

ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Intranasal administration of *Acinetobacter lwoffii* in a murine model of asthma induces IL-6-mediated protection associated with cecal microbiota changes

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Abstract

Background: Early-life exposure to certain environmental bacteria including *Acinetobacter lwoffii* (AL) has been implicated in protection from chronic inflammatory diseases including asthma later in life. However, the underlying mechanisms at the immune-microbe interface remain largely unknown.

Methods: The effects of repeated intranasal AL exposure on local and systemic innate immune responses were investigated in wild-type and *Il6*^{-/-}, *Il10*^{-/-}, and *Il17*^{-/-} mice exposed to ovalbumin-induced allergic airway inflammation. Those investigations were expanded by microbiome analyses. To assess for AL-associated changes in gene expression, the picture arising from animal data was supplemented by in vitro

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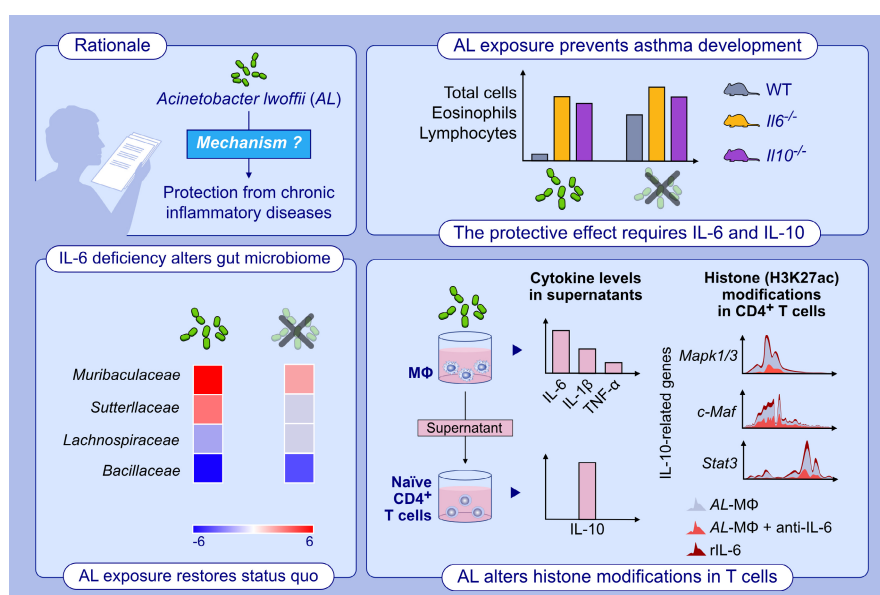
experiments of macrophage and T-cell responses, yielding expression and epigenetic data.

Results: The asthma preventive effect of AL was confirmed in the lung. Repeated intranasal AL administration triggered a proinflammatory immune response particularly characterized by elevated levels of IL-6, and consequently, IL-6 induced IL-10 production in CD4⁺ T-cells. Both IL-6 and IL-10, but not IL-17, were required for asthma protection. AL had a profound impact on the gene regulatory landscape of CD4⁺ T-cells which could be largely recapitulated by recombinant IL-6. AL administration also induced marked changes in the gastrointestinal microbiome but not in the lung microbiome. By comparing the effects on the microbiota according to mouse genotype and AL-treatment status, we have identified microbial taxa that were associated with either disease protection or activity.

Conclusion: These experiments provide a novel mechanism of *Acinetobacter lwoffii*-induced asthma protection operating through IL-6-mediated epigenetic activation of IL-10 production and with associated effects on the intestinal microbiome.

KEYWORDS

adaptive immunity, asthma, epigenomics, innate immunity, microbiota



GRAPHICAL ABSTRACT

This study confirms that IL-6-deficient mice are resistant to asthma-protective effect driven by *Acinetobacter lwoffii* (AL). Downstream of IL-6, the asthma-blocking properties is mediated by IL-10, but not IL-17. In IL-6-deficient mice, exposure to AL increases *Muribaculaceae* and *Sutterellaceae*, and decreases *Lachnospiraceae* and *Bacillaceae*, compared to the unexposed mice, restoring the status quo observed in the wild-type littermates mice. In vitro experiments demonstrate modulations in the epigenetic landscape of histone mark H3K27ac at the promoter regions of the *Il10* pathway-related genes (*Mapk1/3*, *Stat3*, and *c-Maf*) in the CD4⁺ T-cells cultured with AL-conditioned macrophage supernatant (AL-Mφ). These changes were reversed by anti-IL-6 treatment (AL-Mφ + anti-IL-6) and phenocopy by adding rIL-6 alone. Abbreviations: AL, *Acinetobacter lwoffii*; AL-Mφ, AL-conditioned macrophage; *c-Maf*, c-musculoaponeurotic fibrosarcoma transcription factor; IL, interleukin; *Il6*^{-/-}, *Il6* knockout; *Il10*^{-/-}, *Il10* knockout; Mφ, macrophages; *Mapk1/3*, mitogen-activated protein kinase 1/3; rIL-6, recombinant IL-6; *Stat3*, signal transducer and activator of transcription 3; TNF, tumor necrosis factor; WT, wild-type

1 | INTRODUCTION

Referring to the hygiene hypothesis, altered environmental exposures in urbanized countries have modified early-life microbial exposure and microbiome development, which has been associated with

the increased prevalence of allergic diseases such as asthma.¹⁻³ The asthma-protective effects of lifestyle, such as living on traditional farms, have emerged as a potential factor to explain differences in disease incidence in recent years.⁴⁻⁸ This concept is primarily based on epidemiological studies performed in several populations and this

effect has mostly been attributed to respiratory exposure to particular environmental microbes.^{9,10}

A few farm-derived microbes potentially contributing to the protection against asthma and other allergies have been reported, including *Lactococcus lactis*,⁸ *Staphylococcus sciuri*,¹¹ or *Bacillus licheniformis*¹² and, as the most remarkable representative, *Acinetobacter lwoffii* (AL), a Gram-negative bacterium identified in several independent studies.^{8,13,14} as a putatively protective bacterial species.^{8,13,15–17} However, the AL protective effects involve several mechanisms, operating most probably cooperatively or at least in parallel. Those include innate immune activation in the lung shifting the M2 macrophage phenotypes to M2b and thus leading to asthma-protection in OVA-sensitized mice.¹⁸ Several signaling pathways have been linked to these effects such as those related to maternal TLR-mediated immune recognition or epigenetic changes.^{13,14} The mechanisms by which AL or other environmental bacterial exposures protect from asthma remains largely unclear. In this study, we hypothesized that AL acts on both innate and adaptive immune systems through IL-6/IL-10 axis and changes in the epigenetic landscape of T cells, in parallel associated with changes in the gut mucosal microbiome.

2 | MATERIALS AND METHODS

2.1 | Mice

Six- to eight-week-old female BALB/c mice were used in all in vivo experiments. They were kept under specific pathogen-free housing conditions and supplied with water and an ovalbumin (OVA)-free diet ad libitum. All animal experiments were performed in accordance with German and international guidelines and were approved by Regierungspräsidium Gießen, Germany (MR20/2013 and G23/2018). For more details, please, refer to the Appendix S1.

