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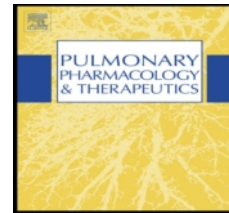
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Authors: Olof Prause, Apostolos Bossios, Elin Silverpil, Stefan Ivanov, Steven Bozinovski, Ross Vlahos, Margareta Sjöstrand, Gary P. Anderson, Anders Lindén, Lung Immunology, Pharmacology Groups



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**IL-17-producing T Lymphocytes in Lung Tissue and in the
Bronchoalveolar Space after Exposure to Endotoxin from *Escherichia coli*
in vivo – Effects of Anti-Inflammatory Pharmacotherapy**

Olof Prause^{1*}, Apostolos Bossios^{1*}, Elin Silverpil¹, Stefan Ivanov¹, Steven Bozinovski², Ross
Vlahos², Margareta Sjöstrand¹, Gary P. Anderson², Anders Lindén¹

**) These two authors contributed equally to this study.*

¹Lung Immunology & Pharmacology Groups,

¹) Department of Internal Medicine/Respiratory Medicine and Allergology,
Institute of Medicine, Sahlgrenska Academy at the University of Gothenburg,
Gothenburg, Sweden.

²) Lung Disease Research Group,

Cooperative Research Centre for Chronic Inflammatory Diseases,
Departments of Pharmacology and Medicine, the University of Melbourne,
Parkville, Victoria, Australia.

Short title.

Pharmacology of IL-17-producing T lymphocytes in lungs

Corresponding author.

Professor Anders Lindén, M.D., Ph.D.

Department of Internal Medicine/Respiratory Medicine & Allergology

Bruna Stråket 11B

Sahlgrenska University Hospital

S- 413 45 Gothenburg, Sweden

e-mail. anders.linden@gu.se

Abstract

Interleukin (IL)-17 may play a critical role for the innate immune response in mammals. However, little is known about its production in T-lymphocytes in comparison with other cells, in lung tissue and in the bronchoalveolar space *in vivo*. Even less is known about the effects of anti-inflammatory pharmacotherapy on this IL-17 production. In this study on mice we show that one single, intranasal exposure to endotoxin from *Escherichia Coli* increases extracellular IL-17 protein in bronchoalveolar (BAL) samples during 3 days, and is accompanied by a local increase in neutrophils and other inflammatory cells. This endotoxin exposure also elevates IL-17 mRNA in lung tissue samples. Moreover, after endotoxin exposure, the absolute number of CD3-positive cells containing intracellular IL-17 protein is increased as well; from a moderate cell number in lung tissue samples and from virtually none in BAL samples; with the number in lung tissue exceeding that observed in BAL samples. Notably, we also demonstrate that among the cells that contain intracellular IL-17 protein after endotoxin exposure, the percentage of CD3-positive cells is similar to that of CD3-negative cells in lung tissue. In contrast, CD3-negative cells dominate among IL-17-containing cells in BAL samples. A high systemic dose of a glucocorticoid receptor agonist attenuates the endotoxin-induced increase in extracellular IL-17 protein in BAL samples, IL-17 mRNA in lung tissue samples, and in IL-17-containing CD3-positive cells in BAL and lung tissue samples. This is also true for the endotoxin-induced accumulation of neutrophils and other inflammatory BAL cells *in vivo*. A systemic dose of a calcineurin-phosphatase inhibitor exerts a less complete and more selective effect on the endotoxin-induced increase in extracellular IL-17 protein and on neutrophils in BAL samples. *In vitro*, endotoxin also increases extracellular IL-17 protein in a co-culture of CD3-positive spleen cells and adherent mononuclear BAL cells; an increase that was inhibited by a glucocorticoid as well as by a calcineurin-phosphatase

inhibitor In conclusion, endotoxin-induced IL-17 production and release from T lymphocytes originates from cells that reside in lung tissue and from cells that have been recruited to the bronchoalveolar space. In both compartments, there is also a substantial number of cells other than T-lymphocytes that produce IL-17 after endotoxin exposure. The sustained IL-17 production from T lymphocytes and the associated neutrophil accumulation may be inhibited non-selectively through glucocorticoid receptor stimulation and more selectively through calcineurin phosphatase inhibition.

Abbreviations: CI, calcium ionophore; COPD, chronic obstructive pulmonary disease; CsA, cyclosporine A; BAL, bronchoalveolar lavage; ELISA, enzyme-linked immunosorbent assay; FCS, foetal calf serum; HBSS, Hanks balanced salt solution; IL, interleukin; i.n., intranasally; i.p., intraperitoneally; LPS, lipopolysaccharide; PBS, phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate

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Introduction

The homodimeric cytokine interleukin (IL)-17 (A) is believed to play a critical role for the innate component of host defence against bacteria in mammalian lungs, through its ability to indirectly mobilize neutrophils (1-4). Based mainly upon studies on blood cells, it has recently been suggested that there is a specific type of lymphocytes, a CD4-positive subset of T helper lymphocytes, named “Th-17”, that is producing IL-17 in mammals and that this subset is phenotypically and functionally different from the Th-1 and Th-2 subsets (5-8). However, it is also known that there are other subsets of T lymphocytes capable of producing IL-17 in response to bacterial stimuli in the lungs, presumably in addition to the “Th-17” subset. These cells include CD4-negative invariant NKT, $\gamma\delta$ and cytotoxic T lymphocytes (8-11).

There may be a pathogenic context for IL-17 *per se* in human lungs; the local concentration of IL-17 is increased in patients with inflammatory lung diseases such as severe asthma and cystic fibrosis as well as in healthy volunteers exposed to organic dust, in association with a local accumulation of neutrophils (12-15). There is also solid experimental evidence from mice that the production and release of IL-17 protein from T lymphocytes is important for endotoxin-induced accumulation of neutrophils in the bronchoalveolar space *in vivo* within a certain time frame and that this release of IL-17 protein requires co-stimulation, by antigen presenting cells such as airway macrophages (1,16,17). However, very little is known about IL-17-producing T lymphocytes in lung tissue in relation to the bronchoalveolar space as well as about the relative contribution of T-lymphocytes and other cellular sources of IL-17 in these tissue compartments. Moreover, given the potentially critical role in pulmonary host defence that has been attributed to IL-17-producing T lymphocytes recently, surprisingly little is known about

the suitable pharmacological strategies for regulating the accumulation and activity of these lymphocytes in lungs *in vivo* (4).

In the current study, we utilized a mouse model of gram-negative bacterial lung infection that is relevant for humans (17,18), to characterize the induced production of IL-17 protein in the collective population of T lymphocytes in lung tissue and in the bronchoalveolar space *in vivo*. In this models, we determined the sensitivity of endotoxin-induced IL-17 production and the associated neutrophil accumulation to anti-inflammatory pharmacotherapy *in vivo*; more specifically, to glucocorticoid receptor stimulation and calcineurin phosphatase inhibition, respectively. In addition, we assessed the relative contribution of T lymphocytes to the endotoxin-induced IL-17 production by comparing the percentage of CD3-positive and CD3-negative cells among cells containing intracellular IL-17 protein *in vivo*. Finally, we determined the direct effect of the two principles of anti-inflammatory pharmacotherapy on endotoxin-induced release of IL-17 protein in isolated T lymphocytes co-cultured with macrophage-like cells from the bronchoalveolar space *in vitro*.

Methods

Animal model

Pathogen-free mice (C57/Bl6, male, 6-8 weeks old, from B&K Universal AB, Stockholm, Sweden) were kept at the animal facilities of the University of Gothenburg under conditions which were approved from the Animal Ethics Committee of Göteborg (Diary number 298/01). The animals received standard laboratory food and water *ad libidum*.

Time course of endotoxin-induced effect on the concentration of extracellular IL-17 protein and neutrophils in the bronchoalveolar space in vivo

We first characterized the time course of the endotoxin-induced effect on the concentration of extracellular IL-17 protein and neutrophils in bronchoalveolar lavage (BAL) samples. To do this, mice were anaesthetized transiently using isofluoran (Apoteksbolaget, Gothenburg, Sweden) and stimulated with endotoxin from a pathogenically-relevant, gram-negative species (LPS: 10 µg, E. coli serotype 026:B6 from Sigma-Aldrich, St. Louis, Missouri, USA, in 50 µl of PBS) in a sub-maximally effective dose intranasally (i.n.) as described previously (17,18). The animals recovered well from each period of anaesthesia and did not display any clinical signs of long term side effects. At 1, 2 and 3 days, respectively, animals were anesthetized using a mixture of ketamine (670 mg/kg) and xylazin (130 mg/kg) (both from Apoteksbolaget, Gothenburg, Sweden) intraperitoneally (i.p.) and then euthanized by bleeding of the right ventricle of the heart.

