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## IL-33 signaling regulates innate IL-17A and IL-22 production via suppression of PGE2 during lung fungal infection

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### Abstract

Members of the IL-1 family play protective and regulatory roles in immune defense against the opportunistic mold *Aspergillus fumigatus*. Here, we investigated the IL-1 family member IL-33 in lung defense against *A. fumigatus*. IL-33 was detected in the naïve lung, which further increased after exposure to *A. fumigatus* in a Dectin-1 independent manner. Mice deficient in the receptor for IL-33 (*Il1rl1*<sup>-/-</sup>) unexpectedly demonstrated enhanced lung clearance of *A. fumigatus*. IL-33 functioned as a negative regulator of multiple inflammatory cytokines, as IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-17A and IL-22 were significantly elevated in fungal-exposed *Il1rl1*<sup>-/-</sup> mice. Subsequently, IL-33 administration to normal mice attenuated fungal-induced IL-17A and IL-22, but not IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 production. IL-33 mediated regulation of IL-17A and IL-22 did not involve the modulation of IL-23 but rather prostaglandin E2 (PGE2); PGE2 was significantly increased in fungal-exposed *Il1rl1*<sup>-/-</sup> mice and normal mice produced less PGE2 after fungal exposure when administered IL-33, suggesting that IL-33 mediated regulation of IL-17A and IL-22 occurred at the level of PGE2. This was confirmed by *in vivo* cyclooxygenase 2 (COX-2) inhibition, which attenuated fungal-induced IL-17A and IL-22, as well as IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 production, in *Il1rl1*<sup>-/-</sup> mice resulting in impaired fungal clearance. We also show that a PGE2 receptor agonist increased, whereas a PGE2 synthase inhibitor decreased, the levels of IL-17A and IL-22, but not IL-1 $\alpha$ , IL-1 $\beta$  and IL-6. This study establishes novel mechanisms of induction of innate IL-17A/IL-22 production via PGE2 and regulation of the PGE2-IL-17A-IL-22 axis via IL-33 signaling during lung fungal exposure.

### Introduction

Concerns over the rise in invasive fungal infections (IFIs) over the last several decades due to modern medical interventions (new, more effective immunosuppressive drugs/regimens) are mounting (1). IFIs caused by *Aspergillus fumigatus* remain one of the most lethal human infectious diseases. Invasive aspergillosis (IA) is particularly insidious due to a combination of increasing antifungal drug resistance, extremely high mortality and an inherent difficulty in diagnosis (2); (3). Although IA is the most devastating aspect of *A. fumigatus* exposure and accounts for 20–40% of IFIs in solid organ and hematopoietic transplantation (4) (5),

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colonization with, or sensitization to, *A. fumigatus* has dramatic effects on lung function in asthmatics (6) and individuals with cystic fibrosis (7).

The IL-1 family of cytokines is comprised of eleven members, seven with pro-inflammatory characteristics (IL-1 $\alpha$ , IL-1 $\beta$ , IL-18, IL-33, IL-36 $\alpha$ , IL-36 $\beta$  and IL-36 $\gamma$ ) and four with anti-inflammatory characteristics (IL-1Ra, IL-36Ra, IL-37 and IL-38). Early studies in experimental models of *A. fumigatus* infection documented the induction of IL-1 family members, such as IL-1 $\beta$  (8) (9). We have further shown that the fungal beta-glucan receptor Dectin-1 drives IL-1 $\alpha$  and IL-1 $\beta$  production in vivo during IA (10). A recent study has demonstrated that IL-1R was essential for survival with IL-1 $\alpha$  promoting leukocyte recruitment and IL-1 $\beta$  promoting anti-fungal activity (11). Studies in humans have shown that *A. fumigatus* strongly induces IL-36 $\gamma$  and IL-36Ra, but not IL-36 $\alpha$ , which were dependent on Dectin-1 and TLR4 (12). Inhibiting IL-36 signaling was found to abrogate the induction of protective Th17 and Th1 responses. With respect to immunopathogenic effects of IL-1 family members during IA, IL-1 $\alpha$  and IL-1 $\beta$  are thought to be the primary drivers of inflammation in CGD via decreased autophagy and increased inflammasome activation (13). Finally, analysis of polymorphisms within the IL-1 family revealed that a SNP in IL-1Ra VNTR2 (rs380092), IL-1 $\alpha$ -889C (rs1800587) and IL-1 $\beta$  (rs1143627) correlates with incidence or risk of developing IA in haematological patients (14).

IL-33 is recognized as one of eleven members of the IL-1 cytokine family, due to its chromosome cluster, structure and processing (reviewed in (15)). IL-33 has been studied in a variety of inflammatory conditions, including allergic asthma, colitis, rheumatoid arthritis and cardiovascular diseases (reviewed in (16)). Likewise, IL-33 has been investigated in a variety of viral (influenza, RSV), bacterial (sepsis, Gram-negative keratitis, Gram-positive skin infection) and fungal (*C. albicans* peritonitis, fungal asthma) infection models (17). By and large, IL-33 mediates the induction of type 2/Th2 responses and suppression of Th1 and inflammatory responses. However, IL-33 has the potential to mediate a diverse array of functions, such as neutrophil recruitment and ROS/RNI induction, depending on the site and type of insult (17). Although IL-33 has yet to be investigated in IA, blockade of IL-33R signaling has been shown to improve fungal asthma severity, putatively through the modulation of pro-allergic responses (18). In the current study, we defined the role of IL-33 in lung defense against IA. Here, we show that IL-33 is detrimental for lung antifungal defense due to its function as a negative regulator of IL-17A and IL-22 via suppression of COX-2/PGE2.

## Materials and Methods

### Mice

WT BL/6 mice, 6 to 8 weeks of age, were obtained from The Jackson Laboratory (Bangor, ME) or Taconic (Hudson, NY). *Il1r1*<sup>-/-</sup> (ST2/IL-33R) mice were a kind gift from Dr. Andrew McKenzie, Cambridge University. *Il1r1*<sup>-/-</sup> mice were obtained from Jackson. All animals were housed in a specific pathogen-free, Association for Assessment and Accreditation of Laboratory Animal Care-certified facility and handled according to Public Health Service Office of Laboratory Animal Welfare policies after review by the UAB Institutional Animal Care and Use Committee.

### Preparation of *A. fumigatus*; *in vivo* challenge; lung fungal burden assessment

*A. fumigatus* isolate 13073 (ATCC, Manassas, VA) was maintained on potato dextrose agar for 5–7 days at 37°C. Conidia were harvested by washing the culture flask with 50 ml of sterile phosphate buffered saline supplemented with 0.1% Tween 20. The conidia were then passed through a sterile 40 µm nylon membrane to remove hyphal fragments and enumerated on a hemacytometer. For challenge, mice were lightly anesthetized with isoflurane and administered  $7 \times 10^7$  *A. fumigatus* conidia in a volume of 50 µl intratracheally as previously described (10) (19). Briefly, mice are held in a vertical, upright position and the tongue is withdrawn from the mouth using forceps. A pipette is used to deliver the 50 µl inoculum to the caudal oropharynx in which normal breathing results in fluid aspiration into the lungs (20). For lung fungal burden analysis, the left lungs were collected at 48 h post-exposure and homogenized in 1 ml of PBS. Total RNA was extracted from 0.1 ml of unclarified lung homogenate using the MasterPure™ yeast RNA purification kit (Epicentre Biotechnologies, Madison, WI), which includes a DNase treatment step to eliminate genomic DNA as previously reported (21). Total RNA was also extracted from serial 1:10 dilutions of live *A. fumigatus* conidia ( $10^1 - 10^9$ ) and DNase treated to form a standard curve. Lung *A. fumigatus* burden was analyzed with real time PCR measurement of the *A. fumigatus* 18S rRNA (GenBank accession number AB008401 (22)) and quantified using a standard curve of *A. fumigatus* conidia as previously described (21). As a validation of the real-time PCR method, heat-killed *A. fumigatus* did not yield a signal by real-time PCR and were unable to grow on potato dextrose agar plates (21). In addition, no amplification controls (i.e. no reverse transcriptase included in the cDNA reaction) yielded a signal of <0.001% by real-time PCR, indicating that the DNase treatment step efficiently eliminated contaminating *A. fumigatus* DNA (as DNA is not predicative of organism viability (23)).