2.2 | Treatment, sensitization, and local allergen challenge

Mice from *Il6*^{-/-}, *Il10*^{-/-}, and *Il17af*^{-/-} strains and their wild-type (WT) littermates were sensitized to OVA by three subcutaneous injections of 10 µg of OVA grade VI (Sigma, Deisenhofen, Germany) diluted in 200 µl of PBS on days 36, 43, and 50. An allergen challenge was performed on 3 consecutive days (62, 63, and 64) by an OVA aerosol (1% wt/vol OVA in PBS) to the airways for 20 min daily to induce an allergic asthma phenotype.^{13,14,19–21} As a control, PBS was used instead of OVA during sensitization and challenge. As pre-exposure prophylaxis, mice were anesthetized with 36 mg/kg of ketamine (Ketamin 10% Medistar GmbH, Germany) plus 4 mg/kg of rompun (Rompun 2% Bayer, Germany). Afterward, the mice received intranasal AL strain F78 (10⁸ CFU in 50 µl of PBS) or PBS alone as a control, every second day for 12 applications in the case of the experiments shown in Figure 1A–D or for 17 applications for the rest of the in vivo investigations (*Il6*^{-/-} vs. WT littermates, *Il10*^{-/-} vs. WT littermates,

Il17^{-/-} vs. WT littermates). The sensitization and challenge regimen began 3 days after the last prophylaxis. AL culture conditions are given in the Appendix S1.

2.3 | Measurements in bronchoalveolar lavage and blood

Mice were sacrificed 2 days after the last OVA challenge with Ketamine (Ketamin 10% Medi star GmbH, Ascheberg, Germany) and Rompun (Rompun 2% Bayer AG, Vienna, Austria). Using a tracheal cannula, Bronchoalveolar Lavage (BAL) was performed using 1 ml of PBS containing 1× protease inhibitor cocktail (Roche, Darmstadt, Germany), and cells enumerated with a cell counter (Casy TT; Schärfe Systems, Burladingen, Germany). BALs were centrifuged for 10 min at 350g. Cell pellets were used for BAL differential cell counts and cell-free supernatants were stored at -20°C for measurements of IL-6, IL-1β, and TNF-α, performed as described in the Appendix S1 according to the described methods.

2.4 | Lung histology

Lungs were fixed with 10% formalin (wt/vol) directly after BAL collection, stored in 10% formalin, then embedded into paraffin, and 3-µm sections were processed with periodic acid-Schiff (PAS) stain to evaluate mucus-producing Goblet cells. PAS-stained sections were quantified using a PC-based Olympus light microscope BX51 equipped with a Cell-F System (Olympus). Eight to ten random images were collected using the same setting for the camera for each lung section under the ×10 objective.^{21,22} Average numbers of PAS⁺ mucus-producing goblet cells and the inflammation score assessed as described in the Appendix S1.

2.5 | Isolation, differentiation, and stimulation of bone marrow macrophages, myeloid dendritic cells and plasmacytoid dendritic cells

The isolation and differentiation of the Bone Marrow (BM) cells into BM-Derived Macrophages (BMDMs), Myeloid Dendritic Cells (MDCs), and plasmacytoid Dendritic Cells (pDCs) are described in the Appendix S1. After differentiation cells were stimulated in vitro with either *E. coli* LPS (10 ng/ml; Sigma) or 1×10⁶ CFU of AL for 24 h, then supernatants were harvested and cytokines measured, as described in Appendix S1.

2.6 | Stimulation of murine peritoneal macrophages and splenic naïve CD4⁺ T-cells

Peritoneal macrophages were isolated as described in the Appendix S1. In the first step, macrophages were cultured in 6-well