After establishing a tracheotomy, mouse airways were washed with PBS (two times 250 µl) to obtain BAL samples. All the recovered BAL samples from one mouse were pooled and kept on ice until centrifugation (at 1000 rpm). After centrifugation, the cell-free BAL fluid was frozen (-80°C) for later analysis of IL-17 protein. The BAL cells were

resuspended in PBS containing 0.03% of bovine serum albumin (BSA) and total cell number was determined.

Differential cell counts were performed on cytospin slides prepared from BAL samples (Cytospin 3; Shandon Life Sciences, Astmor, UK) using May-Grünwald-Giemsa staining. All slides were evaluated in a light microscope (Zeiss Axioplan 2; Carl Zeiss AG, Jena, Germany) at x 100 magnification. We counted 400 cells per sample.

Effect of pharmacotherapy on the concentration of extracellular IL-17 protein and inflammatory cells in the bronchoalveolar space in vivo

Once the time course experiments were conducted and we had confirmed a correlation between the endotoxin-induced increase in the concentration of IL-17 protein and the number of neutrophils in BAL samples (Figure 2), we conducted the corresponding pharmacology experiments. For these pharmacology experiments, we used exactly the same protocol as the time course experiments in which the BAL samples were harvested 2 days after endotoxin exposure (described above) but with one exception; one hour before and 24 hours after intranasal administration of endotoxin (10 µg in 50 µl PBS), the mice were treated with the glucocorticoid receptor agonist dexamethasone (20 µg/mouse and 200 µg/mouse, respectively, constituting doses of 0.77 – 1.18 mg/kg and 7.69 - 11.76 mg/kg, respectively) (19) or the inhibitor of the endogenous calcium/calmodulin-dependent phosphatase calcineurin, cyclosporine A (calcineurin phosphatase inhibition: 500 µg/mouse, constituting a dose of 19.23 – 29.41 mg/kg) (20) or vehicle (PBS) intraperitoneally.

Effect of pharmacotherapy on CD3-positive cells and their intracellular IL-17 protein in lung tissue and in the bronchoalveolar space in vivo

To further address the pharmacology of IL-17-producing T lymphocytes, we conducted flow cytometry analysis of intracellular expression of IL-17 in CD3-positive cells from lung tissue and BAL suspension, respectively. This flow cytometry was performed as described earlier (8). Briefly, BAL was conducted (4 x 250 μ L of PBS with protein secretion blockage buffer 0.5% Golgi Stop; from BD Bioscience, Erembodegem, Belgium) and the BAL sample was processed as described above to determine cell numbers. The lungs were then perfused, excised and placed in buffer solution (HBSS with 0.5% Golgi Stop protein secretion blockage). The lungs were mechanically disrupted in a 100- μ m nylon mesh cell strainer following by filtration (40 μ m) to remove tissue fragments. Then both BAL and lung cells were incubated with a blocking solution (PBS with 2% mouse sera; from Dako, Denmark; and 0.5% Golgi Stop) during 15 minutes in order to block any unspecific binding. The cells were thereafter incubated with a PerCP-conjugated anti-CD3 antibody (clone 145-2C11; from BD Bioscience) or its isotype-matched control antibody (30 min. at 4°C), followed by two washings with wash buffer (PBS with 1% FBS). Cells were then fixed in paraformaldehyde (4%) at room temperature (10 min.), followed by two more washings (1% FCS/PBS). After re-suspension in 2 ml of SAP Buffer (0.1% saponin and 0.05% NaN₃, w/v in HBSS, from Sigma-Aldrich), the cells were incubated during 45 min. with a PE-conjugated rat anti-mouse IL-17 monoclonal antibody (clone TC11-18H10; from BD Bioscience) and its isotype control, at room temperature, followed by two washes with SAP buffer. Finally, the cells were washed (1% FCS/PBS) re-suspended in the same buffer, and analyzed using a FACScan flow cytometer (from BD Bioscience). Ten thousand cells were computed in a list mode and analyzed using the CellQuest Software (from BD Bioscience).

Effect of pharmacotherapy on IL-17 mRNA in lung tissue in vivo

These pharmacology experiments focused on the impact of glucocorticoid receptor stimulation, since this pharmacological intervention displayed the most pronounced effect on extracellular IL-17 protein and neutrophils in BAL samples *in vivo*. Mice were pre-treated with dexamethasone (20 µg/mouse and 200 µg/mouse, respectively) one hour before intranasal exposure to endotoxin (10 µg in 50 µl PBS) (18). At 12 hours, BAL was performed and lungs were perfused with PBS via the right ventricle of the heart. The lungs were removed, snap frozen in liquid nitrogen and kept at -80°C until further processing.

Total RNA was isolated from 15 mg of frozen lung tissue ground to a fine powder under liquid nitrogen using an RNeasy kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The purified total RNA prep was used as a template to generate first-strand cDNA synthesis using Super Script III (Invitrogen, Carlsbader, CA, USA) as described previously (8). The reaction mix containing 1 µg of RNA, 250 ng of random hexamers (Promega, Madison, WI, USA), and 10 mM dNTP mix was diluted to 12 ml in sterile water, heated to 65°C for 5 min and chilled on ice for 1 min. First-strand synthesis was then performed in a 20 µl total reaction volume by adding 50 mM Tris-HCl (pH 8.3), 75mM KCl, 3 mM MgCl₂, 10 mM DTT, 40 U RNaseout and 200 U Superscript III reverse transcriptase enzyme at 50°C for 50 min. The reaction was inactivated by heating at 70°C for 15 min. cDNA was diluted 5-fold in sterile water prior to amplification. Quantitative real-time PCR was performed as described previously (ABI PRISM 7900 HT Sequence Detection System) (21) using validated Assays on Demand Taqman primers for IL-17 from Applied Biosystems-Nordic, Stockholm, Sweden. Briefly, the gene expression for IL-17 was quantified by multiplexing single reactions, where our gene of interest was standardized to control (18S rRNA). An individual sample

from the control group was then arbitrarily assigned as a calibrator against which all other samples are expressed as a fold difference.

Effect of pharmacotherapy on the endotoxin-induced increase in extracellular IL-17 protein in vitro

Negatively selected CD3-positive cells from spleens (lymphocyte-like cells as judged by light microscopy) and adherent mononuclear BAL cells (macrophage-like cells as judged by light microscopy) were isolated and cultured as described previously (17).

Briefly, naïve C57/Bl6 mice were sacrificed, BAL was conducted (4 x 1.0 ml of PBS) and the spleen was surgically removed in each mouse. Adherent mononuclear cells in the BAL sample (macrophage-like cells as judged by light microscopy) were washed with PBS and seeded out (5×10^4 per well) on a 96-well culture plate (model 3072; from BD Bioscience) in cell culture medium (RPMI 1640; from Sigma-Aldrich). Cells were then incubated during 3 hours at 37°C and washed two times to remove non-adherent, non-macrophage cells.

For the enrichment of CD3-positive cells, mouse spleens were minced, red blood cells lysed using a hypotonic solution and remaining cells were then filtrated through a 40 µm cell strainer to obtain a single cell solution. Cells were washed (PBS with 0.5% BSA) and CD3-negative cells were magnetically labelled using a biotin-conjugated antibody cocktail (Pan T-cell isolation kit, Miltenyi Biotec, Bergisch Gladbach, Germany) followed by incubation with streptavidin-coupled microbeads (Miltenyi Biotec). The cells were then washed and passed through a column (Miltenyi Biotec) in a magnetic field. Labelled CD3-negative cells attached to the column while unlabelled CD3-positive cells were eluated and seeded (0.5×10^6 cells/ml) in complete media (RPMI 1640 with FCS 10%, L-glutamine 2 mM, sodium pyruvate 1% and penicillin-streptomycin 100 U/ml

and 100 µg/ml; all from Sigma-Aldrich) together with the adherent mononuclear BAL cells. The mean purity of the CD3-positive cells after magnetic separation was 88% (n=4). After enrichment, the co-culture of cells were pre-treated with either the calcineurin phosphatase inhibitor, cyclosporine A (10^{-6} M) (20) or the glucocorticoid receptor agonist, hydrocortisone (10^{-6} M) (22) or vehicle only. Ethanol was used as solvent for these chemicals; the final concentration of ethanol did not exceed 0.1% and was used in the same concentration in the negative and positive control as well. Hydrocortisone was chosen as glucocorticoid receptor agonist, because it shows a better water-solubility than dexamethasone. Thirty minutes after pre-treatment, cells were stimulated with endotoxin (LPS 100 ng/ml), positive control (calcium ionophore A 23487 [CI] 1 µg/ml plus phorbol 12-myristate 13-acetate [PMA] 2 ng/ml) or negative control (RPMI 1640 only) and incubated (at 37°C with 5% CO₂) during 20 hours, after which the conditioned cell medium was harvested. The cell medium was then centrifuged to remove cells and it was subsequently frozen (-80°C) for later analysis of IL-17 protein.

