

1 **Administration of a Bacterial Lysate to the Airway Compartment Is Sufficient to Inhibit**
2 **Allergen-Induced Lung Eosinophilia in Germ-free Mice**

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12 **Running Title:** Airway-delivered microbial signals inhibit allergen-induced lung eosinophilia

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15 PCT/EP2019/074562, "Method of Treating and/or Preventing Asthma, Asthma Exacerbations,
16 Allergic Asthma and/or Associated Conditions with Microbiota Related to Respiratory
17 Disorders." The rest of the authors have no relevant conflicts of interest.
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1 **Abbreviations:**

2	AHR	airway hyperresponsiveness
3	BAL	bronchoalveolar lavage
4	Ex-GF	Ex-germ free
5	GF	germ-free
6	i.n.	intra-nasal
7	i.p.	intra-peritoneal
8	OVA	ovalbumin
9	qPCR	quantitative polymerase chain reaction
10	RT	reverse transcription
11	SPF	specific pathogen-free

Abstract

The nexus between eosinophils and microbes is attracting increasing attention. We previously showed that airway administration of sterile microbial products contained in dust collected from traditional dairy farms virtually abrogated broncho-alveolar lavage (BAL) eosinophilia and other cardinal asthma phenotypes in allergen-sensitized specific pathogen-free (SPF) mice. Interestingly, comparable inhibition of allergen-induced BAL eosinophilia and promotion of airway barrier integrity were found upon administration of a sterile, pharmacological grade bacterial lysate, OM-85, to the airway compartment of allergen-sensitized SPF mice. Here we asked whether intrinsic properties of airway-delivered microbial products were sufficient to inhibit allergic lung inflammation or whether these effects were mediated by reprogramming of the host microbiota. We compared germ-free (GF) mice and offspring of GF mice associated with healthy mouse gut microbiota and maintained under SPF conditions for multiple generations (Ex-GF mice). These mice were treated intra-nasally with OM-85 and evaluated in the OVA and *Alternaria* models of allergic asthma focusing primarily on BAL eosinophilia. Levels of allergen-induced BAL eosinophilia were comparable in GF and conventionalized Ex-GF mice. Airway administration of the OM-85 bacterial lysate was sufficient to inhibit allergen-induced lung eosinophilia in both Ex-GF and GF mice, suggesting that host microbiota are not required for the protective effects of bacterial products in these models and local airway exposure to microbial products is an effective source of protection. OM-85-dependent inhibition of BAL eosinophilia in GF mice was accompanied by suppression of lung type-2 cytokines and eosinophil-attracting chemokines, suggesting that OM-85 may work at least by decreasing eosinophil lung recruitment.

Key words: microbes, lung, eosinophilia, germ-free mice

Summary sentence: Airway-delivered microbial signals inhibit allergen-induced lung eosinophilia

1 Introduction

2
3 The functions of eosinophils are multiple and complex. Airway eosinophilia is a cardinal
4 signature of allergic asthma in both mice¹ and humans², but eosinophils also play homeostatic
5 and regulatory roles at mucosal barriers and contribute to tissue repair and protection against
6 parasitic infections³. Additional complexities of eosinophil biology are highlighted by the
7 transcriptional and functional heterogeneity of these multi-faceted cells in tissues⁴. The nexus
8 between eosinophils and microbes is also attracting increasing attention. While eosinophils are
9 found in germ-free (GF) mice, implying that microbial cues are dispensable for the development
10 of this lineage, commensal microbes regulate the frequency and function of intestinal
11 eosinophils, thereby influencing tissue repair, allergic sensitization to food antigens⁵ and
12 protection against pathogen intrusion³.

13 We became interested in eosinophils as targets of microbial signals during our studies of
14 asthma protection in microbe-rich environments. Airway administration of microbial products
15 contained in sterile, autoclaved extracts of dust collected from Amish or European traditional
16 dairy farms virtually abrogated broncho-alveolar lavage (BAL) eosinophilia and other cardinal
17 asthma phenotypes in allergen-sensitized specific pathogen-free (SPF) mice^{6, 7}. In line with the
18 microbial nature of the exposure, suppression of BAL eosinophilia depended on Myd88/Trif
19 innate immune signaling⁶ and was accompanied by increased airway epithelial barrier function⁷
20 and profound reprogramming of the lung innate immune transcriptome (Van Linden, DeVries
21 and Vercelli, unpublished). Interestingly, comparable inhibition of allergen-induced BAL
22 eosinophilia and promotion of airway barrier integrity were found upon intra-nasal (i.n.)
23 administration of a sterile, pharmacological grade bacterial lysate, OM-85, to the airway
24 compartment of SPF mice sensitized to allergen systemically (with ovalbumin: OVA) or through
25 the airway mucosa (with *Alternaria alternata*)⁸.