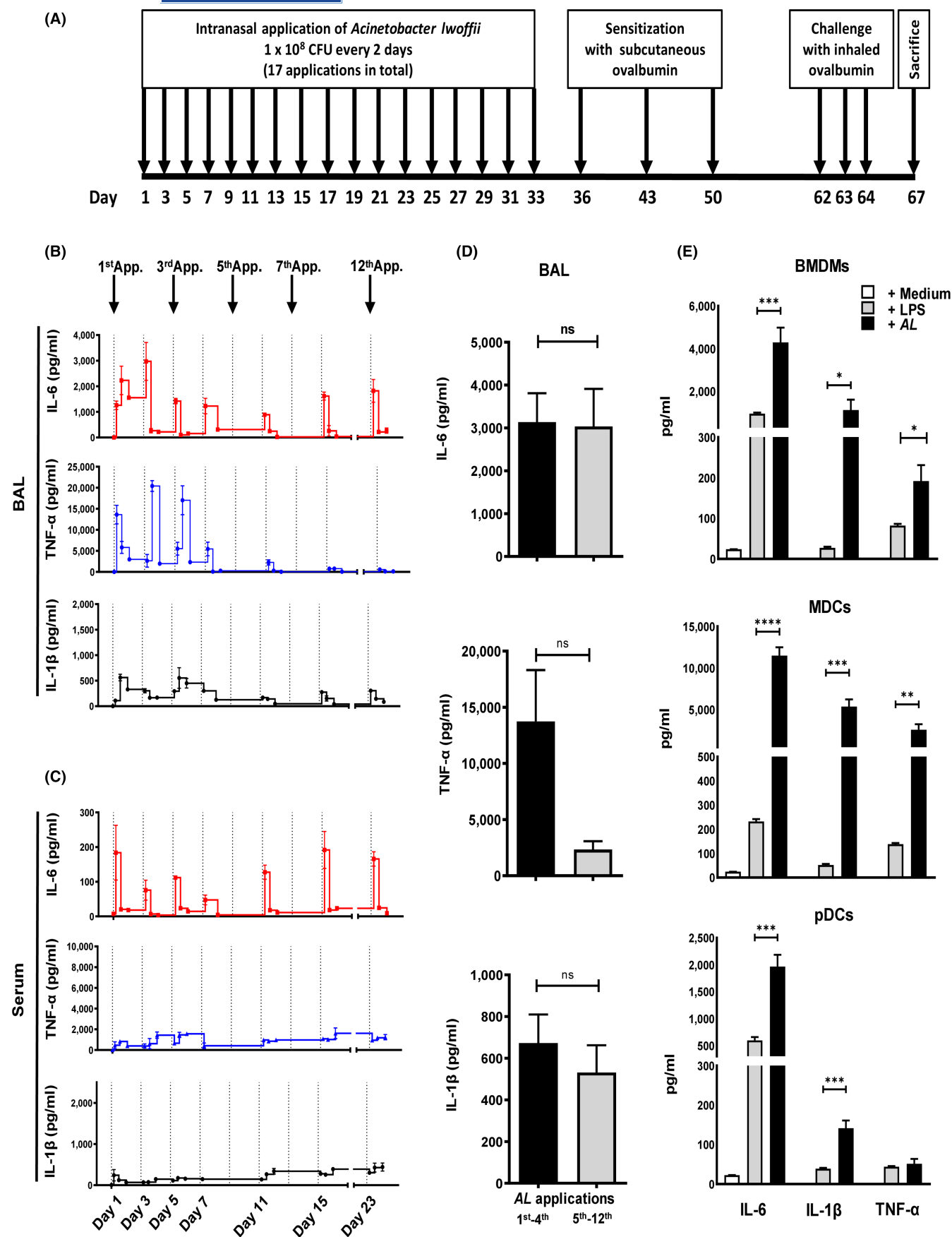


FIGURE 1 Repeated intranasal administration of *Acinetobacter lwoffii* (AL) stimulates chronic IL-6-dependent inflammation in the lung and systemically. (A) Protocol of repeated AL exposures followed by induction of experimental allergic asthma to ovalbumin (OVA). Control groups received intranasal Phosphate-Buffered Saline (PBS) instead of AL or PBS instead of OVA sensitization. (B, C) Bronchoalveolar lavage fluid (BAL) and serum were collected from 2 to 4 mice at 4-, 12-, and 24-h following AL exposure and IL-6, TNF- α , and IL-1 β were measured in cell-free samples. Shown are mean $\pm$ SEM for each time point. (D) Means of peak cytokine productions from 1st to 4th application versus 5th to 12th application for BAL IL-6, TNF- α , and IL-1 β production after AL administration. Shown are mean $\pm$ SEM computed from peak values measured after 4 h of each AL administration for the respective time frame. Data from D are representative of one experiment with different time points ($n = 10$ biologically independent samples for the 1st to 4th application group and $n = 8$ biologically independent samples for the 5th to 12th application group). Significant differences were evaluated using two-tailed unpaired t -test. * $p < .05$. (E) Bone marrow (BM) was collected from naïve WT mice and differentiated in vitro to BM-Derived Macrophages (BMDMs), Myeloid Dendritic Cells (MDCs), and plasmacytoid Dendritic Cells (pDCs). Differentiated cells were subsequently stimulated in vitro for 24 h with either LPS (10 ng/ml), 1×10^6 CFU of AL, or with medium alone as a negative control, supernatants collected, and cytokine levels were measured. Data from E are representative of two pooled independent experiments ($n = 6$ biologically independent samples/group). Shown are mean $\pm$ SEM. Significant differences were tested using unpaired two-tailed t -tests. * $p < .05$; ** $p < .01$; *** $p < .001$; **** $p < .0001$.

plates (0.5×10^6 cells/ml) and stimulated with either LPS (10 ng/ml; Sigma) or 1×10^6 CFU of AL for 24 h. Later, the supernatant was harvested and aliquoted. One aliquot was used to measure IL-6, IL-1 β , and TNF- α using antigen-specific ELISAs as described in the Appendix S1, and another aliquot was used for the second step in which splenic isolated naïve CD4 $^+$ T-cells were co-stimulated in vitro with anti-CD3 (0.5 μ g/ml Biolegend, California CA, USA) and anti-CD28 antibodies (1 μ g/ml; Biolegend) and cultured with the supernatant of AL-stimulated macrophages, there was no direct co-culture of macrophages with T cells. Finally, the AL-stimulated macrophages were washed, and the culture medium was replaced twice with fresh medium + rIL-2. At the end of the experiment, cytokines derived only from T-cells were measured in the final T cell supernatants as described in more detail in the Appendix S1 and in Figure S2A.

2.7 | In vitro Chromatin immunoprecipitation (ChIP) and quantitative analysis of *Il10* mRNA expression in stimulated CD4 $^+$ T-cells

Detailed methodology is given in the Appendix S1.

2.8 | 16S rRNA sequence analysis and microbiome studies

Detailed methodology is described in the Appendix S1.

2.9 | Statistical analysis

Significance differences for the in vivo and in vitro experiments were evaluated using unpaired two-tailed t -tests assuming normal distribution or the Kruskal-Wallis method and Pairwise PERMANOVA for the microbiome data. Detailed methodology is described in the Appendix S1. All statistical methods that were used to calculate significances are available in Table S6.

3 | RESULTS

3.1 | Intranasal administration of AL stimulates chronic IL-6-dependent inflammation

To closely mimic natural exposures, we developed a model with recurrent intranasal AL applications (Figure 1A). Prior studies showed substantial prevention of OVA-induced experimental asthma following early-life AL exposure,^{8,13} operating in a toll-like receptor (TLR)-dependent fashion.¹³ We now show that intranasal AL administration triggers a low-level of pro-inflammatory cytokine responses, detectable both locally in bronchoalveolar lavage fluid (BAL) (Figure 1B) as well as systemically in serum (Figure 1C). Each AL administration triggered a transient elevation of TNF- α , IL-6, and IL-1 β levels, but after several AL courses, this effect was less pronounced for TNF- α , but not for IL-6 or IL-1 β . After AL administration, the elevated IL-1 β levels were sustained in both serum and BAL (Figure 1B–D). When BMDMs, MDCs, or pDCs were stimulated with AL, proinflammatory cytokine production was induced in each cell population, with consistently high IL-6 levels and more striking increases in the MDCs (Figure 1E). AL-exposed conditioned peritoneal macrophages also produced high IL-6 levels compared to relatively small amounts of IL-1 β , or TNF- α (Figure S1A). From these results, we conclude that recurrent intranasal AL exposures induced a substantial nontolerant IL-6 response.

3.2 | *Il6* deficient mice are resistant to the asthma-protective effect mediated by AL

To analyze the contribution of IL-6 to the AL-induced asthma protection, we studied this model in WT mice and their *Il6* $^{-/-}$ littermates. As expected, the *Il6* $^{-/-}$ WT littermates developed the asthma phenotype after OVA challenge, as measured through numbers of total leukocytes including eosinophils, and lymphocytes in BAL, goblet cell metaplasia, and mucosal inflammation (Figure 2A), as well as Th2 cytokines in BAL including IL-5 and IL-13 (Figure 2B). Following exposure to AL prior to the OVA challenge, the experimentally induced

allergic airway inflammation was largely reduced as indicated by lower numbers of leukocytes, eosinophils, and lymphocytes in BAL, as well as BAL levels of pro-allergic cytokines (IL-5 and IL-13) in parallel to augmented levels of anti-allergic cytokine (IL-10) (Figure 2). However, the antibody titers in serum including OVA-specific IgE, IgG1, and IgG2a were not significantly different (Figure S1B). The asthma phenotype was augmented in the *Il6*^{-/-} mice, as indicated by enhancement of the airway inflammatory responses including the total numbers of leukocytes and eosinophils in the BAL, as well as the BAL levels of IL-5 and IL-13, regardless of AL administration (Figure 2A, B) with increases in the inflammation surrounding both the smaller and larger airways (Figure 2C). After AL treatment, goblet cell numbers were significantly reduced in the OVA-sensitized WT mice and *Il6*^{-/-} littermates, to a lesser extent in the latter group

(Figure 2A). However, the OVA-sensitized *Il6*^{-/-} mice were largely resistant to the AL-mediated asthma-protective effect, as reflected by higher numbers of leukocytes and eosinophils and IL-5 in the BAL compared with OVA-sensitized WT mice, regardless of AL exposure (Figure 2A, B). Our results provide evidence that the AL-mediated reduction of OVA challenge-induced asthma requires IL-6 activity in WT mice.