Measurement of cytokines

The concentration of free, soluble mouse IL-17 protein in cell-free BAL samples or conditioned media from cell cultures was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (from R&D Systems). A concentration below the lowest detectable value of the ELISA standard curve (5.4 pg/ml) was assigned the mean value of the lowest concentration of the ELISA standard curve and zero (i.e. 2.7 pg/ml).

Statistical analysis

A correlation analysis of certain data was conducted utilizing Spearman rank correlation test. The Mann-Whitney U-test (preceded by the Kruskal Wallis test for multiple

comparisons) was utilised for comparison of data. Data are presented as mean \pm standard error of the mean (SEM). P -values ≤ 0.05 were considered significant. n refers to the number of independent experiments for each treatment group.

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Results

Time course of endotoxin-induced effect on the concentration of extracellular IL-17 protein and neutrophils in the bronchoalveolar space in vivo

Intranasal stimulation with endotoxin (10 μ g) substantially increased the concentration of free, soluble IL-17 protein in the extracellular compartment of BAL samples (Figure 1) and it also increased the number of neutrophils and other inflammatory cells in the BAL sample (Table 1). The IL-17 concentration peaked at day 1 and gradually decreased over day 2 and 3 in a time-dependent manner (Figure 1). There was no detectable IL-17 protein in mice exposed to the vehicle of endotoxin at either time point (Figure 1). At the time point 2 days after stimulation, we found a statistically significant correlation between the concentration of IL-17 and the number of neutrophils in BAL samples (ρ : 0.63, $p < 0.05$, $n = 11$; Figure 2). This correlation proved statistically significant, even when the most extreme “outlier observation” was excluded (Figure 2 & data not shown).

Table 1.

| Cell type | Negative Control | | | LPS | | |
|-------------|--------------------|--------------------|--------------------|---------------------|---------------------|--------------------|
| | 1 day | 2 days | 3 days | 1 day | 2 days | 3 days |
| Macrophages | 2.16 \pm 0.35 | 2.83 \pm 0.27 | 2.65 \pm 0.93 | 2.89 \pm 0.38 | 3.63 \pm 0.47 | 5.52 \pm 1.02 |
| Lymphocytes | 0.01 \pm 0.01 | 0.01 \pm 0.01 | 0.00 \pm 0.00 | 0.20 \pm 0.04 | 0.59 \pm 0.09 | 1.22 \pm 0.24 |
| Neutrophils | 0.03 \pm 0.02 | 0.00 \pm 0.00 | 0.01 \pm 0.01 | 18.27 \pm 1.53 | 12.82 \pm 0.82 | 5.07 \pm 1.09 |

Legend to table 1. Effect of local exposure to endotoxin (LPS; 10 μ g i.n.) on the number of macrophages, lymphocytes and neutrophils ($\times 10^4$ /ml) in BAL samples from mice at 1, 2 and 3 days, compared to negative control (PBS). Lymphocyte and neutrophil numbers

were increased after treatment with endotoxin ($p < 0.05$); macrophage numbers showed a tendency towards an increase, most pronounced at 3 days. Data presented as mean \pm SEM ($n=4-11$).

Effect of pharmacotherapy on the concentration of extracellular IL-17 protein and inflammatory cells in the bronchoalveolar space in vivo

Pre-treatment with the high dose (200 μ g) of dexamethasone attenuated the endotoxin-induced increase in free, soluble IL-17 protein in the extracellular compartment of BAL samples (Figure 3). In contrast, pre-treatment with the moderate dose (20 μ g) of dexamethasone only partially reduced the endotoxin-induced increase in concentration of IL-17 protein in BAL samples, even though this inhibitory effect was statistically significant as well (Figure 3).

Pre-treatment with cyclosporine A incompletely reduced the endotoxin-induced increase concentration of IL-17 protein in BAL samples, even though this effect was statistically significant (Figure 3). As opposed to the general inhibitory effect on the endotoxin-induced increase in all BAL cells that was exerted by the high dose of the glucocorticoid, cyclosporine A exerted an incomplete but clear decreasing effect that was selective and statistically significant for BAL neutrophils (Table 2).

Table 2.

| | PBS i.n. | LPS i.n. | | | | |
|-------------|-----------------|-----------------|---------------------|----------------------|-----------------|--|
| Cell type | PBS i.p. | PBS i.p. | Dex 20 μ g i.p. | Dex 200 μ g i.p. | CsA i.p. | |
| Macrophages | 3.57 \pm 0.59 | 4.64 \pm 0.34 | 4.36 \pm 0.38 | 2.45 \pm 0.25 | 4.33 \pm 0.51 | |
| Lymphocytes | 0.03 \pm | 1.72 \pm | 1.60 \pm | 0.28 \pm | 1.49 \pm | |

| | 0.00 | 0.20 | 0.24 | 0.26 | 0.30 |
|--------------------|------------------------|-------------------------|-------------------------|------------------------|-------------------------|
| Neutrophils | 0.04 ± 0.03 | 17.68 ± 1.65 | 19.72 ± 3.58 | 0.97 ± 0.52 | 10.31 ± 2.01 |

Legend to table 2. Effect of treatment with dexamethasone (Dex, 20 µg/mouse and 200 µg/mouse, respectively) and cyclosporine A (CsA, 500 µg/mouse), respectively, on cell numbers in BAL samples harvested after endotoxin exposure in mice. The drugs were injected intraperitoneally (i.p.) one hour before and one day after intranasal stimulation with 10 µg endotoxin and numbers of macrophages, lymphocytes and neutrophils (mean ± sem cell number $\times 10^4$ /ml) were determined two days after endotoxin exposure. Data presented as mean ± SEM (n=6-20).

Treatment with the high dose of dexamethasone (200 µg/mouse) substantially decreased the endotoxin-induced increase in macrophages, lymphocytes and neutrophils in BAL samples and these effects were statistically significant. In contrast, treatment with cyclosporine A (CsA) selectively and in a statistically significant manner decreased the numbers of BAL neutrophils only. The low dose of dexamethasone (20 µg/mouse) caused no substantial effect on either cell number in BAL samples.

Effect of pharmacotherapy on CD3-positive cells and their intracellular IL-17 protein in lung tissue and in the bronchoalveolar space in vivo

Intranasal endotoxin exposure increased the total number of inflammatory cells in lung tissue samples, compared with the negative control (47.2 ± 22 vs. $22.3 \pm 5.1 \times 10^6$, $p < 0.05$) and this increase was fully attenuated in a statistically significant manner by the high (200 µg: $23.6 \pm 3.6 \times 10^6$ $p < 0.05$ vs. LPS) but not the moderate (20 µg) dose of dexamethasone or cyclosporine A (data not shown). There was no substantial effect of either treatment on the total number of CD3-positive cells in lung tissue samples (Figure

4A). Interestingly, endotoxin exposure also increased the number of IL-17-containing CD3-positive cells in this compartment, from a reproducibly low to a substantially higher level, and the high dose of dexamethasone (200 µg) attenuated this increase in a statistically-significant manner (mean data in Figure 4B and data from one representative FACS analysis in Figure 4E). The moderate dose of dexamethasone and the one dose of cyclosporine A exerted only weak inhibitory effects; effects that did not prove statistically significant (data not shown).

Similar to the case for lung tissue, intranasal endotoxin exposure increased the total number of BAL cells compared to control (29.2 ± 22 vs. $363.3 \pm 9.3 \times 10^3$ cells, $p < 0.05$). The high dose of dexamethasone ($200 \mu\text{g}; 114.5 \pm 15.2 \times 10^3$ cells) as well as the one dose of cyclosporine A ($243.3 \pm 41.6 \times 10^3$ cells) partially inhibited ($p < 0.05$ compared with LPS for both) this increase in BAL cells. The moderate dose of dexamethasone did not produce any corresponding, statistically significant effect (data not shown). Noteworthy and in contrast to the case in lung tissue, intranasal endotoxin exposure increased both the total number of CD3-positive cells and the number of IL-17-expressing CD3-positive cells in BAL samples, from virtually no to a substantially higher number in both cases and these effects proved statistically significant (Fig 4 C & D). The high dose (200 µg) of dexamethasone totally attenuated these responses to endotoxin, again in a statistically-significant manner (Figure 4 C & D). The moderate dose of dexamethasone and the one dose of cyclosporine A did not produce any corresponding, statistically significant effect.

Percentage of CD3-positive and -negative cells among cells containing intracellular IL-17 protein after endotoxin exposure, in lung tissue and in the bronchoalveolar space in vivo.

In lung tissue, the percentage of CD3-positive cells (57.5 ± 7.6 %) was similar to that of CD3-negative cells (43.3 ± 8.0 %) among all IL-17-containing cells after endotoxin exposure ($p=0.66$, $n=3$). However, in this compartment, the relative mean fluorescence intensity (rMFI, equals signal for monoclonal antibody/signal for isotype control antibody) was higher for CD3-positive (3.0 ± 0.2) compared with CD3-negative (2.4 ± 0.2) IL-17-containing cells and this difference was statistically significant ($p<0.05$, $n=3$).

