

ORIGINAL ARTICLE

A low dose of *Mycoplasma pneumoniae* infection enhances an established allergic inflammation in mice: the role of the prostaglandin E₂ pathway

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Summary

Background Over 40% of chronic stable asthma patients have evidence of respiratory *Mycoplasma pneumoniae* (Mp) infection as detected by PCR, but not by serology and culture, suggesting that a low-level Mp is involved in chronic asthma. However, the role of such a low-level Mp infection in the regulation of allergic inflammation remains unknown.

Objective To determine the impact of a low-level Mp infection in mice with established airway allergic inflammation on allergic responses such as eosinophilia and chemokine eotaxin-2, and the underlying mechanisms [i.e. the prostaglandin E₂ (PGE₂) pathway] since PGE₂ inhalation before an allergen challenge suppressed the eosinophil infiltration in human airways.

Methods BALB/c mouse models of ovalbumin (OVA)-induced allergic asthma with an ensuing low- or high-dose Mp were used to assess IL-4 expression, bronchoalveolar lavage (BAL) eosinophil, eotaxin-2 and PGE₂ levels, and lung mRNA levels of microsomal prostaglandin E synthase-1 (mPGES-1). Primary alveolar macrophages (pAMs) from naïve BALB/c mice were cultured to determine whether Mp-induced PGE₂ or exogenous PGE₂ down-regulates IL-4/IL-13-induced eotaxin-2.

Results Low-dose Mp in allergic mice significantly enhanced IL-4 and eotaxin-2, and moderately promoted lung eosinophilia, whereas high-dose Mp significantly reduced lung eosinophilia and tended to decrease IL-4 and eotaxin-2. Moreover, in both OVA-naïve and allergic mice, lung mPGES-1 mRNA and BAL PGE₂ levels were elevated in mice infected with high-dose, but not low-dose Mp. In pAMs, IL-4/IL-13 significantly increased eotaxin-2, which was reduced by Mp infection accompanied by dose-dependent PGE₂ induction. Exogenous PGE₂ inhibited IL-4/IL-13-induced eotaxin-2 in a dose-dependent manner.

Conclusions This study highlights a novel concept on how different bacterial loads in the lung modify the established allergic airway inflammation and thus interact with an allergen to further induce Th2 responses. That is, unlike high-level Mp, low-level Mp fails to effectively induce PGE₂ to down-regulate allergic responses (e.g. eotaxin-2), thus maintaining or even worsening allergic inflammation in asthmatic airways.

Keywords alveolar macrophages, asthma, eotaxin-2, *Mycoplasma pneumoniae*, PGE₂

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Introduction

Mycoplasma pneumoniae (Mp) has been recognized as a contributing factor in both stable asthma and asthma exacerbations [1–6]. Our previous clinical study has demonstrated that 42% of chronic stable asthma patients had evidence of airway Mp infection detected by means of PCR. However, serology (IgG and IgM) and mycoplasma culture in airway biopsy specimens from the Mp-PCR-

positive asthmatic patients were negative, suggesting that a subset of chronic asthma patients was associated with a low-level Mp infection [1]. However, the impact of such a persistent low-level Mp infection on allergic responses has not been investigated.

Airway inflammation in asthma is skewed towards a T-helper type 2 (Th2) immune response involving Th2 cytokines (e.g. IL-4 and IL-13), chemokines (e.g. eotaxins) and various inflammatory cells such as eosinophils [7, 8].

To date, three eotaxins (eotaxin-1, -2 and -3) have been identified in humans [9–14]. However, only eotaxin-1 and -2 have been found in mice [15]. Elevated eotaxin-1, -2 and -3 are associated with an allergen-induced eosinophilia in asthmatic airways [16–21]. Eotaxin-2-deficient mice have been used to prove the critical role of eotaxin-2 in eosinophil recruitment following allergen challenges [22]. Alveolar macrophages are at the very front of host innate immune responses to environmental insults such as infections. Besides its role in lung defence against pathogens, innate immunity modifies allergic responses [23]. However, how alveolar macrophages bridge innate immunity and allergic response is unclear. For example, it remains to be established whether alveolar macrophages can generate eotaxin-2, thus contributing to an eosinophil infiltration in allergic airways.

Prostaglandins can be induced in various biological processes including an early response to allergen challenge and infection [24–27]. As the most abundant prostaglandin, prostaglandin E_2 (PGE_2) production starts with the release of arachidonic acid (AA) from membrane phospholipids by phospholipase A_2 and AA is converted to PGH_2 by cyclooxygenases COX-1 and COX-2. Then, specific PGE synthases (PGES) convert PGH_2 into different prostaglandins – PGE_2 , D_2 , F_{2a} , and I_2 . Microsomal PGES-1 (mPGES-1) is the only inducible and most abundant isozyme of PGES expressed in lung resident cells including macrophages [28–32]. PGE_2 inhalation before an allergen challenge suppressed allergic inflammation including eosinophil infiltration in human airways [33–35]. However, the mechanisms by which PGE_2 inhibits allergic responses are poorly understood.

