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NLRC4 Inflammasome-Mediated Regulation of Eosinophilic Functions

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
Alum, aluminum hydroxide; ASC, apoptosis-associated speck-like protein containing a

ABSTRACT

Eosinophils play critical roles in the maintenance of homeostasis in innate and adaptive immunity. Although primarily known for their roles in parasitic infections and the development of Th2 cell responses, eosinophils also play complex roles in other immune responses ranging from anti-inflammation to defense against viral and bacterial infections. However, the contributions of pattern recognition receptors in general, and NOD-like receptors (NLRs) in particular, to eosinophil involvement in these immune responses remain relatively underappreciated. Our *in vivo* studies demonstrated that NLRC4 deficient mice had a decreased number of eosinophils and impaired Th2 responses after induction of an allergic airway disease model. Our *in vitro* data, utilizing human eosinophilic EoL-1 cells, suggested that TLR2 induction markedly induced pro-inflammatory responses and inflammasome forming NLRC4 and NLRP3. Moreover, activation by their specific ligands resulted in caspase-1 cleavage and mature IL-1β secretion. Interestingly, Th2 responses such as secretion of IL-5 and IL-13 decreased after transfection of EoL-1 cells with short interfering RNAs targeting human NLRC4. Specific induction of NLRC4 with PAM3CSK4 and flagellin upregulated the expression of IL-5 receptor and expression of Fc epsilon receptors (FcεR1α, FcεR2). Strikingly, activation of the NLRC4 inflammasome also promoted expression of the costimulatory receptor CD80 as well as expression of immunoregulatory receptors PD-L1 and Siglec-8. Concomitant with NLRC4 upregulation, we found an increase in expression and activation of matrix metalloproteinase (MMP)-9, but not MMP-2. Collectively, our results present new potential roles of NLRC4 in mediating a variety of eosinopilic functions.

Keywords: Eosinophil; Pattern recognition receptor; NLR proteins; Inflammasome

INTRODUCTION

Eosinophils, along with mast cells and basophils, play critical roles in Th2 responses by coordinating immune reactions to extracellular parasitic infections and by mediating allergic responses (1). Eosinophils are myeloid in origin, develop in the bone marrow and later migrate into the bloodstream (2). Although they constitute only a small portion of white blood cells, they have potent biological characteristics which activate extensive and destructive physiological and biological processes (3). Eosinophils are armed with granules containing chemokines and cytokines, metalloproteinases, parasite killing cationic proteins,

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CARD; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; co-IP, co-immunoprecipitation; Ct, cycle threshold; DC/Macs, dendritic cells/macrophages; EAD, eosinophil-associated diseases; ECL, electrochemiluminescence; EDN, eosinophil-derived neurotoxin; FLA, flagellin; FSC-A, forward scatter area; FSC-H, forward scatter height; HPRT, hypoxanthine guanine phosphoribosyl transferase; hrIL, human recombinant IL; IL-5Rα, IL-5 receptor alpha; IP, intraperitoneally; LN, lymph node; MMP, matrix metalloproteinase; NAIP, NLR family apoptosis inhibitory protein; NLR, NOD-like receptor; ns, not significant; Ova, ovalbumin; PAMP, pathogen-associated molecular pattern; PRG2, proteoglycan 2; PRR, pattern recognition receptor; RNAi, RNA interference; siRNA, short interfering RNA; SSC-H, side scatter height.

Author Contributions

Conceptualization: Ciraci C; Data curation: Akkaya I, Durmus L, Ciraci C; Formal analysis: Akkaya I, Ciraci C; Funding acquisition: Ciraci C; Investigation: Ciraci C; Methodology: Akkaya I, Oylumlu E, Ozel I, Uzel G, Ciraci C; Project administration: Ciraci C; Resources: Ciraci C; Supervision: Ciraci C; Validation: Oylumlu E, Ozel I, Uzel G; Writing - original draft: Akkaya I, Ciraci C; Writing - review & editing: Ciraci C.

neurotoxins and reactive oxygen species. These granules can be released in a rapid and direct manner when exposed to stimulants (2,4). Eosinophil activation is deeply intertwined with adaptive immunity and particularly with Th2 responses (2,5). IL-4, IL-5, IL-13, IL-25 and IL-33 which are secreted by Th2 cells play critical roles in the maturation, activation and proliferation of eosinophils. Eosinophils also produce these cytokines which further drive Th2 activation (2). Notably, IL-5 is also required for eosinophil maturation and induces the expression of IL-4, IL-6, IL-13 in eosinophil precursors (6). Recent studies show that eosinophils exhibit additional regulatory roles mediated by secretion of IL-1β (7). Sugawara et al. also demonstrated that small intestine resident eosinophils negatively regulate Th17 activation by secreting an IL-1 receptor antagonist under steady state conditions in mice, a process that occurs through GM-CSF receptor signaling (8). Furthermore, several studies have described the association between eosinophils and immune defenses against bacterial, fungal and respiratory viral infections. (9-11).

NOD-like receptors (NLRs) are cytosolic pattern recognition receptors (PRRs) that are activated by either pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (12). A number of NLRs (NLRP1, NLRP3, NLRC4, and NLRP6) form large multimeric protein complexes called inflammasomes that initiate the cleavage and secretion of IL-1β and IL-18 by auto-activated caspase-1 (13). NLRC4, together with NLRP3, are the most studied and well elucidated inflammasome forming NLRs. These 2 NLRs are reportedly activated by gram-negative bacteria such as *Salmonella typhimurium*, *Legionella pneumophila* and *Pseudomonas aeruginosa* (14). Despite having a leucine rich repeat domain, NLRC4 generally requires recognition of bacterial proteins by the upstream NLR family apoptosis inhibitory protein (NAIP) receptor for subsequent inflammasome activation (15). NLRC4 can bind to adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) to interact with pro-caspase-1 and ASC is required for cytokine processing (16).

Eosinophils recognize pathogens primarily through their pattern recognition receptors, especially membrane bound TLRs (17). Certain nematodes have been shown to have a link with bacterial symbionts and their PAMPs can be recognized by TLR2 and TLR4 (18). Additionally, the polysaccharide chitin and secreted molecules of various nematodes are known ligands of TLR2 and TLR4 (19). Apart from bacteria and nematodes, TLR7 in human eosinophils was upregulated upon parainfluenza infection or treatment with TLR7 ligand (9). In contrast, the expression, function and roles of NLRs in shaping eosinophilic functions are poorly understood and largely unknown. Here, we report that NLRC4-deficient mice have significatly fewer eosinophils in the bronchoalveolar lavage fluid (BALF) as compared to wild-type mice following induction of allergic airway disease. We also demonstrate that NLRP3 and NLRC4 are expressed in human EoL-1 eosinophils. Futhermore, we show that NLRC4 expression is induced by PAM3CSK4 and flagellin (FLA) and that this induction, in turn, results in the secretion of IL-1β by human EoL-1 cells. PAM3CSK4 and FLA also induce the expression of proteins involved in eosinophilic responses such as matrix metalloproteinase (MMP)-9.

MATERIALS AND METHODS

Mice

Nlrc4$^{-/-}$ mice have been described previously (20). Mice were backcrossed 10 generations onto a C57BL/6N background. Age and sex matched C57BL/6N mice were purchased from the National Cancer Institute. This study was carried out in accordance with the

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recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Institutional Animal Care and Use Committee at the University of Iowa approved all protocols used in this study.

Induction and evaluation of airway inflammation

Mice were intraperitoneally (IP) sensitized on day 0 with either 2 mg alum (Thermo Fisher Scientific, Waltham, MA, USA) and 20 mg ovalbumin (Ova) or 2 mg alum and PBS. Mice were intranasally challenged with 20 mg Ova in 50 ml PBS on days 15, 16 and 17. Lymph nodes (LNs), lungs, blood, and BALF were harvested on day 19. Bronchoalveolar lavage (BAL) was performed by delivering 1 ml PBS into the airway via tracheal cannula and aspirating the fluid. The lavage was repeated 3 times. Red blood cells were lysed and the cells were stained with trypan blue to determine viability, and total cell counts were calculated using a hemocytometer. Cytospin slides were prepared by H&E staining with HEMA3 (Thermo Fisher Scientific) and numbers of neutrophils, lymphocytes, dendritic cells/macrophages (DC/Macs), and eosinophils were counted. Serum samples were collected on day 19 and Ova-specific IgG1, IgG2c, and total IgE Abs were analysed by ELISA. Siglec F^+ cells were prepared by positive selection from lungs of wild type or $NLRC4^{-/-}$ mice using Siglec F Miltenyi beads per the manufacturer's instructions (Miltenyi Biotec, San Diego, CA, USA). Total 5×10^6 cells were then transferred into $NLRC4^{-/-}$ mice via tail vein injection on day 15 after mice were immunized with aluminum hydroxide (Alum)/Ova or Alum/PBS as described above.