In BAL cells, however, the percentage of CD3-positive cells (24.2 ± 5.9 %) was smaller than that of CD3-negative cells (75.8 ± 5.9 %) among all IL-17-containing cells after endotoxin exposure ($p<0.05$, $n=3$). In analogy, in this compartment, the rMFI was substantially smaller for CD3-positive (3.9 ± 0.2) compared with CD3-negative (18.1 ± 0.4) IL-17-containing cells and this difference was statistically significant ($p<0.05$, $n=3$).

Effect of pharmacotherapy on IL-17 mRNA in lung tissue in vivo

Pre-treatment with the high dose (200 μ g) of dexamethasone totally attenuated the endotoxin-induced increase in mRNA for IL-17 assessed in lung tissue samples harvested 12 hours after exposure and this impact was statistically significant (Figure 5). In contrast, the moderate dose (20 μ g) of dexamethasone did not fully attenuate the corresponding increase in mRNA, even though the effect was statistically significant compared with the negative control ($p<0.05$, $n=3-4$) (Figure 5).

Effect of pharmacotherapy on the endotoxin-induced increase in extracellular IL-17 protein in vitro

Endotoxin stimulation caused a 7.9 ± 1.8 fold increase of the concentration of free, soluble IL-17 protein that was detected in the conditioned medium from the co-cultured CD3-positive spleen cells and adherent mononuclear BAL cells, compared to the negative control group (21.8 ± 4.5 pg/mL) (Figure 6). Treatment with hydrocortisone almost

completely attenuated the endotoxin-induced increase (% of positive control) in IL-17 protein (down to 7 ± 3 %), whereas cyclosporine A only partially reduced it ($36 \pm 10\%$) ($p < 0.05$, $n=4$).

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Discussion

We found that local exposure to endotoxin from the human lung pathogen *Escherichia Coli* (18) causes a sustained increase in extracellular IL-17 protein in BAL samples that lasts at least 3 days. The time course of the concentration of extracellular IL-17 is paralleled by a corresponding time course for neutrophil accumulation in BAL samples and there is a strong correlation between these two parameters 2 days after endotoxin exposure. We also found that, within the same time frame after endotoxin exposure, mRNA for IL-17 in lung tissue is increased, just like the number of CD3-positive cells containing intracellular IL-17 protein among BAL cells and in lung tissue. The endotoxin-induced increase in IL-17-containing CD3-positive cells is from a moderate number in lung tissue samples and from virtually none in BAL samples; with the number in lung tissue clearly exceeding that observed in BAL samples. In addition, we observed that among the cells that contain intracellular IL-17 protein after endotoxin exposure, the percentage of CD3-positive cells is similar to that of CD3-negative cells in lung tissue whereas the percentage of CD3-negative cells exceeds that of CD3-positive cells among IL-17-containing cells in BAL samples. Interestingly, the rMFI, the estimated strength of this signal for intracellular IL-17 protein, was strongest for CD3-positive cells in the lungs and for CD3-negative cells in BAL samples. Moreover, endotoxin exposure causes a marked increase of extracellular IL-17 protein in co-culture of CD3-positive spleen cells and adherent mononuclear BAL cells.

Most previous studies on IL-17 and neutrophils in the lungs have focused on T-lymphocytes as the cellular origin of IL-17 and studies demonstrating an attenuating effect of anti-IL-17-antibodies or the absence of the IL-17 receptor or gene have supported this concept (1-4). However, there is one previous report suggesting that mouse airway neutrophils can produce and release IL-17 protein during certain circumstances (16). In view of these previous findings, the interpretation of our currently demonstrated correlation between IL-17 protein

and the number of BAL neutrophils 2 days after endotoxin exposure is not entirely trivial: We cannot rule out that neutrophils contribute to IL-17 production in the bronchoalveolar space, given that we found that there may be more CD3-negative than CD3-positive IL-17-containing cells in BAL samples after endotoxin exposure, and that these CD3-negative cells display a stronger signal for IL-17 than the CD3-positive cells do. In contrast, in the lung tissue, we found approximately as many CD3-positive as CD3-negative IL-17-containing cells after endotoxin exposure, whereas the CD3-positive cells accounted for the strongest IL-17 signal in this compartment. We interpret these findings as the collective population of T-lymphocytes constituting one substantial source of IL-17 protein in the lungs and that there are other cells as well, possibly neutrophils, that can contribute to this IL-17 production as part of the innate response to a gram-negative bacterial pathogen (1-4,16, 17). The relative contribution of each respective cell population to IL-17 production may be somewhat different for the two compartments that we investigated in the current study; the bronchoalveolar space and the lung tissue. The possibility that neutrophils within the bronchoalveolar space constitute a substantial source of IL-17 protein after exposure to gram-negative bacteria, in addition to T lymphocytes, is indeed intriguing. Even though it is beyond the scope of our current study, this possibility warrants further investigation in future studies (1-4,16, 17).

Because we studied all CD3-positive cells, and not just the CD4-positive subpopulation, our results reflect the response by the collective population of IL-17 producing T lymphocytes in mouse lungs, including CD4-positive Th17 lymphocytes as well as CD4-negative invariant NK T, $\gamma\delta$ and cytotoxic T lymphocytes (8-11). We believe that this is functionally most interesting when evaluating the biology of IL-17, in particular since there is currently no convincing functional data published on IL-17-producing cells other than T lymphocytes in humans (4). Moreover, when specifically targeting all the T lymphocytes in BAL samples with flow cytometry, we found that endotoxin exposure increases the total number of CD3-

positive cells in this compartment, from virtually none to a substantial number, as well as the number of IL-17-containing CD3-positive cells. These findings thus forward the possibility of both recruitment and activation of IL-17-producing T lymphocytes in the bronchoalveolar cells playing a role in the early innate immune response in mammalian lungs.

Even though the bronchoalveolar space represents the primary target site after local endotoxin exposure, the IL-17 protein that is released by endotoxin could hypothetically originate from the surrounding tissue. We therefore analysed the total number and the number of IL-17-containing CD3-positive cells in lung tissue as well. Notably, endotoxin exposure did not exert any substantial effect on the total number of CD3-positive cells in the lung tissue samples, in contrast to what was the case in the BAL samples. However, endotoxin exposure did increase the number of IL-17-containing CD3-positive cells in lung tissue samples, just like in the BAL samples, and after endotoxin exposure, the absolute number of IL-17-containing CD3-positive cells in the lung tissue samples by far exceeded the corresponding number in the BAL samples. Moreover, in lung tissue samples from naïve control mice that were not exposed to endotoxin, we repeatedly detected a population of IL-17-containing CD3-positive cells, whereas this was not the case for the BAL samples. We speculate that these findings illustrate that a small population of IL-17-producing T lymphocytes always reside in the mammalian lung tissue, ready to immediately respond to emerging gram-negative bacterial pathogens, thereby constituting a functionally critical component of local host defence (21,24,25). Mechanistically, this possibility may explain why neutralisation of endogenous IL-17 protein can be so powerful in attenuating endotoxin-induced neutrophil accumulation in the bronchoalveolar space, as previously demonstrated, even though the bronchoalveolar space may contain relatively few IL-17-producing T lymphocytes (1,2,4,16).

When assessing whether targeting glucocorticoid receptors constitute a feasible approach for pharmacologically regulating the accumulation and activity of IL-17-producing T

lymphocytes in lungs in vivo, we did obtain evidence that this is the case but only if a high dose of the agonist is utilized. Thus, we found that a high, but not a moderate, systemic dose of the glucocorticoid receptor agonist dexamethasone effectively reduces the endotoxin-induced increase in extracellular IL-17 protein in BAL samples, IL-17 mRNA in lung tissue samples, total CD3-positive cells as well as IL-17-containing CD3-positive cells in BAL and lung tissue samples. The same is true for the endotoxin-induced, IL-17 associated increase in neutrophils and other inflammatory cells in BAL samples. In addition, we observed a complete attenuating effect exerted by the glucocorticoid receptor agonist hydrocortisone on the endotoxin-induced release of extracellular IL-17 protein in a co-culture of spleen CD3-positive cells co-cultured with adherent mononuclear BAL cells in vitro.

