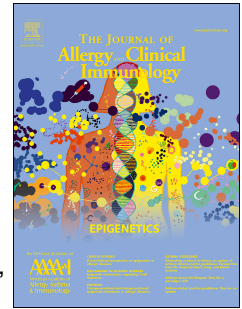


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TLR9-dependent interferon production prevents group 2 innate lymphoid cell-driven airway hyperreactivity

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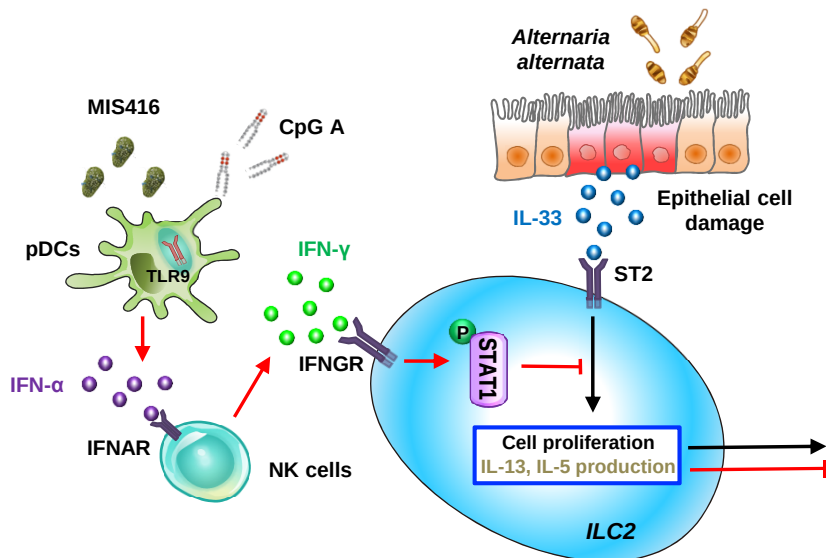
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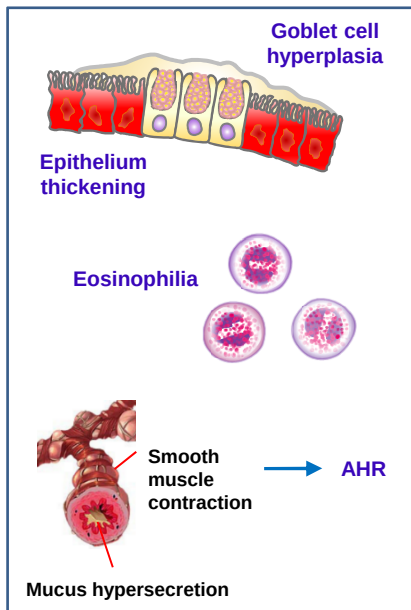
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TLR9 activation by CpG motifs inhibits ILC2-driven asthma through interferon production



Type 2 immunopathology



TLR9: Toll-like receptor 9; MIS416: TLR9 ligand-containing microparticle; pDCs: Plasmacytoid dendritic cells; NK cells: Natural killer cells; ILC2: Group 2 innate lymphoid cells; IFN: Interferons; STAT1: Signal transducer and activator of transcription 1; IFNAR: Interferon α/β receptor; IFNGR: Interferon gamma receptor; AHR: Airway hyperreactivity

TITLE PAGE

Original Article

**TLR9-dependent interferon production prevents group 2 innate lymphoid cell-driven
airway hyperreactivity**

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Author Contribution: C.L.P.T. planned and performed experiments, and wrote the manuscript. A.C.Y.L. helped plan experiments, prepared the materials and mice, and edited the manuscript. P.Y.C. performed the multiplex beads array experiment and assisted with experiments. G.W. provided MIS416 and edited the manuscript. Y.J.C. conceived and initiated the project, planned experiments, and wrote the manuscript.

Competing financial interests

G.W. is an employee at Innate Immunotherapeutics, Auckland, New Zealand. The authors have declared that no competing financial interest exists.

Abstract:

Background: Group 2 innate lymphoid cells (ILC2s) are important mediators of allergic asthma. Bacterial components such as unmethylated CpG DNA, a toll-like receptor 9 (TLR9) agonist, are known to possess beneficial immunomodulatory effects in T cell-mediated chronic asthma. However, their roles in regulating ILC2s remain unclear.

Objective: To determine the role of TLR9 activation in regulating ILC2 function and evaluate the therapeutic utility of an immunomodulatory microparticle containing natural TLR9 ligand (MIS416).

Methods: We evaluated the immunomodulatory effects of CpG A in IL-33-induced airway hyperreactivity (AHR) and airway inflammation. The roles of interferons (IFNs) were examined *in vivo* and *in vitro* using *Stat1*^{-/-} mice and neutralizing antibodies against IFN- γ and IFN- α/β receptor subunit 1 (IFNAR1), and their cellular sources were identified. The therapeutic utility of MIS416 was investigated in the *Alternaria alternata* model of allergic asthma and in humanized NSG mice.

Results: We show that TLR9 activation by CpG A suppresses IL-33-mediated AHR and airway inflammation through inhibition of ILC2s. Activation of TLR9 leads to production of IFN- α , which drives IFN- γ production by NK cells. Importantly, IFN- γ is essential for TLR9-driven suppression and IFN- α cannot compensate for impaired IFN- γ signalling. We further show that IFN- γ directly inhibits ILC2 function through STAT1-dependent mechanism. Finally, we demonstrate the therapeutic potential of MIS416 in *A. alternata*-induced airway inflammation, and validated these findings in humans.

61

62 **Conclusion:** TLR9 activation alleviates ILC2-driven AHR and airway inflammation through
63 direct suppression of cell function. Microparticle-based delivery of TLR9 ligands may serve
64 as a therapeutic strategy for asthma treatment.

Key messages:

- TLR9 activation by CpG A attenuates IL-33-driven AHR and eosinophilia through inhibition of ILC2 function.
- TLR9 activation rapidly triggers innate IFN- γ production by NK cells in a STAT1 and type I IFN-dependent manner.
- IFN- γ , but not type I IFN, is the effector cytokine mediating the suppressive effects of CpG A.
- IFN- γ directly inhibits cytokine production by ILC2s, likely through *socs1* induction.
- Local administration of microparticle-encapsulated natural TLR9 ligand effectively attenuates *A. alternata*-induced airway inflammation and human ILC2 expansion.

Capsule summary: Our findings underscore the critical role of TLR9-induced innate IFN- γ production by NK cells in suppressing ILC2-driven airway inflammation and offer an alternative delivery approach for natural TLR9 ligand through bacterial-derived microparticle encapsulation.

Key words: TLR9; CpG A; group 2 innate lymphoid cells; allergic asthma; MIS416; interferons

Abbreviations:

AHR: Airway hyperreactivity; BALF: Bronchoalveolar lavage fluid; TLR9: Toll-like receptor 9; ILC2: Group 2 innate lymphoid cells; IFN: Interferons; pDC: Plasmacytoid dendritic cells; NK cells: Natural killer cells; RAG: Recombination-activating gene; WT: Wild-type

INTRODUCTION

Asthma is a heterogenous inflammatory disease characterized by chronic inflammation of the airways and airway obstruction, leading to symptoms such as shortness of breath, chest tightness, and wheezing.¹ Approximately 300 million people worldwide are affected by this disorder, which has many different clinical phenotypes although allergen-induced allergic asthma remains the most prevalent form. Hallmark traits of allergic asthma are eosinophilic inflammation in the airways and the presence of serum immunoglobulin E (IgE), both of which are attributed to T helper type 2 (T_H2) cell-derived type 2 cytokines: interleukin (IL)-4, IL-5, and IL-13. Other less common asthma phenotypes are classified as the non-allergic form, including those caused by viral infection, air pollution, and obesity.² As there is no cure for asthma yet, the main aim of current treatment is to provide long-term disease control. However, these treatments only alleviate the symptoms, but do not treat the primary cause of the disease.

Innate lymphoid cells (ILCs) are a group of newly defined non-B, non-T cell lymphocytes that respond rapidly to environmental factors to produce an array of cytokines.³ Group 2 ILCs (ILC2s), in particular, have been implicated in the pathogenesis of asthma.⁴ Studies on fungal allergens,⁵ proteases,⁶ and virus-mediated airway hyperresponsiveness⁷ have all demonstrated a critical role of ILC2s in provoking inflammation. These cells can be rapidly activated by epithelial-derived cytokines such as IL-33, IL-25, and thymic stromal lymphopoietin (TSLP), as well as lipid mediators including leukotriene D4 to produce high levels of T_H2 cytokines IL-13, IL-5, and to a lesser extent IL-4.⁸ ILC2-derived IL-13 and IL-5 contribute to asthma development through the recruitment of eosinophils and by promoting goblet cell hyperplasia and mucus hypersecretion, respectively. In addition, ILC2s can orchestrate the adaptive immune response through its antigen presenting function,⁹ as well as through ILC2-derived IL-13 which induces dendritic cell (DC)-mediated memory T_H2 cell

recruitment after allergen re-challenge.¹⁰ Taken together, ILC2s are important modulator and effector cells in the pathogenesis of asthma.

Microbial components possess immunomodulatory properties that can confer protection against asthma. In line with this notion, studies have shown that Toll-like receptor (TLR) activation by microbial ligands such as LPS (TLR4 ligand) conferred protection against allergic airway diseases.^{11, 12} Bacterial DNA represents another TLR ligand that possesses immunomodulatory properties. The unmethylated CpG motifs found on bacterial DNA are recognized by TLR9,^{13, 14} an intracellular TLR expressed by multiple immune cells including B cells, monocytes, and DCs in mouse,¹⁵ but is restricted to plasmacytoid DCs (pDCs) and B cells in humans.¹⁶ The immunostimulatory properties of synthetic CpG oligodeoxynucleotides (ODNs) have been extensively studied in T cell-mediated allergic asthma models, including cockroach allergen,¹⁷ ovalbumin (OVA),¹⁸ and house dust mites (HDM).¹⁹ In most studies, the protective effects of class B ODNs are attributed to increased Treg cell function^{17, 20} and T_H1-skewed responses^{19, 21}. The role of CpG ODNs in the context of ILC2-driven allergic asthma remains undefined.

MIS416 is a myeloid-targeted immunomodulatory microparticle (2 x 0.5 micron rod-shaped particle) derived from commensal gram positive *Propionibacterium acnes*. MIS416 comprises of cell-wall skeleton-associated muramyl dipeptide (MDP) and bacterial genomic single stranded DNA which activates the intracellular pathogen sensors, nucleotide oligomerization domain 2 (NOD2) and TLR9, respectively.²² Due to its microparticle formulation, uptake is restricted to phagocytic myeloid cells, thus avoiding direct effects on the lymphoid and non-immune compartments. When used as a vaccine adjuvant, MIS416 promoted effective T_H1 adaptive immunity,^{22, 23} an immunological response that has been exploited for the treatment of T_H2-biased allergic responses. Its efficacy in treating allergic diseases, however, has not been elucidated.

Here we demonstrate that TLR9 activation by immunostimulatory CpG A potently suppresses IL-33-mediated airway hyperreactivity (AHR) and lung inflammation through suppression of ILC2 function. Mechanistically, TLR9 signaling results in type I IFN secretion, which in turn promotes IFN- γ production by NK cells. Importantly, IFN- γ , but not type I IFN, is the effector cytokine mediating the inhibitory effects of CpG A, acting directly on ILC2s to suppress cell proliferation and cytokine production. Extrinsic and ILC2-intrinsic STAT1 contribute to type I IFN production and IFN- γ -mediated inhibition of ILC2 function, respectively, culminating in reduced airway inflammation. Finally, we show that the bacterial DNA-containing immunomodulatory microparticle MIS416 can effectively suppress *A. alternata*-driven airway inflammation in mice and type 2 cytokine production by human PBMCs as well as human ILC2 expansion. Overall, this study identifies an innate immune-driven mechanism for CpG A/TLR9 signaling in regulating ILC2 function and demonstrates the potential therapeutic utility of microparticle-based formulation of TLR9 agonist for asthma management or treatment.

METHODS

Mice

BALB/c and C57BL/6 mice were purchased from National Laboratory Animal Center (Taipei, Taiwan). *Rag2*^{-/-} mice were purchased from Taconic Farms (Hudson, NY) whereas *Rag1*^{-/-}, *Rag2/IL2rg*^{-/-}, *Stat1*^{-/-}, and NOD SCID (non-obese diabetic severe combined immunodeficiency) γ C-deficient (NSG) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *Tlr9*^{-/-} mice were a kind gift from Dr Shizuo Akira (Osaka University). *Ifng*^{-/-} mice were a kind gift from Dr Liao Nan-Shih (Institute of Molecular Biology, Academia Sinica). All animals were housed under specific pathogen-free conditions. Experiments were performed with age-matched (6 to 10 weeks) female mice.

Models of lung inflammation

Mice were given 0.1 μ g of recombinant murine IL-33 (Biolegend, San Diego, CA) once daily for 3 days through the intranasal route. Airway inflammation was assessed 3 days later. For the ILC2 adoptive transfer experiment, mice were sacrificed one day after the last IL-33 treatment. In the fungal allergen model, mice were challenged intranasally with 5 μ g of *Alternaria alternata* (Greerlabs, Lenoir, NC) daily for four days. Airway inflammation was assessed 24 hours after the last challenge.

In vivo CpG A or MIS416 treatment

In the pre-treatment protocol, mice were given either CpG A (InvivoGen, San Diego, CA), ctrl ODN (InvivoGen), MIS416, or DNA-deficient MIS416 (empty MIS416 or eMIS416) through the intranasal route, one day and 30 min prior to the first IL-33 or *A. alternata* treatment. CpG A and ctrl ODN were given at 5 μ g per dose whereas MIS416 and eMIS416

were administered at 50 µg per dose. In the post-treatment protocol, mice were given 50 µg MIS416, 30 min after the first and third *A. alternata* challenge.

Neutralizing antibody treatment

To neutralize IFN-γ, mice were injected intraperitoneally with 250 µg of anti-IFN-γ or control IgG antibodies daily for 3 days (Days -1 to 1). CpG A or MIS416 were given on days -1 and 0, followed by IL-33 treatment from days 0 to 2. Anti-IFNAR1, anti-PDCA1 or control IgG antibodies were administered through the intraperitoneal route at a dose of 500 µg per mouse, 24 hours and 30 min before CpG A (5 µg) or MIS416 (50 µg) treatment and sacrificed 24 hours later. All neutralizing antibodies were purchased from BioXcell (West Lebanon, NH).

Measurement of airway AHR

Mice were anesthetized with pentobarbital (Sigma-Aldrich, St Louis, MO) at 100 mg/kg of body weight. Anesthetized mice were tracheotomized, intubated, and mechanically ventilated at a tidal volume of 0.20 mL and a frequency of 150 breathes/min. Lung function was determined by direct measurement of airway resistance in response to increasing methacholine (Sigma-Aldrich) doses (1.25 to 40 mg/mL) via the FinePointe RC system (Buxco Research Systems, Wilmington, NC).

Flow cytometry

Single-cell suspensions were processed for flow cytometry analysis as described in the Methods section in this article's Online Repository.

ILC2 sorting

Murine lung ILC2s were sorted from *Rag2*^{-/-} mice given 0.1 µg of IL-33 for three consecutive days and rested for three days to obtain sufficient numbers for sorting. ILC2s were sorted as CD45⁺ Lin⁻ ST2⁺ cells. Lineage markers used are CD3, CD19, FcεRI, F4/80, CD11b, CD11c, and CD49b. Sorting was performed with a FACS Aria cell sorter (BD Biosciences, Mountain View, CA) with a sorting purity of > 95%. Cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), IL-33, IL-2 and IL-7 (All at 10 ng/mL).

