Title:
A dual role for CRTH2 in acute lung injury

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EJ, AJC, GLC, and SG contributed to the conception of the project. EJ, AJC, RH, DT, NR, and SG contributed to experimental procedures. AJC, RH, and CC contributed to data analysis. EJ, AJC, CDC, DAS, GLC, and SG contributed to writing the manuscript.

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ABSTRACT

Acute respiratory distress syndrome (ARDS) is a life-threatening clinical condition defined by rapid onset respiratory failure following acute lung injury (ALI). Its increased incidence due to COVID-19 and high mortality rate (~40%) make the study of ARDS pathogenesis a crucial research priority. CRTH2 is a G protein-coupled receptor with established roles in type 2 immunity and well-characterized inhibitors. Prior studies have shown it also promotes neutrophilic inflammation, indicating that CRTH2 inhibition may be a potential therapeutic strategy for ARDS. To test this hypothesis, we first examined the expression pattern of CRTH2 on murine neutrophils. We found it is expressed on neutrophils, but only after extravasation into the lung. Next, we showed that extravasated lung neutrophils generate inflammatory responses upon stimulation with the CRTH2-specific agonist DK-PGD2, as demonstrated by reactive oxygen species (ROS) production. This response was abrogated in CRTH2 KO neutrophils. Inhibition of CRTH2 with fevipiprant suppressed baseline ROS production, indicating an autocrine PGD2-CRTH2 signaling loop. We then evaluated the role of CRTH2 in vivo using a murine model of LPS-induced ALI. Despite the pro-inflammatory effects of CRTH2 on neutrophils in vitro, we observed worsening of lung injury in CRTH2-deficient mice in terms of neutrophilic inflammation, vascular leak, and survival. Bulk RNAseq of lung tissue indicated an impairment in type 2 immune signaling; qPCR and ELISA confirmed downregulation of the key type 2 effector cytokine, IL-4. Thus, CRTH2 appears to play a dual role in ALI, directly promoting neutrophil effector responses, but indirectly suppressing lung injury and neutrophilic inflammation through type 2 immunity. These

findings reveal a novel protective function for CRTH2 during lung injury and argue against the use of CRTH2 inhibitors in ARDS.

Keywords:

Neutrophils, CRTH2, acute lung injury, PGD2, type 2 immunity, murine ARDS model

Abbreviations:

ALI – Acute lung injury
ARDS – Acute respiratory distress syndrome
CRTH2 – Chemoattractant receptor-homologous molecule expressed on Th2 cells
KO – Knockout
LPS – Lipopolysaccharide
PGD2 – Prostaglandin D2
RNAseq – Ribonucleic acid sequencing
WBC – White blood cells

Introduction

Acute respiratory distress syndrome (ARDS) is a life-threatening clinical condition defined by rapid onset respiratory failure following acute lung injury (ALI).(1) Due to the COVID-19 pandemic, ARDS has emerged as one of the leading causes of death worldwide.(2) Mortality rates range between 30-40% and no effective disease-modifying therapies have been identified, outside of immunomodulators for early COVID-19.(1) Thus, investigation into ARDS pathogenesis and treatment represents an urgent global research priority.

Chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2, also called DP2, CD294, GPR44, and PTGDR2) is a seven-membrane spanning G-protein coupled receptor (GPCR) expressed on type 2 immune cells such as eosinophils, mast cells, Th2 cells, and type 2 innate lymphoid cells (ILC2s).(3–5) Upon activation with its endogenous ligand, prostaglandin D_2 (PGD_2), CRTH2 signals through $G_i\alpha$, leading to increases in cytosolic calcium and reduction in cAMP levels.(4) In eosinophils, CRTH2 agonism promotes inflammatory responses including degranulation and chemotaxis.(3) CRTH2 ligation similarly activates ILCs and Th2 cells, leading to chemotaxis, suppression of apoptosis, and synthesis of type 2 cytokines including IL-4, IL-5, IL-9, and IL-13.(6–9)

Given its key role in type 2 inflammation, CRTH2 has been investigated as a therapeutic target in allergic diseases such as asthma. Numerous small molecule antagonists have been developed for this purpose, best exemplified by fevipiprant, which advanced to late-stage clinical trials.(10) Phase II studies in asthma showed promising results, as

fevipiprant suppressed peripheral and airway eosinophilia, reduced airway remodeling, improved quality of life, and increased lung function.(11–14) In a follow-up phase III clinical trial, CRTH2 blockade proved safe and efficacious as it significantly improved lung function and asthma control.(15) However, it failed to achieve the primary endpoint of reducing exacerbation frequency, so further development for asthma has been halted.(10)