Cell culture and stimulation

The EoL-1 cell line was used as a model cell line. The cells were cultured in RPMI 1640 medium (PAN-Biotech GmbH, Aidenbach, Germany) supplemented with 10% heat-inactivated newborn calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES and incubated at 37°C and 5% CO_2. To assess the impact of Th2 responses on eosinophilic functions in EoL-1 cells, cells (1.6×10^6/ml) were treated with Th2 cytokines with recombinant human IL-5 (50 ng/ml) or IL-13 (50 ng/ml) separately or co-incubated with IL-5 and IL-13. Cells were lysed 24 h after the treatment. Cells were treated with biologically active recombinant human IL-1β (R&D Systems, Minneapolis, MN, USA) at different doses (100 pg/ml, 1 ng/ml, and 10 ng/ml) for 24 h. IgE stimulation was performed by treating EoL-1 cells with human native IgE (Abcam, Cambridge, UK) at different doses (0.5, 1, 5, 10 μg/ml) for 24 h. To activate inflammasome assembly, cells were stimulated with TLR2 ligand PAM3CSK4 (1 μg/ml; Invivogen, San Diego, CA, USA) as the first signal and treated with ATP (50 mM) for 30 min, cell media were replaced and incubated for another 6 h.

FLA transfection

Cells were transfected with FLA at 4 h post PAM3CSK4 stimulation using lipofectamine 2000 in antibiotic free medium (21). FLA (100 ng/ml; Invivogen) and lipofectamine were incubated for 20 min at room temperature and cells were treated with the mix. Cells were lysed 10 h or 24h after PAM3CSK4 stimulation.

NLRC4 knockdown

NLRC4 short interfering RNA (siRNA) transfection of EoL-1 cells was performed as described previously (22-24). Three siRNAs targeted towards NLRC4 (Cohesion Biosciences, London, UK) were used. Transfection reagent Lipofectamine RNAiMAX (Thermo Fisher Fisher) was utilized in siRNA transfections and siRNAs were diluted in 250 μl Opti-MEM (Gibco, Waltham, MA, USA). A universal negative control siRNA (Stealth RNAi™ siRNA Negative

Control Lo GC; Invitrogen, Waltham, MA, USA), which is not homologous to anything in the vertebrate transcriptome was used to normalize relative gene inhibition of the target gene. Transfection efficiency was evaluated under a fluorescent microscope, using BLOCK-iT™ Alexa Fluor® Red Fluorescent Control (Thermo Fisher Fisher) at 24 postransfection.

BCA protein assay

Thermo Scientific Pierce BCA Protein Assay Kit was used to determine the protein concentrations of lysed cells. Cells were lysed in RIPA buffer. Lysates were precleared by centrifugation. Supernatants were used as protein samples. The absorbance of the standards and unkown samples were measured by using a spectrometer at 562 nm wavelength.

Immunoblotting and co-immunoprecipitation (co-IP)

Lysates (20–50 μg) were prepared in Laemmli buffer containing SDS and denatured at 95°C for 10 min. Electrophoresis was carried out using SDS-PAGE in 10%–12% (w/v) polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). For protein detection, anti-ASC (Enzo Life Sciences, Farmingdale, NY, USA), anti-NAIP (Abcam), anti-NLRC4 (BioLegend, San Diego, CA, USA), anti-NLRP3 (CST, Danvers, MA, USA), anti-caspase-1 (Abcam), anti-IL-1β (CST), anti-FcεR1α (Thermo Fisher Scientific), anti-FcεR2 (St John's Laboratory, London, UK), anti-MMP2 (CST), anti-MMP9 (CST) Abs were used (anti-GAPDH [CST], anti-B-actin [CST], and anti-vinculin [CST] Abs were used as housekeeping proteins). The membrane was visualized after incubating with HRP-conjugated anti-rabbit and anti-mouse IgG Abs (CST) by electrochemiluminescence (ECL; Roche, Mannheim, Germany) using the ChemiDoc XRS+System (Bio-Rad Laboratories). Band intensity was quantified using Image Lab Software (Bio-Rad Laboratories).

EoL-1 cell lysates and anti-human ASC (Enzo Life Sciences) Ab and isotype control (IgG2b, Sigma-Aldrich, St. Louis, MO, USA) were incubated by rotating overnight at 4°C. The Pierce™ Protein A/G Magnetic Beads (Thermo Fisher Scientific) were then added to the Ag/Ab mixture and incubated at room temperature for 1 h with rotation. The precipitated proteins were detected by immunoblotting with anti-human NLRC4 (BioLegend). The following co-IP protocol was performed as described in (24). Samples from the same cell lysates were analyzed separately because of different enhanced chemiluminescence sensitivities.

Flow cytometry

Cell staining with PE anti-human Siglec-8 (BioLegend), PE anti-human CD (CD80) (BioLegend), APC anti-human FcεR1α (BioLegend), APC anti-human CD86 (BioLegend), APC anti-human CD23 (BioLegend), APC anti-human CD63 (BioLegend), PE anti-human PDL1 (BioLegend), PE anti-human IL-5 receptor alpha (IL-5Rα; R&D Systems) were assessed by flow cytometry on an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and data analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA).

Cytokine measurement

ELISA was performed to determine cytokine measurement in supernatants from EoL-1 cells. Nunc MaxiSorp 96 well plates (Thermo Fisher Scientific) were coated with purified capture anti-human Abs specific for the Ag of interest diluted in PBS (1:250) and incubated overnight at 4°C. The wells were washed with PBS-T 3 times and blocked with 10% blocking solution (10% FBS containing PBS) for 1 h at room temperature. Biotin conjugated anti-human Abs for the Ag of interest were diluted in blocking solution (1:250) and incubated for 1–2 h at room

temperature. HRP Avidin D diluted in blocking solution (1:2,000) was added to the wells and incubated for 30 min at room temperature. A 1:1 mixture of TMB Peroxidase Substrate and TMB Peroxidase Substrate Solution B mixture was added to each sample and after the color change was observed, the reaction was stopped by adding 1N HCl. The absorbance was measured at 450 nm with the spectrometer. Data were analyzed using GraphPad Prism 6. Purified anti-human IL-1β, IL-5, IL-6, IL-13 (BioLegend) and biotin-conjugated anti-human IL-1β, IL-5, IL-6, IL-13 (BioLegend) Abs and MMP-9 human ELISA kit (BioLegend) were used for this study.

Real-time RT-PCR

Total RNA was isolated from samples (3 wells from 24 well plates, 3 replicates per each treatment) using RNAquous© (Ambion, Austin, TX, USA) according to manufacturer's instructions. All RNA samples were DNase treated with DNA-Free (Ambion) according to manufacturer's instructions before quantitative PCR.

MMP-9 primers: (F 5′-TTCTCCAGAAGCAACTGTCC-3′, R 5′-TAGGTGATGTTGTGGTGGTG-3′), MMP-2 primers: (F 5′-CCGTGTTTGCCATCTGTTTTAG-3′, R 5′-AGGTTCTCTTGCTGTTTACTT TGGA-3′), hNLRC4 set 1 primers (variant 1, 2, 3): (F 5′-GTGTTCTCCCACAAGTTTGA-3′, R 5′-AGT AACCATTCCCCTTGGTC-3′), hNLRC4 set 2 primers (variant 4): (F 5′-AAGATGAATGAAGAAGAT GCTATAA-3′, R 5′-ATCAAGAATGCTCAGTTTGACC-3′), hNAIP primers: (F 5′-CTGGATAAGTTC CTGTGCCTG-3′, R 5′-AGGATCATACTCAGCTGAAATTTGG-3′), Siglec-8 primers (F 5′-CTGCAG GAAGAAATCGGCA-3′, R 5′-ATGCTCGGTGTGGAGAAGC-3′), proteoglycan 2 (PRG2) primers (F 5′-AAACTCCCCTTACTTCTGGCT-3′, R 5′-GCAGCGTCTTAGCACCCAA-3′) (25), eosinophil-derived neurotoxin (EDN) primers (F 5′-AGATCAACGACGAGACCCTC-3′, R 5′-GCTGAAGGGGT ATGGAGACT-3′), hypoxanthine guanine phosphoribosyl transferase (HPRT) 1 primers (F 5′-GAC CAGTCAACAGGGGACAT-3′, R 5′-AACACTTCGTGGGGTCCTTTTC-3′). Each RT-PCR reaction was performed as previously described (23,24). The mRNA levels for the target gene corrected to those for the housekeeping gene (HPRT) were calculated by subtracting their corresponding cycle threshold (Ct) before and after stimulation using the following formula:

$$\text{Before stimulation, } \Delta Ct_{control} = Ct_{\text{target gene control}} - Ct_{\text{HPRT control}} \quad (1)$$

$$\text{After stimulation, } \Delta Ct_{stimulated} = Ct_{\text{target gene stimulated}} - Ct_{\text{HPRT stimulated}} \quad (2)$$

The fold change in mRNA was determined by: Fold change $2^{Ct(\text{stimulated})-Ct(\text{control})}$. Experiments were performed at least twice, and one representative experiment is depicted. Results were expressed as fold-change in expression of stimulated cells relative to non-stimulated cells.

