



Immunomodulatory effects of feeding with *Bifidobacterium longum* on allergen-induced lung inflammation in the mouse

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ABSTRACT

Background: The intestinal microbiota has important effects on host immune responses and feeding with certain commensal organisms has anti-inflammatory effects in a variety of diseases, including experimental asthma. The aim of the current study was to examine how robust the effects of feeding with the commensal strain, *Bifidobacterium longum* (*Bif*) were on the pulmonary responses to allergen sensitization and challenge.

Methods: BALB/c mice were given two intraperitoneal injections of ovalbumin (10 µg in alum) on days 0 and 7 and were fed daily with *Bif* or vehicle from days 0–14. Challenges with ovalbumin (10 µg) were administered intra-nasally once on day 14 or three times on days 14, 15 and 16 and the lung inflammatory response was assessed one day later.

Results: *Bif* feeding attenuated airway inflammation following a single ovalbumin challenge, reducing bronchoalveolar lavage (BAL) eosinophilia, BAL fluid IL-4 protein and BAL cell IL-4 and IFN-γ mRNA levels. However, BAL fluid IL-5 protein was increased. There was an accompanying increase in lung regulatory T cells assessed by flow cytometry. Responses to triple challenge with ovalbumin were much less affected by *Bif* feeding, including unchanged cytokine levels, ovalbumin-specific IgE and airway hyperresponsiveness to methacholine.

Conclusion: These results show modest immunoregulatory effects of oral feeding with *Bif* with inhibition of certain components of allergen-induced airway inflammation that is associated with the expansion of regulatory T cells in the lungs but that is overcome by repeated allergen exposure.

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1. Introduction

Asthma is characterised by airflow limitation, airway hyper-responsiveness (AHR) and, typically, airway inflammation with the infiltration of eosinophils and activated CD4⁺ T cells. This chronic inflammatory response is believed to be the underlying cause of airway narrowing, subepithelial fibrosis, goblet cell hyperplasia, and increased smooth muscle mass that has been associated with increased asthma severity [1–3]. Several factors are believed to be involved in the development and severity of asthma including diet, environmental exposures and, in particular, allergens [4–6] as well

as genetic predisposition [7]. The causes of atopy or the predisposition to allergic sensitization have been suggested to be related to inappropriate maturation of the immune system [8]. Persistence of a Th2 tendency beyond early life has been attributed to lack of exposure to agents which may lead under normal circumstances to a more Th1 oriented inflammatory response to inhaled soluble antigens or, indeed, no response in the case of exposure to innocuous antigens. These notions have been embodied in the hygiene hypothesis [9].

Evidence is accruing that commensal organisms may play a role in regulating the immune system [10]. Human trials of the administration of commensal organisms have shown efficacy against atopic dermatitis [11], suggesting a possible role for these organisms in other human atopic disease. Recent studies have reported improvement in allergic rhinitis and asthma in children and a reduction in both Th1 and Th2 cytokines [12] and a reduction in systemic Th2 cytokines in atopic adults [13] following *Lactobacillus gasseri* and oligosaccharide and *Bifidobacterium breve*

Abbreviations: AHR, Airway hyperresponsiveness; *Bif*, *Bifidobacterium longum*; BALF, bronchoalveolar lavage fluid; IL, interleukin; OVA, ovalbumin; PBS, phosphate-buffered saline; T regs, regulatory T cells; Th1, T helper 1; Th2, T helper 2; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

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treatments, respectively. In the latter study there was no reduction in sputum eosinophilia and in AHR, despite a reduced IL-5 level systemically. Feeding of normal animals with various commensals has also attenuated allergic inflammation in experimental asthma [14]. Recently we tested the effects of three probiotic bacterial strains in the murine ovalbumin (OVA) models of respiratory and oral allergy [15]. We detected bacterial strain specific effects with induction of Foxp3+ T regs and one of the bacteria, *Bifidobacterium longum* (Bif, AH1206), in particular had a beneficial anti-inflammatory effect. However for commensal organisms to have beneficial effects in asthma they would require to have robust anti-inflammatory effects and to inhibit other functional outcomes as AHR. We therefore evaluated the effects of Bif 1206 feeding in the BALB/c mouse undergoing ovalbumin sensitization and challenge to assess the effect of commensal feeding over a 2 week period, encompassing the sensitization period, on allergen-driven airway inflammation and we contrasted the effects of the probiotic feeding on animals exposed once or three times to ovalbumin. We addressed also the effects of Bif feeding on a variety of outcome measures such as serum ovalbumin-specific immunoglobulin E, airway responsiveness to inhaled aerosolized methacholine, lung cytokine expression levels from protein measurements in bronchoalveolar lavage fluid (BALF) and mRNA expression in BALF cells. We also determined the changes in lung, spleen and Peyer's patch T regs (CD4+CD25+FoxP3+).

2. Materials and methods

2.1. Animals

Male BALB/C mice, 8–10 weeks of age were purchased from Charles River Laboratories (Bicester, UK and St. Constant, Quebec, Canada). The protocols were approved by institutional animal care committees. Mice were immunized twice, 7 days apart, with 10 µg ovalbumin (OVA; Sigma grade V, Sigma–Aldrich, Dorset, UK) intraperitoneally in a suspension of 1 mg Al(OH)₃ (Sigma) in saline. One week after the second injection, mice were challenged with 10 µg OVA in 50 µl of sterile saline intra-nasally under light anaesthesia either once or three times at daily intervals. Bronchoalveolar lavage was performed 24 h after a single and triple challenge. Bronchoalveolar lavage was also performed on control non sensitized mice to establish background cellular levels. Lungs, intrathoracic lymph nodes and spleens were harvested after the single challenge with OVA. Airway hyperresponsiveness (AHR) was measured and serum, lungs, lymph nodes and Peyer's patches were harvested after three challenges with OVA. Control animals were also OVA sensitized and PBS challenged. Four groups were studied; 1. OVA sensitized and OVA challenged, sham fed animals, (OVA/placebo); 2. OVA sensitized and PBS challenged, sham fed animals (Control/placebo); 3. OVA sensitized and OVA challenged, Bif fed animals (OVA/1206); 4. OVA sensitized and PBS challenged Bif fed animals (Control/1206). Animals received 1×10^9 Bif daily orally in a 200 µl suspension. Bif (*B. longum* AH1206) was kindly provided by Dr. B. Kiely (Alimentary Health Ltd., Cork, Ireland).

2.2. Bacterial culture

The bacterial feed solution, BALF and intestinal fecal samples were cultured to determine the presence of *Bifidobacteria*. *Bifidobacteria* were cultured at 37 °C anaerobically using the Anaerogen™ system (Oxoid, Ontario, Canada) for 48 h in deMann, Rogosa and Sharpe agar, (MRS, Oxoid), supplemented with 0.05% cysteine (Sigma, Ontario, Canada) and mupirocin (100 µg/ml)(Sigma). Colonies were gram stained using the Fisher PROTOCOL™ gram stain kit (Fisher Scientific, Ontario, Canada) to confirm Bif identity

by a positive gram stain and the characteristic bifurcated morphology.

2.3. Bronchoalveolar lavage

Lavage was performed using phosphate-buffered saline (PBS) (Invitrogen, Paisley, Scotland), containing 10% FBS (Invitrogen), administered as an aliquot of 0.6 ml and three subsequent aliquots of 1 ml each. The return from the 0.6 ml aliquot was centrifuged and the supernatant was retained for bacterial culture and cytokine analysis. The cell pellets from all aliquots were pooled for cell counts and mRNA analysis (25%). Total viable cells were counted with a haemocytometer and Trypan blue. Differential cell counts were performed on Diff-Quick (Fischer Scientific, Pittsburgh, PA, USA) stained slides. All counts were performed by an investigator blinded to experimental conditions.