The function of CRTH2 outside of type 2 immunity is less understood, but several studies have indicated that it plays a role in neutrophilic inflammation.(16) For instance, CRTH2 inhibition reduced neutrophil recruitment to the lung in a lipopolysaccharide (LPS)-induced model of ALI.(17) Similar results were observed upon pharmacologic blockade of CRTH2 in murine smoking models.(18, 19) Reciprocally, Jandl et al demonstrated that activation of CRTH2 with the isoform-specific agonist, DK-PGD2, exacerbated neutrophilic inflammation during ALI.(20) Outside of the lung, CRTH2 appears to play a similar pro-neutrophilic role, as shown in murine models of cutaneous inflammation.(21, 22)

Thus, we hypothesized that CRTH2 inhibition might ameliorate neutrophil-mediated tissue damage during ALI, and therefore represent a novel therapeutic strategy for ARDS. To evaluate this hypothesis, we studied the expression and function of CRTH2 in isolated murine neutrophils from the lung. We then utilized a model of LPS-induced ALI in CRTH2 knockout mice to investigate the function of CRTH2 in vivo.

Methods

Murine model of acute lung injury

Male and female wild-type and CRTH2 KO C57BL/6 mice (Jackson Laboratories) were bred at Yale University under specific pathogen-free conditions. All animal studies were performed in accordance with Institutional Animal Care and Use Committee (IACUC) approved protocols at Yale University. 8- to 12-week-old age- and gender-matched mice were used for experiments after randomizing to control for litter effects. Intrapulmonary instillation of *Pseudomonas aeruginosa*-derived LPS (Sigma, L9143) via oropharyngeal (OP) aspiration, performance of BAL, measurement of cell counts, and quantification of neutrophil elastase activity were performed as described previously.(23, 24) To induce ALI, 10 μg/g of LPS was delivered intratracheally and mice were sacrificed after 12h.

Flow cytometry

To determine neutrophil counts, total white blood cells (WBCs) were counted on a benchtop hematology analyzer (AcT Diff, Beckman Coulter) and BAL cells were stained with antibodies against Ly6G (Biolegend) and CD45 (Biolegend) prior to evaluation by flow cytometry, as previously described.(23) To quantify CRTH2 surface expression, blood and BAL cells were stained for Ly6G, F4/80 (Biolegend), and CRTH2 (Thermo). An isotype matched antibody was used to control for non-specific CRTH2 staining.

In vitro assay for reactive oxygen species

Neutrophils were collected from mice via BAL 12-24 hours after intratracheal delivery of 5 μg of LPS. Cells were washed twice in PBS, centrifuged at 2600 RCF for 6 minutes, and resuspended at a final concentration of 1.5 x 10^6 cells/mL in Dulbecco's Modified Eagle Medium/F12 without phenol red (Thermo). Wells were loaded with 50 μL of the neutrophil suspension (750,000 total cells). Indicated stimuli were then added together with the fluorogenic substrate dihydrorhodamine 123 (DHR123, Sigma), a detector of superoxide species, at a final concentration of 12.5 μM. Kinetics of reactive oxygen species (ROS) production were quantified with a BioTek Cytation 3 plate reader by measuring fluorescence at 10-minute intervals over 8h (excitation 495 nm; emission 525 nm).

Transcriptomic analysis

Whole lungs were snap-frozen in 500 μL TRIzol buffer (Invitrogen) and homogenized via sonication at 20 kHz for 1 min before RNA isolation was performed using a RNeasy Mini Kit (Qiagen). For qPCR, the iScript cDNA Synthesis Kit (Bio-Rad) was utilized to generate cDNA; 18S rRNA was used as the reference gene. For bulk RNA-seq, quality assessment and cDNA library preparation were performed by the Yale Center for Genomic Analysis prior to rRNA depletion and Illumina-based NovaSeq S2 sequencing using a read length of 2x100 and 35M reads/sample.

Statistical analysis

Statistical analyses were performed using Prism 8 (Graphpad Software Inc). Results are presented as mean ± standard error of the mean (SEM). Data were analyzed using

Student's t-test or one-way ANOVA as appropriate. Data shown are representative of multiple independent repetitions of the experiment. Statistical significance throughout was defined as $P < 0.05$.