3.3 | Downstream of IL-6, the AL protective effect is mediated through IL-10 but not IL-17

Next, we examined whether AL-induced IL-6 modulates CD4⁺ T-cell responses in this model. Supernatants from AL-primed peritoneal

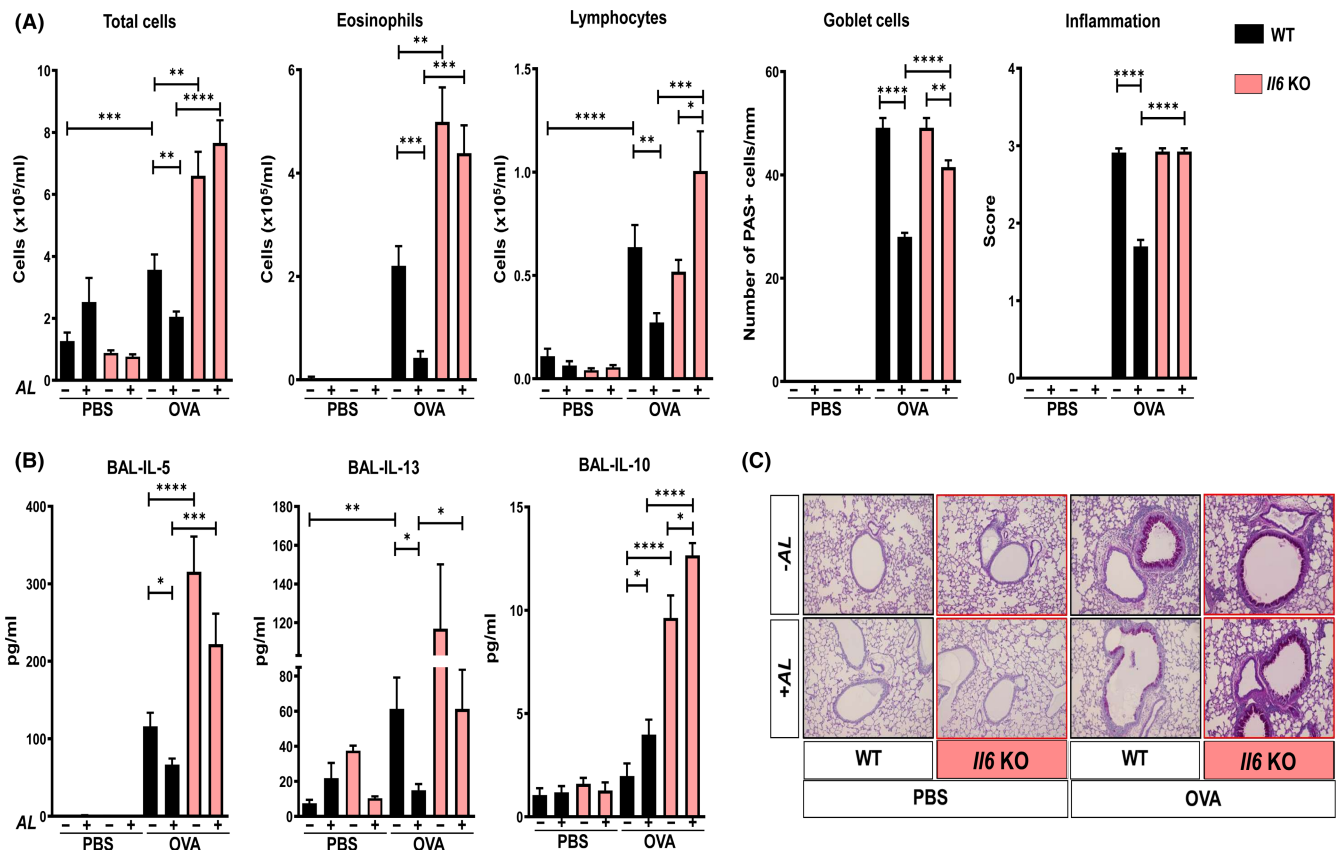


FIGURE 2 *Il6*^{-/-} mice are resistant to the asthma-protective effects provided by AL. *Il6*^{-/-} mice and their WT littermates were exposed to AL as shown in Figure 1A. (A) Differential cell counts were measured in BAL fluids: Total BAL leukocytes, eosinophils, and lymphocytes. Quantitation of mucus-producing goblet cells expressed/millimeter of basement membrane and inflammation score were measured in periodic acid-Schiff (PAS)-stained lung tissue sections as described in Methods. (B) Concentrations of IL-5 and IL-13 and IL-10 were measured in cell-free BAL fluids. (C) Representative PAS-stained lung tissues showing mucus-producing goblet cells in airway inflammation. Tissue samples were taken on day 67. Data in A of total BAL leukocytes, eosinophils, and lymphocytes are representative of three-pooled independent experiments ($n = 15$ biologically independent samples/group except for the PBS *Il6*^{-/-} controls with no OVA which are from two independent experiments with $n \geq 8$ biologically independent samples per group). Data of the Goblet cells and Inflammation score in A are from two-pooled independent experiments ($n = 8$ biologically independent samples/group). Data in B are from three-pooled independent experiments for the IL-5 and IL-13 ($n \geq 15$ biologically independent samples/group) for the IL-10 measurement data are from two-pooled independent experiments ($n \geq 10$ biologically independent samples/group). Only significant differences of the following comparisons between groups were shown (WT-OVA-AL vs. WT-OVA-no AL), (*Il6*^{-/-}-OVA-AL vs. *Il6*^{-/-}-OVA-no AL), (WT-OVA-no AL vs. *Il6*^{-/-}-OVA-no AL), (WT-OVA-AL vs. *Il6*^{-/-}-OVA-AL), and (WT-PBS-no AL vs. WT-OVA-no AL). Shown in A and B are mean $\pm$ SEM. Significant differences in A and B were calculated using unpaired two-tailed *t*-test. * $p < .05$; ** $p < .01$; *** $p < .001$; **** $p < .0001$; PBS, Phosphate-buffered saline; OVA, ovalbumin; AL, *Acinetobacter lwoffii*; +, pretreated with AL; -, pretreated with PBS.

macrophages of the experiment in Figure S1A were incubated in vitro with naïve CD4⁺ T-cells co-stimulated with anti-CD3/anti-CD28 antibodies (the experimental design is described in Figure S2A). The cytokines produced by the CD4⁺ T-cell were measured at the end of the experiment (Figure S2B). Highest concentrations were found for IL-10 and IL-17 proteins (Figure 3A), although other cytokines were secreted to a lesser amount, such as IL-12 and IFN- γ (Figure S2B). Stimulation of naïve CD4⁺ T-cells with recombinant IL-6 (rIL-6) also induced IL-10 production, as well as IL-17, albeit to a much lower extent (Figure 3A). The AL-conditioned macrophage supernatant and rIL-6 induced both IL-10 transcription and translation (Figure 3B). In contrast, preincubation with anti-IL-6 antibody blocked IL-10 induction on both levels (Figure 3B). These studies indicate that a major effect of the AL induction of IL-6 is promoting IL-10 production in CD4⁺ T-cells.

To examine if these in vitro results are of relevance for protection against asthma, we conducted a parallel in vivo experiment using *Il10*^{-/-} mice and their WT littermates (Figure 3C). Results were similar to those observed in the experiment involving the *Il6*^{-/-} mice and their WT littermates (Figure 2). As expected, WT mice that were the littermates the *Il10*^{-/-} mice were susceptible to OVA-challenge-induced asthma with effects blocked by AL administration. However, in the *Il10*^{-/-} mice, AL administration no longer rescued from the asthma phenotype, indicating a pivotal role of IL-10 in the AL-mediated asthma protection. To assess whether IL-17 also has a role in the asthma protection, *Il17*^{-/-} mice were studied in relation to their WT littermates (Figure 3D). However, AL was protective even in the absence of *Il17*. In total, these findings indicate that the AL-mediated asthma protection involves the IL-6-IL-10 axis, but not IL-17.