In the current study, we hypothesized that a high-level Mp induces PGE_2 production in allergic lungs, which in turn suppresses allergic inflammation. In contrast, a low-level Mp would fail to induce PGE_2 , thus maintaining or even worsening allergic inflammation. Therefore, we determined the effects of low- and high-dose Mp infections in ovalbumin (OVA)-challenged mice on airway allergic inflammation including eosinophils, eotaxin-2 and the PGE_2 pathway. Furthermore, we cultured mouse primary alveolar macrophages (pAMs) to determine whether Mp-induced PGE_2 or exogenous PGE_2 down-regulates IL-4/IL-13-induced eotaxin-2 production.

Materials and methods

Mice

Female wild-type BALB/c mice (8–12 weeks) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All the mice were quarantined for 1 week before the experiments and bled to establish that they were free from virus and *M. pulmonis*. The experimental procedures

were approved by our Institutional Animal Care and Use Committee.

Mycoplasma pneumoniae preparation

Mp (strain FH; American Type Culture Collection 15531, Manassas, VA, USA) was grown in SP-4 broth (Remel, Lenexa, KS, USA) for 5 days at 37 °C, 5% CO_2 . The adherent Mp was harvested and prepared as described previously [36]. Mp was re-suspended in saline to yield the indicated concentrations for intranasal inoculation in mice, or in phosphate-buffered saline (PBS) for infecting alveolar macrophages *in vitro*.

Mycoplasma pneumoniae infection in an established mouse allergic asthma model

BALB/c mice were intraperitoneally (i.p.) sensitized twice in a span of 14 days by injection of 20 µg of OVA (Sigma-Aldrich, St Louis, MO, USA) emulsified in 2.25 mg of aluminium hydroxide (Imject[®] Alum; Pierce, Rockford, IL, USA). Two weeks after the last OVA sensitization, mice were placed in a Plexiglas chamber (Ridout Plastics Company Inc., San Diego, CA, USA) and challenged with 1% aerosolized OVA for 20 min using an ultrasonic nebulizer (De Vilbiss, Heston, UK). The OVA challenges were performed once daily for 3 consecutive days.

Two days after the last challenge, mice were anaesthetized i.p. with 0.25 g/kg body weight of Avertine (2, 2, 2-tribromoethanol dissolved in liquid *tert*-amyl alcohol at 0.025 g/mL, Sigma-Aldrich) and inoculated intranasally with 50 µL of Mp at different doses [10^3 or 10^7 colony-forming unit (CFU) per mouse] or 50 µL of saline (sham infection control). OVA-naïve BALB/c mice were similarly treated with Mp or saline as the controls for OVA groups. Mice were killed 7 days after Mp or saline to examine Th2 cytokine IL-4 expression, bronchoalveolar lavage (BAL) eosinophil, eotaxin-2 and PGE_2 levels, as well as lung mPGES-1 mRNA levels.

Bronchoalveolar lavage and lung tissue processing

BAL was collected as described previously [36]. Cell-free BAL fluid was stored at –80 °C for enzyme-linked immunosorbent assay (ELISA) analysis. The cytopins of BAL cells were stained using a Diff-Quick Kit (IMEB Inc., San Marcos, CA, USA), and cell differentials were determined as percentages of 500 counted cells. The lungs were perfused with PBS and excised. The left lung lobe was used for Mp culture. One of the right lobes was used for RNA extraction. The remaining tissue was used for lung enzymatic digestion to obtain a single-cell suspension for flow cytometry and $CD4^+$ T cell isolation [37].

Mycoplasma pneumoniae culture and polymerase chain reaction

To determine whether live Mp existed in mouse lung tissues, we performed a mycoplasma spp. culture as described previously [38, 39]. In brief, the left lung lobe was homogenized in 500 µL of PBS. Ten microlitres of the lung homogenate was placed onto PPLO culture plates (Remel) and incubated at 37 °C, 5% CO₂ for 7 days to count CFU.

A real-time RT-PCR was performed to detect mRNA of Mp-specific 16S rRNA in lung tissues. This assay was performed to further confirm the specificity of Mp culture results and to increase the sensitivity of Mp detection. A positive result indicates the existence of live Mp in the lung.

Flow cytometry of intracellular interleukin-4 production

All the reagents were from BD Biosciences (San Jose, CA, USA) unless specifically indicated. Mouse lung single-cell suspensions were adjusted to a concentration of 1×10^6 cells/mL with 10% fetal bovine serum (FBS) in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Carlsbad, CA, USA). The cells were transferred into 12-well culture plates and stimulated with 10 ng/mL phorbol myristate acetate (Sigma-Aldrich), 500 ng/mL ionomycin (Sigma-Aldrich) and BD GolgiStopTM (1 µL/1 mL) at 37 °C, 5% CO₂ for 6 h. The cells were pre-treated with anti-mouse CD16/CD32 (FcγIII/II receptor) (2.4G2) to block non-antigen-specific binding of antibodies to the FcγIII and FcγII, and possibly FcγI receptors, and then stained with FITC-conjugated anti-mouse CD3 (145-2C11) and APC-conjugated anti-mouse CD4 (RM4-5). After the wash, the cells were fixed and permeabilized using BD Cytofix/Cytoperm Buffer, and were further stained with PE-conjugated anti-mouse IL-4 (11B11) or isotype control rat IgG_{2b}. At least 50 000 viable cells were analysed using a BD FACSCalibur flow cytometer with CellQuest software. The following gating and analysis methods were used to analyse IL-4 protein expression in CD4⁺ T cells. First, only viable lymphocytes were included for analysis. Next, CD4⁺ T cells were specifically identified as CD3 and CD4 double-positive lymphocytes. Lastly, IL-4 protein levels were expressed as the percentage of CD3⁺CD4⁺ T cells expressing IL-4 by subtracting the background from the isotype control staining.