RNAseq analysis was conducted using the *edgeR* package in the R programming language (V.3.6.0, Vienna, Austria), and potential differentially expressed genes ("candidate DEGs") were identified by selecting genes with both a raw p-value of < 0.05 and consistent direction of regulation within sample groups (allowing for no more than one outlier per gene) after TPM normalization and scaling. We performed KEGG pathway analysis and gene ontology (GO) analysis using the Enrichr analysis tool as described by Chen et al.(25)

Results

Flow cytometry demonstrates cell-surface expression of CRTH2 on neutrophils

To assess whether CRTH2 has direct functional effects on neutrophils, we first tested for cellular expression of the receptor. Using flow cytometry we found that CRTH2 was expressed on the surface of F4/80+ cells in the blood (such as monocytes and eosinophils), consistent with prior reports (Fig. 1).(3) We also observed that it was absent from circulating neutrophils. However, after recruitment into the lung, extravasated neutrophils collected from the bronchoalveolar lavage (BAL) showed significant staining for CRTH2, indicating compartment-specific receptor expression.

CRTH2 mediates pro-inflammatory responses in neutrophils in vitro

BAL neutrophils were then tested for functional responses to CRTH2 agonists. To do so we utilized DK-PGD2, which binds selectively to CRTH2 and not DP1 (a closely related prostanoid receptor also capable of binding PGD2).(4) In a microplate-based kinetic fluorescence assay measuring ROS, DK-PGD2 dose-dependently stimulated ROS production in isolated neutrophils. The measured half-maximal effective concentration (EC_{50}) was approximately 40 nM, similar to a previously reported value (54 nM) that was obtained in HEK293 cells expressing human CRTH2.(26) Importantly, BAL neutrophils collected from CRTH2 KO mice showed an absent response to DK-PGD2, confirming receptor specificity.

Next, BAL neutrophils were exposed to the CRTH2 inhibitor, fevipiprant, to evaluate its effects on baseline ROS production, which is substantial in these highly activated

extravasated cells. Strikingly, fevipiprant suppressed ROS at an IC_{50} of only 91 fM, well below inhibitory concentrations reported in the literature (~1 nM).(27) Again, this effect was abolished in CRTH2 KO cells, demonstrating that CRTH2 specifically mediates the suppressive effects of fevipiprant. Of note, neutrophils express all of the enzymes necessary for PGD2 synthesis, including hematopoietic prostaglandin D synthase (H-PGDS), and have been shown to generate PGD2 in vitro in the 10 pM range.(28) Thus, CRTH2 may mediate a previously unrecognized cell-autonomous signaling loop that promotes inflammatory responses in extravasated neutrophils.

CRTH2 deficiency exacerbates lung injury in vivo

Having observed proinflammatory effects of CRTH2 agonism on neutrophils in vitro, we expected CRTH2 deficiency to ameliorate lung injury in vivo. To test this, we used a model of severe ALI in which a lethal dose of LPS was administered intratracheally and mice were sacrificed at the 12h timepoint. Surprisingly, we observed an increase in neutrophil infiltration (Fig. 3A). This was associated with exaggerated neutrophil effector responses, as shown by BAL neutrophil elastase activity (Fig. 3B) and elevated cell-free DNA – a marker of neutrophil extracellular traps (data not shown). Lung damage was also exacerbated in CRTH2-deficient mice, as demonstrated by BAL protein content (Fig. 3C), and survival was severely impaired (Fig. 3D). Histologic differences were mild, as shown in Figure 3E, consistent with the relatively modest increases in BAL neutrophilia in the CRTH2 KO mouse.

Transcriptomic analysis reveals exaggerated neutrophilic inflammation and suppression of Type 2 immunity in CRTH2 KO mice.

