IFN-γ Directly Controls IL-33 Protein Level through a STAT1- and LMP2-dependent Mechanism*

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**Background:** IL-33 levels are regulated through poorly understood cytokine-dependent mechanisms.

**Results:** IFN-γ but not IL-4 down-regulates IL-33 protein by activating STAT1 and LMP2 proteasome, without engaging caspase-1, -3, or -8.

**Conclusion:** Down-regulation of IL-33 protein by IFN-γ requires STAT1 and non-canonical involvement of LMP2 proteasome.

**Significance:** Understanding the mechanisms of IL-33 regulation is important for the development of IL-33-targeting therapies.

IL-33 contributes to disease processes in association with Th1 and Th2 phenotypes. IL-33 mRNA is rapidly regulated, but the fate of synthesized IL-33 protein is unknown. To understand the interplay among IL-33, IFN-γ, and IL-4 proteins, recombinant replication-deficient adenoviruses were produced and used for dual expression of IL-33 and IFN-γ or IL-33 and IL-4. The effects of such dual gene delivery were compared with the effects of similar expression of each of these cytokines alone. In lung fibroblast culture, co-expression of IL-33 and IFN-γ resulted in suppression of the levels of both proteins, whereas co-expression of IL-33 and IL-4 led to mutual elevation. In vivo, co-expression of IL-33 and IFN-γ in the lungs led to attenuation of IL-33 protein levels. Purified IFN-γ also attenuated IL-33 protein in fibroblast culture, suggesting that IFN-γ controls IL-33 protein degradation. Specific inhibition of caspase-1, -3, and -8 had minimal effect on IFN-γ-driven IL-33 protein down-regulation. Pharmacological inhibition, siRNA-mediated silencing, or gene deficiency of STAT1 potently up-regulated IL-33 protein expression levels and attenuated the down-regulating effect of IFN-γ on IL-33. Stimulation with IFN-γ strongly elevated the levels of the LMP2 proteasome subunit, known for its role in IFN-γ-regulated antigen processing, siRNA-mediated silencing of LMP2 expression abrogated the effect of IFN-γ on IL-33. Thus, IFN-γ, IL-4, and IL-33 are engaged in a complex interplay. The down-regulation of IL-33 protein levels by IFN-γ in pulmonary fibroblasts and in the lungs in vivo occurs through STAT1 and non-canonical use of the LMP2 proteasome subunit in a caspase-independent fashion.

Interleukin (IL)-33 is a member of the IL-1 family (1, 2), initially described as a nuclear factor (3) but later shown to also function as a cytokine, and has been implicated in several important lung diseases (4–22). Full-length IL-33 precursor is synthesized as a ~30-kDa precursor consisting of 270 amino acid residues for human IL-33 and 266 amino acid residues for mouse (m) IL-33. This form is almost entirely intracellular and, moreover, intranuclear (3–5, 23). Partial proteolysis of the IL-33 precursor produces a shorter mature form (24, 25), which acts as the IL-33 cytokine when released into the extracellular space and bound to the specific cell surface receptor, a heterodimer of the IL-1RAP and T1/ST2 chains (24, 26). Other proteases, specifically caspases, cleave IL-33, inactivating its function (27, 28). The IL-33-T1/ST2 pathway is centrally involved in Th2-driven processes, including allergies, asthma, anaphylaxis, and parasite expulsion, whereas the intracellular form binds DNA and functions as a regulator of gene expression (3–5, 23–27, 29–31).

IL-33 mRNA is regulated rapidly, within hours (30, 32, 33), whereas the fate of synthesized IL-33 protein is unknown. Abundant evidence suggests that IL-33 is a potent pro-Th2 cytokine (5, 8, 24, 34–40), whereas data on the regulation of IL-33 expression at the mRNA and/or protein levels by IL-4 are limited (41). The interplay between IL-33 and IFN-γ remains a matter of controversy. Various studies have reported a down-regulating (5, 42, 43), a null (44), or an up-regulating (45, 46) effect of IL-33 on IFN-γ levels. Furthermore, there is a dearth of information about the effects of IFN-γ on IL-33. Some evidence suggests an IFN-γ-driven increase in IL-33 mRNA transcription in human skin keratinocytes (32, 33), cardiac myocytes, and fibroblasts (47) but not dermal fibroblasts (32, 33). By contrast, IFN-γ appears to attenuate IL-33 mRNA expression in macrophages (48) and microglial cells (49).

The effects of IFN-γ on expression of mRNA and proteins are broad and diverse and are driven primarily by intracellular signaling through signal transducer and activator of transcrip-

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3 The abbreviations used are: m, mouse (e.g. mIL-33); AdV, adenoviral; NMLF, normal mouse lung fibroblast; qPCR, quantitative PCR; fmk, fluoromethyl ketone; RSV, Rous sarcoma virus; BAL, bronchoalveolar lavage.
IFN-γ Controls IL-33 Levels through STAT1 and LMP2

Stimulation of cells with IFN-γ leads to STAT1 phosphorylation and nuclear translocation from the cytoplasm, with subsequent binding to DNA and regulation of gene expression (51). STAT1 is an important regulator of the expression of numerous genes underlying diverse cellular processes such as the immune response, antiviral protection, and apoptosis (52, 53). Interestingly, IFN-γ increases the expression levels and activity of caspase-1, -3, and -8 (54–58), of which caspase-1 and -3 are known to inactivate IL-33 (27, 28); however, caspase-8, being an initiator caspase, then activates effector caspase-3 and -7 (59, 60).