### Lung cell isolation and culture; inflammatory cytokine analysis; PGE2 analysis

Mice were anesthetized with intra-peritoneal ketamine/xylazine and sacrificed by exsanguination 48 h post-infection. Both lungs were collected and minced in IMDM media (Sigma, St. Louis, MO) supplemented with 1% pen-strep-glut (Mediatech, Herndon, VA), 10% heat inactivated FBS (Invitrogen, Carlsbad, CA) and 0.4 mg/ml polymyxin B (Thermo Fisher), followed by incubation for 60 min with tissue culture-grade type IV collagenase (1 mg/ml; Sigma, St. Louis, MO) in a 37°C orbital shaker at 100 rpm. The cell suspension was filtered through sterile 70 µm and 40 µm nylon filters and red blood cells lysed with ACK buffer (Lonza, Walkersville, MD) to create lung cell preparations. For lung cell cultures, cells were enumerated on a hemacytometer and plated at  $1 \times 10^6$  cells in a volume of 0.2 ml. Supernatants were collected after 24 h, clarified by centrifugation and stored at –80°C. Supernatants were analyzed for protein levels of 32 cytokines and chemokines using Bio-Plex multiplex suspension cytokine array (Bio-Rad Laboratories), according to the manufacturer's instructions (20) (21). The data were analyzed using Bio-Plex Manager software (Bio-Rad Laboratories). IL-22 levels were quantified by ELISA (R&D Systems) (10). PGE2 levels were quantified using the Prostaglandin E2 Parameter Assay Kit (Cat. #KGE004B, R&D Systems). In some experiments, neutrophil, inflammatory monocyte and eosinophil levels in lung digest cells were analyzed by flow cytometry. Cells were washed and Fc receptors were blocked with Mouse BD Fc Block™ (BD Biosciences, San Diego,

CA) at 4°C for 20 min. Thereafter, cells were stained with a single-color LIVE/DEAD® Fixable Dead Cell Stain (Invitrogen) followed by labeling with cell-specific markers (antibodies from BD Biosciences, Biolegend and eBiosciences) (24).

### ***In vivo* treatments (IL-33, celecoxib, IL-17A/IL-22 neutralization)**

In specific experiments, WT BL/6 mice were treated with recombinant murine IL-33 intratracheally. Specifically, mice were challenged with *A. fumigatus* and 6 and 24 h thereafter, administered IL-33 (1 µg in 50 µl; R&D Systems) or PBS intratracheally. In other experiments, WT BL/6 and *Irr1*<sup>-/-</sup> mice were administered celecoxib (Cayman Chemical; 100 mg/kg in 250 µl intraperitoneally) or vehicle (ethanol). Mice received celecoxib or vehicle 24 h prior to *A. fumigatus* challenge and then again at 6 h and 24 h post-challenge. For both treatments, 48 h after *A. fumigatus* challenge, mice were sacrificed and the lungs processed for cell isolation, culture and inflammatory cytokine, IL-22 and PGE2 analysis. For *in vivo* IL-17A and IL-22 neutralization, *Irr1*<sup>-/-</sup> mice were challenged intratracheally with  $7 \times 10^7$  *A. fumigatus* conidia in 50 µl and 24 h thereafter, mice were administered 50 µg of rat anti-mouse IL-17A (Cat. #MAB421) or IL-22 (Cat. #MAB5821) or rat IgG isotype control antibody or polyclonal goat anti-mouse IL-17A (Cat. #AF-421-NA) or IL-22 (Cat. #AF582) or goat IgG isotype control antibody (Cat. #AB-108-C) (all antibodies from R&D Systems) intratracheally. Forty-eight hours after challenge, mice were sacrificed, the left lungs were collected and fungal burden assessed as described above.

### ***In vitro* treatments (PGE2 inhibition, EP receptor agonist)**

In specific experiments, WT BL/6 mice were challenged with *A. fumigatus* and 48 h thereafter, lungs collected and enzymatically digested. Cells were enumerated and plated at  $1 \times 10^6$  cells in a volume of 0.2 ml in the presence of vehicle, an inhibitor of microsomal prostaglandin E synthase-1 (mPGES-1; Cayman Chemical) or misoprostol, a global EP receptor (PGE2 receptor) agonist (Sigma, St. Louis, MO). Supernatants were collected after 24 h, clarified by centrifugation and inflammatory cytokine and IL-22 levels were quantified by Bio-Plex and ELISA (R&D Systems), respectively (10).

### **Statistics**

Data were analyzed using GraphPad Prism® Version 5.0 statistical software (GraphPad Software, San Diego, CA). Comparisons between groups when data were normally distributed were made with the two-tailed unpaired Student's t test. Significance was accepted at a value of  $p < 0.05$ .

## **Results**

### **The lack of IL-33 signaling results in enhanced *A. fumigatus* lung clearance**

IL-33 is detrimental for lung infection with the Th1-requiring fungal pathogen *Cryptococcus neoformans* as a result of its known propensity to induce type 2/Th2 responses (25). IL-33 is protective in a model of *C. albicans* peritoneal sepsis via the induction of heightened neutrophil responses (26). Elimination of *A. fumigatus* from the lung is highly dependent on neutrophils (27). Moreover, there is evidence to suggest that innate type 2 responses may also be beneficial in *A. fumigatus* lung clearance (28). Collectively, host immune responses

initiated by IL-33 would appear to favor *A. fumigatus* lung clearance. In initial studies, we demonstrate that IL-33 is increased in response to acute *A. fumigatus* challenge (Figure 1A), however unlike our previous report with fungal asthma (29), IL-33 did not require Dectin-1 mediated beta-glucan recognition for induction (Figure 1A). To our surprise, mice deficient in the IL-33 receptor (ST2/T1/IL1-RL1; *Il1rl1*<sup>-/-</sup>) demonstrated lower *A. fumigatus* burden in the lung 48 h post-challenge (Figure 1B), suggesting that IL-33 induction functions as a negative regulator of fungal clearance. We did not observe a difference in fungal burden prior to 48 h post-challenge (data not shown). Intriguingly, augmented fungal clearance in *Il1rl1*<sup>-/-</sup> mice at 48 h post-challenge was not a consequence of differential lung cellularity (Figure 1C), as we observed no significant changes in neutrophils and eosinophils (gated on CD11b<sup>+</sup> cells followed by Ly6G<sup>+</sup> cells as neutrophils and Siglec-F<sup>+</sup> cells as eosinophils) (Figure 1D) or inflammatory monocytes (gated on CD11b<sup>+</sup> Ly6C<sup>+</sup> cells followed by gating on CCR2<sup>+</sup> cells) (Figure 1E) (all of which have been implicated in innate lung clearance of *A. fumigatus* (10) (30) (31). Similar data was observed at 12 and 24 h post-infection (Supplemental Figure 1). This observation is supported by no differences in the levels of various chemokines produced by lung digest cells (Figure 1F) and similar levels of inflammatory cells and inflammation on H&E-stained lung tissue sections (Figure 1G). Sequential GMS-stained lung tissue sections also demonstrated lower levels of *A. fumigatus* organisms in *Il1rl1*<sup>-/-</sup> mice (right) compared to WT (left) mice (Figure 1H). Thus, despite the induction of IL-33 after acute *A. fumigatus* exposure, clearance of the organism from the lung is enhanced in the absence of IL-33 signaling.

### IL-33 signaling negatively affects IL-17A and IL-22 after *A. fumigatus* exposure

As we did not observe major cellular changes in the lung between *A. fumigatus* exposed WT and *Il1rl1*<sup>-/-</sup> mice, we examined whether there was a difference in inflammatory factors that could explain improved fungal clearance in the absence of IL-33 signaling. We observed significantly higher levels of IL-1 $\alpha$ , IL-1 $\beta$  and IL-6, a profile that favors the development of IL-17A and IL-22, which were also produced at higher levels by lung digest cells from *Il1rl1*<sup>-/-</sup> mice (Figure 2A). Higher inflammatory cytokine levels in the absence of IL-33 signaling suggested that IL-33 functioned as a negative regulator of these mediators. To confirm this, we challenged mice with *A. fumigatus* followed by treating them with IL-33, which resulted in a ~ 65% reduction in IL-17A (Figure 2B) and ~ 50% reduction in IL-22 production (Figure 2C). Although this suggests that IL-33 treatment could have a negative effect on the clearance of *A. fumigatus* from the lung, we did not observe an effect of IL-33 treatment on fungal burden (Figure 2D). Thus, the absence of IL-1RL1/ST2 signaling results in augmented lung clearance of *A. fumigatus* in the presence of heightened IL-17A and IL-22 production, indicating that signaling through IL-1RL1 serves as a negative regulator of these mediators.

