

AMCoR

Asahikawa Medical College Repository <http://amcor.asahikawa-med.ac.jp/>

International Journal of Pediatric Otorhinolaryngology (2007) 71(2):297-305.

Effects of macrolides on antigen presentation and cytokine production by dendritic cells and T lymphocytes

Ishida, Yoshiya ; Abe, Yusuke ; Harabuchi, Yasuaki

I. Title page

Title: Effects of Macrolides on Antigen Presentation and Cytokine Production by Dendritic Cells and T lymphocytes.

Running Title: Effects of Macrolides on Dendritic Cells

Key words: Macrolides, Dendritic Cells, Human, T Lymphocytes, Immune responses

Affiliation: Asahikawa Medical College, Department of Otolaryngology-Head and Neck Surgery

Authors: Yoshiya Ishida, Yusuke Abe, Yasuaki Harabuchi

Mailing address for all authors:

Department of Otolaryngology-Head and Neck Surgery, Asahikawa Medical College, Midorigaoka

E2-1-1-1, Asahikawa, Hokkaido, 078-8510, Japan

Phone: +81-166-68-2554; Fax: +81-166-68-2559

Mailing address for correspondence and reprints:

Yoshiya Ishida: Department of Otolaryngology-Head and Neck Surgery, Asahikawa Medical

College, Midorigaoka E2-1-1-1, Asahikawa, Hokkaido, 078-8510, Japan

Phone: +81-166-68-2554; Fax: +81-166-68-2559

E-mail: ishida@asahikawa-med.ac.jp

II. Abstract

Macrolides are effective therapeutic agents for chronic respiratory tract diseases, such as chronic sinusitis, sinobronchial syndrome and diffuse panbronchiolitis. Although only limited information is available about their mechanisms, suppression of various inflammatory cytokines (IL-8, etc.) and some transcription factors has been reported to be involved. Non-typeable *Haemophilus influenzae* (NTHI) is one of the most important pathogens of the respiratory tract. P6 is one of the outer membrane proteins of NTHI and the target antigen of protective antibodies. To analyze the influence of macrolides on human dendritic cells (DCs), we treated DCs with macrolides and used them as antigen-presenting cells (APCs). Clarithromycin, roxithromycin and prednisolone suppressed the *in vitro* proliferative response of CD4⁺ T cells to P6 and also the production of cytokines. As a control, we also cultured DCs alone and exposed them to the medicament, while conversely culturing T cells without adding any drugs to the cultures. The results showed similar tendencies for suppression of immune responses. These findings suggest that macrolides suppress the antigen-specific immune responses of DCs *in vitro*.

III. Text

Introduction

It is thought that a vicious circle prevails in chronic respiratory tract inflammatory diseases such as chronic sinusitis and diffuse panbronchiolitis, involving infection by pathogenic microbes on the one hand and the body's immune response on the other. Pathogens disrupt the local defense mechanisms and thus become established locally, and this induces an immune response on the part of the host. Especially in the case of inflammation having IL-8 and neutrophils as key elements, the local defense mechanisms are further disrupted by esterases and active enzymes, the clearance of the pathogens is interfered with, and a vicious circle results [1].

Fourteen-member lactone-ring macrolide antimicrobial agents are used as immunomodulators in the treatment of such chronic respiratory tract inflammatory diseases as chronic sinusitis, sinobronchial syndrome and diffuse panbronchiolitis, etc., and they have been reported to be useful in this application [2-6]. It is said that low-dose long-term chemotherapy of macrolides for these chronic respiratory tract inflammatory diseases stops the excessive immune response of the host and makes it possible to eliminate the vicious circle. There have been multiple reports that the underlying mechanism of macrolides in this role is that they inhibit the production of IL-8 and various other inflammatory cytokines [7-11], while it has also been reported that macrolides show inhibitory effects at the levels of mRNA and NF- κ B and AP-1 transcription factors [12-17]. Iino et al. demonstrated that the expression of a costimulator of antigen-presenting

cells was inhibited by macrolides [18, 19]. In addition, Asano et al. showed that macrolides specifically inhibit Th2 cytokines [20, 21]. However, there remain many facets of the action mechanism underlying the anti-inflammatory effects of macrolides that are not fully understood.

Dendritic cells (DCs) are distributed in many tissues of the body, and it is known that they play an important role in inducing immune responses by carrying out antigen presentation to T cells. In addition, in recent years it has been shown that DCs are also involved in immunologic tolerance, and attention has thus been given to the aspect of DCs as immunoregulatory cells. It has also recently been shown that culture of CD14⁺ human peripheral blood monocytes in the presence of GM-SF and IL-4 for about 7 days is capable of inducing differentiation of DCs, and research on DCs themselves has become vigorous [22]. Nevertheless, there have still been few reports of studies of the effects of macrolides on human DCs.

Haemophilus influenzae is an important bacterial causative organism of chronic airway inflammatory diseases. One of the outer membrane proteins of this bacterium is the P6 protein, which is a commonly shared antigen of all *Haemophilus influenzae* strains. P6 is a target antigen for induction of infection-preventing antibodies, and it has also received much attention as the target of vaccine therapy [23-26].

The objective of the present study was to investigate the effects of macrolides on such antigen-specific immune responses of CD4⁺ T cells as their proliferative response and their cytokines production (IL-8 and Th1/2 cytokines). These investigations were carried out using DCs generated from peripheral blood and the P6 outer membrane protein of *Haemophilus influenzae*.

Methods

P6 was purified from NTHi 1479 strain by the previously described method with some modifications [27-29]. Endotoxin was not detected by TOXICOLOR[®] (SEIKAGAKU CORPORATION, Tokyo, Japan) in the purified P6 preparation.

DCs were used as antigen-presenting cells (APCs), which were generated from CD14-positive monocytes as previously reported [22]. PBMCs were isolated from the peripheral blood of five healthy male donors by gradient centrifugation (Ficoll Paque Plus[®]; Amersham Pharmacia Biotech, Piscataway, NJ, USA). CD14-positive monocytes were then purified from the PBMCs by positive immunoselection using an anti-CD14 antibody coupled onto magnetic microbeads (MACS[®]; Miltenyi Biotec, Auburn, CA, USA). The CD14-positive monocytes were cultured at 1×10^6 cells/ml in the presence of 50 ng/ml of IL-4 (PeproTec, London, UK) and 50 ng/ml of GM-CSF (PeproTec, London, UK) in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Equitech-bio, Ingram, TX, USA), 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml Penicillin G (PCG) and 100 µg/ml streptomycin. After 7 days of culture, the nonadherent cells (DCs) were harvested and used as APCs.

Clarithromycin (kindly provided by Taisho Pharmaceutical Co., Ltd.) and roxithromycin (a kind gift of Eisai Co., Ltd.) were employed as macrolides, while prednisolone (kindly provided by Shionogi & Co., Ltd.) was used as a control of immunosuppression.

After irradiation of 96-well plates with 4,000 rads, the DCs were dispensed into the wells at

5×10^3 /well, and then the test drugs were added to the wells in three concentrations of 10.0 $\mu\text{g/mL}$, 1.0 $\mu\text{g/mL}$ and 0.1 $\mu\text{g/mL}$. P6 protein was then added to each well at 10.0 $\mu\text{g/mL}$, and the plates were incubated in RPMI1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin 2×10^{-5} 2ME and 10% heat-inactivated human AB serum (SIGMA-ALDRICH, St. Louis, MO, USA) for 2 hours at 37°C in a 5% CO₂, humidified atmosphere. Next, CD4+ T cells that had been separately isolated by the magnetic beads method (MACS[®]; Miltenyi Biotec, Auburn, CA, USA) were added to each well (still containing the added drug and/or P6 protein), and the supernatants were collected after 48 h. The concentrations of IFN- γ , IL-4, IL-5, IL-8 in the supernatants were assayed by ELISA (Biosciences, San Diego, CA, USA).

