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TLR3-driven IFN-β antagonizes STAT5-activating cytokines and suppresses innate type 2 response in the lung

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Abstract:	Background Group 2 innate lymphoid cells (ILC2s) are involved in type 2 immune responses in mucosal organs and are associated with various allergic diseases in humans. Studies are needed to understand the molecules and pathways that control ILC2s. Objective The aim of this study was to develop a mouse model that limits the innate type 2 immune response in the lung and to investigate the immunologic mechanisms involved in regulation of lung ILC2s. Methods Naïve BALB/c mice were administered various toll-like receptor (TLR) agonists and exposed intranasally (i.n.) to the fungal allergen Alternaria alternata . The mechanisms of ILC2 suppression were investigated using gene knockout mice and a blocking antibody. Cellular and molecular mechanisms were analyzed using cultures of lung cells and isolated lung ILC2s. Results Among the TLR agonists tested, polyinosinic-polycytidylic acid [poly (I:C)] effectively inhibited innate type 2 response to A. alternata . Poly (I:C) promoted production of interferon (IFN)-α, -β, and -γ, and its inhibitory effects were dependent on the IFN-α/β receptor pathway. IFN-β was 100-times more potent than IFN-α at inhibiting type 2 cytokine production by lung ILC2s. Signal transducer and activator of transcription 5 (STAT5)-activating cytokines, including interleukin-2 (IL-2), IL-7, and thymic stromal lymphopoietin (TSLP), but not IL-33, promoted survival and proliferation of lung ILC2s in vitro , while IFN-β blocked these effects. Expression of the transcription factor GATA3, which is critical for differentiation and maintenance of ILC2s, was inhibited by IFN-β.

	Conclusion IFN-β blocks the effects of STAT5-activating cytokines on lung ILC2s and inhibits their survival and effector functions. Administration of IFN-β may provide a new strategy to treat diseases involving ILC2s.

TLR3-driven IFN-β antagonizes STAT5-activating cytokines and suppresses innate type 2 response in the lung

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ABSTRACT

Background: Group 2 innate lymphoid cells (ILC2s) are involved in type 2 immune responses in mucosal organs and are associated with various allergic diseases in humans. Studies are needed to understand the molecules and pathways that control ILC2s.

Objective: The aim of this study was to develop a mouse model that limits the innate type 2 immune response in the lung and to investigate the immunologic mechanisms involved in regulation of lung ILC2s.

Methods: Naïve BALB/c mice were administered various toll-like receptor (TLR) agonists and exposed intranasally (i.n.) to the fungal allergen *Alternaria alternata*. The mechanisms of ILC2 suppression were investigated using gene knockout mice and a blocking antibody. Cellular and molecular mechanisms were analyzed using cultures of lung cells and isolated lung ILC2s.

Results: Among the TLR agonists tested, polyinosinic-polycytidylic acid [poly (I:C)] effectively inhibited innate type 2 response to *A. alternata*. Poly (I:C) promoted production of interferon (IFN)-α, -β, and -γ, and its inhibitory effects were dependent on the IFN-α/β receptor pathway. IFN-β was 100-times more potent than IFN-α at inhibiting type 2 cytokine production by lung ILC2s. Signal transducer and activator of transcription 5 (STAT5)-activating cytokines, including interleukin-2 (IL-2), IL-7, and thymic stromal lymphopoietin (TSLP), but not IL-33, promoted survival and proliferation of lung ILC2s *in vitro*, while IFN-β blocked these effects. Expression of the transcription factor GATA3, which is critical for differentiation and maintenance of ILC2s, was inhibited by IFN-β.

Conclusion: IFN-β blocks the effects of STAT5-activating cytokines on lung ILC2s and inhibits their survival and effector functions. Administration of IFN-β may provide a new strategy to treat diseases involving ILC2s.

Key Messages

- Poly (I:C) inhibits allergen-induced innate type 2 responses in the lung that are mediated by ILC2s.
- The inhibitory effects of poly (I:C) are dependent on the IFN-α/β receptor pathway.
- IFN-β inhibits proliferation and survival of lung ILC2s induced by STAT5-activating cytokines.
- The ability of IL-7 to promote GATA3 expression in ILC2s is blocked by IFN-β.

Capsule Summary

Using mouse models, Tei *et al.* demonstrate that IFN-β effectively inhibits proliferation and survival of lung ILC2s that are promoted by STAT5-activating cytokines and suppresses allergen-induced innate type 2 responses in the lung.

Keywords

Group 2 innate lymphoid cell, lung, poly (I:C), IL-5, IL-7, IL-13, IL-33, TSLP, IFN-β

Abbreviations

7-AAD: 7-aminoactinomycin D; Ab: antibody; BAL: bronchoalveolar lavage; CFSE: carboxyfluorescein succinimidyl; FACS: fluorescence-activated cell sorting; FBS: fetal bovine serum; GAS: IFN-γ-activated site; IFNAR: interferon-α/β receptor; IFNGR: interferon-γ receptor; ILC2: group 2 innate lymphoid cell; IL: interleukin; i.n.: intranasally; i.p.: intraperitoneally; ISRE: IFN-stimulated response; Lin^-: lineage-negative; NK: natural killer; PBS: phosphate-buffered saline; pDCs: plasmacytoid dendritic cells; poly (I:C): polyinosinic-polycytidylic acid; PRRs: pattern recognition receptors; RT: room temperature; SEM: standard error of the mean; STAT: signal transducer and activator of transcription; Th2: type 2 helper T; TLRs: toll-like receptors; TSLP: thymic stromal lymphopoietin; WT: wild-type

INTRODUCTION

The chronic inflammation of airways observed in allergic asthma is characterized by increased expression of type-2 cytokines, such as interleukin (IL)-5 and IL-13, and infiltration of eosinophils.[1] Multiple cell types coordinate the inflammatory response, including $CD4^+$ T cells, mast cells, eosinophils, and neutrophils.[2] Group 2 innate lymphoid cells (ILC2s) recently were found to reside in mucosal organs and contribute to type 2 immune responses and tissue inflammation.[3, 4, 5] ILC2s do not express antigen-specific receptors; instead, they are directly activated by cytokines, such as IL-33, IL-25, and thymic stromal lymphopoietin (TSLP), derived from epithelial cells and other cell types, and rapidly produce large quantities of IL-5 and IL-13.[6, 7] ILC2s also promote the development of antigen-specific type 2 helper (Th2) $CD4^+$ T cells.[8, 9] Increased numbers and activation of ILC2s are associated with asthma, allergic rhinitis, and chronic rhinosinusitis (CRS) in humans.[7] More recently, glucocorticoid-resistant ILC2s have been found to be increased in airway tissues and peripheral blood in patients with asthma and correlated with the severity of disease.[10] Furthermore, human asthma is associated with polymorphisms of genes related to ILC2s, including *IL33*, *IL1RL1*, *IL7R*, *RORA*, and *IL2RB*.[11, 12, 13] These previous studies provide abundant and important information to explain how ILC2s are activated and how they might be involved in the pathophysiology of human diseases. However, less is known about the molecules and mechanisms that regulate or suppress ILC2s.

Pattern recognition receptors (PRRs) recognize microorganisms and their components and promote anti-microbial immunity and Th1- or Th17-type adaptive immune responses, while potentially antagonizing the Th2-type immune responses associated with allergic asthma.[14] A recent study showed that an environment rich in microbes was associated with lower rates of asthma in humans[15], and environmental endotoxin has been shown to protect against allergic

immune responses.[16] Toll-like receptors (TLRs) are PRRs and recognize a variety of microbial components, such as lipopolysaccharide (recognized by TLR4), lipopeptides (recognized by TLR2/1 and TLR2/6), flagellin (recognized by TLR5), unmethylated CpG motifs in DNA (recognized by TLR9), and RNA (recognized by TLRs 3, 7, and 8).[17] More recently, TLR activation was found to suppress ILC2-mediated innate type 2 immune responses. For example, administration of TLR agonists, such as TLR7/8 agonist R848 and TLR9 agonist CpG, inhibited IL-33- or allergen-induced innate type 2 responses in mouse lungs.[18, 19] Interferon (IFN)-α produced by plasmacytoid dendritic cells (pDCs) likely contributes to the inhibitory effects of TLR agonists by suppressing ILC2s directly[18] or by promoting IFN-γ production by natural killer (NK) cells.[19] These observations suggest that TLR agonists could be used to examine the immunological mechanisms controlling ILC2s.

The objective of this project was to identify the molecules and pathways that suppress lung ILC2s. Following in the footsteps of previous studies, we took a straightforward approach by administrating various TLR agonists into the airway of naive mice and examining the responses to the airborne allergen fungus *Alternaria alternata*. We found that IFN-β, which is induced by poly (I:C), inhibits the action of signal transducer and activator of transcription 5 (STAT5)-activating cytokines, such as IL-7 and TSLP, which promote survival and proliferation of lung ILC2s. The results provide new insight into how homeostasis and activation of ILC2s are controlled in the lung tissues and suggest potential strategies that could be used to regulate ILC2s in asthma and other allergic airway diseases.

METHODS

Mice

Wild-type (WT) BALB/c, C57BL/6, *Ifngr1*$^{-/-}$, and *Ifnar1*$^{-/-}$ mice (C57BL/6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME). The WT C57BL/6 mice and *Ifngr1*$^{-/-}$ or *Ifnar1*$^{-/-}$ mice were housed in the same room at least for 1 week before the start of experiments. The IL-5-reporter C.129S4(B6)-*Il5*^{tm1Ktk} (*Il5*^{Venus}) mice[20] were a gift from Dr. Kiyoshi Takatsu, Toyama University, Japan, and were maintained in the Mayo Clinic animal facility. All mice used in the experiments were female and in the age range of 6–12 weeks. All animal experiments and handling procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee and performed according to the Committee guidelines.

Reagents

Fluorescently labeled antibodies (Abs) to CD3 (145-2C11), CD25 (PC51), CD44 (IM7), CD16/CD32 (2.4G2), CD14 (rmC5-3), CD45R/B220 (RA3-6B2), and IgG2a isotype control were purchased from BD Biosciences (San Jose, CA). Fluorescently labeled Abs to GATA3 (TWAJ) and IgG1 isotype control were purchased from eBioscience (San Diego, CA). Fluorescently labeled Annexin-V, IgG2b isotype control, and 7-AAD viability staining solution were purchased from BioLegend (San Diego, CA). Ghost Dye™ Red 780 was purchased from TONBO Biosciences (San Diego, CA). Recombinant mouse proteins, including IL-2, IL-7, IL-25, TSLP, and IFN-β were from R&D Systems (Minneapolis, MN). Mouse IL-33 and IFN-α2 were from eBioscience, and mouse IFN-γ was from PeproTech (Rocky Hill, NJ). High molecular weight poly (I:C), CpG A (ODN1585), and R848 were from InvivoGen (San Diego, CA). The culture filtrate extract of *A. alternata* was from Greer Laboratories (Lenoir, NC); the extract

contained detectable, but minimal, amounts of endotoxin (i.e., 3 ng endotoxin/mg extract). Anti-IFNAR1 (MAR1-5A3) and isotype-matched control IgG for blocking experiments were purchased from BioXcell (West Lebanon, NH).

Mouse models of innate type 2 immune responses

Generally, naïve BALB/c or C57BL/6 mice were administered TLR agonists intranasally (i.n.) and then exposed i.n. to *A. alternata* extract. The timing and frequency for administration of TLR agonists and *A. alternata* were optimized for the purpose of each experiment. We collected bronchoalveolar lavage (BAL) fluids and lung tissues for immunologic analyses. The trachea was cannulated to collect BAL fluids, and lavage was performed in triplicate using Hank's Balanced Salt Solution (HBSS; 0.5, 0.25, and 0.25 ml, respectively). Cell numbers were counted, and differentials were determined in cytospin preparations stained with Wright-Giemsa stain. More than 200 cells were counted using conventional morphologic criteria. The BAL fluid supernatants were stored at -20 °C for cytokine assays. The lungs were homogenized in 0.5 ml of PBS, and centrifuged at 10,000 × g at 4 °C for 15 min. The supernatants were analyzed for total protein concentration with the Pierce™ BCA Protein Assay kit (Thermo Fisher, Rockford, IL) and for cytokine levels (see below).

