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Endogenous IL-17 as a Mediator of Neutrophil Recruitment Caused by Endotoxin Exposure in Mouse Airways

Masahide Miyamoto, Olof Prause, Margareta Sjöstrand, Martti Laan, Jan Lötvall, and Anders Lindén

We have previously demonstrated that administration of the recently described cytokine IL-17 in rat airways in vivo recruits and activates neutrophils locally. In the current study, we examined whether endogenous IL-17 is involved in mediating neutrophil recruitment caused by endotoxin exposure in mouse airways. Our in vivo data show that local endotoxin exposure causes the release of free, soluble IL-17 protein 6 h later. Systemic pretreatment with a neutralizing anti-IL-17 Ab almost completely inhibits neutrophil recruitment 24 h, but not 6 h, after endotoxin exposure in the airways. Pretreatment with neutralizing anti-IL-6 and anti-macrophage inflammatory protein (MIP)-2 Abs inhibits neutrophil recruitment caused by local endotoxin exposure and IL-17, respectively. Our in vitro data show that endotoxin exposure stimulates the release of soluble IL-17 protein in T lymphocytes harvested from lung and spleen, respectively, and that this cytokine release requires coculture with airway macrophages. Intracellular IL-17 protein is detected in T lymphocytes from spleen but not in airway macrophages after coculture and stimulation of these two cell types. Finally, anti-IL-17 does not alter endotoxin-induced release of IL-6 and MIP-2 from T lymphocytes and airway macrophages in coculture. In conclusion, our results indicate that endotoxin exposure causes the release of IL-17 from T lymphocytes and that this cytokine release requires the presence of macrophages. Once released, endogenous IL-17 acts in part by inducing local release of neutrophil-mobilizing cytokines such as IL-6 and MIP-2, from nonlymphocyte, nonmacrophage cells, and this contributes to recruitment of neutrophils in the airways. These IL-17-related mechanisms constitute potential targets for pharmacotherapy against exaggerated neutrophil recruitment in airway disease.


A n exaggerated recruitment and subsequent activation of neutrophils is likely to be important for the development and course of several inflammatory diseases in the airways and lungs, such as severe asthma, chronic obstructive pulmonary disease, cystic fibrosis, and acute respiratory distress syndrome (1–9). The mechanisms by which neutrophils contribute to these diseases may involve the release of proteolytic enzymes, such as neutrophil elastase, and free oxygen radicals (3, 10–19). When released, these compounds can cause bronchoconstriction, bronchial hyperreactivity, hypersecretion, epithelial damage, and tissue remodeling in the airways.

The recruitment of neutrophils in the airways involves several cytokines, including the neutrophil activator IL-6 and the neutrophil chemoattractant IL-8 (1, 2, 8, 20–35). Both IL-6 and IL-8 can be produced by several types of cells present in the airway wall, including airway epithelial and smooth muscle cells, endothelial cells, fibroblasts, and monocytes/macrophages (26, 29–35). However, the cellular mechanisms that orchestrate the release of IL-6 and IL-8 in the airways are not fully understood.

It is known that subsets of T lymphocytes orchestrate the recruitment of eosinophils in response to allergen in sensitized airways (36). Interestingly, there is also evidence that certain subsets of T lymphocytes orchestrate the recruitment of neutrophils in response to allergen in sensitized airways (36). However, little is known about the molecular mechanism(s) by which subsets of T lymphocytes orchestrate neutrophil recruitment in the airways in response to stimuli other than allergens.

The cytokine IL-17 is a 17-kDa molecule that is produced by CD4+ and CD8+ subsets of T lymphocytes from humans and mice in vitro (37–39). In a recent study we demonstrated that exogenous human (h) IL-17 protein recruits neutrophils in rat airways in vivo, in part via the induced release of a rat correlate to IL-8, macrophage inflammatory protein (MIP)-2 (40, 41). We also demonstrated that the corresponding effect of exogenous hIL-17 on human bronchial epithelial cells in vitro is mediated by induced de novo synthesis and functionally significant release of hIL-8 protein. However, until now it has not been known whether endogenous IL-17 protein is important for the neutrophil recruitment in response to a nonallergic stimuli such as local endotoxin (LPS) exposure in the airways in vivo (8, 42–44).