Pulmonary CD4⁺ T cell isolation

Single-cell suspensions of mouse lungs were processed to purify CD4⁺ T cells using a CD4 T cell isolation kit and Mini-MACS columns (Miltenyi Biotec., Auburn, CA, USA). Flow cytometry confirmed the purity of CD4⁺ T cells to be

≥95%. The isolated CD4⁺ T cells were lysed using TRIzol (Invitrogen) for total RNA extraction.

Mouse primary alveolar macrophages enrichment and culture

Naïve BALB/c mice were killed by exposure to CO₂. The lungs were lavaged thrice by injections of 1 mL of ice-cold $1 \times$ Hanks' balance salt solution (Invitrogen) supplemented with 1% penicillin/streptomycin solution (HyClone, Logan, UT, USA) and 0.6 mM EDTA (Sigma-Aldrich). BAL cells were collected by centrifugation at 300 g for 10 min at 4 °C. Cell pellets were suspended with 10% FBS in RPMI 1640 medium (Invitrogen), seeded in triplicate (5×10^5 cells/well) per condition in 48-well culture plates, and allowed to adhere to the plate for 2 h at 37 °C, 5% CO₂. More than 99% of adherent cells were identified as macrophages using light microscopy with Diff-Quick staining (IMEB Inc.). Thereafter, the adherent cells were gently washed once in PBS and treated with 10% FBS in RPMI 1640 medium supplemented with recombinant mouse IL-4 and IL-13 proteins (R&D Systems, Minneapolis, MN, USA) or bovine serum albumin (BSA, non-specific protein control, Sigma-Aldrich), followed by Mp, PGE₂ (Cayman Chemical, Ann Arbor, MI, USA), or corresponding controls as detailed in the figure legends. At the end of cultures, cell culture supernatants were collected for eotaxin-2 or PGE₂ measurement.

Enzyme-linked immunosorbent assay

Eotaxin-2 levels in the mouse BAL fluid or cell culture supernatants were determined using the mouse CCL24/Eotaxin-2 DuoSet ELISA kit (R&D Systems) as per the manufacturer's instruction. PGE₂ levels were quantified using reagents purchased from Cayman Chemical and performed using the ELISA Tech (Aurora, CO, USA).

Quantitative real-time reverse transcriptase-polymerase chain reaction

Primers and probes were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA) and are listed as below. Mouse IL-4 (GenBank accession number, NM_021283): forward, 5'-ACAGGA GAAGGGACGCCAT-3'; reverse, 5'-GAAGCCCTACAGAC GA-GCTCA-3'; probe, 5'-TCCTCACAGCAACGAAGAACCACA-3'. Mouse mPGES-1 (GenBank accession number, NM_008969): forward, 5'-TTCCAAGTGGGCTCACA AG-AGT-3'; reverse, 5'-ATCCACAGCCACATGCAGAA CA-3'; probe, 5'-TGGCCGGATTGGTGGAGGTAGGAACCTT TGA-3'.

Quantitative real-time RT-PCR was performed as described previously [37]. Briefly, real-time PCR was

performed using the ABI Prism[®] 7700 sequence detection system (Applied Biosystems). The 25 µL PCR contained 30 ng of cDNA, 100 nM fluogenic probe, 100 nM primers and other components from the TaqMan[®] RT-PCR kit (Applied Biosystems). 18S rRNA was evaluated as an internal positive control. The comparative cycle of threshold (C_t) method was used to demonstrate the relative mRNA levels of the target genes.

Statistical analysis

Normally distributed data were presented as means $\pm$ SEM and compared using the ANOVA test. Non-normally distributed data were expressed as medians with interquartile (25–75%) ranges and compared using the Wilcoxon rank-sum test. $P < 0.05$ was considered significant.

Results

Mycoplasma pneumoniae qualification by culture and polymerase chain reaction in mouse lungs

No Mp was detected in saline control (sham infection) mice with or without an allergen challenge by Mp culture or PCR. On day 7 post-Mp infection (day 9 after the last OVA challenge), allergic mice with a high-dose Mp infection had significantly higher levels of lung tissue Mp burden (10-fold) than non-allergic mice by culture [$50 (9-188) \times 10^2$ vs. $4 (0-7) \times 10^2$ CFU/left lung, $P = 0.02$]. Mice infected with high-dose Mp were 100% positive for Mp 16S rRNA as confirmed by PCR. In contrast, we did not find Mp by culture in both OVA-naïve and allergic mice with a low-dose Mp infection. However, 83.3% of the mice infected with low-dose Mp were positive for Mp 16S rRNA by PCR.