Also, among genes that are activated by IFN-γ through STAT1 is a proteasome subunit, LMP2 (also known as subunit β1i) (61–63), a 23-kDa structural component of the 20S immunoproteasome (64, 65). LMP2 plays an important role in protein degradation, contributing to production of antigenic peptides that are presented by MHC class I molecules (66–69). This classical view of immunoproteasome function is being extended currently to include non-immune functions such as protein degradation other than MHC antigen processing, which is more similar to the functions fulfilled by constitutive/standard proteasomes (70, 71). For example, LMP2 is expressed and further induced by IFN-γ in neurons (72, 73) and may contribute to the pathophysiology of Huntington (72) and Alzheimer (73) diseases. LMP2 deficiency in mice leads to a higher degree of motor function and increased body weight (74), muscle mass loss and contractile impairment in the heart (75), and loss of ischemic preconditioning-induced cardioprotection (76), with impaired degradation of tensin homologue deleted on chromosome 10 (PTEN) (75, 76). LMP2 is also involved in regulating the proliferation of multiple myeloma cells (71) and the survival and expansion of T cells (77). Furthermore, LMP2 protects against oxidative damage by degrading oxidized proteins (78, 79).

To assess the interplay between IL-33 and IL-4 as well as IL-33 and IFN-γ in cell culture and in the complexity of a realistic in vivo environment, we utilized gene delivery of either IL-33 and IL-4 or IL-33 and IFN-γ to mouse lungs in vivo using dual expression, replication-deficient adenoviral (AdV) constructs. To isolate the effects on IL-33 protein levels, protein expression in this system was driven by non-mammalian cytomegalovirus (CMV) and Rous sarcoma virus (RSV) promoters rather than by natural promoters. The effects of these dual expression constructs were characterized in comparison with similar constructs encoding each of these cytokines alone. Subsequent experiments addressed the molecular mechanisms of the IFN-γ effects on IL-33 levels.

**EXPERIMENTAL PROCEDURES**

Reagents, Cell Culture, and Molecular Biology Techniques—Mouse embryonic fibroblast cell line NIH3T3 was purchased from the American Type Culture Collection (Manassas, VA) and cultured per the supplier’s recommendations. Primary normal mouse lung fibroblasts (NMLFs) were propagated from wild-type female C57BL/6 mice. STAT1−/− mouse lung fibroblasts were a kind gift from Dr. Matthew B. Frieman (University of Maryland School of Medicine). Cells were maintained in T75 culture flasks in a humidified atmosphere of 5% CO₂ at a temperature of 37°C in RPMI 1640 medium supplemented with 10% bovine calf serum, 2 mM glutamine, 2 mM sodium pyruvate, and 50 mg/liter gentamicin (all from Invitrogen). For experiments, cells were cultured in 6-well plates in the same medium. Cell viability and cell numbers were validated using CellTiter-Fluor™ (Promega, Madison, WI) as described previously (80).

All recombinant cytokines used for cell activation were purchased from R&D Systems (Minneapolis, MN). Concentrations of IL-33, IFN-γ, and IL-4 proteins in cell lysates and supernatants were tested in ELISAs (R&D Systems). Supplies for Western blotting assays were purchased from R&D Systems and Millipore, and Western blotting assays were performed as described previously (5, 80–90). STAT1 or LMP2 siRNAs and the corresponding non-targeting scrambled control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and transfection of cultured cells with siRNAs was performed by electroporation using Amxax Nucleofector (Lonza, Walkersville, MD).

For reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR), total cellular RNA was isolated using TRIzol reagent (Ambion, Grand Island, NY), and complementary DNA was synthesized from 1–5 μg of RNA using a RT² First Strand cDNA kit (Qiagen, Germantown, MD) according to the manufacturer’s protocol. Primers for human (catalogue number PPH17375E) and mouse (PPM32527A) IL-33, human (PPH05666E) and mouse (PPM72041A) 18S rRNA, mouse LMP2 (PPM41820A), and mouse STAT1 (PPM04025F) were obtained from SABiosciences/Qiagen (Valencia, CA). RT-qPCR was performed on an Applied Biosystems StepOne Plus PCR system (Carlsbad, CA) using RT² SYBR Green qPCR Mastermix (SABiosciences) according to the manufacturer’s directions.

Cell-permeable, irreversible inhibitors of caspase-1 (Z-W-E(OMe)-H-D(OMe)-fmk), caspase-3 (Z-D(OMe)-E(Ome)-V-D(OMe)-fmk), and caspase-8 (Z-I-E(Ome)-T-D(OMe)-fmk) activity were all obtained from R&D Systems and used in cell culture at a concentration of 120 μM. Cells were pretreated with these inhibitors for 4 h before further manipulations and cultured for the times indicated below with inhibitor-containing medium replaced every 24 h. The pharmacological STAT inhibitor nifuroxazide (a nitrofuran-based antibiotic that effectively suppresses the activation of cellular STAT1/3/5 transcription activity (Millipore, Billerica, MA, catalogue number 481984)) was used in cell culture at a 300 μM concentration. Cells were pretreated with nifuroxazide for 1 h before further processing, and the inhibitor remained in the medium throughout the entire duration of the experiment. A STAT1-responsive promoter-firefly luciferase reporter construct (SABiosciences Qiagen, catalogue number CCS-009L) was utilized to monitor IFN-γ-induced signaling pathway activity in the cells. Positive control (constitutive luciferase expression) and negative control (non-inducible luciferase-encoding) plasmids were tested in parallel. NIH3T3 cells transfected with these constructs were treated with 100 ng/ml recombinant (r)IFN-γ, 300 μM nifuroxazide, or their combination for 24 h and luciferase luminescence measured.
A known activator of IL-33 production acting through TLR3 (30), polyinosinic-polycytidylic acid (poly(I-C)) (catalogue number P0913, Sigma-Aldrich) was used at 100 μg/ml for 24 h to stimulate NMLF in culture with or without 100 ng/ml rIFN-γ.

Construction, Validation, and Use of Recombinant Adenovirus Vectors—The AdV constructs in this study are summarized in Table 1. These constructs were designed for gene delivery of mIFN-γ or mIL-4 under control of the CMV promoter in the E1 region of the adenoviral genome. EGFP was also encoded in the E1 region of each construct under control of the RSV promoter. AdV constructs encoding the full-length mIL-33 gene under the RSV promoter were used in the majority of experiments; an AdV construct encoding the full-length mIL-33 gene under control of the CMV promoter was used where specifically indicated in the Results section.