Isolation of human peripheral blood mononuclear cells (PBMCs)

Whole blood was obtained from healthy male donors (Age 20 to 30). PBMCs were isolated by separation over a Histopaque 1077 (Sigma-Aldrich) density gradient and cultured in RPMI 1460 supplemented with IL-2 alone or with IL-33 (Both at 50 ng/mL) in the absence or presence of MIS416 (0.1, 1 or 10 µg/mL) for 72 hours.

Humanized mice

Adoptive transfer of PBMCs into NSG mice was performed as previously described.²⁴ Briefly, PBMCs were isolated from whole blood by separation over a Histopaque density gradient and injected intravenously into NSG mice at a density of 1×10^7 cells/mouse, followed by three daily intraperitoneal injections of recombinant human IL-33, IL-2, and IL-7 (All at 500 ng/mouse/day). MIS416 (50 µg) was administered intranasally right before the first and second cytokine injections. Mice were sacrificed 24 hours later.

Statistical tests

Statistical analyses were performed using Prism 6 (GraphPad Prism software, San Diego, CA). Two-way analysis of variance (ANOVA) and Student's two-tailed *t*-test were used to

determine statistical significance between groups, where P values of < 0.05 were considered to be significant.

Study approval

Animal studies were approved by Academia Sinica Institutional Animal Care and Use Committee (IACUC), and all experiments were performed according to the guidelines of IACUC. Informed consent was obtained from each donor as requested and approved by Academia Sinica Institutional Review Board (AS-IRB-BM-15054).

Additional information on methods can be found in the Methods section in this article's Online Repository.

RESULTS

TLR9 agonist, CpG A, suppresses IL-33-mediated acute AHR and lung inflammation

Activation of TLR9 by CpG ODNs has been demonstrated to exert protective effects in antigen-induced respiratory allergic responses.²¹ However, its effect in acute models of asthma has not been described. To this end, we pre-treated mice with CpG A (ODN 1858) through the intranasal route, followed by intranasal administration of IL-33 to activate ILC2s (Fig 1, A). We found that CpG A-mediated TLR9 activation significantly inhibited IL-33-induced AHR ($P < 0.001$; Fig 1, B; solid compared with open triangles) and eosinophil infiltration in the BALF (Fig 1, C). Associated was the suppression of lung IL-13 and IL-5, key effector cytokines that regulate AHR and airway eosinophilia (Fig 1, D). To determine whether this was the result of a failure of IL-33-dependant ILC2 expansion, we analyzed the proportion of ILC2s in the lungs after treatment and found that mice treated with CpG A and IL-33 had lower percentage (Fig 1, E) and total number (Fig 1, F) of lung ILC2s compared with IL-33-treated mice. The suppressive effects were found to be CpG-dependent as non-CpG ODN (control ODN 1585) failed to suppress IL-33-induced AHR (Fig E1, A) and eosinophilia (Fig E1, B). Likewise, IL-33-driven lung ILC2 expansion (Fig E1, C) and type 2 cytokine production were unaffected (Fig E1, D).

To exclude the role of adaptive immunity, we used *Rag2*^{-/-} mice and observed that CpG A-treated *Rag2*^{-/-} mice developed lower AHR ($P < 0.001$; Fig 1, G; solid compared with open triangles) and BALF eosinophil count (Fig 1, H) than their PBS-treated counterparts when exposed to IL-33. Likewise, ILC2 expansion was abrogated by CpG A, as determined by reduction in total lung ILC2 numbers (Fig 1, I). Moreover, CpG A partially suppressed IL-13 and IL-5 production by lung ILC2s (Fig 1, J-K). Collectively, these data imply that inhibition

of ILC2 expansion and function by CpG A accounted for the lack of lung hyperreactivity and inflammation, and that suppression was independent of adaptive immunity.

TLR9 activation by CpG A induces the production of IFN- γ by NK cells through type I IFN signaling

Activation of TLR9 by CpG A leads to production of type I and II IFNs, as well as IL-12.^{25, 26} We measured BALF levels of these cytokines at different time-points and found that type I IFN (IFN- α), type II IFN (IFN- γ), and IL-12p40, but not bioactive IL-12p70, were induced by CpG A (Fig 2, A and B). Kinetically, both IFN- α and IFN- γ production peaked at 24 hours (Fig 2, A), whereas IL-12p40 levels increased continuously throughout the observation period (Fig 2, B). The mRNA level of IFN- α , however, was induced 1 hour post-CpG A treatment, and increased gradually over time, peaking at 16 hours (Fig E2, A). Meanwhile, IFN-responsive genes (*Mx1*, *Ifit1*, *Ccl2* and *Cxcl10*) were induced as early as 3 h post-CpG A treatment and peaked between 16 to 24 hours (Fig E2, B-E).

Both type I IFNs and IL-12 can induce IFN- γ production.^{27, 28} Since IFN- α , but not IL-12 was induced, we further examined whether type I IFN signaling is essential for IFN- γ production. To block type I IFN signaling, we employed anti-IFNAR1 neutralizing antibody and found that this completely abrogated IFN- γ production (Fig 2, C), implying that IFN- γ production relied on type I IFN signaling. Consistent with previous studies,²⁹ we observed that BM-pDCs produced high levels of IFN- α in response to CpG A (Fig 2, D). To confirm the role of pDCs, we used anti-PDCA1 neutralizing antibody to deplete these cells. Depletion of pDCs markedly reduced BALF IFN- γ production (Fig 2, E). Taken together, these data indicate that pDCs and their associated cytokines are crucial for IFN- γ -production.

Among the immune cell subsets, T cells and NK cells can be induced by type I IFNs to produce IFN- γ .^{30, 31} Therefore, to identify the major producers of IFN- γ , we first analyzed the

levels of IFN- γ in CpG A-treated *Rag2*^{-/-} (T and B cell-deficient) and *Rag2/Il2rg*^{-/-} mice (lacking all lymphocytes, including NK cells, but have intact myeloid component). CpG A triggered IFN- γ production in *Rag2*^{-/-} mice, but not *Rag2/Il2rg*^{-/-} mice (Fig 2, *F*), in both BALF and lungs, suggesting that adaptive and myeloid components are expendable, and innate lymphoid cells are likely the major producers of IFN- γ . Flow cytometry analysis revealed NK cells (CD3⁻ NK1.1⁺ T-bet⁺ Eomes⁺) to be the major IFN- γ -producing cells (Fig 2, *G*). In line with this, NK cells were also activated by CpG A treatment, as determined by CD69 expression (Fig 2, *H*). Notably, we found that CpG A-mediated NK cell activation and IFN- γ production were dependent on type I IFN signaling, as anti-IFNAR1 treatment attenuated IFN- γ production (Fig 2, *I* (upper panel) and *J*) and inhibited NK cell activation (Fig 2, *I* (lower panel) and *K*).

IFN- γ mediates the inhibitory effects of CpG A

To examine the role of IFN- γ in TLR9-mediated protection, we administered anti-IFN- γ antibody concurrently with CpG A, followed by IL-33 treatment (Fig 3, *A*). Neutralization of IFN- γ abolished the suppressive effects of CpG A and restored AHR ($P > 0.05$; Fig 3, *B*; solid circles compared with solid triangles) and associated eosinophilia (Fig 3, *C*). Likewise, lung levels of IL-13 and IL-5 were significantly higher in these mice compared with mice given CpG A and IL-33 only (Fig 3, *D*). Total lung ILC2 number was also completely restored (Fig 3, *E*). To further substantiate these findings, we used *Ifng*^{-/-} mice and showed that CpG A failed to suppress BALF eosinophilia (Fig 3, *F*) and lung ILC2 cell expansion (Fig 3, *G*). Overall, these data support the notion that IFN- γ is the effector cytokine that governs the protective effects of CpG A.

A recent study demonstrated that IFN- γ can directly inhibit mesenteric ILC2 cells.³² In accordance with the aforementioned study, we found that IFN- γ suppressed lung ILC2 cell

proliferation (Fig 4, A) and type 2 cytokine production (Fig 4, B) without affecting viability (Fig 4, C). IL-33 can activate Akt and mitogen-activated protein kinases (MAPK).^{33, 34} However, phosphorylation levels of these kinases were unaltered by IFN- γ treatment (Fig 4, D), suggesting that inhibition likely occurs downstream of these kinases.

STAT1 is essential for CpG A-mediated inhibition of ILC2s

STAT1 is a major transcription factor for both type I and II IFN signaling.³⁵ IFN- γ treatment has been shown to induce STAT1 expression in ILC2s, and STAT1 directs the suppressive effects of IFN- γ *in vitro*.³² We found that IFN- γ induced STAT1 phosphorylation in sorted lung ILC2s (Fig 4, E). Consistent with published reports,^{32, 36} STAT1 deficiency impaired the ability of IFN- γ to inhibit cytokine production (Fig 4, F). Additionally, IFN- γ treatment induced *socs1* expression in a STAT1-dependent manner (Fig 4, G).

Next, we examined whether STAT1 is critical for CpG A-mediated inhibition. To investigate this, we employed *Stat1*^{-/-} mice and found that STAT1 deficiency abolished the suppressive effects of CpG A in terms of AHR ($P > 0.05$; Fig 5, A; solid compared with open triangles), BALF eosinophil count (Fig 5, B), and lung ILC2 expansion (Fig 5, C). Accordingly, CpG A failed to suppress type 2 cytokine production in these mice (Fig 5, D). This is attributed to impaired IFN- α and IFN- γ production, as determined by cytokine measurement in the BALF (Fig 5, E).

To determine whether ILC2-intrinsic STAT1 signaling is important for CpG A-mediated inhibition, we sorted and adoptively transferred WT and *Stat1*^{-/-} lung ILC2s into CD45.1 recipient mice prior to treatment with IL-33 and CpG A (Fig 5, F). Both WT and *Stat1*^{-/-} ILC2s reconstituted equally in the BALF of recipient mice and expressed GATA3 (Fig 5, G). However, the inhibitory effect of CpG A on ILC2 cytokine production was

partially attenuated in *Stat1*^{-/-} donor ILC2s as compared to their WT counterparts (Fig 5, *H* and *I*), suggesting a role for ILC2 intrinsic STAT1.

MIS416, a TLR9 ligand-containing immunomodulatory microparticle is specifically taken up by myeloid cells

To explore the therapeutic applicability of TLR9 signaling in treating ILC2-driven airway inflammation, we utilized an immunomodulatory microparticle called MIS416, which comprises of *Propionibacterium acnes* DNA (TLR9 ligand) and MDP (NOD2 ligand) cross-linked to the cell-wall skeleton (Fig E3, *A*). Due to its rod shape and microparticle size, MIS416 is only sequestered by phagocytic immune cells such as macrophages, monocytes, and DCs, thus limiting uptake by nonimmune cells or lymphocytes.²⁶ To confirm the myeloid-specificity of MIS416 in the lungs, we treated mice with fluorescent-labeled MIS416 (AF488-MIS416 or AF647-MIS416) for 1, 3, or 5 days and performed flow cytometry analysis to study the kinetics of MIS416 uptake by different myeloid and lymphoid subsets (Fig E3, *B*). As shown in Fig 6A and B, MIS416 can be uptake by myeloid cells, mainly AMs, but not lymphoid and nonimmune cells (CD45⁻ population). Additionally, MIS416 also induced expansion of myeloid cells, most notably pDCs, whereas AM population only expanded on day 5 (Fig 6, *C*). Since AMs were the major cells that uptake MIS416, we examined their function by using clodronate to deplete these cells (Fig E3, *C*). AM depletion did not affect AHR (Fig E3, *D*) and BALF eosinophil count (Fig E3, *E*), suggesting that they are dispensable for suppression.

In line with the restricted uptake of MIS416 by phagocytic cells, we did not observe any uptake by ILC2s (Fig E4, *A*), which can only undertake endocytosis.¹⁰ Furthermore, *in vitro* analysis of cultured lung ILC2s revealed that MIS416 did not affect ILC2 proliferation

(Fig E4, B) and type 2 cytokine production at both protein (Fig E4, C) and mRNA (Fig E4, D) levels. These observations verify that MIS416 had no direct effect on ILC2s.

MIS416 antagonizes IL-33-driven airway inflammation through ILC2 inhibition

To investigate the effects of MIS416 on ILC2-driven AHR and airway inflammation, we pre-treated mice with MIS416 according to the CpG A protocol in Fig 1A, followed by IL-33 treatment. Similar to CpG A, MIS416 suppressed IL-33-induced AHR ($P < 0.001$; Fig 6, D; solid circles compared with solid triangles) and BALF eosinophil count (Fig 6, E). Importantly, MIS416 deficient in TLR9 ligand (termed empty MIS416 or eMIS416) failed to suppress AHR ($P > 0.05$; Fig 6, D; solid circles compared with solid squares) and BALF eosinophil count (Fig 6, E). Similar findings were observed in *Tlr9*^{-/-} mice (Fig E5, A to C), thus affirming the importance of TLR9 signaling for the protective effects of MIS416. We also ruled out the role of adaptive immunity by demonstrating that MIS416 attenuated AHR ($P < 0.001$; Fig E5, D; solid square compared with open circles), BALF eosinophil count (Fig E5, E), and ILC2 induction (Fig E5, F) in the lungs of *Rag2*^{-/-} mice.

In addition to inhibiting AHR and eosinophilia, we found that MIS416 almost completely abolished IL-13 and IL-5 production (Fig E6, A). Like CpG A, this was attributed to suppression of ILC2 expansion (Fig E6, B (upper panel) and C) and cytokine production (Fig E6, B (lower panel) and D). Consistent with these observations, MIS416 ameliorated IL-33-induced airway thickening and mucus hypersecretion, assessed by H&E and PAS staining, respectively (Fig E6, E).

MIS416-driven inhibition requires IFN- γ signaling

As with CpG A, MIS416 treatment induced IFN- α (Fig E7, A) and IFN- γ (Fig E7, B) in the lungs on day 1 post-MIS416 treatment. To confirm the importance of IFN- γ signaling in

MIS416-mediated suppression, we blocked IFN- γ signaling using anti-IFN- γ neutralizing antibody. This resulted in failure of MIS416 to inhibit AHR ($P > 0.05$; Fig 6, *F*; solid rhombus compared with solid triangle) and BALF eosinophilia (Fig 6, *G*). Moreover, in the absence of IFN- γ signaling, IL-33-induced lung pathology was not reversed by MIS416 (Fig 6, *H*), providing further evidence which supports a role for IFN- γ signaling in MIS416-driven inhibition.

Similar to a previous study that showed that MIS416 induces IFN- α production by human pDCs,³⁷ we found that MIS416 triggered murine BM-pDCs, but not BM-cDCs, to produce high levels of IFN- α in a dose-dependent manner (Fig E7, *C*). Mechanistically, depletion of pDCs abrogated IFN- α production *in vivo* (Fig E7, *D*), whereas blocking type I IFN signaling abolished IFN- γ production (Fig E7, *E*), implicating the pDC/type I IFN/IFN- γ axis. Similar to CpG A, IFN- γ production by MIS416 was NK cell-dependent, based on the observations that IFN- γ production was induced by MIS416 in *Rag2*^{-/-} mice (Fig E7, *F*), but not *Rag2/Il2rg*^{-/-} mice (Fig E7, *G*), and that the percentage (Fig E7, *H*) and total number (Fig E7, *I*) of IFN- γ ⁺ NK cells increased in response to MIS416. Collectively, these data confirms that MIS416 works in a similar manner as CpG A.