Low-dose Mycoplasma pneumoniae infection enhanced, whereas high-dose *Mycoplasma pneumoniae* infection tended to reduce T-helper type 2 cytokine interleukin-4 in established allergic mouse lungs

IL-4 protein was undetectable in BAL fluid of all the mice on day 7 post-Mp or saline (day 9 post-OVA challenge). However, as shown in Fig. 1a, a low-dose Mp infection in allergic mice significantly increased lung tissue IL-4 mRNA levels compared with saline controls as well as mice with a high-dose Mp infection. Moreover, significant up-regulation of IL-4 mRNA levels was found in isolated lung CD4⁺ T cells from allergic mice with a low-dose Mp infection compared with saline controls and a high-dose Mp-infected mice (Fig. 1b). Unlike low-dose Mp infection, high-dose infection did not exert such effects on IL-4 mRNA levels in either the whole lung tissue or isolated lung CD4⁺ T cells. In addition, as shown in Fig. 1c, a low-dose Mp infection in allergic mice

tended to increase intracellular IL-4 protein in lung CD4⁺ T cells compared with saline controls ($2.8 \pm 0.5\%$ vs. $1.9 \pm 0.5\%$, $P = 0.08$). As compared with the high-dose Mp infection, the low-dose Mp infection significantly increased IL-4 protein in lung CD4⁺ T cells ($1.5 \pm 0.4\%$ vs. $2.8 \pm 0.5\%$, $P = 0.01$).

Low-dose Mycoplasma pneumoniae infection maintained whereas high-dose *Mycoplasma pneumoniae* infection decreased bronchoalveolar lavage eosinophils in established allergic mouse lungs

As shown in Table 1, high-dose as well as low-dose Mp infection in both OVA-naïve and allergic mice increased the total BAL leucocyte counts compared with the corresponding saline controls. A significant BAL eosinophil influx existed in OVA-challenged mice as compared with OVA-naïve mice [77 (74–85)% vs. 0 (0–1)%, $P < 0.0001$]. In allergic mice, compared with saline controls, a high-dose Mp infection significantly reduced BAL eosinophils [77 (74–85)% vs. 50 (44–53)%, $P < 0.0001$], whereas a low-dose Mp infection slightly increased BAL eosinophils [77 (74–85)% vs. 85 (79–85)%, $P = 0.09$].

Although the eosinophil influx was the focus of this study, we also qualified other inflammatory cells in BAL. Briefly, on day 7 post-Mp infection, high-dose Mp, but not low-dose Mp, significantly increased the percentages of macrophages and lymphocytes in both allergic and OVA-naïve mice. The percentage of neutrophils was only significantly increased after a high-dose Mp infection in OVA-naïve mice.

Low-dose Mycoplasma pneumoniae infection enhanced, whereas high-dose *Mycoplasma pneumoniae* infection tended to reduce bronchoalveolar lavage eotaxin-2 levels in established allergic mouse lungs

As low-dose vs. high-dose Mp infection in allergic mice demonstrated opposite effects on BAL eosinophil levels, we determined whether levels of eotaxins differed among the various groups of mice. Eotaxin-1 was undetectable in BAL fluid in all the mice on day 7 post-Mp or saline. However, eotaxin-2 levels were significantly elevated in the OVA-challenged mice compared with the OVA-naïve mice. In allergic mice, a low-dose Mp infection significantly increased BAL eotaxin-2 levels compared with the saline controls (470 ± 40 vs. 337 ± 47 pg/mL, $P = 0.03$). In contrast, a high-dose Mp infection in allergic mice decreased BAL eotaxin-2 levels compared with the saline controls, although it did not reach a statistically significant level (230 ± 30 vs. 337 ± 47 pg/mL, $P = 0.08$). However, compared with a low-dose Mp infection, a high-dose Mp infection significantly decreased the levels of BAL eotaxin-2 in allergic mice (470 ± 40 vs. 230 ± 30 pg/mL, $P = 0.0005$) (Fig. 2).