For the current study we hypothesized that endogenous IL-17 protein mediates neutrophil recruitment in response to local endotoxin exposure in the airways and that this IL-17 acts in part via the induced release of neutrophil-associated cytokines from cells in...
the airway wall. We now present evidence supporting this hypothesis, obtained using local exposure to endotoxin from *Escherichia coli* as a stimulus of neutrophil recruitment in mouse airways in vivo. We also identify inflammatory cells capable of releasing mouse (m)IL-17 protein in response to endotoxin exposure.

**Materials and Methods**

**Animals**

We used specific pathogen-free C57BL/6 mice (male, 6–8 wk old, weight range 17–26 g; B&K Universal, Stockholm, Sweden). Mice were kept in individually ventilated racks and received standard laboratory food plus water ad libitum at the Animal Care Facility of Göteborg University (Göteborg, Sweden). The experiments were approved by the Animal Ethics Committee in Göteborg, Sweden (diary numbers 310/98 and 298/01).

**Aneasthesia and euthanasia**

Before intranasal (IN) or i.p. administration of either endotoxin, cytokines and/or Abs, the mice were transiently anesthetized using CO2 gas (AGA Gas, Göteborg, Sweden). By the end of experiments and before the collection of bronchoalveolar lavage (BAL), blood, and tissue samples, the animals were anesthetized i.p. using a mixture of xylazine (130 mg/kg in 0.1 ml sterile PBS) and ketamine (670 mg/kg in 0.1 ml PBS) (Apoteksbolaget, Göteborg, Sweden). After reaching deep anesthesia, mice were euthanized by opening the chest and bleeding the right ventricle of the heart.

**Local endotoxin exposure**

Various doses (2–200 μg, depending on experiment, in 25 μl PBS) of endotoxin (LPS, *E. coli* serotype 055:B5; Sigma-Aldrich, St. Louis, MO) or vehicle (25 μl PBS) were administered IN using a micro pipette (45).

**Systemic blockade of IL-17**

Twenty-four hours before endotoxin exposure, a specific, monoclonal anti-mIL-17 Ab (anti-IL-17b; R&D Systems, Abingdon, England) was administered i.p. (100 μg in 0.5 ml PBS) followed by 0.2 ml of air to ascertain that all Abs solution reached the peritoneal cavity. As control, an isotype Ab (rat (r)IgG2a; BD Pharmingen, Heidelberg, Germany) was administered i.p. (100 μg in 0.5 ml of PBS).

**Local stimulation with IL-17**

Exogenous mIL-17 protein (R&D Systems) was administered IN (3 μg in 25 μl PBS) of this recombinant protein contained <1 ng of endotoxin per mg mIL-17, according to batch analysis by the manufacturer.

**Systemic blockade of neutrophil-associated cytokines**

Specific, mAbs against mIL-6 and/or MIP-2 (100 μg in 0.5 ml PBS; R&D Systems) and/or corresponding isotype control Abs (rIgGl for anti-mIL-6 Ab, rIgG2b for anti-MIP-2 Ab) were instilled i.p. 24 h before endotoxin exposure.

**Blood samples**

Samples of venous blood (0.6 ml) were harvested from the right ventricle of the heart during the euthanasia procedure (described above) by the end of each experiment. Blood smear samples were prepared for each mouse.

**Bronchoalveolar lavage**

After euthanasia, a tracheotomy was performed and BAL was conducted (2 × 0.25 ml of PBS) via a tracheal cannula. This was followed by gentle aspiration. The recovered BAL suspension was pooled and kept on ice until centrifugation (1000 rpm, 10 min, 4°C; using model 5403; Eppendorf-Netheler, Hamburg, Germany). The total number (i.e., concentration) of cells was determined using resuspended cell pellets from BAL suspension. The cell-free BAL fluid supernatant was frozen for later analysis (~8°C).