MIS416 potently suppresses *A. alternata*-induced AHR and type 2 inflammation

To further evaluate the therapeutic benefit of MIS416 in inhibiting ILC2-mediated type 2 lung inflammation, we used a clinically relevant model, *A. alternata*. In this model, we performed pre- and post-treatment regimens by exposing mice to MIS416 before or after the first *A. alternata* challenge (Fig 7, *A*), which allows us to evaluate the prophylactic and therapeutic potentials of MIS416, respectively. We found that both treatment potently suppressed *A. alternata*-induced AHR ($P < 0.001$ for both treatment regimens; Fig 7, *B*). *A. alternata*-driven type 2 inflammatory markers were also suppressed, as evidenced by the

decrease in BALF eosinophil infiltration (Fig 7, C) and lung IL-5 and IL-13 levels (Fig 7, D) in MIS416-treated mice, regardless of the treatment regimen. Flow cytometry analysis further revealed that *A. alternata* significantly increased lung ILC2 percentage (Fig 7, E) and number (Fig 7, F), and both pre- and post-treatment with MIS416 ameliorated the induction.

MIS416 suppresses type 2 cytokine production by human PBMCs and human ILC2 expansion

To evaluate the translational aspects of our findings, we first examined whether MIS416 can inhibit type 2 cytokine production by ILC2s in PBMCs. *In vitro* culture of PBMCs with IL-33 and IL-2 promotes antigen-independent production of type 2 cytokines by human ILC2s.³⁸ Using similar protocol, we cultured PBMCs from three healthy donors in the presence or absence of MIS416, and observed that MIS416 suppressed cytokine production in all donors, although to varying degrees of potency (Fig 8, A). To determine whether MIS416 inhibits human ILC2 expansion, we adoptively transferred PBMCs into NSG mice prior to treatment with MIS416 and IL-33 (Fig 8, B). Flow cytometry analysis revealed partial reduction in the percentage (Fig 8, C) and total number (Fig 8, D) of IL-33-activated human ILC2s in the lungs of mice treated with MIS416 as compared to their PBS-treated counterparts.

DISCUSSION

In this study, we show that TLR9 activation by CpG A protects against ILC2-driven AHR and airway inflammation through type I IFN/IFN- γ signaling axis in a STAT1-dependent manner. Importantly, NK cell-derived IFN- γ , but not type I IFNs, play a critical effector role in mediating the suppressive effects of CpG A. In a proof-of-concept study, we further show that TLR9 activation by the therapeutic microparticle MIS416 abolished *A. alternata*-induced AHR and type 2 inflammation through inhibition of ILC2s. Notably, MIS416 inhibited human ILC2 expansion and IL-33-induced type 2 cytokine production in PBMC culture. This study thus highlights an allergen independent, innate-driven mechanism for suppression of acute airway inflammation triggered by ILC2s, unlike previous studies that implicate a role for adaptive immunity.^{18, 19, 39}

Synthetic CpG-ODNs have been of interest as immunomodulatory agents for asthma treatment due to their anti-allergic effects in experimental models of asthma.^{40, 41} CpG ODNs potently prevented the development of allergen-induced AHR and BALF eosinophilia in both mouse and primate models of asthma,^{17, 42, 43} as well as features of airway remodeling including basement membrane thickening and goblet cell metaplasia.⁴³ When used as an adjuvant for specific antigen immunotherapy, CpG ODNs reversed established eosinophilic airway inflammation and AHR.^{44, 45} Moreover, treatment with CpG ODNs increased resistance to respiratory syncytial virus infection and prevented virus-induced asthma exacerbation.⁴² Nevertheless, in a model of severe asthma induced by triple allergen, CpG ODN treatment failed to attenuate AHR and airway remodeling although IgE and eosinophil levels in the lung were suppressed.⁴⁶ In our study however, TLR9 activation significantly suppressed AHR and type 2 inflammation induced by *A. alternata*, a fungal allergen associated with severe asthma.^{47, 48} This suggests that despite causing similar asthma features, the different biologic mechanisms triggered by different allergens or combination of allergens

may affect the efficacy of CpG ODNs, further underscoring the heterogeneity of asthma and the importance of targeted therapy.

Mechanistically, the inhibitory effects of CpG ODNs have been attributed in part to the induction of IFN- γ and IL-12, which creates a T_H1-oriented milieu that counteracts T_H2 inflammation.⁴⁹ In addition, CpG-ODN also induces IL-10 production by regulatory T cells (Tregs) and interstitial macrophages which can suppress T_H2 immune response,^{50, 51} although TLR9 activation in the presence of 1 α ,25-dihydrovitamin D3 inhibits the regulatory function of Tregs.⁵² In this study, we found elevated levels of IFN- α , IFN- γ , and IL-12p40, but not bioactive IL-12p70 in the BALF and lungs of CpG A-treated mice. We also did not detect IL-10 (data not shown), further supporting our findings that CpG A exerts its effect through IFN responses. Contrary to a recent finding that the TLR7 agonist, R848, induces rapid increase of IFN- α (peaking at 1 h) in the BALF,⁵³ CpG A-induced IFN- α showed delayed and overlapping kinetics with IFN- γ , peaking at 24 hours. However, the mRNA level of IFN- α and IFN-responsive genes were induced within the first 3 hours post-CpG A treatment. This indicates the induction of an earlier but weaker type I IFN response that was amplified over time. Indeed, CpG A has been shown to induce a delayed type I IFN kinetics which require autocrine type I IFN signaling in a STAT1-dependent manner.^{29, 54} In support of this, we found that STAT1-deficiency blocked IFN- α production in response to CpG A. We also observed that CpG A was less effective in inhibiting IL-33-induced cytokine production by *Stat1*^{-/-} ILC2s as compared to WT ILC2s. Hence, these data suggest that both extrinsic and ILC2-intrinsic STAT1 regulates the inhibitory effects of CpG A.

Both type I and II IFNs have been shown to directly inhibit IL-33-activated ILC2s *in vitro*.^{32, 36} *In vivo*, TLR7- and influenza A-induced type I IFN signaling have been reported to suppress ILC2s.^{36, 53} Both stimuli also trigger IFN- γ ,^{55, 56} but the interplay between both IFNs has not been elucidated in the context of ILC2s. We found that type I IFNs are crucial for

IFN- γ production by NK cells given that IFNAR blockade markedly reduced IFN- γ production. This finding is consistent with previous reports that indicate a stimulatory role for type I IFNs in IFN- γ production by NK cells.^{28, 31} Importantly, IFN- γ is the effector cytokine mediating the suppressive effect of CpG A and type I IFNs cannot compensate for impaired IFN- γ signaling. It has been reported that lung ILC2s express the IFN- γ receptor, IFNGR1, but not type I IFN receptor, IFNAR1.^{32, 36} Nonetheless, IFNAR1 expression can be induced by IL-2 in culture.²⁶ Therefore, the lack of IFNAR1 receptor expression may account for the inability of lung ILC2s to respond to type I IFNs in the absence of IFN- γ *in vivo*, but not *in vitro*. Mechanistically, IFN- γ did not inhibit phosphorylation of p38 and ERK, both of which have been shown to play prominent roles in ILC2 proliferation and cytokine production,^{57, 58} but increased *socs1* mRNA level in a STAT1-dependent manner. A previous study has shown that knockdown of *socs1* enhanced mRNA and protein expression levels of IL-13 and IL-5 in ILC2s, suggesting a limiting role for this protein.⁵⁹ Hence, *socs1* may play a role in IFN- γ -mediated suppression.

We also demonstrate the therapeutic efficacy of the bacteria-derived microparticle MIS416 in both mouse and human ILC2s. Immune-modifying treatments that mimic infection with natural pathogens have attracted much attention from researchers, leading to the development of virus-like particles such as CpG DNA-containing QbG10 as a possible treatment strategy for asthma,⁶⁰ given that free CpG ODNs result in non-specific side effects.⁶¹ Despite showing promising results in treating patients with mild-to-moderate asthma, QbG10 failed to show efficacy in treating moderate-to-severe asthma, and this was attributed in part to its subcutaneous route of delivery.^{60, 62} Here, we show that intranasal administration of MIS416 effectively prevented allergen-induced AHR and eosinophilia, and suppressed lung ILC2 expansion through similar mechanisms as CpG A. This result highlights the feasibility of local administration of microparticle formulation, which is less

invasive than subcutaneous injection and allows for direct targeting of the lung mucosal surfaces, as an alternative strategy to deliver bacterial DNA.

When administered intranasally, MIS416 was found to be taken up primarily by AMs, and to a lesser extent, cDCs and pDCs. This is not unexpected, given that AMs are the first line of phagocytic defense in the lower airways,⁶³ and that they are approximately 50 times more numerous than pDCs and cDCs. However, AMs do not express TLR9,⁶⁴ and therefore cannot be activated by bacterial DNA. We observed a small population of MIS416⁺ pDCs, followed by cell expansion on day 3 post-treatment. We interpret these data as resident pDCs responding initially to phagocytosed MIS416, which then trigger an altered systemic immune cell trafficking orchestrated by type I IFN-dependent autocrine and paracrine innate signaling and amplification of IFN- γ signaling pathway. This leads to asynchronous changes in individual cell subset number during the course of the MIS416 response. Despite the poor phagocytic property of pDCs, we show that they readily produce IFN- α when exposed to MIS416 *in vitro*. This data is in agreement with findings on the characterization of MIS416 mechanism of action in human leukocytes.²² This study demonstrated that MIS416-induced IFN- α was mostly derived from pDCs, despite showing lower MIS416 uptake compared to other blood phagocytes, such as monocyte DCs and monocytes. Furthermore, this activity was blocked by chloroquine, an endosomal/lysosomal inhibitor known to inhibit TLR9 signaling,⁶⁵ underscoring the importance of TLR9 activation in these cells.

Finally, we demonstrate that MIS416 strongly suppressed human T_H2 responses based on PBMC cell-based assay. By performing adoptive transfer of PBMC into NSG mice, we provide evidence that MIS416 also suppressed human ILC2 expansion in response to IL-33. These findings, along with the fact that MIS416 reliably induces a robust type I/II IFN response in humans,³⁷ indicate that this pathway is conserved between mouse and human. This study thus demonstrates that immune modulation by MIS416 may offer an easily

administered treatment option for asthma. Importantly, MIS416 may offer an advantage over currently prescribed corticosteroids, since by comparison, MIS416 acts upstream of inflammatory cell signaling rather than directly on pathway signaling intermediates. Moreover, the outcome from a recent phase 2b human trial for secondary progressive multiple sclerosis (SPMS) patients further substantiates the previously established safety profile of MIS416.^{66, 67}

In conclusion, our study highlights the role of an allergen-independent, innate immune-driven effector function triggered by TLR9 signaling, which robustly counteracts the rapidly activated ILC2s through NK cell-derived IFN- γ . Notably, IFN- γ is the effector cytokine mediating the suppressive effects of both TLR9 agonists, and type I IFNs cannot compensate for impaired IFN- γ signaling. From the therapeutic standpoint, we demonstrate the potential therapeutic utility of microparticle-based formulation for local delivery of TLR9 agonists as a means to overcome the limitations associated with other candidate TLR9 based therapeutics and their lack of target cell specificity.

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FIGURE LEGENDS

Figure 1: CpG A suppresses IL-33-induced AHR and type 2 inflammation independent of adaptive immunity

A, Schematic diagram of CpG A treatment (5 μ g) in BALB/c (**B-F**) or *Rag2*^{-/-} mice (**G-J**) treated with 0.1 μ g IL-33 through the intranasal (i.n.) route. **B**, Lung resistance of BALB/c mice in response to increasing doses of methacholine. **C**, Total eosinophils in BALF of BALB/c mice. **D**, IL-5 and IL-13 levels in lung homogenates of BALB/c mice. **E**, Representative flow diagram showing lung ILC2 population (Lin⁻ ST2⁺ ICOS⁺ GATA3⁺ CD127⁺) from BALB/c mice, gated from viable CD45⁺ cells. Representative histograms are shown, whereby grey solid lines: Isotype-matched control and blue solid lines: Antibody staining. **F**, Total number of lung ILC2s from BALB/c mice, assessed as in **E**. **G**, Lung resistance of *Rag2*^{-/-} mice in response to increasing doses of methacholine. **H**, Total eosinophils in BALF of *Rag2*^{-/-} mice. **I**, Total number of lung ILC2s from *Rag2*^{-/-} mice, assessed as in **E**. **J**, Representative flow diagram showing lung IL-13⁺ IL-5⁺ ILC2s from *Rag2*^{-/-} mice, gated as viable CD45⁺ Lin⁻ ST2⁺ cells. **K**, Percentage of IL-13⁺ IL-5⁺ ILC2s, assessed as in **J**. Data are presented as means $\pm$ SEMs of 2 or 3 independent experiments (n = 4-7 each). **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

Figure 2: CpG A induces IFN- γ production by NK cells in a type I IFN-dependent manner

A-B, C57BL/6 mice received 5 μ g CpG A intranasally and sacrificed at the indicated time points. IFN- α , IFN- γ (**A**), and IL-12 (p40 and p70 form) (**B**) levels in BALF. **C**, IFN- γ level in BALF of mice treated with 500 μ g anti-IFNAR1 or control IgG antibody (Ab) and CpG A (5 μ g). **D**, IFN- α level in the supernatant of BM-pDCs treated with 1 μ M CpG A for 24

hours. **E**, IFN- γ level in BALF of mice treated as in **C**, but with 500 μ g anti-PDCA1. **F**, IFN- γ level in BALF of *Rag2*^{-/-} and *Rag2/Il2rg*^{-/-} mice after 24 hour treatment with CpG A (5 μ g). **G-H**, Representative flow diagram showing IFN- γ ⁺ (**G**) and CD69⁺ (**H**) NK cells (NK1.1⁺ T-bet⁺ Eomes⁺) after treatment as in **F**. **I**, Representative flow diagram showing IFN- γ ⁺ and CD69⁺ NK cells after treatment as in **C**. For histograms, grey solid lines: Isotype-matched control whereas blue solid lines: Antibody staining. **J-K**, Total IFN- γ ⁺ (**J**) and CD69⁺ (**K**) NK cells, assessed as in **I**. Data are shown as means $\pm$ SEMs of 2 independent experiments (n = 4-5 each). *n.s.*, Not significant. **P*<0.05, ***P*<0.01 and ****P*<0.001 (* denotes statistical significance for IFN- γ and IL-12p40; # denotes statistical significance for IFN- α).

Figure 3: CpG A-mediated inhibition is IFN- γ -dependent

A, Schematic diagram showing the treatment protocol for anti-IFN- γ neutralizing antibody (250 μ g) or control IgG antibody (Ab) and CpG A (5 μ g) in BALB/c mice exposed to 0.1 μ g IL-33 (i.n., intranasal; i.p., intraperitoneal). **B**, Lung resistance in response to increasing doses of methacholine. **C**, Total eosinophils in BALF. **D**, IL-13 and IL-5 levels in lung homogenates of mice. **E**, Total number of lung ILC2s, assessed as in Figure 1E. **F-G**, *Ifng*^{-/-} mice were treated with CpG A and IL-33 as in Figure 1A. **F**, Total eosinophils in BALF. **G**, Total number of lung ILC2s, assessed as in Figure 1E. Data are presented as means $\pm$ SEMs of 2 independent experiments (n = 4-6 each). *n.s.*, Not significant. **P*<0.05, ***P*<0.01 and ****P*<0.001.