3.4 | AL alters the landscape of histone modifications in CD4⁺ T-cells mainly via IL-6

We have previously shown that AL administration leads to epigenetic changes in CD4⁺, but not CD8⁺ or NK T-cells.¹⁴ Therefore, we presumed that the AL-triggered IL-10 production is mediated through dynamic chromatin modifications of CD4⁺ T-cells. To capture the alterations in the epigenetic profile of the CD4⁺ T-cells under differing in vitro stimulation conditions, we examined the histone modification H3K27ac acetylation that is associated with active transcription.²³ 6.1%, 9.7%, and 7.9% gains of H3K27ac-annotated peaks were observed after incubation of naïve CD4⁺ T-cells with rIL-6 only, AL-conditioned macrophage supernatant and anti-IL-6 antibodies, respectively (Figure 4A–C). Remarkably, a large proportion (88.3%) of the gained H3K27ac-annotated peaks of the AL-conditioned macrophage supernatant were fused within the peaks of the rIL-6 stimulation condition (Figure 4D), indicating that the majority of the AL-conditioned macrophage effects attribute to the potential IL-6 role. In contrast, the H3K27ac annotated peaks of histone modifications were similar for the three negative controls (medium alone, unconditioned macrophage supernatant or unconditioned macrophage supernatant pretreated with anti-IL-6 antibodies) (Figure S3A).

To connect the identified peaks with their associated genes, we focused mainly on H3K27ac in the vicinity of gene promoters (i.e., TSS $\pm$ 1 kb). Notably, following incubation with either AL-conditioned macrophage supernatant or rIL-6, we detected changes in H3K27ac for genes required for *Il10* gene expression^{24–28} including enrichment of H3K27ac at the promoter regions of *Mapk1*, *Mapk3*, *Stat3*, and *c-Maf*, as well as at the *Il10* gene promoter (Figure 4D, E; top and middle line). Conversely, the H3K27ac mark was largely depleted at these gene promoters after pretreatment of the supernatant with anti-IL-6 antibody (Figure 4E; bottom line). These changes were highly specific since other inflammation-related genes such as *Foxp3*, *Ifng*, *Ifngr1*, *Ifngr2*, and *Il3* remained unaffected in all treatment conditions (Figure S3B).

We observed concordant molecular pathways for the H3K27ac-enriched genes in CD4⁺ T-cells for both AL-conditioned macrophage supernatants and rIL-6 treatment including MAPK signaling pathways, which maintain *Il10* expression in CD4⁺ T-cells^{26–28} (Figure 4F). The pathway analysis of the top 10 significant terms showed a robust interconnection between IL-6 and MAPK signaling pathways with the rest of the pathways (Figure 4F, G). In contrast, those pathways were not detected when CD4⁺ T-cells were stimulated with AL-conditioned macrophage supernatant pretreated with anti-IL-6 antibody. Overall, our results indicate that AL modulates the epigenetic landscape in CD4⁺ T-cells via macrophage-derived cytokines with a major IL-6 role.

3.5 | Impact of AL exposure on the microbiome compositions

Since hosts were exposed to AL by intranasal inhalation, we hypothesized that AL might mediate the asthma protective effects through changes in the host microbiome, whether in the airways or in the gut. To understand the impact of AL on microbiome compositions, experiments were conducted in the three cytokine-knockout (KO) mice and their WT littermates with or without AL exposure and/or OVA sensitization.

First, we examined α -diversity at sacrifice in the cecal contents of the groups of experimental mice. No differences in α -diversity were significant, regardless of genetic background, treatment, sensitization, or experiment (Tables S1 and S2; Figure S4A). For the lung samples, after removing contaminants, sequence depth was low, and no comparisons between exposures were significantly different (Tables S1 and S2; Figure S4B). Overall, AL exposure had no measurable impact on the cecal and lung microbiome α -diversity.

Next, we examined the microbiota β -diversity. As determined by analysis of Bray–Curtis and weighted UniFrac distance matrices, the microbiota of the mice in the three experiments were significantly different from each other, reflecting their differing founder populations (Figure S4C). The *Il10*^{-/-} mice and their WT littermates significantly differed from each other (Figure S4D, G, respectively), as did the *Il17*^{-/-} and their WT littermates (Figure S4E, H), indicating