GenBank™ consensus sequences for mIL-33, mIFN-γ, and mIL-4 were used to artificially synthesize (GenScript, Piscataway, NJ) DNA fragments corresponding to the respective cDNAs. All restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA) and used as recommended by the manufacturer. All AdV were constructed using the RAPAd technology (ViraQuest, North Liberty, IA) described elsewhere (91).

The AdV-CMV-IL33, AdV-IL4, and AdV-IFN constructs were created and validated as reported previously for similar constructs (5, 85–88, 90). Briefly, the DNA fragments were transferred into the pVQAd5CMVK-Npa shuttle plasmid. Then, 15 μg of the shuttle plasmid and 4 μg of RAPAd backbone (ViraQuest) were digested independently with PacI. The two linear plasmids were then transfected using CaCl2 medium described previously and were shown to induce IL-33 expression (92, 93). Briefly, IL-33-, IFN-γ-, IL-4-, IL33&IFN-γ, and IL33&IL4-encoding or NULL vectors were suspended in sterile PBS and dialyzed through a 300,000-kD cutoff membrane to remove the A195 buffer that was used for cryopreservation of the viruses. After a minor anterior midline neck incision was made to make the trachea visible, a MicroSprayer (Penn-Century, Philadelphia, PA) was inserted intratracheally through the mouth, and 50 μl of the viral suspension (5 × 108 pfu/mouse) was instilled. Control mice were instilled with 50 μl of 0.9% NaCl containing AdV-NULL viral suspension. On days 3, 7, 14, and 21 following intratracheal instillation, mice were euthanized by CO2 asphyxiation followed by cervical dislocation.

To assess the effect of IFN-γ on naturally induced IL-33 expression, intratracheal instillations of 0.075 units of bleomycin (Sigma-Aldrich) or PBS control were performed as described previously and were shown to induce IL-33 expression in the lungs (4). On day 14, mice were euthanized, lungs isolated, and lung tissue digested with collagenase (Sigma-Aldrich). Non-adherent cells were discarded after overnight incubation in a plastic cell culture dish, and adherent cells were treated with or without 100 ng/ml rIFN-γ, which was followed by ELISA of cell lysates for IL-33.

To obtain bronchoalveolar lavage (BAL) fluid, the animals were euthanized, and lung lavage was performed immediately postmortem through an 18-gauge, blunt-end needle secured in the trachea. Two instillations and withdrawals of 1 ml of PBS each time were performed in each animal. The two aliquots of BAL fluid were pooled, and the cells were separated by centrifugation. The BAL cells were stained with a Protocol Hema 3 staining set (Fisher). Differential cell counts using morphologic criteria were performed by authors who were blinded to the identity of the samples.
**IFN-γ Controls IL-33 Levels through STAT1 and LMP2**

To obtain lung homogenates for ELISA, the animals were euthanized, and the lungs were extracted and snap-frozen in liquid nitrogen, thawed in 0.5 ml of PBS containing a protease inhibitor mixture (Sigma), and further homogenized in a glass homogenizer. The solid tissue was separated by centrifugation, and the supernatant was used for ELISA of mouse IL-33 and mouse IFN-γ (R&D Systems).

**Statistical Analyses**—Data are reported as mean ± S.D. values. Differences between groups were evaluated with Student’s two-tailed unequal variance t test, and p values less than 0.05 were considered statistically significant. To distinguish the potentiating or inhibitory effects of two treatments combined, the observed change induced by the combined factors was compared with the sum of changes induced by each of the factors alone.

**RESULTS**

**Regulation of IL-33 Expression by IFN-γ and IL-4 in Cultured Cells**—We and others have shown that IL-33 regulates Th1 and cytokine patterns and corresponding phenotypic manifestations in cell culture and in vivo (5, 8, 24, 34–40). The goal of the initial experiments in this study was to assess whether rIFN-γ or rIL-4, prototypic Th1 and Th2 cytokines, respectively, in turn affects the overall natural expression of the IL-33 gene in cell culture. Primary NMLFs or primary human keratinocytes were treated with 10 ng/ml IFN-γ or 100 ng/ml IL-4 and harvested after 6 and 12 h. Recombinant mouse or recombinant human cytokines were used for these experiments in a species-specific fashion. These cytokine concentrations were determined earlier to be optimal for the induction of molecular changes in fibroblast (80–82) and keratinocyte (32, 33) cultures. Changes in IL-33 gene expression were assessed with RT-qPCR. Treatment with rIFN-γ, but not rIL-4, significantly attenuated the steady-state levels of IL-33 mRNA, leading to a 100–1000-fold decrease (p < 0.05, Fig. 1A). This was in contrast to observations in the HaCat keratinocyte cell line and in primary human keratinocytes in which a similar treatment with rIFN-γ increased the steady-state levels of IL-33 mRNA 7.8 ± 1.1-fold and 3.1 ± 1.0-fold, respectively (p < 0.05 in both cases), which was consistent with previous reports by others for keratinocytes (32, 33). These observations collectively suggest that IFN-γ-driven regulation of IL-33 gene expression is diverse and cell type-dependent. Assessing the levels of IL-33 protein in these experiments was challenging, because the levels of protein expression were too low to be reliably measured by ELISA or Western blotting in the cell culture supernates or lysates of these primary cells. Additional experiments demonstrated a down-regulating effect of rIFN-γ on naturally increased IL-33 protein levels in primary cells, as discussed below (Fig. 2).