26 Although they pointed to eosinophils as targets of microbial exposures, these experiments did
27 not reveal whether intrinsic properties of airway-delivered microbial products were sufficient to
28 inhibit allergic lung inflammation or whether these effects were mediated by reprogramming of
29 the host microbiota. To address this question, we associated GF mice from birth with healthy
30 mouse gut microbiota or treated these mice i.n. with the OM-85 bacterial lysate. Allergen-
31 induced responses were evaluated in the OVA and *Alternaria* asthma models, focusing primarily
32 on BAL eosinophilia.

1 Methods

2 **Mice** Balb/c GF mice (Taconic) were bred and housed in sterile isolators at the University of
3 Arizona Gnotobiotic Animal Facility with unlimited access to autoclaved food (2019S, Inotiv) and
4 water. GF status of the colony was monitored routinely by anaerobic and aerobic culture of stool
5 samples. A colony of Ex-GF Balb/c mice was established by transferring GF mice to the SPF
6 rooms of the University of Arizona Animal Facility and exposing them for 2-3 wks to soiled
7 bedding from a sentinel cage. Ex-GF mice were bred and their offspring were maintained under
8 SPF conditions for multiple generations (>1.5 yrs). These mice were SPF for murine norovirus
9 (MNV), mouse hepatitis virus (MHV), minute virus of mice (MVM), mouse parvovirus (MPV),
10 Theiler's murine encephalomyelitis virus (TMV), rotavirus, Sendai virus, pneumonia virus of
11 Mice (PVM), reovirus type-3 (Reo3), lymphocytic choriomeningitis virus (LCMV), Ectromelia,
12 mouse adenovirus (MAV)-1 and -2, polyoma virus, *Helicobacter*, *Rodentibacter*, *M. pulmonis*,
13 *CAR Bacillus*, *C. rodentium*, *C. piliforme*, *C. kutscheri*, *Salmonella*, fur mites, lice, nematodes,
14 cestodes, protozoa, and microsporidia. All experimental procedures were approved by the
15 University of Arizona Institutional Animal Care and Use Committee (IACUC).

16 **Bacterial 16S rRNA gene qPCR** One week before each experiment, GF mice were
17 transferred into ISOcage P cages (Tecniplast), where all experimental procedures were
18 performed. Fecal samples were collected immediately after transfer, weekly during the
19 experiment, and immediately before mice were sacrificed. A bacterial 16S rRNA gene
20 quantitative polymerase chain reaction (qPCR)⁹ was performed on these fecal samples. DNA
21 was extracted using the DNeasy PowerSoil Pro kit (Qiagen) and quantified with a Qubit 2.0
22 Fluorometer (Life Technologies). qPCR reactions were performed using the SYBR green
23 Perfecta FastMix (QuantaBio) and run in triplicate on an Applied Biosystems 7900 instrument.
24 The bacterial 16S rRNA gene was amplified using eubacterial universal primers 926F (5'-
25 AAACCTCAAAGGAATTGACGG-3') and 1062R (5'-CTCACRRCACGAGCTGAC-3')⁹. A standard
26 curve was generated using dilutions of 16S qPCR standards (Zymo Research Corp, CA)
27 covering a 32 - 3.22⁰⁷ copy range. Ct values were converted to copies/reaction¹⁰. 16S rRNA
28 gene copy numbers were presented as copy numbers/ng of DNA. No template mix and fecal
29 DNA from SPF mice were included as controls.

30 **Allergic asthma models** Experiments in the OVA model were performed as previously
31 described⁷, with minor modifications. Briefly, to compare OVA-induced responses in GF and Ex-
32 GF mice, 7-8 week old GF and Ex-GF Balb/c mice were sensitized intra-peritoneally (i.p.) with
33 OVA (chicken egg white albumin, Sigma: 50 µg) + Alum (Imject Alum, Thermo Fisher: 150 µl) at

day 0 and 7, challenged i.n. with OVA (100 µg) at day 14-16, and sacrificed at day 21. To assess the effects of OM-85 on OVA-induced BAL eosinophilia in GF Balb/c mice, 7-8 week old animals were sensitized with OVA (50 µg) + Alum (150 µl) i.p. at day 0 and 9, challenged i.n. with OVA (100 µg) at day 22-24, and sacrificed at day 29. OM-85 (1 mg in 0.9% sterile saline/treatment x 9 treatments) was administered i.n. (25 µl/nostril) under 1-5% isoflurane anesthesia every 2-3 days from day 0 to day +17.