To understand the apparent discrepancy between the pro-neutrophilic effects of CRTH2 in vitro and its anti-inflammatory effects in vivo, we undertook bulk RNA sequencing (RNAseq) of lung tissue. As shown in Figure 4A, there were marked transcriptional differences in WT vs CRTH2 KO lungs. Consistent with our cellular and biochemical analyses (Fig. 3A-B), neutrophilic gene expression was highly upregulated (Fig. 4B). This was manifested by elevated expression of lymphocyte-derived cytokines (e.g. *il17a*), chemokines (e.g. *cxcl1*), and neutrophil effector proteins (e.g. *mmp8/9* and *camp*). We next conducted KEGG pathway mapping and GO enrichment analysis to identify candidate anti-inflammatory pathways that may be impaired in the CRTH2 KO mouse. This approach implicated decreased type 2 signaling – a finding that fits well with the established role of CRTH2 in type 2 immunity. Indeed, we observed downregulation of IL-4 and several downstream genes including *cldn2*, *muc5b*, *ptgs1* and *retnlg* – a central marker of alternatively-activated macrophages (AAM). A second AAM marker, *cxcl13*, was also decreased in CRTH2-deficient mice. To confirm the diminished expression of IL-4, we evaluated transcription in the lung and protein levels in the BAL; both were significantly decreased (Fig. 4D-E).

Discussion

The role of CRTH2 in neutrophilic inflammation has been understudied. This gap may be attributable in part to early reports demonstrating absent expression of CRTH2 on human blood neutrophils,(3) leading investigators to focus on type 2 immune cells such as eosinophils instead. However, our results indicate that CRTH2 is in fact expressed neutrophils, specifically after extravasation into the lung. Interestingly, one prior study similarly demonstrated tissue compartment-dependent expression of CRTH2; in patients with cystic fibrosis, neutrophils from blood were negative for the receptor, while sputum neutrophils stained positively.(29) Thus, CRTH2 appears to be upregulated on neutrophils during extravasation into the lung in mice and humans. Further studies will be needed to evaluate if this phenomenon is generalizable to extra-pulmonary tissues as well.

Using a functional assay for ROS production we demonstrated that CRTH2 mediates pro-inflammatory responses in extravasated lung neutrophils. The CRTH2 agonist, DK-PGD2, elicited neutrophil activation at a nanomolar EC_{50}, similar to previously reported values.(26) However, the more striking observation was that CRTH2 antagonism with fevipiprant inhibited ROS production in the femtomolar range, a rarely observed level of pharmacologic potency. Neutrophils are known to synthesize and secrete PGD2, the endogenous ligand of CRTH2, at picomolar concentrations during swarming – a patterned response in which neutrophils seal off infected areas of tissue to prevent pathogen spread.(28) In addition, DK-PGD2 has been shown to promote chemotaxis in bone marrow-derived murine neutrophils.(22) Taking this literature precedent together with our findings, we propose the existence of a cell-autonomous PGD2-CRTH2

signaling mechanism that may promote neutrophil swarming and/or recruitment into the tissue. Indeed, leukotriene B4 – another neutrophil-derived eicosanoid – is known to mediate neutrophil recruitment via autocrine signaling during inflammation.(30)

Despite the direct pro-inflammatory effects on neutrophils, CRTH2 served a predominantly anti-inflammatory role in vivo during severe ALI. Although our mechanistic studies are preliminary, they provide support for the model shown in Figure 5, which holds that CRTH2 deletion leads to impaired type 2 immune signaling. This may be mediated by Th2 cells or type 2 innate lymphoid cells (ILC2s), which are the major sources of IL-4, IL-5, and IL13. Our data specifically indicate a deficiency in IL-4, which mediates tissue protection during ALI through multiple mechanisms. First, it acts directly on neutrophils via IL-4Ra to suppress their recruitment and activation.(31) Second, it promotes epithelial repair after injury.(32) Third, it elicits differentiation of AAMs, a subset of macrophages that promotes tissue repair and resolution of inflammation.(33) Our transcriptomic data provides some evidence for diminished AAM differentiation, as expression of the core AAM marker *retnlg* (also known as FIZZ1 and RELM-α) was markedly reduced. Furthermore, a recent study demonstrated that CRTH2 deficient mice showed lower numbers of CD206^{+} AAMs following bleomycin-induced lung injury.(34, 35) Direct cellular analysis of lung macrophages in our model will be necessary to evaluate this question.

In summary, our findings indicate a dual role for CRTH2 during ALI: direct stimulation of inflammatory neutrophil responses, and indirect suppression of lung immunopathology via IL-4. They also demonstrate a second receptor mechanism through which PGD2 affords tissue protection, in addition to the known PGD2-DP1 signaling axis.(36) While

these mechanisms would argue against CRTH2 antagonism as a therapeutic strategy in ARDS, it may prove useful for milder neutrophil-driven lung diseases such as chronic obstructive pulmonary disease, cystic fibrosis, and neutrophilic asthma. Further studies in relevant animal models will be necessary to test this hypothesis.