**Cell differential counts in BAL and blood samples**

Cell differential counts were performed on cytospin slides prepared from BAL fluid (Cytospin 3; Shandon Life Science, Astmorf, U.K.) and on smear slides from blood, using May–Grunwald-Giemsa staining. All slides were evaluated in a microscope (Zeiss Axiosplan 2; Zeiss, Jena, Germany) at ×1000 magnification. Those cell counts were conducted on 400 cells in BAL samples and on 200 cells in blood samples.
ml; R&D Systems) was used to detect the captured extracellular IL-17 protein. Spots were visualized using AV-HPR enzyme and 3-amino-9-ethylcarbazole substrate. The plates were washed in water to stop the reaction. Spots were enumerated using a dissecting microscope at magnification of ×40 (CETI, Antwerpen, Belgium).

**Detection of intracellular IL-17 protein with immunohistochemistry (IHC)**

IHC staining of intracellular mIL-17 protein was performed in negatively selected spleen CD3⁺ lymphocytes. The protein release from these cells was blocked using 0.1% GolgiPlug (BD PharMingen) during the last 6 h of culture. The CD3⁺ lymphocytes were fixed immediately after incubation in 2% formaldehyde, and thereafter cytopsin preparations were prepared. The airway macrophage fraction was fixed directly on the culture slides in the same way. The cytoplasmic slides were stored wrapped in aluminum foil at ~84°C.

The slides were washed, and endogenous peroxidase was blocked with glucose oxidase solution according to manufacturer’s instructions (Sigma-Aldrich). Dilutions were performed using 0.05% BSA solution in PBS (Sigma-Aldrich), and incubations were performed at room temperature. After blocking unspecific binding using rabbit serum (DAKO, Glostrup, Denmark), the slides were incubated overnight with a monoclonal anti-mIL-17 Ab (BHH01, 10 μg/ml; R&D Systems) or isotype control Ab (IgG2α, 10 μg/ml; BD PharMingen). After washings, the slides were incubated with rabbit anti-mIgL1-HRP Ab (61-0120; Zymed Laboratories, San Francisco, CA) for 1 h followed by more washing. The Abs were then visualized by the 3,3'-diaminobenzidine-chromogen system (DAKO). After additional washing in distilled water, the preparations were counterstained with Mayer’s hematoxylin (DAKO) and subsequently washed in tap water, dehydrated, and mounted.

**Data presentation and statistical analysis**

All data are presented as mean (SEM) unless otherwise stated. The Spearman rank correlation was used for correlation analysis of data. Statistical analysis of differences between groups was conducted using the Wilcoxon signed rank test for single, paired comparisons and Mann-Whitney U test for multiple comparisons. All p values refer to the Wilcoxon signed rank test or the Mann-Whitney U test. All n values refer to the number of independent experiments for each treatment group, unless otherwise stated.

**Results**

**Neutrophils and soluble IL-17 protein in BAL fluid after local endotoxin exposure**

IN administration of endotoxin dose dependently increased neutrophils in BAL fluid 24 h after administration (Fig. 1A). According to the dose-response data, 10 μg of endotoxin per mouse should produce a submaximum, reproducible response. Therefore, this dose was used in most subsequent endotoxin exposures.

Two different doses of endotoxin IN markedly increased the concentration of soluble mIL-17 protein in cellfree concentrated BAL fluid 6 h after administration (Fig. 1B). There was no detectable mIL-17 protein in the cellfree concentrated BAL fluid from mice exposed to vehicle at this time point.

**Soluble IL-17 protein from T lymphocytes after endotoxin exposure in vitro**

As measured with EIA, addition of endotoxin (100 ng/ml) caused a small increase in the concentration of soluble mIL-17 protein in conditioned cell medium from the monoculture of positively selected CD3⁺ spleen lymphocytes in vitro (Fig. 2A). This was not the case in medium from a monoculture of negatively selected CD3⁺ spleen lymphocytes (Fig. 2B). However, negatively selected as well as positively selected CD3⁺ spleen lymphocytes responded to endotoxin (100 ng/ml) when cocultured with airway macrophages, with a clear increase in the concentration of soluble mIL-17 protein (Fig. 2, A and B). For positively selected CD3⁺ spleen lymphocytes in coculture, this release constituted 107% (17) of the positive control response to CI plus PMA. The corresponding response of negatively selected spleen CD3⁺ cells in coculture with airway macrophages was 113% (30). The monoculture of airway macrophages did not display any substantial release of soluble mIL-17 protein in the conditioned cell medium after stimulation with CI plus PMA (6.4 (1.5) pg/ml, n = 3).