### PGE2, but not IL-23, is increased in the absence of IL-33 signaling

IL-23 is the most recognized positive regulator of IL-17A and IL-22 production and we have previously reported that IL-23 drives IL-17A and IL-22 production in the lung after *A. fumigatus* exposure (24) (19). We therefore speculated that the increased production of IL-17A and IL-22 in the absence of IL-33 signaling was most likely the result of increased IL-23. However, *Il1rl1*<sup>-/-</sup> mice had similar levels of IL-23 in the lung as WT mice (Figure

3A). There was also no difference in IL-23 production by *A. fumigatus* stimulated BMDCs between WT and *Il1rl1*<sup>-/-</sup> mice (Supplemental Figure 2). This data suggested that IL-17A and IL-22 were elevated in *Il1rl1*<sup>-/-</sup> mice via an IL-23 independent mechanism. PGE2 has been recently described as potentiator of innate IL-22 production (32), therefore we questioned whether PGE2 was modulated in the absence of IL-33 signaling. Results show that at baseline, lung cells from naïve WT and *Il1rl1*<sup>-/-</sup> mice produced similar levels of PGE2 (Figure 3B). However, upon exposure to *A. fumigatus*, lung cells from *Il1rl1*<sup>-/-</sup> mice demonstrated significantly higher PGE2 production (Figure 3B). This data suggests IL-33 signaling regulates PGE2 production during *A. fumigatus* exposure. We next examined mice treated with IL-33 after *A. fumigatus* exposure and found that, like IL-17A and IL-22, PGE2 levels were significantly lower (Figure 3C). Thus, IL-33 is a negative regulator of PGE2, which may function as a positive regulator of IL-17A and IL-22 production during lung fungal exposure.

### **Cyclooxygenase-2 inhibition results in attenuated inflammatory cytokine production in *Il1rl1*<sup>-/-</sup> mice after *A. fumigatus* exposure**

Prostaglandin-endoperoxide synthase 2 (i.e. cyclooxygenase 2, COX-2) initiates the conversion of arachidonic acid to multiple prostaglandins, including PGE2. A recent study in humans reported that individuals with various systemic inflammatory syndromes demonstrated decreased COX-2 and PGE2 expression coupled with lower IL-22 levels (32). Employing an experimental murine model of LPS-induced systemic inflammation, this report demonstrated that PGE2-EP4 signaling promoted homeostasis of type 3 innate lymphoid cells (ILC3s) and drove them to produce IL-22 (32). To determine if this axis also functioned in the induction of lung-derived IL-22, as well as other inflammatory mediators, we examined the effects of COX-2 inhibition. Treating *A. fumigatus* exposed *Il1rl1*<sup>-/-</sup> mice with Celecoxib/Celebrex®, a COX-2 selective NSAID, significantly lowered IL-1 $\alpha$  (Figure 4A), IL-1 $\beta$  (Figure 4B), IL-6 (Figure 4C), IL-17A (Figure 4D) and IL-22 (Figure 4E) production. Consequently, the attenuation of these mediators resulted in less efficient fungal clearance in *Il1rl1*<sup>-/-</sup> mice (Figure 4F). Despite our previous reports showing a role for IL-17A and IL-22 in lung defense against *A. fumigatus* (24) (19), neutralization of both mediators in *Il1rl1*<sup>-/-</sup> mice unexpectedly did not affect their ability to clear *A. fumigatus* from the lung (Figure 4G). The effects of COX-2 inhibition were also observed in WT mice (for example, IL-22 levels were 504  $\pm$  67 pg/ml vs. 166  $\pm$  61 pg/ml in vehicle-treated (n = 4) vs. Celebrex-treated mice (n = 4), respectively; P = 0.001). Thus, inhibition of COX-2 signaling in the absence of IL1RL1 during *A. fumigatus* exposure compromises lung production of multiple inflammatory mediators leading to impaired fungal clearance.

### **PGE2 supports production of IL-17A and IL-22, but not IL-6, IL-1 $\alpha$ and IL-1 $\beta$ , by lung cells from *A. fumigatus* exposed mice**

Although PGE2 is a product of COX-2 signaling, it is unclear whether PGE2 itself affects inflammatory mediator production. To address this, we cultured lung cells from infected mice with an inhibitor of microsomal prostaglandin E synthase-1 (mPGES-1), which converts the COX-2 product PGH2 into the biologically active PGE2. This demonstrated that specific inhibition of PGE2 attenuated IL-17A and IL-22 production (Figure 5A), but not IL-6, IL-1 $\alpha$  and IL-1 $\beta$  (Figure 5B). Conversely, treating lung cells from infected mice

with the global EP receptor (PGE2 receptor) agonist misoprostol increased production of IL-6, IL-17A and IL-22 production (Figure 5C) over vehicle treated lung cells, but not IL-1 $\alpha$  and IL-1 $\beta$  (Figure 5D). Misoprostol was not effective at inducing these mediators in lung cells from naïve mice (Supplemental Figure 3). Collectively, our data reveals that PGE2 specifically promotes the production of IL-17A and IL-22 during lung fungal exposure.

### **Increased IL-1 $\alpha$ and IL-1 $\beta$ signaling is not the mechanism associated with enhanced PGE2 and IL-22 production in *Il1rl1*<sup>-/-</sup> mice after *A. fumigatus* exposure**

Data thus far indicates that the level of PGE2 directly correlates with the level of IL-17A and IL-22 after *A. fumigatus* exposure. However, it is not clear why PGE2 is elevated in *Il1rl1*<sup>-/-</sup> mice after *A. fumigatus* exposure. In addition to IL-17A and IL-22, data in Figure 2A showed that IL-1 $\alpha$  and IL-1 $\beta$  were also significantly elevated in *Il1rl1*<sup>-/-</sup> mice after *A. fumigatus* exposure. IL-1 $\beta$  has previously been implicated in PGE2 induction (33). In turn, mice deficient in the receptor for IL-1 $\alpha$  and IL-1 $\beta$  demonstrated ~ 60% reduction in PGE2 levels (Figure 6A) as well as a profound reduction in the production of IL-22 (Figure 6B), suggesting that the increase in IL-1 $\alpha$  and/or IL-1 $\beta$  in the absence of IL-33 signaling was potentially responsible for the increase in PGE2 and thus IL-22 production. To confirm this, we determined whether IL-1 $\alpha$  and IL-1 $\beta$  levels were modulated in mice that received IL-33 after *A. fumigatus* challenge. Despite IL-33 signaling impairing PGE2 (Figure 3C) and IL-22 (Figure 2C) production, IL-33 administration had no effect on the levels of IL-1 $\alpha$  or IL-1 $\beta$  (Figure 6C). There was also no synergy between IL-1 $\beta$  and PGE2 receptor stimulation, as the addition of IL-1 $\beta$  in conjunction with misoprostol in lung cells from *A. fumigatus* infected mice did not augment production of IL-22 (Supplemental Figure 3). Thus, the mechanism behind elevated PGE2 in the absence of IL-33 signaling is not a result of enhanced IL-1 $\alpha$  and IL-1 $\beta$  production.