In the *Alternaria* model, 7-8 week old GF and Ex-GF Balb/c mice were sensitized i.n. with *Alternaria alternata* extracts (Greer Laboratories: 50 µg of dry weight in 50 µl of saline) at day 0 and 1, challenged i.n. with *Alternaria* extracts (25 µg of dry weight in 50 µl) at day 21-23, and sacrificed at day 26. OM-85 (1 mg in 0.9% sterile saline/treatment x 14 treatments) was administered i.n. (25 µl/nostril) under 1-5% isoflurane anesthesia every 2-3 days from day -14 to day +16⁸. In all models, terminal assessments included invasively measured lung function (airway hyperresponsiveness: AHR), BAL cellularity with differentials and levels of lung cytokine and chemokine mRNA measured by reverse transcription (RT)-qPCR^{7, 8}.

Statistical analyses Experiments were designed to test the hypothesis, based on prior knowledge, that early association with healthy mouse gut microbiota or i.n. administration of microbial products affect asthma-related phenotypes. Therefore, statistical analyses focused on the comparison between mice treated with allergen + microbes or microbial products and mice treated with allergen alone. Saline-treated groups served as experimental controls. Inter-group differences were assessed by an unpaired, two-tailed *t* test after determining the normality of sample value distribution with the Shapiro-Wilk test. Unless otherwise stated, P-values <0.05 were considered statistically significant. AHR dose-response curves were compared using linear mixed-effects models, with treatment group and dosage of bronchoconstrictor as independent variables (fixed effects) and using each mouse identifier to account for repeated measures within each subject (random effects)^{7, 8}.

Results

Association with healthy gut microbiota from birth inhibits allergen-induced AHR in GF

mice To investigate whether host microbiota mediate the asthma-protective effects of airway-delivered microbial products, we used Balb/c GF mice as well as offspring of GF mice initially exposed for 2-3 wks to microbiota from healthy SPF mice of the same strain and then maintained under SPF conditions for multiple generations (>1.5 yrs) (Ex-GF mice). Thus, all Ex-GF mice discussed herein were genetically identical to the GF ones and acquired their microbiota from birth, that is, during the critical age window that allows the transferred microbiota to fully mature and exert their immunomodulatory effects¹¹.

We then compared GF and Ex-GF mice in two distinct models of allergic asthma, monitoring lung function (AHR) and BAL eosinophilia. **Fig. 1A** shows that AHR elicited by i.p. sensitization with OVA + Alum followed by i.n. challenge with OVA alone was significantly ($P=0.0003$) reduced in Ex-GF relative to GF mice. Similarly, AHR was significantly ($P=0.03$) less intense in Ex-GF than GF mice sensitized and challenged with the aeroallergen *Alternaria alternata* (**Fig. 1B**). The latter data were especially significant because *Alternaria*, a protease-rich fungus, is strongly associated with the development of allergic asthma in children and adults¹². In mice, i.n. exposure to *Alternaria* extracts in the absence of adjuvants induces allergic lung inflammation that closely recapitulates the human phenotype and reflects the activation of innate and adaptive immune pathways triggered by damage to the airway epithelial barrier^{8, 13, 14}. Our current results demonstrated that microbial conventionalization from birth protected mice from alterations in lung function linked to the lack of microbial colonization.

Allergen-induced BAL eosinophilia in GF mice is unaffected by early association with healthy mouse gut microbiota but is inhibited by airway administration of a bacterial lysate

The next set of experiments compared allergen-induced BAL eosinophilia in GF and Ex-GF mice. Intense eosinophilia was detected in OVA-sensitized mice, but to our surprise no significant differences were found between GF and Ex-GF animals ($P=0.35$, **Fig. 2, left panel**). Similarly, GF and Ex-GF mice developed robust but comparable *Alternaria*-dependent BAL eosinophilia ($P=0.7$, **Fig. 2, right panel**). *Alternaria*-sensitized GF and Ex-GF mice also harbored closely comparable proportions ($13.6 \pm 4.1\%$ in GF mice vs. $15.9 \pm 4.6\%$ in Ex-GF mice) and numbers (not shown) of BAL eosinophils with ring-shaped nuclei, which are thought to represent less mature, potentially homeostatic cells¹⁵.

