Interleukin-33 promotes type 1 cytokine expression via p38 MAPK in human natural killer cells

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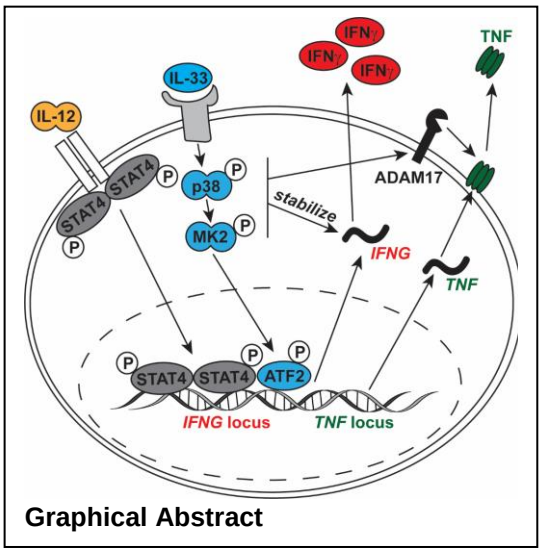

Graphical Abstract

This study tests the hypothesis that activation of mitogen-activated protein kinase (MAPK) by physiologically-relevant concentrations of interleukin-33 (IL-33) contributes to enhanced cytokine expression by IL-12 stimulated human natural killer (NK) cells. While IL-33 canonically triggers type 2 cytokine responses, this cytokine can also synergize with type 1 cytokines like IL-12 to provoke interferon-gamma (IFN-γ). We show that picogram concentrations of IL-12 and IL-33 are sufficient to promote robust secretion of IFN-γ by human NK cells that greatly exceeds responses to either cytokine alone. Nanogram doses of IL-33, potentially consistent with levels in tissue microenvironments, synergize with IL-12 to induce secretion of additional cytokines, including tumor necrosis factor (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-33-induced activation of the p38 MAPK pathway in human NK cells is crucial for enhanced release of IFN-γ and TNF in response to IL-12. Mechanistically, IL-33-induced p38 MAPK signalling enhances stability of *IFNG* transcripts and triggers ADAM17-mediated cleavage of TNF from the cell surface. These data support our hypothesis and suggest that altered sensitivity of NK cells to IL-12 in the presence of IL-33 may have important consequences in diseases associated with mixed cytokine milieus, like asthma and chronic obstructive pulmonary disease.

Innate lymphoid cell | p38 MAPK | IL-12 | NK cell | IL-33 | IFN-γ | GM-CSF | TNF | synergy | asthma | COPD

Introduction

Natural killer (NK) cells play a key role in the clearance of virus-infected cells and the pathogenesis of many diseases[1-3]. The type 1 cytokine interleukin-12 (IL-12) provokes a canonical interferon-gamma (IFN-γ) response by NK cells[4]. Other type 1 cytokines, including IL-15 and IL-18, enhance IL-12-induced IFN-γ release by NK cells[5-8]. This type 1 cytokine synergy can also promote enhanced release of tumor necrosis factor-alpha (TNF) and granulocyte macrophage colony stimulating factor (GM-CSF). Thus, type 1 cytokine rich environments associated with immune insults such as infection or injury can stimulate cytokine responses from NK cells that further promote type 1 responses. For example, in the experimental model of *Leishmania infantum,* the combination of IL-12 and IL-18 is critical for NK-cell IFN-γ expression[9].

A central dogma of cytokine biology holds that type 1 cytokines (e.g. IL-12) suppress type 2 cytokine responses, while type 2 cytokines (e.g. IL-4) correspondingly suppress type 1 responses[10, 11]. Thus, type 2 cytokines should putatively suppress NK-cell production of IFN-γ. Yet the prototypical typical type 2 cytokine IL-4, alone or in combination with IL-12, triggered high levels of IFN-γ expression by mouse NK cells[12 13]. Another type 2 cytokine, IL-33, can enhance IL-12-induced production of IFN-γ by both NK and NKT cells[14-16]. Thus, NK cells in type 2 cytokine rich environments may exhibit hypersensitive IFN-γ responses following IL-12-inducing infections or insults.

In the present study, we confirm that primary human NK cells treated with a combination of IL-33 and IL-12 *ex vivo* produce high levels of IFN-γ[14] and we extend this observation to near physiological picogram concentrations of these cytokines. Mechanistically, we implicate the p38 mitogen-activated protein kinase (MAPK) pathway in IL-33-mediated enhancement of IL-12-induced responses of human NK cells and uncover a role for ADAM17 in enhanced release of TNF under these stimulatory conditions. These data provide mechanistic insights into how IL-33-rich inflammatory milieus may directly enhance proinflammatory activities of NK cells.