To examine the effects of TLR agonists on production of type 1 and type 2 IFNs in the lung, naïve BALB/c mice were administrated 25 μg of poly (I:C), R848, or CpG A intranasally (i.n.), and lungs were collected after 6 h or at times indicated. IFN-α, IFN-β, and IFN-γ levels in lung tissues were analyzed by ELISA. To examine the effects of TLR agonists on innate type 2 responses, naïve BALB/c mice or *Il5*^{Venus} reporter mice were pretreated i.n. once with poly (I:C) or PBS (as a control) at 24 h prior to the administration of *A. alternata*. Mice were then

administered 50 μg of *A. alternata* extract i.n., and BAL fluids and lungs were collected 4.5 h later. To examine the roles of IFN receptors, naïve BALB/c mice were administered anti-IFNAR1 or control IgG i.n. (50 μg) and intraperitoneally (i.p.) (250 μg) together with i.n. poly (I:C) (25 μg). After 24 h, mice were administered *A. alternata* extract i.n. and euthanized 4.5 h later. Alternatively, naïve WT C57BL/6 mice or *Ifngr1*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice were administered poly (I:C) i.n. 24 h prior to administration of *A. alternata* extract. To examine the direct effects of IFN-β, naïve BALB/c mice were also administered IFN-β (1 μg/dose) or PBS i.n. for 3 consecutive days prior to administration of *A. alternata* extract. Finally, in some experiments, naïve BALB/c mice were administered poly (I:C) and *A. alternata* extract i.n. every 3 days for 6 days. Mice were euthanized 24 h after the last administration of *A. alternata* extract, and BAL fluids and lungs were collected for analyses.

Lung single cell culture

Lung single cell culture was used to examine ILC2 cytokine production in the presence of other immune cells in the lung. Lungs were collected from naïve BALB/c mice, and lung single-cell suspensions were obtained in buffer containing a cocktail of collagenases (Liberase TM, Roche Diagnostics, Indianapolis, IN) and Gentlemax Dissociator (Milteny Biotec) as described previously.[21] Red blood cells were lysed with ammonium chloride/potassium lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA), and cells were resuspended in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (FBS) (RPMI 1640 medium). To examine the cytokine production, lung single cell suspensions were cultured at 1.0×10^6 cells/ml with 10 ng/ml IL-33 with or without serial dilutions or 100 ng/ml for each of IFN-α, IFN-β, IFN-γ, or IFN-λ in 48-well tissue culture

plates for 4 days at 37 ºC and 5% CO_2. Supernatants were collected and analyzed for IL-5 and IL-13 by ELISA.

Lung ILC2 sorting and culture

To isolate lung ILC2s, naive BALB/c mice were injected i.p. with a cocktail of IL-25 and IL-33 (400 ng/dose each), once daily for 3 days. Twenty-four hours after the last injection, lungs were collected, and a lung single-cell suspension was generated as described above. In some experiments, lungs were collected from naïve BALB/c mice without prior treatment with cytokines. Cells was enriched for ILC2s using EasySep Mouse ILC2 Enrichment Kit (STEMCELL Technologies, Vancouver BC, Canada) according to manufacturer's protocol. After staining with FITC-conjugated Abs to CD3, CD14, CD16/CD32, B220, PerCP Cy5.5-conjugated anti-CD44, and APC-conjugated anti-CD25, lung ILC2s were sorted by fluorescence-activated cell sorting (FACS) using BD FACSAria® as lineage-negative (Lin^-)$CD25^+CD44^{high}$ cells as described previously.[21] Sorted lung ILC2s were cultured with indicated concentration of cytokines, including IL-33, IL-7, IL-2, TSLP, IFN-α, IFN-β, or IFN-γ, at 5.0×10^4 to 1.0×10^5 cells/ml in RPMI 1640 medium in round-bottomed 96-well tissue culture plates for up to 4 days. For the cell proliferation assay, sorted lung ILC2s were labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) before culture. After 4 days, CFSE dilution was analyzed using a BD FACSCanto® flow cytometer.

FACS analyses

Flow cytometry was used to analyze apoptosis and death of ILC2s as well as expression of GATA3 protein. After a 3-day culture or for another indicated period, lung ILC2s were stained

with fluorescently labeled Annexin-V and 7-AAD viability-staining solution following the protocol recommended by the manufacture. For analysis of GATA3 protein expression, cells were stained with Ghost Dye™ Red 780 fixable cell viability dye and permeabilized with a Foxp3/transcription factor staining kit (eBioscience). Cells were then staining with PE-conjugated anti-GATA3 Ab or control IgG and analyzed with a BD FACSCanto® flow cytometer by gating separately on Ghost Dye™ Red-positive or -negative cells.

NanoString and quantitative polymerase chain reaction (PCR) gene expression assays

For NanoString™ gene expression analysis, isolated lung ILC2s were cultured with medium alone or with 10 ng/ml IL-7 for 16 h. Total RNA was purified from ILC2s with TRIzol and PureLink RNA Mini Kit columns (Thermo Fisher Scientific, Waltham, MA). mRNA was probed with the nCounter® analysis platform (NanoString Technologies, Seattle, WA) by using a Mouse Immunology Profiling Panel and following the protocol recommended by the manufacture. Data were analyzed with the nSolver™ Analysis Software package.

For quantitative PCR, total RNA was purified from lung ILC2s with TRIzol and PureLink RNA Mini Kit columns as described above, and cDNA was reverse transcribed with iScript (Bio-Rad Laboratories, Hercules, Calif). mRNA transcripts for *Gata3* were quantified by real-time PCR using TaqMan Gene Expression Arrays and TaqMan Universal PCR Master Mix (Thermo Fisher Scientific) as per the manufacturer's instructions. Data were normalized to the levels of gene expression in ILC2s cultured with medium alone.

ELISAs

The levels of IL-5, IL-13, IFN-α, IFN-β, IFN-γ, and IFN-λ were measured by

Quantikine ELISA kits (R&D Systems). Cytokine concentrations in the cell supernatants were measured by DuoSet ELISA kits (R&D Systems) for IL-5 and IL-13. All ELISAs were performed as per manufacturer's instructions.

Statistics

Data are presented as the mean ± standard error of the mean (SEM) for the numbers of mice or experiments as indicated. Statistics were performed using paired and unpaired Student *t* test, ANOVA, or repeated measures ANOVA as appropriate for each set of experimental conditions; $p<0.05$ was considered significant.

RESULTS

TLR3 agonist poly (I:C) effectively inhibits innate type 2 immune response

Previous studies showed that the fungus *A. alternata* induces innate type 2 immune responses in the lungs of mice[21]. TLR agonists, such as R848 (TLR7/8 agonist) and CpG (TLR9 agonist), were shown recently to inhibit IL-33- or allergen-induced innate type 2 responses in mice.[18,19] To establish a mouse model to investigate the molecules and pathways that suppress ILC2s in the lung *in vivo*, we pretreated naïve BALB/c mice i.n. with two different doses (5 μg and 25 μg) of poly (I:C) (TLR3 agonist), R848, and CpG A. Twenty-four hours later, mice were exposed to *A. alternata* extract, and the lungs were analyzed for type 2 cytokines 4.5 h later (Fig. 1A). Administration of *A. alternata* increased lung levels of IL-5 and IL-13 (Fig. 1B), which is consistent with previous observations.[21, 22] These cytokine responses were significantly inhibited in mice pretreated with 5 μg of poly (I:C) ($p<0.05$) and further reduced in those treated with 25 μg of poly (I:C) ($p<0.05$). In this model, R848 showed modest effects, and CpG A inhibited the response only at the 25-μg dose ($p<0.05$).

To examine whether the inhibition by poly (I:C) is mediated by ILC2s, we examined IL-5venus reporter mice. Naïve *Il5*^{Venus} reporter mice were pretreated with poly (I:C) i.n. and then exposed i.n. to *A. alternata* extract. After 4.5 h, the expression of IL-5venus was analyzed as an indicator of *Il5* transcription by gating on the lung ILC2 population (Lin$^-$CD25$^+$CD44^{high}), as previously described.[21, 23] When mice were exposed to *A. alternata*, the proportion of IL-5-expressing ILC2s increased (Fig. 1C); however, IL-5-expressing ILC2s significantly decreased in mice pretreated with poly (I:C). Mean fluorescence intensity (MFI) verified that IL-5venus expression in ILC2s was significantly increased by *A. alternata* exposure, and this effect was suppressed by pretreatment with poly (I:C) (Fig. 1D, $p<0.05$).

We next examined whether poly (I:C) affects allergen-induced airway inflammation. Naïve BALB/c mice were exposed to poly (I:C) (50 μg/dose) and *A. alternata* (50 μg/dose) three times over a period of 6 days (Fig. 1E); poly (I:C) was administrated 1-day prior to each administration of *A. alternata*. When exposed to PBS or poly (I:C) alone, no eosinophils or neutrophils were detectable in BAL fluids (Fig. 1F); however, the number of eosinophils in the BAL fluid significantly increased in mice exposed to *A. alternata*, and the airway eosinophilia was nearly abolished in mice treated with poly (I:C) ($p<0.01$). A slight increase in neutrophils was also observed in mice administered *A. alternata* with poly (I:C) compared to those administered *A. alternata* alone. Similarly, the levels of IL-5 and IL-13 in lungs increased in mice exposed to *A. alternata* and were inhibited by administration of poly (I:C) (Fig. 1G, $p<0.05$). Taken together, these findings suggest that poly (I:C) effectively inhibits allergen-induced ILC2 production of type 2 cytokines and eosinophilic airway inflammation.

Type 1 interferons mediate the inhibitory effect of poly (I:C) in vivo

Given that poly (I:C) inhibits the ILC2-mediated innate type 2 immune response to *A. alternata* in the lung, we next investigated the mechanism. Previous studies showed that i.n. administration of R848 or CpG promotes production of IFN-α and IFN-γ in the lung[18, 19], so we first measured lung levels of type 1 and type 2 IFNs at 6 h after i.n. administration of poly (I:C), R848, or CpG A (25 μg for each). Roughly equal amounts of IFN-γ were produced by all three TLR agonists tested (Fig. 2A). Poly (I:C) also robustly induced both IFN-α and IFN-β, whereas R848 induced modest levels of these cytokines. In contrast, CpG A failed to induce IFNα or IFN-β. A kinetic study showed that when poly (I:C) was administered, the lung levels of IFN-α, IFN-β, and IFN-γ reached a plateau at 6 h and remained elevated for at least 24 h (Fig. 2B).

283 To evaluate which IFNs are involved in poly (I:C)-mediated suppression of ILC2s *in*
284 *vivo*, we used *Ifnar1*$^{-/-}$ and *Ifngr1*$^{-/-}$ mice that are deficient in receptors for IFN α/β and IFN-γ,
285 respectively (Fig. 2C). In WT C57BL/6 mice, lung levels of type 2 cytokines increased after i.n.
286 exposure to *A. alternata*, and poly (I:C) significantly reduced these cytokines ($p<0.01$).
287 Importantly, *Ifnar1*$^{-/-}$ mice reversed the inhibitory effect of poly (I:C) (Fig. 2D), suggesting a
288 critical role for the IFNAR1 pathway to inhibit the ILC2 response. In contrast, *Ifngr1*$^{-/-}$ mice
289 failed to reverse the inhibitory effects of poly (I:C) (Fig. 2E).
290 We verified the roles for IFNAR by using a blocking Ab. The WT BALB/c mice were
291 treated with anti-IFNAR1 Ab or control IgG at the same time as i.n. administration of poly (I:C)
292 (Fig. 2F) and then exposed to *A. alternata* extract 24 h later. *A. alternata*-induced type 2 cytokine
293 responses were inhibited by poly (I:C) in mice given isotype control IgG; however, the anti-
294 IFNAR1 blocking Ab reversed the inhibitory effects (Fig. 2G). Together, these results suggest
295 that type 1 IFNs, but not IFN-γ, mediate the inhibitory effects of poly (I:C) *in vivo*. Furthermore,
296 the involvement of type 1 IFNs was observed in both BALB/c and C57BL/6 mice, suggesting
297 that their effects are not limited to a certain mouse strain.
298
299 *IFN-β potently inhibits innate type 2 responses in the lung*
300 We next examined the effects of ligands for IFNAR, namely IFN-α and -β, on type 2
301 immune responses in the lungs by using *in vitro* and *in vivo* models. First, we obtained single-
302 cell suspensions of lungs from naïve BALB/c mice, which maintained the composition of various
303 cell types in the lung. Cells were cultured with IL-33, which promotes a ILC2-mediated type 2
304 cytokine response[21], with serial dilutions of IFNs for 4 days. Without IL-33, IL-5 and IL-13 were
305 undetectable in culture supernatants (data not shown). When cultured with IL-33 at 1 ng/ml,

large quantities of IL-5 and IL-13 were detectable, and the levels were reduced by IFN-β, with an IC_{50} of approximately 100 pg/ml for both IL-5 and IL-13 (Fig. 3A). IFN-β nearly abolished type 2 cytokines at 1 ng/ml. IL-33-induced cytokine production was also inhibited partially by IFN-α at 100 ng/ml, whereas lower concentrations of IFN-α appear to enhance type 2 cytokine response. When lung single cell cultures were treated with IL-33 at 10 ng/ml, even higher concentrations of IL-5 and IL-13 were detectable in the culture supernatants. IFN-β inhibited these cytokine responses with IC50 of <100 pg/ml. IFN-α also partially inhibited type 2 cytokine production in a concentration-dependent manner, but IFN-β was approximately 100-fold more potent than IFN-α.

