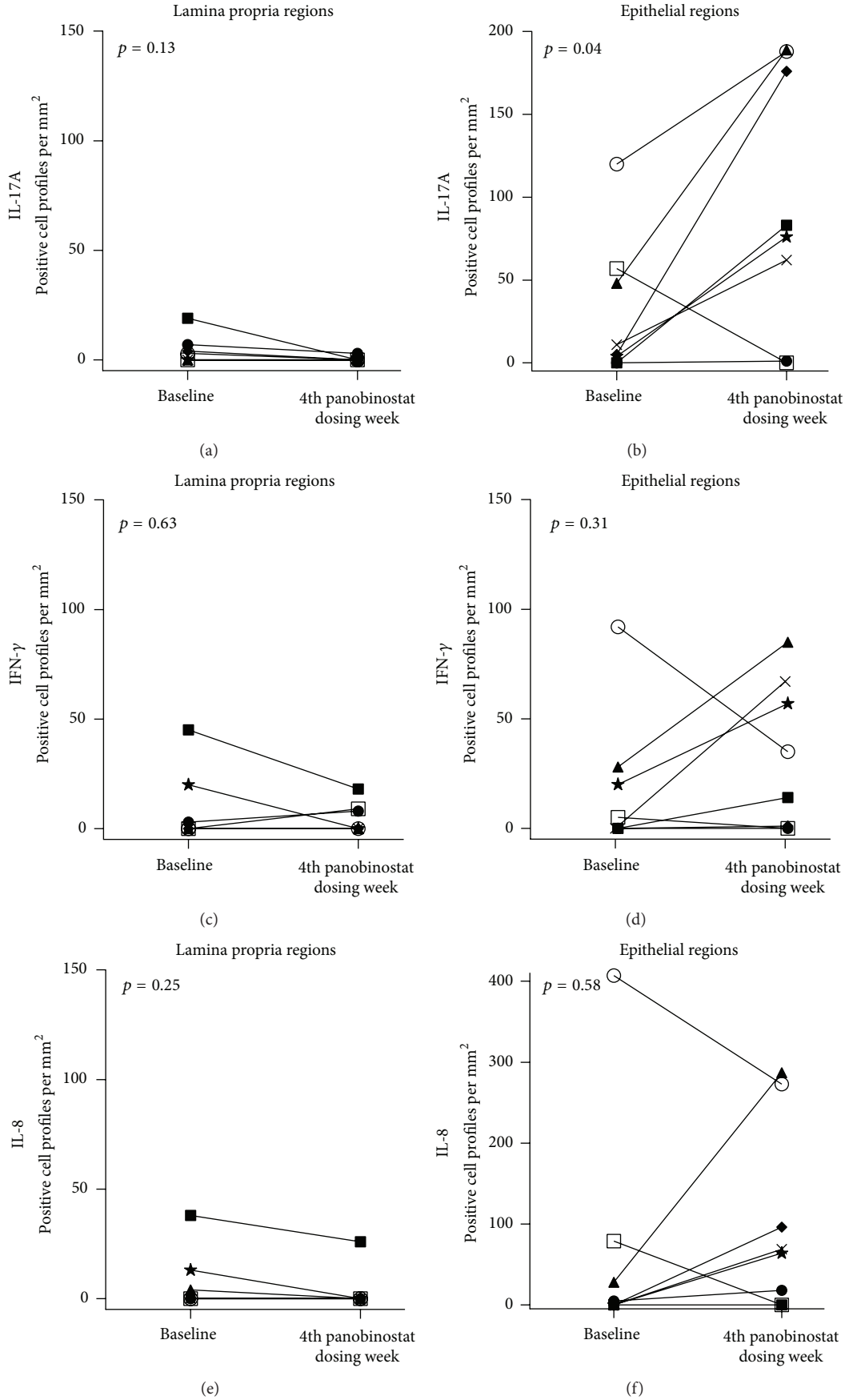


Figure 5: Continued.