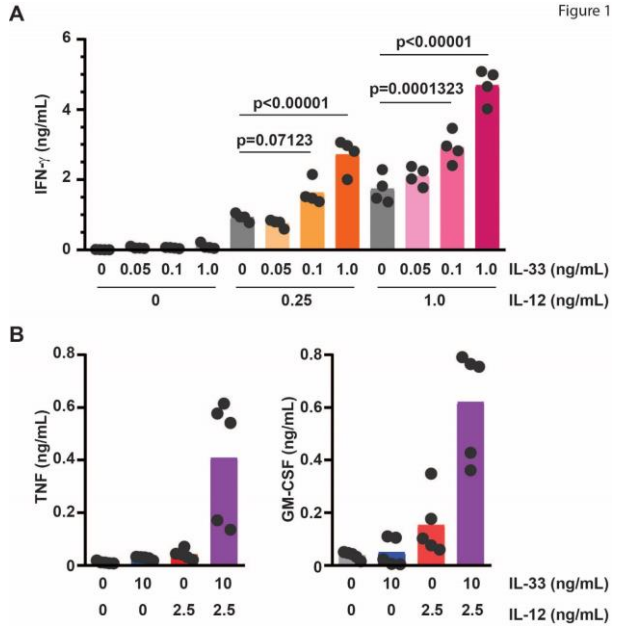

Figure 1. IL-33 enhances IL-12-induced cytokine expression in primary human NK cells. Enriched primary human NK cells were stimulated with combinations of IL-12 and IL-33 (see methods) for 16 hours prior to ELISA determination of (**A**) IFN-γ, (**B**) TNF or GM-CSF expression in cell-free supernatant. Results from one representative iteration of (**A**) ten or (**B**) five similar experiments using cells from different blood donors is shown (bars represent mean). Contributions of each cytokine and synergistic interactions determined by two-way ANOVA. Multiple comparisons of ordinary one-way ANOVA used to compare columns in part **A**.

Results

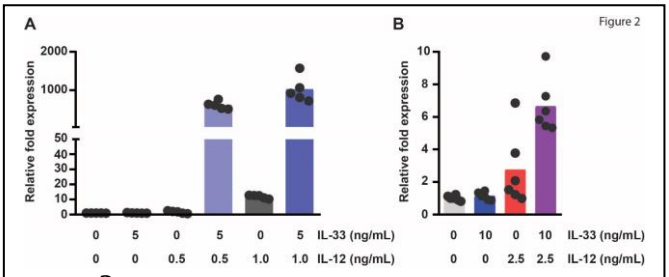

Figure 2. Increased *IFNG* and *TNF* expression in IL-12/IL-33 stimulated NK cells. Enriched primary human NK cells (three different donors, three replicates per experiment) were stimulated with combinations of IL-12 and IL-33 for 6 hours prior to qRT-PCR determination of (**A**) IFN-γ or (**B**) *TNF*. Contributions of each cytokine and synergistic interactions determined by two-way ANOVA.

IL-33 enhances IL-12-induced cytokine expression in primary human NK cells.

Primary NK cells isolated from the blood of healthy de-identified adults (n=10) secrete IFN-γ in response to doses of IL-12 as low as 250 pg/mL (**Figure 1A**). In contrast, production of IFN-γ by these cells was barely detectable after stimulation with IL-33 alone, even at doses as high as ≥1 ng/mL (**Figure 1A**). However, 100 pg/mL or more of IL-33 markedly enhanced (1.7- to 2.9-fold) IL-12-elicited IFN-γ protein expression (**Figure 1A**), with synergistic interactions between IL-12 and IL-33 contributing significantly (p<0.00001, two-way ANOVA) to the overall variation in IFN-γ expression.

High concentrations of IL-33 (10 ng/mL) additionally provoked expression of TNF and GM-CSF when administered in combination with IL-12 (**Figure 1B**). A substantial fraction of the variance in TNF (72.12%, p=0.0003, two-way ANOVA) and GM-CSF (82.49%, p=0.00002, two-way ANOVA) expression was attributable to synergistic interactions between IL-33 and IL-12 (**Figure 1B**).

We also observed a >100-fold enhancement of IL-12-induced *IFNG* mRNA expression following co-stimulation with IL-33 (90.89% of variance attributable to interactions between IL-33 and IL-12, p<0.00001, two-way ANOVA) (**Figure 2A**). *TNF* transcript expression was increased ~2.5-fold by the combination of IL-12 and IL-33 in comparison to IL-12 alone (17.68% of variance between groups attributable to interactions between IL-12 and IL-33, p<0.00001, two-way ANOVA) (**Figure 2B**).