2.4. Measurement of in vivo respiratory mechanics

Measurements of mechanics were made after three challenges only as airway hyperresponsiveness is inconsistently induced by a single challenge. For this purpose, mice were anesthetized with xylazine (10 mg/kg) and pentobarbital sodium (30 mg/kg) and paralysed with pancuronium bromide (1.2 ml/kg) intra-peritoneally. Mice were tracheostomized using an 18-gauge tracheal cannula and ventilated with a tidal volume of 10 ml/kg at a PEEP of 3 cmH₂O at a frequency of 150/minute with a small animal ventilator (Flexivent, Scireq Inc. Montreal, Canada). Respiratory system resistance (R_{RS}) and elastance (E_{RS}) were measured using the forced oscillation technique [16]. Measurements were obtained at baseline and after the delivery for 5 s periods aerosols of increasing concentrations (20, 40, 80, 160 mg/ml in PBS) of methacholine (MCh; Sigma) generated via an ultrasonic nebulizer (Aeroneb Lab, Aerogen Ltd, Ireland). Eight to ten measurements were acquired following each aerosol of MCh so as to permit the peak response to be identified.

2.5. Lung, lymph node, Peyer's patch and spleen cell isolation

Lungs and Peyer's patches were isolated and placed in 0.28 Wunsch units/ml of a purified collagenase-thermolysin blend (Liberase blendzyme-2) at 37 °C for 30 min. Cell digests were filtered through a 70 µm cell strainer and centrifuged at 500 g × 10 minutes. Single cell suspensions were generated from spleen and lung-draining lymph nodes by mechanical disruption of tissues and subsequent filtering through a 70 µm cell strainer. Lung and splenic erythrocytes were lysed (Mouse Erythrocyte Lysing Kit, RnD Systems, Abingdon, UK). Splenocytes were seeded in 96 well tissue culture plates (Sarstedt, Newton, USA) at 2×10^5 cells per well. Single cell suspensions were stimulated for 48 h with anti-CD3 (10 µg/ml) and anti-CD28 (2 mg/ml) antibodies (BD Biosciences, Oxford, UK) at 37 °C and 5% CO₂ humidified atmosphere. Cell supernatants were stored at –80 °C for analysis of cytokine production.

2.6. Bronchoalveolar lavage fluid and cell supernatant cytokine assays

IFN-γ, IL-1-β, IL-2, IL-4, IL-5, KC (CXCL1), IL-10, IL-12p40 and TNF-α levels were quantified using an ultrasensitive electrochemiluminescence multiplex system (Sector 2400 imager, Meso Scale Discovery, Gaithersburg, MD, USA). The sensitivity of detection was approximately 1 pg/ml.

2.7. Measurement of OVA-IgE levels in serum

Following assessment of airway responses to methacholine (after three OVA challenges), blood was collected by exsanguination. Serum was stored at -20°C . A Mouse OVA-IgE ELISA kit (MD Bioproducts, MN, USA) (sensitivity < 3.8 ng/ml, IgG cross-reactivity $< 0.01\%$) was used to measure OVA-specific IgE.

2.8. T cell assessment by flow cytometry

Single cell suspensions from lung tissue, spleens, Peyer's patches and lung-draining lymph nodes of mice were obtained as outlined above. Monoclonal antibodies to CD4 (clone GK1.5), CD25 (clone 7D4) were used to label cells for T cell subset analysis (BD BioSciences, Oxford, UK). Antibodies to the transcription factor Foxp3 (clone FJK-16s) (eBioscience, San Diego, USA) were used to label permeabilized cells. Cellular phenotypes were assessed by flow cytometry (FACSCalibur, BD Biosciences) and commercial software (BD CellQuest).

2.9. mRNA expression of pro-inflammatory cytokines/mediators in BALF cells

BALF samples were lysed in 50 μl of Sidestep Lysis and stabilisation buffer (Stratagene, Netherlands) and stored at -70°C . Complementary DNA (cDNA) was synthesised using 1 μl of lysate incubated with 300 ng of random nanomers, 1 μl AffinityScript RT, RNase Block enzyme mixture, in a volume of 20 μl with the provided buffer (Stratagene). Synthesis conditions used were 25°C for 5 min, 42°C for 45 min, 95°C for 5 min and 4°C for 5 min. PCR primers and probes (Supplemental Table 1) were designed using the Universal ProbeLibrary Assay Design Centre (<https://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp>). β -actin was used as a housekeeping gene. Amplification reactions contained 1 μl cDNA, 10 μl of the $2\times$ FastStart TaqMan[®] Probe Master (Roche), 900 nM of each primer and 250 nM of probe mix and were brought up to a total of 20 μl by the addition of RNase-free water. All reactions were performed in triplicate using the LightCycler 480 System (Roche). Positive and negative controls were also included. The $2^{-\Delta\Delta\text{Ct}}$ method [17] was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments. Pooled cDNA from all groups were used as a calibrator control for analysis of differential gene expression.

2.10. Statistical analyses

Data are expressed throughout as the mean $\pm$ standard error of the mean. Comparison among means was performed using Student's *T* test when single comparisons were performed and ANOVA with Tukey's post hoc test for multiple comparisons. When data were not normally distributed the analyses were performed on log transformed data.

3. Results

3.1. Viability of Bifidobacteria following feeding

To determine the viability of *Bif* following feeding, we performed plate counts on the fed *Bif* solution, feces and the BALF of mice. *Bif* was detectable in the feed ($1 \times 10^9 \pm 1 \times 10^7$ colony forming units (CFU/200 μl dose)) and in the fecal pellets ($4 \times 10^7 \pm 1 \times 10^6$ CFU/pellet) (Supplemental Table 2). No *Bif* was detectable in the BALF.

3.2. Effects of *B. longum* feeding on BALF cells after OVA challenge

To assess whether the effect of *Bif* feeding on airway inflammation was affected by the number of allergen exposures we measured BALF cellular and cytokine responses following a single and triple OVA challenge. At 24 h after challenge with OVA there was a significant increase in total cells in the BALF ($P < 0.01$, Fig. 1A), that was largely attributable to eosinophils ($P < 0.001$, Fig. 1B). *Bif* feeding inhibited both the increase in total cells ($P < 0.05$) and in eosinophils in the single challenged group ($P < 0.01$) but not in the triple challenged group. OVA challenge evoked BALF neutrophilia that was unaffected by *Bif* feeding in either challenge group (Fig. 1C). *Bif* feeding had no effect on the lymphocyte numbers following a single OVA or saline challenge. However mice that were fed *Bif* and received three saline challenges did have fewer BALF lymphocytes, nonetheless this effect was not observed in the triple OVA challenged mice ($P < 0.01$, Fig. 1D). There was a slight increase in macrophages in the triple OVA challenged *Bif* fed group when compared to the those that were fed *Bif* and received three saline challenges ($P < 0.05$, Supplemental Table 3). OVA sensitized but saline challenged mice displayed a similar BALF cell profile compared to non sensitized control mice (see Supplemental Table 3, Naïve BALF).

3.3. Effects of *B. longum* feeding on pro-inflammatory mediator protein levels in BALF

We evaluated BALF Th1 and Th2 cytokine levels by multiplex assay. Following a single or triple challenge with OVA there was a significant increase in IL-4 ($P < 0.001$, Fig. 2A) and the increase was attenuated by *Bif* feeding in the triple challenge group ($P < 0.05$ Fig. 2A). IL-5 was also significantly increased after one and three OVA challenges ($P < 0.001$ and $P < 0.05$, Fig. 2B). The increase in IL-5 after a single OVA challenge was augmented after *Bif* feeding ($P < 0.05$) but was unaffected by *Bif* feeding in the triple challenged group. IFN- γ was also significantly increased in the single and triple OVA challenged *Bif* fed groups compared to the *Bif* fed controls ($P < 0.001$, $P < 0.05$, Fig. 2C). IL-10 increased after a single OVA challenge ($P < 0.001$) and after the triple challenge ($P < 0.05$) but was not affected by *Bif* feeding (Fig. 2D). However, *Bif* feeding significantly reduced the level of IL-10 after a single challenge with saline ($P < 0.01$).

The neutrophil chemoattractant KC was increased significantly after one OVA challenge ($P < 0.001$) but not three OVA challenges (Fig. 3A). *Bif* feeding did not affect post OVA challenge levels. However KC was lower in the *Bif* fed animals that received three saline challenges ($P < 0.05$, Fig. 3A). IL-1 β increased after a single OVA challenge and was further increased ($P < 0.01$) in *Bif* fed OVA challenged animals (Fig. 3B). There were no significant differences in IL-1 β among the animals, fed *Bif* or placebo, undergoing three challenges of saline or OVA. TNF- α , IL-2 and IL-12 levels were also analysed; a single OVA challenge increased all these cytokines in both placebo fed and *Bif* fed animals, with the exception of IL-2 in the *Bif* fed, where higher IL-2 levels were present in the *Bif* fed single saline challenged mice. Three OVA challenges resulted in an increase in IL-12 in the *Bif* fed mice only (see Supplemental Table 3).