Interestingly, marked differences in response patterns were observed when GF and Ex-GF mice received a sterile bacterial lysate, OM-85, directly through the airways. Indeed, i.n.

administration of OM-85 was sufficient to strongly and significantly reduce *Alternaria*-dependent BAL eosinophilia not only, as expected⁸, in Ex-GF mice (P=0.001) but also in GF animals (P=0.002) (**Fig. 2, right panel**). OVA-induced BAL eosinophilia was also significantly (P=0.012) suppressed in OM-85-treated GF mice (**Fig. 2, center panel**), demonstrating that the effects of the bacterial lysate are not allergen- or model-specific.

Airway administration of a bacterial lysate inhibits Alternaria-induced expression of type-2 cytokines and eosinophil chemoattractants in the lung

To begin investigating the mechanisms through which airway delivery of a bacterial lysate inhibits allergen-induced BAL eosinophilia in mice lacking microbiota, we compared cytokine and chemokine expression in the lungs of *Alternaria*-immunized GF and Ex-GF mice that did or did not receive OM-85 intra-nasally. The type-2 cytokines *Il5* and *Il13* have powerful effects on eosinophil biology: IL-5 promotes eosinophil production, priming, and survival, and IL-13 induces lung cells to produce eotaxins (*Ccl11* and *Ccl24*), which attract circulating eosinophils to the tissue¹⁶. Lung levels of *Alternaria*-induced *Il5* and *Il13* mRNA were closely comparable in GF and Ex-GF mice (P=0.5 and P=0.9, respectively). In contrast, expression of both *Il5* and *Il13* was markedly reduced in the lungs of GF mice treated i.n. with OM-85 (P=0.00001 and P=0.00009, respectively). Consistent with our previous results in SPF mice⁸, a strong reduction in type-2 cytokines was detected in the lungs of OM-85-treated Ex-GF mice (P=0.00004 for *Il5* and P=0.0003 for *Il13*; **Fig. 3A and B**).

We next focused on the eosinophil chemoattractants *Ccl11* and *Ccl24* because they are both regulated by IL-5 and IL-13, bind CCR3 and promote eosinophil migration to tissues, albeit with distinct temporal patterns¹⁷. While GF and Ex-GF mice sensitized and challenged with *Alternaria* alone exhibited no significant differences in *Ccl11* and *Ccl24* mRNA levels (P=0.17 and P=0.15, respectively), GF mice treated intra-nasally with OM-85 had markedly lower lung expression of both chemokines relative to *Alternaria*-sensitized animals (P=0.00009 and P=0.00000007, respectively). Like type-2 cytokines, *Ccl11* expression was significantly reduced in the lungs of Ex-GF mice treated with *Alternaria*+OM-85 compared to Ex-GF mice that had received *Alternaria* alone (P=0.003); the OM-85-induced decrease in *Ccl24* closely approached statistical significance (P=0.017, **Fig. 3C and D**).

Discussion

Our data demonstrate that administration of a bacterial lysate to the airway compartment is sufficient to inhibit OVA- and *Alternaria*-induced lung eosinophilia in GF mice, implying that host microbiota are not required for the protective effects of bacterial products in these models of allergic airway inflammation. Our results also suggest that local airway exposure to microbial products is especially effective in conferring protection. Mechanistically, suppression of allergen-induced eosinophil recruitment to the lung may be important for the OM-85-dependent inhibition of BAL eosinophilia in GF animals.