We next examined the role of IFN-β in an innate type 2 response in an *in vivo* model. We administrated IFN-β i.n. for 3 consecutive days, and mice were then exposed i.n. to *A. alternata* extract (Fig. 3B). *A. alternata* induced robust production of type 2 cytokines at 4.5 h in both BAL fluids and lung tissues, and IFN-β significantly reduced the cytokine levels (Fig. 3C, $p<0.05$ and $p<0.01$). We also found that the number of ILC2s in the lung was significantly reduced in mice treated with IFN-β and exposed to *A. alternata* as compared to those treated with PBS and then exposed to *A. alternata* (Figs. 3D and 3E, $p<0.01$). These findings suggest that exogenous IFN-β inhibits type 2 cytokine production by lung ILC2s *in vitro* and *in vivo*, and that IFN-β is likely to be the key cytokine that mediates inhibition in mice treated with poly (I:C), as shown by its potency compared to IFN-α.

IFN-β inhibits ILC2 proliferation induced by STAT-5-activating cytokines

To address the mechanisms involved in regulation of ILC2s by IFN-β, we moved to *in*

vitro models using lung ILC2s purified by FACS. IL-7 is indispensable for development of ILCs[6, 24, 25] and often used in combination with IL-33 to stimulate cytokine production by ILC2s in culture.[26, 27] Therefore, we isolated ILC2s from the lungs of BALB/c mice and stimulated them with IL-33 alone or IL-33 plus IL-7 in the presence of IFN-β for 4 days; IFN-γ was used as a control. Although the amounts of type 2 cytokines produced by ILC2s stimulated with IL-33 alone or IL-7 alone were modest, IL-33 and IL-7 together induced large quantities of IL-5 and IL-13 (Supplemental Fig. E1). When ILC2s were stimulated with IL-33 alone, production of IL-5 and IL-13 was partially inhibited by IFN-γ, but not by IFN-β (Fig. 4A, $p<0.01$). In contrast, when ILC2s were stimulated with IL-33 plus IL-7, IFN-β significantly inhibited type 2 cytokine production in a concentration-dependent manner (Fig. 4B, $p<0.05$ and $p<0.01$). In this condition, IFN-β and IFN-γ at 100 ng/ml showed roughly comparable inhibitory effects.

These observations led us to speculate that the mechanisms of ILC2 suppression by IFN-β and IFN-γ are different and that IFN-β may control the effects of IL-7, rather than the effects of IL-33, on ILC2s. To address this question directly, we examined the proliferation of ILC2s by isolating lung ILC2s, labeling them with CFSE, and culturing the cells for 4 days with IL-7 or IL-33. As compared to medium alone, IL-7 induced proliferation of ILC2s, resulting in robust dilution of CFSE (Fig. 4C) over 4 days. Other STAT5-activating cytokines, including IL-2 and TSLP[28] , also induced ILC2 proliferation. In contrast, IL-33 alone induced minimal proliferation of ILC2s, and the combination of IL-33 and STAT5-activating cytokines showed comparable effects as STAT5-activating cytokines alone.

Given the robust activities of IL-7 and other STAT5-activating cytokines on ILC2 proliferation, we examined the effects of IFN-β and IFN-γ. CFSE-labeled ILC2s were cultured with each cytokine in the presence of interferons, and CFSE dilution was measured as an

indicator of ILC2 proliferation. Both IL-7 and IL-2 induced robust proliferation of ILC2s (Fig. 4D and 4E), which was inhibited by IFN-β nearly to the baseline level (i.e., medium alone, $P<0.01$). Although IFN-γ also inhibited IL-2- and IL-7-induced proliferation of ILC2s, its effects were weaker than those by IFN-β, especially when ILC2s were cultured with IL-2. TSLP induced modest proliferation of ILC2s, which was inhibited both by IFN-β or IFN-γ. When ILC2s were cultured with IL-33 plus STAT5-activated cytokines, they proliferated vigorously (Supplemental Fig. E2A), and this proliferation was inhibited strongly by IFN-β but weakly by IFN-γ (Supplemental Fig. E2A and E2B). Altogether, these findings suggest that STAT5-activating cytokines, but not IL-33, promote proliferation of lung ILC2s *in vitro* and that IFN-β inhibits the effects of these cytokines.

IFN-β suppresses IL-7-induced survival of lung ILC2s

To explore the effects of IFN-β on ILC2s further, we examined their viability by flow cytometry. Isolated lung ILC2s were cultured with medium alone, IL-33, or IL-7 with or without IFN-β for 72 h and stained with Annexin V and 7-aminoactinomycin D (7-AAD). In culture with medium alone without any growth factors, approximately 70% of ILC2s became apoptotic and then necrotic, as indicated by cellular membranes that were permeable to 7-AAD (Fig. 5A) and flow cytometry scatter plots. IL-33 showed minimal effects on the distribution of these apoptotic and necrotic cells. In contrast, IL-7 significantly inhibited apoptosis of ILC2s, resulting in 80% viable cells over a period of 72 h of culture (Fig. 5A and 5B). Importantly, in the presence of IFN-β, a large proportion of cells became apoptotic and necrotic even in the presence of IL-7. In contrast, IFN-β showed minimal effects on apoptosis when ILC2s were cultured with medium alone or with IL-33, suggesting that IFN-β disrupted the molecular pathway activated by IL-7 to

maintain survival of ILC2s *in vitro*.

To examine the effects of IL-7 on lung ILC2s at molecular levels, we analyzed their gene expression using a NanoString® assay. Isolated lung ILC2s were cultured with medium alone or with IL-7 for 16 h. Using unsupervised heat map analysis, IL-7 was shown to promote expression of a number of genes while it inhibited fewer genes (Fig. 6A). According to dot plots, upregulated genes included *Il5*, *Il13, Icos, Il2ra, Il2rb,* and *Gata3* (Fig. 6B). Increased expression of *Gata3* was also verified by quantitative RT-PCR (Fig. 6C). It has been reported previously that GATA3 is indispensable for differentiation and maintenance of ILC2s.[24, 25, 29] Therefore, we examined GATA3 protein expression in ILC2s by flow cytometry by gating separately on alive (i.e., negative for Ghost Dye Red 780 staining) and dead cells. When cultured with medium alone for 72 h, approximately 50% of live cells lost their expression of GATA3 (Fig. 6D). In contrast, when cultured with IL-7, a majority of live ILC2s expressed GATA3. Dead cells, detected as the Ghost-positive population, did not express GATA3 regardless of whether they were cultured with medium alone or IL-7. Collectively, IL-7 enhanced survival of isolated ILC2s *in vitro* and the effects were inhibited significantly by IFN-β. IL-7 promoted expression of GATA3, which may explain the supportive effects of IL-7 on lung ILC2s.

GATA3 expression in lung ILC2s is regulated reciprocally by STAT5-activating cytokines and IFN-β

Finally, to examine the molecular mechanisms involved in IFN-β-mediated suppression of ILC2s, we examined the effects of various cytokines on GATA3 protein expression using the same approach as described above; GATA3 protein was analyzed by gating on viable cells. IL-7 enhanced expression of GATA3 within the live ILC2 population (Fig. 7A), resulting in increased

MFI of GATA3 staining (Fig. 7B) and a smaller proportion of GATA3-negative cells (Fig. 7C) compared to ILC2s cultured with medium alone. Similarly, IL-2 and TSLP enhanced GATA3 expression. The ranked order of STAT5-activating cytokines which promoted GATA3 expression was IL-7>IL-2>TSLP, as indicated by GATA3 MFI. In contrast, IL-33 showed no effects on GATA3 expression. A kinetic study showed that lung ILC2s lose their expression of GATA3 over 72 hours when they were cultured *in vitro* with medium alone (Supplemental Figure E3). In contrast, IL-7 enhanced GATA3 expression, resulting in increased GATA3 MFI in the same time period.

We then examined the effects of IFN-β on GATA3 expression in lung ILC2s by culturing them with IL-7, as a representative of STAT5-activating cytokines. Again, IL-7 enhanced expression of GATA3 in ILC2s compared with those cultured with medium alone (Fig. 7D-F), and IFN-β significantly inhibited GATA3 expression to levels roughly comparable to ILC2s cultured without IL-7 ($p<0.01$). IFN-γ also partially inhibited GATA3 expression, but not as strongly as IFN-β. The inhibitory effects of IFN-β on IL-7-induced GATA3 expression were reproduced in a kinetic study (Supplemental Figure E3). Altogether, these results suggest that STAT5-activating cytokines enhance expression of GATA3 by lung ILC2s, whereas IFN-β blocks the effects of these cytokines.

Finally, we investigated the effects of IFN-β on GATA3 expression *in vivo*. Naïve BALB/c mice were administrated PBS or IFN-β i.n. for 3 consecutive days, and lungs were analyzed 24 h after the last administration of IFN-β by gating on the $Lin^{-}CD25^{+}CD44^{high}$ ILC2 population (Fig. 8A, 8B). We found that GATA3 protein levels in ILC2s were significantly lower in mice administered IFN-β than those administered PBS (Fig. 8C, $p<0.01$), consistent with decreased cytokine production by those lung ILC2s when they are exposed to *Alternaria in vivo*

(Fig. 3). On the other hand, the number of ILC2s was not significantly affected by IFN-β (Fig. 8D)

DISCUSSION

The objective of this project was to identify a strategy to suppress innate type 2 immunity, which could potentially be used to treat patients with allergic airway diseases. We found that activation of TLR3 by poly (I:C) induces IFN-α, -β, and -γ in the lung of naïve mice and suppresses ILC2-mediated allergic airway inflammation. Previous reports showed that activation of TLR7/8 and TLR9 by R848 and CpG A, respectively, suppressed ILC2-driven airway inflammation by producing IFN-α or IFN-γ.[18, 19] It has also been shown that poly (I:C) ameliorates *Aspergillus flavus*-induced type 2 immune response in the lung.[30] Our observations add to this knowledge by demonstrating that poly (I:C)-mediated IFN-β plays a major role in suppressing lung ILC2s and allergic airway inflammation. Indeed, we compared the effects of poly (I:C), R848, and CpG A in parallel in an acute innate type 2 response model and found that the poly (I:C) was the most effective at suppressing *A. alternata*-induced production of type 2 cytokines (Fig. 1). The results of *Ifnra1*-deficient mice and blocking Abs support the roles for IFNRA and its ligands IFN-α and -β. Results of *in vitro* culture experiments show that IFN-β was more than 100x more potent than IFN-α. Altogether, these results lead us to conclude that the IFN-β that activates the IFNAR-pathway likely play a major role in inhibiting the innate type 2 response to *A. alternata* exposure *in vivo*.