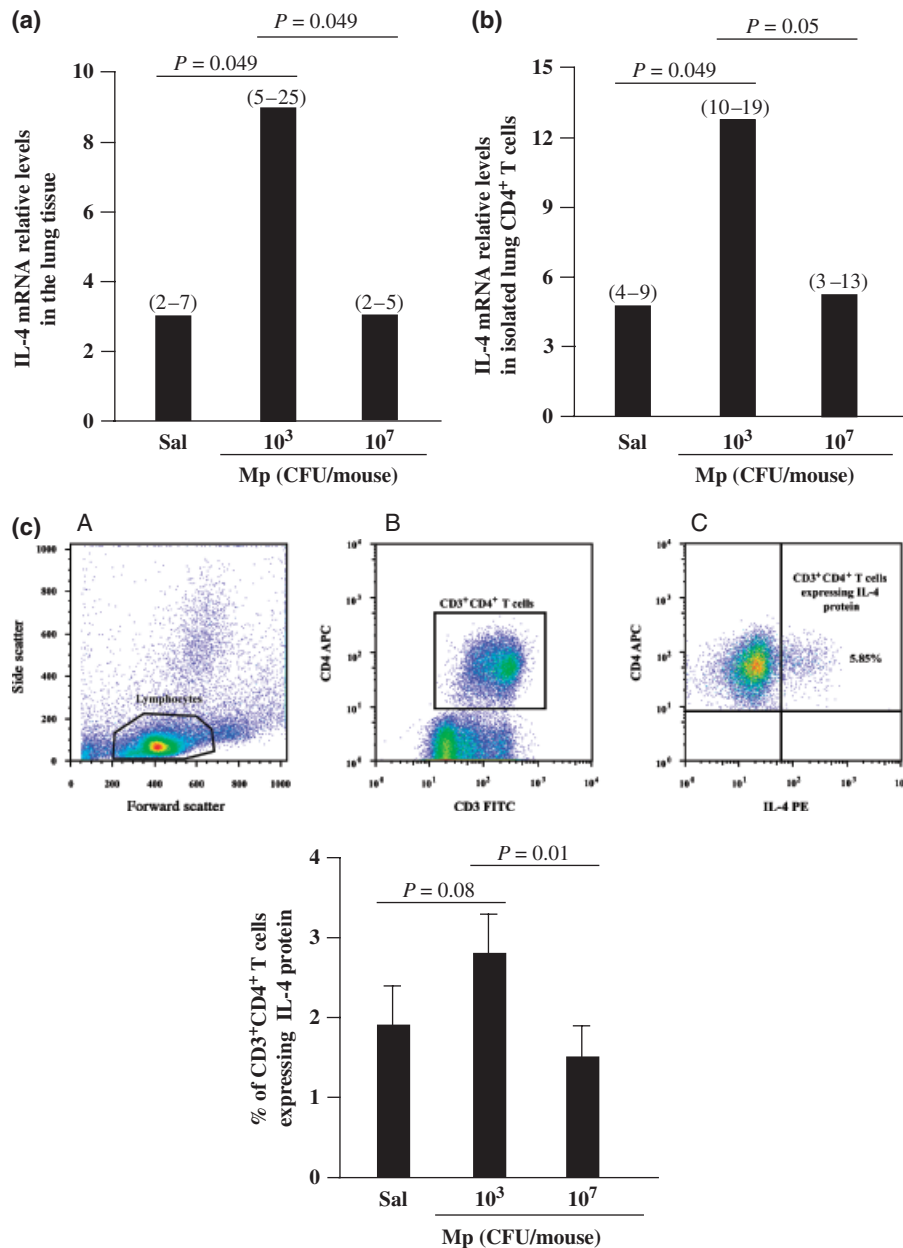


Fig. 1. Low-dose *Mycoplasma pneumoniae* infection in allergic mice enhances IL-4 expression. Intranasal inoculation of low-dose (10³ CFU/mouse), high-dose (10⁷ CFU/mouse) Mp, or saline was performed in ovalbumin-allergic mouse models ($n = 6$ /group) as described in "Materials and methods". Lungs were collected on day 7 post-Mp or -saline. (a) IL-4 mRNA relative levels in the lung tissue. (b) IL-4 mRNA relative levels in isolated lung CD4⁺ T cells. Gene expression was evaluated using quantitative real-time RT-PCR. Data are expressed as medians (25–75% range). (c) Upper panel: representative dot-plot of gating and data analysis of the CD4⁺ T cells expressing IL-4 protein in whole lung cells were quantified using intracellular cytokine staining and flow cytometry (A: gating for viable lymphocytes; B: gating for CD3⁺CD4⁺ T cells; C: % of CD3⁺CD4⁺ T cells expressing IL-4 protein). Lower panel: The percentages of CD3⁺CD4⁺ T cells expressing IL-4 protein. Data are expressed as means ± SEM. CFU, colony-forming unit; Mp, *Mycoplasma pneumoniae*; Sal, saline.

Low-dose Mycoplasma pneumoniae infection in allergic mice failed to activate the prostaglandin E₂ pathway, whereas high-dose Mycoplasma pneumoniae infection did

In OVA-naïve mice, Mp infection induced expression of lung mPGES-1 mRNA in a dose-dependent manner (data not shown). As shown in Fig. 3, in allergic mice, lung mPGES-1 mRNA levels were also significantly elevated in

mice infected with a high-dose, but not a low-dose, Mp infection.

In allergic mice, high-dose Mp increased the PGE₂ levels in BAL fluid as compared with saline controls (407 ± 111 vs. 227 ± 89 pg/mL), but this increase did not reach a statistical difference ($P = 0.3$). Low-dose Mp did not change the PGE₂ levels compared with saline controls (167 ± 38 vs. 227 ± 89 pg/mL, $P = 0.8$).

Table 1. Bronchoalveolar lavage cell profiles

Groups	Total leukocytes ($\times 10^4$ cells/mL)	Eosinophils (%)	Macrophages (%)	Lymphocytes (%)	Neutrophils (%)
OVA+Mp 10^7	55 (30–96)	50 (44–53)*	22 (21–23)*	26 (21–32)*	2 (1–3)
OVA+Mp 10^3	46 (42–58)	85 (79–85)	14 (13–17)	0.5 (0.2–5)	0.5 (0.1–1)
OVA+saline	39 (17–70)	77 (74–85) [±]	13 (10–16)	8 (1–13)	2 (1–3)
Mp 10^7	15 (11–22) [±]	1 (0.3–2)	86 (74–90) [±]	5 (2–8) [±]	8 (5–20) [±]
Mp 10^3	7 (5–12)	0.1 (0–0.3)	96 (95–97)	0.9 (0–1)	3 (3–5)
Saline	4 (3–5)	0 (0–1)	98 (97–98)	1 (1–2)	1 (0.4–1)

Data are expressed as medians (25–75% range), $n = 6$ mice/group.

* $P < 0.05$, compared with OVA+saline group by Wilcoxon's rank-sum test.

[±] $P < 0.05$, compared with saline group by Wilcoxon's rank-sum test.

OVA, ovalbumin; Mp, *Mycoplasma pneumoniae*.