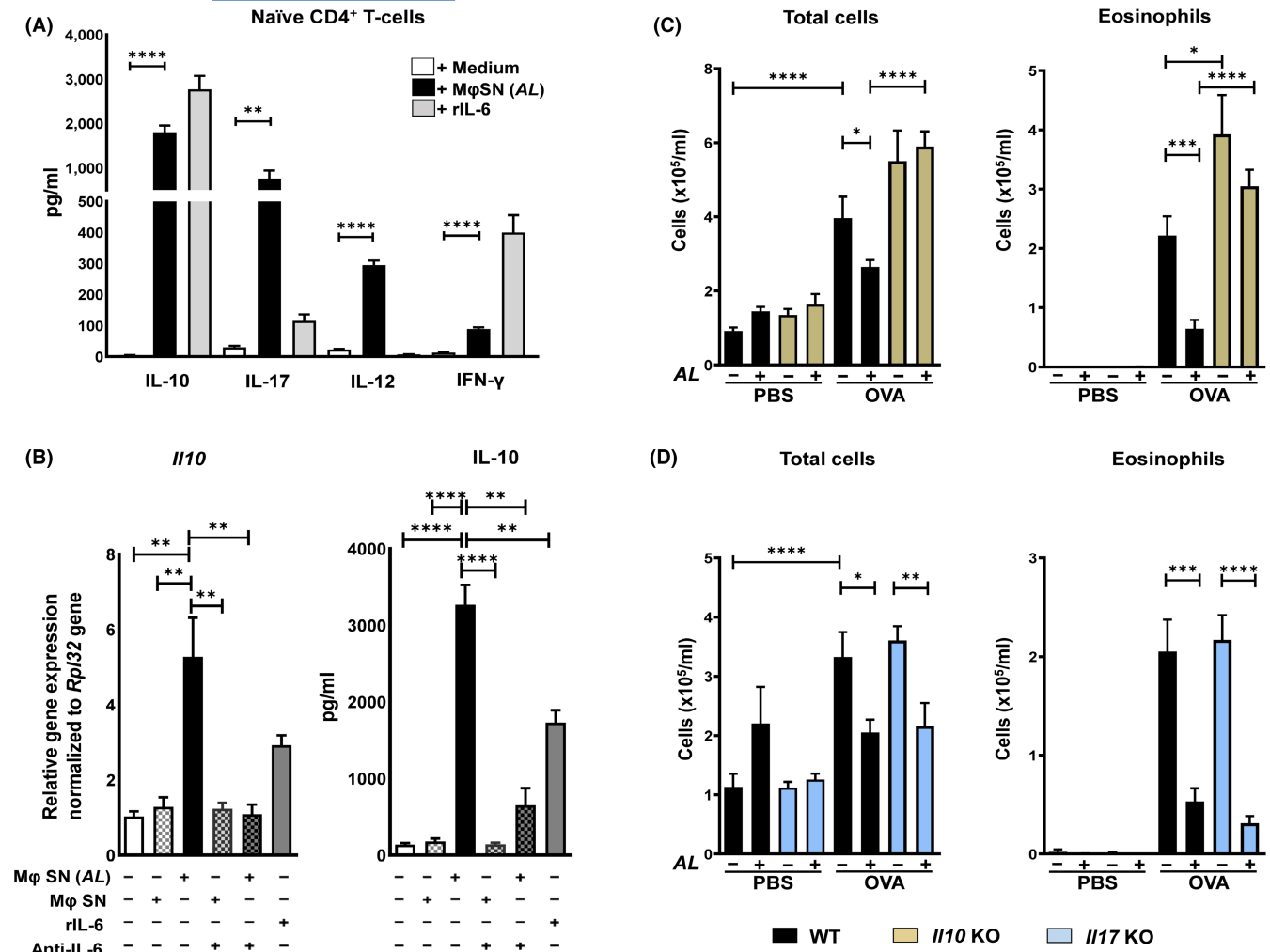


FIGURE 3 The AL-triggered allergic airways protective effect is mediated through IL-6 and IL-10, but not IL-17. (A) Naïve splenic CD4⁺ T-cells derived from WT mice were co-stimulated with anti-CD3/anti-CD28 monoclonal antibodies and cultured with AL-conditioned peritoneal macrophage supernatant or recombinant IL-6 (rIL-6), as described in the Methods section. Medium alone was used as a negative control. Cytokines were measured in culture supernatants after 5 days. (B) Relative gene expression of the *Il10* mRNA normalized to the housekeeping gene *Rpl32*, and IL-10 protein production levels of the CD4⁺ T-cell cultured with AL-conditioned or unconditioned macrophage supernatants alone or preincubated with anti-IL-6 antibody, recombinant IL-6 alone, or medium, used as a negative control. (C, D) *Il10*^{-/-} and *Il17*^{-/-} mice and their WT littermates were exposed to AL as shown in Figure 1A. Differential cell counts were measured in BAL fluids, with total BAL leukocytes and eosinophils presented. Data in A are representative of two-pooled independent experiments ($n = 6$ biologically independent samples/group). Data in B are representative of one experiment ($n = 4$ biologically independent samples/group). Data in C are from two-pooled independent experiments ($n = 12$ biologically independent samples/group for WT and $n \geq 8$ biologically independent samples/group for *Il10*^{-/-}). Data in D are from three-pooled independent experiments ($n = 19$ biologically independent samples/group for WT, and from two-pooled independent experiments, $n = 8$ biologically independent samples/group for *Il17*^{-/-}). For c and d only significant differences of the following comparisons between groups were shown (WT-OVA-AL vs. WT-OVA-no AL), (IL-6 KO-OVA-AL vs. IL-6 KO-OVA-no AL), (WT-OVA-no AL vs. IL-6 KO-OVA-no AL), (WT-OVA-AL vs. IL-6 KO-OVA-AL), and (WT-PBS-no AL vs. WT-OVA-no A). Shown in A–D are Mean $\pm$ SEM. Significant differences in A–D were calculated using two-tailed unpaired two-tailed t-tests. * $p < .05$; ** $p < .01$; *** $p < .001$, **** $p < .0001$. PBS, Phosphate-buffered saline; OVA, ovalbumin; AL, *Acinetobacter lwoffii*; +, pretreated with AL; -, pretreated with PBS.

the genotype effects on the microbiome, but the *Il6*^{-/-} mice and their WT littermates did not differ (Figure S4F, I).

We then examined the effects on community structure (β -diversity) in the lung and in the intestine after the experimental introductions and challenge. In both WT and cytokine-KO strains, there were minimal effects on the lung community structure resulting from either AL administration or OVA challenge (Tables S3

and S4). In contrast, in the caecum, AL intranasal administration had strong effects on β -diversity in all three cytokine-KO strains following sensitization with OVA (Table S4). In contrast, in the WT mice, AL administration only minimally affected β -diversity (Table S3). Taken together, these data indicate significant effects of the AL exposure on the cecal microbiota community structure, essentially exclusively in the cytokine-KO mice following OVA sensitization.