Zymography

MMP-9 and MMP-2 secretion and activity were investigated by zymogram analysis. Cell supernatants were electrophoresed into 7.5% (w/v) polyacrylamide gel copolymerized with gelatin (3 mg/ml). Supernatants were electrophoresed under nonreducing conditions. After electrophoresis, gels were incubated 2 times for 30 min in Triton X-100 at room temperature and then incubated in zymogram activation solution for 2–4 nights at 37°C. After incubation, gels were stained with Coomassie staining solution for 2 h and visualized using the ChemiDoc XRS+System (Bio-Rad Laboratories). Band intensity was quantified using Image Lab Software (Bio-Rad Laboratories).

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Statistical analysis

Statistics were performed using unpaired Student's 2-tailed t-test or 2-way ANOVA. We performed a nonparametric Mann-Whitney *U* test for BAL counts and serum Ab levels.

RESULTS

NLRC4 is expressed and inducible in EoL-1 eosinophils

The human eosinophilic leukemia cell line (EoL-1) has been employed as a model cell line for studying eosinophilic functions (25). The similarities and comparability of this cell line with human primary eosinophils have already been established (26). Even though NLRC4 is expressed in multiple innate immune cells, its functional role in eosinophils is largely unknown. Quantitative analysis of gene expression and immunoblotting experiments revealed the basal expression of the inflammasome forming molecules NLRC4 and NLRP3 in EoL-1 cells (**Fig. 1**). Unlike mice, the human *NLRC4* gene has 4 isoforms; therefore, we utilized 2 primer sets to enable detection of all *NLRC4* isomers (**Fig. 1A**). Additionally, we showed that NAIP, ASC and caspase-1, the other members of the NLRC4 inflammasome, are expressed in EoL-1 cells (**Fig. 1B and C**). Based on the two-signal model (27-29), we utilized PAM3CSK4 as the priming signal (signal 1) and FLA from *S. typhimirium* as the activation signal (signal 2) for NLRC4; PAM3CSK4 (signal 1) and ATP (signal 2) were utilized for NLRP3. Stimulation of EoL-1 cells with PAM3CSK4 and FLA led to the upregulation of NLRC4. We then performed several experiments to determine if this upregulation of NLRC4 led to formation of functional NLRC4 inflammasomes and recognition of bacterial molecular patterns (**Fig. 1B**) (30,31). Although, we did not detect any change in NLRP3 and ASC protein expression by immunoblotting, IL-1β and active caspase-1 protein levels were increased after EoL-1 cells were stimulated with PAM3CSK3 and ATP (**Fig. 1C**). Furthermore, we measured a significant increase in the production of extracellular IL-1β, but not IL-6 cytokines by ELISA after treating EoL-1 cells with either NLRC4 or NLRP3 ligands (signals 1 and 2) (**Fig. 1D**), suggesting that NLRC4 and NLRP3 inflammasomes can be activated and are functional in EoL-1 cell line (**Fig. 1D**).

Additionally, we confirmed the inflammasome formation by examining the interaction between NLRC4 and ASC adaptor protein by co-IP and detected that endogenous NLRC4 protein precipitated with the ASC after treated with NLRC4 ligands but did not interact with ASC in the non-treated cells (**Fig. 1E**). Although, IL-1β levels in EoL-1 eosinophils are low, it is biologically relevant and data are consistent with the current literature (7,8). In contrast, IL-33, an important mediator of Th2 responses and eosinophil activation (32), was not secreted by EoL-1 cells at basal levels or upon stimulation (data not shown). To verify that NLRC4 activation was indeed the reason for the IL-1β secretion upon FLA transfection, NLRC4 mRNA expression was downregulated by RNA interference (RNAi) technology. Human EoL-1 eosinophils transfected with multiple NLRC4-target siRNAs exhibited 45% decrease in NLRC4 mRNA expression by quantitative PCR (**Fig. 1E**), and this partial NLRC4 knockdown was sufficient to ablate IL-1β secretion, further confirming the NLRC4's role in IL-1β secretion in EoL-1 cells (**Fig. 1F**). Since bacterial FLA is a cognate ligand of TLR5 (33), we included extracellular FLA in the experimental groups as well to examine whether the cytokine production is via NLRC4. In this case, EoL-1 cells were stimulated with FLA without transfection as TLR5 protein is a transmembrane protein which recognizes its ligand via its extracellular domain (33). Interestingly, induction through TLR5 did not affect the caspase-1 and IL-1β cleavage and secretion (**Fig. 1G**).

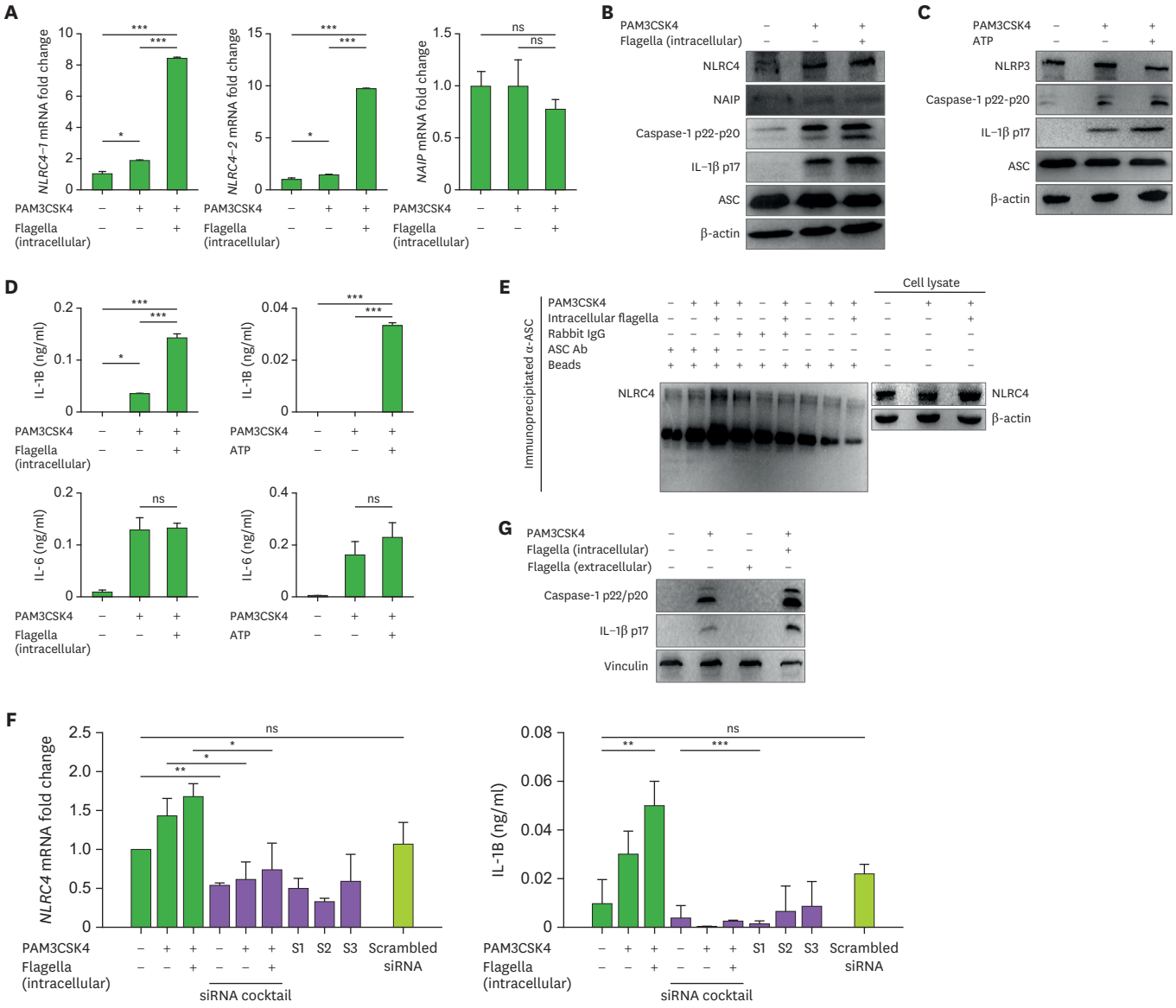

Figure 1. NLRC4 is expressed and inducible in EoL-1 cells. NLRC4 and NAIP mRNA expression in EoL-1 eosinophils treated with TLR2 ligand PAMCSK4 (1 ug/ml) as the first signal and transfected with FLA (100 ng/ml) as the second signal for NLRC4 activation (A). Values represent the mean±SD and experiments were carried out in triplicates. Student's t-test shows the significant difference between stimulated and nonstimulated cells.Immunoblot analysis of EoL-1 cells treated with PAMCSK4 (1 ug/ml) for 4 h and transfected with 100 ng/ml FLA and incubated for another 20 hours (B) or 5 mM ATP for 30 min (C); media was replaced with fresh media and cells were further incubated for another 6 h. EoL-1 cytokine secretion into culture media following PAM3CSK4, PAMCSK4+FLA or PAM3CSK4+ATP treatment was measured by ELISA (D). Cell lysates were prepared and immunoprecipitated with anti-ASC Ab, then followed by precipitation with protein-A/G-magnetic beads. Blots were probed with an anti-NLRC4 Ab and visualized using ECL. Middle panels: controls. Right lane: cell lysates were separated by SDS-PAGE and immunoblotted (E). Human EoL-1 eosinophils were transfected with scrambled siRNA or 3 different siRNAs targeting *NLRC4* (S1, S2, S3) or co-transfected with the combination of S1, S2 and S3 siRNAs. A 24 h after transfection, cells were stimulated with NLRC4 agonist and IL-1β secretion was measured by ELISA (F) (scr: scramble siRNA, siRNA cocktail= S1+S2+S3). Immunoblot analysis of cleaved caspase-1 and IL-1β in EoL-1 cells treated with PAMCSK4 and extracellular or transfected FLA (G). Values represent the mean±SD of 2 separate experiments.
*p<0.05, **p<0.01, ***p<0.001.