To more reliably assess the effect of IFN-γ on IL-33 protein, both NMLF and NIH3T3 cultures were infected with AdV-RSV-IL33 or AdV-CMV-IL33 and treated 48 h later with rIFN-γ at 10 ng/ml or 100 ng/ml, as indicated in Fig. 1, B and C. The expression of IL-33 mRNA in these cultures was not inhibited by IFN-γ (p > 0.05), which is understandable considering that the expression was driven by the recombinant CMV or RSV promoters and not by a native IL-33 promoter. Although infecting control cultures with AdV-NULL led to a mild eleva-
IFN-γ Controls IL-33 Levels through STAT1 and LMP2

NMLF and NIH3T3 cultures infected with AdV-NULL, AdV-IFNγ, and AdV-IL4. Thus, IFN-γ directly down-regulates IL-33, but not EGFP, protein levels. Further experiments focused on this regulatory aspect and on the IL-33-driven down-regulation of IFN-γ expression.

In separate experiments, NMLF cultures were infected with AdV constructs encoding IL-33, IFN-γ, or IL-4 alone or, alternatively, dual expressers of IL-33 and IFN-γ or IL-33 and IL-4, as described in Table 1. The levels of protein expression were assessed 48 h later with an ELISA of cell lysates and supernates (Fig. 3, C–F). IL-33 remained intracellular, as expected for the full-length form of the protein based on previous results (5), whereas IFN-γ and IL-4 proteins were secreted into cell culture supernates as expected for this cytokines. Consistent with the findings presented in Fig. 3, A and B, co-delivery of IL-33 and IFN-γ with the dual expresser AdV caused significant attenuation in the levels of both of these cytokines in the cell culture supernates and cell lysates (p < 0.05, Fig. 3, C and D). By contrast, co-delivery of IL-33 and IL-4 led to significant potentiation of the levels of both cytokines in the cell culture supernates (p < 0.05, Fig. 3, E and F). The latter mutual potentiating effects of IL-4 and IL-33 were unlikely to be due to an increase in the production of endogenous cytokines, because (a) IL-4 or IL-33 gene delivery alone had no such effects, and (b) separate experiments in which NMLF cell cultures were stimulated with rIFN failed to show any increase in natural IL-33 expression (Fig. 1A).

However, the possibility cannot be excluded that exogenous IL-33 regulates the expression of endogenous IL-33 more potently in the presence of IL-4. Moreover, it is possible that IL-33 and IL-4 regulate each other’s protein stability and turnover. Although interesting, this observation of mutual potentiation between IL-33 and IL-4 was not pursued, to preserve the focus on IFN-γ-driven IL-33 protein attenuation. Nevertheless, these data are relevant to the observations on the IFN-γ-driven IL-33 protein attenuation because they confirm that the inhibition of IL-33 is not an artifact of the dual expresser AdV system.

The inhibitory effect of IFN-γ on IL-33 production upon AdV delivery of both cytokines with the AdV-IL33&IFNγ dual expresser was even more pronounced (Fig. 3C) than in the rIFN-γ-treated AdV-IL33-infected cultures (Fig. 3, A and B). Such potent attenuation of IL-33 levels may be due to higher local concentrations of IFN-γ in and around the IL-33-expressing cells. To validate these ELISA findings, Western blotting analyses of cell lysates were performed with anti-IL-33 antibodies (Fig. 4). The levels of IL-33 protein in the cells infected with the dual expresser AdV-IL33&IFNγ were substantially lower than the IL-33 protein levels in the cells infected with equal amounts of AdV-IL33. Co-expression of IL-33 and IL-4 with the dual expresser AdV did not affect the level of IL-33 produced (Fig. 4). Thus, in addition to down-regulating natural IL-33 mRNA levels (Fig. 1A), IFN-γ down-regulates IL-33 protein levels in cell culture (Figs. 1C, 3, A–C, and 4) even in the absence of IL-33 mRNA down-regulation (Fig. 1B). IL-4 does not have such an inhibitory effect on IL-33 (Figs. 1A and 3, E and F).

Regulation of Naturally Overexpressed IL-33 Protein by IFN-γ—To assess whether the observed attenuating effect of IFN-γ on IL-33 is relevant to the natural regulation of IL-33

FIGURE 2. Changes in IL-33 protein level (mean ± S. D.) in cell lysates measured by ELISA in duplicate cultures repeated three times with consistent results. A, adherent lung cells obtained from mice challenged with bleomycin (solid lines) or PBS (dashed lines) were cultured either without (Ctrl) or with 100 ng/ml rIFN-γ for 24 h. B, NMLFs were cultured with rIFN-γ (100 ng/ml), poly(I:C) (100 μg/ml), or their combination, as indicated, for 24 h. NMLFs cultured with no additives were used as a control.
protein levels, two independent approaches were used. First, assessment of the ability of bleomycin to induce IL-33 expression \textit{in vivo} (4) was employed. Mice were challenged with intratracheal bleomycin or PBS, lung cells isolated as described under “Experimental Procedures,” and adherent lung cells cultured with or without rIFN-$\gamma$/H9253 followed by ELISA of cell lysates (Fig. 2A). The results showed that the bleomycin challenge \textit{in vivo} caused an increase in IL-33 protein levels. **FIGURE 3.** Changes in IL-33 (A, B, C, and E), IFN-$\gamma$ (D), and IL-4 (F) protein levels with mean $\pm$ S.D. as measured by ELISA in triplicate cultures. Each experiment was repeated on 2–5 separate occasions with similar results. A and B, NIH3T3 fibroblasts were infected with AdV-RSV-IL33 (A), AdV-CMV-IL33 (B), or AdV-NULL control, as indicated. After 48 h, cells were treated with the indicated concentrations of rIFN-$\gamma$ for an additional 72 h before the assays. Data for cell lysates are shown; IL-33 was undetectable in cell culture supernates. C–F, primary NMLFs were infected with AdV encoding the indicated proteins, and cell lysates (black bars) and supernates (gray bars) were collected and analyzed 48 h post-infection. Asterisks mark significant ($p < 0.05$) differences in the levels of indicated cytokines between dual and single gene delivery.