After culture under each of the above sets of conditions for 7 more days, the incorporation of [3H]-thymidine (Amersham Pharmacia Biotech, Buckinghamshire, England) was measured in order to evaluate the proliferative response under each set of condition. Eighteen hours before harvest, 0.5 $\mu\text{Ci/well}$ [3H]-thymidine was added to each well. Then the lymphocytes were harvested on glass-fiber filters, and the incorporated radioactivity (cpm) of the harvested cells was measured by liquid scintillation counting.

For the ELISA and the determination of the proliferative response, the wells for each condition were prepared in triplicate, and the mean values were determined. The graphs show the error bars for the mean ± 1 SD of the specimens from the five donors. Paired two group t-test was used to compare mean values. In addition, for investigation of the effects of the drugs on the DCs, only DCs were incubated with one of the drugs for the first 48 hr, after which the drug was eliminated,

and then CD4+ T cells were added and investigated in the same manner as above.

Results

Figure 1 presents the bar graphs of the results for the measurements of the proliferative response. The mean CPM resulting from stimulation with P6 protein alone was 20230.8. In comparison with when no macrolide was added to the cell (lymphocytes + DCs) cultures, both roxithromycin and clarithromycin showed concentration-dependent inhibition of the proliferative response. Each of these macrolides showed statistically significant inhibition of the response when present at a concentration of 1.0 µg/mL or higher ($p < 0.05$). Addition of prednisolone at each of the tested concentrations also resulted in statistically significant concentration-dependent inhibition of the proliferative response ($p < 0.05$).

Figure 2 presents the results of assay of the IFN- γ concentration in the cell (CD4+ T cells+ DCs) cultures. Stimulation with P6 protein alone resulted in production of IFN- γ to a mean concentration of 2370.4 pg/mL, but this was significantly inhibited by each tested concentration of roxithromycin ($p < 0.05$). Clarithromycin showed statistically significant inhibition of IFN- γ production only when added at a concentration of 10 µg/mL ($p < 0.05$). As in the case of the proliferative response, prednisolone showed statistically significant concentration-dependent inhibition of the production of IFN- γ at each tested concentration ($p < 0.05$).

The IL-4 concentration are presented in Fig. 3. It is seen that stimulation with P6 protein alone resulted in almost no IL-4 production. The concentration of IL-4 was significantly increased

by the presence of clarithromycin at 10 µg/mL and also by prednisolone at 10 µg/mL ($p < 0.05$).

Roxithromycin did not induce a statistically significant change in the IL-4 production, but there was a tendency for IL-4 to be increased at each roxithromycin concentration.

Figure 4 presents the data for the IL-5 concentration in the cell (CD4+ T cells+ DCs) cultures. Although low in comparison with IFN- γ , stimulation with P6 protein alone induced IL-5 production to a mean concentration of 390.7 pg/mL. Both roxithromycin and clarithromycin at 10 µg/mL inhibited the production of IL-5 such that its concentration was below the limit of detection. Prednisolone also suppressed IL-5 production below the limit of detection at each of the concentrations at which it was added.

The IL-8 concentration in the cultures of lymphocytes + DCs are shown in Fig. 5. P6 stimulation resulted in mean production of 2818.9 pg/mL of IL-8. Both roxithromycin and clarithromycin showed concentration-dependent inhibition of the production of IL-8, each with statistical significance at 10 µg/mL. Prednisolone significantly inhibited IL-8 production when added at concentrations of 1.0 µg/mL and 10 µg/mL.

The drugs were added to cultures of only DCs, as well, and then the proliferative response (Fig. 6) and the productions of IFN- γ (Fig. 7) and IL-8 (Fig. 8) were investigated. No statistically significant differences were observed, but as with the above results, there were tendencies for the proliferative response and the productions of IFN- γ and IL-8 to be inhibited by the added drugs in a dose-dependent manner.

Discussion

Fourteen-member lactone-ring macrolide antimicrobial agents are employed in the treatment of chronic respiratory tract inflammatory diseases since they show immunomodulating activity, and their usefulness in that application has been reported [2-6]. With regard to the underlying mechanism of macrolides in this role, they have been reported to inhibit the production of mRNA for IL-8 and various other inflammatory cytokines [7-11], and there have also been reports that macrolides show inhibitory effects at the levels of the NF- κ B and AP-1 transcription factors [12-17]. There have been reports of studies of the effects of macrolides on Langerhans cells, epithelial cells and fibroblasts, but to date there have been few reports of studies involving the effects of these drugs on induced DCs. In addition, although the effects of macrolides on the immune responses of CD4⁺ T cells to nonspecific stimuli such as TCR (anti-CD3) and LPS have been reported [21], few studies have been carried out in relation to the immune response to actual cell components of causative bacteria. In consideration of this background, we designed the present experiments using DCs and the P6 protein to investigate the effects of macrolides on the antigen-specific immune responses of CD4⁺ T cells.

The macrolides we used were roxithromycin and clarithromycin, two so-called new macrolides. The new macrolides are said to have immunomodulatory activity [2-6]. We did not use erythromycin in this study, but it can be thought that it will be necessary to compare various drugs (roxithromycin, clarithromycin and other macrolides) with erythromycin in future studies. In addition, we selected prednisolone as a control drug showing inhibition of immune responses [7].

The concentrations of the macrolides and prednisolone that were used in this study were selected in consideration of the concentrations they show in the blood when administered orally to human patients. The usual clinical doses of these macrolides in low dose long-term chemotherapy are 150 mg in the case of roxithromycin and 200 mg for clarithromycin. In addition, the maximum plasma concentration (C_{max}) after oral administration of 150 mg of roxithromycin is 6.8 µg/mL [30], while that after clarithromycin 200 mg per os (po) is 1.16 µg/mL [31]. Accordingly, we selected three concentrations for use in this study: 0.1 µg/mL, 1.0 µg/mL and 10 µg/mL, for both roxithromycin and clarithromycin. Similarly, prednisolone administered po in a 5-mg dose yields a C_{max} of 0.105 µg/mL, while a 50-mg po dose shows a C_{max} of 0.66 µg/mL [32]. We thus decided to use prednisolone at the same three concentration levels as the two macrolides.

As an index of the immune response of CD4⁺ T cells, we evaluated the proliferative response by measuring the incorporation of [³H]-thymidine into the cells. Both roxithromycin and clarithromycin at concentrations of 1.0 µg/mL and higher showed statistically significant inhibition of the proliferative response in comparison with when no macrolide was added to the cell cultures. As noted above, the C_{max} of each drug was higher than 1.0 µg/mL, and it can be surmised that these drugs would show a sufficient inhibitory effect even when employed in a dose that is actually used.

In earlier studies carried out by our group, we demonstrated that when using DCs and stimulating CD4⁺ T cells with P6 protein, the T cells preferentially produced IFN-γ. Accordingly, in the present study we investigated the effects of macrolides on cytokine production by measuring

the T cells' and DCs' IFN- γ production using an ELISA technique. Roxithromycin was found to inhibit IFN- γ production at each of its added concentrations, whereas clarithromycin showed statistically significant inhibition of IFN- γ production only when added to the cell culture at a concentration of 10 $\mu\text{g}/\text{mL}$. The data for clarithromycin showed considerable scattering, resulting in statistically significant inhibition being found only for the 10- $\mu\text{g}/\text{mL}$ concentration, but as in the case of the proliferative response both of the macrolides showed dose-dependent inhibition of IFN- γ production. In addition, it has been reported that macrolides also affect the Th1/2 balance, and for this reason we assayed the productions of IL-4 and IL-5 by T cells as Th2 cytokines [20, 21]. In our earlier studies using DCs and stimulating CD4⁺ T cells with P6 protein, we found that almost no IL-4 was produced, while there was slight production of IL-5. In the present study, IL-5 showed a tendency to be inhibited by both roxithromycin and clarithromycin, whereas IL-4 production conversely showed a tendency to be increased. It might be thought that this increase in IL-4 can be explained as a relative acceleration of the Th2 response due to inhibition of the Th1 response. However, IL-5 is also a Th2 cytokine, and its production was inhibited, and this difference in the effects on IL-4 and IL-5 needs to be investigated in future studies. This phenomenon may not be changing the balance of Th1/Th2, and may be showing the immunomodulatory effects which became superfluous.