Figure 4: IFN- γ inhibits ILC2 proliferation and cytokine production in a STAT1-dependent manner

ILC2s sorted from the lungs of IL-33-treated wild-type (WT) or *Stat1*^{-/-} mice were treated with 10 ng/mL IL-33 in the absence or presence of 10 ng/mL IFN- γ at the indicated

concentrations for 48 hours unless specified otherwise. All cultures contained IL-2 and IL-7 (Both at 10 ng/mL). **A**, Proliferation of WT ILC2s, assessed by CFSE after 6-day treatment. **B**, IL-13 and IL-5 levels in culture supernatants of WT ILC2s. **C**, Representative flow diagram showing propidium iodide (PI) and annexin V positive WT ILC2s. **D**, Fold change in MFI of phosphorylated Akt, ERK, JNK, and p38 in WT ILC2s after 10-min treatment. **E**, Fold change in MFI of phosphorylated STAT1 in WT ILC2s after 10-min treatment. **F**, IL-13 and IL-5 levels in culture supernatants of WT and *Stat1*^{-/-} ILC2s. **G**, *socs1* mRNA expression in WT and *Stat1*^{-/-} ILC2s after 6-hour treatment. Data are shown as means $\pm$ SDs from 1 of 3 independent experiments (n=3 wells) with consistent findings. *n.s.*, Not significant. **P*<0.05, ***P*<0.01 and ****P*<0.001 (compared with IL-33-treated group).

Figure 5: ILC2-intrinsic and extrinsic STAT1 is required for the suppressive effect of CpG A

A-D, *Stat1*^{-/-} mice were treated with 5 μ g CpG A and 0.1 μ g IL-33 as in Figure 1A. **A**, Lung resistance in response to increasing doses of methacholine. **B**, Total eosinophils in BALF. **C**, Total number of lung ILC2s, assessed as in Figure 1E. **D**, IL-13 and IL-5 levels in lung homogenates of mice. **E**, IFN- α and IFN- γ levels in BALF of C57BL/6 and *Stat1*^{-/-} mice treated with 5 μ g CpG A and sacrificed 24 hours later. **F**, Schematic diagram showing CpG A (5 μ g) and IL-33 (0.1 μ g) treatment in recipient CD45.1 mice following adoptive transfer of ILC2s sorted from C57BL/6 (WT) or *Stat1*^{-/-} mice (i.e., intratracheal; i.n. intranasal). **G**, Representative flow diagram showing WT and *Stat1*^{-/-} donor ILC2s (gated as viable CD45.2⁺ GATA3⁺ cells) from mice treated as in **F**. Representative histograms are shown, whereby grey solid lines: Isotype-matched control and blue solid lines: Antibody staining. **H**, Representative flow diagram showing IL-13- and IL-5-producing donor ILC2s (gated as viable CD45.2⁺ cells) from mice treated as in **F**. **I**, Percentage of IL-13⁺ IL-5⁺ ILC2s,

assessed as in **H**. Data are shown as means $\pm$ SEMs from 2 or 3 independent experiments (n=4-8). *n.s.*, Not significant. * P <0.05, ** P <0.01 and *** P <0.001.

Figure 6: Myeloid-specific MIS416 inhibits IL-33-mediated AHR and lung inflammation in a TLR9 and IFN- γ -dependent manner

A-B, Representative flow diagram showing the kinetics of MIS416 uptake by myeloid (**A**) and lymphoid (**B**) cells in the lungs of BALB/c mice on days 1, 3, and 5 after MIS416 treatment, assessed as in Figure **E2B**. Representative histograms are shown, whereby grey solid lines: Isotype-matched control and blue solid lines: Antibody staining. (AM: Alveolar macrophages, IM: Interstitial macrophages, cDC: Conventional dendritic cells, pDC: Plasmacytoid dendritic cells). **C**, Absolute number of DCs (pDCs and cDCs), macrophages (AMs and IMs), and monocytes, assessed as in Figure **E2B**. **D-E**, BALB/c mice were treated as in Figure **1A**, with 50 μ g MIS416 or eMIS416 being used in replacement of CpG A. **D**, Lung resistance in response to increasing doses of methacholine. **E**, Total eosinophils in BALF. **F-H**, BALB/c mice were treated as in Figure **3A**, with MIS416 (50 μ g) being used in replacement of CpG A. **F**, Lung resistance in response to increasing doses of methacholine. **G**, Total eosinophils in BALF. **H**, Representative images of H&E- and PAS-stained histologic sections of the lungs of mice (*Scale bars* = 200 μ m). Data are shown as means $\pm$ SEMs of 3 independent experiments (n=7-9 each). *n.s.*, Not significant. * P <0.05, ** P <0.01 and *** P <0.001.

Figure 7: MIS416 suppresses *A. alternata*-induced AHR and type 2 inflammation

A, Schematic diagram of MIS416 (50 μ g) pre-treatment and post-treatment regimens via intranasal (i.n.) delivery in *A. alternata* (5 μ g) model of airway inflammation in BALB/c mice. **B**, Lung resistance in response to increasing doses of methacholine. **C**, Total

eosinophils in BALF. **D**, IL-5 and IL-13 levels in lung homogenates of mice. **E**, Representative flow diagram showing lung ILC2 population (Lin⁻ Thy1.2⁺ ST2⁺ ICOS⁺), gated from viable CD45⁺ cells. **F**, Total number of lung ILC2s, assessed as in **E**. Data are shown as means $\pm$ SEMs of 2 independent experiments (n=6-8 each). ** P <0.01 and *** P <0.0001.

Figure 8: MIS416 inhibits type 2 cytokine production by human PBMCs and human ILC2 expansion

A, IL-13 and IL-5 levels in the supernatants of PBMCs (from 3 healthy donors) treated with 50 ng/mL IL-33 in the presence or absence of MIS416 at the indicated concentrations. **B**, Schematic diagram showing IL-33/IL-2/IL-7 (500 ng each) and MIS416 (50 μ g) treatment in NSG mice following adoptive transfer of PBMCs from healthy donors (i.n., intranasal; i.p., intraperitoneal; i.v., intravenous). **C**, Representative flow diagram showing human ILC2 population (CD45⁺ Lin⁻ CD127⁺ CRTH2⁺) in the lungs of NSG mice. **D**, Percentage of human ILC2s in the lungs per mouse. Data are shown as means $\pm$ SDs (n = 3 wells) for **A** and means $\pm$ SEMs (n=4) for **D**. *n.s.*, Not significant. * P <0.05, ** P <0.01 and *** P <0.0001.

Figure 1

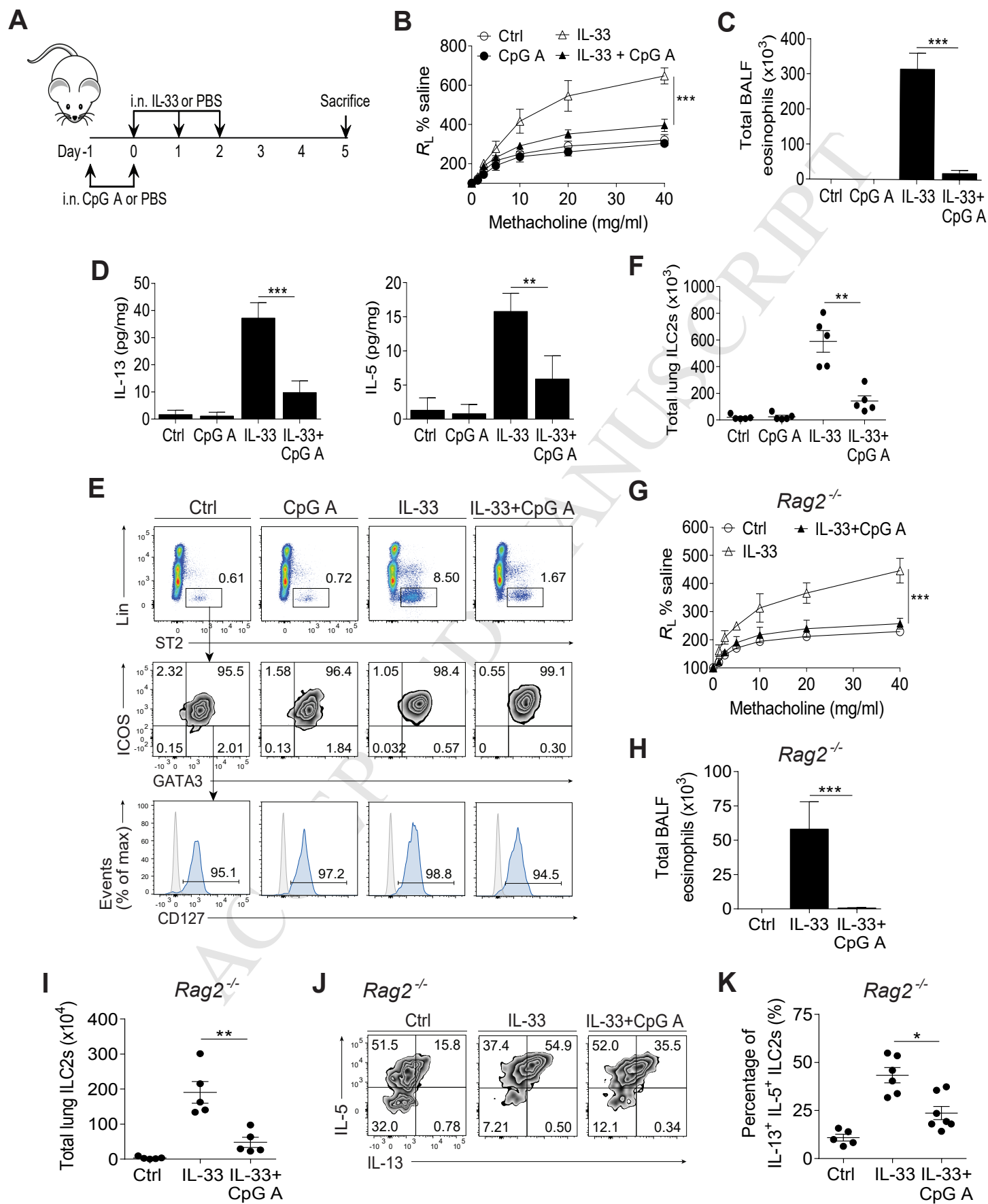


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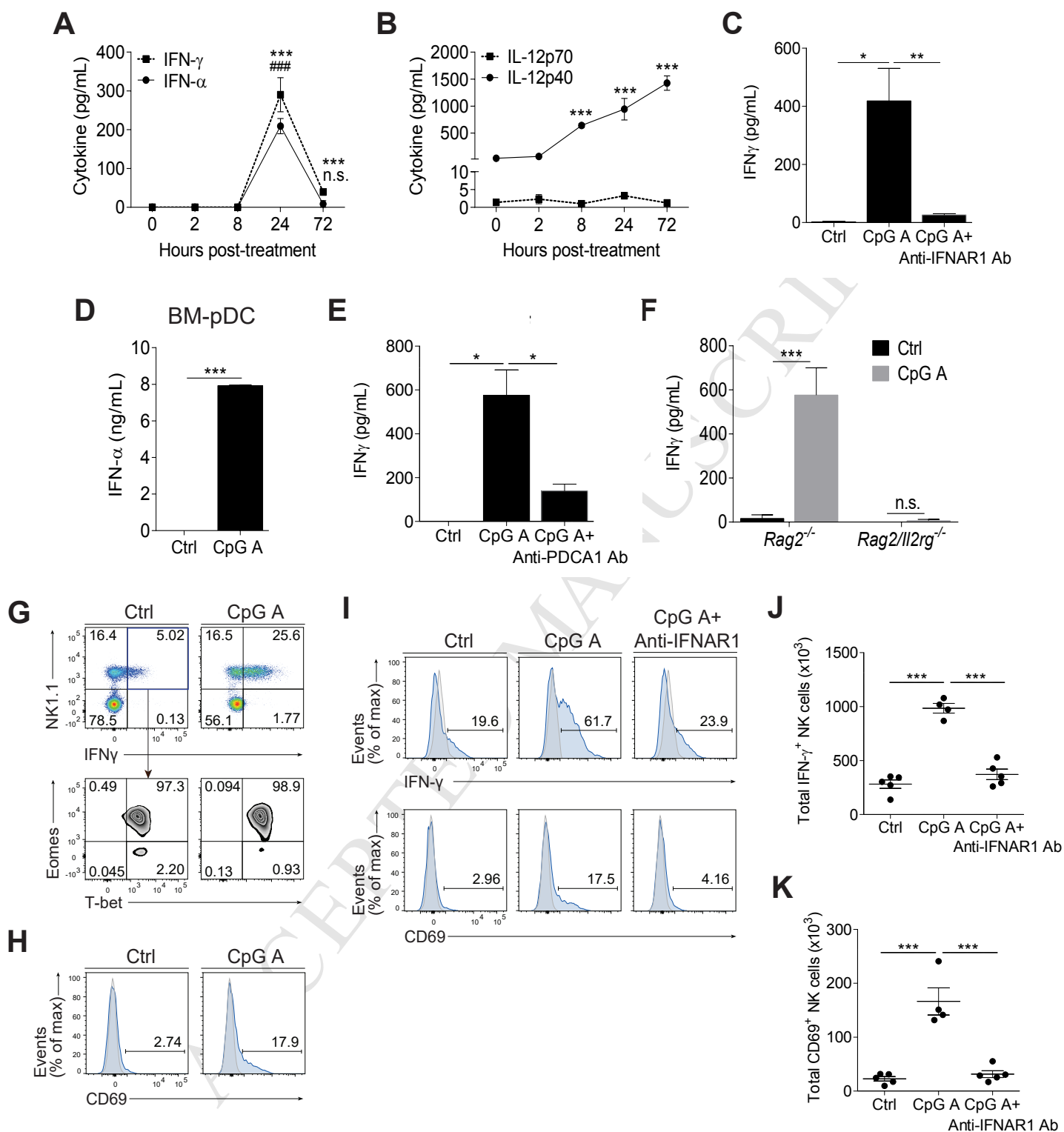


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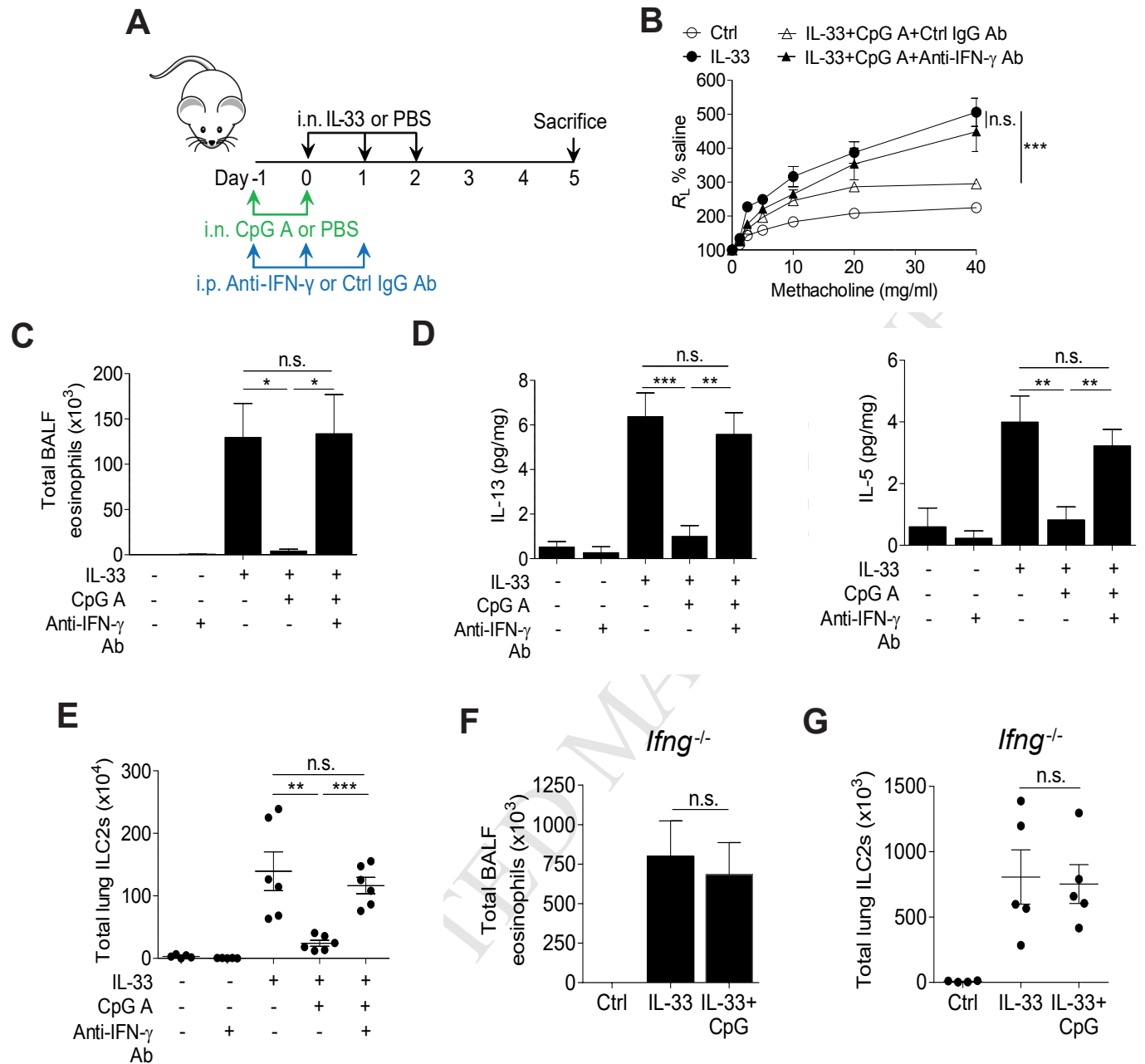