**TABLE 1** AdV constructs used in the \textit{in vivo} and cell culture experiments

| AdV             | E1-CMV | E3-RSV |
|-----------------|--------|--------|
| NULL            | NULL   | EGFP   |
| IL33 or RSV-IL33| NULL   | miIL-33|
| CMV-IL33        | miIL-33| EGFP   |
| IFN-$\gamma$    | miIFN-$\gamma$ | miIL-33 |
| IL4             | miIL-4 | EGFP   |
| IL33&IFN-$\gamma$| miIFN-$\gamma$ | miIL-33 |
| IL33&IL4        | miIL-4 | miIL-33|

**FIGURE 4.** Western blotting for IL-33 (upper gel) and $\beta$-actin (lower gel) of NMLF lysates infected with AdV-NULL (lane 1), AdV-RSV-IL33 (lane 2), AdV-IFN-$\gamma$ (lane 3), AdV-IL33&IFN-$\gamma$ (lane 4), AdV-IL33&IL4 (lane 5), or AdV-CMV-IL33 (lane 6). Loading of the AdV-CMV-IL33-infected sample was adjusted to compensate for the higher activity of CMV-driven, as opposed to RSV-driven, IL-33 production (compare the vertical scales in Fig. 1, A and B). This experiment was repeated twice with consistent results.
levels in pulmonary cells, whereas treatment with rIFN-γ in culture had an attenuating effect on the elevated IL-33 protein levels (p<0.05, Fig. 2A). In the second set of experiments, NMLF derived from unmanipulated mouse lungs were cultured with poly(I-C) (100 μg/ml), a known natural activator of IL-33 acting through TLR3 (30), in the presence or absence of rIFN-γ, as described under “Experimental Procedures” (Fig. 2B). Again, IFN-γ attenuated the stimulating effect of poly(I-C) on IL-33 protein expression levels measured by ELISA in cell lysates (p<0.05, Fig. 2B). Together, the data in Figs. 1 and 2 suggest that IFN-γ attenuates the levels of IL-33 protein whether artificially expressed by a recombinant construct or naturally expressed from the endogenous promoter.

**Regulation of IL-33 Expression by IFN-γ and IL-4 in Vivo**—The experimental results detailed above suggest that IFN-γ down-regulates IL-33 protein expression in fibroblasts, whereas its effect on IL-33 mRNA varies depending on cell type (down-regulation in fibroblasts versus up-regulation in keratinocytes). To assess the relevance of these findings to the complex, realistic, in vivo situation, subsequent gene delivery studies were performed in experimental mice. To ensure co-localization of IL-33 expression and simultaneous IFN-γ or IL-4 expression, dual expresser AdV constructs encoding IL-33 and IFN-γ, or IL-33 and IL-4, were utilized in comparison with control AdV constructs encoding a single cytokine, as shown in Table 1. WT C57BL/6 mice were infected intratracheally with IL33, IFNγ, IL4, IL33&IFNγ, and IL33&IL4 AdV constructs as described previously (5, 85–88, 90). Mice instilled intratracheally with AdV constructs showed no signs of morbidity such as body weight loss, ruffled fur, dehydration, diarrhea, hunched posture, or decreased motor activity at any time post-infection. Animals were euthanized on days 7 and 14, and analyses were performed as described below. These times were selected based on our previous experience with this AdV gene delivery system, in which the highest expression of the delivered gene(s) was observed on day 7 followed by a decline on day 14 and residual expression on days 21–28 (5, 85–88, 90).

Elevation in the levels of cytokines in lung homogenates was observed following gene delivery. As expected, the production of pulmonary cytokines was elevated following gene delivery. Infection of mice with AdV-RSV-IL33 led to elevated expression of IL-33 in the lungs (Fig. 5A, B, and C), and gene delivery of IFN-γ by itself caused a modest but statistically significant elevation in endogenous IL-33 production (Fig. 5A). Remarkably, simultaneous delivery of IL-33 and IFN-γ with the dual expresser AdV did not lead to an elevation in IL-33 protein production (Fig. 5A), although the expression levels of IFN-γ protein remained high with or without simultaneously delivered IL-33 (Fig. 5B). That IL-33 was not elevated in mice infected with the dual AdV-IL33&IFNγ was not a result of a defect in the virus design, construction, or use, because IL-33 mRNA expression was successfully driven by delivery of the same construct (Fig. 1, B and C).

In contrast to the observations made in cell culture (Fig. 3D), the suppressive effect of IL-33 delivery on IFN-γ expression in the dual expresser system in vivo was not observed (Fig. 5B), the tendency toward lower levels of IFN-γ in the dual expresser system did not reach significance (p>0.05). Also, in contrast to observations made in cell culture (Fig. 3E), gene delivery of IL-4 alone in vivo potently stimulated expression of endogenous IL-33 to levels greater than achieved by direct gene delivery of IL-33 (Fig. 5C). Further contradicting our findings in cell
**IFN-γ Controls IL-33 Levels through STAT1 and LMP2**

**FIGURE 6.** Changes in the cellular composition of BAL induced by gene delivery of IL-33, IL-4, IFN-γ, IL-33 and IFN-γ, or IL-33 and IL-4 to the lungs of C57BL/6 mice in vivo (mean ± S.D.), on days 7 (A and C) and 14 (B and D). Data are shown as mean ± S.D. Cell counts, 5–5 mice/group. Asterisks indicate significant differences (p < 0.05) between AdV-IL33&IFNγ- and AdV-IFNγ-challenged mice or between AdV-IL33&IL4 and AdV-IL4-challenged mice. Double daggers indicate significant differences (p < 0.05) between AdV-IL33-, AdV-IFNγ-, AdV-IL4-, AdV-IL33&IFNγ-, or AdV-IL33&IL4- and AdV-NULL-challenged mice.

in culture (Fig. 3E), the combined delivery of IL-33 and IL-4 with the dual expresser AdV did not lead to a potentiating effect on IL-33 expression (Fig. 5C), yet the potentiating effect on IL-4 protein production in the dual expresser system was reproduced on day 14 after gene delivery (compare Fig. 5D with Fig. 3F). The findings in Fig. 5, B–D, although interesting and likely biomedically important, were not pursued further in the current study, to preserve the focus on the finding of a consistent inhibitory effect of IFN-γ on IL-33 protein in cell culture and in vivo.