Th2 cytokines, IL-5 and IL-13 might be operating through NLRC4 in EoL-1 eosinophils

IL-5 is one of the key cytokines in eosinophilic functions. Considered a critical element of Th2 responses, IL-5 can be secreted by Th2 cells, mast cells, ILC2 cells and also eosinophils (34). IL-5 binds to the high affinity IL-5Rα, which triggers signaling pathways leading to a

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multiplicity of responses, from proliferation to cytotoxicity (34). Yet the relationship between NLRC4 and IL-5 signaling in eosinophilic functions remains unclear. Hence, we initially examined the IL5/NLRC4 axis *in vivo* by IP injecting wild-type and NLRC4 deficient mice with mouse recombinant IL-5, and then harvesting cells from the intraperitoneal cavities at 24 and 72 h post injection. Interestingly, the number of eosinophils recruited to the site of infection were significantly lower in NLRC4-deficient mice than in wild-type mice at both time points (**Fig. 2A**). Next, to examine the expression and inducibility of NLRC4 in human EoL-1 eosinophils *in vitro*, we treated EoL-1 cells with human recombinant IL (hrIL)-5 and hrIL-13. We then investigated the extent to which NLRC4 promoted the production of IL-1β, an inflammasome dependent cytokine. To determine the NLRC4 dependency of Th2 immune responses, we utilized siRNAs directed at the NLRC4 and transfected EoL-1 cells with these

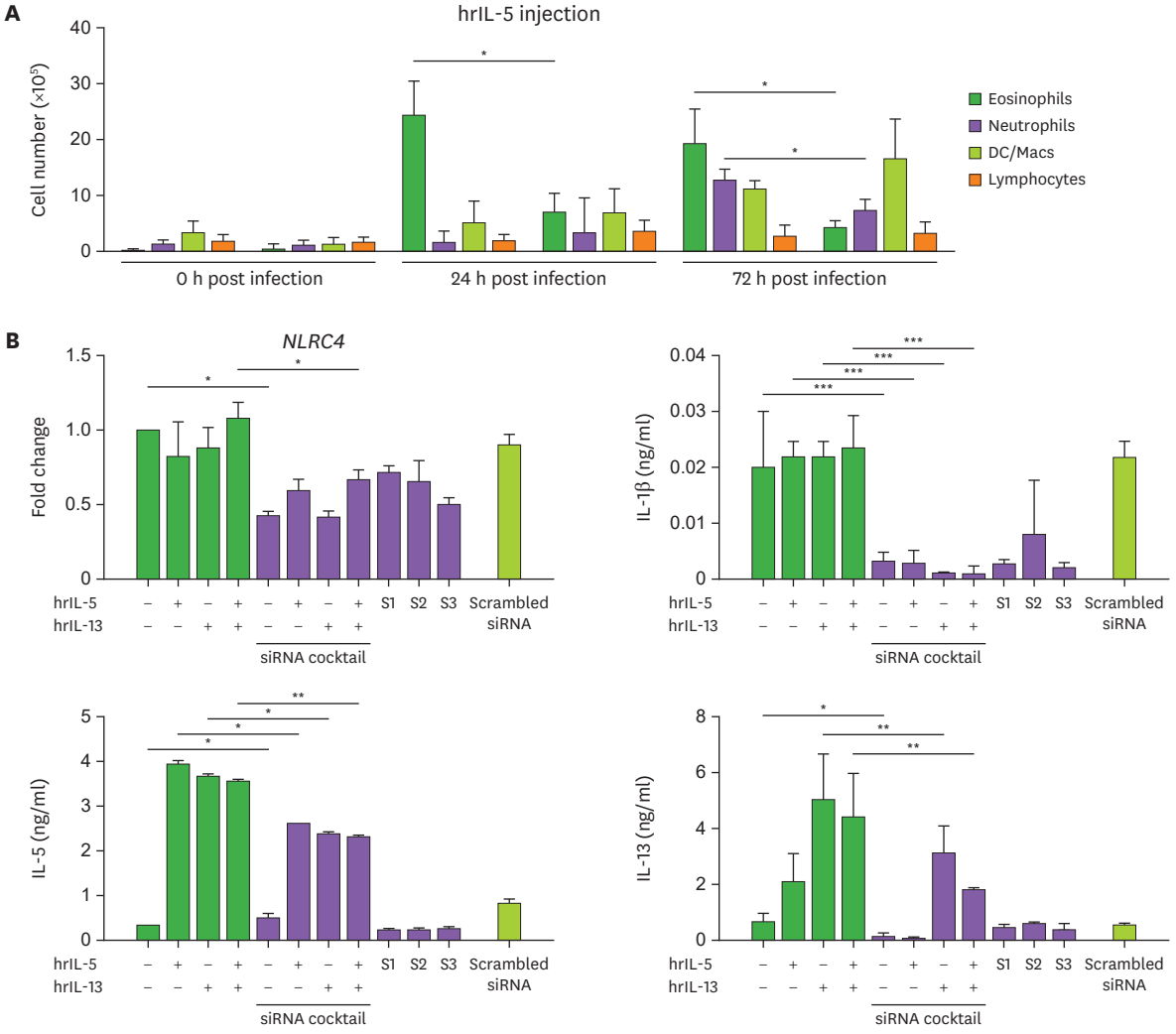

Figure 2. Th2 cytokines promote NLRC4 expression and IL-1β secretion. Wild-type mice were IP injected with mouse hrIL-5 on day 0. On day 1 and 3, 5 ml of cold PBS was delivered into the peritoneal cavity and fluid was gently aspirated from peritoneum. Numbers of neutrophils, lymphocytes, DC/Macs, and eosinophils were counted (A). Values represent the mean±SD of two separate experiments. NLRC4 mRNA and protein expression in EoL-1 eosinophils treated with either hrIL-5 or hrIL-13, or both (B). RNAi mediates downregulation of the human *NLRC4* gene. Human EoL-1 eosinophils were transfected with scrambled siRNA, 3 different siRNA targeting *NLRC4* (S1, S2, S3) or co-transfected with the combination of S1, S2 and S3 siRNAs. A 24 h after transfection, EoL-1 cells were treated with 5 ng/ml hrIL-5 or hrIL-13 or, both. Cells and supernatants were harvested following 24 h stimulation and NLRC4 mRNA was analyzed by quantitative PCR. IL-1β, IL-5 and IL-13 secretions were measured by ELISA (added concentrations of recombinant cytokines were substracted from the total concentrations of hrIL-5 and hrIL-13 in treatment groups) (C). Experiments were carried out in duplicate. Values represent the mean±SE of 3 separate experiments.
Asterisks show the significant difference between negative siRNA transfected and siRNA transfected: *p<0.05, **p<0.01, ***p<0.001.

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siRNAs. Transfection of cells with siRNAs targeting NLRC4 resulted in a 55% reduction in NLRC4 mRNA expression (**Fig. 2B**). NLRC4 knockdown diminished IL-1β, IL-5, and IL-13 secretions from EoL-1 cells induced with hrIL-5 and hrIL-13 treatment (**Fig. 2B**).