## **Discussion**

In this body of work, we demonstrate that despite induction of IL-33 in the lung in response to *A. fumigatus*, IL-33 signaling negatively regulated immune responsiveness to *A. fumigatus*, as evidenced by mice deficient in IL-33 receptor signaling (*ST2*; *Il1rl1*<sup>-/-</sup> mice) displaying lower fungal burden post-challenge. Assessment of potential mechanisms to explain enhanced fungal clearance in the absence of IL-33 signaling revealed a specific profile of elevated cytokines, IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-17A and IL-22. Despite observing elevated levels of these mediators, they did not correlate with any changes in lung cellularity, an observation itself supported by the lack of changes in the levels of multiple chemokines. As a result, we focused on the IL-17A/IL-22 axis, due to their known abilities to induce soluble antimicrobial responses from the lung epithelium (34) and our previous work documenting a protective role for both IL-17A and IL-22 in host defense against *A. fumigatus* (24) (19). An intriguing finding from the current study was that IL-33 functioned as a negative regulator of IL-17A and IL-22. To date, there have been relatively few cytokines identified as negative regulators of IL-17A and IL-22. The most well-recognized include TGF- $\beta$ , which promotes IRF4-mediated suppression of IL-17A and IL-22 (35). Others include IL-27, which promotes c-Maf mediated inhibition of IL-17A and IL-22, and IL-25, which favors type 2 cytokine induction and consequently, IL-17A and IL-22

suppression. Overall, our findings suggest the addition of IL-33 to this list, as the absence of IL-1RL1/ST2 signaling results in heightened IL-17A and IL-22 production, whereas increasing IL-33 levels results in decreased IL-17A and IL-22 production, indicating that IL-33 signaling through IL-1RL1 serves as a negative regulator of IL-17A and IL-22.

Based on our previous work (24) (19), our hypothesis behind elevated IL-17A and IL-22 levels in the absence of IL-33 signaling was that IL-33 negatively regulated IL-23, which we have shown to drive IL-17A and IL-22 production in the lung (24) (19). However, this was found not to be the case, resulting in a search for an IL-23 independent mechanism of enhanced IL-17A and IL-22 production. A role for PGE2 in the generation of IL-17A and IL-22 has been controversial. It has been reported that PGE2 may enhance IL-17A production from previously polarized Th17 cells or memory Th17 cells (36) (37), however recent data shows that PGE2 has the complete opposite (i.e. negative) effect on Th17 generation from naïve T cells (38). In this latter study, the fungal pathogen *C. neoformans* was shown to produce its own PGE2, which negatively affected IL-17A production but had no effect on IL-22 (38). Other studies showed that anti-CD3 activation of human PBMCs in the presence of exogenous PGE2 promoted IL-17A production but significantly dampened IL-22 production (39). Human PBMCs stimulated with the fungus *Candida albicans* demonstrated increased IL-17A and IL-22 production that were slightly affected by the NSAID diclofenac ( $P < 0.05$  for IL-17A, IL-22 was slightly lower but not statistically significant (40)). In this report, EP receptor agonists were moderately effective at restoring NSAID-inhibited IL-17A production, although the effects on IL-22 were not determined. However, as described earlier, a recent report demonstrated that the COX2/PGE2 axis functioned in the generation of innate IL-22 production in both humans and mice (32). Here, using an experimental model of LPS-induced systemic inflammation, this report demonstrated that PGE2-EP4 signaling promoted homeostasis of type 3 innate lymphoid cells (ILC3s) and drove them to produce IL-22. We found that similar to IL-17A and IL-22, PGE2 levels were elevated in *Il1rl1*<sup>-/-</sup> mice and treating mice with IL-33 resulted in the lowering of both IL-17A and IL-22 and PGE2 levels in the lung. Although IL-17A and IL-22 levels were reduced after IL-33 treatment (~ two-thirds and one-half, respectively), they were not completely eliminated. Moreover, IL-33 treatment had no effect on the levels of IL-1 $\alpha$ , IL-1 $\beta$  and IL-6. Consequently, we did not observe an effect of IL-33 treatment on *A. fumigatus* lung clearance. Based on these results, we hypothesize that IL-33 treatment does not facilitate a level of immunosuppression that compromises fungal clearance. In contrast, when targeting PGE2 via COX-2 inhibition, IL-17A and IL-22 levels, as well as IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 levels, in *Il1rl1*<sup>-/-</sup> mice were significantly reduced, which correlated with reduced *A. fumigatus* lung clearance. An unexpected finding from our work was that neutralization of IL-17A and IL-22 in *Il1rl1*<sup>-/-</sup> mice did not impair *A. fumigatus* lung clearance. Based on observations with IL-33 treatment (reduced IL-17A and IL-22 levels, intact IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 levels and no effect on fungal clearance) and Celecoxib/ Celebrex® treatment (reduced IL-17A, IL-22, IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 levels and a negative effect on fungal clearance), the mechanism of resistance in *Il1rl1*<sup>-/-</sup> mice appears to be quite complex. Specifically, intact (IL-33 treatment) or elevated (*Il1rl1*<sup>-/-</sup> mice) IL-1 $\alpha$ , IL-1 $\beta$  and/or IL-6 may compensate in some manner when IL-17A and IL-22 levels are manipulated. It would be of interest in future studies to cross *Il1rl1*<sup>-/-</sup> mice with *Il1r*<sup>-/-</sup>



mice, which would eliminate IL-1 $\alpha$  and IL-1 $\beta$  signaling in tandem with IL-33 signaling and may result in mice deficient in both IL-17A and IL-22 induction as well as IL-1 $\alpha$  and IL-1 $\beta$  signaling.

When targeting PGE2 via COX-2 inhibition, IL-17A and IL-22 levels, as well as IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 levels, in *Il1rl1*<sup>-/-</sup> mice were significantly reduced, further suggesting that COX-2 mediated PGE2 contributed in some way to the heightened IL-17A and IL-22 production observed in these mice. To confirm that PGE2 had the specific ability to induce IL-17A and IL-22, addition of a PGE2 agonist to lung cells from *A. fumigatus* exposed mice increased IL-6, IL-17A and IL-22 production, but not IL-1 $\alpha$ , IL-1 $\beta$ . Conversely, specific inhibition of PGE2 had a negative effect on IL-17A and IL-22 production from lung cells, but not IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 production. The novelty in our findings is not only that the COX-2/PGE2 axis drives innate IL-17A and IL-22 production in a mucosal tissue (i.e. the lung), suggesting a conserved mechanism of IL-17A and IL-22 induction, but also identifies putative upstream regulators of the PGE2-IL-17A-IL-22 axis, i.e. IL-33 signaling. A question arising from our findings is how does IL-33 signaling regulate PGE2 induction and thus control the level of inflammatory cytokines during fungal exposure. IL-1 signaling was a prime candidate responsible for increased PGE2 in *Il1rl1*<sup>-/-</sup> mice, as IL-1 $\alpha$  and IL-1 $\beta$  were elevated in these mice after fungal exposure and IL-1 signaling is known to induce PGE2 (41) (42). However, despite a profound reduction in IL-17A and IL-22 levels, IL-1 receptor deficiency resulted in only a moderate reduction in PGE2 production. Moreover, treating mice with IL-33 did not attenuate the levels of IL-1 $\alpha$  and IL-1 $\beta$  despite significantly lower PGE2 levels. Collectively, these results suggest that other IL-1 independent factors were responsible for elevated PGE2 levels in *Il1rl1*<sup>-/-</sup> mice. To this end, we assessed additional factors that were increased in *A. fumigatus* exposed *Il1rl1*<sup>-/-</sup> mice, yet were decreased in mice that were treated with IL-33. This analysis identified the cytokines IL-3, IL-17A and IFN- $\gamma$ . Although published reports suggest that each of these may induce PGE2 (43) (44) (45) (46) (47), neutralization of IL-3, IL-17A or IFN- $\gamma$  in lung cell cultures from *Il1rl1*<sup>-/-</sup> mice did not result in attenuated PGE2 or IL-22 production (Supplemental Figure 4). Based on these observations, we hypothesize that a soluble factor such as a cytokine is an unlikely candidate for heightened PGE2 production in the absence of IL-33 signaling. IL1RL1 (and thus IL-33) has been shown to regulate TLR2 responses, as macrophages from *Il1rl1*<sup>-/-</sup> mice show hyperresponsiveness when stimulated with bacterial lipoprotein (48). As we have previously reported a small role for TLR2 in alveolar macrophages responses to *A. fumigatus* (49), it is possible that enhanced TLR2 signaling in *Il1rl1*<sup>-/-</sup> mice contribute to higher PGE2 and IL-17A and IL-22 induction in some manner. Indeed, fungal recognition via TLR2 can drive PGE2 production (50). To this end, we cannot exclude the possibility that IL-33 may affect the expression or function of other PRRs, namely the beta-glucan receptor Dectin-1. Another study has shown that macrophages from mice deficient in *Atf3*, an ATF/CREB basic leucine zipper (bZip) transcription factor induced during cellular stress have enhanced COX-2 signaling and PGE2 production after stimulation with zymosan (51). Ongoing studies are interrogating the role of IL-33 in *Atf3* induction as well as its effect on Dectin-1.