IL-8 is a cytokine that is produced by monocyte and macrophage lineages and is involved in the migration of neutrophils and T cells [11, 12, 15, 16]. Accordingly, under the conditions of our present studies, it can be surmised that IL-8 is produced mainly by the DCs. Both roxithromycin

and clarithromycin showed concentration-dependent inhibition of the production of IL-8, each with statistical significance at 10 µg/mL. Therefore, it can be surmised that macrolides possess activity by which they significantly inhibit the production of IL-8 by DCs. As was noted in our introductory comments to this paper, it can be thought that this inhibition of IL-8 production is extremely important from the viewpoint of disrupting the vicious circle considered to prevail in chronic respiratory tract inflammatory diseases.

These studies described above were carried out using our experimental system in which DCs and T cells are incubated in mixed culture and both are exposed to the actions of macrolides. As a control, we also cultured DCs alone and exposed them to the drugs, while conversely culturing T cells without adding any drugs to the cultures. No statistically significant differences were found between these two sets of conditions, although there were similar tendencies for inhibition of the immune response (i.e., proliferative response, IFN- γ and IL-8 production). These results indicate that macrolides also exert immunomodulatory effects directly on DCs, as well.

The following three conclusions can be drawn on the basis of the experimental results discussed above. (1) The immune responses of CD4⁺ T cells to stimulation by P6 protein are inhibited by macrolides. (2) Investigation of the effects of the macrolides on cytokines revealed a tendency for Th1 responses to be inhibited. (3) The macrolides showed a tendency to inhibit the immune responses of even DCs. In future studies it will be necessary to carry out a more detailed analysis of the mechanisms underlying the immunomodulatory effects of macrolides on DCs.

Conclusions

Studies were carried out to elucidate the immune responses of DCs and CD4+ T cells originating from healthy adult volunteers to the P6 protein when the cells were exposed to macrolides. Both roxithromycin and clarithromycin showed inhibition of the proliferative response of the CD4+ T cells to P6 protein that was similar to the inhibition shown by prednisolone. Each of the tested drugs showed concentration-dependent inhibition of the productions of IFN- γ , IL-5 and IL-8, but production of IL-4 was accelerated in a concentration-dependent manner by each of the drugs. The drugs showed a similar tendency to inhibit the immune responses of even the DCs when they were cultured alone, without CD4+ T cells.

References

- [1] Cole PJ. Microbial-host interactions in the airways in chronic respiratory infection. *Clin Ther* 1991;13:194-8.
- [2] Sawaki M, Mikami R, Mikasa K, Kunimatsu M, Ito S, Narita N. [The long term chemotherapy with erythromycin in chronic lower respiratory tract infections--second report: including cases with *Pseudomonas* infections]. *Kansenshogaku Zasshi* 1986;60:45-50.
- [3] Yamamoto M, Kondo A, Tamura M, Izumi T, Ina Y, Noda M. [Long-term therapeutic effects of erythromycin and newquinolone antibacterial agents on diffuse panbronchiolitis]. *Nihon Kyobu Shikkan Gakkai Zasshi* 1990;28:1305-13.
- [4] Kudoh S, Uetake T, Hagiwara K, Hirayama M, Hus LH, Kimura H, et al. [Clinical effects of low-dose long-term erythromycin chemotherapy on diffuse panbronchiolitis]. *Nihon Kyobu Shikkan Gakkai Zasshi* 1987;25:632-42.
- [5] Nagai H, Shishido H, Yoneda R, Yamaguchi E, Tamura A, Kurashima A. Long-term low-dose administration of erythromycin to patients with diffuse panbronchiolitis. *Respiration* 1991;58:145-9.
- [6] Fujii T, Kadota J, Kawakami K, Iida K, Shirai R, Kaseda M, et al. Long term effect of erythromycin therapy in patients with chronic *Pseudomonas aeruginosa* infection. *Thorax* 1995;50:1246-52.
- [7] Wallwork B, Coman W, Feron F, Mackay-Sim A, Cervin A. Clarithromycin and prednisolone

inhibit cytokine production in chronic rhinosinusitis. *Laryngoscope* 2002;112:1827-30.

[8] Iino Y, Toriyama M, Kudo K, Natori Y, Yuo A. Erythromycin inhibition of lipopolysaccharide-stimulated tumor necrosis factor alpha production by human monocytes in vitro. *Ann Otol Rhinol Laryngol Suppl* 1992;157:16-20.

[9] Takeshita K, Yamagishi I, Harada M, Otomo S, Nakagawa T, Mizushima Y. Immunological and anti-inflammatory effects of clarithromycin: inhibition of interleukin 1 production of murine peritoneal macrophages. *Drugs Exp Clin Res* 1989;15:527-33.

[10] Fujita K, Shimizu T, Majima Y, Sakakura Y. Effects of macrolides on interleukin-8 secretion from human nasal epithelial cells. *Eur Arch Otorhinolaryngol* 2000;257:199-204.

[11] Suzuki H, Shimomura A, Ikeda K, Furukawa M, Oshima T, Takasaka T. Inhibitory effect of macrolides on interleukin-8 secretion from cultured human nasal epithelial cells. *Laryngoscope* 1997;107:1661-6.

[12] Abe S, Nakamura H, Inoue S, Takeda H, Saito H, Kato S, et al. Interleukin-8 gene repression by clarithromycin is mediated by the activator protein-1 binding site in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2000;22:51-60.

[13] Desaki M, Takizawa H, Ohtoshi T, Kasama T, Kobayashi K, Sunazuka T, et al. Erythromycin suppresses nuclear factor-kappaB and activator protein-1 activation in human bronchial epithelial cells. *Biochem Biophys Res Commun* 2000;267:124-8.

[14] Mukaida N, Gussella GL, Kasahara T, Ko Y, Zachariae CO, Kawai T, et al. Molecular analysis of the inhibition of interleukin-8 production by dexamethasone in a human fibrosarcoma cell line.

Immunology 1992;75:674-9.

[15] Mukaida N, Morita M, Ishikawa Y, Rice N, Okamoto S, Kasahara T, et al. Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-kappa B is target for glucocorticoid-mediated interleukin 8 gene repression. *J Biol Chem* 1994;269:13289-95.

[16] Okamoto S, Mukaida N, Yasumoto K, Rice N, Ishikawa Y, Horiguchi H, et al. The interleukin-8 AP-1 and kappa B-like sites are genetic end targets of FK506-sensitive pathway accompanied by calcium mobilization. *J Biol Chem* 1994;269:8582-9.

[17] Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2001;2:675-80.

[18] Iino Y, Sasaki Y, Kojima C, Miyazawa T. Effect of macrolides on the expression of HLA-DR and costimulatory molecules on antigen-presenting cells in nasal polyps. *Ann Otol Rhinol Laryngol* 2001;110:457-63.

[19] Keicho N, Kudoh S, Yotsumoto H, Akagawa KS. Antilymphocytic activity of erythromycin distinct from that of FK506 or cyclosporin A. *J Antibiot (Tokyo)* 1993;46:1406-13.