Collectively, our observations on the impact of dexamethasone in vivo and hydrocortisone in vitro are compatible with a high systemic dose of the glucocorticoid directly targeting the endotoxin-induced activity in IL-17-producing T lymphocytes in the lungs, as well as the recruitment in to the bronchoalveolar space of all inflammatory cells including IL-17-producing T lymphocytes. Given the fact that previous in vivo studies have shown that dexamethasone inhibits delayed type hypersensitivity, which depends upon the activity of T lymphocytes, as well as olive oil-induced inflammation, which depends upon neutrophil activity, it is possible that systemic glucocorticoid receptor stimulation inhibits endotoxin-induced neutrophil-accumulation at many different levels simultaneously, including inhibition of T lymphocytes of the memory helper subset (26,27). In line with an effect “down-stream” of IL-17, we have previously shown that glucocorticoid receptor stimulation results in inhibition of the C-X-C chemokine release that occurs in response to IL-17 in the lungs, in human bronchial epithelial cells and in mice in vivo (28,29). However, it remains unclear to what extent glucocorticoid receptor stimulation can more specifically inhibit the specific signalling pathway “up-stream” of the IL-17-producing T lymphocytes, including event involving T lymphocytes of the memory helper subset, even though it is known that endotoxin

acts via toll-like receptor (TLR)-4 on antigen-presenting cells and via the subsequent production and release of IL-23, a specific activator of IL-17-producing T lymphocytes in the lungs (1,2,3,4,8,23,24).

We believe that our current findings may have clinical implications, since it is traditionally known that the innate immune response is relatively glucocorticoid-insensitive (25). This is because IL-17-producing T lymphocytes may hypothetically constitute a link between a “glucocorticoid-insensitive” innate immunity and a “glucocorticoid-sensitive” adaptive immunity. Our findings that systemic glucocorticoid receptor stimulation bears the potential to completely attenuate the endotoxin-induced production and release of IL-17 protein in the lungs *in vivo*, as well as the associated neutrophil accumulation, is potentially problematic and promising at the same time. On one hand, our findings implicate that the early, neutrophilic component of host defence in the lungs may actually be impaired by systemic stimulation of glucocorticoid receptors, if a really high dose of the glucocorticoid receptor agonist is utilised. On the other hand, a very high systemic dose of the particular agonist may be required, and useful, to down-regulate an excess activation of the neutrophilic component of host defence in mammalian lungs.

In contrast to the case for glucocorticoid receptor stimulation, we found that calcineurin phosphatase inhibition by cyclosporine A exerts a more selective and incomplete effect on the endotoxin-induced increase in extracellular IL-17 protein and on neutrophils in BAL samples, in the one dose given. In line with this incomplete inhibition, the utilised calcineurin phosphatase inhibitor caused a weak effect on IL-17-containing CD3-positive cells in lung tissue and in BAL samples; an effect that did prove to be statistically significant. Finally, the incomplete but statistically significant inhibitory effect of the calcineurin phosphatase inhibitor on IL-17 release was confirmed in the co-culture of endotoxin-stimulated CD3-positive spleen cells and adherent mononuclear BAL cells.

Even though the current study is the first one conducted in a lung context, two previous studies on isolated cells have demonstrated that the calcineurin phosphatase inhibitor cyclosporine A does inhibit induced IL-17 production in human CD4-positive cells (30,31) In these *in vitro* studies on cells harvested from blood, the IL-17 production was induced by either IL-15 or by calcium ionophore plus PMA. Moreover, in another previous *in vitro* study on mouse lymphocytes from spleens and lymph nodes, it was demonstrated that induced production of IL-17 is sensitive to cyclosporine A as well; in this particular *in vitro* study, IL-17 production was induced by anti-CD3 antibodies, yet another artificial stimulus (32). To these previous observations, we now add our *in vivo* and *in vitro* data suggesting that cyclosporine A exerts a substantial but incomplete inhibitory effect on the innate immune response to a gram-negative bacterial stimulus in the lungs by directly acting on and inhibiting the IL-17 release from T lymphocytes recruited to the bronchoalveolar space and residing in lung tissue, as well as the associated accumulation of neutrophils. This means that targeting endogenous calcineurin phosphatase may constitute a pharmacotherapeutic strategy by which an excess activation of the early neutrophil component of host defence in the lungs can be down-regulated, in a relatively selective manner without totally attenuating this critical response (2,4,16,17).

In conclusion, this experimental study on a mouse model indicates that a component from a bacterial pathogen that can cause pneumonia in humans (18) *per se* does induce sustained IL-17 production and release from T lymphocytes that reside in lung tissue and are recruited to the bronchoalveolar space *in vivo*. In addition, there is a population of cells other than T lymphocytes that is likely to contribute to IL-17 production in lung tissue and in the bronchoalveolar space. The endotoxin-induced IL-17 production, and the associated neutrophil accumulation *in vivo*, can be inhibited by anti-inflammatory pharmacotherapy. Non-selective inhibition can be achieved through systemic stimulation of glucocorticoid receptors, if a high dose of the agonist is utilised. More selective, but less complete inhibition

can be achieved through systemic inhibition of endogenous calcineurin phosphatase. Due to their IL-17-inhibiting capacity in the lungs *in vivo* and in isolated T lymphocytes *in vitro*, both these approaches deserve a more in-depth clinical evaluation in neutrophilic lung disorders such as acute lung allograft rejection, ARDS, severe asthma, COPD and cystic fibrosis; disorders where moderate doses of glucocorticoid receptor agonists seem insufficient as anti-inflammatory treatment (2,4,33-37).

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Legends

Figure 1. Concentration of free, soluble interleukin (IL)-17 protein in the extracellular compartment of mouse BAL samples at 1, 2 and 3 days, respectively, after exposure to endotoxin (10 μg intranasally, i.n., white columns) compared to negative control (PBS, black columns) ($p < 0.05$, $n = 3-9$). The concentration of IL-17 correlated negatively to time after endotoxin exposure ($\rho: -0.7$; $p < 0.01$, $n = 10-11$). The detection limit for IL-17 was 5.4 pg/ml. Data presented as mean \pm SEM. ($n = 4-11$).

Figure 2. Concentration of free, soluble IL-17 protein in the extracellular compartment versus number of neutrophils in mouse BAL samples 2 days after exposure to endotoxin (10 μg i.n.) Data presented as mean \pm SEM ($\rho: 0.63$; $p < 0.05$, $n = 15$).

Figure 3. Effect of pre-treatment (i.p.) with the glucocorticoid receptor agonist dexamethasone (Dex, 20 μg and 200 μg , respectively) and the calcineurin-phosphatase inhibitor cyclosporine A (CsA, 500 μg) on the concentration of free, soluble IL-17 protein in the extracellular compartment of mouse BAL samples harvested 2 days after exposure to endotoxin (10 μg). In all samples from negative controls, the concentration of IL-17 protein was below the detection limit. Data presented as mean \pm SEM ($* = p < 0.05$; $n = 6-20$).

Figure 4. Effect of pre-treatment (i.p.) with dexamethasone (Dex, 200 or 200 μg) and the calcineurin phosphatase inhibitor, cyclosporine A (CsA, 500 μg) on the total number of CD3-positive cells and on IL-17-containing CD3-positive cells in mouse lung tissue (A,B) and BAL samples (C,D) harvested two days after exposure to endotoxin (10 μg) compared to negative and positive control. Data presented as mean \pm SEM ($* = p \leq 0.05$;

n=3). E) Representative lungs FACS plots from animals treated with PBS, endotoxin or endotoxin and a high dose of dexamethasone (200 $\mu\text{g}/\text{mouse}$). Lungs were removed from animals, disrupted mechanically and stained for CD3/IL-17 PE or with isotype control antibodies. In all plots, gating was done in the CD3-positive cell population. The numbers in the boxes represent the percentage of gated CD3-positive cells. The left column represent CD3-positive cells stained with isotype control (IgG1 PE) and right column represent CD3-positive cells stained with IL-17 PE.

Figure 5. Relative expression (%18S rRNA) of mRNA for IL-17 in mouse lung tissue samples after intranasal exposure to endotoxin only (10 μg , white column) as well as with intraperitoneal (i.p.) pre-treatment with dexamethasone (striped column: 20 μg of dexamethasone; black column: 200 μg of dexamethasone). Data presented as mean \pm SEM (*= $p \leq 0.05$; n=4).

Figure 6. Effect of pre-treatment with the glucocorticoid receptor agonist hydrocortisone (HC, 10^{-6}M) and cyclosporine A (CsA, 10^{-6}M) on the concentration of free, soluble IL-17 protein in conditioned medium from the extracellular compartment of a co-culture of CD3-positive spleen cells and adherent mononuclear BAL cells from mice, after stimulation with endotoxin (LPS, 100 ng/ml) *in vitro*. Data presented as mean \pm SEM (n=4).