The observations in this study are consistent previous observations, which indicated that R848 and CpG A inhibit allergen-induced innate type 2 response through the IFN-α and IFN-γ pathways.[18, 19] Rather, our findings demonstrate the diversity of IFN responses depending on the nature of TLRs involved. Agonists for TLR7/8 and TLR9 activate pDCs, which results in their production of IFN-α.[18, 19] On the other hand, pDCs do not express the poly (I:C) receptor TLR3.[31] Although the identification of the targets for poly (I:C) was not the primary goal of this

project, we speculate that either airway epithelial cells or CD8α$^+$ conventional DCs might be involved. IFN-β is expressed by bronchial epithelial cells in response to rhinovirus infection.[32] The CD8α$^+$ population of conventional DCs express TLR3, but not TLR7.[31, 33] We also found previously that bone marrow-derived conventional DCs produce IFN-β when they are stimulated with poly (I:C) *in vitro*.[34] Thus, microbes may activate distinct sets of IFNs, depending on their nature (i.e., dsRNA virus, ssRNA virus, or bacteria), TLRs, and innate immune cells. Such redundant mechanisms may represent fine-tuning of anti-microbial immune responses and regulation of allergic immune responses.

By comparing three interferons, including IFN-α, -β, and -γ, we found unique features and a potent capacity of IFN-β to suppress lung ILC2s. IFN-β failed to inhibit type 2 cytokine response when ILC2s were stimulated with IL-33 alone, but it did inhibit ILC2s when they were stimulated with IL-33 and IL-7 (Fig. 4). IFN-β was effective at suppressing the responses of ILC2s to IL-7 and other STAT5-activating cytokines, including proliferation, survival, and GATA3 expression (Fig. 4, Fig.5 and Fig. 7), but IFN-γ showed only modest effects. ILC2s are known to express receptors for all three interferons[26], but the downstream signaling pathways are likely distinct. For example, type 1 IFNs generally promote the IFN-stimulated gene factor 3, consisting of STAT1, STAT2, and IRF9, which binds IFN-stimulated response (ISRE) elements in DNA.[35] On the other hand, type 2 IFN or IFN-γ predominantly signal through a STAT1 homodimer, which binds IFN-γ-activated site (GAS) elements.[35] Although IFN-α and IFN-β share the IFNAR1 and IFNAR2 receptor complex, they are known to generate distinct biological outcomes in various cell types. This is likely due to variable affinities to the receptor complex.[36, 37] Indeed, IFN-α binds IFNAR1 and IFNAR2 at affinities of 0.5 to 5 μM and 0.4 to 5 nM, respectively; IFN-β binds these receptors with affinities of 0.1 μM and 0.1 nM, respectively.[38] In

addition, cell lines with low IFNAR1 expression respond to IFN-β but not to IFN-α.[39] Thus, the unique and potent effects of IFN-β in regulating lung ILC2s, as compared to other interferons, can be explained by its affinity to the receptor and downstream signals.

Although the molecular targets of IFN-β on ILC2s have not been fully understood, we speculate that GATA3 might be involved. GATA3 has been shown to be indispensable for differentiation, maintenance, and function of ILC2s in both in mice and humans.[24, 25] We found that IL-7 and other STAT5-activating cytokines, but not IL-33, enhanced expression of GATA3 protein by lung ILC2s *in vitro*, and IFN-β inhibited GATA3 expression both *in vitro* and *in vivo*. Our observations are consistent with a previous report in human peripheral blood ILC2s showing that TSLP, but not IL-33, increases GATA3 expression.[25] In $CD4^+$ T cells, GATA3 expression is induced by the TCR signals and IL-4.[40, 41] However, the mechanisms involved in the expression of GATA3 in mature ILC2s have remained an enigma. Our findings suggest that GATA3 expression in lung ILC2s is controlled by the balance between the activities of two key transcription factors, STAT5 and STAT1/2 (as a downstream of IFN-β). This model is consistent with the observation in $CD4^+$ T cells that STAT5 activators promote expression of GATA3 in differentiated Th2 cells.[28] IFN-β, but not IFN-γ, suppressed GATA3 during Th2 cell development and in fully committed Th2 cells.[42] In addition, the level of STAT5 activation likely plays a central role in homeostasis and the functions of ILCs in general.[43] Further studies in the molecular mechanisms involved in GATA3 expression in ILC2s in mucosal organs and roles of STAT protein(s) in the process will likely provide important information to understand the immunobiology of ILC2s and to find ways to control them.

IFN-β response is compromised in bronchial epithelial cells from patients with asthma.[32] In this study, we found that exogenous IFN-β, when administered i.n., suppresses ILC2

expression of GATA3 (Fig. 8) and allergen-induced innate type 2 responses in mouse models (Fig. 3). Human asthma is associated with polymorphisms of genes associated with ILC2s, including *IL33*, *IL1RL1*, *IL7R*, *RORA*, and *IL2RB*.[11, 12, 13] Therefore, IFN-β can be considered an option to treat patients with asthma. Indeed, a clinical trial was performed to examine the efficacy of inhaled IFN-β in asthma patients who are accompanied by cold symptoms as IFN-β might promote anti-viral immune responses.[44] The study found that inhaled IFN-β was safe and reduce medication use to treat cold-induced asthma exacerbations. Furthermore, systemic treatment with IFN-β has been well tolerated and is currently used to treat patients with relapsing multiple sclerosis.[45, 46, 47] Therefore, more clinical trials with IFN-β are warranted to examine the clinical efficacy of IFN-β in asthma, to optimize the regimens, and to develop biomarkers to identify patients who are most benefitted from the treatment.

In conclusion, we found that TLR3-driven IFN-β inhibits allergen-induced ILC2-mediated airway inflammation and that IFN-β blocks GATA3 expression, proliferation, and survival in lung ILC2s that are promoted by STAT5-activating cytokines. The inhibitory effects of IFN-γ on ILC2 production of cytokines have been reported previously.[48, 26, 19] The results our study suggest that IFN-β suppresses ILC2s likely through a distinct mechanism(s) from IFN-γ. Further studies to understand the inhibitory mechanisms of ILC2s will likely provide renewed understanding of the immunobiology of this unique cell type and novel therapeutic options for asthma and allergic airway diseases.

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642 **FIGURE LEGENDS**

643 **Figure 1.** TLR3 agonist poly (I:C) inhibits *Alternaria alternata*-induced innate type 2 response.
644 (A) Experimental model. Naïve BALB/c mice were pretreated intranasally (i.n.) with PBS or
645 indicated doses of TLR agonists, and exposed i.n. to *A. alternata* extract or PBS 24 h later. At 4.5
646 h, lungs were collected. (B) Lung levels of type 2 cytokines were analyzed by ELISA. *$p<0.05$
647 compared with mice pretreated with PBS and exposed i.n. to *A. alternata*. Data are presented as
648 the mean ± SEM (n = 2 in each group). (C) IL-5venus mice were pretreated with poly (I:C) (50
649 μg) or PBS i.n., and exposed 24 h later to *A. alternata* or PBS. At 6 h, lung single-cell
650 suspensions were analyzed by gating on $Lin^{-}C25^{+}CD44^{high}$ lung ILC2s (upper panels) and for
651 expression of IL-5venus protein (lower panels). Representative scattergrams are shown. (D)
652 Mean fluorescence intensity (MFI) of IL-5venus in ILC2s is presented. *$p<0.05$ between the
653 groups is indicated by horizontal lines. Data are presented as the mean ± SEM (n = 2-3 in each
654 group) and are representative of two experiments. (E) Experimental model. Naïve BALB/c mice
655 were exposed i.n. to *A. alternata* or PBS every 3 days for 6 days with or without poly (I:C) (50
656 μg/dose). Twenty-four hours after the last *A. alternata* exposure, BAL fluids and lungs were
657 collected. (F) Cell numbers and differentials in BAL fluids were analyzed. (G) Lung levels of
658 type 2 cytokines were analyzed by ELISA. *$p<0.05$, **$p<0.01$ between the groups indicated by
659 horizontal lines. Data are presented as the mean ± SEM (n = 4 in each group).
660
661 **Figure 2.** The inhibitory effects of poly (I:C) is mediated by type 1 IFNs. (A) Naïve BALB/c
662 mice were administrated 25 μg poly (I:C), R848, or CpG A i.n., and lungs were collected at 6 h.
663 Lung levels of interferons were analyzed by ELISA. The intact group indicates naïve mice
664 without any manipulations. *$p<0.05$, **$p<0.01$ compared to mice administered PBS. Data are

presented as the mean ± SEM (n = 3 in each group). (B) Naïve BALB/c mice were administrated 50 μg poly (I:C) or PBS. Kinetic changes in the lung levels of interferons were analyzed. Data are presented as the mean ± SEM (n = 3 in each group). (C) Experimental model. Similar to Figure 1A, naïve WT C57BL/6 mice or *Ifnar1*$^{-/-}$ or *Ifngr1*$^{-/-}$ mice were pretreated i.n. with 50 μg poly (I:C), and exposed i.n. to *Alternaria alternata* 24 h later. At 4.5 h, lung levels of type 2 cytokines were analyzed by ELISA. (D, E) The results with *Ifnar1*$^{-/-}$ and *Ifngr1*$^{-/-}$ mice are presented in Panel D and Panel E, respectively. Data are presented as the mean ± SEM (n = 5 in each group) and are representative of two experiments. **$p<0.01$ between the groups indicated by horizontal lines. (F) Experimental model. Naïve BALB/c mice were pretreated with 50 μg poly (I:C) i.n. together with i.p. (250 μg) and i.n. (50 μg) administration of anti-IFNAR1 blocking antibody and exposed to *A. alternata* extract i.n. 24 h later. At 4.5 h, lung levels of type 2 cytokines were analyzed by ELISA. (G) Lung levels of type 2 cytokines are presented. *$p<0.05$, **$p<0.01$ between the groups indicated by horizontal lines. Data are presented as the mean ± SEM (n = 3 in each group) and are representative of three experiments.

Figure 3. IFN-β effectively inhibits IL-33- or allergen-induced innate type 2 response in the lung. (A) Lung single-cell suspensions were cultured with indicated concentrations of IL-33 with serial dilution of IFN-α or IFN-β for 96 h. The levels of IL-5 and IL-13 in the supernatants were determined by ELISA. Data are presented as the mean ± SEM (n = 3 in each group) and are representative of three experiments. *$p<0.05$, compared with cells cultured without IFNs. (B) Experimental model. Naïve BALB/c mice were administrated IFN-β (500 ng/dose) or PBS for 3 consecutive days i.n., and then exposed *Alternaria alternata* extract i.n. At 4.5 h, lungs were collected for analysis. (C) BAL and lung levels of IL-5 and IL-13 were analyzed by ELISA. Data

688 are presented as the mean ± SEM (n = 4 in each group). *p<0.05, **p<0.01 between the groups
689 indicated by horizontal lines. (D) ILC2s in the lung were analyzed by gating on the Lin^-
690 $CD25^+CD44^{high}$ population by flow cytometry. Representative scattergrams are shown. (E) The
691 number of ILC2 cells are presented. Data are presented as the mean ± SEM (n = 4 in each
692 group). **p<0.01 between the groups indicated by horizontal lines.
693
694 **Figure 4.** IFN-β inhibits type 2 cytokine production and proliferation of lung ILC2s. (A and B).
695 Isolated lung ILC2s were cultured with medium alone, IL-33 (10 ng/ml) (Panel A), or IL-33 plus
696 IL-7 (10 ng/ml each) (Panel B) for 96 h with serial dilutions of IFN-β or 100 ng/ml IFN-γ. The
697 levels of IL-5 and IL-13 in the supernatants were determined by ELISA. Data are presented as
698 the mean ± SEM (n = 3) and are representative of three experiments. *p<0.05, **p<0.01
699 compared with cells cultured without IFNs. (C) CFSE-labeled lung ILC2s were cultured with
700 medium alone, IL-7, IL-2, and TSLP with or without IL-33 (10 ng/ml each) for 96 h. Dilutions of
701 CFSE were analyzed by flow cytometry. Representative histograms are shown. (D) CFSE-
702 labeled lung ILC2s were cultured as described above with or without 10 ng/ml IFN-β and IFN-γ.
703 Dilutions of CFSE were analyzed by flow cytometry. Representative histograms are shown. (E)
704 MFI of CFSE dilution is presented. Data are presented as the mean ± SEM (n = 2) and are
705 representative of three experiments. *p<0.05, **p<0.01 between the groups indicated by
706 horizontal lines.
707
708 **Figure 5.** IFN-β induces apoptosis of lung ILC2s. (A) Isolated lung ILC2s were cultured with
709 medium alone, IL-33, or IL-7 with or without IFN-β (10 ng/ml each) for 72 h. Cell viability was
710 analyzed by staining with Annexin-V and 7-AAD, followed by flow cytometry. (A)