GF mice associated with human gut microbiota are often used to study causal relationships between altered microbiomes and host pathology. Insightful results have been provided by studies in intestinal inflammatory disease models (reviewed in Ref. ¹⁸). In contrast, work on lung disease, and asthma in particular, has produced mixed results. For instance, GF mice associated with potentially protective human bacterial taxa exhibited only modest attenuation of allergic airway inflammation and no significant inhibition of allergen-induced BAL eosinophilia. Effects on AHR were not tested¹⁹. A concern raised by inter-species microbial association is that it may lead to incomplete and/or selective colonization and thus may not adequately recapitulate the compositional and functional features of the transferred inoculum and the phenotypic characteristics of its donor¹⁸. To address this concern and provide proof-of-concept evidence about a link between microbes and lung eosinophilia, we associated GF mice with gut microbiota from healthy SPF mice or treated GF mice i.n. with a sterile pharmacological grade bacterial lysate, OM-85. Surprisingly, the short-term airway exposure of adult GF mice to the bacterial lysate succeeded in suppressing airway eosinophilia, type-2 cytokines and eosinophil-chemoattractants in the lung, but these effects were not observed in Ex-GF mice. The reasons behind these findings are unclear at this time. The Ex-GF mice used in our experiments were colonized with healthy mouse gut microbiota from birth, and the colony was maintained under SPF conditions for over a year. Changes in microbial composition over this time and/or incomplete reconstitution of the airway microbiome might have led to the emergence of communities with metabolic profiles unable to fully support protective lung immune responses to asthma-promoting stimuli. However, the finding that AHR was attenuated in Ex-GF mice in two distinct allergic asthma models militates against this possibility. It is also notable that throughout multiple experiments performed in parallel over more than one year, i.n. administration of OM-85 induced a robust and comparable average reduction of *Alternaria*-dependent BAL eosinophilia in Ex-GF and SPF mice (2.3- and 3.3-fold, respectively). However, the average reduction of

1 eosinophilia observed in GF mice treated with OM-85 during the same period was 6.7-fold - that
2 is, even stronger.

3 We hypothesize that the route through which microbial signals were delivered, and the ability of
4 these signals to target the airway epithelium may underlie the remarkable sensitivity of GF
5 airways to microbial products. Indeed, we recently showed that the OM-85 bacterial lysate
6 restores barrier function in airway epithelial cells exposed to *Alternaria*⁸. Similarly, microbial farm
7 dust products with asthma-protective activity boosted barrier function in serum-starved airway
8 epithelium⁷. We speculate that the barrier restorative properties of microbial products may be
9 especially crucial in the airways, where epithelial cell turnover is approximately 10-fold lower
10 than in other locations such as the gut²⁰, and even more so in the airways of GF mice, which
11 lack the resident microbial communities normally contributing to epithelial barrier integrity²¹. In
12 this scenario, the concentrations of microbial products (including metabolites) required to jump-
13 start eosinophilia-limiting responses might be achieved more readily and efficiently by directly
14 treating GF airways with sterile bacterial lysates or products. While the primary cellular and
15 molecular mechanisms underlying these effects require further investigation, our data already
16 suggest that testing complex mixtures of microbial metabolites in GF animals might provide a
17 powerful tool to characterize bioactive substances in the absence of confounding factors derived
18 from the host microbiota.

Legends

Fig.1. GF mice associated with healthy gut microbiota from birth (Ex-GF mice) exhibit attenuated AHR in two allergic asthma models. GF (black symbols) and Ex-GF (grey symbols) Balb/c mice were sensitized and challenged with OVA (**A**) or *Alternaria alternata* extracts (**B**). Shown are invasive measurements of total airway resistance (Rrs) from two independent experiments for each model. Numbers of mice/group are indicated in the figure captions. Symbols and bars denote means and standard errors of the mean (SEM), respectively. Statistical differences between dose-response curves of total airway resistance in GF and Ex-GF mice were assessed by linear mixed-effects models. ALT: *Alternaria*

Fig.2. Airway administration of a bacterial lysate but not early association with healthy mouse gut microbiota inhibits allergen-induced BAL eosinophilia in GF mice BAL cellularity was measured in GF (black bars) and Ex-GF (grey bars) Balb/c mice sensitized and challenged with OVA (**left and center panel**) or *Alternaria alternata* (**right panel**). Additional groups of mice (hatched bars) received allergen + 9 (OVA model) or 14 (*Alternaria* model) i.n. OM-85 treatments (1 mg/mouse/treatment). Shown are total numbers of BAL eosinophils/mouse (mean and SEM) from two independent experiments for each model. Numbers of mice/group are indicated in the figure captions. Statistical differences between groups were assessed by an unpaired, two-tailed *t* test after evaluating the normality of sample value distributions. Bolded P-values remained significant after Bonferroni correction ($P < 0.016$). ALT: *Alternaria*