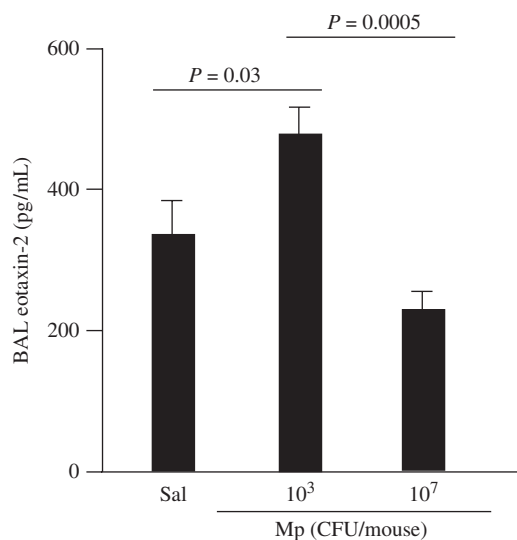


Fig. 2. Low-dose Mp infection in allergic mice enhances eotaxin-2 production. Intranasal inoculation of low-dose (10^3 CFU/mouse), high-dose (10^7 CFU/mouse) Mp, or saline was performed in ovalbumin-allergic mouse models ($n = 6$ /group) as described in "Materials and methods". The BAL fluid was collected on day 7 post-Mp or -saline for eotaxin-2 measurement using an ELISA. Data are expressed as means $\pm$ SEM. BAL, bronchoalveolar lavage; CFU, colony-forming unit; Mp, *Mycoplasma pneumoniae*; Sal, saline.

Prostaglandin E_2 inhibited interleukin-4/interleukin-13-induced eotaxin-2 production in mouse primary alveolar macrophages

To further test whether a high-dose Mp infection inhibits eotaxin-2 production through the PGE_2 pathway, pAMs from OVA-naïve BALB/c mice were isolated and treated *in vitro* with recombinant mouse IL-4 and IL-13 or BSA before the Mp or PBS treatment. Regardless of the doses, Mp alone did not induce eotaxin-2 production (data not shown). As shown in Fig. 4a, pAMs constitutively released a low level of eotaxin-2. IL-4/IL-13 significantly increased eotaxin-2 production compared with BSA con-

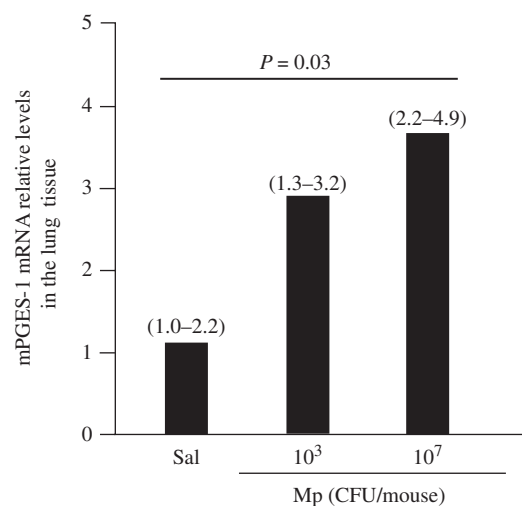


Fig. 3. High-dose Mp infection in allergic mice induces lung mPGES-1 mRNA expression. Intranasal inoculation of low-dose (10^3 CFU/mouse), high-dose (10^7 CFU/mouse) Mp, or saline was performed in OVA-allergic mouse models ($n = 6$ /group) as described in "Materials and methods". Lungs were collected on day 7 post-Mp or -saline for mPGES-1 mRNA quantification by quantitative real-time RT-PCR. Data are expressed as medians (25–75% range). CFU, colony-forming unit; Mp, *Mycoplasma pneumoniae*; mPGES-1, microsomal prostaglandin E synthase-1; Sal, saline.

trols. Similar to *in vivo* data, low-dose Mp (0.01 CFU/cell) in IL-4/IL-13-treated cells failed to reduce eotaxin-2 compared with PBS controls. However, high doses of Mp (0.1, 1 and 10 CFU/cell) markedly down-regulated IL-4/IL-13-induced eotaxin-2 production in a dose-dependent manner. In contrast to its effects on eotaxin-2, Mp infection in IL-4/IL-13-treated cells dose-dependently increased PGE_2 production (Fig. 4b).

To examine whether the PGE_2 directly inhibits eotaxin-2 production, exogenous PGE_2 (1–1000 nM) was added to IL-4/IL-13- or BSA-pre-treated mouse pAMs. As shown in Fig. 5, PGE_2 inhibited IL-4/IL-13-induced eotaxin-2 production in a dose-dependent manner.