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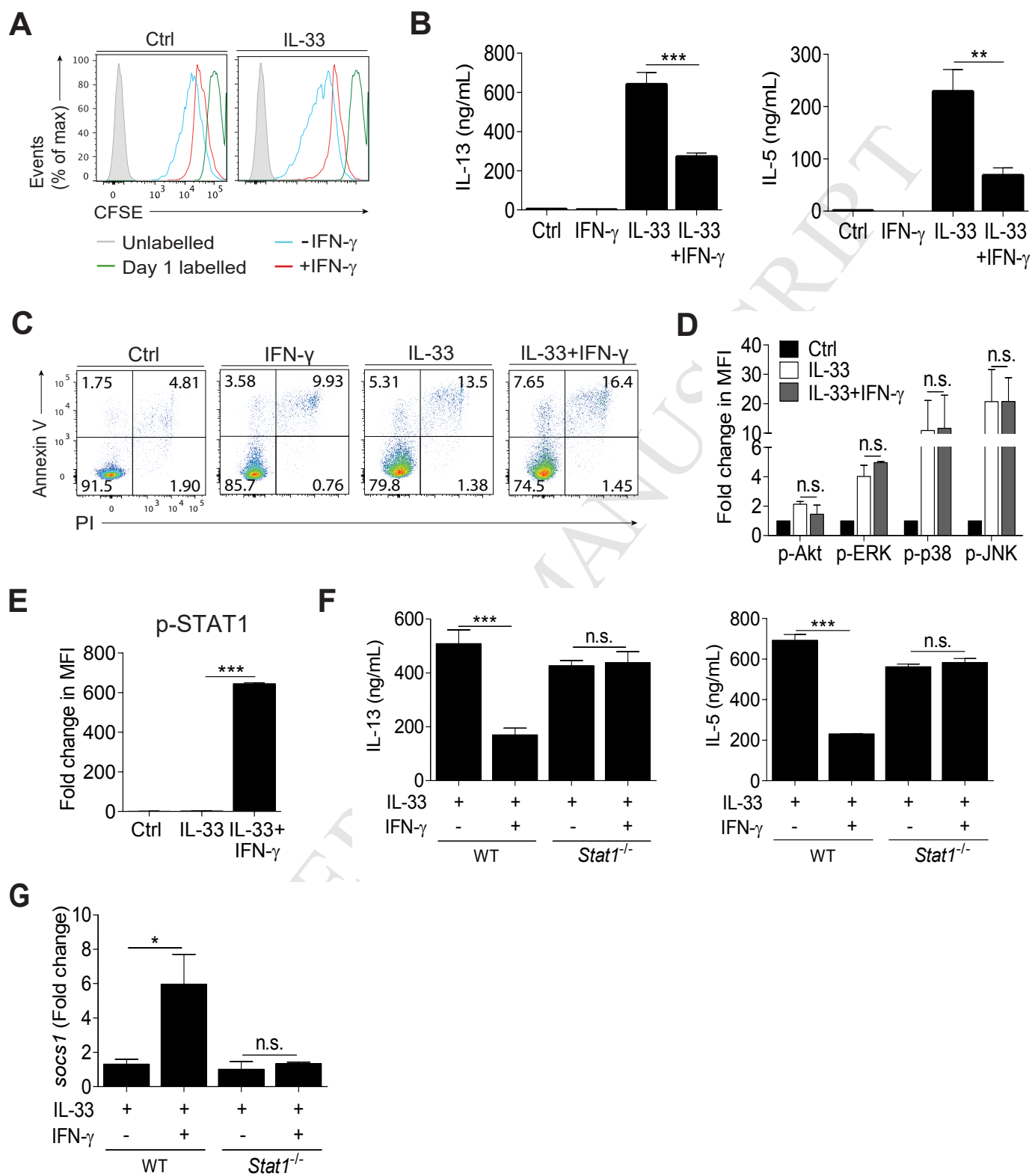


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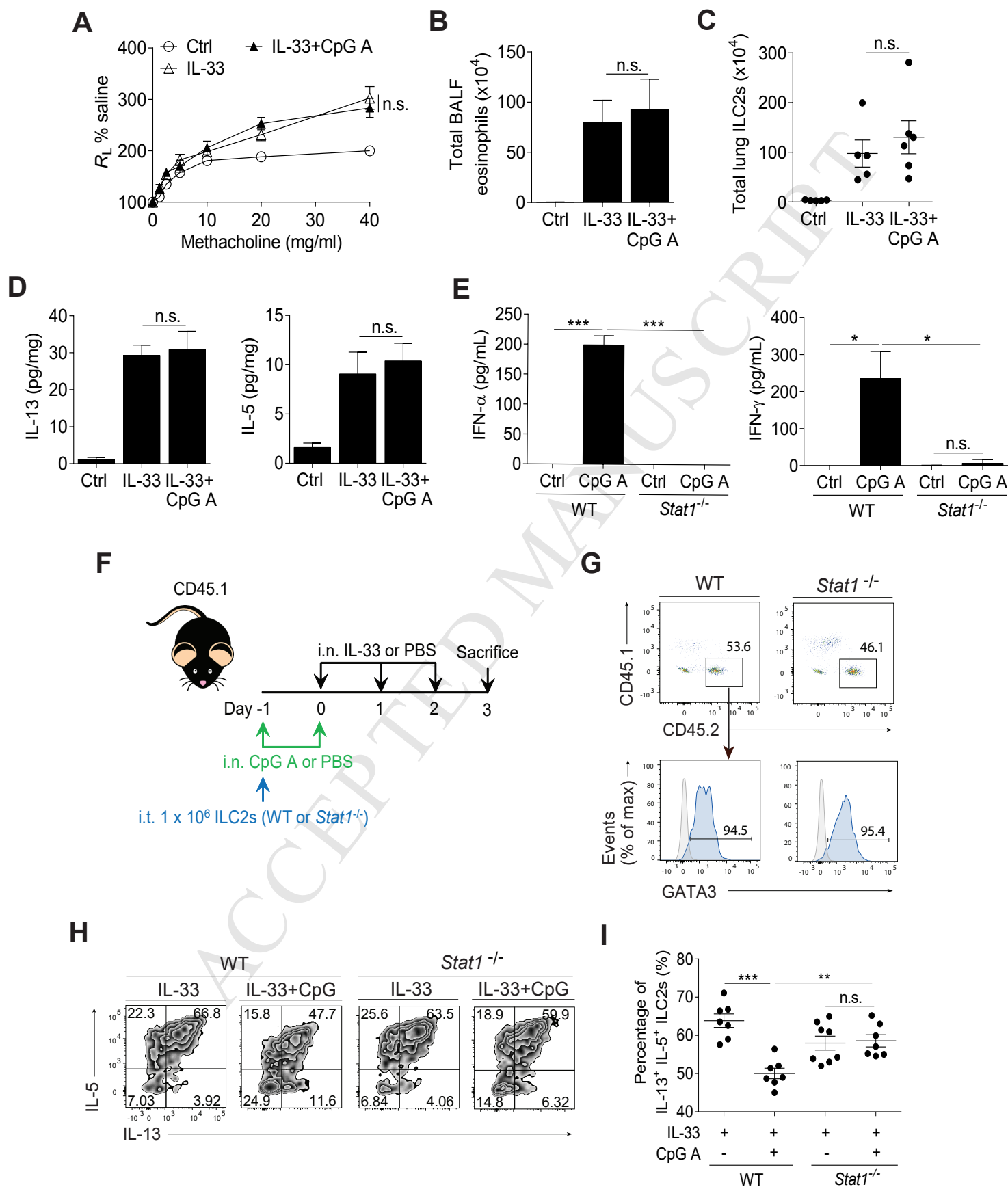


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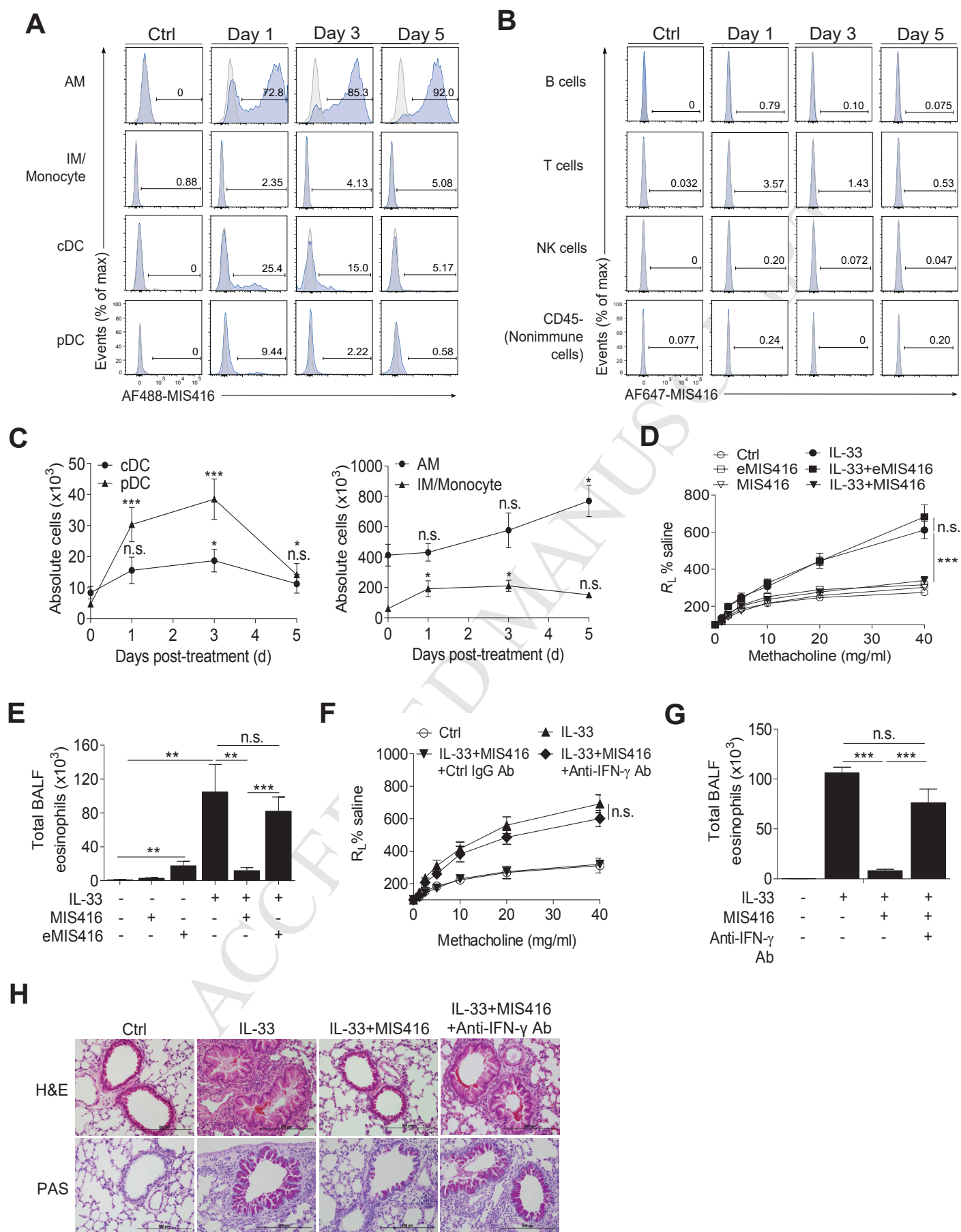


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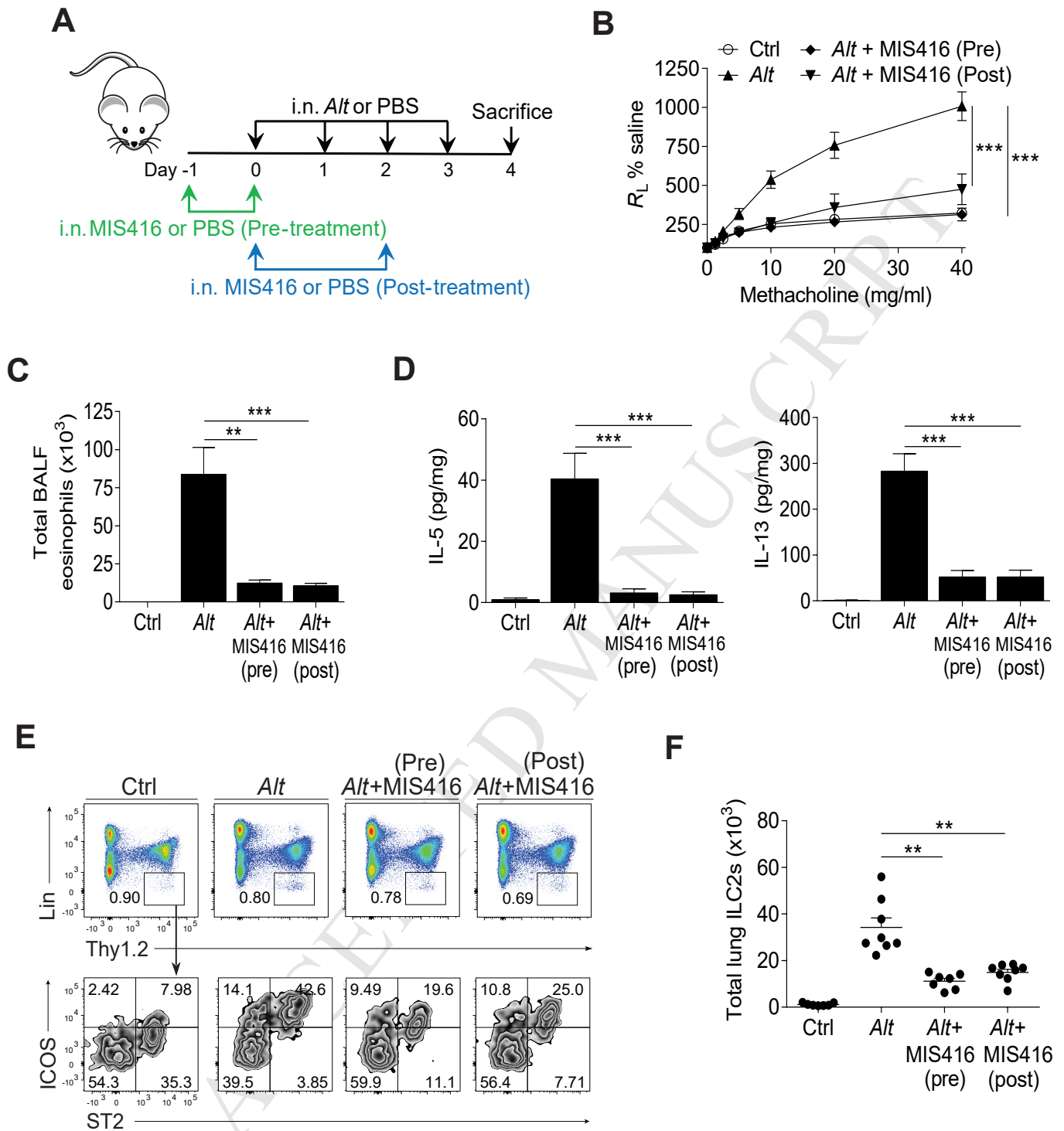