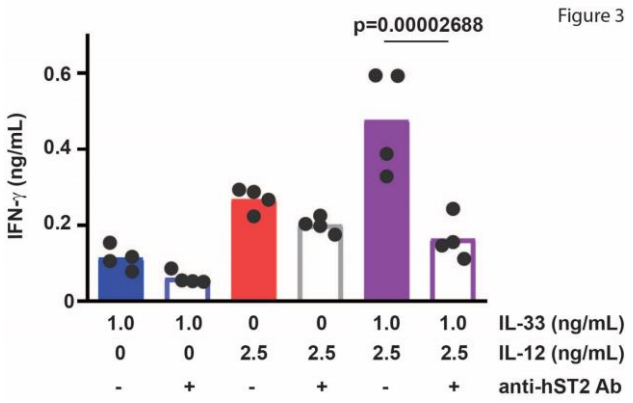

Figure 3. Role of ST2 in IL-33-mediated enhancement of IL-12 responsiveness of NK cells. Enriched primary human NK cells were pre-treated with 1 μg anti-ST2 (filled bars) antibody (or isotype, unfilled bars) one hour prior to stimulation with combinations of IL-12 and IL-33 for 16 hours prior to ELISA determination of IFN-γ (columns represent mean). Multiple comparisons of ordinary one-way ANOVA were used to compare columns.

Antibody blockade of the IL-33 receptor, ST2, abrogated the enhancing effect of IL-33 on IL-12-induced IFN-γ expression, consistent with previous studies[14]. However, ST2 blockade had no measurable effect on IFN-γ induced by IL-12 alone (**Figure 3**).

IL-33 enhances IFN-γ and TNF release via activation of p38 MAPK.

In macrophages, IL-33 stimulates activation of p38 MAPK[17, 18]. To determine if IL-33 activates p38 MAPK in human NK cells, we measured phosphorylation of p38 MAPK and downstream targets of this kinase by flow cytometry. Primary human NK cells were cultured and stimulated with IL-12, IL-33 or both cytokines. Phosphorylation of p38 MAPK (Thr180, Tyr182) and activating transcription factor 2 (ATF2, Thr71) were measured by flow cytometry. IL-33 stimulation, irrespective of the presence of IL-12, stimulated p38 MAPK phosphorylation within 5 minutes of cytokine exposure (**Figure 4A**). Levels of phospho-p38 MAPK remained elevated for at least 30 minutes post-stimulation with IL-33. Likewise, IL-33 stimulation induced the phosphorylation of ATF2, albeit with slower kinetics (**Figure 4B**). In contrast, IL-12 but not IL-33 induced phosphorylation (Tyr693) of STAT4 (**Figure 5**). The extent of IL-33-induced phospho-p38 MAPK (**Figure 4A**) and phospho-ATF2 (**Figure 4B**), as well as IL-12-induced phospho-STAT4 (**Figure 5**), were not measurably altered over this time scale by co-administration of the other cytokine.

To determine the role of p38 MAPK in IL-33 enhancement of IL-12-induced cytokines, a specific p38 MAPK inhibitor, R-1503[17], was employed. NK cells were pre-treated with R-1503 (0 to 500 nM) for 150 minutes prior to stimulation with IL-33 (10 ng/ml) and IL-12 (1 ng/ml) for 16 hours. None of the concentrations of inhibitors used in this study