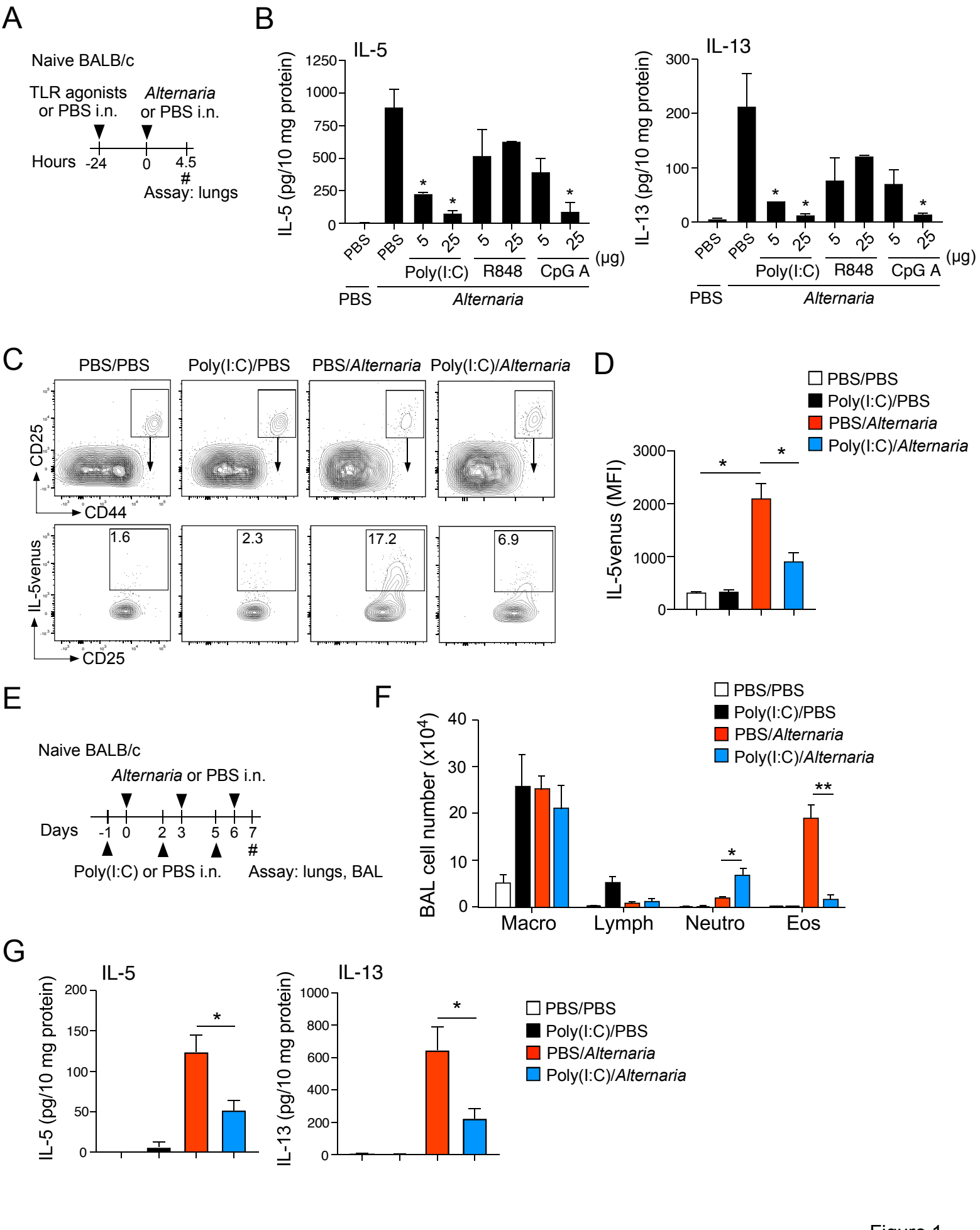
Representative scattergrams are shown. (B) Proportions of live (Annexin $V^-7\text{-}AAD^-$), apoptotic (Annexin $V^+7\text{-}AAD^-$), and necrotic (Annexin $V^+7\text{-}AAD^+$) cells are shown. Data are presented as the mean ± SEM (n = 2) and are representative of three experiments. **p<0.01 between the groups indicated by horizonal lines.

Figure 6. IL-7 induces GATA3 mRNA and protein expression in lung ILC2s. (A) Isolated lung ILC2s were cultured with medium alone or with 10 ng/ml IL-7 for 16 h. mRNA was collected and analyzed by the Nanostring® assay. (A) The results of unsupervised heat map analysis are shown. Sample numbers indicate a paired and separate experiment. (B) Scatter plots of all the analyzed genes are shown. Red dots indicate notable genes. (C) Isolated lung ILC2s were cultured with medium alone or with 10 ng/ml IL-7 for 16 h. mRNA was analyzed by real-time RT-PCR, and the results were normalized to the cells cultured with medium alone. (D) Isolated lung ILC2s were cultured with medium alone or with 10 ng/ml IL-7 for 72 h. Cells were stained with Ghost Dye Red 780, followed by staining for GATA3 protein. Cells were analyzed by flow cytometry by gating separately on live cells (Ghost Dye Red 780-negative) and dead cells (Ghost Dye Red 780-positive). Representative scatter grams and histograms are shown.

Figure 7. IFN-β inhibits GATA3 expression induced by STAT5-activating cytokines in lung ILC2s. (A) Isolated lung ILC2s were cultured with medium alone, IL-7, IL-2, TSLP or IL-33 (10 ng/each) for 72 h, and GATA3 protein expression in live ILC2s (i.e., Ghost Dye Red 780-negative) was analyzed by flow cytometry as described in Fig. 6D. Representative histograms and scattergrams are shown. (B) MFI for GATA3 protein. (C) Proportion of GATA3-negative cells among the live cells. Data are presented as the mean ± SEM (n = 2). *p<0.05, **p<0.01

compared with cells cultured with medium alone. (D) Isolated lung ILC2s were cultured with medium alone or IL-7 with or without 10 ng/ml IFN-β or IFN-γ for 72 h, and GATA3 protein expression in live ILC2s (i.e., Ghost Dye Red 780-negative) was analyzed by flow cytometry. (E) MFI for GATA3 protein. (F) Proportion of GATA3-negative cells among the live cells. Data are presented as the mean ± SEM (n = 3) and are representative of two experiments. *$p<0.05$, **$p<0.01$ between the groups indicated by horizontal lines.

Figure 8. IFN-β inhibits GATA3 expression in lung ILC2s *in vivo*. (A) Experimental model. Similar to Figure 3B, naïve BALB/c were administered i.n. IFN-β (1 μg/dose) or PBS for 3 consecutive days. At 24 h after the last administration, GATA3 protein expression in lung ILC2s was analyzed by gating on the $Lin^{-}CD25^{+}CD44^{high}$ population. (B) Representative scattergrams and histograms are shown. (C) MFI for GATA3 protein and proportion of GATA3-negative cells within the lung ILC2 population. (D) Proportion of lung ILC2s within the Lin^{-} cell population is shown (n.s., not significant). Data are presented as the mean ± SEM (n = 3-4 in each group) and are representative of two experiments. **$p<0.01$ between the groups indicated by horizontal lines.



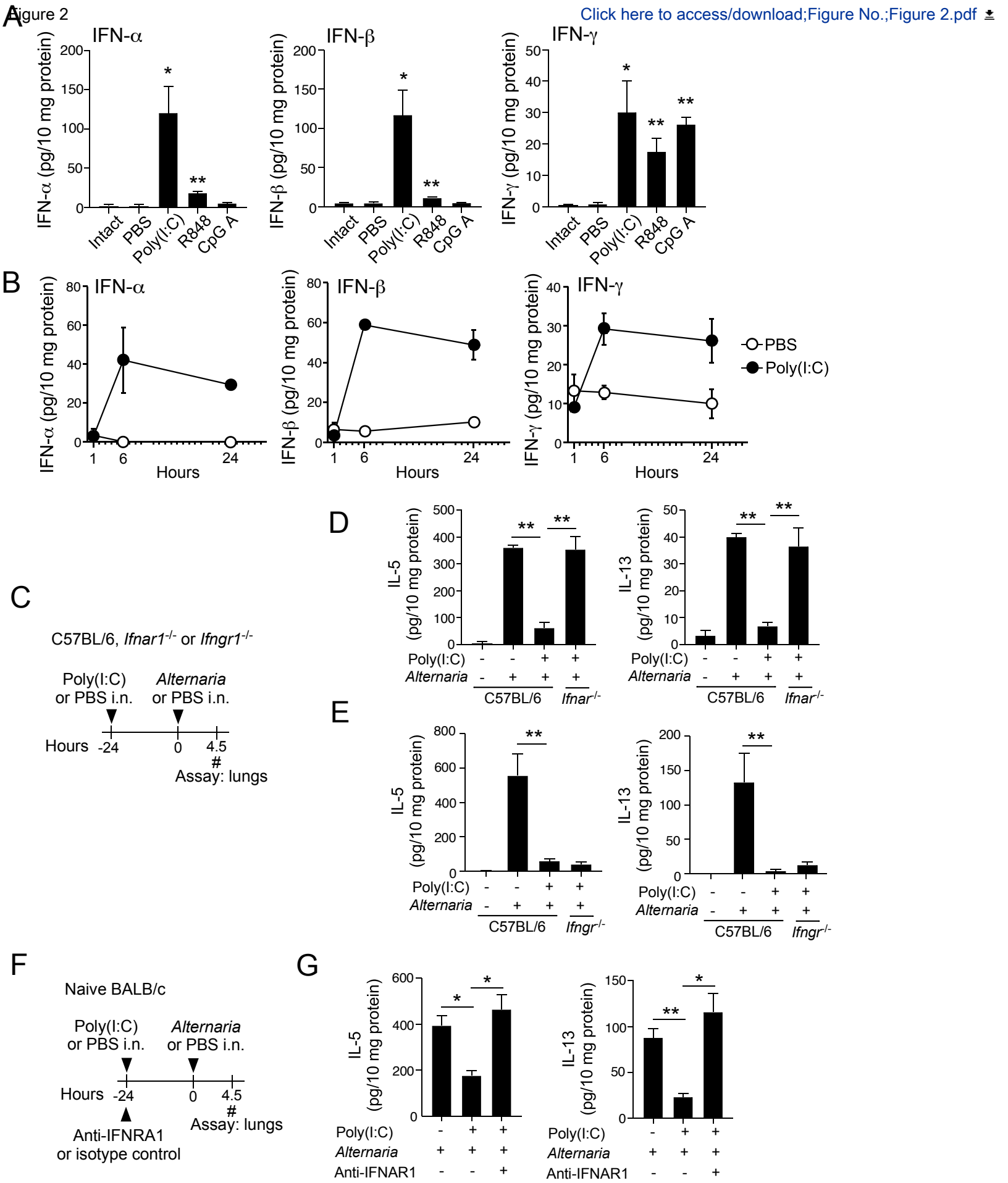
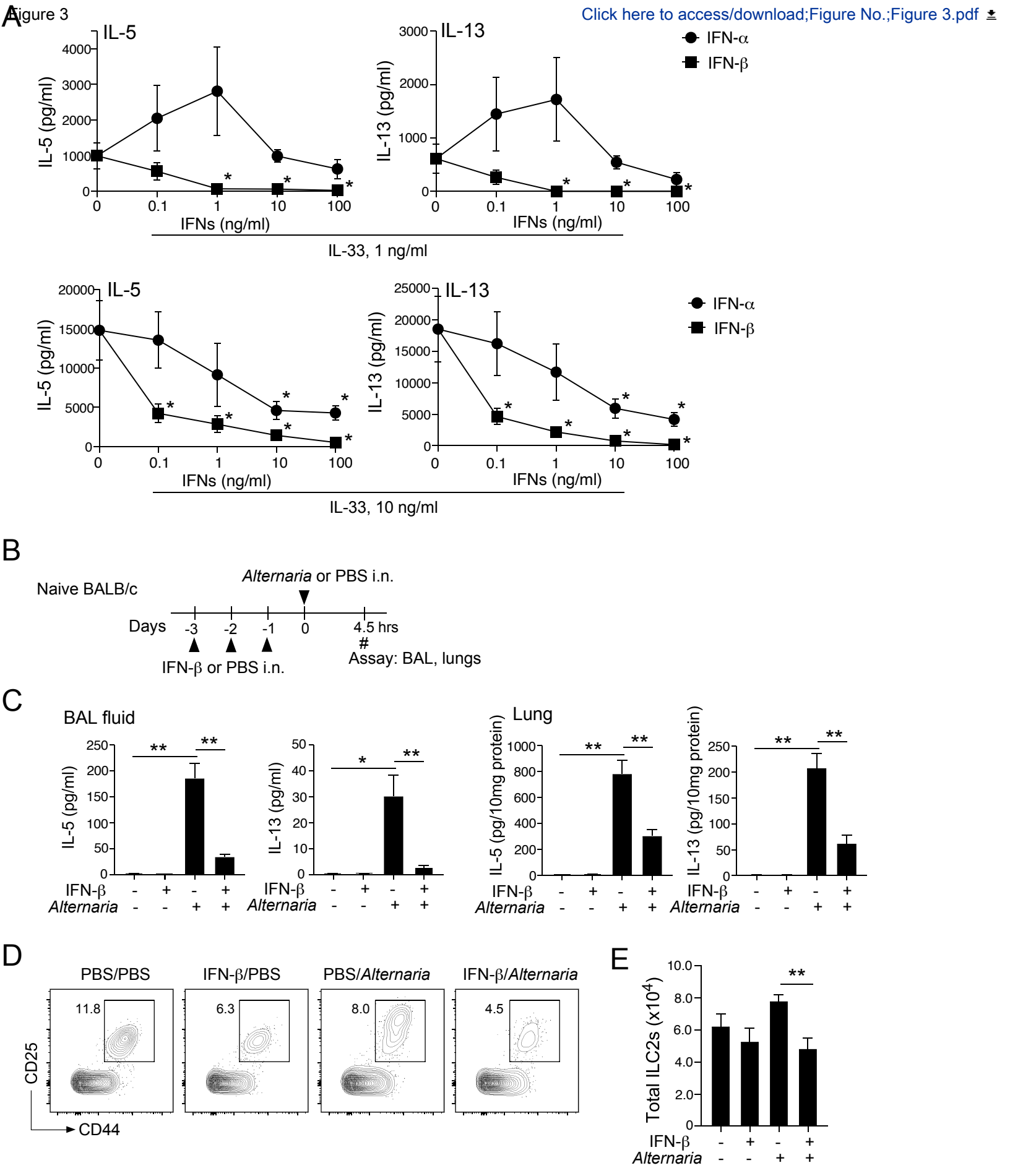