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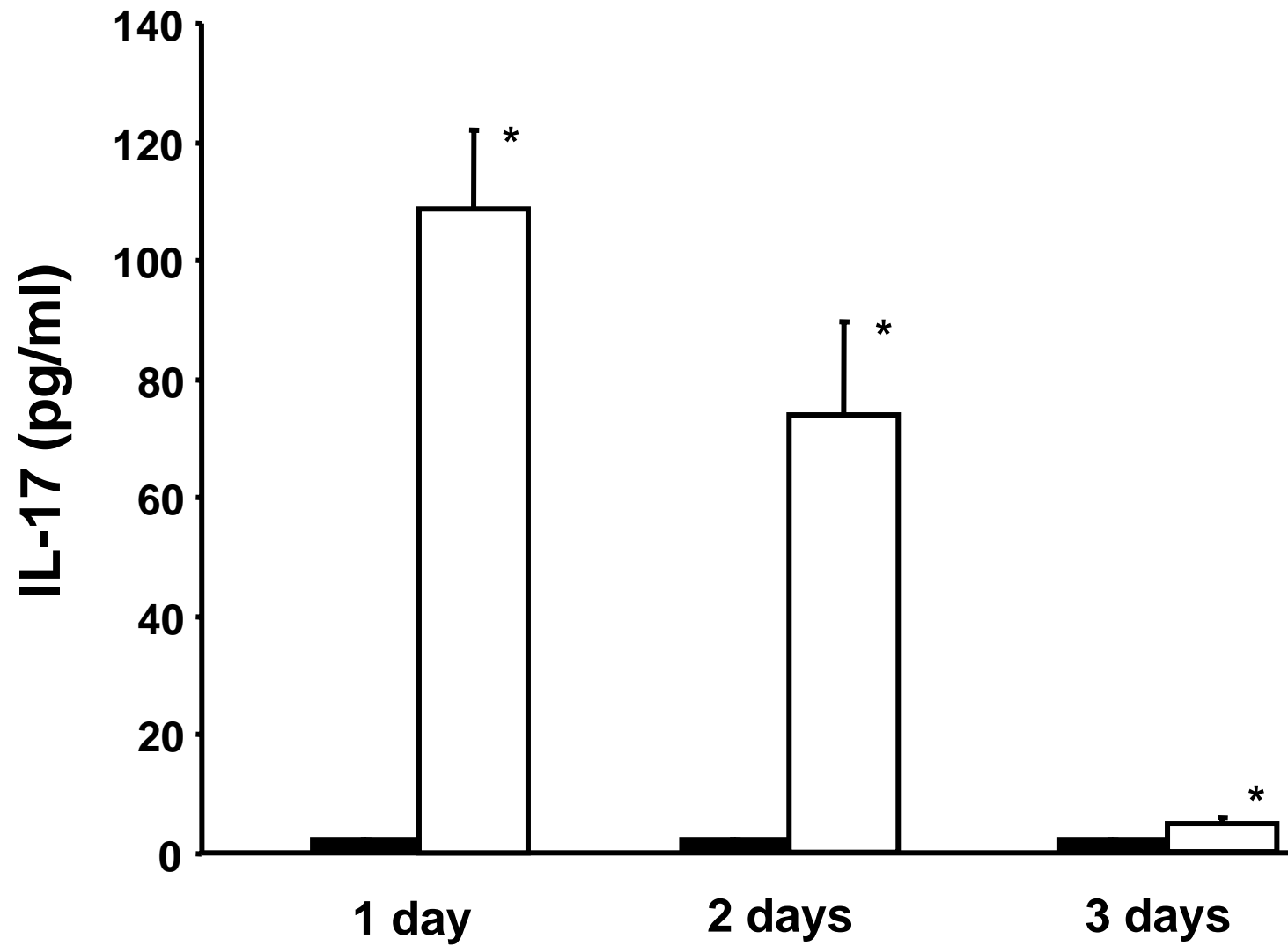
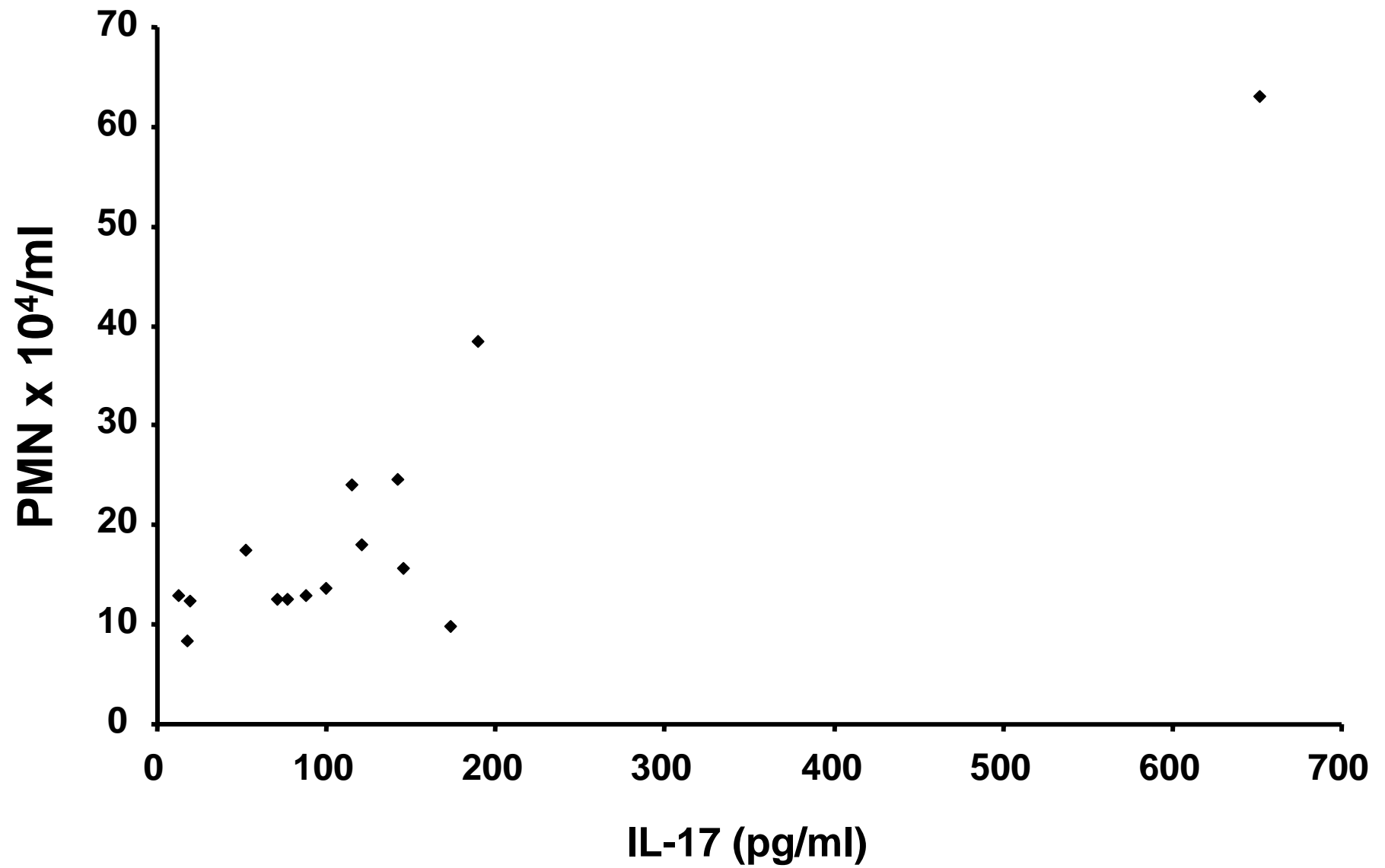
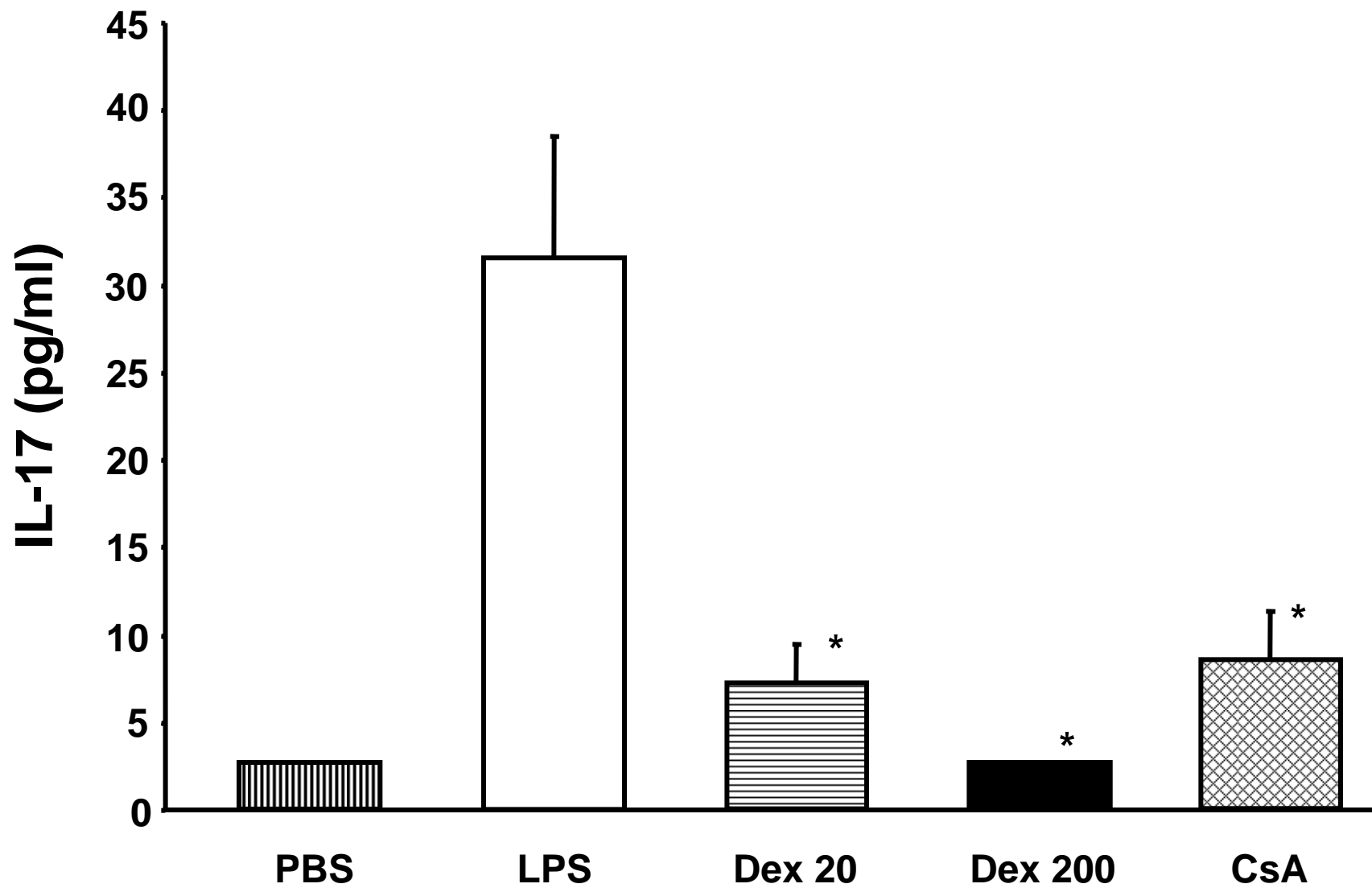