3.4. Effects of Bifidobacterium feeding on lung CD4+, CD25+, FoxP3+ cells

To evaluate effect of *Bif* feeding on the lung T cell populations, lung tissue digests were analysed by flow cytometry. OVA exposure increased the number of CD4+CD25+FoxP3+ T cells (T reg) and *Bif* feeding further significantly increased these cells in the single but not the triple challenged groups (Fig. 4A–E). The

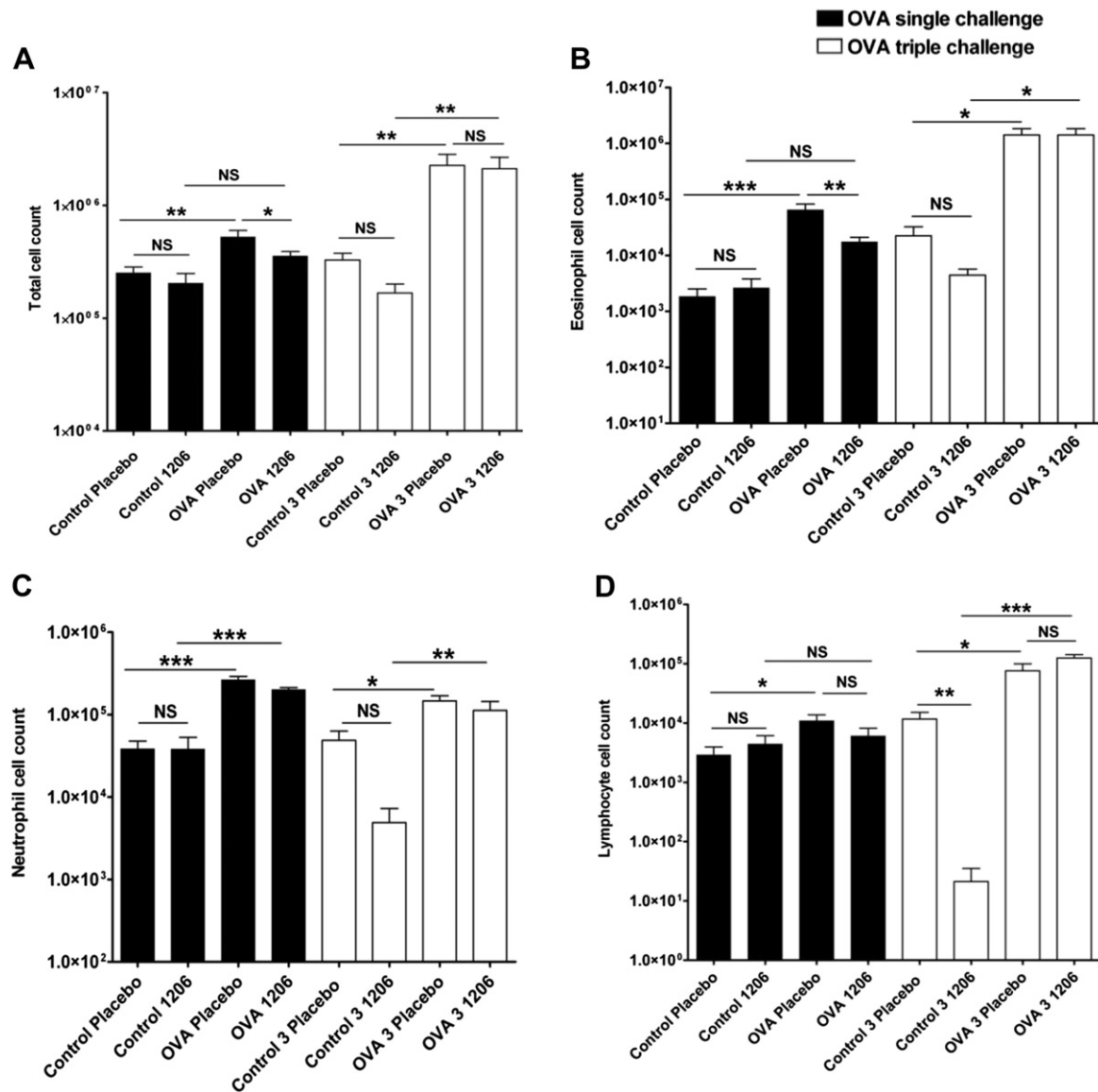


Fig. 1. Effect of *Bifidobacterium longum* 1206 feeding on the cellular content of bronchoalveolar lavage fluid (BALF) following single and triple OVA challenge (A) total cell count (A), eosinophil count (B), neutrophil (C) and lymphocyte of BALF. OVA single challenge (dark bars) $n = 10$, OVA triple challenge (light bars) $n = 8$. Values are shown as Mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS denotes non-significant.

CD4+CD25+FoxP3⁺ population was also increased following OVA challenge (Fig. 4F), and this cell population was significantly increased in the *Bif* fed triple challenged group ($P < 0.05$). The CD4+CD25⁺FoxP3⁺ population was increased following single and triple OVA challenge in the *Bif* fed groups (Fig. 4G).

3.5. Effects of *Bifidobacterium* feeding on pro-inflammatory mediator mRNA expression in BALF cells following a single OVA challenge

In order to assess the immunomodulatory effects of *Bif* feeding on cells in the bronchoalveolar compartment specifically we used qRT-PCR to examine the expression of the prototypical Th1 and Th2 cytokines, IFN- γ and IL-4, and several pro-inflammatory mediators reflecting activation of macrophages and mast cells (leukotriene C₄ synthase (LTC₄S)), neutrophils (neutrophilic granule protein (NGP)) and eosinophils (eosinophil-associated, ribonuclease A family, member 2 (EAR2)). We focused on changes after a single challenge

as *Bif* feeding appeared to have greater effects in the animals undergoing a single challenge. IL-4 and IFN- γ mRNA expression was significantly up-regulated in BALF cells after OVA challenge ($P < 0.001$, Fig. 5A; $P < 0.01$, Fig. 5B, respectively) and was significantly inhibited by *Bif* feeding. A small increase in LTC₄S and a substantial increase in NGP expression were observed after OVA challenge and were both prevented by *Bif* feeding ($P < 0.05$ and $P < 0.01$, Fig. 5C and D). Ear-2 mRNA expression was not increased after OVA challenge nor after feeding. IL-5 mRNA expression was undetectable in BALF cells.

3.6. Effects of *Bifidobacterium* feeding on spleen, Peyer's patch and lymph node T regs and on splenocyte cytokine production following OVA challenge

To assess the systemic effects of *Bif* feeding we analysed the spleen and intrathoracic lymph nodes by flow cytometry. The CD4+CD25+FoxP3⁺ T cell populations of the spleen and the