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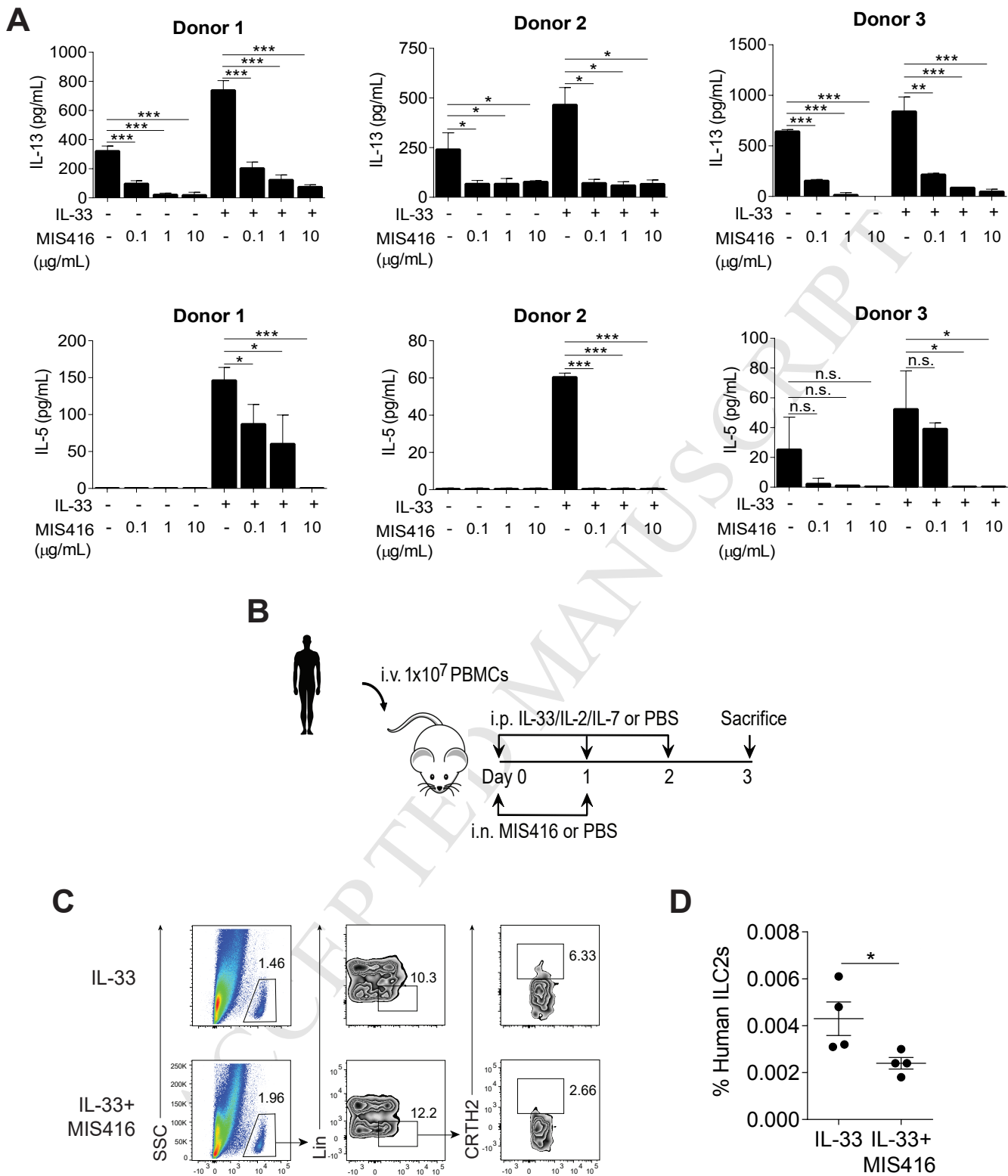


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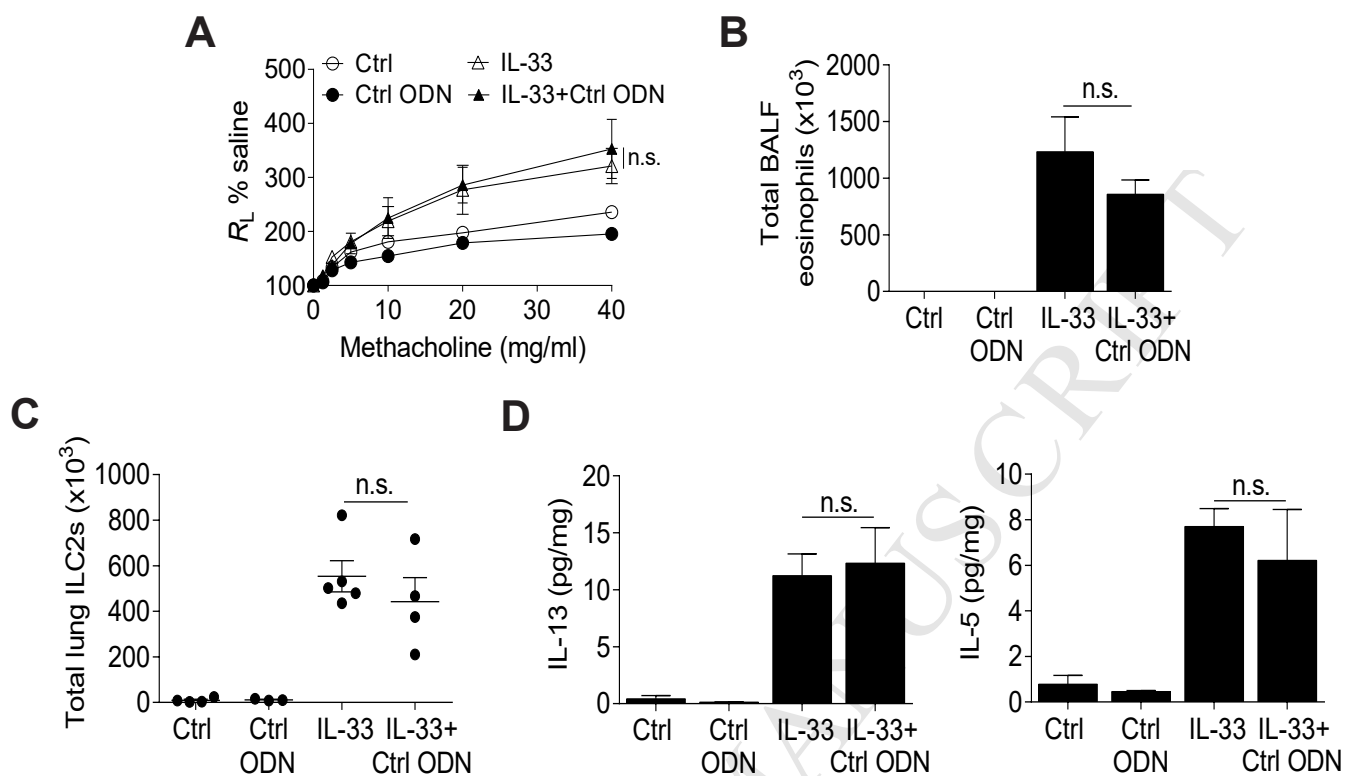


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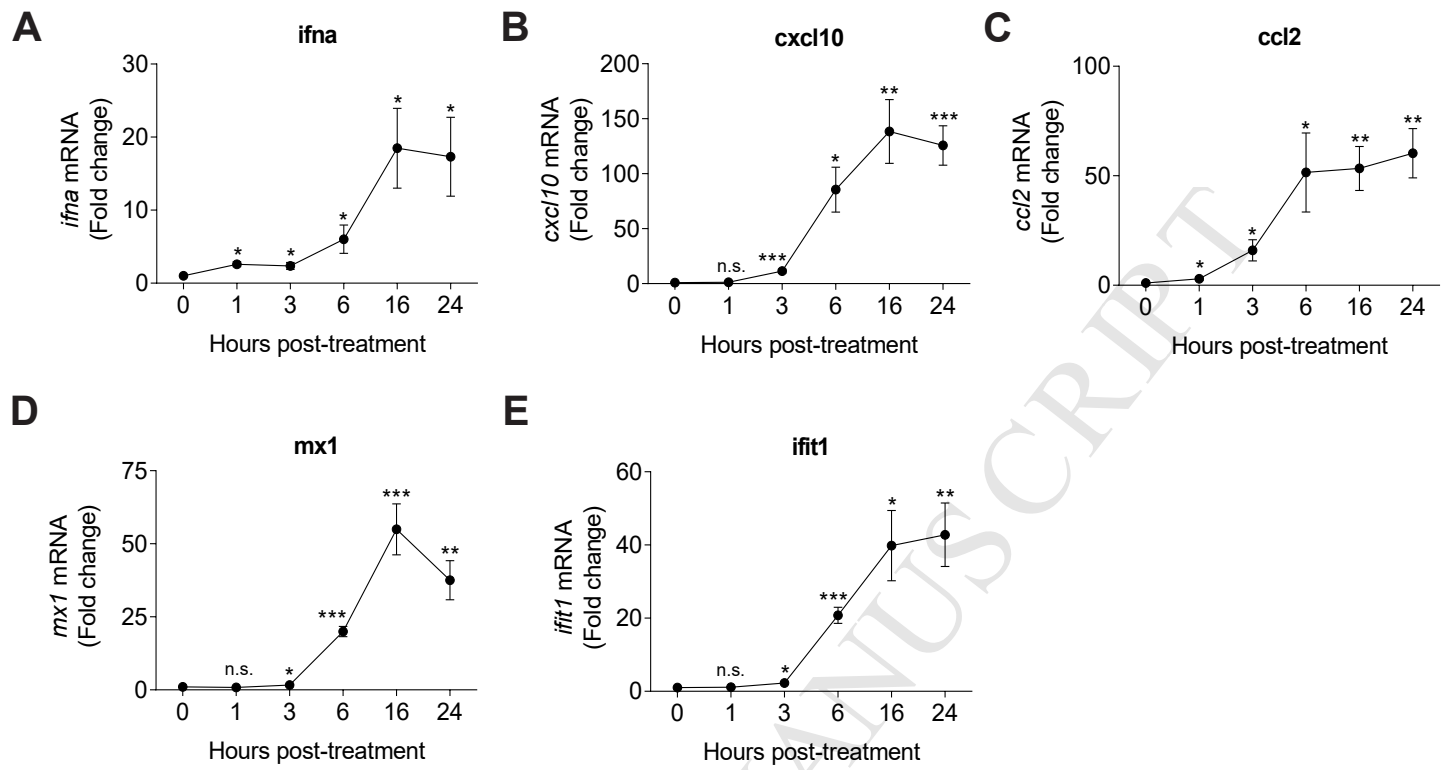
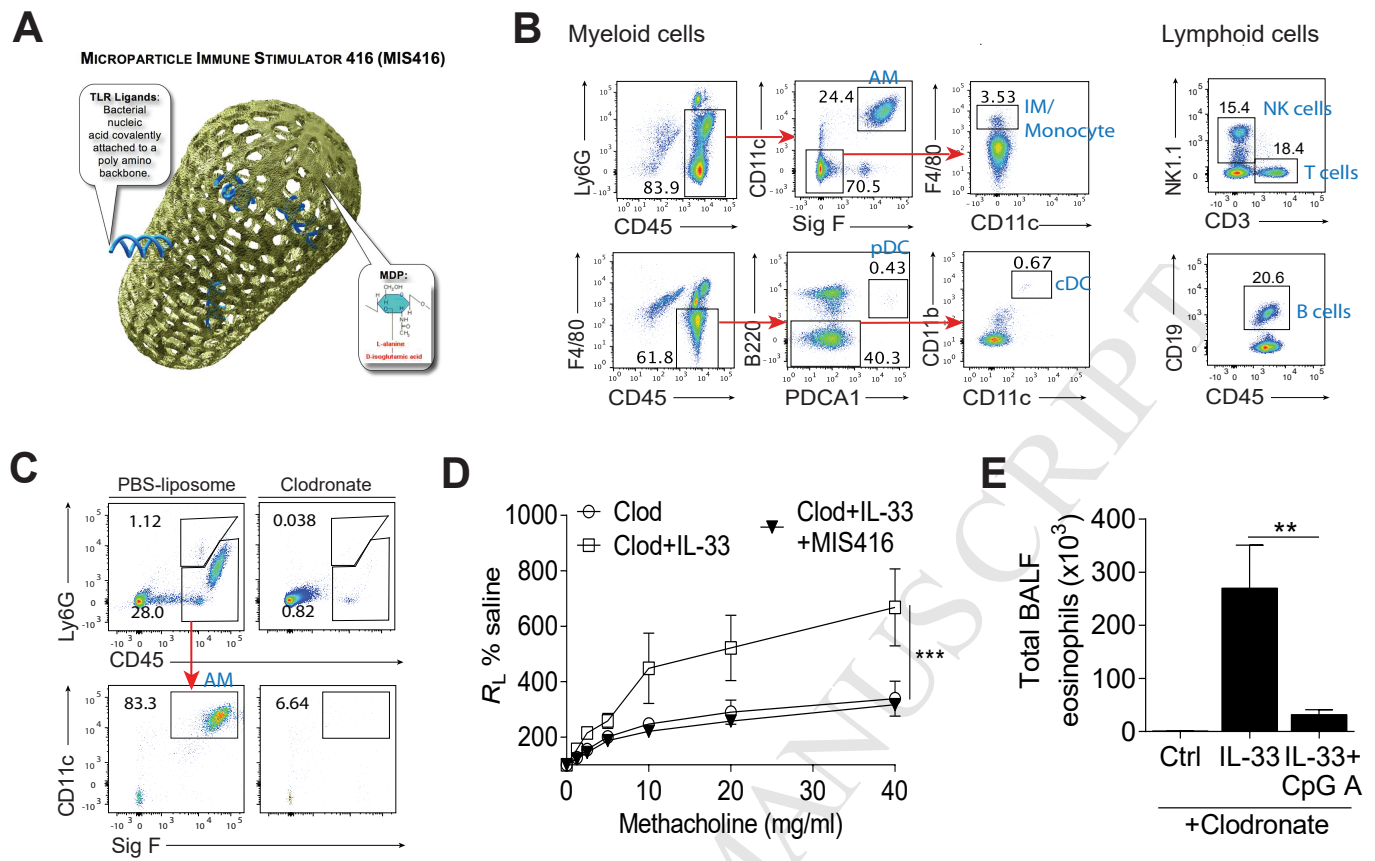