In conclusion, this is the first report identifying a novel role for IL-33 in the regulation of IL-17A and IL-22 production in the lung after fungal exposure. The absence of IL-33

signaling resulted in enhanced IL-17A and IL-22 production in the presence of enhanced PGE2 levels, whereas the opposite was true in mice administered IL-33. Inhibition of COX-2 signaling *in vivo* resulted in lower IL-17A and IL-22 induction and impaired fungal clearance. A specific agonist of PGE2 receptors augmented, while specific inhibition of PGE2 generation impaired, IL-17A and IL-22 production. Overall, the current body of work adds depth to our understanding of the role specific IL-1 family members play in IL-17A and IL-22 regulation and innate lung defense. Moreover, these findings lay the foundation for determining whether IL-33 signaling affects PGE2 and/or IL-17A and IL-22 induction in other IL-17A/IL-22 dependent models of inflammation, such as colitis and psoriasis, or infection models requiring IL-17A/IL-22 for defense, such as Gram-negative bacterial pneumonia and influenza.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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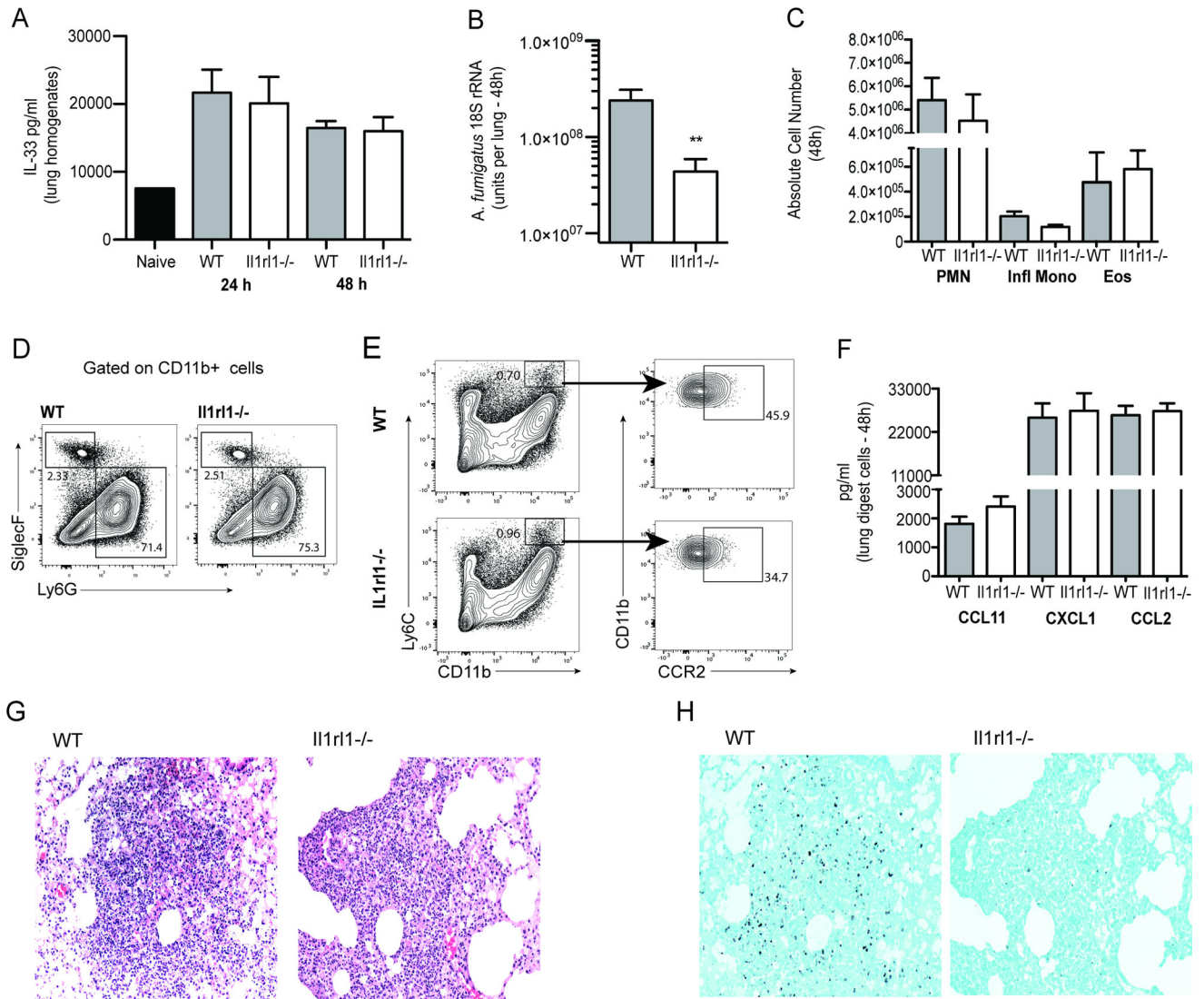
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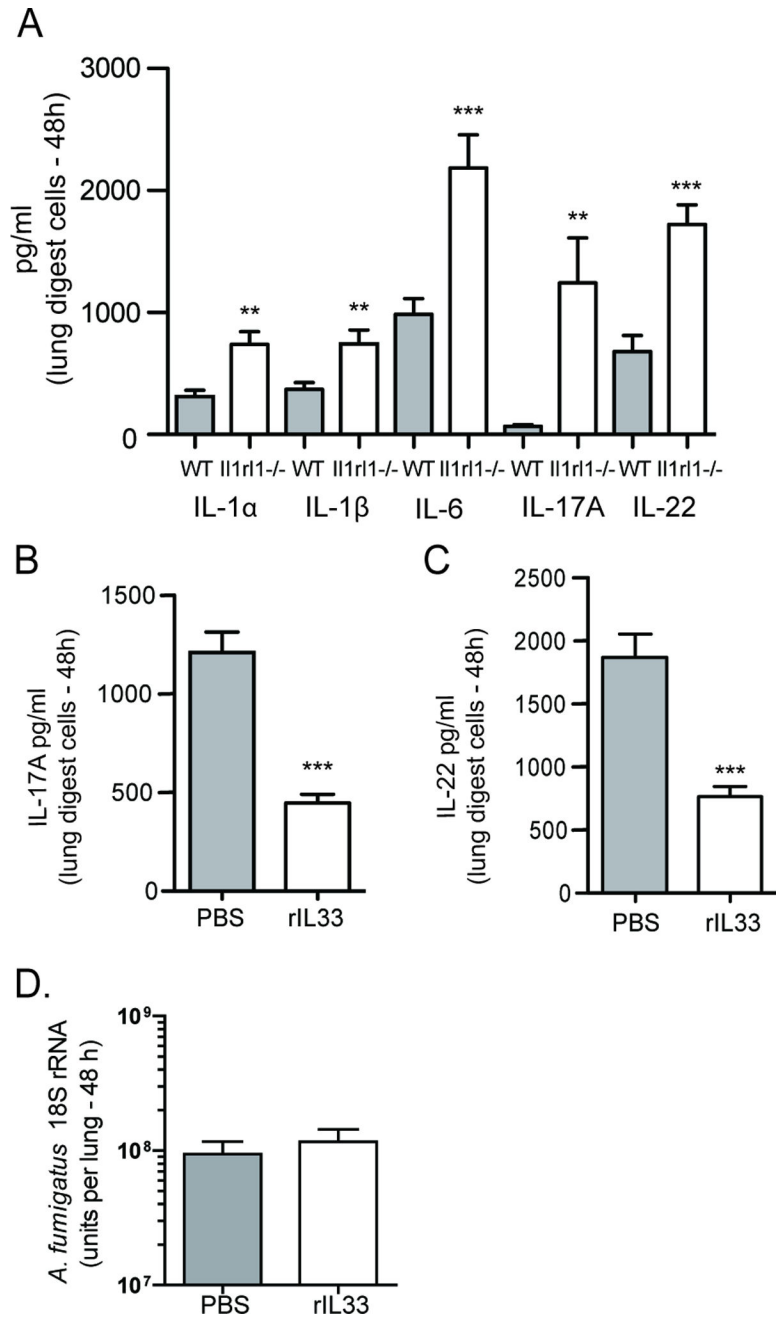
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**Figure 1. The lack of IL-33 signaling results in enhanced *A. fumigatus* lung clearance** (A) C57BL/6 wild-type and Dectin-1 deficient (*Clec7a*<sup>-/-</sup>) mice were challenged intratracheally with *A. fumigatus* conidia (Af) and 48 h post-exposure, whole lungs were collected and IL-33 levels quantified in clarified lung homogenates by ELISA. The Figure illustrates cumulative data from two to three independent studies (n = 3–5 mice per group, per study). (B) C57BL/6 wild-type (WT) and *Il1rl1*<sup>-/-</sup> (IL-1RL1; ST2 deficient) mice were challenged intratracheally with *A. fumigatus* conidia and 48 h after exposure, lung fungal burden was assessed by real-time PCR analysis of *A. fumigatus* 18S rRNA levels. The Figure illustrates cumulative data from two independent studies (n = 4–5 mice per group, per study). Data are expressed as mean *A. fumigatus* 18S rRNA + SEM. (C) Cumulative flow cytometric data from two independent studies (n = 2–4 mice per group, per study). Data are expressed as absolute number of live cells in lung digests. (D/E) Lung cells were isolated via enzymatic digestion, Fc-blocked, stained with a live/dead staining kit and thereafter stained with fluorochrome-conjugated CD11c, CD11b, Ly6G, Ly6C and Siglec F. Representative flow cytometric plots are included for (D) neutrophils and eosinophils (gated on CD11b+