NLRC4 induction regulates eosinophilic protein expression in human EoL-1 cells

In addition to their well studied roles in defense against extracellular parasites and in the generation of Th2 responses, numerous studies have reported that eosinophils also have immunoregulatory functions in the gastrointestinal tract (8), as well as roles in the activation of Th1 and Th17 responses against bacterial, fungal and viral infections (11,35,36). Eosinophil phenotyping based on gene profiling; the role of eosinophils in tissues including intestine, respiratory track and esophagus; determination of biomarkers of eosinophil activation, (de) granulation and tissue load and a better understanding of the role of eosinophils in human diseases remain important pursuits. Given the basal expression and activation of NLRC4 in eosinophilic EoL-1 cells, we evaluated the biological processes that shape eosinophilic functions. Strikingly, NLRC4 activation by treatment with PAM3CSK4 upregulated the protein expression of both the high and low affinity IgE receptors FcεR1α and FcεR2 (**Fig. 3A-C**). The low affinity receptor FcεR2 was also upregulated by TLR signaling alone. These broad but important effects of TLRs in mediating the activation of eosinophilic cells are somewhat expected considering that TLRs are known to have roles in pathogen recognition by eosinophils (17,19). More importantly, flow cytometry analyses showed that both TLR and NLRC4 induction in EoL-1 cells resulted in increased surface expression of IL-5Rα, revealing a reciprocal molecular relationship between NLRC4 and IL-5–IL-5Rα signaling and expression (**Fig. 3B**). In contrast, NLRP3 induction had no significant effect on FcεRs and IL-5Rα expression (**Fig. 3A and B**). Here, we also included the exctracellularly recognized FLA to further verify the inflammasome dependent proteins, and concluded that not only IL-1β and caspase-1, but also FcεRs are secreted through NLRC4 inflammasome but not TLR5 signaling (**Figs. 1G** and **3C**). These data also suggest that PRRs (NLRC4 and TLR signaling) may impact the interaction between eosinophils and humoral immunity involving B lymphocytes, e.g., the role of IL-6 in aiding the survival of B-cells (37). Furthermore, NLRC4 activation upregulated the human eosinophilic marker Siglec-8 which has an anti-inflammatory function in eosinophil activation and survival (**Fig. 3D and E**) (38,39), indicating the involvement of NLRC4 in negative feedback mechanisms as well.

Eosinophils are known to have Ag presenting roles and to express the costimulatory receptors CD80 and CD86. They can also physically interact with T cells (40). The effect of pattern recognition receptors on costimulatory receptors expressed on eosinophils are not well studied. Our flow cytometry results revealed that, similar to DC/Macs (18,41,42), the surface expression of CD80 was upregulated in EoL-1 upon TLR induction (**Fig. 3F**). On one hand, this upregulation was further enhanced upon NLRC4 activation in a manner independent of NLRP3 as its induction did not alter CD80 expression (**Fig. 3F**). On the other hand, CD86 was constitutively expressed in EoL-1 cells. The expression of costimulatory molecules on human Eol-1 eosinophils suggest that these cells could function as immunoregulatory cells involved in the release of cytokines.

IL-1β might coordinate the immunoregulatory functions of EoL-1 eosinophils

To elucidate the molecular mechanisms behind CD80 upregulation by NLRC4 activation, we investigated 2 potential downstream effectors of the NLRC4 inflammasome; caspase-1 activation and IL-1β secretion. To address whether NLRC4 activation is solely responsible for eosinophils' Ag presenting cell features, we treated human EoL-1 eosinophils with hrIL-1β or conditioned media to detect the broad effects of secreted molecules and possible autocrine effects following the TLR and NLRC4 induction. Given that the immunoregulatory receptor PD-L1 has critical

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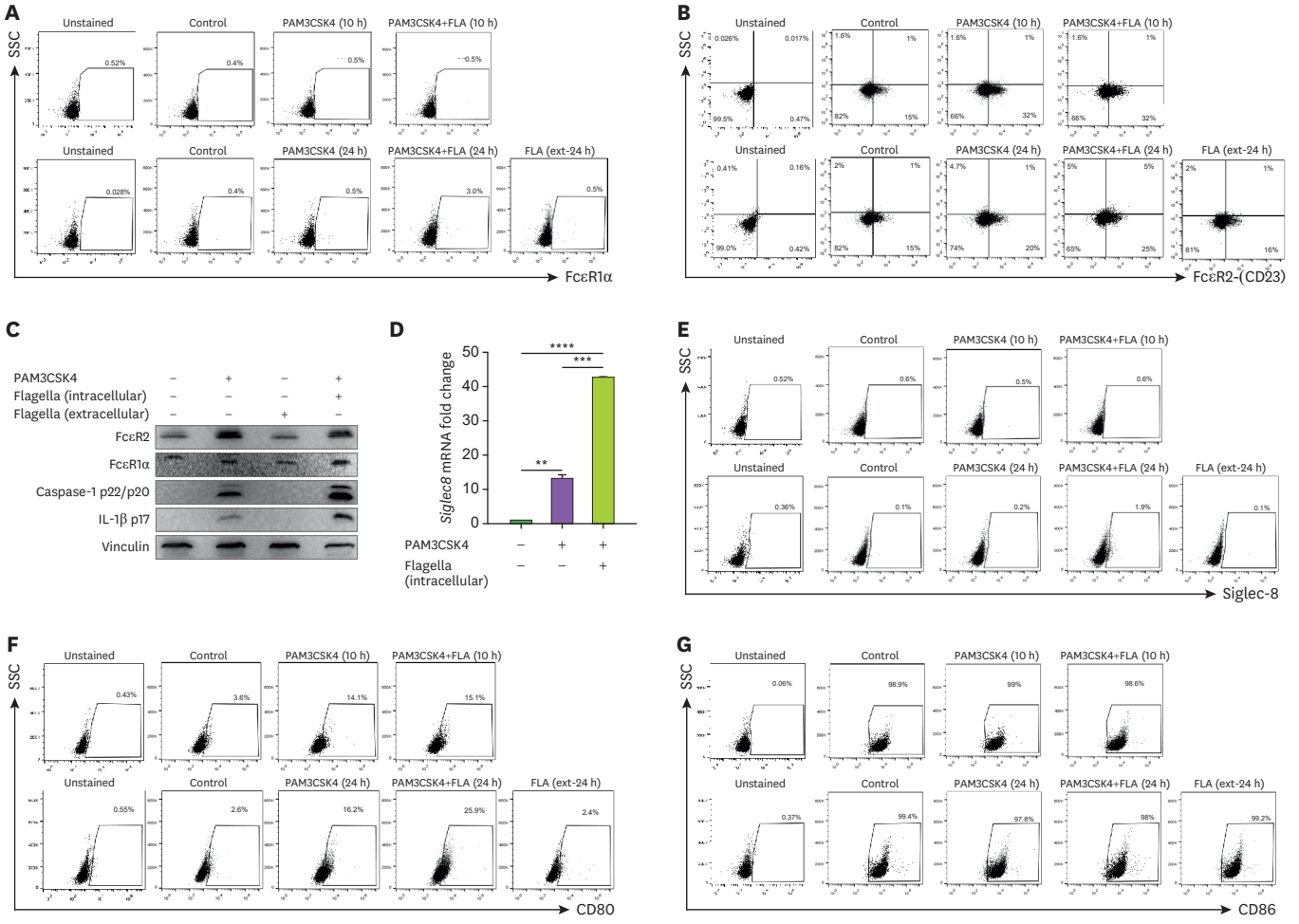

Figure 3. TLR2 and NLRC4 induction promotes a number of eosinophilic receptors. Surface expression of FcεR1α (A) and FcεR2 (B) on EoL-1 eosinophils treated with TLR2 ligand PAM3CSK4 (1 ug/ml) for 4 h, transfected with FLA (100 ng/ml) and incubated for another 20 h. After the treatment of PAM3CSK4 for 4 h, cells were also treated with 5 mM ATP for 30 min; media were replaced with fresh media and cells were further incubated for another 20 h. Cells were harvested at 24 h poststimulation and analyzed by flow cytometry. FcεR1α and FcεR2 protein expressions after treatment with PAM3CSK4, PAM3CSK4 plus FLA or FLA alone. Cell lysates were separated by SDS-PAGE, and immunoblotted (C). The mRNA expression of the eosinophilic marker Siglec-8 is on EoL cells upon NLRC4 induction by quantitative PCR (D). Values represent the mean±SD of 2 independent experiment. Surface expression of Siglec-8 (E), CD80 (F), CD86 (G) on cells treated with PAM3CSK4, PAM3CSK4, and FLA or FLA only for 24 h by flow cytometry. Eol-1 cells were gated on the basis of size and granularity using FSC-H/SSC-H by first removing debris and doublet cells using FSC-A/FSC-H. Single cells were sub-gated using FcεR1α (APC). Percentages within the gates indicate the proportion of FcεR1α (APC) expressing cells in the Eol-1 cell population (A), IL-5Rα (PE) and FcεR2 (APC) co-expressing cells (B), Siglec-8 (PE) (E), and CD80 (FITC) (F). Results are representative of three independent experiments.
ext, extracellular.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

roles in the interaction with T cells, we measured PD-L1 as well as CD80 surface expressions by flow cytometry (**Fig. 4**) (10). In parallel with CD80, PD-L1 surface expression increased upon TLR and NLRC4 induction (**Fig. 4**). Therefore, similar to increased Siglec-8 expression, upregulation of PD-L1 suggests a negative feedback mechanism which might be necessary for balancing the highly potent biological effects of eosinophils. In addition, treatment with hrIL-1β or conditioned media suppressed the effects of NLRC4 induction which might be promoting *de novo* expression of IL-1β at the time of incubation. It is possible that we did not detect a significant increase in CD80 or PD-L1 surface expressions after hrIL-1β or conditioned media treatment because the IL-1β dose and exposure time in comparison to *de novo* IL-1β expression in response to NLRC4 induction or any other cell secreted molecule in the conditioned media might have reversed the impact on the expression patterns of these receptors.