As analyzed with ELISPOT assay (cells pooled from seven mice, used in three to five parallel experiments), positively selected CD3⁺ lung lymphocytes cocultured with airway macrophages displayed a substantial increase in released soluble mIL-17 protein in response to CI plus PMA (CI plus PMA: 19.0 (2.8) spots compared with 6.0 (1.6) spots for vehicle, p < 0.01). The monoculture of positively selected CD3⁺ lung lymphocytes did not display any substantial release of soluble mIL-17 protein (CI plus PMA: 7.2 (1.7) spots compared with 3.7 (1.2) spots for vehicle, p > 0.05). Using the same ELISPOT assay, preliminary experiments indicated that airway macrophages in monoculture were unable to release soluble mIL-17 protein after stimulation with CI plus PMA (data not shown).

**IHC staining of intracellular IL-17 protein in negatively selected spleen CD3⁺ lymphocytes, after coculture with airway macrophages, revealed a higher relative number of IL-17-staining cells after addition of endotoxin than after addition of vehicle (Fig. 3, A and B). Replacing the specific anti-mIL-17ab with an isotype control Ab did not reveal any corresponding unspecific staining of**
CD3⁺ spleen lymphocytes (Fig. 3C). In contrast, airway macrophages did not display any specific staining for intracellular mIL-17 protein after coculture with negatively selected CD3⁺ spleen lymphocytes (Fig. 3, D–F).

**Neutrophils in BAL fluid and in blood after systemic blockade of IL-17**

The i.p. administration of a specific anti-mIL-17 Ab dose dependently attenuated the increase in neutrophils in BAL fluid 24 h after administration of endotoxin IN, whereas the isotype control Ab did not (Fig. 4A). This inhibitory effect was selective for neutrophils (Table I). However, the anti-mIL-17 Ab did not cause any corresponding inhibitory effect on the number of neutrophils in BAL fluid (Fig. 4B) 6 h after endotoxin IN. Nor did the anti-mIL-17 Ab produce any substantial effect on the induced increase in neutrophils in blood 24 h after endotoxin IN (Fig. 4C). There was no pronounced difference in the recovery volume of BAL fluid or in the weight of mice for the different treatment groups (data not shown).

**Neutrophils in BAL fluid and in blood after local stimulation with IL-17**

IN administration of exogenous mIL-17 protein markedly increased the number of neutrophils in BAL fluid, from 0.01 (0.00) × 10⁴ neutrophils/ml (vehicle) to 1.79 (0.80) × 10⁴ neutrophils/ml (mIL-17) 12 h after administration, and this effect was statistically significant (p = 0.0005, n = 5–14). In contrast, exogenous mIL-17 did not display any pronounced effect (p > 0.05, n = 5–14) on neutrophils in blood at the same time point (2.05 (0.54) and 2.15 (0.22) × 10⁴ neutrophils/ml after vehicle and mIL-17, respectively).

**Neutrophils in BAL fluid and in blood after systemic blockade of neutrophil-associated cytokines**

The i.p. administration of either a specific anti-mIL-6 Ab or a specific anti-MIP-2 Ab, or both, markedly reduced the induced increase in neutrophils in BAL fluid (Fig. 5A) harvested 24 h after endotoxin IN. In contrast, these Abs did not exert any corresponding effect on neutrophils in blood (Fig. 5B).

**Neutrophil-associated cytokines in BAL fluid after systemic blockade of IL-17**

IN administration of endotoxin markedly increased the concentration of mIL-6 (Fig. 6A) and MIP-2 (Fig. 6B) in cell-free BAL fluid after 24 h. The i.p. administration of a specific anti-mIL-17 Ab before administration of endotoxin IN caused substantial inhibition of this increase in mIL-6 and MIP-2 at 24 h after endotoxin IN (Fig. 6, A and B). In contrast, administration of the anti-mIL-17 Ab i.p. did not cause a corresponding inhibition of mIL-6 (346.7 (143.1) for LPS plus IL-17ab compared with 594.8 (210.1) for LPS plus IgG2a; p > 0.05) and 1.3 (0.5) for the negative control PBS plus IgG2a or MIP-2 (82.9 (41.8) for LPS plus IL-17ab compared with 174.8 (103.8) for LPS plus IgG2a; p > 0.05 and 3.4 (0.6) for the negative control PBS plus IgG2a) in cellfree BAL fluid (pg/ml) harvested 6 h after administration of endotoxin IN.