Fig. 3: Airway administration of a bacterial lysate but not early association with healthy mouse gut microbiota inhibits *Alternaria*-induced expression of type-2 cytokines and eosinophil-attracting chemokines in the lung. mRNA levels of type-2 cytokines (*Il5*, **A**; *Il13*, **B**) and eosinophil chemo-attractants (*Ccl11*, **C**; *Ccl24*, **D**) were measured by qRT-PCR in the lungs of GF (black bars) and Ex-GF (grey bars) Balb/c mice sensitized and challenged with *Alternaria*. Additional groups of mice (hatched bars) received *Alternaria* + 14 i.n. administrations of OM-85 (1 mg/mouse/treatment). Shown are cytokine and chemokine levels (mean and SEM) from two independent experiments. Numbers of mice/group are indicated in the figure captions. Statistical differences between groups were assessed by an unpaired, two-tailed *t* test after evaluating the normality of sample value distributions. Bolded P-values remained significant after Bonferroni correction ($P < 0.016$). ALT: *Alternaria*

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Fig. 1

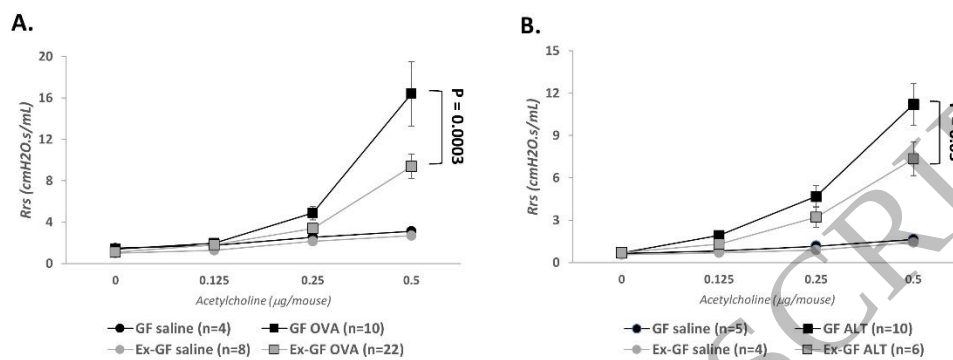


Figure 1
165x93 mm (x DPI)

Fig. 2

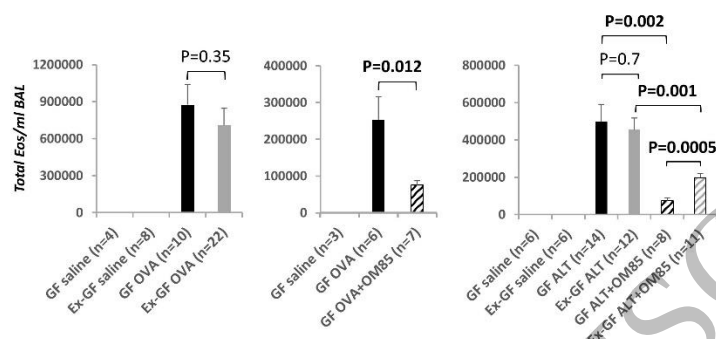


Figure 2
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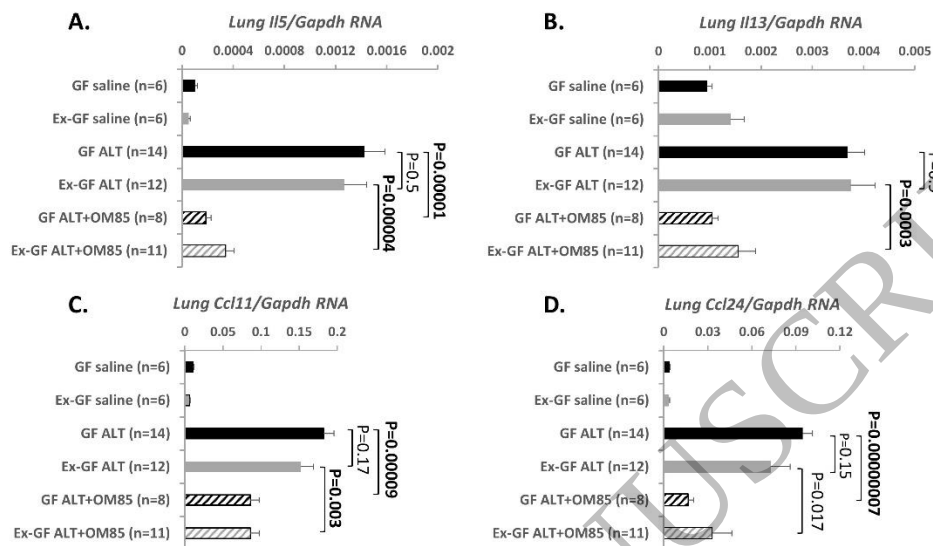
Fig. 3

Figure 3
165x93 mm (x DPI)