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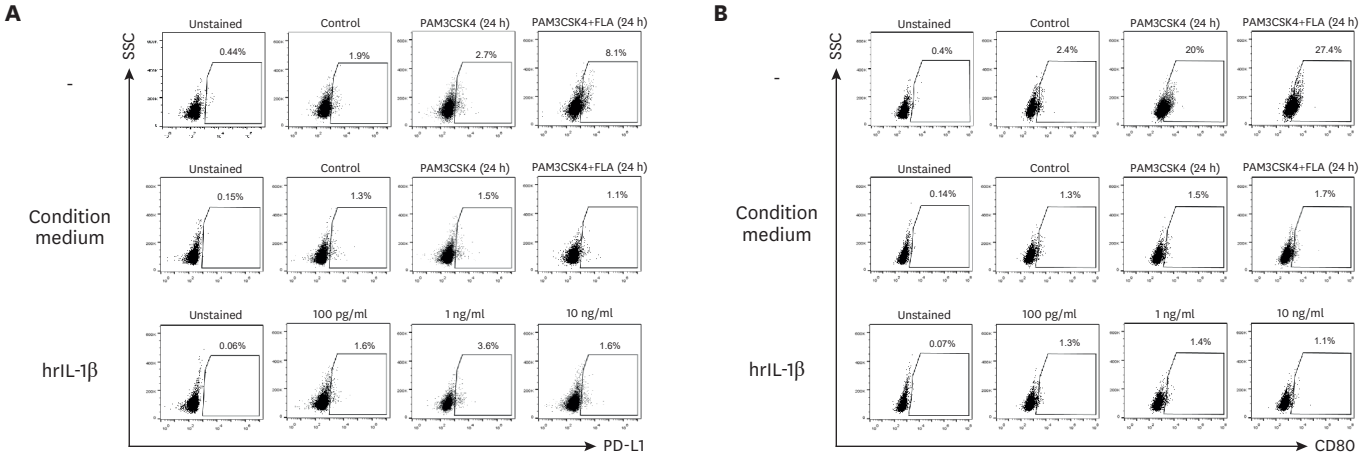

Figure 4. hrIL-1β and conditioned media suppress expression of NLRC4-induced co-stimulatiory molecules. Surface expression of PD-L1 (A) and CD80 (B) by flow cytometry. Cells were treated with TLR2 or NLRC4 agonist in the presence of conditioned media or hrIL-1β (100 pg/ml, 1 ng/ml, 10 ng/ml). Results are representative of 2 independent experiments. Eol-1 cells were initially identified on the basis of size and granularity using FSC-H/SSC-H by first removing debris and doublet cells using FSC-A/FSC-H. Single cells were sub-gated using T cell costimulatory molecule CD80 (FITC) and critical immune checkpoint PD-L1 (PE). Percentages within the gates indicate the proportion of PD-L1 (PE) and CD80 (FITC) expressing cells in the Eol-1 cell population.

NLRC4 inflammasome promotes degranulation and MMP-9 expression and secretion, but not MMP-2

CD63, a potential surface marker for eosinophil degranulation, is a transmembrane-4 glycoprotein and a member of the tetraspanin superfamily. We took advantage of this marker to investigate the degranulation of EoL-1 eosinophils following NLRC4 induction with PAM3CSK4 and FLA by using flow cytometry to measure its expression (**Fig. 5A**). Our results demonstrated that induction through TLR2 alone and NLRC4 further increased CD63 surface expression, suggesting degranulation in response to NLRC4 activation (**Fig. 5A**). Additionally, we measured the mRNA expression levels of PRG2 (aka major basic protein) and EDN (**Fig. 5A**), as these proteins are secreted during degranulation (2). MMPs are zinc dependent proteases that play various roles in tissue remodeling and degradation of extracellular matrix proteins in acute and chronic lung diseases (43-45). In addition to their roles in tissue repair-wound healing, MMPs can also play roles in angiogenesis and metastasis (46). Previous studies demonstrated that MMP-9 correlates with airway remodeling and asthma associated with eosinophils (47,48). Since MMPs have an important biological significance in eosinophilic functions associated with inflammation, tissue remodeling and homeostasis, regulation of MMPs in eosinophilic responses has been the focus of numerous studies (43,49). For these reasons, we investigated the ability of EoL-1 cells to produce and secrete active MMP-2 and MMP-9 upon stimulation with PAM3CSK4 and FLA. We also studied the production of MMP-2 and MMP-9 in EoL-1 eosinophils by Western blotting. Quantitative analysis revealed that MMP-2 and MMP-9 mRNA expression was significantly upregulated following induction of TLR alone and NLRC4 in EoL-1 cells (**Fig. 5B**). Gelatin zymography and extracellular MMP-9 measurement by ELISA also showed an increased gelatinolytic activity of MMP-9 in both TLR2 and NLRC4 stimulated EoL-1 eosinophils; however, we did not detect such a significant change in MMP-2 protein expression or in its proteolytic activity (**Fig. 5C and D**). To further verify the increase in MMP-9 gelatinolyctic activity through NLRC4 induction, we stimulated EoL-1 cells with native human IgE and Ova specific IgE to activate the cells via different pathways. Interestingly, stimulation with native IgE and immune complexes did not significantly alter MMP-9's proteolytic activity (**Fig. 5E**).

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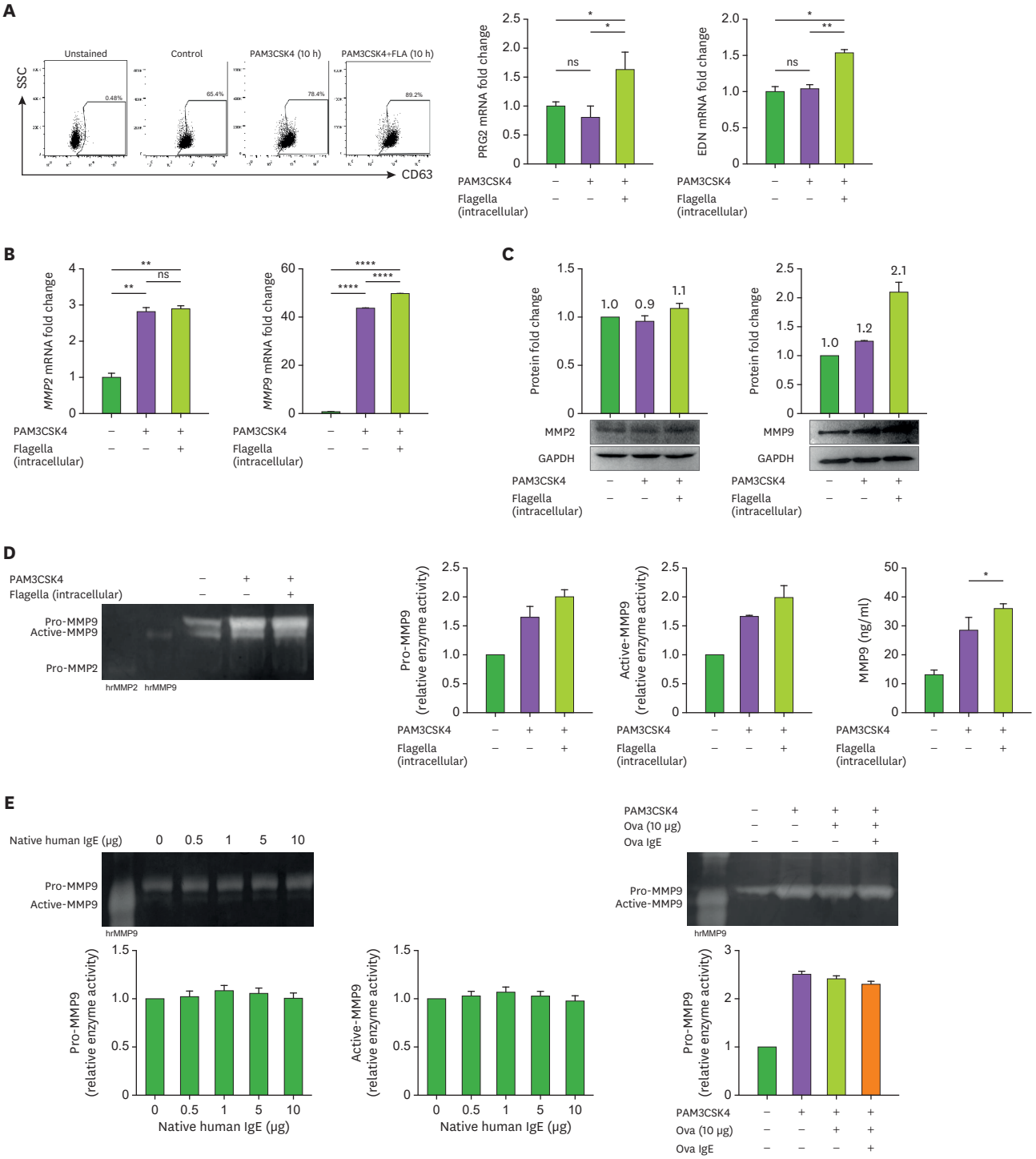