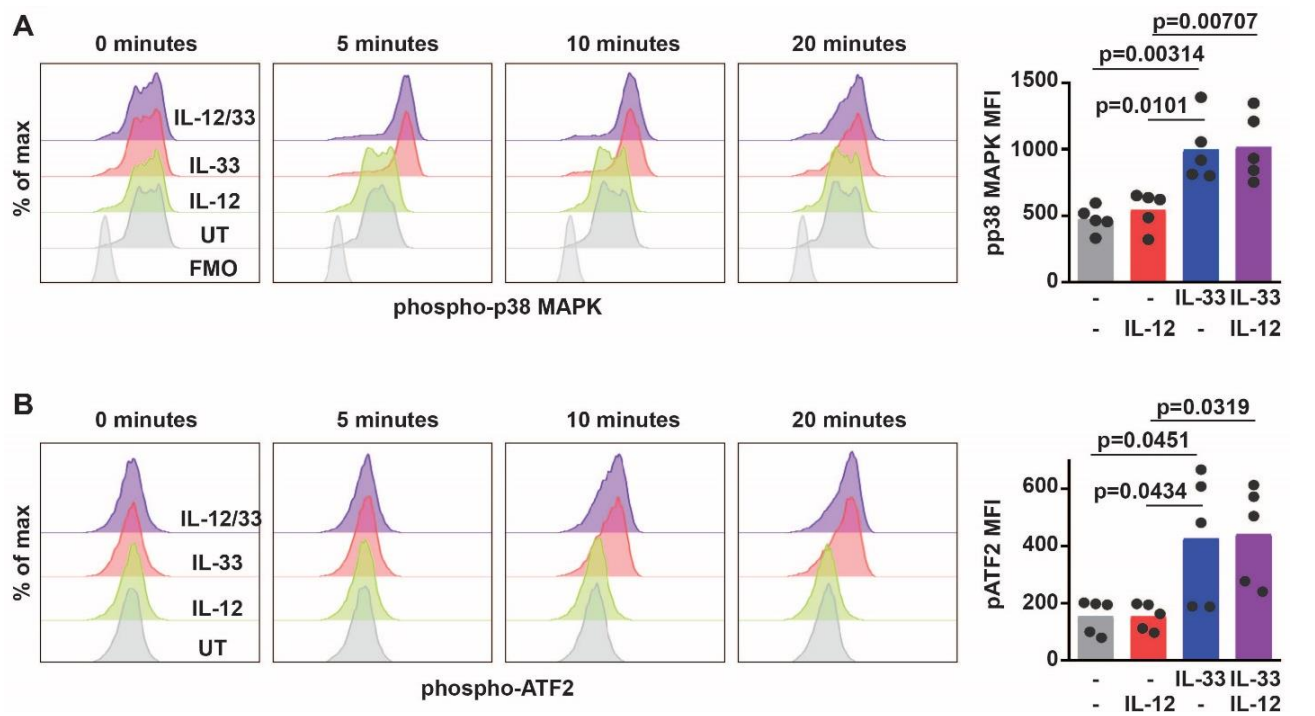

Figure 4. IL-33 activates p38 MAPK in human NK cells. Human NK cells (four different donors, duplicates per experiment) were left untreated (UT) or incubated with IL-12 or IL-33 for 5 to 120 minutes prior to phosphoflow measurement of intracellular **(A)** phospho-p38 MAPK, **(B)** phospho-ATF2, or **(C)** phosphor-STAT4 levels. Representative histogram plots are shown, along with graphs of median of fluorescence intensity (MFI). Data represents means ± SEMs. Multiple comparisons of ordinary one-way ANOVA shown.

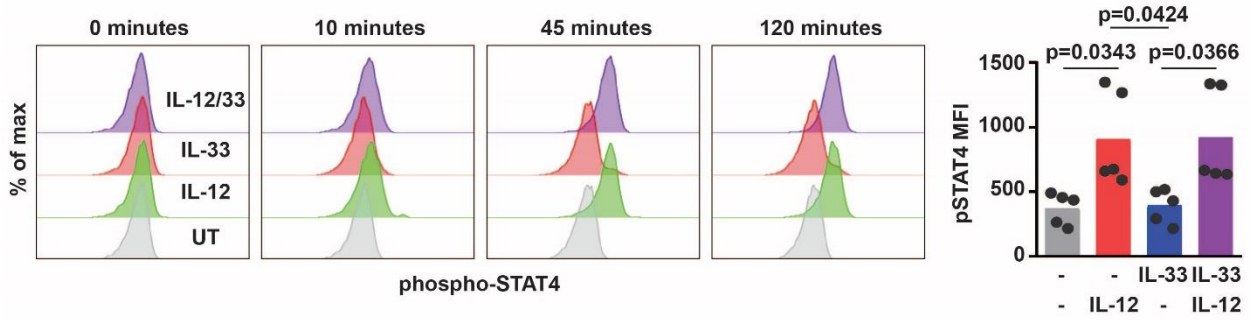

Figure 5. IL-33 does not interfere IL-12 STAT4 phosphorylation. Human NK cells (four different donors, duplicates per experiment) were left untreated (UT) or incubated with IL-12 or IL-33 for 10 to 120 minutes prior to phosphoflow measurement of phosphorylated STAT4 levels. Representative histogram plots are shown, along with graphs of median of fluorescence intensity (MFI). Multiple comparisons of ordinary one-way ANOVA were used to compare columns.