cells followed by Ly6G<sup>+</sup> cells as neutrophils and Siglec-F<sup>+</sup> cells as eosinophils) and (E) inflammatory monocytes (gated on CD11b<sup>+</sup> Ly6C<sup>+</sup> cells followed by gating on CCR2<sup>+</sup> cells). (F) C57BL/6 wild-type (WT) and *Il1rl1*<sup>-/-</sup> (IL-1RL1; ST2 deficient) mice were challenged intratracheally with *A. fumigatus* conidia and 48 h after exposure, the right lungs were collected, enzymatically digested and unfractionated lung cells cultured in triplicate for 24 h. CCL11/eotaxin, CXCL1/KC and CCL3/MIP-1 $\alpha$  levels were quantified in clarified co-culture supernatants by Bio-Plex. The Figure illustrates cumulative data from four independent studies (n = 1–2 mice per group, per study). (G) Representative H&E-stained lung sections from wild-type (left) and *Il1rl1*<sup>-/-</sup> (right) mice challenged intratracheally with *A. fumigatus* conidia for 48 h. Original magnification of 200X. (H) Representative GMS-stained lung sections from wild-type (left) and *Il1rl1*<sup>-/-</sup> (right) mice challenged intratracheally with *A. fumigatus* conidia for 48 h. Original magnification of 200X. For all graphs, \*\* represents a P value of < 0.01, respectively (Unpaired two-tailed Student's t test).



**Figure 2. IL-33 signaling negatively affects IL-17A and IL-22 production after *A. fumigatus* exposure**  
 C57BL/6 wild-type (WT) and *Il1rl1*<sup>-/-</sup> (IL-1RL1; ST2 deficient) mice were challenged intratracheally with *A. fumigatus* conidia and 48 h after exposure, the right lungs were collected, enzymatically digested and unfractionated lung cells cultured in triplicate for 24 h. (A) IL-1α, IL-1β, IL-6, IL-17A and IL-22 levels were quantified in clarified co-culture supernatants by Bio-Plex or ELISA. The Figure illustrates cumulative data from four independent studies (n = 1–2 mice per group, per study). (B/C) C57BL/6 wild-type mice were challenged with *A. fumigatus* and 6 and 24 h thereafter, administered IL-33 (1 μg in 50 μl) or PBS intratracheally. Forty-eight h after exposure, the right lungs were collected,



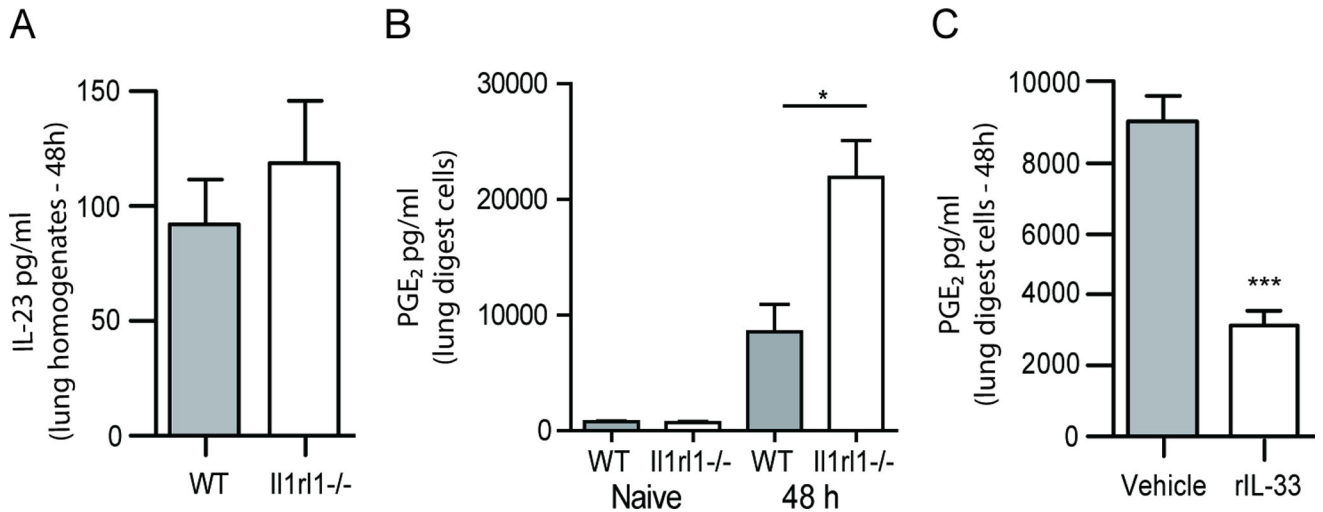
enzymatically digested and unfractionated lung cells cultured in triplicate for 24 h. (B) IL-17A and (C) IL-22 levels in co-culture supernatants were quantified by Bio-Plex or ELISA. The Figure illustrates cumulative data from three independent studies (n = 1–2 mice per group, per study). For all graphs, \*\* or \*\*\* represent a P value of < 0.01 or 0.001, respectively (Unpaired two-tailed Student's t test). (D) C57BL/6 wild-type mice were challenged with *A. fumigatus* and 6 and 24 h thereafter, administered IL-33 (1 µg in 50 µl) or PBS intratracheally. Forty-eight h after exposure, lung fungal burden was assessed by real-time PCR analysis of *A. fumigatus* 18S rRNA levels. The Figure illustrates cumulative data from four independent studies (n = 3–4 mice per group, per study). Data are expressed as mean *A. fumigatus* 18S rRNA + SEM.