[20] Asano K, Kamakazu K, Hisamitsu T, Suzaki H. Modulation of Th2 type cytokine production from human peripheral blood leukocytes by a macrolide antibiotic, roxithromycin, in vitro. *Int Immunopharmacol* 2001;1:1913-21.

[21] Seto H, Asano K, Susaki H. [Inhibitory effect of roxithromycin, a macrolide antibiotic, on Th2 cytokine production]. *Jpn J Antibiot* 2001;54 Suppl C:76-9.

[22] Kobayashi H, Wood M, Song Y, Appella E, Celis E. Defining promiscuous MHC class II

helper T-cell epitopes for the HER2/neu tumor antigen. *Cancer Res* 2000;60:5228-36.

[23] Harabuchi Y, Faden H, Yamanaka N, Duffy L, Wolf J, Krystofik D. Human milk secretory IgA antibody to nontypeable *Haemophilus influenzae*: possible protective effects against nasopharyngeal colonization. *J Pediatr* 1994;124:193-8.

[24] Harabuchi Y, Faden H, Yamanaka N, Duffy L, Wolf J, Krystofik D. Nasopharyngeal colonization with nontypeable *Haemophilus influenzae* and recurrent otitis media. Tonawanda/Williamsville Pediatrics. *J Infect Dis* 1994;170:862-6.

[25] Kodama H, Faden H, Harabuchi Y, Kataura A, Bernstein JM, Brodsky L. Cellular immune response of adenoidal and tonsillar lymphocytes to the P6 outer membrane protein of non-typeable *Haemophilus influenzae* and its relation to otitis media. *Acta Otolaryngol* 1999;119:377-83.

[26] Abe Y, Murphy TF, Sethi S, Faden HS, Dmochowski J, Harabuchi Y, et al. Lymphocyte proliferative response to P6 of *Haemophilus influenzae* is associated with relative protection from exacerbations of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2002;165:967-71.

[27] Karalus RJ, Murphy TF. Purification and characterization of outer membrane protein P6, a vaccine antigen of non-typeable *Haemophilus influenzae*. *FEMS Immunol Med Microbiol* 1999;26:159-66.

[28] Munson RS, Jr., Granoff DM. Purification and partial characterization of outer membrane proteins P5 and P6 from *Haemophilus influenzae* type b. *Infect Immun* 1985;49:544-9.

[29] Badr WH, Loghmanee D, Karalus RJ, Murphy TF, Thanavala Y. Immunization of mice with

P6 of nontypeable Haemophilus influenzae: kinetics of the antibody response and IgG subclasses.

Vaccine 1999;18:29-37.

[30] Koyama M, Tateno M, Shiotsuka M, Yamamoto T, Hirayama M, Saitoh K, et al. Absorption, metabolism and excretion of RU 28965 in humans. Chemotherapy 1988;36:164-83.

[31] Suwa T, Urano H, Kodama T, Nakamura M. Metabolic fate of TE-031 (A-56268) (VIII). Chemotherapy 1988;36:921-32.

[32] Tanaka T, Miyachi Y. Measurement of synthetic steroid, and meaning on clinical. Rinsho 1978;4:822-23.

Figure Legends

Fig. 1: The *in vitro* proliferative responses of CD4⁺ T cells, which were stimulated with dendritic cells and P6 protein, were analyzed. The mean CPM with P6 protein but without any drug was 20230.8. The proliferative response was suppressed by clarithromycin and roxithromycin compared to without any macrolide, in a concentration-dependent manner. With both macrolides, the CPM was significantly suppressed at 1.0 µg/mL or more ($p < 0.05$). The proliferative response was significantly suppressed by prednisolone at each concentration, in a concentration-dependent manner ($p < 0.05$).

Fig. 2: IFN- γ production by mixed CD4⁺/DC cultures in the presence of clarithromycin, roxithromycin and prednisolone was analyzed compared to without any drug. The average production of IFN- γ was 2370.4 pg/mL without any drug. That production of IFN- γ was suppressed by roxithromycin at each tested concentration ($p < 0.05$). With clarithromycin the production was suppressed only at 10 µg/mL ($p < 0.05$). The production of IFN- γ was suppressed by prednisolone at each concentration, in a concentration-dependent manner ($p < 0.05$).

Fig. 3: IL-4 production by mixed CD4⁺/DC cultures in the presence of clarithromycin, roxithromycin and prednisolone was analyzed compared to without any drug. IL-4 production hardly occurred in response to P6 protein stimulation without any drug, but IL-4 was increased

with 10- μ g/mL clarithromycin ($p<0.05$) and 10- μ g/ml prednisolone ($p<0.05$). Although a significant difference was not shown with roxithromycin, there was a tendency for IL-4 production to increase with the concentration.

Fig. 4: IL-5 production by mixed CD4⁺/DC cultures in the presence of clarithromycin, roxithromycin and prednisolone was analyzed compared to without any drug. The average IL-5 production with P6 protein stimulation without any drug was 390.7 pg/ml. With 10- μ g/mL roxithromycin and 10- μ g/mL clarithromycin, the production of IL-5 was suppressed, and it was below the limit of detection. In the presence of prednisolone, production of IL-5 was suppressed at each concentration, and it was below the limit of detection.

Fig. 5: IL-8 production by mixed CD4⁺/DC cultures in the presence of clarithromycin, roxithromycin and prednisolone was analyzed compared to without any drug. The average IL-8 production with P6 protein stimulation without any drug was 2818.9 pg/mL. With 10 μ g/mL clarithromycin and 10 μ g/mL roxithromycin, production of IL-8 was reduced in a concentration-dependent manner, with statistical significance ($p<0.05$). In presence of prednisolone (10 μ g/mL and 1.0 μ g/mL), production of IL-8 was reduced in a concentration-dependent manner ($p<0.05$).

Fig. 6: The *in vitro* proliferative response of CD4⁺ T cells, which were stimulated with DCs and P6

protein, was analyzed (only the dendritic cells were exposed to drugs). The CPM average with P6 protein but without any drug was 4793.7. The proliferative response was suppressed in the presence of DCs, which had been exposed to clarithromycin and roxithromycin, compared to without any drug; this suppression was concentration-dependent. These proliferative responses (CPM) were suppressed in dose dependent manner (n.s.), respectively.

Fig. 7: IFN- γ production by mixed CD4+/DC cultures was analyzed in the presence of clarithromycin, roxithromycin and prednisolone compared to without any drug (only the dendritic cells were exposed to drugs). The average production of IFN- γ was 2121.5 pg/mL in without any drug. That production of IFN- γ was suppressed at each clarithromycin concentration (n.s.). The production of IFN- γ was suppressed at each prednisolone concentration and showed concentration dependence (n.s.).

Fig. 8: IL-8 production by mixed CD4+/DC cultures was analyzed in the presence of clarithromycin, roxithromycin and prednisolone compared to without any drug (only the dendritic cells were exposed to drugs). The average IL-8 production with P6 protein stimulation but without any drug was 2060.7 pg/mL. In presence of clarithromycin, roxithromycin and prednisolone, production of IL-8 was reduced in a concentration-dependent manner (n.s.).