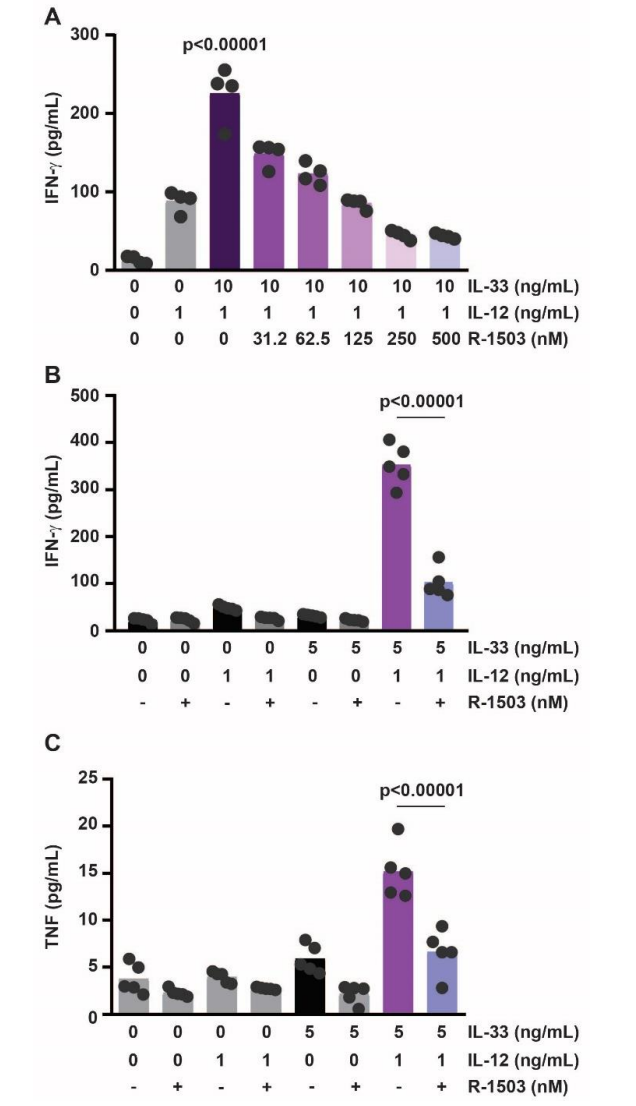

Figure 6. p38 MAPK inhibition abrogates IL-33-enhancement of IL-12-induced activity. (A) IFN-γ production by human NK cells (one representative of 5 donors tested) following 150-minute pretreatment with vehicle (0.1% DMSO) or R-1503 (0-500 nM) and subsequent stimulation with IL-12 and IL-33. **(B and C)** Effect of pre-treatment with 125 nM R-1503 on levels of IFN-γ and TNF in cell-free supernatant collected 6 hours after cytokine stimulation. Multiple comparisons of ordinary one-way ANOVA were used to compare columns.

measurably affected cell viability (data not shown). A dose of 125 nM R-1503 restored expression of IFN-γ in response to IL-12 plus IL-33 to levels seen with IL-12 alone (**Figure 6A**). This concentration of R-1503 did not measurably impact IL-12-induced IFN-γ expression in the absence of IL-33 (**Figure 6B**). Addition of R-1503 did diminished the enhancing effect of IL-33 on IL-12-induced TNF expression (**Figure 6C**).

IL-18 can enhance IL-12-provoked IFN-γ production by promoting *IFNG* transcript stability, which is mediated by p38 MAPK signaling[19]. To determine whether IL-33 exerts a similar effect, human NK cells were incubated with IL-12 alone or in combination with IL-33 for 6 hours in conjunction with pretreatment with R-1503 or vehicle control. Four hours after cytokine administration, cultures were treated with actinomycin D (5 μg/ml) to halt *de novo* transcription, thereby permitting measurement of decay of existing *IFNG* transcripts. Roughly 80% of IL-12-induced *IFNG* transcripts decayed by 60 minutes after actinomycin D treatment (**Figure 7)**. Unexpectedly, the p38 MAPK inhibitor R-1503 enhanced *IFNG* transcript stability (40% decay by 60 minutes). Importantly, a combination of IL-12 and IL-33 had the strongest enhancing effect of *IFNG* transcript stability (20% decay at 60 minutes), and this effect was largely abrogated by R-1503 (40% decay at 60 minutes).

MAPKMAPK (MK2) contributes to IL-33 enhancement of IL-12-induced IFN-γ.

Previous studies showed that MAPKMAPK **(**MK2) is down-stream of p38 MAPK, and that MK2 mediates inflammatory responses[20, 21]. Addition of a specific MK2 inhibitor (MK2 IV) to NK-cell cultures diminished the enhancing effect of IL-33 on IL-12-stimulated IFN-γ production (**Figure 8**). In contrast to R-1503, no dose of MK2 IV that we tested completely abrogated IL-33 enhancement of IFN-γ

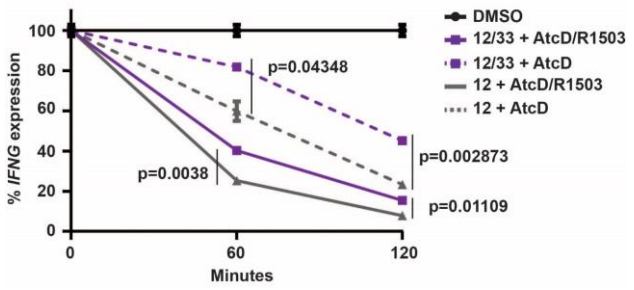

Figure 7. p38 MAPK inhibition disrupts IL-33-mediated IFNG mRNA stability. NK cells were pretreated with R-1503 or DMSO 0.1% and then stimulated with IL-12 (2.5 ng/ml, ▲) or with IL-12/33 (2.5 and 10 ng/ml respectively, ■) for 4 hours. After 4 hours DMSO 0.1% (black solid line), Actinomycin D (ActD 5 µg/ml, dashed lines) or Actinomycin D with R-1503 (solid lines) were added. IL-12 cells (grey), IL-12/33 treated cells (purple). *IFNG* expression was measured by qRT-PCR. Each plot represents one of three independent experiments. Multiple comparisons of ordinary two-way ANOVA were used to compare cell treatments within time mentioned time points.

expression. Thus, MK2 contributes to the enhancing effect of IL-33 but does not fully account for the effects mediated by p38 MAPK.