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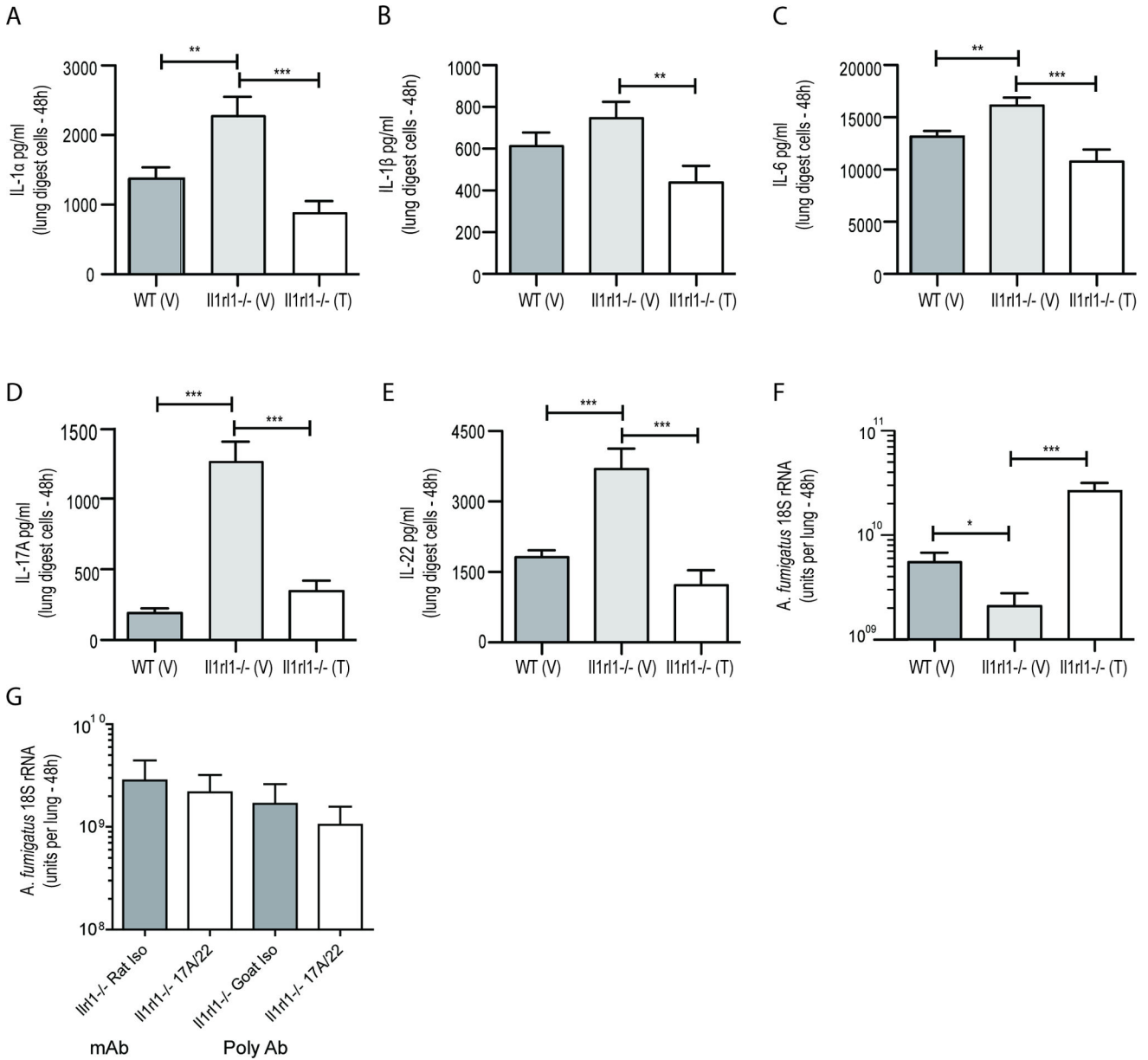
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**Figure 3. PGE<sub>2</sub>, but not IL-23, is increased in the absence of IL-33 signaling**

(A) C57BL/6 wild-type (WT) and *Il1rl1*<sup>-/-</sup> (IL-1RL1; ST2 deficient) mice were challenged intratracheally with *A. fumigatus* conidia and 48 h after exposure, whole lungs were collected and IL-23 levels quantified in clarified lung homogenates by ELISA. The Figure illustrates cumulative data from two independent studies (n = 4–5 mice per group, per study). (B) C57BL/6 wild-type (WT) and *Il1rl1*<sup>-/-</sup> (IL-1RL1; ST2 deficient) mice were challenged intratracheally with *A. fumigatus* conidia and 48 h after exposure, the right lungs were collected, enzymatically digested and unfractionated lung cells cultured in triplicate for 24 h. PGE<sub>2</sub> levels were quantified in clarified co-culture supernatants by EIA. The Figures illustrates cumulative data from three independent studies (n = 1–2 mice per group, per study). (C) C57BL/6 wild-type mice were challenged with *A. fumigatus* and 6 and 24 h thereafter, administered IL-33 (1 μg in 50 μl) or PBS intratracheally. Forty-eight h after exposure, the right lungs were collected, enzymatically digested and unfractionated lung cells cultured in triplicate for 24 h. PGE<sub>2</sub> levels were quantified in clarified co-culture supernatants by EIA. The Figure illustrates cumulative data from three independent studies (n = 1–2 mice per group, per study). For all graphs, \* or \*\*\* represent a P value of < 0.05 or 0.001, respectively (Unpaired two-tailed Student's t test).



**Figure 4. Cyclooxygenase-2 inhibition results in attenuated inflammatory cytokine production in *Il1rl1*<sup>-/-</sup> mice after *A. fumigatus* exposure**

(A/B/C/D/E) C57BL/6 wild-type (WT) and *Il1rl1*<sup>-/-</sup> (IL-1RL1; ST2 deficient) mice were administered Celecoxib intraperitoneally (100 mg/kg in 250  $\mu$ l; denoted as “T” for treated) or Vehicle (ethanol; denoted as “V” for vehicle) 24 h prior to *A. fumigatus* challenge and then again at 6 h and 24 h post-challenge. Thirty-six to forty-eight h after *A. fumigatus* challenge, mice were sacrificed, the right lungs were collected, enzymatically digested and unfractionated lung cells cultured for 24 h. (A) IL-1 $\alpha$ , (B) IL-1 $\beta$ , (C) IL-6, (D) IL-17A and (E) IL-22 levels in co-culture supernatants were quantified by Bio-Plex or ELISA. The Figure illustrates cumulative data from four independent studies (n = 3–4 mice per group, per study). (F) Mice were treated as in (A) and 48 h after exposure, lung fungal burden was assessed by real-time PCR analysis of *A. fumigatus* 18S rRNA levels. The Figure illustrates

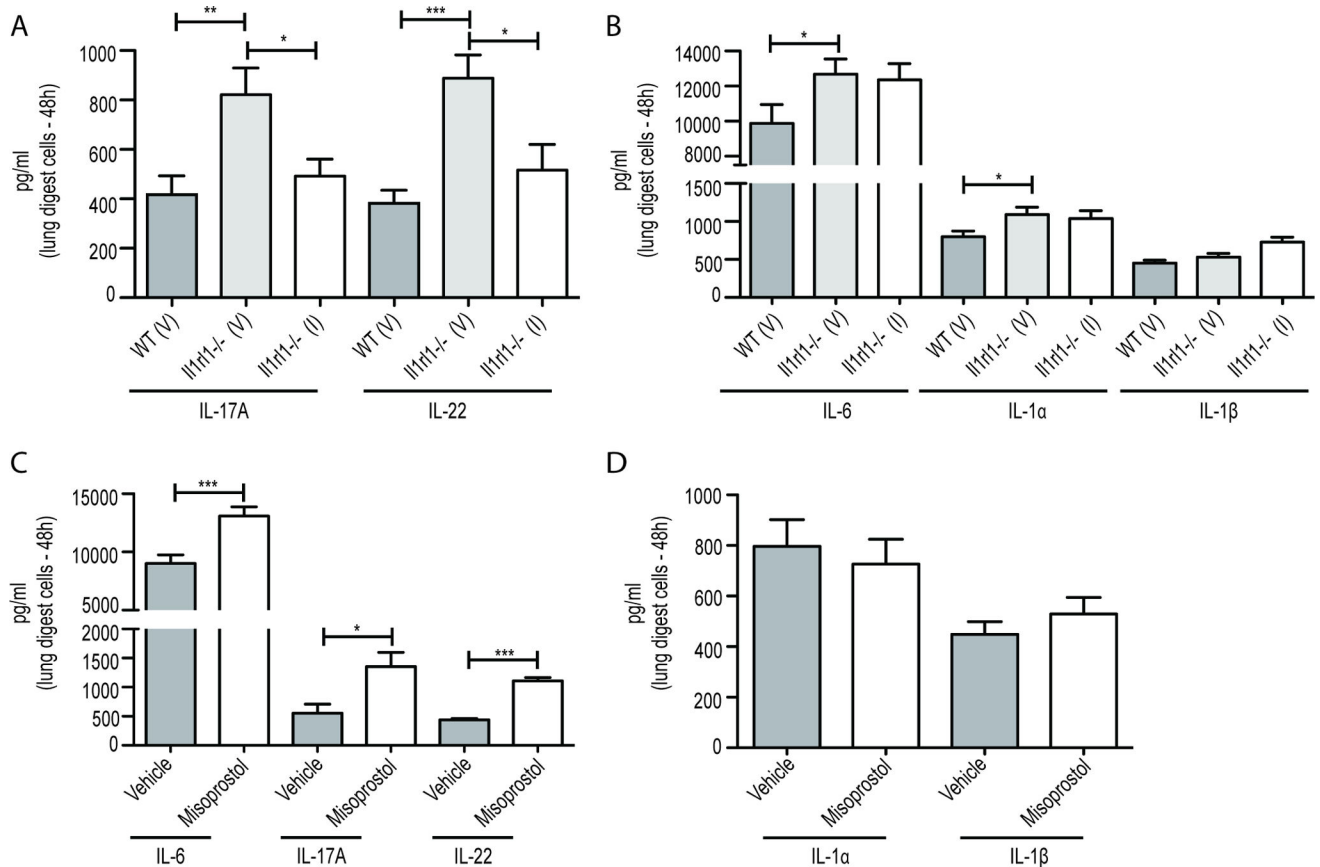
cumulative data from two independent studies (n = 4 mice per group, per study). For all graphs, \* or \*\*\* represent a P value of < 0.05 or 0.001, respectively (Unpaired two-tailed Student's t test). (G) *Il1rl1*<sup>-/-</sup> (IL-1RL1; ST2 deficient) mice were challenged intratracheally with *A. fumigatus* conidia and 24 h thereafter, mice were administered 50 µg of rat anti-mouse IL-17A or IL-22 (R&D Systems) or rat IgG isotype control antibody or goat anti-mouse IL-17A or IL-22 (R&D Systems) or goat IgG isotype control antibody. Forty-eight hours after challenge, mice were sacrificed, the left lungs were collected and fungal burden assessed. The Figure illustrates cumulative data from two to three independent studies (n = 3–4 mice per group). Data are expressed as mean *A. fumigatus* 18S rRNA + SEM.