**Blockade of IL-17 protein and release of neutrophil-associated cytokines in vitro**

As measured with EIA, blockade with an anti-mIL-17 Ab did not exert any substantial effect on the concentration of either IL-6 (anti-mIL-17 Ab: 4162 (814); and vehicle: 4530 (972) pg/ml) or MIP-2 (anti-mIL-17 Ab: 19525 (6675); and vehicle: 17846 (8300) pg/ml) (p > 0.05, n = 3 for both cytokines) in the conditioned cell medium from the coculture of negatively selected CD3⁺ spleen lymphocytes plus airway macrophages exposed to endotoxin (100 ng/ml) in vitro.

**Discussion**

Mechanisms controlling the neutrophil response to endotoxin exposure are probably relevant to several types of diseases in the airways and in the lungs, including plain, Gram-negative bacterial infection, and more complex conditions such as asthma, chronic obstructive pulmonary disease, and acute respiratory distress syndrome (7, 8, 25, 42–44). Our current study now suggests that endogenous IL-17 is important in mediating neutrophil recruitment during a certain time interval after local endotoxin exposure. Using mice in vivo, we demonstrate that local exposure to endotoxin increases the concentration of endogenous mIL-17 protein in the airways within 6 h. We also show that systemic blockade of mIL-17 with a specific anti-mIL-17 Ab inhibits neutrophil recruitment in the airways, occurring not at 6 h but at 24 h after endotoxin exposure. However, this systemic blockade of mIL-17 does not decrease the concentration of circulating neutrophils in venous blood at the same blood point. We also show that local stimulation with exogenous mIL-17 recruits neutrophils in the airways within 12 h. These findings indicate that local release of endogenous IL-17 can occur and subsequently recruit neutrophils within 24 h after local endotoxin exposure. In fact, our data indicate that endogenous IL-17 is required for the neutrophil response 24 h after...
endotoxin exposure in the airways. In contrast, this IL-17 is not required for the corresponding neutrophil response at an earlier time point (6 h); other mediators may be involved in orchestrating neutrophil-recruiting cytokines at this early time point. We also present evidence that endogenous IL-17 does not determine the concentration of circulating neutrophils in blood, at least not 24 h after local endotoxin exposure. Another recent study has shown that local stimulation with endotoxin increases IL-17 mRNA in mouse lung tissue within 6 h and this makes it very likely that endotoxin exposure causes not only release but also de novo synthesis of IL-17 protein in the airways within a 6-h time frame (47).

**FIGURE 3.** IHC staining for intracellular mIL-17 protein in negatively selected mCD3⁺ spleen lymphocytes and airway macrophages using a specific detecting, monoclonal anti-mIL-17 Ab. CD3⁺ lymphocytes and airway macrophages from mice were cocultured during 20 h, prior to separation and staining. A and B, CD3⁺ lymphocytes after administration of vehicle and endotoxin, respectively, stained with an anti-mIL-17ab (see Materials and Methods). C, CD3⁺ lymphocytes after administration of endotoxin stained with isotype control Ab (see Materials and Methods). D–F, Corresponding data for airway macrophages. The percentage of anti-mIL-17-positive cells (of 500 counted) was 0.4, 1.0, 0, 0.4, 0.4, and 0%, for A–F, respectively.
It is well known that subsets of T lymphocytes are involved in directing not only cellular but also functional responses to allergen in sensitized airways, leading to the development of bronchial hyperresponsiveness for example (48). Interestingly, there is also evidence that subsets of T lymphocytes are also involved in determining bronchial hyperresponsiveness in nonallergic airways (49). In both cases it appears that at least the CD4^+ subset of T lymphocytes plays an important role. Our current in vitro data on lung and spleen T lymphocytes (CD3^+ /H11001/H11000/H11004/H11003) from mice now show that some of these cells are capable of releasing mIL-17 protein in response to endotoxin exposure and, as judged in vitro, this release is substantial (50); the endotoxin-induced IL-17 release from T lymphocytes is comparable to that of the very potent control stimulus CI plus PMA in the positive control group. It appears that this IL-17 release originates from the CD3^+/H11001/H11000/H11004/H11003 lymphocytes, and this release requires costimulation by APC (airway macrophages). This is because our IHC data on intracellular IL-17 protein show a clear,