IL-33 and IL-12 regulate TNF release via ADAM17.

TNF is shed from the cell membrane via activity of ADAM-17[22]. We hypothesize that increased TNF secretion by NK cells stimulated with a combination of IL-12 and IL-33 (**Figure 1B**) is a result of elevated ADAM-17 cleavage of cell-surface TNF. Addition of the ADAM17 inhibitor (TAPI-1)[23] to NK-cell cultures resulted in reduction of secreted TNF from 97.09±10.36 to 45.79±5.15 pg/ml (ELISA) in response to IL-33 and IL-12 stimulation (**Figure 9A**). In contrast, R-1503 more completely ablated the enhancing effect of IL-33 on IL-12-induced TNF secretion. Thus, enhanced TNF secretion in response to IL-12 and IL-33 is at least partially dependent on ADAM-17, which may function downstream of p38 MAPK.

While secreted TNF secretion increased in response to the combination of IL-33 and IL-12, cell-associated TNF was reduced 1.88-fold to levels near those induced by IL-12 alone (**Figure 9B**). These flow cytometry measurements were made in the absence of brefeldin-A or other secretion inhibitors, thus reflected TNF naturally retained by the cells. Importantly, reduced TNF secretion by TAPI-I-treated, IL-12 and IL-33 stimulated cells (**Figure 9A**), was associated with a 1.98-fold increase in cell-associated TNF to levels observed with IL-12 alone (**Figure 9B**). Thus, IL-33-stimulated ADAM17 activity contributes to TNF secretion at the expense of cell-associated TNF, likely via cleavage of TNF from the cell surface.

Discussion

We augment previous observations in mouse[15, 16] and human[14] NK cells by showing that near-physiological (e.g. pM) concentrations[24, 25] of the type 2 cytokine IL-33 trigger hypersensitivity of primary human NK cells to the type I cytokine IL-12. In addition, superphysiological concentrations of IL-33 and IL-12 synergistically induced the release of TNF and GM-CSF, suggesting that high concentrations of these cytokines in a tissue microenvironment may have even stronger effects on NK-cell function. IL-33-mediated potentiation of IFN-γ and TNF secretion depended on p38 MAPK, and to a lesser extent, the downstream kinase MK2. Moreover, enhanced TNF secretion was partially attributable to ADAM17. These results highlight the possibility for exaggerated cytokine secretion by human NK cells in physiological setting that are characterized by dual exposure to type 1 (IL-12) and type 2 (IL-33) cytokines.

The type 1 cytokine, IL-18, enhances expression of *IFNG* by IL-12-stimulated human NK cells[26] in part via p38 MAPK stabilization of *IFNG* transcripts[19]. Our data suggest that a type 2 cytokine, IL-33, similarly enhances IL-12-induced IFN-γ expression by human NK cells. Moreover, IL-33 amplification of IFN-γ is dependent on p38 MAPK activity and associated with increased stability of *IFNG* mRNA. The p38 MAPK regulates inflammatory responses via phosphorylation of downstream mediators, including MK2[27, 28]. Yet, MK2 inhibitors had only a partial inhibitory effect in contrast to the abrogation of IL-12/IL-33 synergistic interactions following p38 MAPK inhibition. This finding implies that other p38 MAPK-stimulated mediators, including ATF2, may

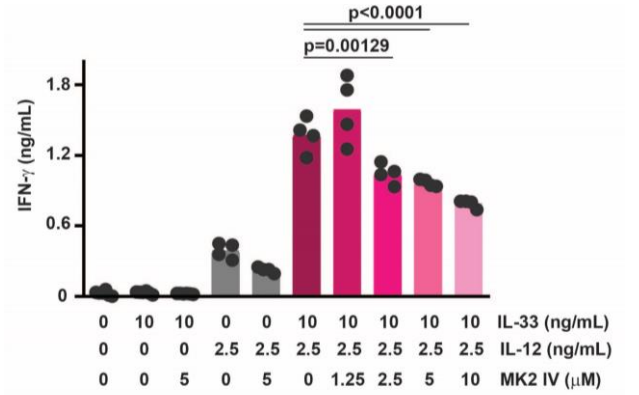

Figure 8. MK2 inhibition interferes IL-33 enhancement of IL-12 induced activity. IFN-γ production by human NK cells (one representative of 5 donors tested) following 150-minute pretreatment with vehicle (0.1% DMSO) or various concentrations of MK2 IV (0-10 µM) and subsequent stimulation with IL-12 and IL-33 for 16 hours, IFN-γ levels were measured in cell free supernatant.