Fig. 1

Proliferative assay

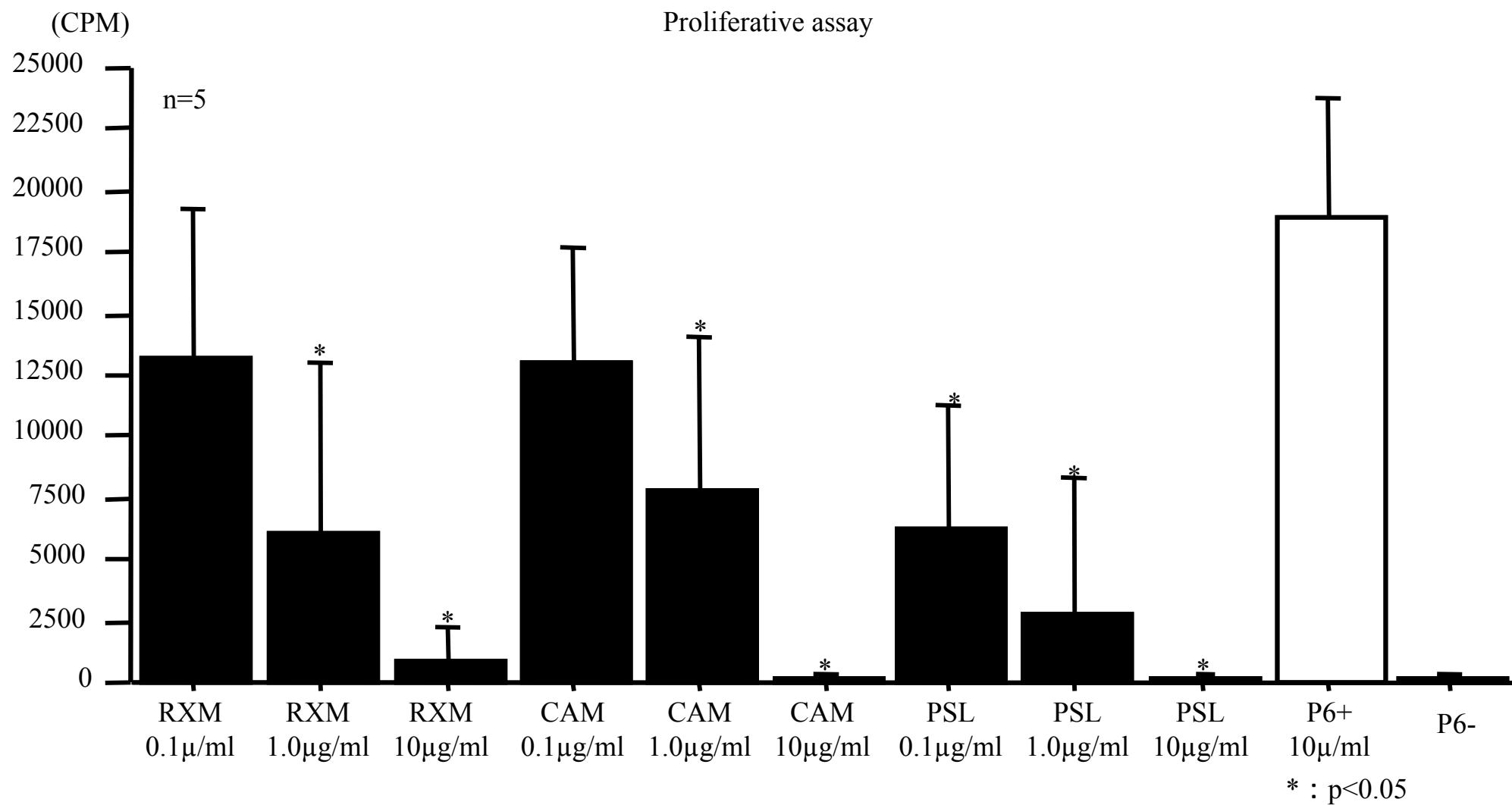


Fig. 2

IFN- γ

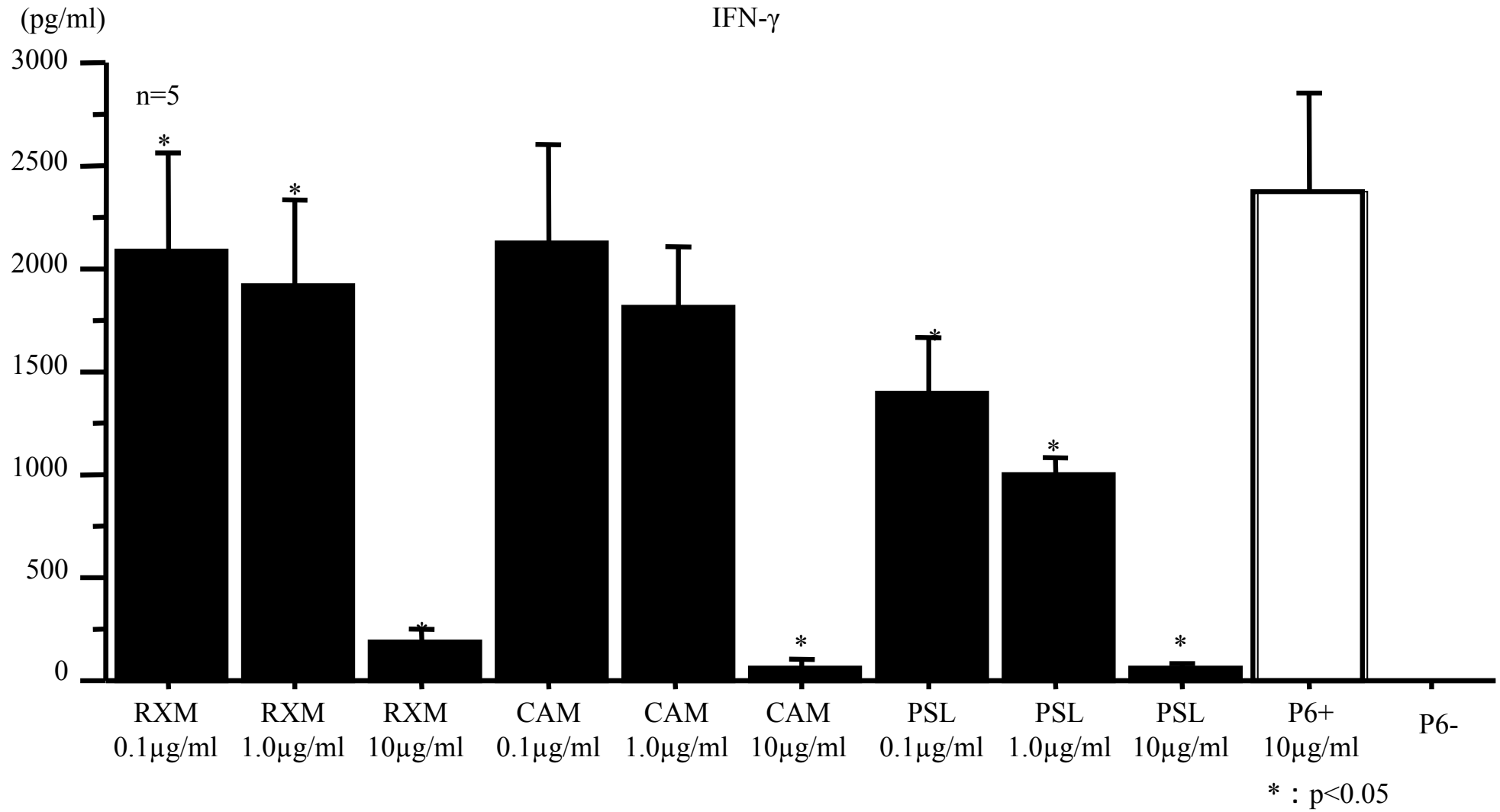