**FIGURE 4.** Effect of systemic blockade of IL-17 on neutrophils in BAL fluid and in blood after local endotoxin exposure in mice in vivo. A, Results from BAL fluid 24 h after administration of endotoxin (LPS: 10 μg) or vehicle (PBS) IN, with (IL-17ab: 3–100 μg) and without (IgG2a) pretreatment with an anti-mIL-17 Ab (rho = −0.4, p = 0.006, total n = 59) i.p. The negative and positive control groups were pretreated with a rat isotype Ab (IgG2a) i.p., n = 5–34. B, Results from BAL fluid 6 h after administration of endotoxin presented in analogy with the results in A (IL-17ab: 100 μg in B), n = 6–7 NS, p > 0.05. C, Results from venous blood presented in analogy with the results in A. n = 10–24. Data presented as mean with SEM.

It is well known that subsets of T lymphocytes are involved in directing not only cellular but also functional responses to allergen in sensitized airways, leading to the development of bronchial hyperresponsiveness for example (48). Interestingly, there is also evidence that subsets of T lymphocytes are also involved in determining bronchial hyperresponsiveness in nonallergic airways (49).

**FIGURE 5.** Effect of systemic blockade of neutrophil-associated cytokines on neutrophils in BAL fluid and in blood after local endotoxin exposure in mice in vivo. A, Results from BAL fluid harvested 24 h after administration of endotoxin (LPS: 10 μg) IN, with and without pretreatment with an anti-mIL-6 Ab (IL-6ab) or an anti-MIP-2 Ab (MIP-2ab) i.p., or both these Abs. For comparison, the neutrophil concentration in BAL fluid from vehicle-exposed mice (IgG2a plus PBS) is presented. The negative (IgG2a plus PBS) and positive (IgG2a plus LPS) control groups were pretreated with corresponding rat isotype Abs (IgG2a) i.p. B, Results from venous blood presented in analogy with the results in A. Data presented as mean with SEM. n = 9–17; *, p < 0.05; **, p < 0.01.

In both cases it appears that at least the CD4^+ subset of T lymphocytes plays an important role. Our current in vitro data on lung and spleen T lymphocytes (CD3^+) from mice now show that some of these cells are capable of releasing mIL-17 protein in response to endotoxin exposure and, as judged in vitro, this release is substantial (50); the endotoxin-induced IL-17 release from T lymphocytes is comparable to that of the very potent control stimulus CI plus PMA in the positive control group. It appears that this IL-17 release originates from the CD3^+ lymphocytes, and this release requires costimulation by APC (airway macrophages). This is because our IHC data on intracellular IL-17 protein show a clear,

<table>
<thead>
<tr>
<th>Cell Type</th>
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<th>IgG2a + LPS (34)</th>
<th>IL-17ab + LPS^8 (25)</th>
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<td>32.92 (3.36)</td>
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<td>3.75 (0.63)</td>
<td>3.65 (1.24)</td>
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<tr>
<td>Other cells</td>
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<td>0.34 (0.09)</td>
<td>0.18 (0.05)</td>
</tr>
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</table>

^8 All cell data are presented as mean (SEM) x 10^6 cells/ml.
^8 IL-17ab + LPS represents data obtained after pretreatment with 100 μg of a mIL-17 Ab (IL-17ab) i.p. Control groups were pretreated with a rat isotype Ab (IgG2a). All corresponding data on neutrophils in BAL fluid are presented in Fig 4A.

Table I. BAL cell differential count in mice after systemic blockade of IL-17

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Endotoxin, Neutrophil Recruitment, and IL-17
specific signal in CD3+ lymphocytes, but not in airway macrophages, after these cells were cocultured together in vitro. In support of this, our EIA and ELISPOT data on extracellular IL-17 phages, after these cells were cocultured together in vitro. In support of T lymphocytes co-

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References