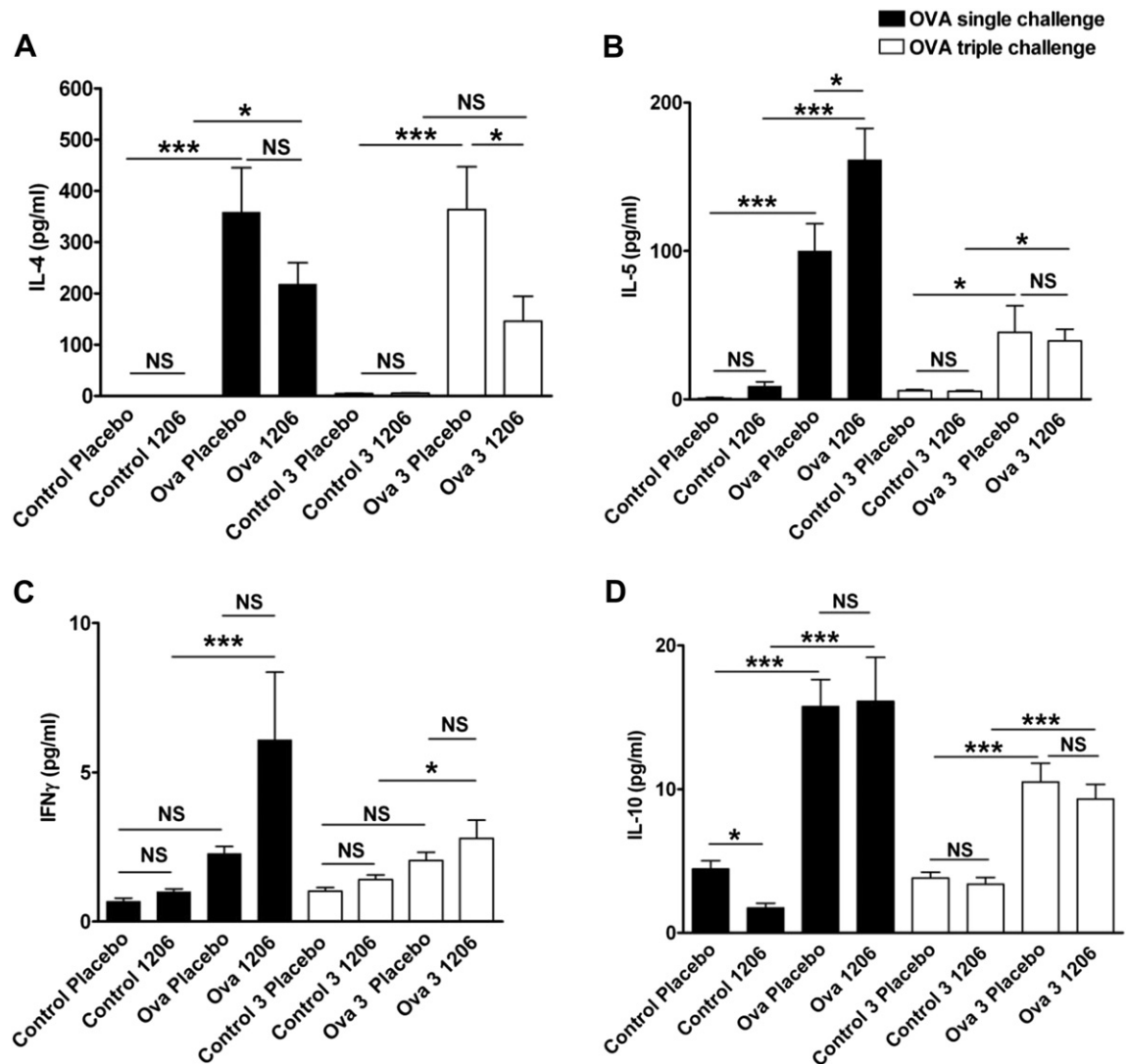


Fig. 2. Effect of *Bifidobacterium longum* 1206 feeding on lung BALF cytokines, IL-4 (A), IL-5 (B), IFN- γ (C) and IL-10 (D) following OVA challenge. Protein levels were measured by ELISA. OVA single challenge (dark bars), $n = 10$, OVA triple challenge (light bars), $n = 8$. Values are shown as Mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS denotes non-significant.

intrathoracic lymph nodes were unaffected by *Bif* feeding with or without OVA challenge (Supplemental Fig. 1A and B). Similar to the spleen and the draining lymph nodes, we saw no significant differences among treatment groups in the T regs in the Peyer's patches (Supplemental Fig. 1C). We tested the functionality of the splenic T cell response by performing anti-CD3/anti-CD28 stimulations on isolated splenocytes. IL-10 was increased in the *Bif* fed OVA challenged group ($P < 0.01$, Supplemental Fig. 2A) and TNF- α was significantly decreased in both the *Bif* fed saline and OVA challenged groups ($P < 0.001$, Supplemental Fig. 2A,B). There was no effect on IL-1 β production (Supplemental Fig. 2C) whereas both IL-4 and IFN- γ were elevated in the *Bif* fed OVA challenged group ($P < 0.01$, $P < 0.001$, Supplemental Fig. 2D and E, respectively). No changes in the levels of KC (CXCL1), IL-2, IL-12 and IL-5 were observed in stimulated splenocytes of either placebo or *Bif* fed animals (Supplemental Table 3).

3.7. Effects of *Bifidobacterium* feeding on airway hyperresponsiveness (AHR) and serum OVA-specific IgE following a triple challenge

The effects of *Bif* feeding on OVA-specific IgE and OVA-induced AHR were assessed in animals that were challenged three times

with OVA. The elevations in IgE seen in the OVA challenged animals were unaffected by *Bif* feeding (Fig. 6A). AHR was induced by OVA challenge (Fig. 6B). Data for changes in E_{RS} only are shown but similar changes were seen in R_{RS} (See Supplemental Fig. 3). The AHR induced by OVA challenge was not reduced by *Bif* feeding and indeed may have been somewhat augmented at the highest concentration of methacholine administered.

4. Discussion

In the current study we examined the effects of feeding ovalbumin-sensitized mice with a *B. longum* strain of commensal bacterium on the inflammatory response to OVA challenge and on markers of immune modulation locally and systemically. A systemic immunostimulatory effect of *Bif* feeding was evidenced by elevations of IL-10, IL-4 and IFN- γ following anti-CD3/anti-CD28 stimulation of splenic T cells. The effects on the lung compartment differed in that OVA-induced increases in IL-4 were attenuated by *Bif* feeding but BAL fluid IL-5 following a single OVA challenge was accentuated by *Bif* feeding despite the reduction in BAL fluid eosinophilia. However the inhibitory effect of *Bif* feeding on BALF eosinophilia was no longer present after three challenges

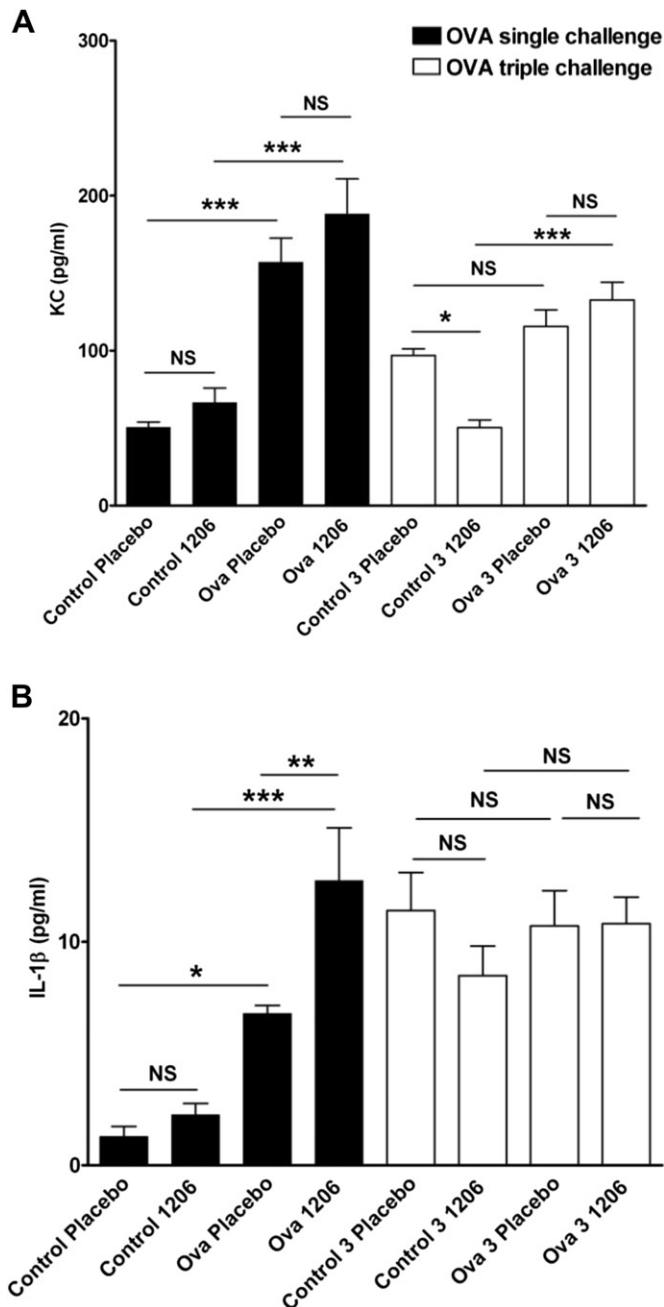


Fig. 3. Effect of *Bifidobacterium longum* 1206 feeding on lung BALF cytokines, KC (A) and IL-1 β (B) following OVA challenge. Protein levels were measured by ELISA OVA single challenge (dark bars), $n = 10$, OVA triple challenge (light bars), $n = 8$. Values are shown as Mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS denotes non-significant.

with OVA. No alteration of OVA-specific IgE or attenuation of OVA-induced AHR was demonstrable following *Bif* feeding.