Fig. 3

IL-4

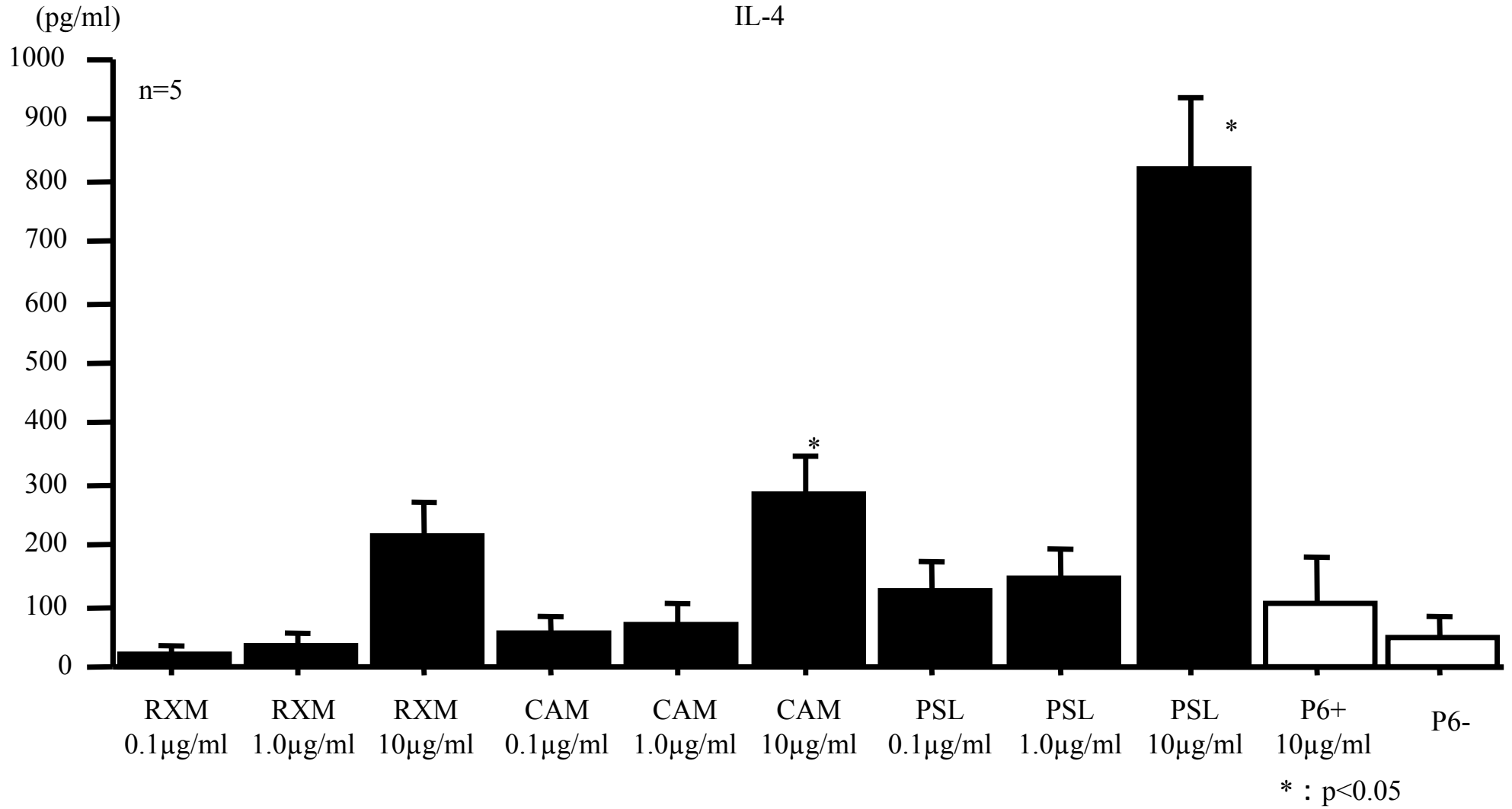


Fig. 4

IL-5

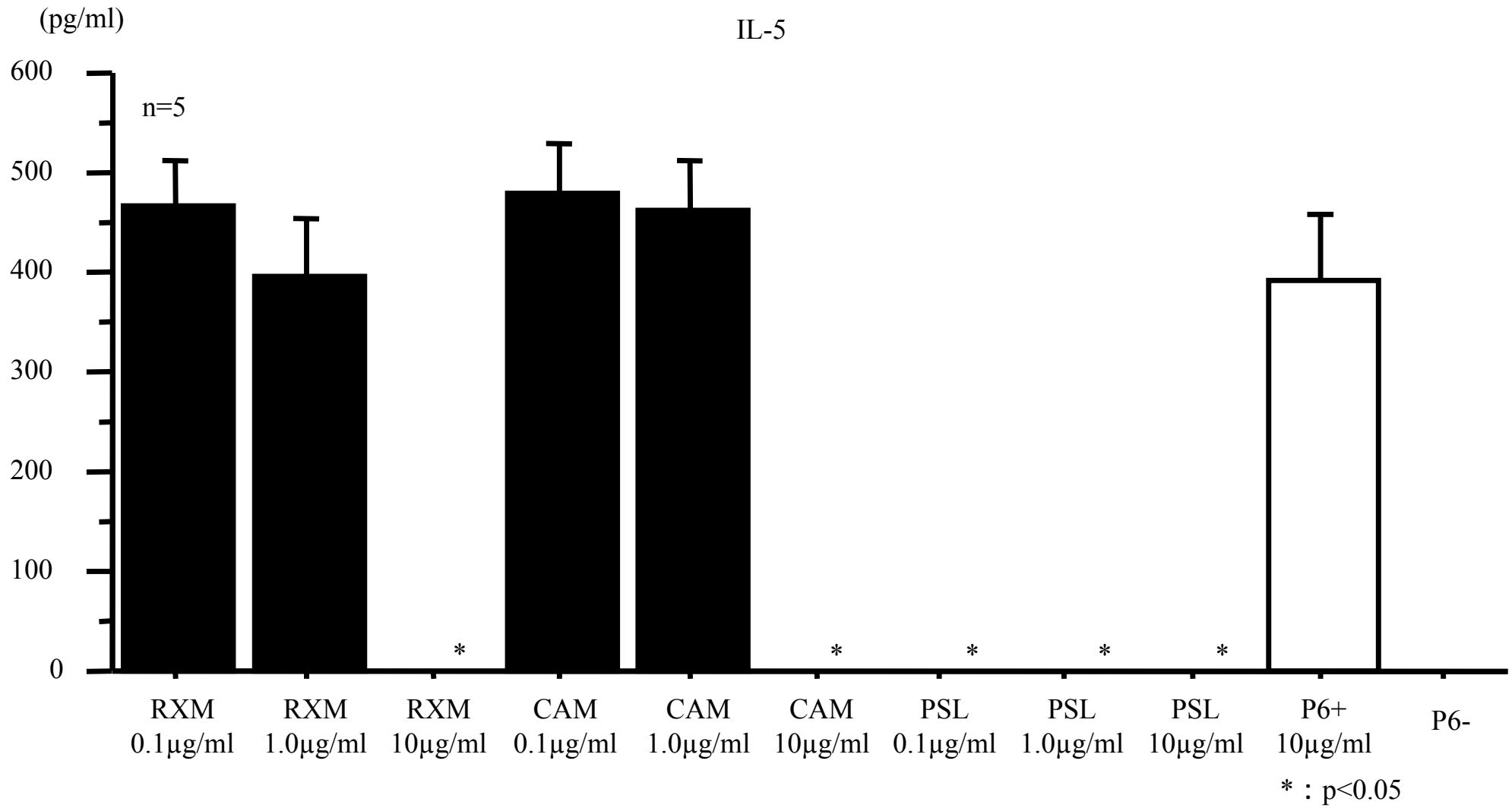