Figure 1

**Figure 2**

**Figure 3**

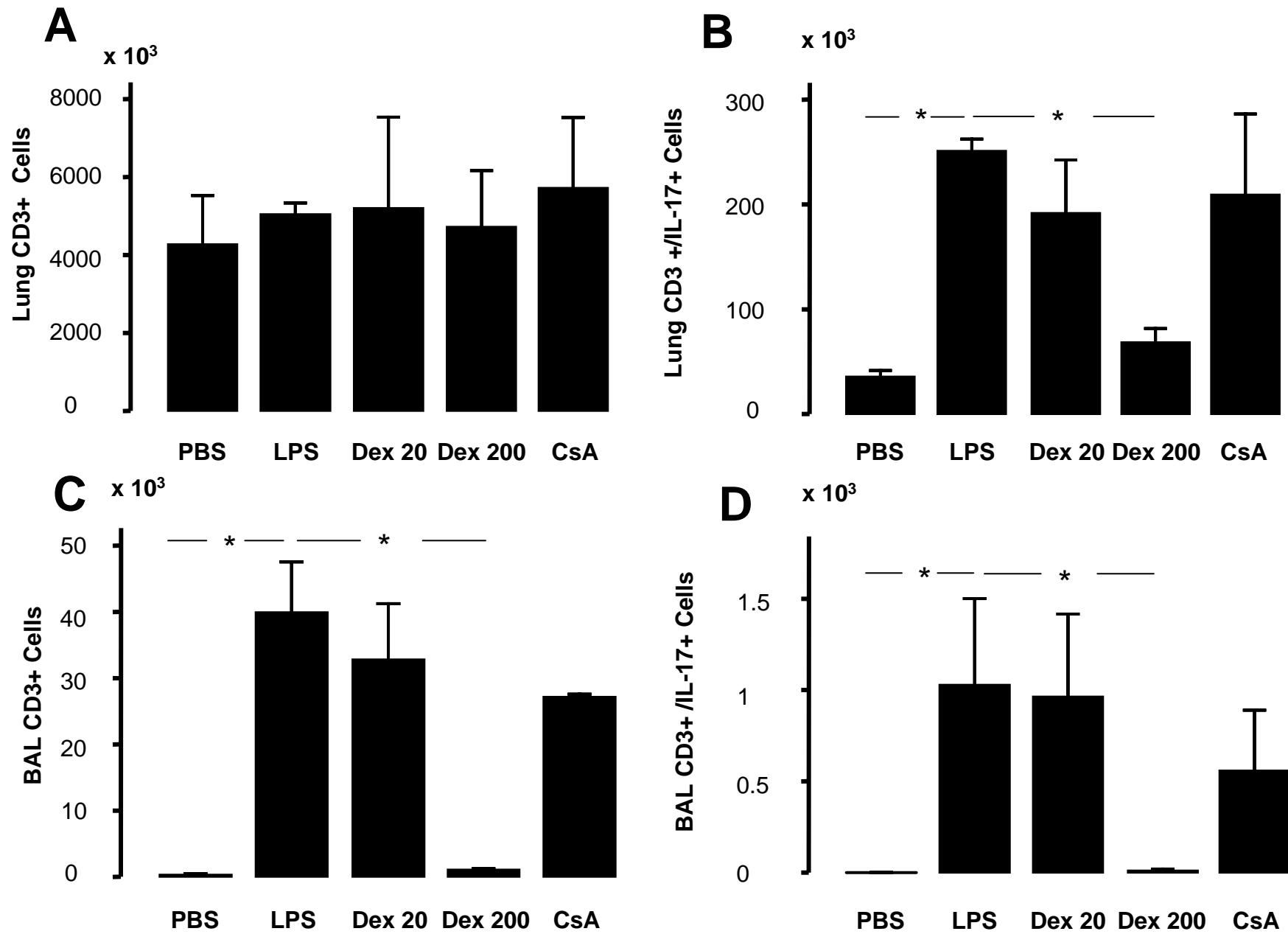
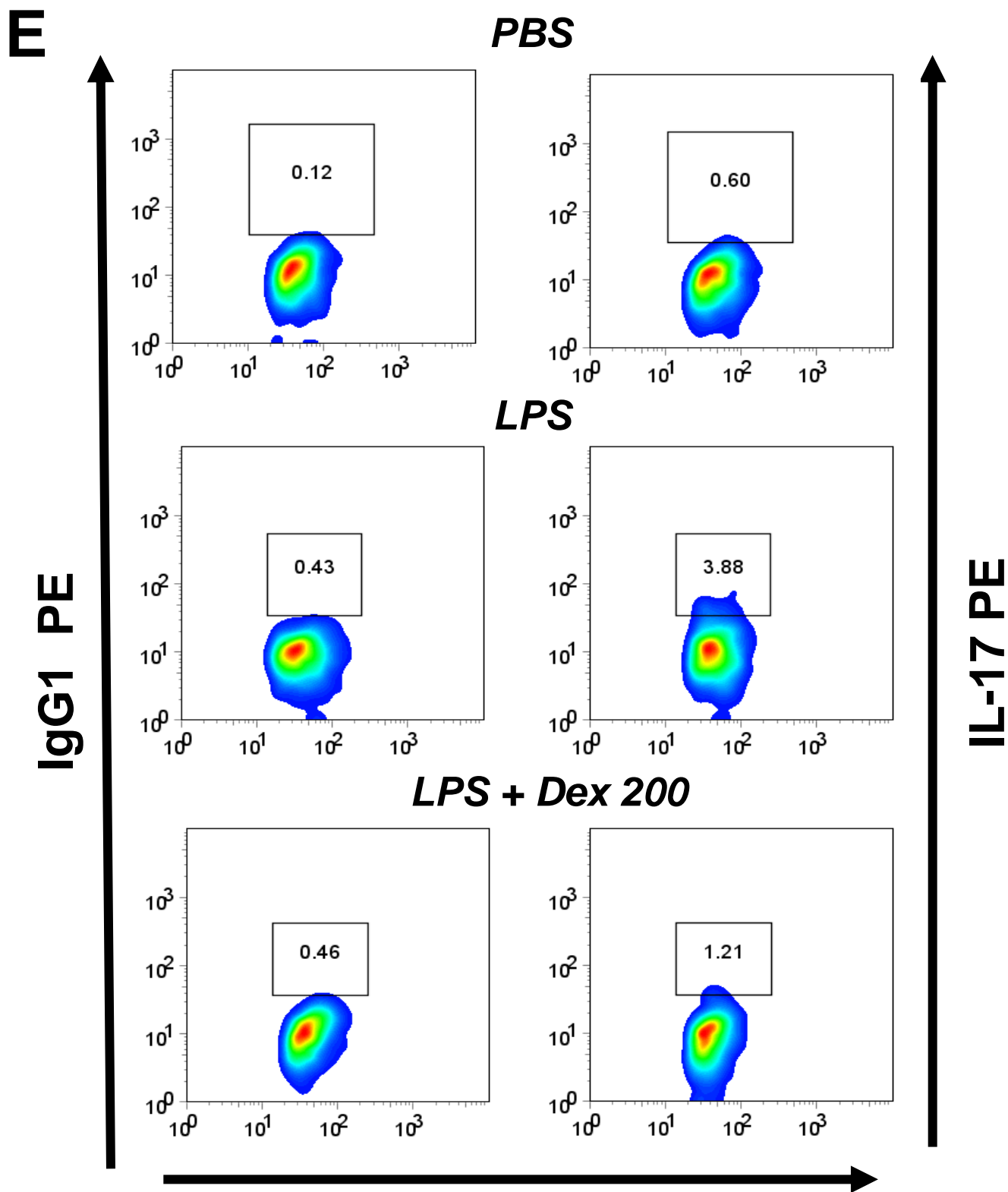