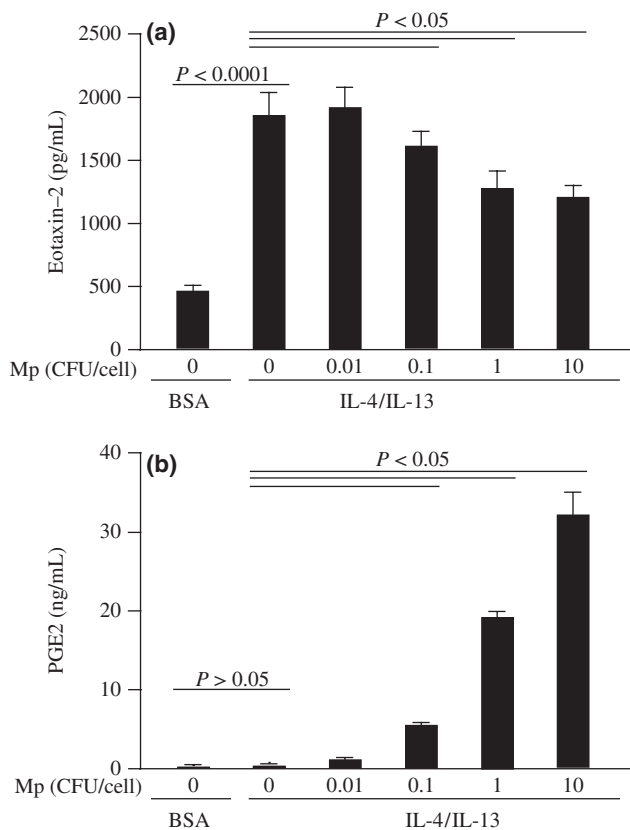


Fig. 4. Mp infection inhibits eotaxin-2, but increases PGE₂ production in IL-4/IL-13-treated alveolar macrophages. BAL cells from naïve BALB/c mice were seeded in triplicate (5×10^5 cells/well) per condition into 48-well culture plates, and incubated for 2 h at 37 °C, 5% CO₂. Thereafter, adherent cells (alveolar macrophages) were washed in phosphate-buffered saline and treated with recombinant mouse IL-4 and IL-13 (10 ng/mL each) or BSA (20 ng/mL) 2 h before PBS or Mp infection (0.01, 0.1, 1 and 10 CFU/cell), and further incubated for 24 h. Cell culture supernatants were collected for eotaxin-2 (a) or PGE₂ (b) ELISA. Data are from five independent experiments and expressed as means $\pm$ SEM. BSA, bovine serum albumin; CFU, colony-forming unit; Mp, *Mycoplasma pneumoniae*; PGE₂, prostaglandin E₂.

Discussion

The results from the present study suggest that a low-level Mp infection in established allergic airways may maintain or even enhance Th2 responses partly due to insufficient production of the immunomodulatory mediator PGE₂ following an Mp infection.

The current allergic mouse models support our previous clinical findings in that Mp in chronic stable asthmatics can only be detected by PCR, but not by culture, and yet Mp-PCR-positive asthmatics present an enhanced allergic response [1]. In the present study, we did not find any live Mp in the allergic mouse lung by culture on day 7 post a low-dose Mp infection (10^3 CFU) although 83.3% of the mice infected with a low dose of Mp were positive for Mp 16S rRNA by PCR. However, in our preliminary experi-

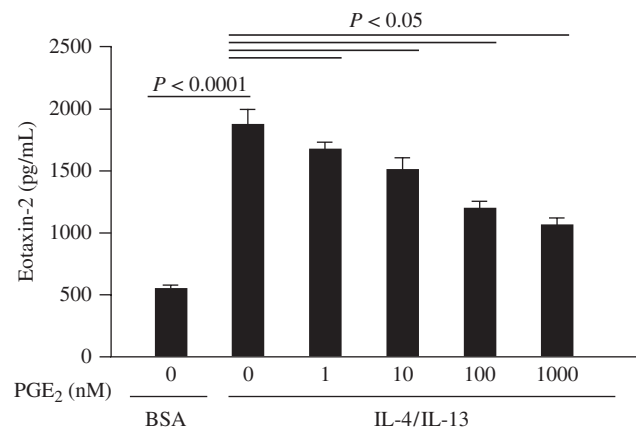


Fig. 5. Exogenous PGE₂ directly inhibits IL-4/IL-13-induced eotaxin-2 production in alveolar macrophages. BAL cells from naïve BALB/c mice were seeded in triplicate (5×10^5 cells/well) per condition into 48-well culture plates, and incubated for 2 h at 37 °C, 5% CO₂. Thereafter, adherent cells were washed in phosphate-buffered saline and treated with recombinant mouse IL-4 and IL-13 (10 ng/mL each) or BSA (20 ng/mL) 2 h before dimethyl sulfoxide (DMSO, control) or PGE₂ (1–1000 nM) in DMSO, and further incubated for 24 h. Cell culture supernatants were collected for eotaxin-2 ELISA. Data are from three independent experiments and expressed as means $\pm$ SEM. BSA, bovine serum albumin; PGE₂, prostaglandin E₂.

ments, we could find Mp by culture after a low-dose Mp infection in OVA-naïve mice at the very early time-point (e.g. 4 h), but not at the later time-points such as 24 h and 7 days. Furthermore, we found that a low dose of live Mp, but not heat-killed Mp, increased BAL neutrophils as compared with saline controls at 24 h [live Mp, $5.28 (1.67\text{--}20.13) \times 10^4$ /mL; heat-killed Mp, $2.11 (1.35\text{--}4.35) \times 10^4$ /mL, and saline, $2.16 (1.05\text{--}4.08) \times 10^4$ /mL]. These results suggest that a low level of live Mp intranasal inoculation indeed reached the lung compartment to induce an inflammatory response in our current mouse models.