Figure 2



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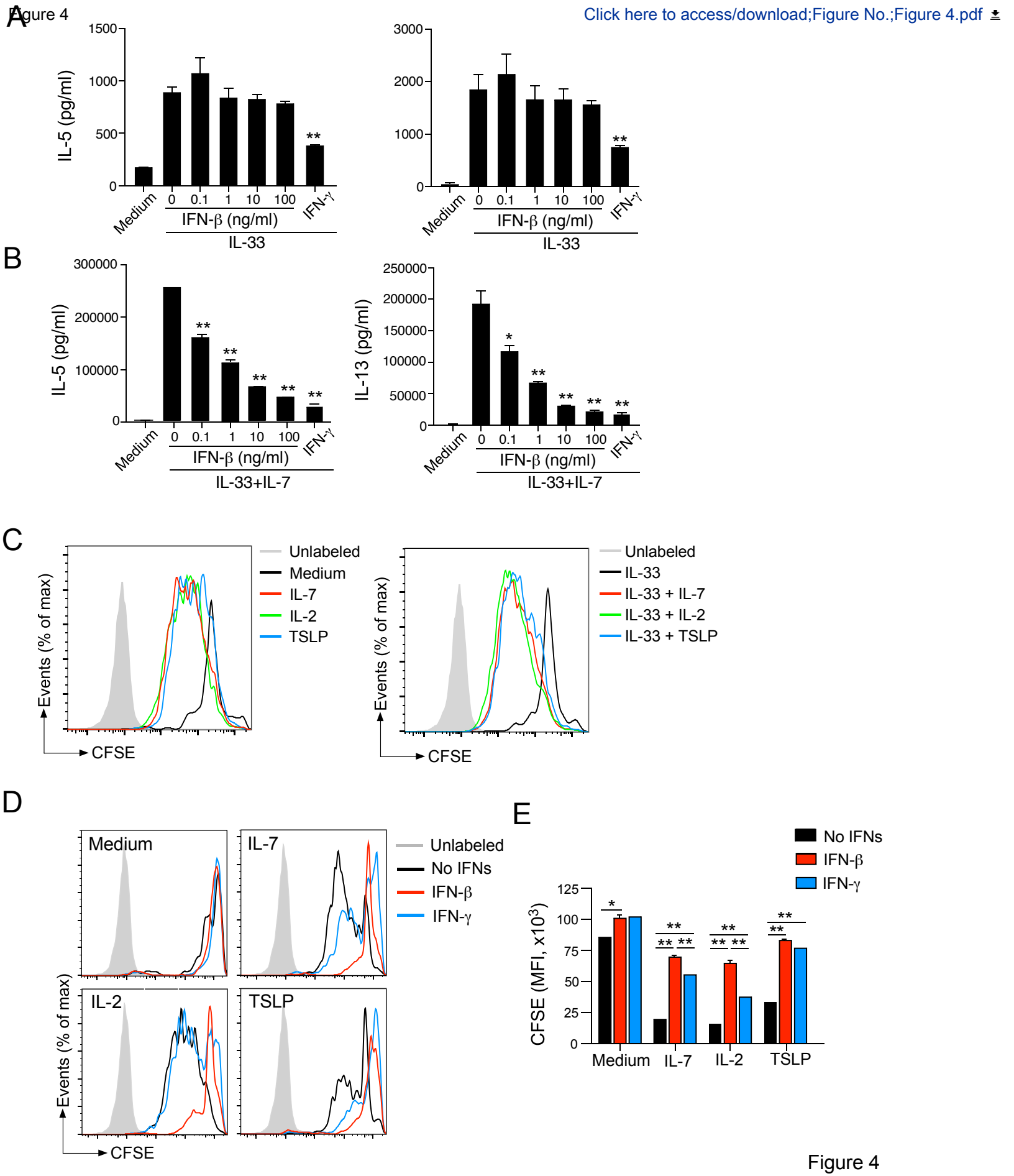

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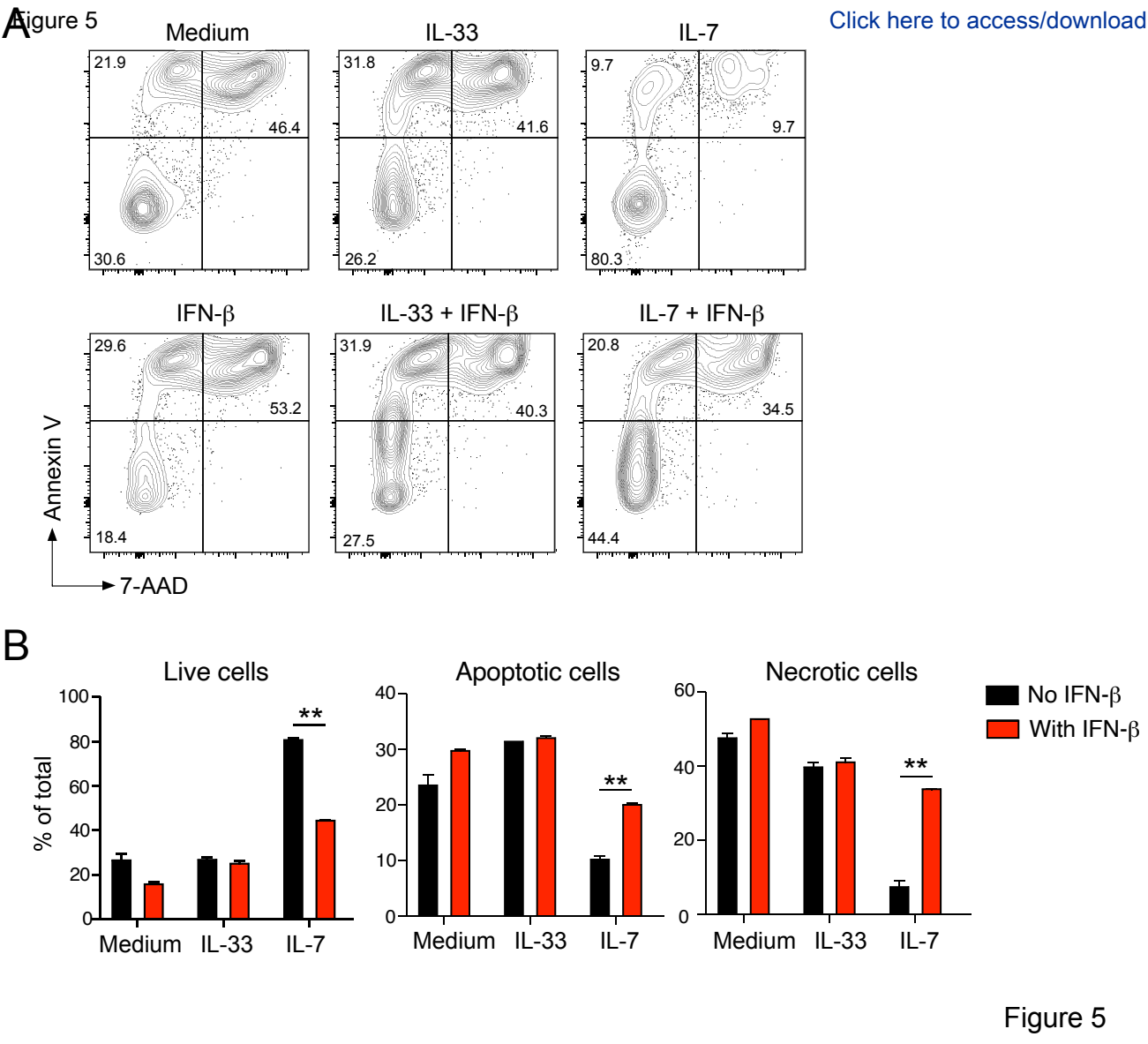

