Interleukin (IL)-23 Stimulates IFN-γ Secretion by CD56bright Natural Killer Cells and Enhances IL-18-Driven Dendritic Cells Activation

Andrea Ziblat1, Sol Y. Nuñez1‡, Ximena Lucia Raffo Iraolagoitia1†‡, Raúl German Spallanzani1†, Nicolás I. Torres1, Jessica M. Sierra1, Florencia Secchiari1, Carolina I. Domaica1, Mercedes B. Fuertes1 and Norberto W. Zwirner1,2*

1Instituto de Biología y Medicina Experimental (IBYME-CONICET), Laboratorio de Fisiopatología de la Inmunidad Innata, Buenos Aires, Argentina, 2Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

Interleukin (IL)-23 is a member of the IL-12 family of cytokines that, as the other members of this family, is secreted by monocytes, macrophages, and dendritic cells (DC) upon recognition of bacterial, viral, and fungal components. IL-23 is critical during immunity against acute infections, and it is also involved in the development of autoimmune diseases. Although immunoregulatory effects of IL-23 on mouse natural killer (NK) cells have been described, the effect of IL-23 on human NK cells remains ill-defined. In this study, we observed that monocytes stimulated with LPS secreted IL-23 and that blockade of this cytokine during monocyte and NK cell coculture led to a diminished production of IFN-γ by NK cells. Accordingly, rIL-23-induced NK cell activation and stimulated IFN-γ production by CD56bright NK cells. This effect involved MEK1/MEK2, JNK, PI3K, mammalian target of rapamycin, and NF-κB, but not STAT-1, STAT-3, nor p38 MAPK pathways. Moreover, while NK cell-mediated cytotoxicity remained unaltered, antibody-dependent cellular cytotoxicity (ADCC) was enhanced after IL-23 stimulation. In addition, IL-23 displayed a synergistic effect with IL-18 for IFN-γ production by both CD56bright and CD56dim NK cells, and this effect was due to a priming effect of IL-23 for IL-18 responsiveness. Furthermore, NK cells pre-stimulated with IL-18 promoted an increase in CD86 expression and IL-12 secretion by DC treated with LPS, and IL-23 potentiated these effects. Moreover, IL-23-driven enhancement of NK cell “helper” function was dependent on NK cell-derived IFN-γ. Therefore, our results suggest that IL-23 may trigger NK cell-mediated “helper” effects on adaptive immunity, shaping T cell responses during different pathological situations through the regulation of DC maturation.

Keywords: natural killer cells, interleukin-23, interleukin-18, IFN-γ, dendritic cells

INTRODUCTION

Natural killer (NK) cells constitute a subgroup of type 1 innate lymphoid cells that are key players during immunity against intracellular pathogens and tumors due to their cytotoxicity and the secretion of IFN-γ and other pro-inflammatory cytokines (1–3). In humans, they are subdivided into two subpopulations based on the relative expression of CD56 and CD16 (4). Almost 90% of peripheral blood NK cells are CD56dimCD16+ (CD56dim), and although they can produce cytokines upon
activation (5, 6), their principal effector function is the cytotoxic activity displayed against susceptible target cells (7, 8). The rest of NK cells in blood are CD56brightCD16dim—CD56dim—, which are mainly producers of cytokines (4, 8) and are more abundant in secondary lymphoid organs where they exert immunoregulatory functions (9). NK cells become activated after direct recognition of infected or tumor cells through a vast array of activating receptors (10) and by cytokines such as interleukin (IL)-12, IL-15, and IL-18 (11, 12) or their combination with PAMPs (13). Monocytes, macrophages, and dendritic cells (DC) are the major producers of these cytokines during their crosstalk with NK cells (14–16). As a result of this bidirectional crosstalk, in some circumstances, activated NK cells kill immature DC, favoring the selection of immunogenic DC necessary for an appropriate immune response (17, 18). In addition, DC stimulate NK cell activation while NK cells enhance macrophage activation and promote DC maturation, and NKp30 and cytokines have been involved in both events (19, 20). Therefore, NK cells skew the adaptive immune response toward a T helper (Th 1) and cytotoxic T lymphocyte (CTL) profiles, both essential for an effective antitumor and antiviral immune response (21–24). Also, the crosstalk between DC and NK cells has been involved in the promotion and protection of autoimmune conditions (25–27).