Figure legends

Figure 1: CRTH2 is expressed on neutrophils after extravasation into the lung. Blood and BAL were collected from mice with ALI. Cells were stained for Ly6G (indicating neutrophils), F4/80 (indicating monocytes, macrophages, and eosinophils), and CRTH2. An isotype antibody for CRTH2 was used to control for non-specific binding. F4/80^{+} cells were CRTH2 positive. Neutrophils only demonstrated CRTH2 expression after extravasation into the lung.

Figure 2: CRTH2 mediates pro-inflammatory responses in lung neutrophils. 5 μg of LPS was delivered intratracheally to induce lung neutrophilia. BAL was collected and isolated neutrophils were incubated with DHR123, a fluorescent sensor for ROS. The CRTH2 agonist DK-PGD2, or the antagonist feviprant, was added and fluorescence was monitored over time. DK-PGD2 induced dose dependent increases in ROS, while fevipiprant suppressed ROS production. Both effects were abolished in CRTH2 KO neutrophils.

Figure 3: CRTH2 is protective during acute lung injury. 200 μg of LPS was delivered intratracheally to induce severe ALI. **A,** BAL WBC and neutrophil counts were significantly increased in CRTH2 KO mice; there was also a trend towards a higher percentage of neutrophils. **B,** Neutrophil elastase activity, measured using a fluorescent kinetic assay, was higher in the BAL of CRTH2 KO mice. **C,** BAL total protein, measured via BCA assay, was elevated in CRTH2 KO mice. **D,** Survival was impaired in CRTH2 KO mice. **E,** Histopathologic injury was mildly increased in CRTH2 KO mice.

Figure 4: Transcriptomic analysis suggests that Il-4 may mediate the protective effect of CRTH2 during acute lung injury. 200 μg of LPS was delivered intratracheally to induce severe ALI and bulk RNAseq was performed on lung tissue. **A,** A heat map of differentially expressed genes indicates transcriptional changes in CRTH2 KO mice. Genes associated with neutrophilic inflammation were significantly upregulated in CRTH2 KO mice **(B)**, while genes associated with type 2 immunity were downregulated **(C)**. Differences were statistically significant, except where indicated by Ψ ($p = 0.06$) and φ ($p = 0.10$). CRTH2 KO mice showed decreased lung expression of IL-4 at the RNA **(D)** and protein level **(E)**.

Figure 5: Proposed model describing the dual role of CRTH2 in acute lung injury. CRTH2 expressed on extravasated neutrophils mediates pro-inflammatory responses. These may be augmented through an autocrine PGD2-CRTH2 signaling mechanism. Histotoxic neutrophil effectors contribute to lung injury (i.e. epithelial damage, exudative edema, etc). CRTH2 expressed on other cell types – possibly Th2 and ILC2 cells – mediates IL-4 expression. IL-4 suppresses neutrophil activation, promotes tissue protective epithelial responses, and elicits AAM differentiation, which together result in diminished lung injury.

Figure 1: CRTH2 is expressed on neutrophils after extravasation into the lung.

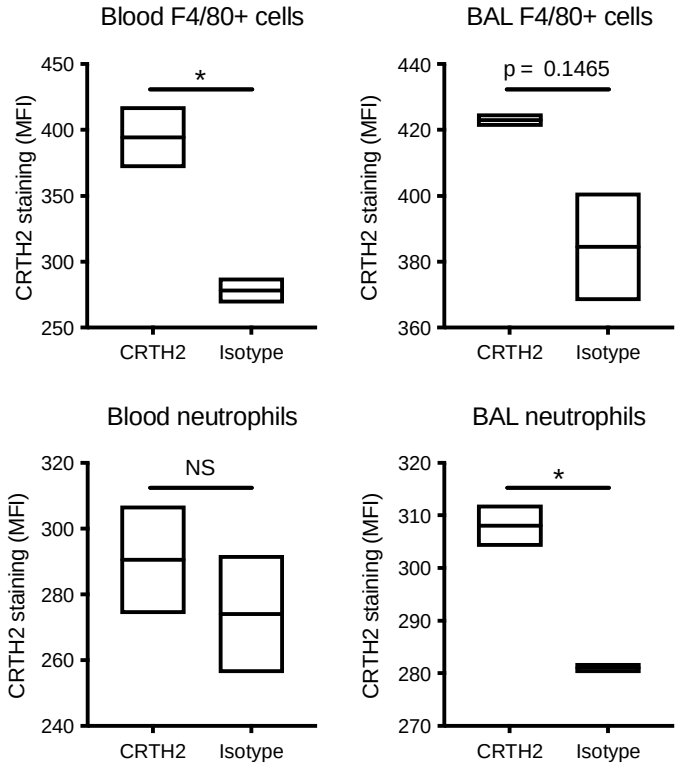


Figure 2: CRTH2 mediates pro-inflammatory responses in lung neutrophils.