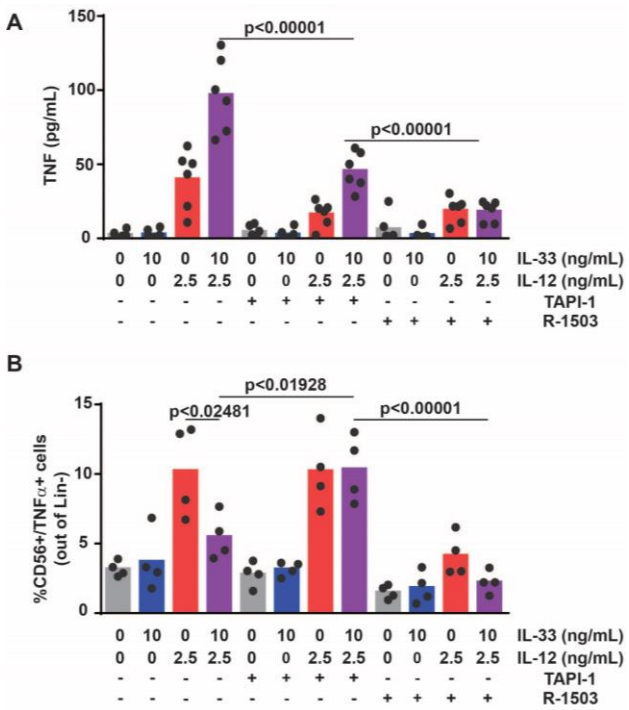

Figure 9. IL-33 and IL-12 regulate TNF release via ADAM17. Enriched human NK cells (from four donors in duplicates per condition) were pretreated with 0.1% DMSO, R-1503 (0.25 µM) or TAPI-1 (8 µM) and then stimulated in the presence of cytokines for 16 hours. (**A**) Secreted TNF expression was measured by ELISA while **(B)** cell-associated TNF was measured by flow cytometry. Multiple comparisons of ordinary one-way ANOVA were used to compare columns.

also be crucial for the enhancing effect of IL-33. In fact, IL-33 rapidly induced the phosphorylation of p38 MAPK and ATF2 in human NK cells. Of note, p38 MAPK-induced activation of ATF2 initiates activator protein 1 (AP-1) complex-mediated transcription of inflammatory cytokines, including IFN-γ[29-31]. Coupled with the lack of apparent effects of IL-33 on IL-12-induced phosphorylation of STAT4, our results highlight the possibility that IL-33 enhances IFN-γ via enhanced AP-1-dependent promotion of transcription.

In addition to p38 MAPK-driven intracellular signaling leading to transcription factor activation, p38 MAPK can also directly phosphorylate membrane proteins like ADAM17[32]. The activation of ADAM17 by p38 MAPK can promote shedding of TNF[17, 33]. Our data are consistent with these possibilities, revealing p38 MAPK-dependent enhancement of TNF expression coupled to ADAM17-dependent release of cell-associated TNF. Collectively, our data suggest that p38 MAPK govern several molecular pathways which regulates inflammatory cytokine release in human NK cells.

The ability of type 2 cytokines to mediate IFN-γ release is not limited to IL-33. Past studies demonstrated that IL-4 enhance IFN-γ production in murine IL-12 and IL-15 stimulated NK cells[13, 34]. We also observed IL-4 or IL-13 enhancement of IL-12-induced IFN-γ production by human NK cells (D.O. and S.N.W. unpublished observations). Whereas IL-4 and IL-13 predominately signal via activation of STAT6, both cytokines appear capable of triggering the p38 MAPK pathway[35]. In fact, synergy between IL-4 and IL-12 in mouse NK cells is partially dependent on p38 MAPK[13]. Whether distinct type 2 cytokines enhance IL-12-induced IFN-γ production via similar or distinct mechanisms remains to be determined.

In pathologies such as asthma and COPD, IL-33 play a central role in disease pathology, those pathologies were shown to be exacerbated by respiratory viral infection which mediates the release of type 1 cytokines such as IL-12[36-38]. Thus, we speculate that NK cells derived from the blood or tissues of patients exhibiting elevated levels of IL-33 would be more sensitive to *ex vivo* IL-12 stimulation. As an example, cigarette smoke induces epithelial damage resulting in increased expression of IL-33[15, 38], which provokes hypersensitive IFN-γ production by mouse NK cells in response to IL-12[39]. Our initial data suggest that NK cells from smokers do exhibit greater sensitivity to IL-12 in terms of IFN-γ expression than NK cells derived from healthy, non-smokers (D.O., M.B., and S.N.W unpublished observations). We speculate that elevated IL-33 levels in smokers or asthmatic patients provokes hypersensitivity to type 1 inflammatory cues triggered by virus infection. Thus, smokers exposed to virus infections likely produce high, potentially pathogenic expression of IFN-γ. Our findings provide new insights about potential functional hypersensitivity of NK cells in type 2 cytokine rich inflammatory milieus such as asthma and COPD as well as in smokers.