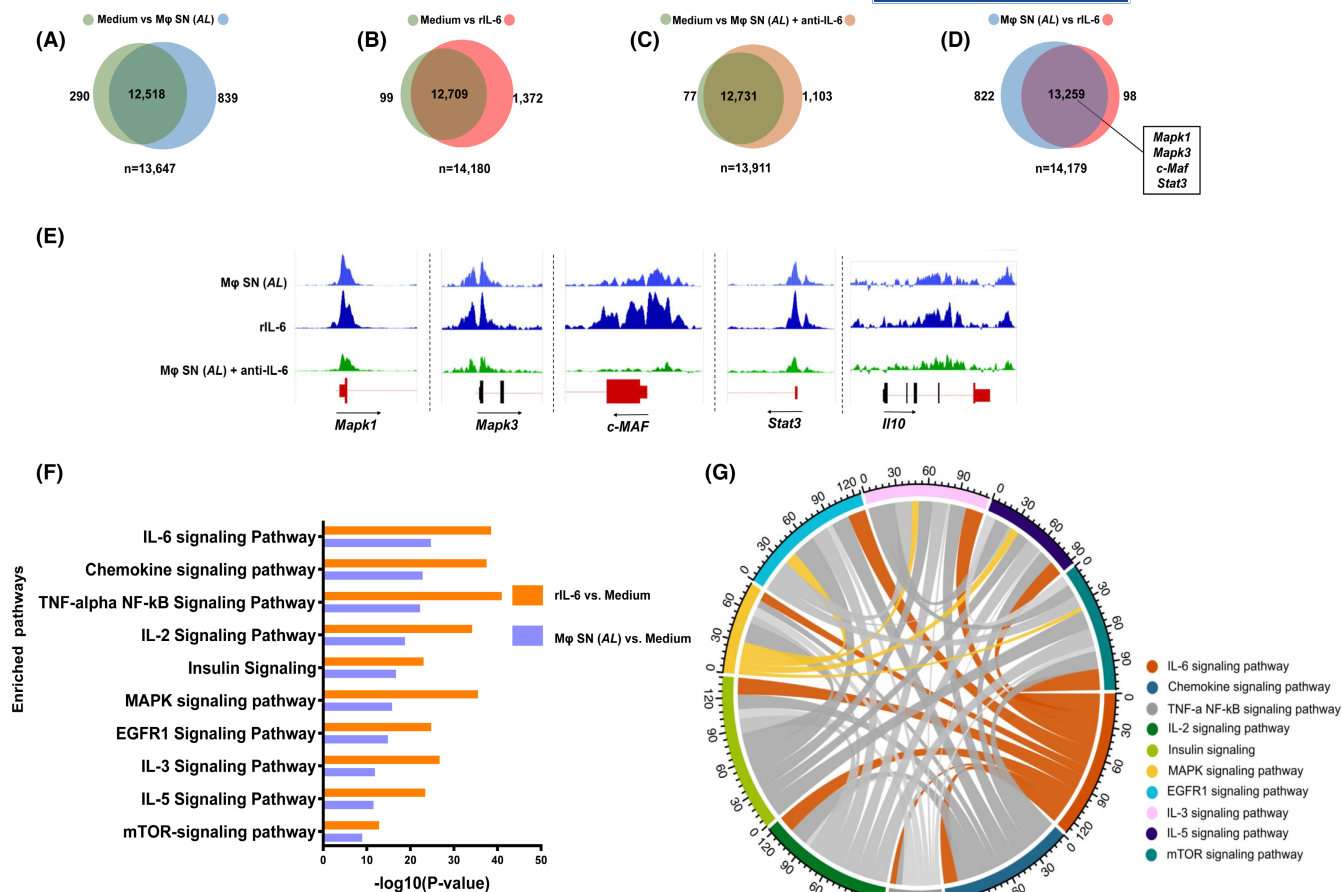


FIGURE 4 AL alters the landscape of histone modifications in CD4⁺ T-cells mainly via IL-6. (A–C) Venn Diagram showing the numbers of overlapped gene-annotated peaks for the active histone mark H3K27ac in CD4⁺ T-cells treated with (A) medium compared to AL-conditioned macrophage supernatant or (B) medium compared to rIL-6 or (C) AL-conditioned macrophage supernatant pretreated with anti-IL-6 antibody compared to medium. (D) Venn Diagram showing the numbers of overlapped of the unique-gained gene-annotated peaks of the AL-conditioned macrophage supernatant and rIL-6. Peaks are associated with genes based on the AnnotatR database. (E) Representative images of the Integrative Genomics Viewer (IGV) of H3K27ac peaks in CD4⁺ T-cells incubated with AL-conditioned macrophage supernatant (top line), rIL-6 (middle line), and AL-conditioned macrophage supernatant pretreated with anti-IL-6 antibody (bottom line), after subtracting H3K27ac modification profile of the medium from all conditions. Images depict the histone modifications at the promoter regions of the *Il10* pathway-related genes *Mapk1*, *Mapk3*, *Stat3*, and *c-MAF* and the *Il10* gene. (F) Functional molecular pathway enrichment analysis of the histone mark H3K27ac in CD4⁺ T-cells using KEGG_2019 and WikiPathways_2019 databases for mice. Pathways depicted found to be enriched following incubation with AL-conditioned macrophage supernatant or rIL-6 each compared to medium. (G) Pathway network analysis was performed as described in the Methods section. The sample processing and numbers are as described in the Methods section.

Next, to address the basis of the differential β -diversities, we assessed the taxa differing significantly in the WT and the KO mice (Table S5; Figure S5). Comparing the wild-type and their respective *Il6*^{−/−} and *Il10*^{−/−} littermates, there were numerous taxonomic differences (Figure S5 panels A and B), but few between WT and their *Il17*^{−/−} littermates (panel C). When AL was introduced, differences between the *Il6*^{−/−} mice and their WT littermates were lost, and reduced for the other two KOs. OVA sensitization led to complex differential effects between WT mice and their KO littermates, but AL introduction led to reduced differences. Thus, AL introduction narrowed the cecal differences between the WT mice and their respective cytokine-KO littermates, across all three mutant mouse lines.

Next, we addressed microbiome composition in relation to asthma protection. In the *Il6*^{−/−} mice, in which the asthma protection from

AL was lost, *Lachnospiraceae* (including *Roseburia*) and *Ruminococcus* were more abundant, and *Muribaculaceae* and *Parasutterella* less abundant compared to their WT littermates (Table S5; Figure S5 panel A). Results were similar for the *Il10*^{−/−} mice vs. their WT littermates (Figure S5 panel B), in total suggesting that the balance between these indicated Firmicutes and Bacteroidetes is influenced by AL, with resultant effects on asthma phenotypes. These organisms were not differential in the *Il17*^{−/−} mice (Table S5; Figure S5 panel C), consistent with no difference in asthma phenotype between the *Il17*^{−/−} mice and their WT littermates.

AL-exposed and unexposed WT mice showed several significant taxonomic differences (Figure S6 panels A, B, and C), including increased *Roseburia* and *Blautia* in the IL-6, decreased *Lactobacillus* and *Ruminococcaceae* in the IL-10 and increased *Mucispirillum* in the IL-17

mice, but the biological significance of these differences is uncertain. However, the lack of substantial microbiome change following AL exposure in the WT mice (Table S3), despite the observed improvement in asthma, suggest that these changes (Figure 5, Figure S5A–D) are not pathophysiologically relevant.

Finally, we examined the effect of adding AL on the KO mouse microbiota (Figure 5) (Figures S5, S6). In the *Il6*^{-/-} mice (Figure 5A), exposure to AL (with or without OVA) increased *Muribaculaceae* and *Sutterellaceae* and related taxa, and decreased *Aeribacillus* and *Lachnospiraceae* (group NKA4A136) compared to the unexposed mice, in essence restoring toward the WT. Among the *Il10*^{-/-} mice (Figure 5B), AL exposure led to increased *Erysipelatrichaceae*, *Bilophila*, and *Muribaculaceae* among others, and decreased *Lachnospiraceae* (group NKA4A136), among others, compared to the unexposed mice, paralleling the restorations in the *Il6*^{-/-} mice. Thus, these identified taxa represent dynamic AL-responsive elements in the microbiota, but without direct evidence of pathophysiological roles.

4 | DISCUSSION

In this study, we demonstrate in the OVA challenge model that AL administration prevents the development of asthma, regardless of the specific intestinal microbiota composition of the mouse group studied. Here, we describe a novel function for IL-6 in ameliorating asthma, contrasting with the effects of the elevated IL-6 concentrations observed in asthma exacerbation,^{29–33} as well as with the tolerance developed after persistent lipopolysaccharide

exposure.^{34–40} The three independent experiments, performed in cytokine KO and WT mice, reaffirm the asthma-blocking properties of AL. However, losing the protective effects in the *Il6*^{-/-} and *Il10*^{-/-} deficient mice indicates that the AL effect requires their activity, and they operate sequentially in this regard. The results clearly show that IL-17 is not involved in the protective pathway. Initially, the repeated intranasal AL administration triggered persistent inflammation, originating locally in the airways and extending systemically at least to the small degree measured. Over time, the hosts became tolerant to the repeated AL exposures, but IL-6 responses remained.

IL-6 plays a role in inducing IL-10 production in naïve CD4⁺ T-cells, an effect mediated by a profound change in the gene regulatory landscape of CD4⁺ T-cells. Our findings confirm that bacterial interactions can modify cellular phenotypes through epigenetic mechanisms.^{14,41} To gain a mechanistic understanding of the key role of IL-6 and IL-10 on the epigenetic level, we employed the H3K27ac marks in the promoter and enhancer regions.^{42,43} Administering rIL-6 can phenocopy much of the altered H3K27ac profile induced by AL-conditioned macrophage supernatants, as observed by the similarity in the enhanced H3K27ac-annotated peaks, and the increases in the H3K27 acetylation in the promoter regions of the genes required for *Il10* gene expression such as *Mapk1*, *Mapk3*, *Stat3*, and *c-Maf*, whereas those changes were reversed by anti-IL-6 treatment. Both KEGG (Kyoto Encyclopedia of Genes and Genomes) and WP (WikiPathways) databases indicate enrichment of MAPK, IL-6, and other cytokine and chemokine signaling pathways, highlighting the importance of the IL-6–IL-10 axis in this tolerance induction initiated by AL. The persistent chronic inflammatory responses induced by AL generate resilience against