Fig. 5

IL-8

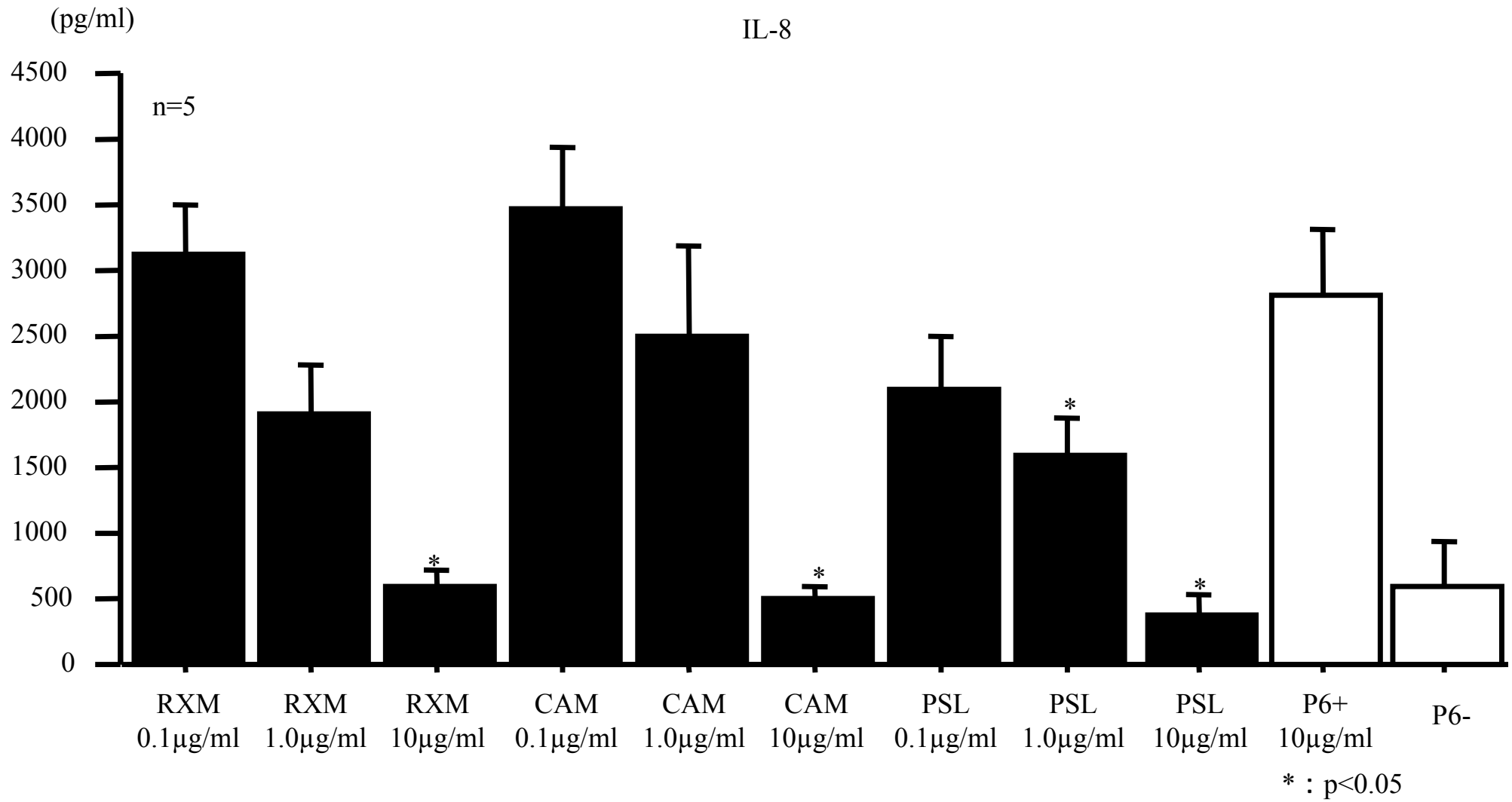
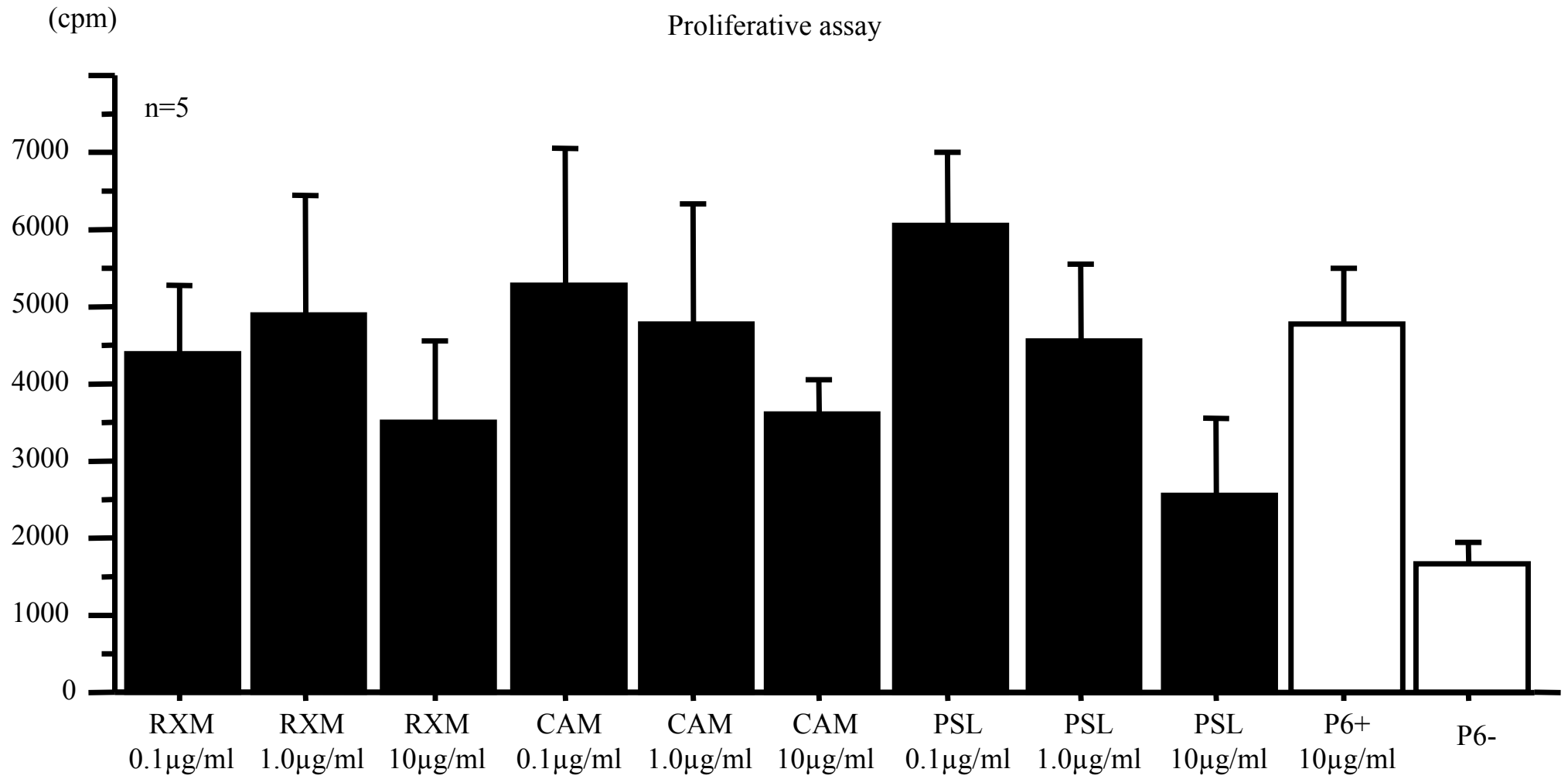