Bif 1206 was previously evaluated by our group in murine models of respiratory and oral allergy [15]. These studies demonstrated an anti-inflammatory effect in mice undergoing two allergen challenges. We wished to evaluate if the effect of *Bif* 1206 feeding in the OVA allergy model was robust and stable following repeated challenge and whether the effects were accompanied by attenuation of AHR. Similar to our initial observations BALF eosinophilia was reduced following a single challenge in *Bif* fed mice but the inhibitory effect was overcome by a triple challenge. In addition we did not find the previously observed reduction in TNF- α in BALF.

The immunomodulatory effects of commensal organisms on allergic pulmonary responses are closely related to the organisms administered. *Lactobacillus reuteri* but not *Lactobacillus salivarius* has been shown to inhibit allergic airway responses in the sensitized mouse [14]. *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* have also been demonstrated to be efficacious in reducing AHR and IgE levels [16]. The duration and timing of feeding are also determinants of anti-inflammatory efficacy. Forsythe and colleagues found that a period of feeding of at least 9 days was required for significant inhibition of airway eosinophilia and AHR to be observed [14] whereas *L. rhamnosus* GG and *B. lactis* were tested from the neonatal period on an alternate day schedule and lasting 8 weeks through the period of sensitization and challenge [16]. A recent study by Hougee et al. [17] has demonstrated strain-dependent immunomodulatory effects of the probiotic bacterium, *B. breve*. Although there are similarities in the anti-inflammatory effects of the above mentioned organisms the different strains vary as to their effects on BALF cytokines. *B. breve* reportedly reduces IL-4, IL-5, IL-10 and IFN- γ in BALF [16] whereas we noted only a decrease in IL-4 but increases in IL-5, IFN- γ and IL-1 β . The increase in IL-5 is also in contrast with observations by Forsythe and colleagues [14]. Differences in feeding protocols and in the organisms used likely account for the differences in findings.

The mechanism of the *Bif* feeding in inhibiting the allergen-induced increase in eosinophilia induced by a single allergen challenge is not clear in our study. IL-5, the principal Th2 cytokine responsible for eosinophilic airway inflammation in the mouse [18,19] was in fact elevated further by *Bif* feeding. The observed increase in IFN- γ protein in the BALF is a potential explanation for the inhibition of eosinophilia. IFN- γ potently inhibits Th2 responses and mice deficient in IFN- γ or the Th1 master transcription factor T-bet have exaggerated experimental asthma phenotypes [20,21]. In addition to its usual inhibitory effects on Th2 responses IFN- γ may increase the phagocytosis of eosinophils by alveolar macrophages, accelerating their clearance from the airspaces of allergen challenged animals [22]. Alternatively *Bif* feeding may have affected chemoattractants for eosinophils, such as eotaxin [23] or derivatives of arachidonic acid metabolism (leukotriene-B $_4$, cysteinyl leukotrienes, or 5-oxo-eicosatetraenoic acid) [24,25].

There was also evidence of the modulation of the function of BALF cells by *Bif* feeding; reductions in the expression levels of IL-4, IFN- γ , LTC $_4$ synthase and NGP were detectable by RT-PCR suggesting attenuated lymphocyte, macrophage, mast cell and neutrophil activation despite lack of an effect of *Bif* feeding on the numbers of these cells in single challenged animals. The increase in IFN- γ protein in the BAL fluid of *Bif* fed and allergen challenged animals appears to have originated from cells within the airway tissues since the transcript was reduced in the BALF cells, indicating different effects of *Bif* feeding on different lung compartments. Indeed IL-5 mRNA levels were undetectable in the BALF cells while the protein levels were enhanced by *Bif*. IL-1 β was stimulated by *Bif* feeding in conjunction with OVA challenge, further indicating that the immunomodulatory effects are mixed, with elements of inhibition and stimulation of pro-inflammatory cytokines associated with innate and adaptive immune pathways.

Anti-inflammatory effects of probiotics have been previously attributed to the recruitment of regulatory T cells [15,16]. In order to assess the possible immunomodulatory role of regulatory T cells in the lungs we assessed CD4+CD25+FoxP3+ cells in lung digests. T regs increased in the lungs following a single OVA challenge and this increase was accentuated by *Bif* feeding, suggesting a possible mechanistic link. The lack of an inhibitory effect of *Bif* feeding on IFN- γ indicates that if indeed the mechanism involves T regs the inhibitory effect of the T regs was selective for the Th2 cells perhaps

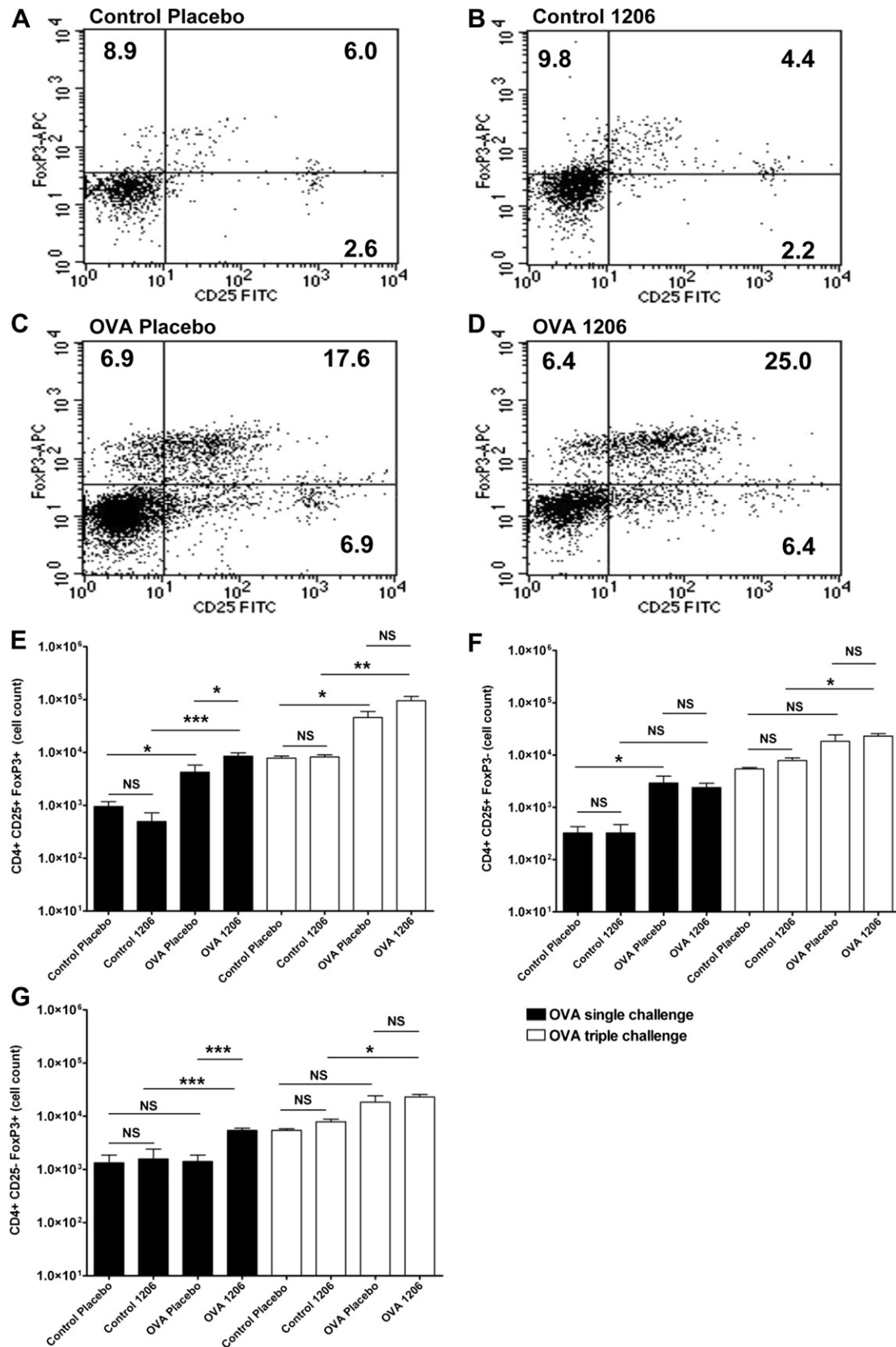


Fig. 4. Flow cytometric analysis of CD4, CD25, FoxP3 T cells in lung digests following *Bifidobacterium longum* 1206 feeding. Representative scatter plots of the CD4, CD25, FoxP3 T cell profile following saline challenge in placebo (A) and *Bif* fed (B) mice and OVA challenge in placebo (C) and *Bif* fed (D) mice. Cell count of CD4⁺CD25⁺FoxP3⁺ T cells (E), CD4⁺CD25⁺FoxP3⁻ (F) and CD4⁺CD25⁻FoxP3⁺ (G) populations. OVA single challenge (dark bars) $n = 10$, OVA triple challenge (light bars) $n = 8$. Values are shown as Mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS denotes non-significant.