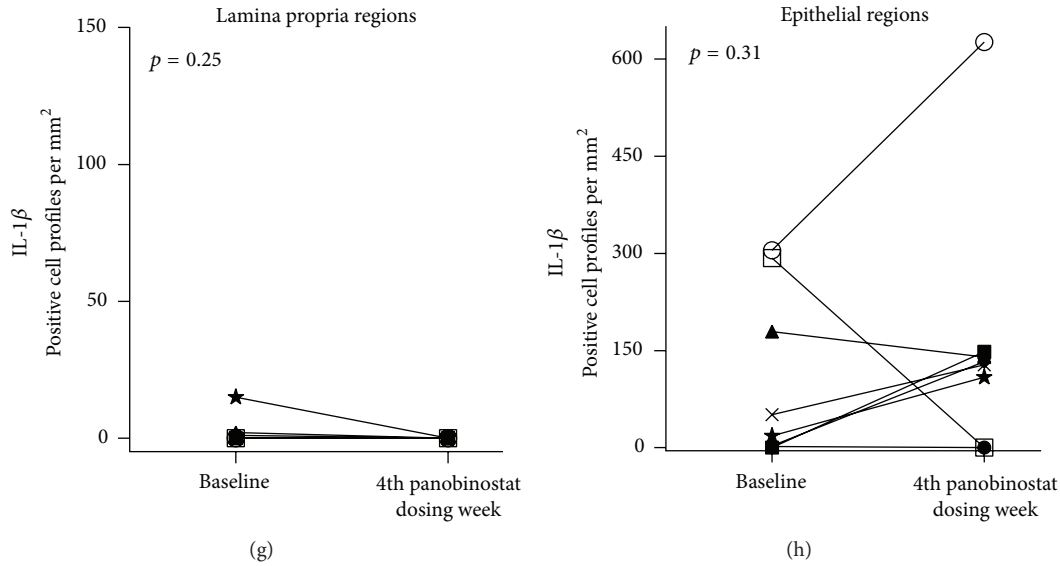


Figure 5: Epithelial region of intestines harbored the cells exhibiting IL-17A mRNA expression increase during panobinostat treatment. *In situ* hybridization for cytokine mRNA was performed with RNAScope technology. (a–h) Plots depict the number of cell profiles producing mRNA for IL-17A (a and b), IFN-$\gamma$ (c and d), IL-8 (e and f), and IL-1$\beta$ (g and h) at baseline and during panobinostat. Left column depicts positive cell profiles per mm$^2$ within the lamina propria regions. Right column depicts positive cell profiles per mm$^2$ within the epithelial regions. Patient $\triangle$ was excluded from the final analyses due to insufficient tissue for both optimization and assay performance. Each patient participant is represented in all figures by the same distinct symbol. Wilcoxon matched-pairs signed rank tests were used to generate the reported $p$ values.

production in either the lamina propria or the epithelial regions of the intestine (Figures 5(c)–5(h)). The results for intracellular and *in situ* IL-17A expression (Figures 3(e), 3(f), 4, 5(a), and 5(b)) may appear to be contradictory at first glance. The likely explanation is that these observations highlight key methodological differences in the two assays. Specifically, the intracellular cytokine secretion assay was performed on isolated LPMCs. The sample preparation used for this analysis excluded the intraepithelial layer from assessment. In contrast, the *in situ* hybridization technique allowed quantitation throughout both the lamina propria and epithelial regions. Using this method and taking the stated assumptions into account, we were able to determine that the epithelial regions were the anatomical location of the increased IL-17A expression (Figure 5(b)).

IL-17A expression in the intestines is frequently associated with inflammatory processes, such as in inflammatory bowel disease [15, 37–41]. However, IL-17A also induces robust production of antimicrobial peptides important for maintaining the intestinal epithelial barrier [42]. IL-17A is produced by intestinal innate lymphoid cells to orchestrate mucosal barrier function [13], exhibits anti-inflammatory behavior in the context of cultured human colonic epithelial cells [43], and ameliorates inflammation in rodent colitis models [43, 44]. Thus the data presented here lead us to hypothesize that the panobinostat-induced upregulation in IL-17A mRNA observed in the intestinal epithelium is associated with mucosal barrier restoration. Such activity would be consistent with the general anti-inflammatory activity of panobinostat in these same patients [25], reduced LPMC T cell activation (Figures 2(a)–2(d)), reduced levels of IFN-$\gamma$ production following *ex vivo* stimulation (Figures 3(a)–3(d)), and the unchanged intestinal IFN-$\gamma$, IL-8, and IL1$\beta$ mRNA levels (Figures 5(c)–5(h)). Future studies are necessary to confirm that the panobinostat-induced IL-17A expression in the intestinal epithelium is indicative of mucosal barrier restoration and a reduction of chronic immune activation in HIV patients during suppressive ART. With such confirmation, perhaps panobinostat (or similarly acting compounds) could become therapeutic options for reducing morbidity associated with chronic inflammation in these individuals.

*3.3. Conclusions.* It is essential that conclusions are cautiously drawn from these data as they are derived from a single-arm phase I/II clinical study with increased peripheral blood cell-associated unspliced-HIV-1 RNA during panobinostat treatment as the primary endpoint. Nevertheless, these data do lend themselves to a key conclusion. Panobinostat has a clear biological impact in the intestines of HIV-1 patients as shown by the decreased CD69$^+$ intestinal T cell frequency and increased IL-17A expression in the intestinal epithelium associated with panobinostat treatment. The significance of this last finding could be profound if future studies confirm that panobinostat dosing leads to improved mucosal barrier function in the intestines of HIV patients during suppressive ART.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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