Total and differential BAL cell counts were assessed in mice following adenoviral single or dual gene delivery of IL-33, IFN-γ, and IL-4 (Fig. 6). In all cases, there was a significant (p < 0.05) elevation in lymphocytes on days 7 and 14 compared with AdV-NULL-challenged mice, although the effects of IFN-γ or IL-4 expression on BAL lymphocytes and overall cellularity substantially exceeded those of IL-33 (Fig. 6). In light of these findings, it is not surprising that the effects of combined IL-33 and IFN-γ or IL-33 and IL-4 delivery did not substantially exceed those of IFN-γ or IL-4 delivery alone. The effects of gene delivery on BAL neutrophils, although statistically significant, were, overall, modest (Fig. 6). Somewhat unexpectedly, there was a delay in elevation of BAL lymphocytes and eosinophils induced by the co-expression of IL-33 and IL-4 compared with IL-4 delivery alone on day 7, but these differences disappeared by day 14 (Fig. 6, C and D).

**Caspase-1, -3, and -8 Have a Limited Effect on IFN-γ-driven IL-33 Protein Down-regulation**—Subsequent experiments addressed the possible mechanisms by which IFN-γ might down-regulate IL-33 protein in cell culture and in vivo (Figs. 2C, 4, and 5A), even when IL-33 mRNA was not significantly suppressed (Fig. 1, B and C). Strong evidence suggests that IL-33 protein is degraded by caspase-3 and -7 (27) and that IFN-γ activates caspase-1, -3, and -8 in various cell types (54, 56–58). We hypothesized that the observed attenuating effect of IFN-γ on IL-33 protein level may be mediated by IFN-γ-driven activation of caspases. To assess this possibility, NIH3T3 fibroblasts infected with AdV constructs encoding IL-33, IFN-γ, or both IL-33 and IFN-γ were cultured with and without irreversible pharmacological inhibitors of caspase-1, -3, or -8 (Fig. 7). These experiments revealed that these inhibitors had limited, if any, effect on AdV-driven IL-33 expression in the absence of simultaneous IFN-γ expression (Fig. 7, A–C). Furthermore, there was a limited, if any, effect on IFN-γ-driven down-regulation in IL-33 protein levels, as the levels of IL-33 were significantly lower (p < 0.05) in cells infected with IL-33 and IFN-γ dual expresser AdV than in cells infected with AdV-IL33 with or without specific caspase inhibitors present (Fig. 7, A–C). Considering the relatively high levels of IFN-γ expression in this system (Fig. 3D, 200 ng/ml), similar treatment of the cells with these caspase inhibitors was tested with a lower concentration of rIFN-γ (10 ng/ml). These inhibitors did not have a noticeable effect on AdV-driven IL-33 expression, but they did neutralize the effect of this relatively low concentration of IFN-γ on IL-33 protein levels (Fig. 7D). Considering that specific inhibition of caspase-1, -3, or -8 did not affect IL-33 protein levels and affected IFN-γ-stimulated IL-33 protein down-regulation only at relatively low concentrations of IFN-γ, caspase-mediated IL-33 degradation was not considered a major mechanism responsible for the IFN-γ effect on IL-33 protein levels.
**IFN-γ Controls IL-33 Levels through STAT1 and LMP2**

**FIGURE 7. Changes in IL-33 protein level (mean ± S.D.) in cell lysates measured by ELISA in triplicate cultures.** Each experiment was repeated on at least six independent occasions, with similar results. A–C, NIH3T3 fibroblasts were treated with specific inhibitors of caspase-1 (Z-WEHD-FMK), -3 (Z-DEVD-FMK), or -8 (Z-ITED-FMK) for 4 h and then infected with AdV constructs encoding IL33, IFNγ, or IL33&IFNγ and incubated for an additional 72 h (black bars). D, NIH3T3 fibroblast cultures, 48 h after infection with AdV-IL33, were treated with caspase-1 (Z-WEHD-FMK), -3 (Z-DEVD-FMK), or -8 (Z-ITED-FMK) inhibitors for 4 h and then activated with rIFN-γ (10 ng/ml) for an additional 72 h (black bars). Similarly processed cultures without rIFN-γ activation were used as controls (gray bars).

*IFN-γ-driven IL-33 Protein Down-regulation Depends on STAT1.*—In contrast to the findings in the experiments with caspase inhibition described above, inhibition of STAT1 signaling had an overt effect both on IL-33 protein levels and on suppression by IFN-γ (Fig. 8). Pharmacological inhibition with nifuroxazide led to a strong increase in IL-33 protein levels in AdV-IL33-infected cells and completely abrogated the effect of IFN-γ in cells infected with the dual expression AdV-IL33&IFNγ (Fig. 8A). These observations suggested that even without stimulation with IFN-γ, basal STAT1 activity plays a role in controlling the basal levels of IL-33 protein, likely by mediating protein degradation and thus maintaining cellular homeostasis. Experiments with a STAT1-responsive promoter-firefly luciferase reporter have indeed confirmed a higher basal expression but not its activity, did not eliminate IFN-γ-driven IL-33 protein down-regulation (Fig. 8D). To further evaluate the effect of STAT1 on IL-33 protein levels, STAT1−/− primary mouse lung fibroblasts were used in cell culture experiments. Equal numbers (2.5 × 10⁵) of WT and STAT1−/− cells were plated for the experiment. The cells were infected with AdVs encoding IL-33, IL-33, and IFN-γ or infected with AdV-IL33 and treated further with rIFN-γ (10 ng/ml). Cell viability assays showed no difference in cell proliferation at the end of the 48-h window period. WT mouse lung fibroblasts were used as a control. Germ line deficiency of STAT1 led to greater levels of IL-33 expression and attenuated the effect of IFN-γ on IL-33 protein levels (Fig. 8E). Specifically, STAT1−/− cells showed a significant 1.6-fold increase in the level of IL-33 protein after gene delivery of IL-33, whereas the differences in the IL-33 protein levels with or without IFN-γ present were not significant (Fig. 8E). Nevertheless, the decreased effect of IFN-γ on IL-33 protein level was preserved in the control group (Fig. 8E).
Thus, STAT1 plays a central role in the regulation of basal and IFN-γ-controlled levels of IL-33 protein.