Figure 5. Degranulation in EoL-1 cells after TLR2 and NLRC4 agonist treament. Surface expression of CD63 by flow cytometry, MPB and EDN mRNA expression was measured by quantitative PCR (A). MMP-2 and MMP-9 mRNA expression was measured by quantitative PCR after treatment with either TLR2 or NLRC4 agonists (B). MMP-2 and MMP-9 protein expression after treatment with either TLR2 or NLRC4 agonists. Cell lysates were separated by SDS-PAGE, and immunoblotted (C). Pro- and active MMP-9 enzyme activity by zymogram assay and extracellularly secreted MMP-9 by ELISA (D). Cells were treated with either TLR2 or NLRC4 agonists, native human IgE (0, 0.5, 1, 5, 10 μg) or Ova specific IgE. Immune complexes were prepared at a ratio of 1:10 (Ova:anti-Ova IgE), supernatants were collected and enzyme activity was measured by Gelatin Zymography. Results are representative of at least two independent experiments. Eol-1 cells were initially gated on the basis of size and granularity using FSC-H/SSC-H by first removing debris and doublet cells using FSC-A/FSC-H. Single cells were sub-gated using CD63 (APC) and subsequently degranulated cells were discriminated by the expression of CD63 (APC). Percentages within the gates indicate the proportion of CD63 (APC) expressing cells in the Eol-1 cell population.
*p<0.05, **p<0.01, ****p<0.0001.

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NLRC4 deficiency impairs Th2 responses in a mouse model of allergic airway disease

NLRs are capable of regulating the production of cytokines from T cells through interactions with DC/Macs (12). The role of inflammasome dependent IL-1β and IL-18 in regulating Th1 and Th17 responses has been investigated utilizing several different disease models. Because these studies are mostly limited to NLRP3 inflammasomes, less is known about the roles of NLRC4 in modulating T cell responses. To addess this deficiency, we used a mouse allergic airway disease model. Mice were immunized IP with Alum, an adjuvant that induces a robust Th2 response, in the presense of either (Ova) or PBS. Mice were subsequently challenged intranasally with Ova on days 15, 16 and 17 and sacrificed on day 19. Airway inflammation was assessed by examining the immune cell composition of BALF and by restimulating T cells with Ova. Insterestingly, the number of eosinophils were markedly decreased in NLRC4-deficient mice as compared to wild-type mice (**Fig. 6A**). We did not see a significant change in the number of neutrophils, lymphocytes, or macrophages in the BALFs of NLRC4-deficient and wild-type mice (**Fig. 6A**). We also measured the Th1 (IFN-γ), Th2 (IL-4, IL-5, and IL-13) and Th17 (IL-17) cytokine responses after *ex vivo* re-stimulation of lung-draining mediastinal LNs with Ova. IL-4, IL-5, and IL-13 cytokine production in LN cells from NLRC4-deficient mice was significantly diminished when compared to wild-type mice (**Fig. 6B**). However, upon restimulation, IFN-γ and IL-17A cytokine levels were comparable in NLRC4-deficient and wild-type mice (**Fig. 6B**). As expected and consistent with the literature (50), Alum/Ova induced Th1 and Th17 responses were much weaker than Th2 respones, consistent with the literature. Furthermore, IL-4, IL-5, and IL-13 cytokines were partially and significantly recovered after wild-type or NLRC4-deficient eosinophil transfer into NLRC4-deficient mice (**Fig. 6C**). We also evaluated the extent of airway inflammation by measuring ovalbumin-specific IgG1, IgG2c and IgE immunoglobulin levels in the serum of both mice groups. IgE levels were significantly lower in NLRC4-deficient mice than wild-type mice; however, differences in IgG1 and IgG2c levels between the two mice groups were not statistically significant (**Fig. 6C**). Taken together, and consistent with decreased eosinophilic influx into the lungs of NLRC4 knockout mice injected with Alum/Ova, LN cells secreted reduced levels of Th2 cytokines upon induction of airway inflammation.

DISCUSSION

Eosinophils have critical functional roles in Th2 responses and defense against extracellular parasitic infections (51). Their potentially potent inflammatory and damaging impacts in the body are generally offset by their relatively low number and tightly regulated proliferation and activation mechanisms. Eosinophils generally recognize parasitic pathogens through PRRs, most notably TLRs (17). Nonetheless, few studies focus on the effects of PRRs on eosinophilic functions and their relationship with the Th2 responses. Moreover, their low abundance makes eosinophils more difficult to study compared to many other immune cell types. For these reasons, we used the EoL-1 model cell line to study eosinophilic characteristics. Here, we report that EoL-1 cells displayed a number of specific characteristics of human eosinophils. Together with their morphological similarities to eosinophils, EoL-1 cells express IL-5Rα, a critically important receptor for the survival and activation of eosinophils, as well as the allergy related IgE low affinity receptor FcεR2 which is likewise expressed on human eosinophils (1,52). Moreover, EoL-1 cells also express the high affinity IgE receptor FcεR1α along with Siglec-8, the eosinophilic marker reported to have anti-inflammatory effects on eosinophil activation and maturation (53,54), upon induction. Therefore, EoL-1 cells are an ideal model for the investigation of eosinophilic functions.

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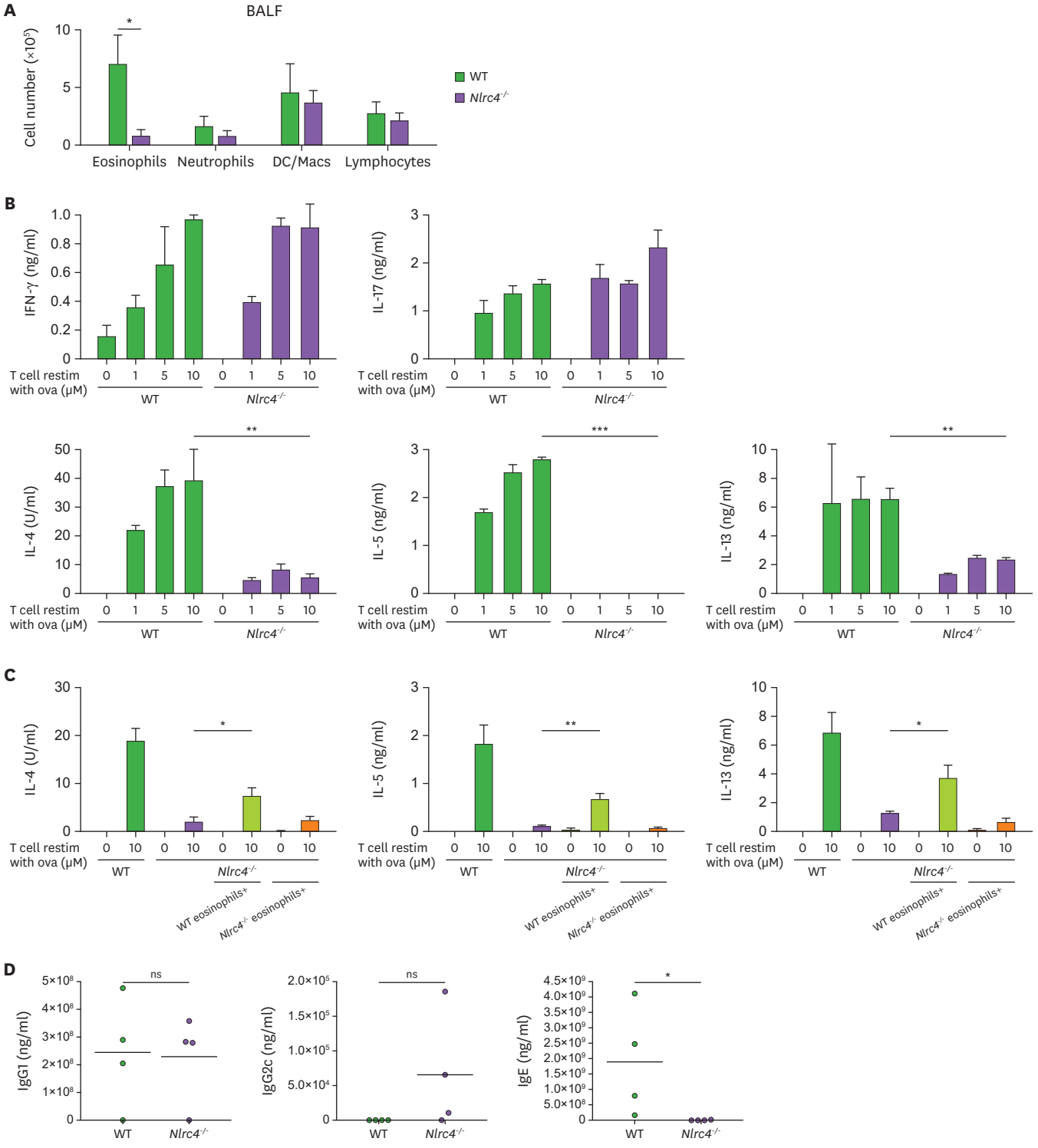