Materials and Methods

Human cells. De-identified blood samples were obtained from healthy donors, as defined by Hoxworth Blood Center guidelines (https://hoxworth.org/donors/eligibility.html), with the approval of Cincinnati Children's Hospital Medical Center Institutional Review Board. Peripheral blood mononuclear (PBMCs) cells were

isolated on a ficoll-hystopaque (GE, Marlborough, MA) gradient, and NK cells were enriched by negative selection using the NK-cell isolation kit immune magnetic beads per manufacturer's protocol (Miltentyi Biotec, San Diego, CA). Enriched cells were >90% $CD56^{+}$ $CD3^{neg}$ NK cells as determined by flow cytometry.

In vitro culture of NK cells. Enriched NK cells were cultured in SCGM media (Cellgenix®, Freiburg, Germany) supplemented with IL-2 (400 U/ml, Peprotech, Rocky Hill, NJ), 10% human serum (Sigma, St. Louis, MO), 10% heat-inactivated fetal bovine serum, 100U/ml penicillin, 100 U/ml streptomycin, 1% non-essential amino acids, 1% sodium pyruvate, 2mM L-Glutamine and 10 mM HEPES. In experiments, NK cells were either used directly after blood isolation or after 7 to 30 days of *ex vivo* culture. Each scenario produced similar results.

Antibodies. The following conjugated antibodies were used in the described studies: Brilliant violet (BV) 421-CD56 (5.1H11), BV711-CD16 (3G8), AlexaFluor (AF) 647-NKp46 (9E2), PE-NKp44 (P44-8) were purchased from Biolegend® (San Diego ,CA); Brilliant Ultra violet (BUV) 395-CD3 (SK7), V500-CD3 (SP34-2), BUV797-CD69 (FN50), and BUV-CD62L (SK11) were purchased from Becton and Dickinson (San Diego, CA); eFluor710-NKG2D (1D11) from Invitrogen (Carlsbad, CA); and mouse anti-human ST2 (AF523) from R&D (Minneapolis, MN). Antibodies were used at doses titrated in our lab.

Cytokine stimulation. Enriched NK cells (unless mentioned otherwise, 5×10^4 per well in triplicate per condition) were cultured overnight in the presence of 60 U/ml of IL-2. Various concentrations of IL-12, IL-18 or IL-33 or (Peprotech®, Rocky Hill, NJ) were added to cultures for 6 to 16 hours. Cell-free supernatant was collected and analyzed by ELISA for levels of human IFN-γ, TNF and GM-CSF (Invitrogen, Waltham, MA). For inhibition of mitogen-activated protein kinase (MAPK) activity, 125 nM (measured IC_{50}) of the selective p38 MAPK inhibitor Pamapimod (R-1503, Selleckchem, Houston, TX) was added 150 minutes prior to cytokine stimulation. The inhibition of MK2 (p38/mitogen-activated protein kinase-activated protein kinase 2) was performed with 5 μM MK2 IV (CAYMAN chemical, Ann Arbor, MI). The inhibition of ADAM-17 (a disintegrin and metalloprotease-17) was performed by adding 8 μM TAPI-1 (Merck Millipore, Burlington, MA). TAPI-1 and MK2 IV were added 60 minutes before cytokine stimulation. At the tested concentrations, none of the tested inhibitor affected cell viability (data not shown).

mRNA stability assay. Primary human NK cells were cultured at 1×10^5 cell per well. Cells were treated with media (0.1% DMSO), IL-12 (2.5 ng/ml) with or without IL-33 (10 ng/ml). Actinomycin D (5 μg/mL, Sigma) was added to cell cultures 4 hours after cytokine treatment, and RNA was isolated before addition of actinomycin D and 2 hours after addition of actinomycin D. RNA was extracted from NK cells with RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The detection of *IFNG* and *TNF* mRNA expression was performed by using TaqMan® probes (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

Statistical analysis. We performed statistical analyses using GraphPad Prism 8.01. We used two-way ANOVA to identify the contribution to multiple variables to an experimental measurement. We used one-way ANOVA to perform multiple comparisons between experimental conditions. The specific statistical analysis test used is indicated in each figure legend. A threshold of p=0.1 was set, and values greater than that threshold are not reported.

Author contribution

DEO: conceptualization, investigation, data curation, writing-original draft. AA, PCA, and DK: investigation, data curation, writing-editing. LCK: data curation, writing-editing. MB: data curation, writing-editing. SNW: conceptualization, data curation, writing-original draft, study supervision.

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