**Effect of Proteasome LMP2 Subunit Inhibition on IFN-γ-driven IL-33 Degradation**—Considering the known regulation of the LMP2 proteasome subunit by IFN-γ, further experiments tested the possibility that the levels of IL-33 protein may be lowered through proteasome-dependent degradation. RT-qPCR experiments revealed that treatment of fibroblasts with 100 ng/ml rIFN-γ, 300 μM nifuroxazide (NF), or their combination, as indicated, for 24 h, led to a 14-fold and 26.5-fold increase in LMP2 mRNA expression at 24 and 48 h post-treatment, respectively (repeated on two separate occasions with consistent results). Western blotting assay showed that infections of cultured fibroblasts with either AdV-NULL or AdV-IL33 did not, whereas infections with AdV-IFNγ or AdV-IL33&IFNγ did, lead to a substantial increase in LMP2 expression (Fig. 9A).

Next, the expression levels of LMP2 in the lung homogenates of the mice infected with corresponding AdVs were assessed (Fig. 9B). Again, expression of IFN-γ, either alone or in combination with IL-33, led to a substantial increase in LMP2 expression (Fig. 9B). To determine whether the previously observed IFN-γ-driven, STAT1-dependent, IL-33 protein down-regulation occurs via proteasome degradation, LMP2 gene expression was attenuated by RNA interference. Western blotting confirmed an attenuation of LMP2 protein level following transfection of cells with LMP2 siRNA but not scrambled siRNA (Fig. 9C).

IL-33 protein levels were measured by ELISA in fibroblasts transfected with LMP2 siRNA or non-targeting scrambled siRNA as a control and then infected with AdV constructs encoding IL33, IFN-γ, IL33&IFNγ, or NULL. Silencing of LMP2 significantly increased the expression levels of IL-33 protein (p < 0.05) and abrogated the IFN-γ-driven down-regulation of IL-33 protein levels (Fig. 9D). These findings suggest that LMP2 plays an
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IFN-\(\gamma\) Controls IL-33 Levels through STAT1 and LMP2

![FIGURE 9. A, Western blot analysis of LMP2 (upper gel) and \(\beta\)-actin (lower gel) in cell culture lysates of NIH3T3 fibroblasts infected with AdV-NULL (lane 1), AdV-IFN-\(\gamma\) (lane 2), AdV-IL33 (lane 3), or AdV-IL33&IFN-\(\gamma\) (lane 4). B, Western blot analysis of LMP2 (upper gel) and \(\beta\)-actin (lower gel) of whole lung homogenates after intratracheal instillation of AdV-NULL (lane 1), AdV-IFN-\(\gamma\) (lane 2), AdV-IL33 (lane 3), AdV-IL33&IFN-\(\gamma\) (lane 4). C, Western blot analysis of LMP2 (upper gel) and \(\beta\)-actin (lower gel) in cell culture lysates of NIH3T3 fibroblasts transfected with control scrambled (lane 1) or LMP2 siRNA (lane 2) and treated with 100 ng/ml of rIFN-\(\gamma\). D, changes in IL-33 protein level \(\pm\) S.D. measured in cell culture lysates by ELISA in triplicate cultures; each experiment was repeated twice with similar results. NIH3T3 fibroblasts were transfected with 500 \(\mu\)g siRNA against LMP2 or non-targeting scrambled siRNA. At 48 h post-transfection cells were infected with AdV constructs encoding IL-33, IFN-\(\gamma\), or IL-33 and IFN-\(\gamma\) genes and incubated for an additional 72 h. Cells without previous transfection with siRNAs but with delivery of corresponding genes were used as a control.

important role in the regulation of IL-33 protein homeostasis. Therefore, we concluded that IFN-\(\gamma\) down-regulates IL-33 protein level via STAT1-dependent, LMP2 proteasome-mediated degradation.

DISCUSSION

This work assessed the complexity of the regulatory interplay between IL-33 and IFN-\(\gamma\) as well as between IL-33 and IL-4 in mouse lungs \(in vivo\) and in cell culture. AdV-driven dual gene delivery of IL-33 and IFN-\(\gamma\) or of IL-33 and IL-4 was utilized in comparison with AdV delivery of each of these cytokines alone as well as with AdV-NULL delivery as control experiments. In parallel experiments, recombinant IFN-\(\gamma\) and IL-4 were used in cell culture to validate the results obtained with AdV gene delivery of these prototypic Th1 and Th2 cytokines, respectively.

Recombinant IFN-\(\gamma\) strongly down-regulated basal levels of IL-33 mRNA in cultured primary lung fibroblasts (Fig. 1A), although IL-33 protein levels in these cultures were too low to be reliably measured. When IL-33 protein was expressed at high levels in cultured fibroblasts through AdV gene delivery, rIFN-\(\gamma\) significantly down-regulated IL-33 protein levels (Fig. 3, A and B). However, the levels of IL-33 mRNA in these cells remained essentially unaffected (Fig. 1, B and C), likely because IL-33 expression was driven by the non-mammalian promoters RSV and CMV. Such suppression of IL-33 protein levels by IFN-\(\gamma\) was observed independently of the promoter driving the expression of IL-33 (Fig. 3, A and B). A similar treatment of AdV-NULL-infected NIH3T3 cells with rIFN-\(\gamma\) had no effect on the RSV-driven expression of EGFP, as the ELISAs showed similar EGFP levels in cell lysates of control and IFN-\(\gamma\)-treated cultures. rIFN-\(\gamma\) also attenuated the levels of naturally induced IL-33 protein (Fig. 2).