Figure E3



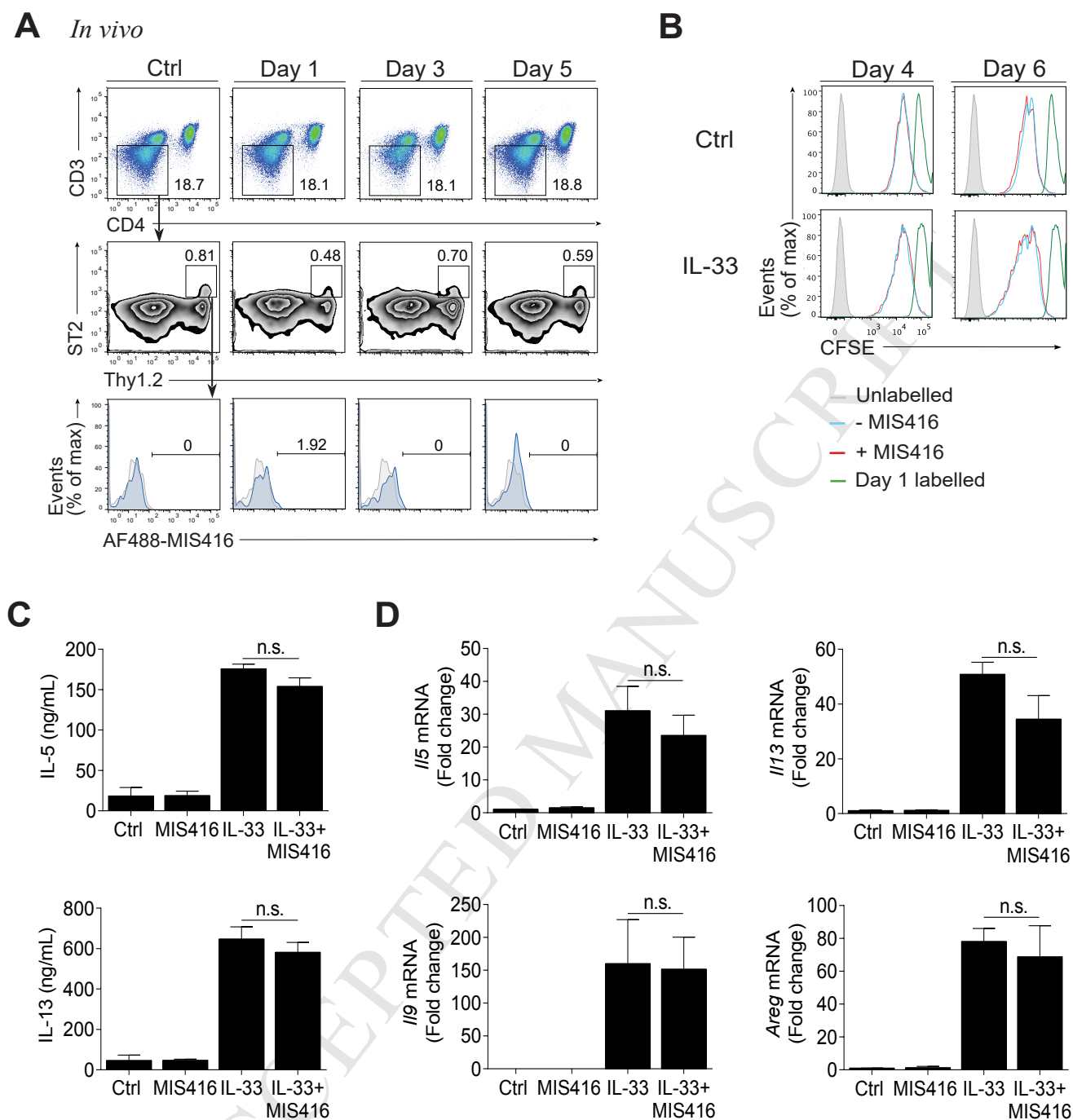


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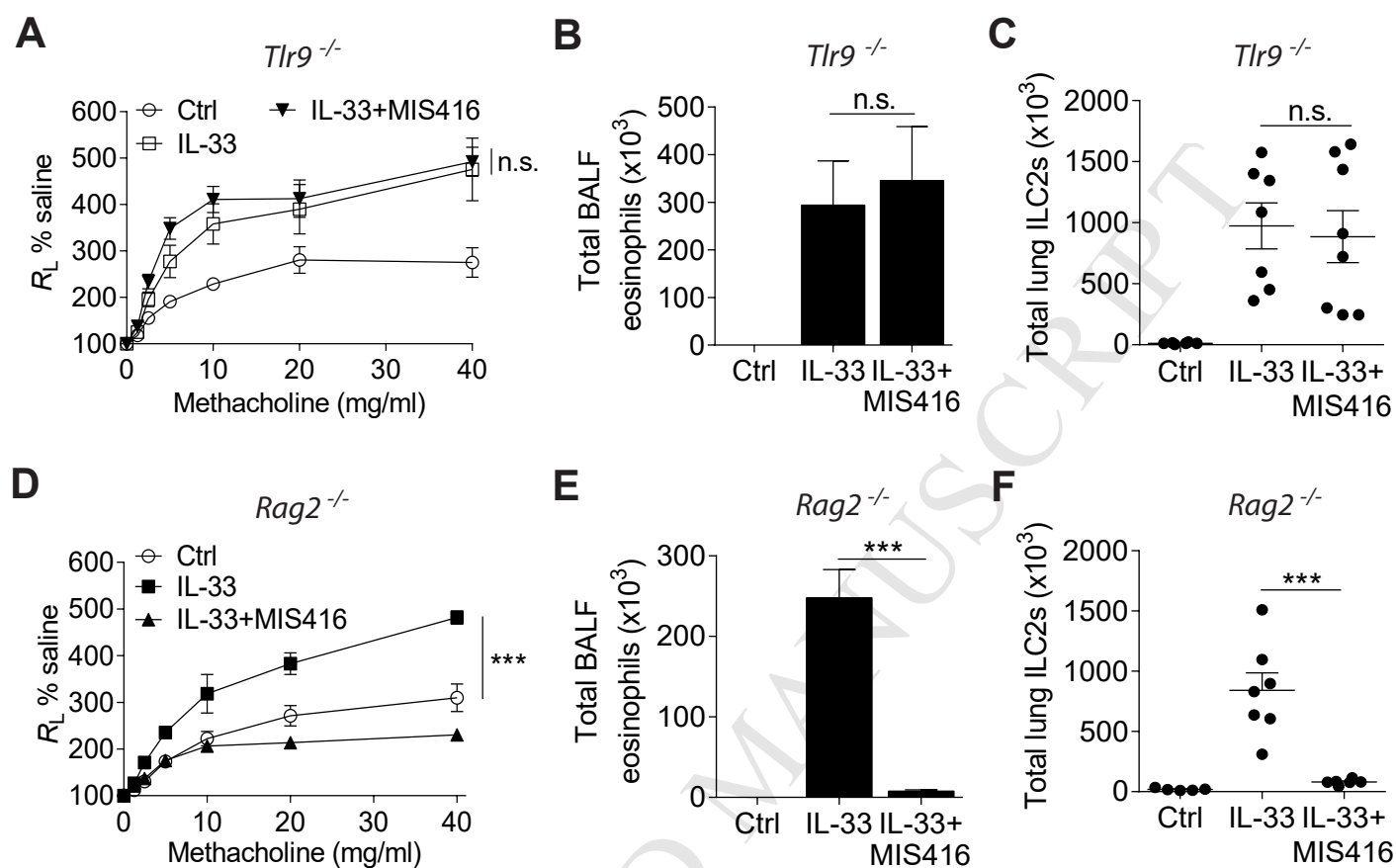


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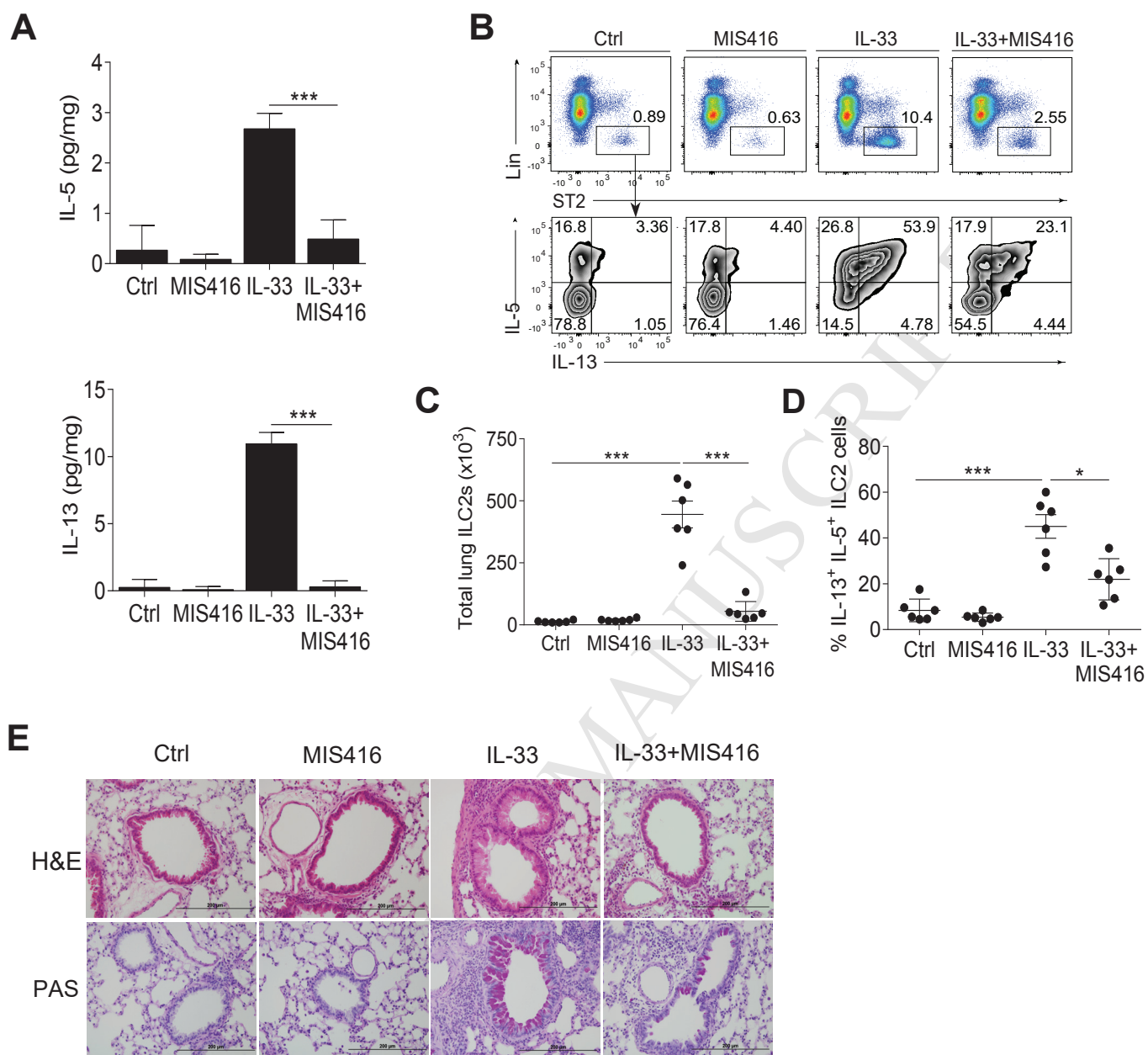
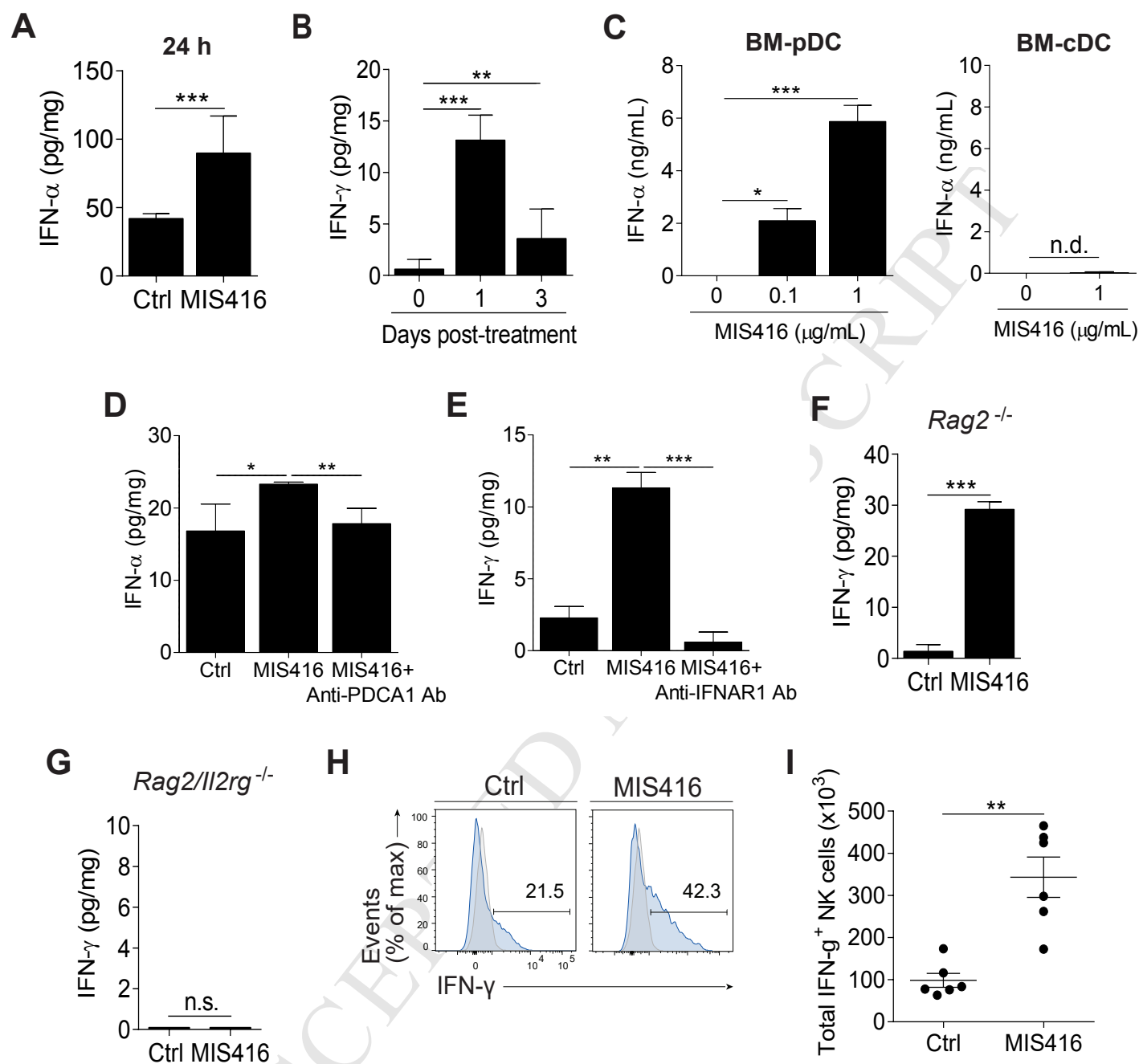


Figure E7



ONLINE REPOSITORY

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Original Article

TLR9-dependent interferon production prevents group 2 innate lymphoid cell-driven airway hyperreactivity

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METHODS

Collection of bronchoalveolar lavage fluid (BALF)

Mice were tracheotomized and BALF was collected by instilling 1 mL PBS supplemented with 2% fetal calf serum (FCS) (HyClone, Logan, UT) (PBS/2% FCS) through the tracheal cannula, followed by aspiration and a second lavage of the same volume. BALF was pooled and pelleted by centrifugation at 400 x *g* for 5 min. BALF cells were resuspended in PBS/2% FCS and fixed onto cytopsin slides via centrifugation. Slides were stained with Diff-Quik solution (Polysciences Inc, Warrington, PA) and BALF differential cell count was performed.