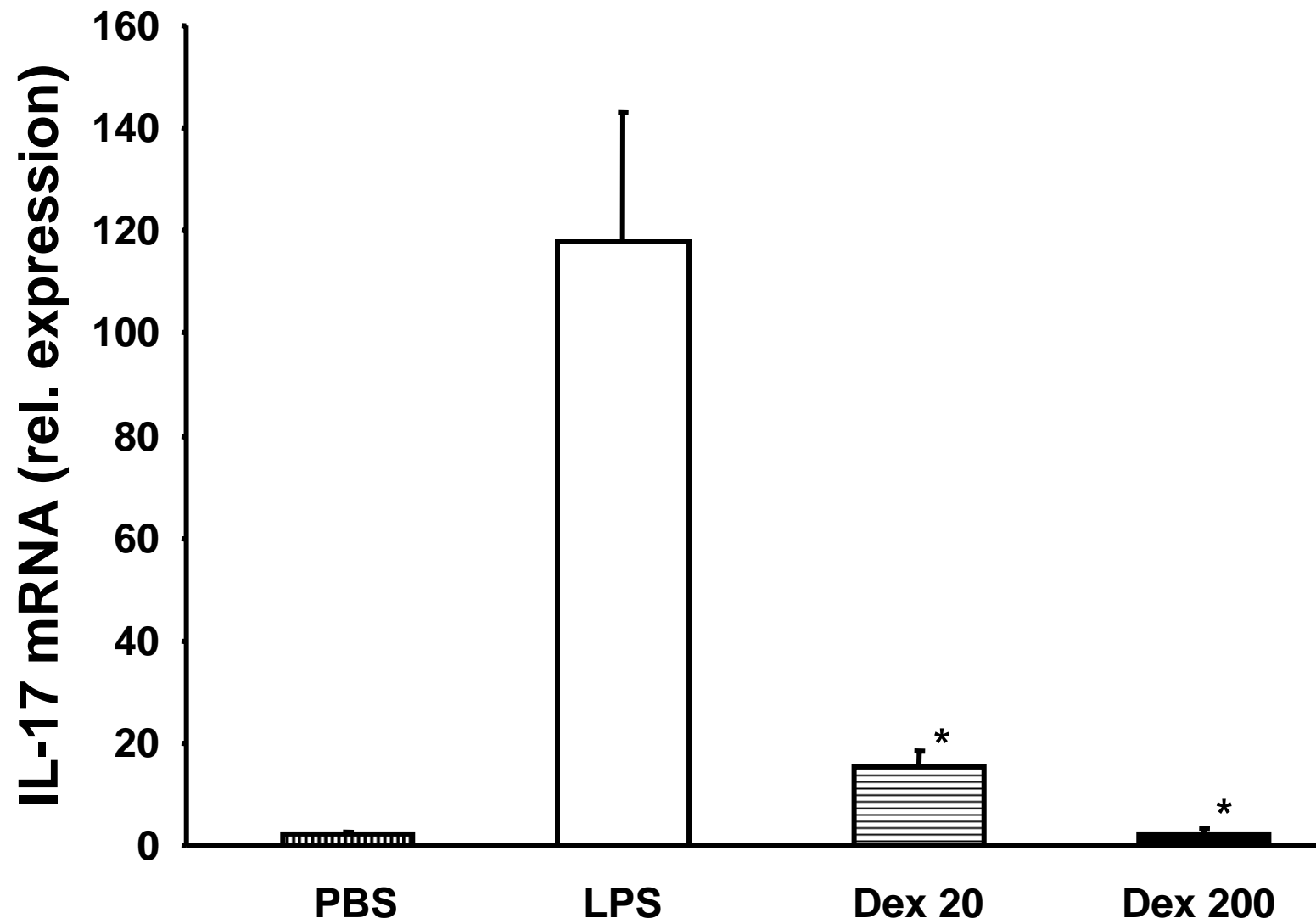
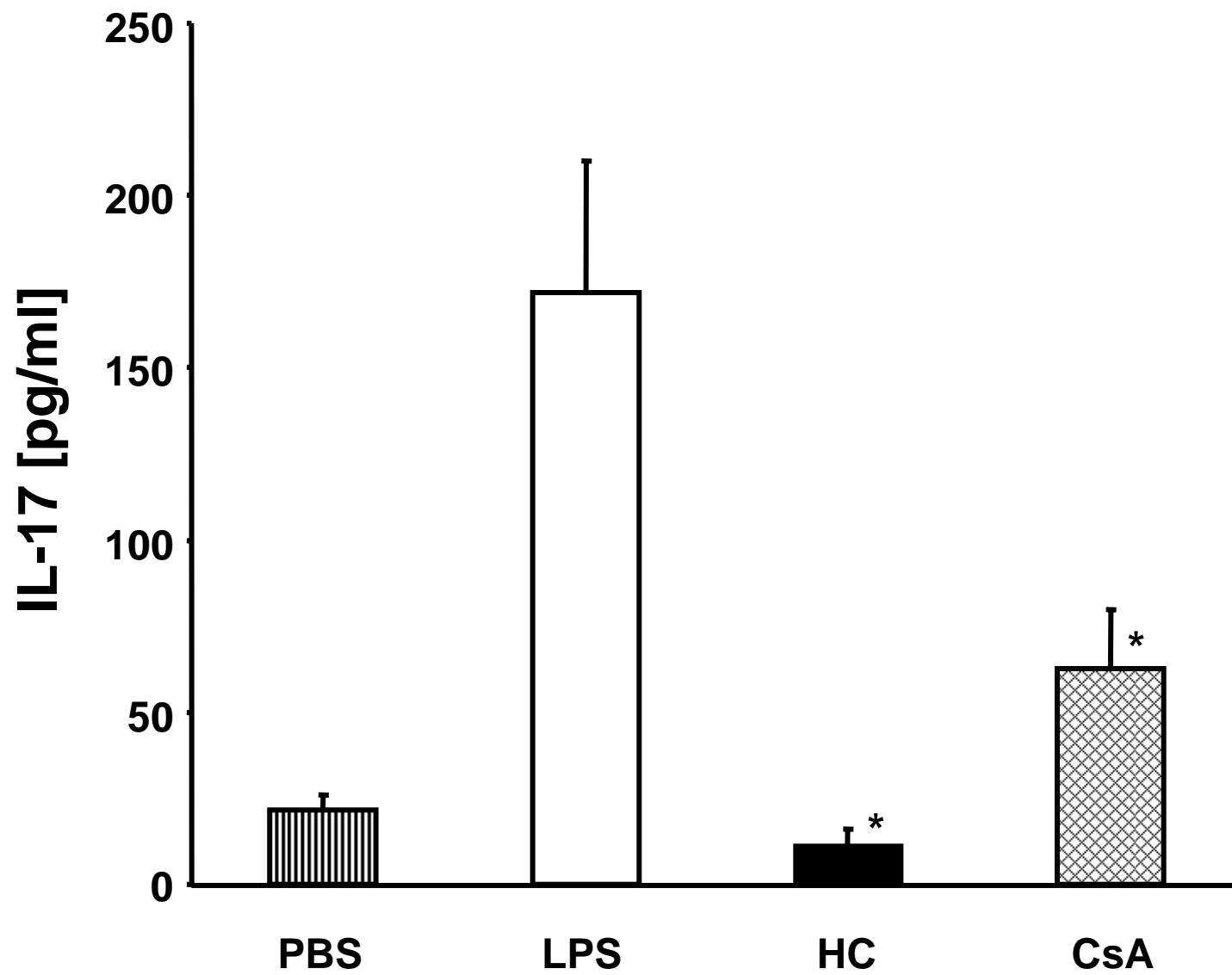


Figure 4



CD3 PercP
Figure 4e

**Figure 5**

**Figure 6**