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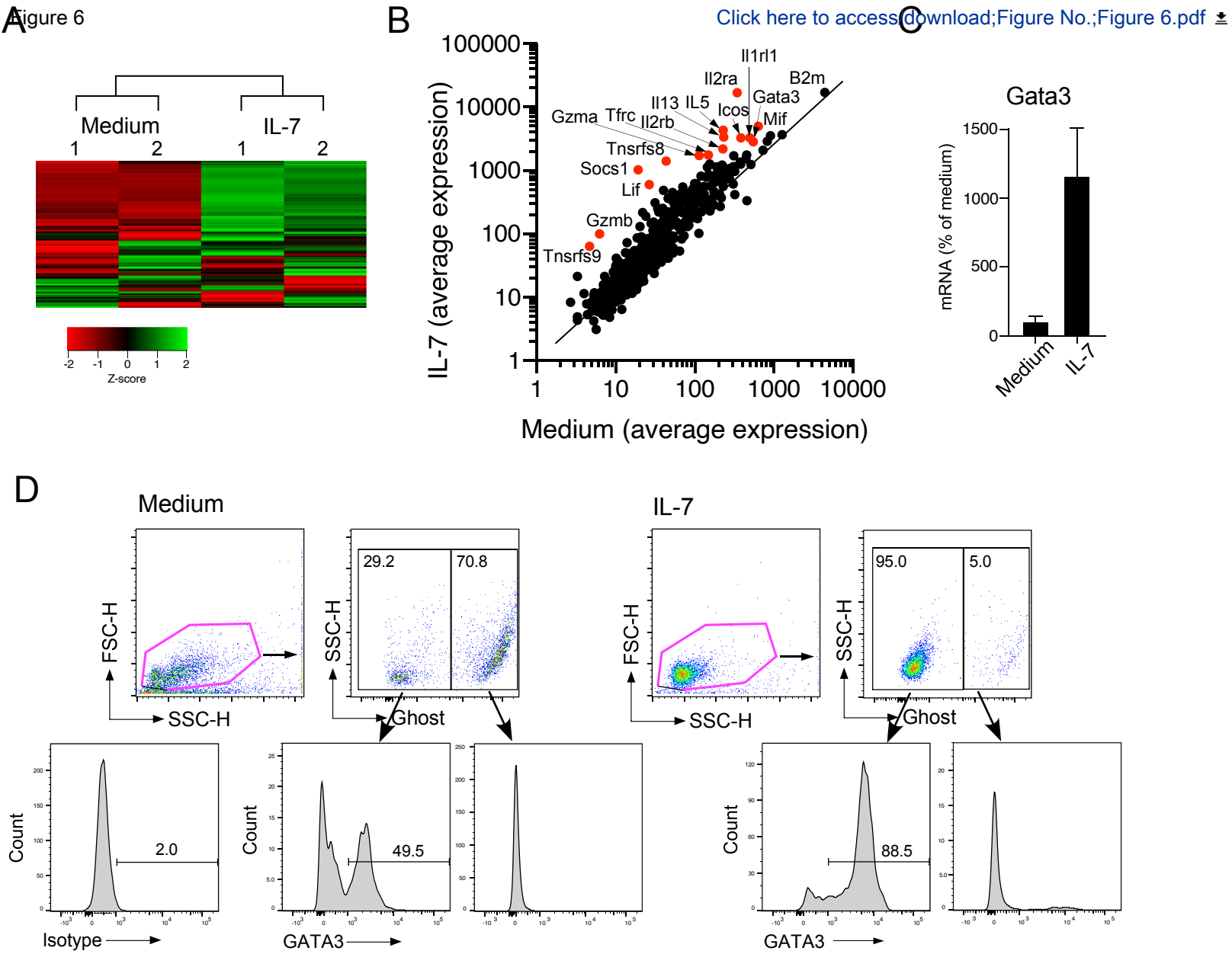


Figure 6

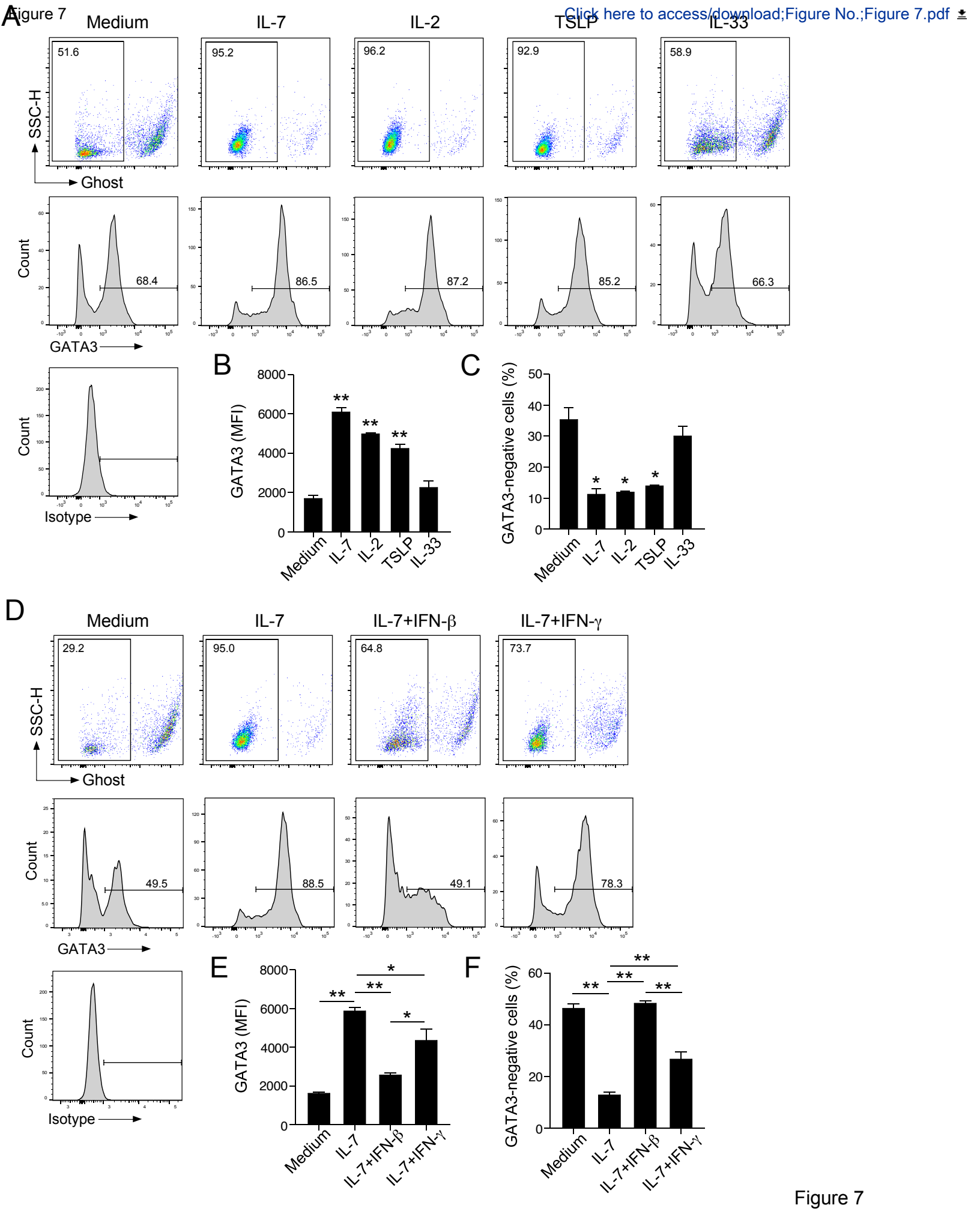


Figure 7

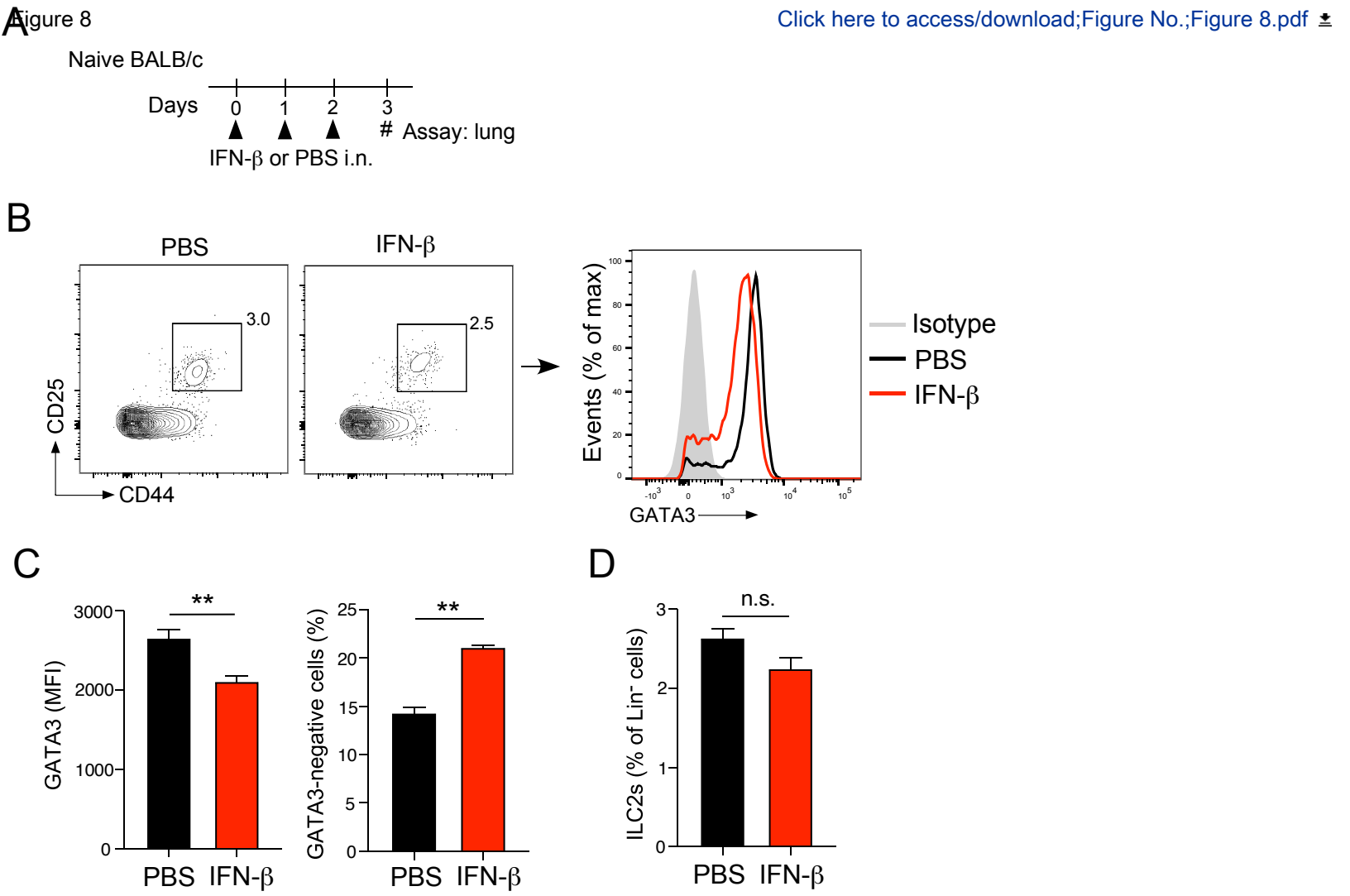
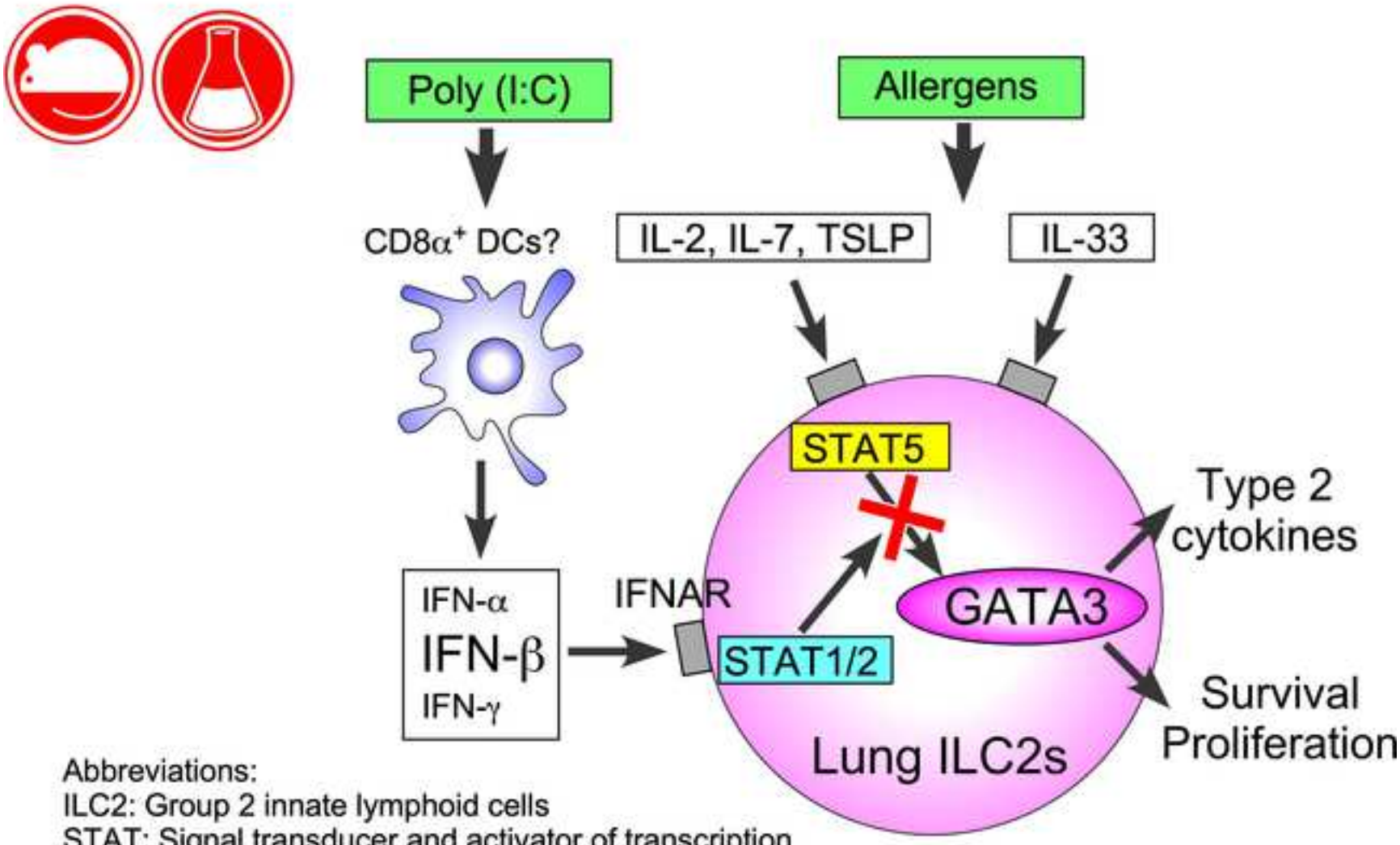