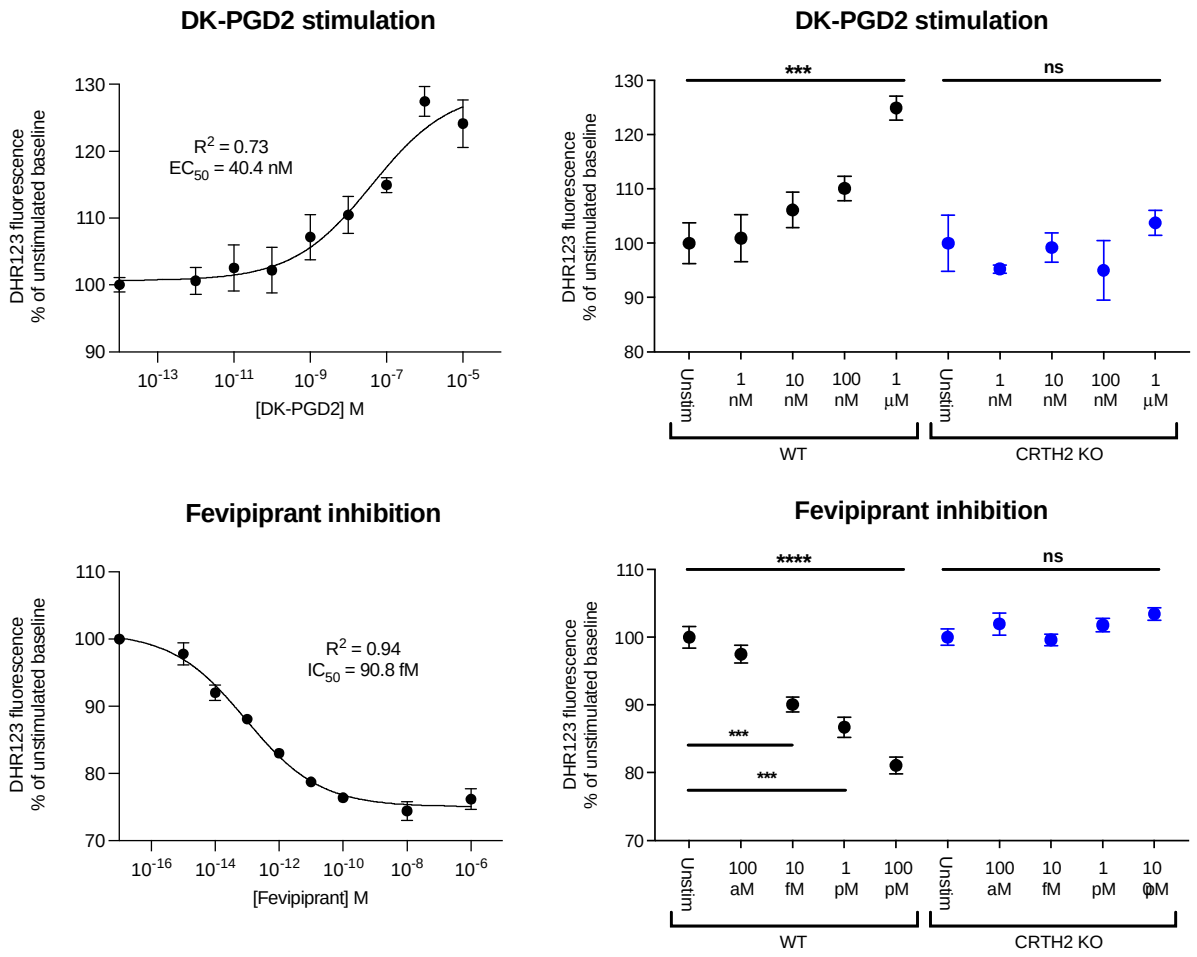


Figure 3: CRTH2 is protective during acute lung injury.