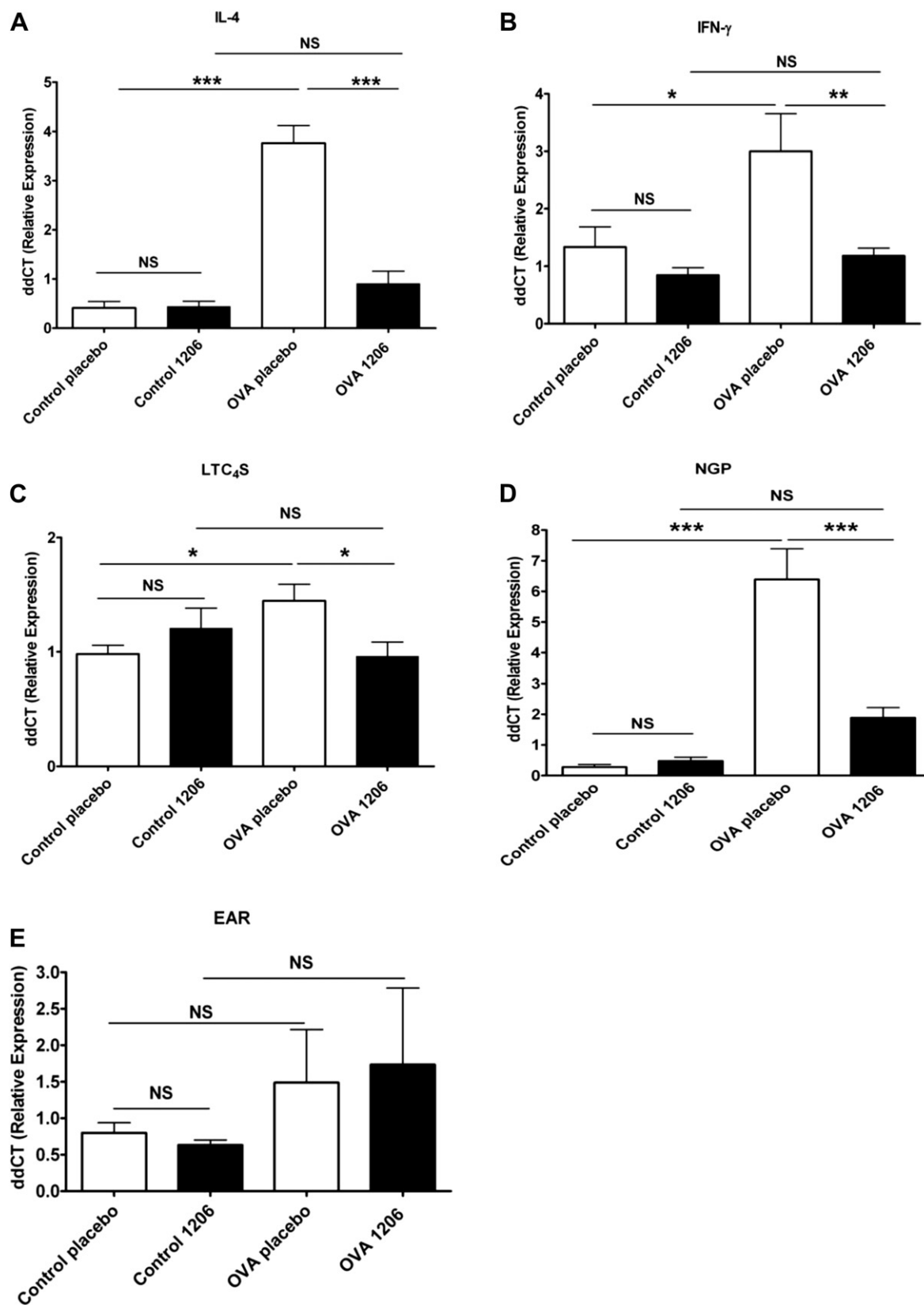


Fig. 5. qRT-PCR analysis of pro-inflammatory mediator and activation marker expression in the bronchoalveolar lavage fluid (BALF) cells following *Bifidobacterium longum* 1206 feeding. IL-4 (A), IFN-γ (B), LTC₄S (C), NGP (D) and EAR (E) mRNA levels following a single OVA challenge. *Bif* 1206 fed (dark bars), placebo fed (light bars), *n* = 10. Values are shown as Mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, NS denotes non-significant.

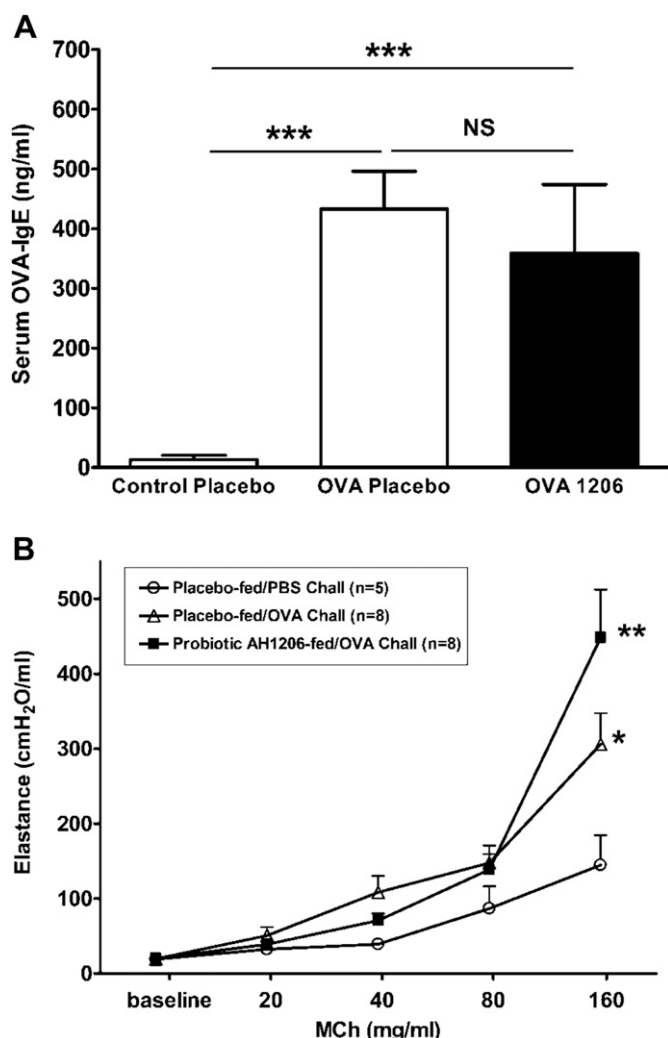


Fig. 6. Effect of *Bifidobacterium longum* 1206 feeding on OVA-specific IgE (A) and airway hyperresponsiveness (AHR) and elastance (B) following OVA challenge. *Bif* 1206 fed (dark bar, $n = 8$), placebo fed (light bars); non challenged, $n = 5$; triple OVA challenged, $n = 8$. Values are shown as Mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS denotes non-significant.

due to the T cell subsets occupying different compartments within the lungs. The increase in T regs with three OVA challenges was not modified further by *Bif* feeding, consistent with the lack of inhibition of allergic inflammation under these conditions.