Similar to the observed suppression of the IL-33 protein levels by exposure to rIFN-\(\gamma\), infection of primary pulmonary mouse fibroblasts with the dual expresser construct AdV-IL33&IFN-\(\gamma\) resulted in significantly lower expression of both IL-33 protein and IFN-\(\gamma\) protein compared with similar infections with AdV-IL33 and AdV-IFN-\(\gamma\) (Figs. 2, C and D, and 3). In contrast to the mutually inhibitory interplay between IL-33 and IFN-\(\gamma\) proteins in this system, similar delivery of IL-33 and IL-4 resulted in mutual potentiation of the expression levels of these protein compared with the corresponding single-cytokine expresser AdV infections (Fig. 3, E and F); this latter observation was not pursued further in this work to preserve the focus on IFN-\(\gamma\)-driven IL-33 protein attenuation. However, the contrasting regulation of IL-33 protein levels by IFN-\(\gamma\) and IL-4 suggests that the observed effects are not artifacts of the dual expresser AdV gene delivery system. Consistent with the previous observations (3, 5), IL-33 remained intracellular in these cultures, whereas both IFN-\(\gamma\) and IL-4 were readily found in cell culture supernates and cell lysates (Fig. 3, C–F).

The finding of the down-regulating effect of IFN-\(\gamma\) on the IL-33 mRNA (Fig. 1A) and protein (Fig. 3) levels appears to contradict the previous reports of an up-regulating effect of IFN-\(\gamma\) on IL-33 mRNA in skin keratinocytes (32, 33), cardiac myocytes, and fibroblasts (47), yet it is consistent with the previously observed apparent attenuating effect of IFN-\(\gamma\) on IL-33 mRNA in macrophages and microglial cells (48, 49). To clarify the Th1 and Th2 regulation of the IL-33 protein expression in the complexity of a realistic \(in vivo\) environment, AdV-mediated gene delivery was utilized (Fig. 5). Consistent with our
IFN-γ Controls IL-33 Levels through STAT1 and LMP2

observed in cell culture (Figs. 2 and 4), expression of IFN-γ in vivo suppressed IL-33 protein levels, whereas IL-33 and IL-4 mutually potentiated each other’s expression (Fig. 5). Thus, IFN-γ down-regulates, whereas IL-4 up-regulates, IL-33 protein expression levels in lung cell culture and in the lungs in vivo. However, such regulation may be different depending on cell type or tissue origin of the cells (32, 33, 47–49).

The subsequent experiments addressed the possibility that IFN-γ accelerates IL-33 protein degradation. Although caspases are thought to play a critical role in degrading IL-33 (27, 28), our results suggest that caspases are unlikely to be major mediators of IFN-γ-driven IL-33 protein degradation, as specific inhibitors of caspase-1, -3, and -8 have minimal, if any, effect on this process (Fig. 7). By contrast, inhibition of STAT1, the central mediator of IFN-γ signaling, with either nifuroxazole or siRNA or a germ line deficiency of STAT1 had a significant effect on the basal IL-33 protein levels as well as on their IFN-γ-driven regulation (Fig. 8). Furthermore, reduced expression of LMP2, a well known downstream target of the IFN-γ–STAT1 pathway, also strongly increased the basal levels of IL-33 protein and abrogated the inhibitory effect of IFN-γ (Fig. 9). This proteasome subunit is known not only to play a major role in the degradation of the peptides presented by MHC class I molecules (66–69) but is also a critical contributor to many non-immune functions (70–79). Thus, IFN-γ attenuates the IL-33 protein levels by engaging STAT1 and the LMP2 proteasome subunit but not caspases.

The implications of these findings are severalfold. First, an important implication of these observations has to do with the central role of IL-33 in immune deviation toward Th2 responses (94, 95). Our results suggest that IL-33 is rapidly and efficiently costimulated at the protein level by the prototypic Th1 cytokine IFN-γ, thus adding a new level of complexity to Th1-Th2 regulatory cross-talk. Second, these data show that, although IL-33 protein degradation can occur through a caspase-dependent mechanism, the realistic regulation of IL-33 protein levels involves a proteasome-dependent process. These observations reinforce the notion that the LMP2 subunit functions not only as an immunoproteasome component but also as part of the conventional proteasome machinery, particularly when regulated by IFN-γ. Third, although recombinant IFN-γ therapies have thus far shown limited effectiveness, there is a possibility that the lack of efficacy was due to rapid clearance of the injected cytokine combined with inefficient blood tissue transfer, which led to limited bioavailability of IFN-γ in the target tissues. Perhaps if the half-life and tissue bioavailability of IFN-γ in vivo can be increased by a better delivery system, it may be used for attenuating a spectrum of IL-33-dependent diseases (4–22). Indeed, recently improved methods of IFN-γ gene delivery in vivo have produced encouraging results (96, 97). Our observations suggest that it may be possible to utilize IFN-γ gene delivery in vivo to treat not only Th2 diseases propelled by the mature IL-33-T1/ST2 receptor axis but also the distinct inflammatory and fibrotic processes induced by elevated levels of full-length IL-33 (4, 5).

In summary, IFN-γ, IL-4, and IL-33 are engaged in a complex interplay in which IFN-γ attenuates elevated IL-33 protein levels in pulmonary fibroblasts and in the lungs in vivo, whereas IL-4 further elevates IL-33 protein levels. The suppressive effect of IFN-γ on IL-33 is mediated through the STAT1/LMP2 proteasome pathway, with a limited engagement of caspase-1, -3, and -8.

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