In the present study, we found that high-level and low-level Mp infections differently regulated the established allergic inflammation. Specifically, in allergic mice, a low-level Mp infection was found to enhance allergic responses, including eotaxin-2, eosinophils and IL-4. In contrast, a high-level Mp infection significantly decreased lung eosinophils, and tended to reduce eotaxin-2 and IL-4 production. Our findings have extended previous reports about the differential effects of various levels of microbial exposure on allergic inflammation. For example, inhaled lipopolysaccharide (LPS) at a very low level during allergen sensitization acted as an adjuvant to trigger Th2 polarization and thus induce allergic airway responses. However, when inhaled at doses 1000-fold higher than those promoting a Th2 response, LPS induced a Th1-predominant response, and prevented an airway eosinophilic inflammation in OVA-allergic mice [40]. Recently, Schröder et al. [41] have shown that a mild pulmonary infection of

Chlamydia pneumoniae, an intracellular pathogen, favoured the development of allergic sensitization in mice, but severe infection did not. Therefore, the exposure level of infectious agents may be an important factor in determining the type or the magnitude of an inflammatory response in an allergic setting.

In the current study, we have shown for the first time that alveolar macrophages are one of the important cellular sources for eotaxin-2. Mouse pAMs constitutively release a low level of eotaxin-2, which alone may not be sufficient to recruit eosinophils because naïve mice with or without Mp infection have virtually no eosinophil infiltration in the lung. Th2 cytokine (e.g. IL-4 and IL-13) stimulation further enhances the production of eotaxin-2 in cultured mouse pAMs. Moreover, in allergic mice, a low-dose Mp infection significantly increased the eotaxin-2 production, accompanied by a moderate increase of eosinophil influx, whereas a high-dose Mp infection did the opposite. This finding is in agreement with previous observations that eotaxin-2 co-ordinates the recruitment of eosinophils into the allergic airways after an allergen challenge [16, 21, 22, 42–47]. Based on our *in vivo* and *in vitro* findings, we propose that alveolar macrophage-derived eotaxin-2 may partly contribute to eosinophilia in allergic airways with a low level of persistent bacterial (e.g. Mp) infection. However, we realize that, in addition to eotaxin-2, other chemokines (e.g. RANTES) also contribute to eosinophil recruitment into inflammatory sites in allergic airways, which have not been investigated in the current study.

Our results indicate that eotaxin-2 production could be regulated through the PGE₂ pathway in Mp-infected allergic lungs or Th2 cytokine-treated alveolar macrophages. First, levels of lung mPGES-1 mRNA were elevated, whereas BAL eosinophils and eotaxin-2 were reduced in allergic mice infected with high-dose, but not low-dose Mp. Of note, PGE₂ is synthesized *de novo* and released into the extracellular space when cells are activated [48, 49]. *In vivo*, the half-life of PGE₂ is approximately 30 s in the circulatory system because PGE₂ is rapidly converted into an inactive metabolite (13,14-dihydro-15-keto PGE₂) by the prostaglandin 15-dehydrogenase pathway [48–50]. This may further explain why PGE₂ levels were not significantly different between allergic mice infected with low-dose and high-dose Mp although, we observed a significant difference of lung mPGES-1 mRNA in these mice. Second, IL-4/IL-13 significantly increased eotaxin-2 production in cultured mouse alveolar macrophages, which was reduced by high doses of Mp infection, coupled with dose-dependent PGE₂ production. Third, exogenous PGE₂ directly inhibited IL-4/IL-13-induced eotaxin-2 production in a dose-dependent manner in mouse alveolar macrophages. Therefore, Mp-induced PGE₂ may attenuate eotaxin-2 production, thus reducing lung eosinophil influx into the established

allergic airways. Nevertheless, we are aware that the role of PGE₂ in regulation of eosinophils in allergic mice infected with Mp, especially at low doses, needs to be further investigated. Our data are in line with previous studies showing an inhibitory role of PGE₂ in allergen-induced inflammation in human subjects [33–35]. We are aware that besides its inhibitory effects on eotaxin-2, PGE₂ may exert an anti-allergic function through other mechanisms. For example, PGE₂ has been shown to block PGD₂ release or Th2 cell activation [35, 51]. In addition, previous studies have reported that PGE₂ acts by binding to four subtypes of PGE receptors (EP₁–EP₄) on the surfaces of specific types of cells [52]. The PGE₂–EP₃ pathway is an important negative modulator of allergic reactions in mouse OVA-allergic asthma models [53]. Moreover, PGE₂ has been shown to directly suppress pro-inflammatory mediators in human blood monocyte-derived macrophages through the EP₄ receptor [54]. Minami et al. [55] further revealed that PGE₂–EP₄ signalling inhibits macrophage activation following pro-inflammatory stimulation by augmenting NF-κB1 p105 protein stability through EP₄-associated protein (EPRAP). The potential role of PGE₂–EP₄ signalling in inhibiting eotaxin-2 production is worthy pursuing in a future study.

In summary, this study highlights a novel concept on how differing bacterial loads in the lung modify the established allergic airway inflammation, and thus interact with an allergen to further induce Th2 responses. That is, unlike high-level Mp, low-level Mp infection fails to effectively induce immunomodulatory mediator PGE₂ to down-regulate allergic responses such as eosinophil chemokine eotaxin-2, thus maintaining or even worsening allergic inflammation in asthmatic airways. These results also suggest a novel role of PGE₂ as an endogenous inhibitor of the established allergic inflammation in the context of airway bacterial infections.

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