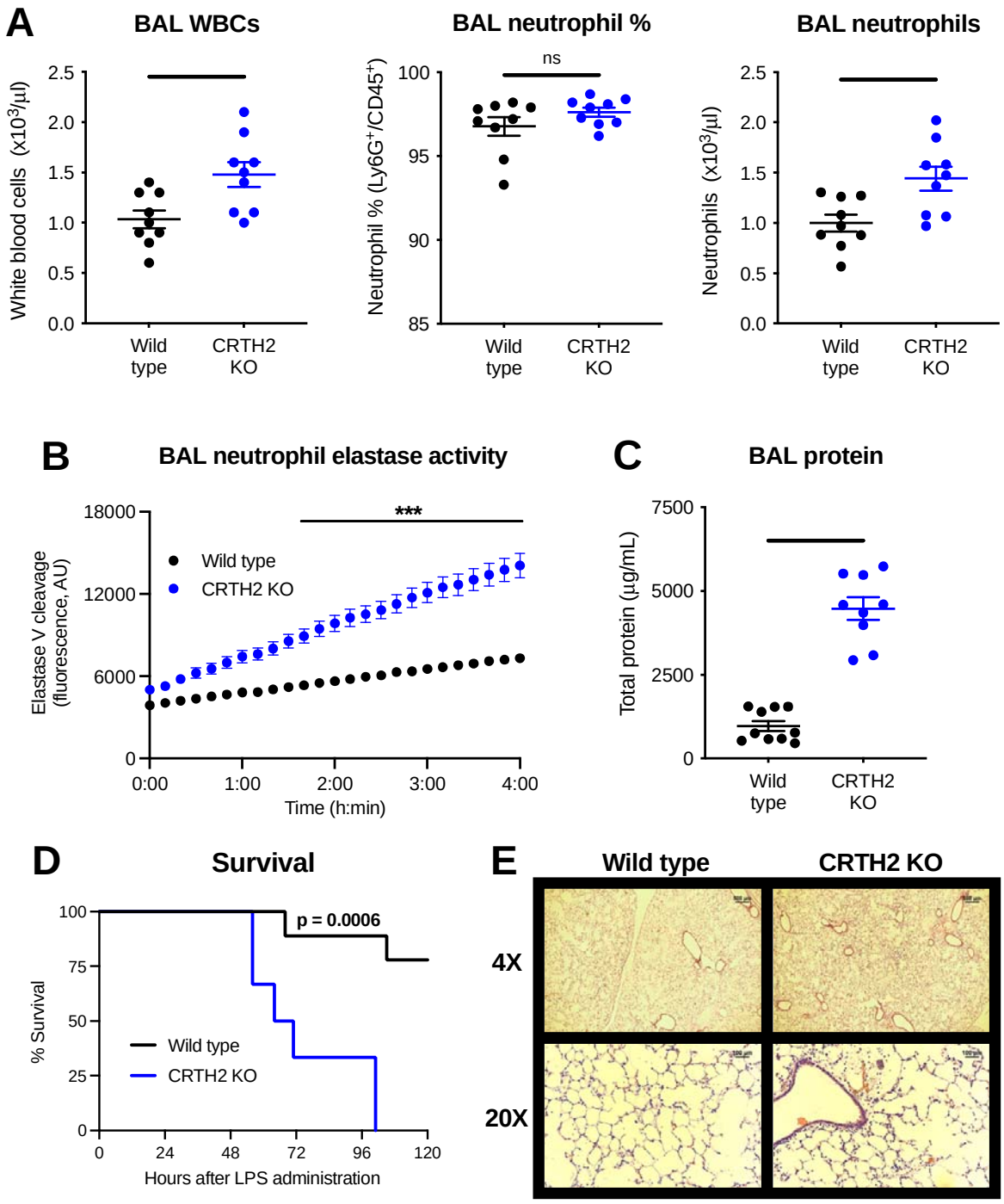


Figure 4: Transcriptomic analysis suggests that Il-4 may mediate the protective effect of CRTH2 during acute lung injury.