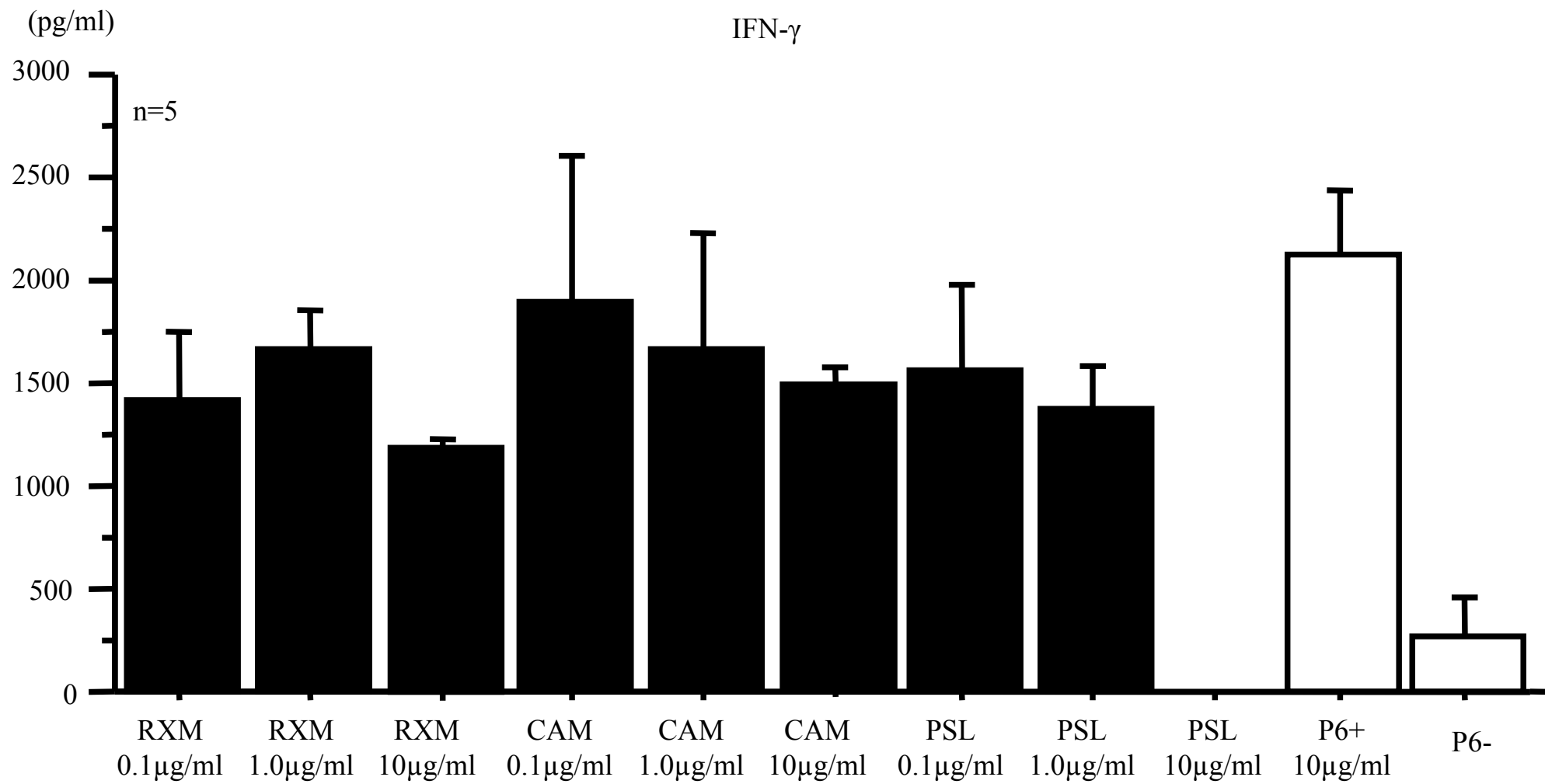
Fig. 6



* : p<0.05

Fig. 7

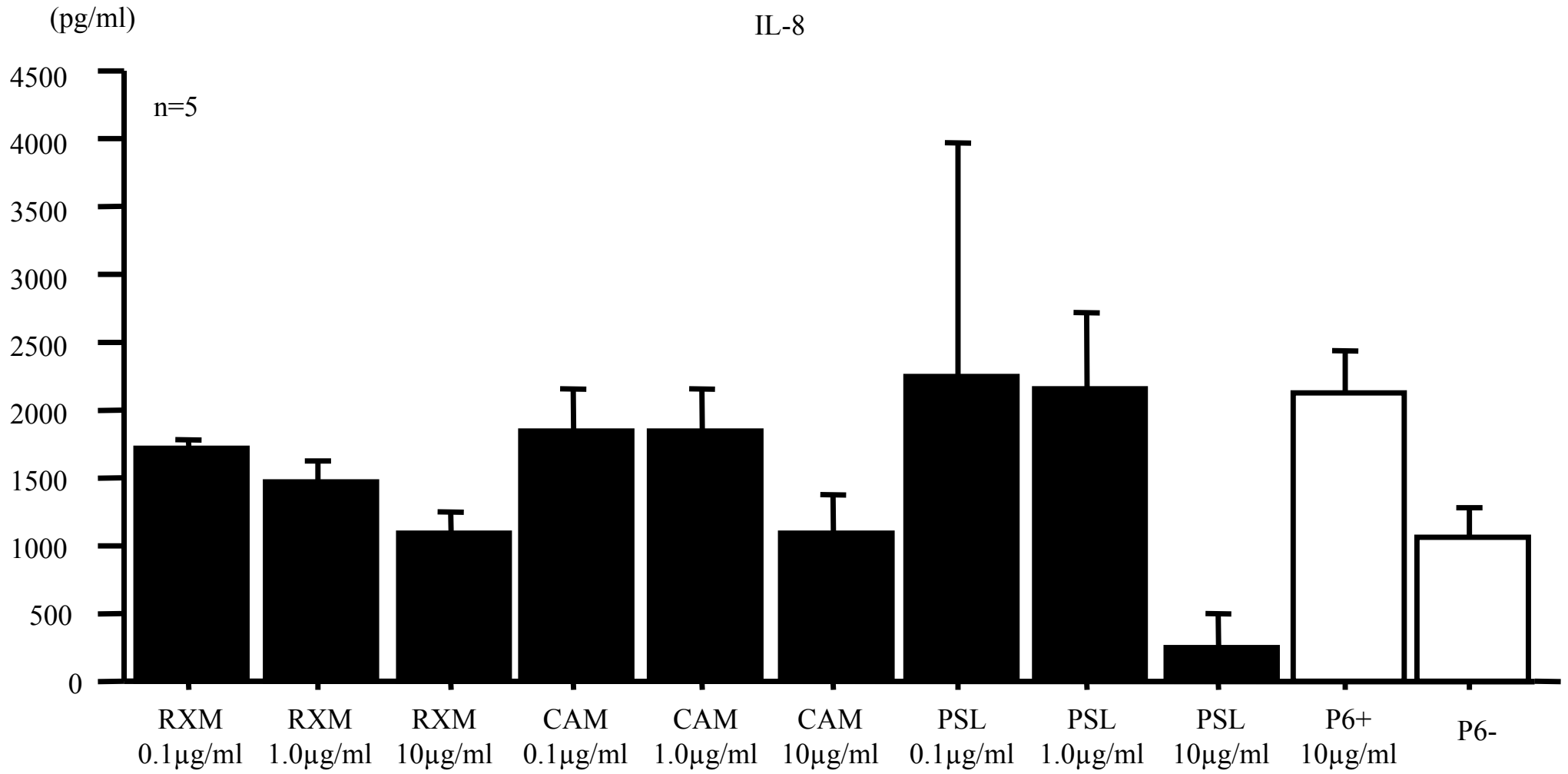
IFN- γ



* : p<0.05

Fig. 8

IL-8



* : p<0.05