Next we examined whether the mechanism by which oral feeding of *Bif* affects the lung T reg population may involve *Bif*-induced immune responses initiated in the Peyer's patches and in the spleen. Intestinal bacteria appear to indirectly affect T cell fate by modulating the dendritic cell response via an interaction with the intestinal epithelium, principally in the Peyer's patches [26]. Intestinal specific CD103⁺ dendritic cells have been shown to be responsible for the induction of T reg cells in the presence of TGF- β and retinoic acid [27]. T regs reduce AHR and vascular remodeling following repeated antigen challenge in the mouse [28]. Karimi et al. found that the adoptive transfer of the CD4⁺CD25⁺ cells isolated from the spleen inhibited the responses to allergen sensitization and challenge, suggesting the possibility that migration of splenic T regs to the lungs may occur and account for the inhibitory effects observed [29]. However we did not detect any difference in the levels of T regs in either spleen or Peyer's patches in mice fed *Bif* prior to and during OVA challenge. Furthermore the adoptive transfer of T regs demonstrates only the plausibility of the

hypothesis that they are responsible for immunomodulation by probiotics and does not prove their participation in the phenomenon. Indeed the effects of feeding on the regulation of pro-inflammatory cytokines in the spleen differed from the lung. *Bif* feeding enhanced the increase in IL-4, IL-10 and IFN- γ production by anti-CD3/anti-CD28 stimulated spleen cells. The net systemic effect of *Bif* feeding was therefore immunostimulatory. The increase of both BALF IL-5 protein levels and lung Foxp3⁺ T reg cells may correlate with recent observations that IL-5 induces antigen specific T regs [30]. Perhaps such immunostimulatory properties may account for the unexpected increase in mortality that was observed in a clinical trial of probiotics in acute pancreatitis [31].

In conclusion, feeding with a *B. longum* strain, AH1206, led to an altered inflammatory response to a single but not repeated allergen challenge in the mouse with modulation of pro-inflammatory cytokines that suggest effects on the innate immune response and an attenuated Th2 inflammation. An increase in T reg cells was observed in the lungs when *Bif* feeding was combined with allergen sensitization and a single challenge. However no changes in allergen-induced airway hyperresponsiveness or in specific IgE were associated with *Bif* feeding. Inconsistencies with previous published results suggest that the effects of commensal feeding on allergen-induced airway responses may be quite sensitive to the organism and the feeding protocols.

Authors contributions

JM; Conceived of and designed the study, performed BALF and differential analysis, and assisted with the writing of the manuscript.

JMS; Aided in study design the study performed BALF, mRNA analysis, protein analysis, cultured bacteria, isolated lymph nodes, spleens and Peyer's patches, performed statistical analyses, compiled the data, drafted and wrote the manuscript.

FS; Aided in study design and manuscript preparation.

COM; performed flow cytometry analysis on lung, lymph node and splenic Foxp3⁺ T cell populations.

KHS; Performed measurements of airway responses to methacholine, measured serum IgE and performed flow cytometry.

BS; Aided in study design, performed splenocytes assays, MSD protein analysis on supernatants.

HKQ; Aided in AHR, BALF sample preparation and serum analysis.

Conflict of interest/disclosures

The bacterial strain *B. longum* AH1206 was provided by Alimentary Health Ltd., Cork, Ireland. J. Mac Sharry is a co-inventor on a patent application related to the use of the *B. longum* AH1206 strain in inflammatory disease, International Application No.: PCT/IE2008/000033.

All other authors state no conflict of interest with regard to the body of work submitted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.pupt.2012.05.011>.

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Supplemental Table 1; Gene specific primers and probe combinations assayed by qRT-PCR

Gene ID	NCBI ID	Primer Sequence (5' - 3')		Probelibrary probe number
B-actin	NM_007393.3	L	ctaaggccaaccgtgaaaag	64
		R	accagaggcatacagggaaca	
IL4	NM_021283.1	L	catcggcattttgaaggag	2
		R	acgtttggcacatccatctc	
IL5	NM_010558.1	L	acattgaccgcaaaaagag	91
		R	atccaggaactgcctctgc	
IFNγ	NM_008337.3	L	tcaaaagagttccttatgtgccta	69
		R	tacgaggacggagagctgtt	
LTC ₄ S	NM_008521.1	L	ctcttctggctaccgtcacc	7
		R	aagcccttcgtgcagagat	
NGP	NM_008694.2	L	gcctaaagactgcgacttcc	52
		R	tgaagaattccctgtgcaa	
EAR2	NM_007895.2	L	caaccagccctaagttccac	18
		R	tgaggcaagcattaggacaa	
TNFα	NM_013693.2	L	ctgtagcccacgtcgtagc	25
		R	ttgagatccatgccgttg	
TGFβ	NM_011577.1	L	tggagcaacatgtggaactc	72
		R	cagcagccggttaccaag	
GATA-3	NM_008091.3	L	cttatcaagccaagcgaag	77
		R	cccattagcgttcctcctc	

Supplemental Table 2; Viability of Bifidobacterium from feed, faecal and BALF samples.

Sample (n =7)	Bifidobacterium (Colony forming units/CFU)	
	Mean	Standard Error (+/-)
Feed (200 µl dose)	1.00E+09	1.00E+07
Intestinal Faecal sample	4.00E+07	1.00E+06
BALF from 1206 Fed Mice	0.00E+00	0.00E+00

Values are shown as Mean ± SEM.

Supplemental Table 3; Effect of Bifidobacterium feeding on BALF; Cell, Protein, mRNA; Lung cell populations and Splenocyte stimulations.

	Sample	BALF							
	Assay	Cytopsin	MSD		qRT-PCR				
		<i>(x 10⁵)</i>	<i>(pg/ml)</i>			<i>(ddCT)</i>			
Single challenge		Macrophage	TNFα	IL-2	IL-12	TNFα	TGFβ	GATA-3	
Control placebo		2.09 +/- 0.33	13.74 +/- 1.39	1.89 +/-0.87	50 +/- 4.99	0.53 +/- 0.12	0.52 +/- 0.23	0.50 +/- 0.16	
Control 1206		1.58 +/- 0.35	17.89 +/- 3.63	6.77 +/- 1.37	63.64 +/- 17.58	0.78 +/- 0.15	0.85 +/- 0.35	0.94 +/- 0.27	
OVA placebo		1.84 +/- 0.36	65.84 +/- 10.71	13.81 +/- 1.53	193.2 +/- 13.89	2.18 +/- 0.58	2.34 +/- 0.65	4.40 +/- 2.2	
OVA 1206		1.39 +/- 0.27	84.61 +/- 14.01	10.59 +/- 1.36	164.2 +/- 17.02	2.21 +/- 0.40	2.96 +/- 1.68	2.10 +/- 1.1	
Triple challenge	(n =8)	Macrophage	TNFα	IL-2	IL-12				
Control placebo		2.46 +/- 0.39	17.61 +/- 11.45	8.60 +/- 0.98	185 +/- 10.03	ND	ND	ND	
Control 1206		1.54 +/- 0.31	18.98 +/- 12.02	8.37 +/- 0.97	128.3 +/- 14.09	ND	ND	ND	
OVA placebo		6.32 +/- 1.3	77.98 +/- 29.98	12.09 +/- 1.17	401.9 +/- 48.47	ND	ND	ND	
OVA 1206		5.63 +/- 1.46	20.51 +/- 8.47	11.71 +/- 0.83	646.5 +/- 261.3	ND	ND	ND	
	Sample	Splenocyte stimulation CD3/CD28				Naive BALF			
	Assay	MSD				Cytopsin	(n =10)		
		<i>(pg/ml)</i>					<i>(x 10⁴)</i>		
Single challenge	(n =10)	KC (CXCL1)	IL-2	IL-12	IL-5	Total Cell Count	25 +/- 2.4		
Control placebo		33.53 +/- 3.21	3965 +/- 669.3	47.06 +/- 4.31	20.93 +/- 7.32	Eosinophil	0.29 +/- 0.05		
Control 1206		37.85 +/- 3.86	2602 +/- 704	44.79 +/- 11.49	22.6 +/- 5.5	Neutrophil	3.8 +/- 0.4		
OVA placebo		38.33 +/- 5.43	3622 +/- 861.6	52.01 +/- 6.46	22.12 +/- 4.72	Lymphocyte	0.16+/- 0.04		
OVA 1206		35.52 +/- 8.84	4312 +/- 533.7	56.22 +/- 5.22	42.81 +/- 6.34	Macrophage	20.9 +/- 2.5		
Triple challenge	(n =8)	KC (CXCL1)	IL-2	IL-12	IL-5				
Control placebo		ND	ND	ND	ND				
Control 1206		ND	ND	ND	ND				
OVA placebo		ND	ND	ND	ND				
OVA 1206		ND	ND	ND	ND				

Values are shown as Mean ± SEM. Statistical conditions are italicised, ANOVA Tukey’s multiple comparison test, * P<0.05, ** P<0.01, *** P< 0.001.