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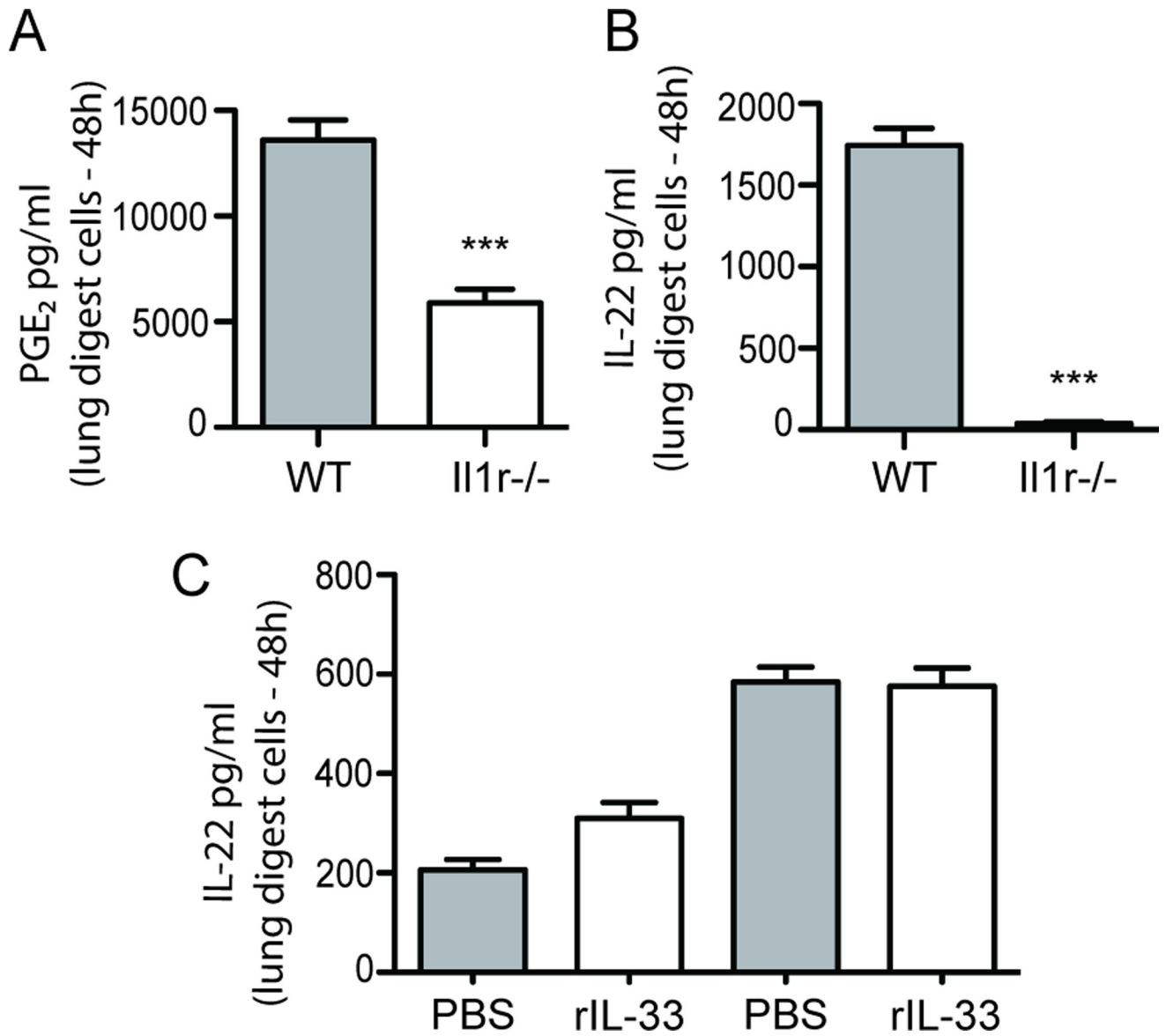
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**Figure 5. PGE2 supports IL-17A and IL-22, but not IL-6, IL-1α and IL-1β, by lung cells from *A. fumigatus* exposed mice**

(A/B) C57BL/6 wild-type (WT) and *Il1rl1*<sup>-/-</sup> (IL-1RL1; ST2 deficient) mice were challenged with *A. fumigatus* conidia and 48 h after exposure, mice were sacrificed, the right lungs were collected, enzymatically digested and unfractionated lung cells cultured in the presence of the mPGES inhibitor (denoted as “I” for inhibitor) or vehicle (DMSO; denoted as “V” for vehicle) in triplicate for 24 h. (A) IL-17A and IL-22 levels and (B) IL-6, IL-1α and IL-1β levels in co-culture supernatants were quantified by Bio-Plex or ELISA. The Figure illustrates cumulative data from three independent studies (n = 1–2 mice per group, per study). (C/D) C57BL/6 wild-type (WT) mice were exposed and cells isolated as in (A). Cells were cultured in the presence of misoprostol or vehicle in triplicate for 24 h. (C) IL-6, IL-17A and IL-22 levels and (D) IL-1α and IL-1β levels in co-culture supernatants were quantified by Bio-Plex or ELISA. The Figure illustrates cumulative data from two independent studies (n = 1–2 mice per group, per study). For all graphs, \* or \*\*\* represent a P value of < 0.05 or 0.001, respectively (Unpaired two-tailed Student’s t test).



**Figure 6. Increased IL-1R signaling is not the mechanism associated with enhanced PGE<sub>2</sub> and IL-22 in *Il1r1*<sup>-/-</sup> mice after *A. fumigatus* exposure**  
 C57BL/6 wild-type (WT) and *Il1r1*<sup>-/-</sup> (IL-1R1 deficient) mice were challenged intratracheally with *A. fumigatus* conidia and 48 h after exposure, the right lungs were collected, enzymatically digested and unfractionated lung cells cultured in triplicate for 24 h. (A) PGE<sub>2</sub> levels were quantified in clarified co-culture supernatants by EIA and (B) IL-22 levels were quantified in clarified co-culture supernatants by ELISA. The Figures illustrates cumulative data from four independent studies (n = 1–2 mice per group, per study). (C) C57BL/6 wild-type mice were challenged with *A. fumigatus* and 6 and 24 h thereafter, administered IL-33 (1 μg in 50 μl) or PBS intratracheally. Forty-eight h after exposure, the right lungs were collected, enzymatically digested and unfractionated lung cells cultured in triplicate for 24 h. IL-1α and IL-1β levels were quantified in clarified co-culture supernatants by Bio-Plex. The Figure illustrates cumulative data from two independent

studies (n = 1–2 mice per group, per study). For all graphs, \*\*\* represents a P value of < 0.001, respectively (Unpaired two-tailed Student's t test).

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