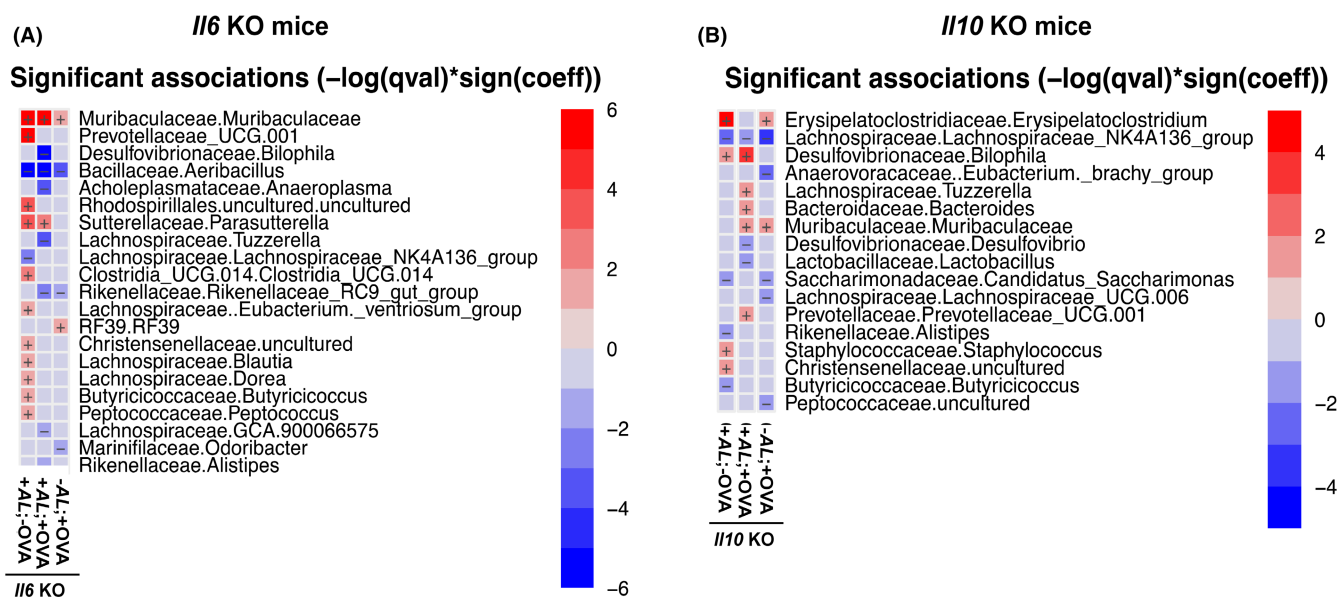


FIGURE 5 Heatmap representing genus level taxa in the KO mouse samples from the IL-6 and IL-10 experiments that are significantly different between treatment groups. (A) Significant associations in the *Il6*^{-/-} mice. (B) Significant associations in the *Il10*^{-/-} mice. Each row represents a different taxon and each column a different treatment time point. Log₁₀ taxon relative abundance is shown using red-blue scales. Significantly different taxa were inferred by MaAsLin2 analysis. All comparisons shown are of animals not pretreated with *Acinetobacter lwoffii* (AL) and not subjected to ovalbumin (OVA)-model versus the specified treatment group. +AL, pretreated with AL; -AL, not pretreated with AL; +OVA, subjected to OVA-model; -OVA, not subjected to OVA-model.

asthma development, providing a novel and unexpected benefit of persistent immune activation, however, this effect was observed for AL and we did not test other microbes.

Although AL was no longer detectable by sacrifice at day 67, the earlier exposure to AL changed the immunological milieu. One possibility is that the effects were perpetuated by downstream changes in the intestinal microbiota. Favoring this hypothesis are the consistent findings in the *Il6*^{-/-} and *Il10*^{-/-} mice, with *Ruminococcaceae* and *Lachnospiraceae* blooms and *Parasutterella* and *Muribaculaceae* inhibition vis-a-vis their WT littermates, despite the highly different compositions in the founding microbiota. As such, changes in these taxa may underlie the pro-asthmatic phenotype in the *Il6*^{-/-} and *Il10*^{-/-} mice, with AL restoring toward the status quo ante.

Lachnospiraceae, major producers of short-chain fatty acids, have been proposed as asthma-protective microbes,^{44,45} although particular genera and species within this family implicated in disease have differed.⁴⁶ In this study, two *Lachnospiraceae* genera (*Blautia* and *Roseburia*) were significantly increased in the IL-6 WT mice, consistent with the expected asthma-protective role, whereas three other *Lachnospiraceae* genera (*Blautia*, *Dorea*, and [*Eubacterium*]_{ventriosum_group}) were significantly increased in *Il6*^{-/-} mice, opposite to expectations. These dichotomous findings suggest that IL-6 phenotype has the greatest bearing on asthma phenotype, with microbial effects secondary.

The AL asthma protection associated with increased *Muribaculaceae* (previously called *S24-7*⁴⁷) and other *Bacteroides* species is consistent with their critical roles in early life immunological development.^{48–50} *Muribaculaceae* have been associated with alleviation of allergic lung inflammation⁵¹ food allergy^{52,53} and with type 1 diabetes protection.⁵⁴ Our observations pertaining to *Parasutterella* are consistent with beneficial metabolic roles of these species.⁵⁵ Although AL was introduced via the airways, and not detected in the lower intestine at the time of sacrifice, the microbiota differences observed were specific to the cecal contents and not observed in the lung. Either the early exposure to AL directly affected the gut microbiota, or AL induced changes in the immunological milieu that altered the intestinal microbiome.

Differences in mouse genotype affecting immunological properties have been long known,^{56–58} but we now understand that intestinal microbiome differences also can affect asthma development.^{59–61} One strength of our studies is the comparison of KO and WT mice from the same founder populations, which minimizes confounding based on microbiome differences. However, in all three KO lines compared to their WT littermates, there were clear differences in the taxa present that based on our study design can now be ascribed to genotype differences.

In conclusion, these experiments demonstrate a new mechanism by which environmental bacteria can prevent asthma (Graphical abstract). The asthma-protective bacterium AL triggers a local pro-inflammatory response in the airways characterized by sustained systemic IL-6 increases, with subsequent epigenetic modifications in CD4⁺ T-cells leading to IL-10 induction. A consequence of the

compounded IL-6 and IL-10 activity is perturbation of the gastrointestinal microbiome, with specific taxa significantly associated with either disease activity or protection. The structure of our experiments provides evidence that these differences in taxa abundance are consequent to the AL treatment, potentially playing an intermediate role in the observed asthma phenotypes. This mechanism linking inflammatory triggers with changes in commensal microbial populations might be useful for developing novel asthma preventive strategies. Such questions can be examined in future studies involving the reconstitution of germ-free mice with AL-perturbed or control microbiota.

AUTHOR CONTRIBUTIONS

H.R., M.B., D.P.P., and B.A.A. conceptualized and directed the study, supervised the experiments, analyzed and interpreted the data. B.A.A., H.H., D.K., and F.A. conducted and analyzed mouse in vivo studies. Z.G., B.A.A. analyzed and interpreted microbiome data. F.A., M.P., A.G., C.D., H.G., B.A.A., D.P.P., H.R., and J.T. designed and performed ChIP-seq experiments and bioinformatics analysis. J.E. and P.L.G., conducted a confocal imaging experiment. A.K. conducted a DCs experiment. H.R., M.B., and B.A.A. wrote the manuscript and drafted the figures.

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CONFLICT OF INTEREST

H.R. has received research support from Mead Johnson Nutrition and Beckman Coulter, has received speaker's honoraria from Allergopharma, Novartis, Thermo Fisher, Danone, Mead Johnson

Nutrition and Bencard Allergie, and has been a consultant for Bencard Allergie and Secarna Pharmaceuticals (co-founder). All other authors have declared that no conflict of interest exists.

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SUPPORTING INFORMATION

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