Control placebo vs. OVA placebo is denoted by 'a';

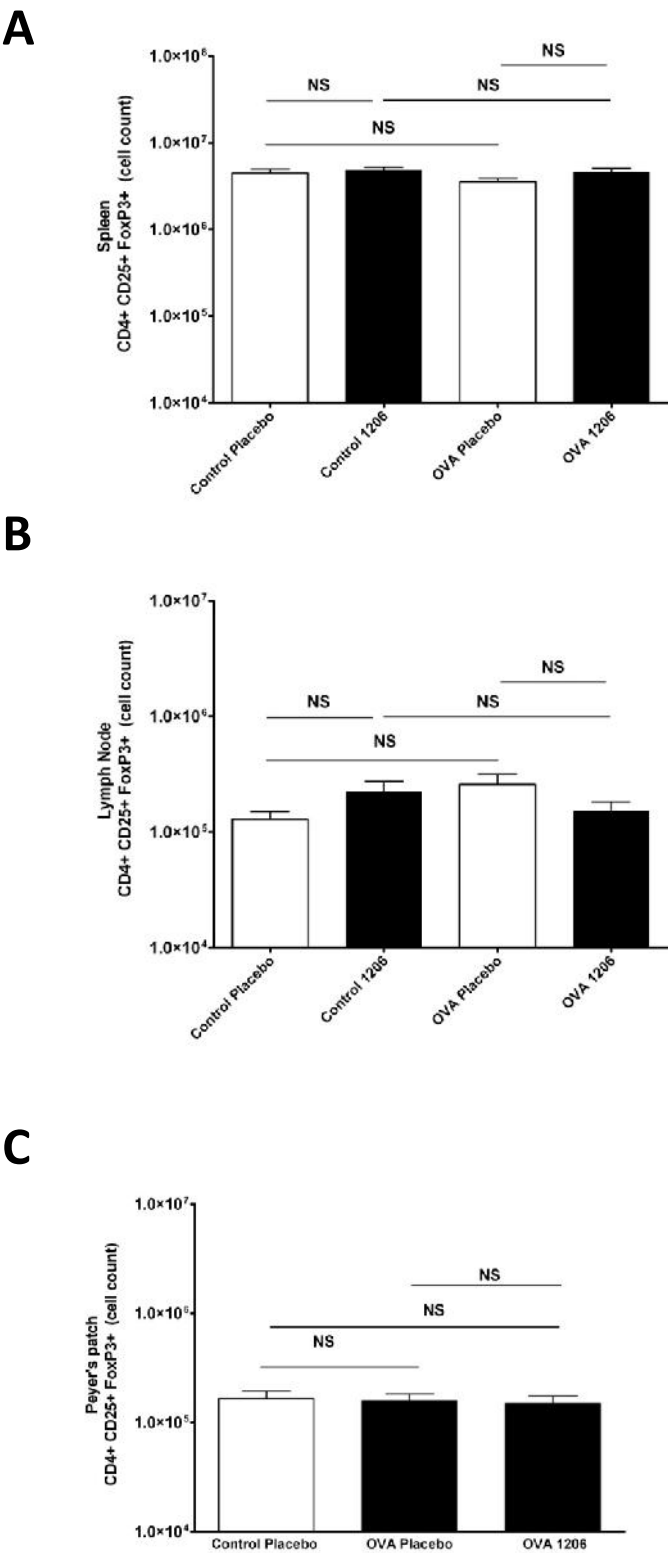
Control 1206 vs. OVA 1206 is denoted by 'b'

OVA placebo vs. OVA 1206 by' c' .

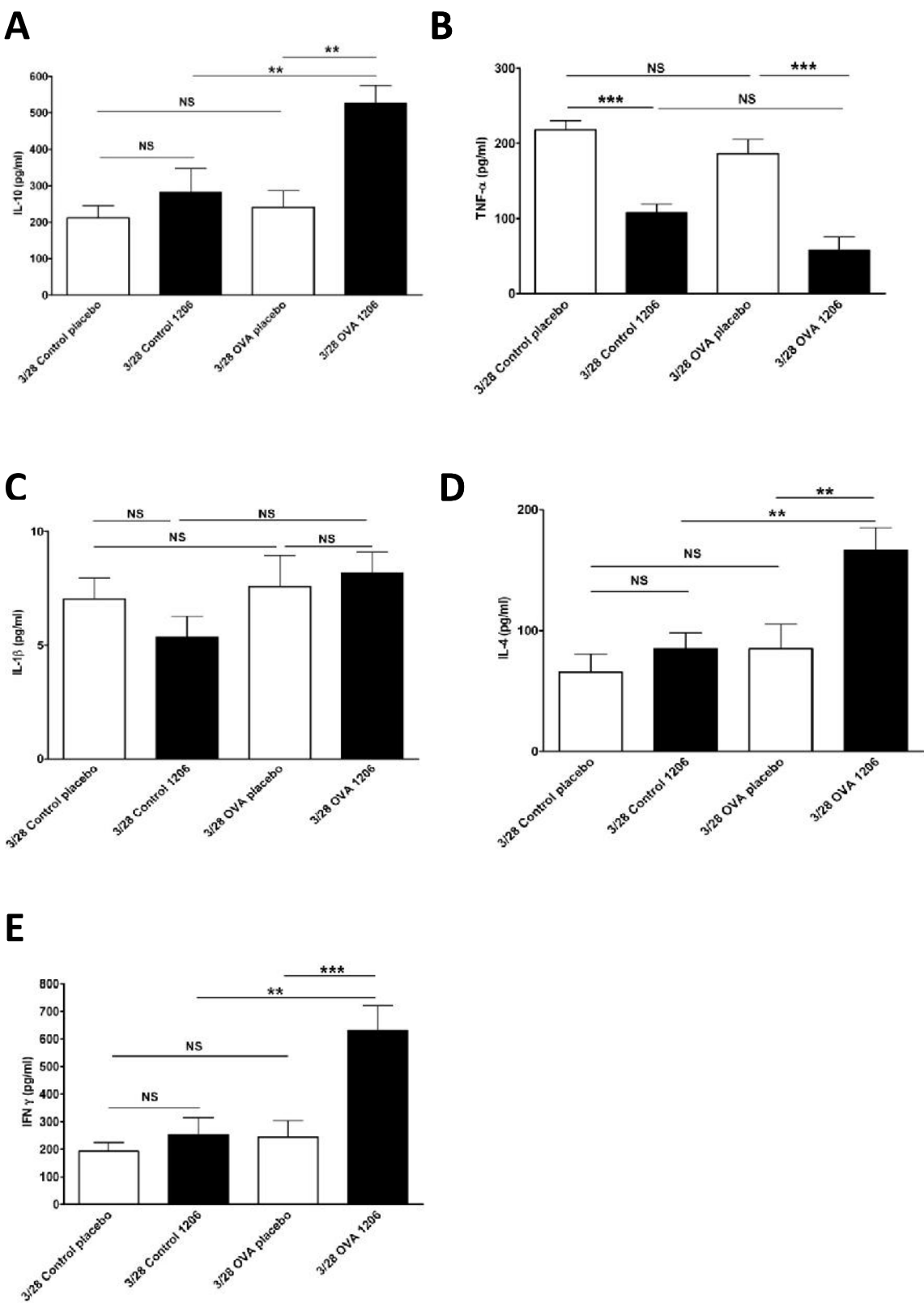
Control placebo vs, Control 1206 is denoted by 'd'.

ND = Not done.

Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3