Antibodies, cytokines and reagents

All antibodies were obtained from Biolegend unless specified otherwise. The following antibodies were used for flow cytometry staining of murine samples at 1:400 dilution, unless specified otherwise; CD3 ϵ (145-2C11), Fc ϵ RI (MAR-1), CD49b (DX5), CD11c (N418), CD11b (M1/70), F4/80 (BM8), CD127 (A7R34; 1:100), CD19 (6D5), Ly6G (1A8; 1:200), ST2 (DIH9; 1:200 dilution), B220 (RA3-6B2; 1:200 dilution), PDCA1 (927; 1:200 dilution), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), NK1.1 (PK136), ICOS (c398.4A; 1:200 dilution), CD127 (A7R34; 1:100 dilution), CD4 (GK1.5; 1:200 dilution), IFN- γ (XMG1.2; 1:50), and GATA3 (16E10A23; 1:20 dilution). Thy1.2 (53-2.1), CD69 (H1.2F3; 1:200 dilution), Tbet (eBio4B10; 1:50 dilution), Eomes (Dan11mag; 1:100 dilution), IL-13 (eBio13A; 1:50 dilution), and IL-5 (TRFK5; 1:50 dilution) were purchased from eBioscience (San Diego, CA). Siglec F (E50-2440; 1:200 dilution) was purchased from BD Biosciences (Mountain View, CA). Human ILC2s were stained with the following antibodies at 1:20 dilution, unless specified otherwise; Human lineage cocktail (CD3, CD14, CD16, CD19, CD20, CD56), Fc ϵ RI (CRA1), CD3 (HIT3a), TCR β (IP26), CD45 (HI30), CRTH2 (BM16),

and CD127 (A019D5) (eBioscience). Fluorochrome-conjugated fixable viability dye (FVD) and Ki-67 were purchased from eBioscience. Mouse (anti-CD16/32) and human TruStain FcX (Fc blocking solution) were purchased from Biolegend. Recombinant mouse IL-33 and IL-2 were purchased from Biolegend whereas recombinant mouse IL-7 was obtained from Peprotech (Rocky Hill, NJ). Recombinant human IL-33 and IL-7 were purchased from Biolegend whereas IL-2 was purchased from eBioscience. Anti-mouse IFN γ (XMG1.2), anti-PDCA1 (BST2) and anti-IFNAR1 (MAR1-5A3) and were purchased from BioXcell (West Lebanon, NH). Phorbol 12-myristate 13-acetate (PMA) and ionomycin were obtained from Sigma-Aldrich (St Louis, MO). Brefeldin A, Annexin V Apoptosis Detection kit, and BD Cytofix/Cytoperm™ kit were purchased from BD Biosciences. Foxp3 Transcription Factor Staining kit was purchased from eBioscience. Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Cayman Chemical (Ann Arbor, MI).

Lung sample processing for flow cytometry

Whole lungs were minced and incubated in DMEM medium containing 0.1% (vol/vol) DNase I (Worthington Biochemicals, Lakewood, NJ) and 1.6 mg/mL collagenase IV (Worthington Biochemicals) for 40 min at 37°C. Tissues were then filtered through a 70- μ m mesh to obtain single cell suspensions. Red blood cells were removed using ACK lysing buffer (Gibco Laboratories, Grand Island, NY) and single cell suspensions were resuspended in the appropriate buffer for further processing.

Flow cytometry

For surface staining, single-cell suspensions were first stained with FVD, followed by pre-incubation with anti-mouse CD16/32 or anti-human TruStain FcX blocking antibody. Cells were then stained with the appropriate surface antibodies. For intracellular cytokine staining,

single cell suspensions from the lungs of mice were stimulated with 100 ng/mL PMA, 1 μ g/mL ionomycin, and 1 μ g/mL Brefeldin A in DMEM for 6 hours. After surface staining, cells were fixed and permeabilized with BD Cytofix/Cytoperm solution, and further stained intracellularly with anti-mouse IL-13, IL-5, or IFN- γ . For Ki-67 and transcription factor staining, Foxp3 staining buffer set was used according to the manufacturer's instructions. For detection of apoptotic cells, cells were stained using Annexin V Apoptosis Detection kit according to the manufacturer's recommendations. After staining, cells were washed and resuspended in PBS/2% FCS and analyzed by flow cytometry. Data were acquired on LSR II (BD Biosciences) and analyzed with FlowJo v.10.1 software (TreeStar).

***In vitro* ILC2 culture**

Freshly sorted lung ILC2s were cultured as previously described,¹ with modifications. Briefly, ILC2s were expanded for 6 days in RPMI 1640 supplemented with 10% FBS, IL-33, IL-2, and IL-7 (all at 10 ng/mL). Cells were rested for 3 days in complete RPMI 1640 medium supplemented with IL-2 and IL-7 (both at 10 ng/mL) prior to each experiment.

CFSE labelling

For CFSE labelling, cultured ILC2s (1.5×10^4 cells/well in 150 μ L of culture medium) were incubated with 5 μ M CFSE for 5 days according to the manufacturer's protocol.

Bone marrow-derived plasmacytoid and conventional DCs

pDCs and cDCs were derived from bone marrow (BM) cells as previously described^{2,3}, with modifications. In brief, BM cells were cultured in RPMI 1640 medium containing 200 ng/mL hu-Flt3 ligand for 9 days at a density of 1.5×10^6 cells/well, in 6-well plates. DCs were harvested by collecting non-adherent cells. DCs were sorted into CD11c⁺ B220⁺ CD11b⁻

(BM-pDCs) and CD11c⁺ B220⁻CD11b^{hi} (BM-cDCs) subsets (purity > 95%) and cultured in RPMI 1640 medium.

Adoptive transfer of ILC2s into CD45.1 mice

Lung ILC2s were sorted from WT or *Stat1*^{-/-} mice and expanded for 4 days in RPMI 1640 medium supplemented with IL-2, IL-7, and IL-33 (all at 10 ng/mL), followed by 3-day rest in RPMI 1640 medium containing only IL-2 and IL-7 (both at 10 ng/mL). Cells were adoptively transferred into CD45.1 mice through the intratracheal route at a density of 1×10^6 cells/mouse, 6 hours prior to the first intranasal MIS416 treatment. MIS416 was administered 1 day and 30 min before the first intranasal IL-33 treatment. IL-33 was administered daily for 3 days and mice were sacrificed 24 hours after the last IL-33 treatment. BALF cells were collected and analyzed by flow cytometry.

Lung histology

Lungs were fixed with 4% formaldehyde (Merck, Darmstadt, Germany) and gradually dehydrated with 30%, 50%, and 70% ethanol (J.T. Baker). Lungs were embedded in paraffin and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) stain. Images were acquired with an Olympus CX31 microscope (Olympus Corp, Tokyo, Japan).

Preparation of lung homogenate for ELISA

Whole lungs were minced and homogenized by sonication with Bioruptor® Plus (Diagenode, Liège, Belgium) in protein lysis buffer (containing 20 mM HEPES HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1% NP-40, 0.5% sodium deoxycholate, 1x protease cocktail inhibitor, and 1x phosphatase inhibitors II and III). Lung lysates were obtained by

centrifugation at 20,000 *g* for 30 min at 4°C and quantified using BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA).

ELISA

IFN- α , IFN- γ , IL-13, and IL-5 concentrations in either culture supernatants of murine or human ILC2s (7.5×10^4 cells/well in 150 μ L of culture medium in triplicates), lung homogenates or BALF of mice were determined using ELISA kits from Biolegend, with the exception of mouse IL-13 (eBioscience). Cytokine levels in lung homogenates were presented as amount of cytokines per mg of total lung protein.

Quantitative real-time PCR (qPCR) analysis

Total RNA from cultured ILC2s (1×10^6 cells/well in 1 mL of culture medium in triplicates) was extracted using Quick-RNATM MicroPrep (Zymo Research, Irvine, CA). One μ g of cDNA was synthesized using the High-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA), and qPCR was performed on TOptical 96 real-time PCR thermal cycler (Biometra, Göttingen, Germany). Reactions were run in triplicates and samples were normalized to GAPDH expression and expressed as fold change relative to control.

Multiplex beads assay analysis

Phosphorylation of Akt (pS473), ERK (pT185/pY187), JNK (pT183/pY185), p38 (pT180/pY182) and STAT1 (Tyr701) in ILC2 culture (2×10^6 cells/well in 2 mL of culture medium) was analyzed using MILLIPLEX[®] MAP kit (Millipore) according to the manufacturer's instructions. Data were acquired on the Bioplex[®] Suspension Array System

149 (Bio-Rad Laboratories, Hercules, CA), and analyzed using the Bioplex Manager™ 6.1
150 software.

FIGURE LEGENDS

Figure E1: Ctrl ODN does not inhibit IL-33-induced AHR and type 2 inflammation

BALB/c mice were pre-treated with ctrl ODN (5 µg) prior to intranasal administration of 0.1 µg IL-33, as in Figure 1A. **A**, Lung resistance in response to increasing doses of methacholine. **B**, Total eosinophils in BALF. **C**, Total number of lung ILC2s, assessed as in Figure 1E. **D**, IL-5 and IL-13 levels in lung homogenates of mice. Data are presented as means ± SEMs of 2 independent experiments (n = 3-5 each). *n.s.*, Not significant.

Figure E2: CpG A induces IFN-α and IFN-responsive mRNA expression

(A-E) C57BL/6 mice received 5 µg CpG A intranasally on day 0 and sacrificed at different time points as indicated. *Ifna* (A), *Cxcl10* (B) *Ccl2* (C), *Mx1* (D) and *Ifit1* (E) mRNA expression in the lungs of mice after treatment. Data are presented as means ± SEMs of 2 independent experiments (n = 6-7 each). *n.s.*, Not significant. **P*<0.05, ***P*<0.01 and ****P*<0.0001.

Figure E3: Alveolar macrophages are dispensable for the suppressive effect of MIS416

A, MIS416 structure. **B**, Gating strategy for myeloid cell subsets; AMs (CD45⁺ Ly6G⁻ CD11c⁺ Siglec F⁺), IM/monocytes (CD45⁺ Ly6G⁻ Siglec F⁻ CD11c⁻ F4/80⁺), pDC (CD45⁺ F4/80⁻ B220⁺ PDCA1⁺) and cDC (CD45⁺ F4/80⁻ B220⁻ PDCA1⁻ CD11c⁺ CD11b⁺) and lymphoid cell subsets; T cells (CD45⁺ CD3⁺), B cells (CD45⁺ CD19⁺), and NK cells (CD45⁺ CD3⁻ NK1.1⁺). Non-hematopoietic cells are defined as CD45⁻ cells. All subsets are gated from viable cells. **C**, Representative flow diagram showing AM population (CD45⁺ Ly6G⁻ CD11c⁺ SigF⁺) in BALF of mice 48 hours after treatment with PBS-liposome (vehicle) or clodronate liposome (250 µg). **D-E**, BALB/c mice were given 250 µg/mouse of PBS-liposome (vehicle) or clodronate liposome, 3 days and 1 hour before MIS416 treatment.

MIS416 and IL-33 were administered as in Figure 1A, with MIS416 being used in replacement of CpG A. **D**, Lung resistance in response to increasing doses of methacholine. **E**, Total eosinophils in BALF. Data are presented as means $\pm$ SEMs of 2 independent experiments ($n = 3-5$ each). *n.s.*, Not significant. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$.

Figure E4: MIS416 does not act directly on ILC2s

A, Representative flow diagram showing MIS416 uptake by ILC2s ($CD3^- CD4^- Thy1.2^+ ST2^+$) from BALB/c mice treated with 50 μ g of AF488-MIS416 for 1, 3, or 5 days. Grey solid lines: Isotype-matched control and blue solid lines: Antibody staining. **B-D**, ILC2s were sorted from the lungs of IL-33-treated *Rag2^{-/-}* mice and cultured with 10 ng/mL IL-33 in the absence or presence of 20 μ g/mL MIS416. All cultures contained IL-2 and IL-7 (Both at 10 ng/mL). **B**, Proliferation of ILC2s, assessed by CFSE after 4- or 6-day treatment. **C**, IL-13 and IL-5 levels in the supernatants of cultured ILC2s after 48-hour treatment. **D**, *Il13*, *Il5*, *Il9* and *Areg* mRNA expression after 6-hour treatment. Data are shown as means $\pm$ SDs from 1 of 3 independent experiments ($n = 3$ wells) with consistent findings. *n.s.*, Not significant.

Figure E5: MIS416-mediated inhibition is dependent of TLR9 but independent of adaptive immunity

A-C, *Tlr9^{-/-}* mice received 50 μ g MIS416 and 0.1 μ g IL-33 as in Figure 1A, with MIS416 being used in replacement of CpG A. **A**, Lung resistance in response to increasing doses of methacholine. **B**, Total eosinophils in BALF. **C**, Total number of lung ILC2s, assessed as in Figure 1E. **D-F**, *Rag2^{-/-}* mice received 50 μ g MIS416 and 0.1 μ g IL-33 through the intranasal route. **A**, **D**, Lung resistance in response to increasing doses of methacholine. **E**, Total eosinophils in BALF. **F**, Total number of lung ILC2s, assessed as in Figure 1E. Data are

presented as means $\pm$ SEMs of 3 independent experiments ($n = 5-8$ each). *n.s.*, Not significant. *** $P < 0.001$.

Figure E6: MIS416 inhibits ILC2 accumulation and type 2 cytokine production

BALB/c mice received 50 μ g MIS416 and 0.1 μ g IL-33 through the intranasal (i.n.) route as in Figure 1A, with MIS416 being used in replacement of CpG A. **A**, IL-5 and IL-13 levels in lung homogenates of mice. **B**, Representative flow diagram showing lung ILC2 population ($\text{Lin}^- \text{ST2}^+ \text{IL-13}^+ \text{IL-5}^+$), gated from viable CD45^+ cells. **C-D**, Total number of lung ILC2s ($\text{Lin}^- \text{ST2}^+$) (**C**) and percentage IL-13 $^+$ IL-5 $^+$ ILC2s (Gated from $\text{Lin}^- \text{ST2}^+$ cells) (**D**) per mouse, assessed as in **B**. **E**, Representative images of H&E- and PAS-stained histologic sections of the lungs of mice (*Scale bars* = 200 μ m). Data are shown as means $\pm$ SEMs of 2 independent experiments ($n = 6$ each). * $P < 0.05$ and *** $P < 0.0001$.

Figure E7: MS416 induces IFN- γ production by NK cells through pDC-derived IFN- α

A-B, BALB/c mice received 50 μ g MIS416 intranasally and sacrificed at the indicated time-points. IFN- α (**A**) and IFN- γ (**B**) levels in lung homogenates of mice. **C**, IFN- α level in the supernatant of BM-pDCs and BM-cDCs treated with MIS416 for 24 hours at the indicated concentrations. **D**, IFN- α level in lung homogenates of BALB/c mice receiving 500 μ g anti-PDCA1 or control IgG antibody (Ab) intraperitoneally, 24 hours and 30 min prior to MIS416 treatment (50 μ g). Mice were sacrificed 24 hours later. **E**, IFN- γ level in lung homogenates of BALB/c mice receiving 500 μ g anti-IFNAR1 or control IgG Ab and MIS416 as in **D**. **F-G**, IFN- γ level in lung homogenates of *Rag2* $^{-/-}$ (**F**) and *Rag2/Il2rg* $^{-/-}$ (**G**) mice receiving MIS416 as in **A**. **H**, Representative flow diagram showing IFN- γ $^+$ NK cells (Gated as $\text{NK1.1}^+ \text{T-bet}^+ \text{Eomes}^+$), gated from viable $\text{CD45}^+ \text{CD3}^-$ cells. **I**, Total IFN- γ $^+$ NK cells, assessed as in **H**.

225 Data are presented as means $\pm$ SEMs of 3 independent experiments (n = 5-6 each). *n.s.*, Not
226 significant. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

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