Figure 6. Th2 cytokine response is impaired in *NLRC4*$^{-/-}$ mice. Wild-type and *NLRC4*$^{-/-}$ mice were injected IP with either Alum/PBS or Alum/Ova on day 0; mice were intranasally challenged with Ova on days 15, 16 and 17. Two days after the last intranasal challenge, differential cell counts in BALF were analyzed (A). Lung draining LN were harvested and restimulated *in vitro* with or without Ova (10 μM) for 72 h and cytokine levels in the supernatants measured by ELISA. Values represent the mean±SD and are representative of three separate experiments each with a minimum of 3 mice per group (B). Eosinophils from wild-type or *NLRC4*$^{-/-}$ mice were transferred into *NLRC4*$^{-/-}$ mice on day 15 after mice were immunized with Alum/PBS or Alum/Ova, Th2 cytokines were assessed by ELISA 4 days later (C). Ova-specific IgG1 and IgG2c levels in serum were measured by ELISA. Values represent the mean±SD of three separate experiments (n=3–5 mice per group) (D).
*p<0.05, **p<0.01, ***p<0.001.

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Allergic airway inflammation is an inflammatory disease associated with the recruitment of inflammatory cells, especially eosinophils, to the pulmonary airways, along with increased levels of Th2 cytokines (particularly IL-4, IL-5, and IL-13) and occurs very early in an immune response within LNs (55). Eosinophils directly process and present Ag to $CD4^+$ T cells, thereby boosting Th2 differentiation and clonal expansion. These attributes are essential functions of eosinophils in the generation of Th2 responses as evidenced by the diminished Th2 immunity in acute and chronic mouse models of allergic airway inflammation in eosinophil deficient mice (56,57). Given that we are examining the roles of eosinophils regulated by NLRC4, we utilized the allergic airway inflammation model for its reproducibility, robustness and quantifiable readouts including BAL counts for eosinophils, serum Ab levels and LN Th2 cytokine measurements.

As previously mentioned, eosinophils mediate inflammatory responses upon pathogen recognition through PRRs, especially TLRs. However, the role of NLRs in these functional processes are largely unknown and have not attracted much attention to date. This fact motivated us to focus on NLRs. Since NLRC4 and NLRP3 are the more widely studied receptors of this family and since both are essential components of multimeric inflammasome complexes, we concentrated on these 2 receptors with a greater emphasis on NLRC4. Importantly, we demonstrated that eosinophil like EoL-1 cells not only express NLRC4 and NLRP3 proteins, but inflammasome structures are activated in these cells upon treatment with NLRC4 and NLRP3 ligands. Treatment with NLRC4 and NLRP3 ligands bacterial FLA and ATP, respectively, led to caspase-1 and IL-1β cleavage in EoL-1 cells. Interestingly, NLRC4 mRNA and protein expression are elevated upon treatment with two signals (PAM3CSK4 stimulation and FLA transfection). Together with the inflammasome activation, EoL-1 cells secreted the pro-inflammatory cytokine IL-1β. Strikingly, NLRC4 knockdown diminished IL-1β secretion, an observation that points to a direct role for NLRC4 activation in inflammasome formation and caspase-1 and IL-1β cleavage. TLR activation mediates other pro-inflammatory pathways that are inflammasome independent, such as IL-6, and as expected, IL-6 was not differentially expressed after stimulation with NLRC4 ligand. Even though EoL-1 cells have been shown to express TLR5 mRNA (data not shown) and protein, IL-1β expression was not differentially expressed when treated with FLA alone, a cognate ligand of TLR5. Despite the low levels of IL-1β and IL-6 production in this cell line, secretion of these cytokines can play roles in maintaining a balance between Th2 and Th17 responses during an infection since IL-1β and IL-6 are the mediators of Th17 activation and eosinophils are known to be the components of the Th2 response. Additionally, MMP-9 expression and secretion were also promoted upon TLR and NLRC4 induction, an additional outcome of the impact of pattern recognition receptors on regulating the potent eosinophilic responses in a broad sense. As the regulation of MMPs could be involved in eosinophil egress into the BAL (58), NLRC4 regulation of MMP-9 might have roles in BAL recruitment in a mouse model of allergic disease.

Apart from the promotion of pro-inflammatory responses, in EoL-1 cells, NLRC4 and TLR activation contributed to the development of eosinophilic functions. Induction with PAM3CSK4 stimulation and FLA transfection promoted increases in the expression of the eosinophilic marker IL-5Rα. Furthermore, the TLR activation also led to the enhancement of the allergic responses since low affinity Fc epsilon receptor FcεR2 expression levels were significantly augmented upon treatment with TLR2 ligand. Moreover, NLRC4 activation upregulated the expression of the high affinity Fc epsilon receptor FcεR1α, Siglec 8 and FcεR2 whereas NLRP3 activation or TLR5 induction had no such an impact on the expression of

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these receptors. Also, we observed a reciprocal relationship between NLRC4 and IL-5Rα. EoL-1 cells expressed higher levels of NLRC4 protein when cells were stimulated with IL-5 and the number of IL-5Rα expressing EoL-1 cells increased upon NLRC4 activation. The effect of NLRC4 activation on eosinophilic functions provides new insights to the regulation of allergic responses but also generates new questions. The reciprocal relationship between IL-5Rα and NLRC4 activation and the positive effects of TLR signaling present PRRs and NLRC4 inflammasome as new potential mediators of allergic and eosinophilic responses.

In human monocytes and macrophages, alternative inflammasome pathways have been shown to promote caspase and IL-1β cleavage and secretion through LPS stimulation alone and furthermore, this occurs through an MyD88 independent and caspase-8 dependent pathway, but not through other TLR ligands such as PAM3CSK4 (59). Data presented here show that IL-1β is cleaved into its biologically active form in EoL-1 eosinophils through TLR2 activation. The constitutive cleavage of caspase-1 or mechanisms downstream the MyD88 independent TLR pathways must also be investigated to elucidate this unique phenomenon.

The Ag presentation capability of eosinophils, mainly upon activation, has been reported (2). Moreover, any potential interaction between eosinophils and Th2 cells resulting from NLR activation and inflammasome formation is essential in the characterization of eosinophilic responses. EoL-1 cells exhibited the characteristics of Ag presenting cells by expressing the costimulatory receptors CD80 and CD86. Strikingly, almost all of the EoL-1 population expressed CD86 under steady conditions. Furthermore, as expected, CD80 expression was upregulated upon TLR activation (60) and interestingly, NLRC4 and NLRP3 activation led to an additional increase in CD80 expression, suggesting a broader role for NLRs in inflammasome activation.

Although eosinophils are one of the least abundant subpopulations of cells in blood circulation, their low number is compensated for by their production of granules with a highly potent content. Moreover, their involvement in a broad spectrum of pathological conditions such as acute and chronic infections, cancer and thrombosis make them an important research topic. Upon activation, eosinophils secrete their granule content which then determines the course of allergic, inflammatory, and immunoregulatory responses. To elucidate the possible mechanisms of promoting impact of NLRC4 activation on CD80, we formulated 2 possible hypotheses: i) caspase-1 cleavage through NLRC4 activation results in further activation of downstream caspase proteins and subsequent activation of transcription factors that result, in turn, in upregulated gene expression of the CD80; ii) IL-1β secretion acts as a autocrine signal in EoL-1 cells and upregulates CD80 expression. However, human recombinant IL-1β treatment or conditioned media treatments supressed the response we measured upon NLRC4 activation. This might be due to the dose and time of hr IL-1β treatment or another cell secreted molecule in EoL cell culture media.

It is possible to speculate that NLRC4 is more intimately related to allergic responses while TLR signaling promotes more general, global immune responses in EoL-1 cells. Nonetheless, it may be concluded that PRRs in general take part in promoting the expression of several allergy related and eosinophil specific genes. Thus, this study also raises further questions on how the expression patterns are affected through the signaling of PRRs and other immunoreceptors (IL-5Rα, effect of IgE binding to Fc epsilon receptors) and how the gene expression profiles of the NLRs are regulated.

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The heterogeneity of eosinophil-associated diseases (EAD), the complexity of tissue involvement, the limited number of animal models as well as the low number of eosinophils in blood, are all factors that pose technical difficulties in working with eosinophils and hinder progress in eosinophil basic research. Furthermore, clinical trials and drug development studies on EADs are limited (61,62). Therefore, design of new drugs targeting eosinophils for selective eradication and to generate better treatment options for EAD diseases should be prioritized. Future studies will be extended to primary human eosinophils from EAD patients and to parasitic and viral infection models. These approaches would constitute a more complex framework for investigating and understanding the interactive relationship between PRRs and eosinophilic functions.

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