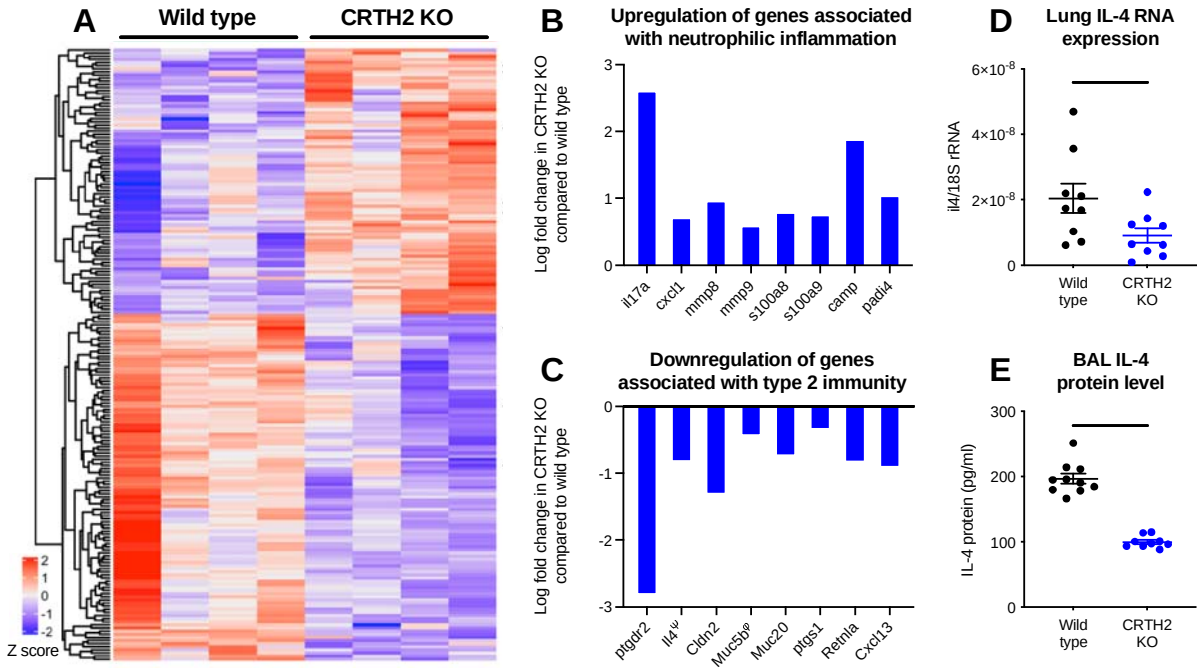
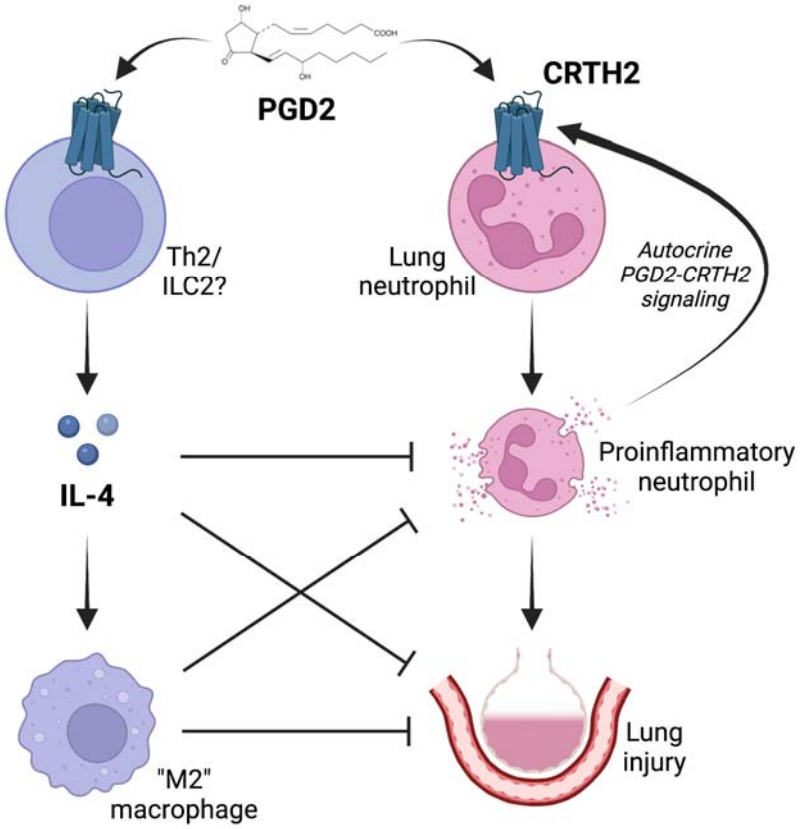


Figure 5: Proposed model describing the dual role of CRTH2 in acute lung injury.



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