Figure 8

Graphical abstract

IFN-β antagonizes the activities of STAT5-activating cytokines to promote GATA3 expression, survival and effector functions of lung ILC2s



Abbreviations:
ILC2: Group 2 innate lymphoid cells
STAT: Signal transducer and activator of transcription
IFNAR: Interferon-α/β receptor

ONLINE REPOSITORY

TLR3-driven IFN-β antagonizes STAT5-activating cytokines and suppresses innate type 2 response in the lung

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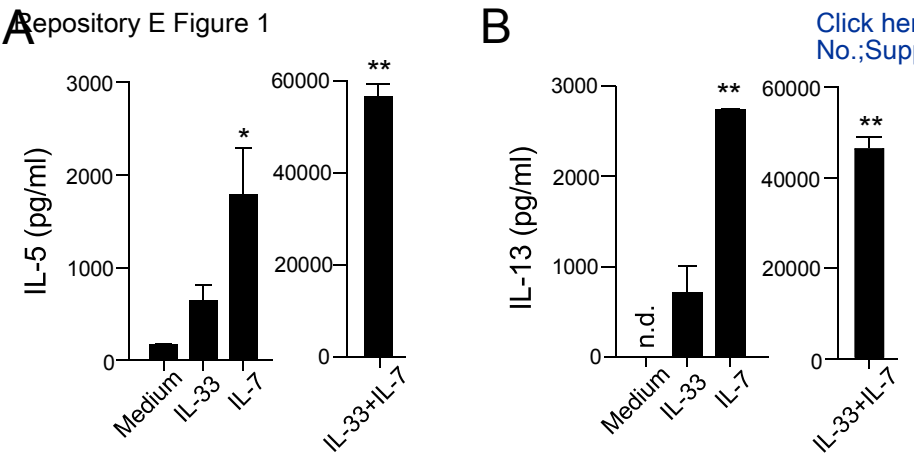
LEGENDS FOR SUPPLEMENTAL FIGURES

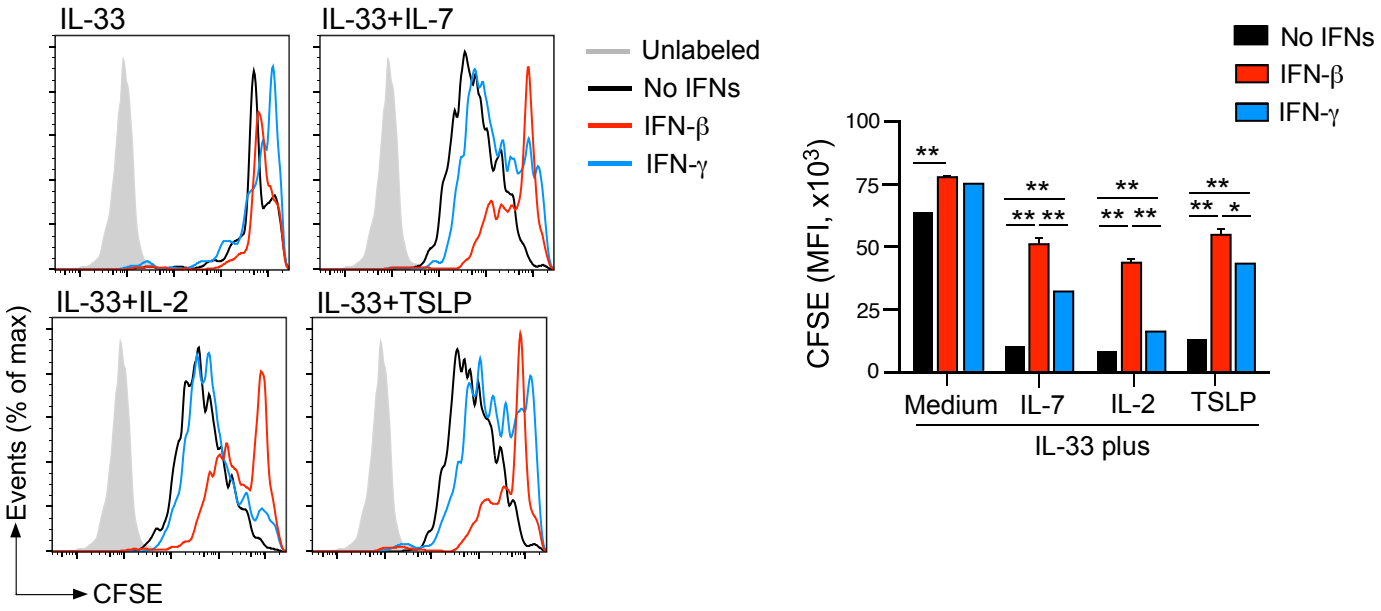
Supplemental Figure E1. IL-33 and IL-7 synergistically promote type 2 cytokine production by lung ILC2s. Isolated lung ILC2s were cultured with medium alone, IL-33, IL-7, or IL-33 plus IL-7 (10 ng/ml each) for 96 h. The levels of IL-5 and IL-13 in the supernatants were determined by ELISA. Data are presented as the mean ± SEM (n = 2 in each group) and are representative of two experiments. *$p<0.05$, **$p<0.01$ compared to the cells cultured with medium alone. n.d. (not detectable)

Supplemental Figure E2. IFN-β inhibits proliferation of lung ILC2s cultured with IL-7 plus IL-33. CFSE-labeled lung ILC2s were cultured with IL-33 alone or IL-33 plus IL-7, IL-2, or TSLP (10 ng/ml each) for 96 h. Dilutions of CFSE were analyzed by flow cytometry. (A) Representative histograms are shown. (B) MFI of CFSE dilution. Data are presented as the mean ± SEM (n = 2) and are representative of three experiments. *$p<0.05$, **$p<0.01$ between the groups indicated by horizontal lines.

Supplemental Figure E3. IL-7 enhances expression of GATA3 protein in lung ILC2s *in vitro*. Isolated lung ILC2s were cultured with medium alone, IL-7, or IL-7 plus IFN-β (10 ng/ml each) for 24, 48, and 72 h. The levels of GATA3 protein in ILC2s were analyzed by intracellular staining and flow cytometry. (A) Representative scattergrams and histograms are shown. (B) The kinetic changes in MFI for GATA3 protein are presented.

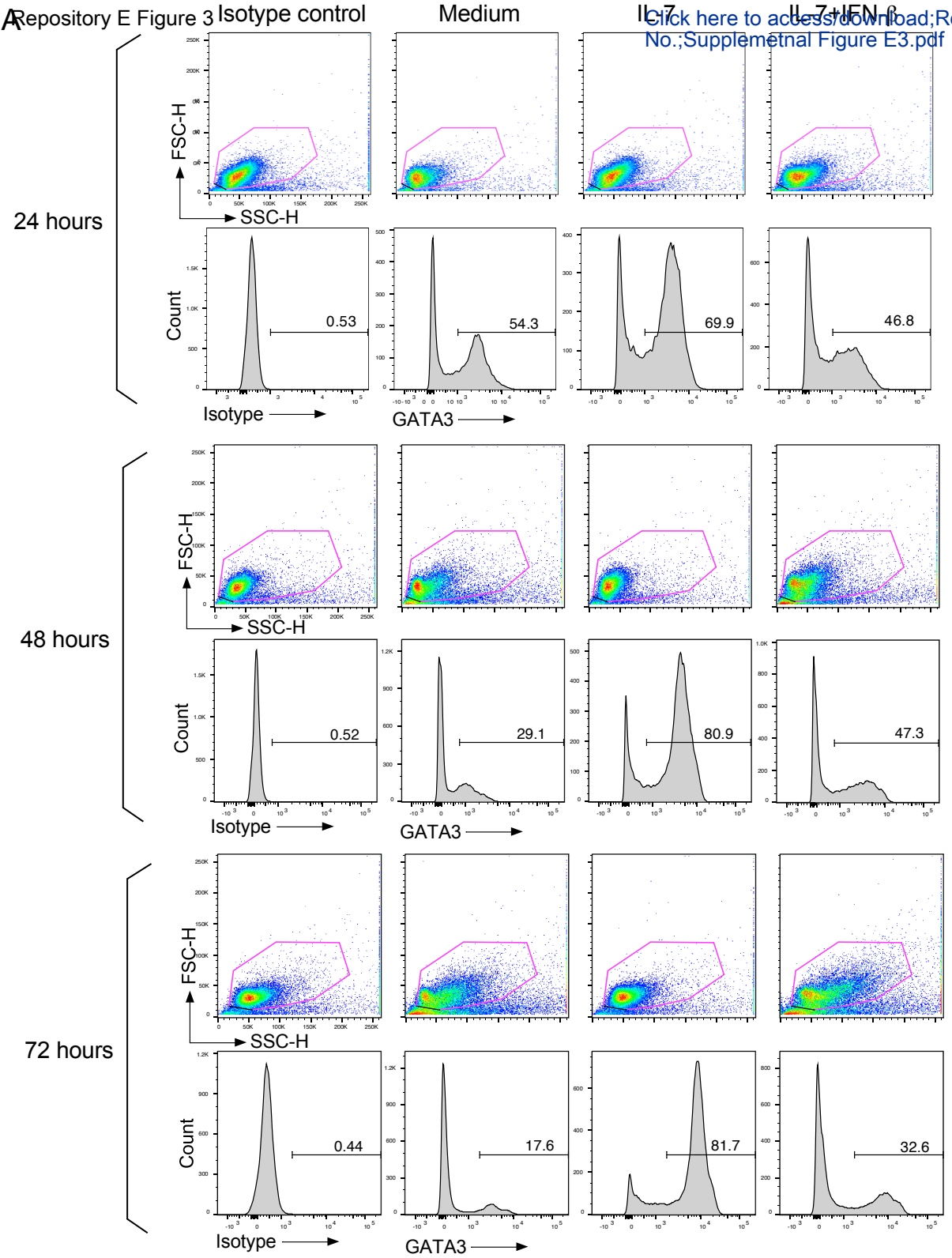


Suplemental Figure E1



Supplemental Figure E2

A



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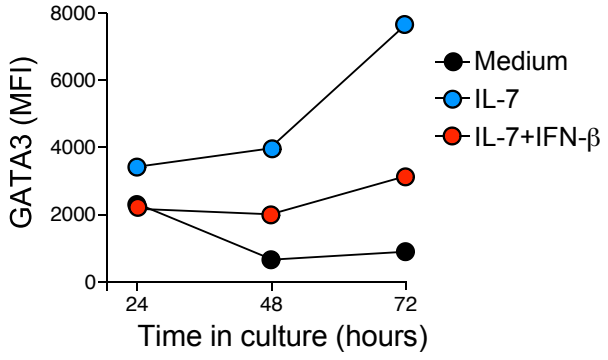

Supplemental Figure E3