Interleukin-12 plays a major role in NK cell activation in response to pathogens and tumors (28, 29). It belongs to an extended family of cytokines that share cytokine and receptor subunits and display overlapping functions (30, 31). Recently, we demonstrated that IL-27, another member of IL-12 family, induces human NK cell-mediated cytotoxicity, IFN-γ production, and potentiates antibody-dependent NK cell-mediated cytotoxicity (ADCC) (32). In addition, IL-27 synergizes with IL-18 for the stimulation of NK cell effector functions (32–34). IL-23 is another member of the IL-12 family composed by the IL-12p40 and p19 subunits that signals through a heterodimeric receptor composed by the IL-12Rβ1 and the IL-23R chains (35, 36). Similarly to IL-12 and IL-27, IL-23 is secreted by monocytes, macrophages, and DC in response to bacterial, viral, and fungal components (37, 38). IL-23 increases IFN-γ production by human CD4+ T cells (35, 39), and it is also involved in survival, expansion, and activation of Th17 cells (31). Data obtained in mice indicate that IL-23 can both inhibit (40) and activate (41, 42) NK cell responses. However, IL-23 effects on human NK cells remain ill-defined. Therefore, in this work, we explored the role of IL-23 on human NK cell effector functions and demonstrated that it stimulated IFN-γ secretion by CD56bright, but not CD56dim cells, primed NK cells for IL-18-driven IFN-γ production, and that NK cells co-stimulated with IL-23 and IL-18 enhanced IL-12 secretion and CD86 expression on DC in an IFN-γ-dependent manner.

MATERIALS AND METHODS

Antibodies and Reagents

Human rIL-2 and rIL-15 (PeproTech), rIL-18 (MBL International), rIL-23 (eBioscience), rGM-CSF (Sigma), and rIL-4 (R&D) were used. Cells were incubated with fluorochrome-coupled mAb against the following human molecules: CD25 (BC96), CD1a (HI149), NKP46 (9E2), NKP30 (P30-15), NKP44 (P44-8), NKP80 (5D12), IFN-γ (4S.B3), T-bet (4B10), CD178 (FasL, NOK-1), CD14 (HCD14), CD83 (HB15e), CD16 (3G8), unlabeled anti-IFN-γ (NIB42), fluorochrome-labeled, and unlabeled isotype-matched controls (IC) from Biolegend; CD226 (DNAM-1, DX11), CD69 (FN50) and CD154 (CD40L, TRAP1) from BD Pharmingen; CD56 (N901) from Beckman Coulter; NKG2C (134591), TRAIL (71908), TIGIT (741182), CD85j (ILT2, 292305), IL-18Rα (H44), IL-18Rβ (132029), and IL-23R (218213) from R&D; CD3 (UCHT-1), HLA-DR (L243), and CD86 (IT2.2) from Tonbo; Eomes (WD1928) from e Bioscience; unlabeled anti-IL-23p19 (B-Z23) from Abcam. The following reagents were used at the indicated concentrations: the inhibitor of c-Jun N-terminal kinase (JNK) SP600125 (20 µM, Calbiochem); the Janus kinase 2 (Jak2) inhibitor AG490 (25 µM, Calbiochem); the p38 MAP kinase inhibitor SB202190 (10 µM, Calbiochem); the inhibitor of phosphoinositide 3-kinase (PI3K) Ly294002 (2 µM, Sigma); the inhibitor of MEK1/MEK2 kinases (MAPKs) U0126 (5 µM, Sigma); the inhibitor of cytokine-induced IκBα phosphorylation BAY11-7082 (1 µM, Sigma); the inhibitor of the mammalian target of rapamycin (mTOR) rapamycin (5 nM, Sigma); the inhibitor of STAT1 Fludarabine (0.1 µg/ml, Fludara® Schering). LPS (E. coli 0111:B4 strain, Sigma) was used at 0.1 or 1 µg/ml. The dose of each pharmacological inhibitor used in the experiments was established in previous work (13, 32) and did not affect NK cell viability. Rituximab (RTX, Roche) and normal human polyclonal IgG (IgG2500, Purissimus, Argentina) were used at 10 µg/ml.

Monocytes, DC, and NK Cells

Buffy coats from healthy volunteers were provided by the Blood Bank of the “Carlos Durand” Hospital or by the “Complejo Médico Churuquina-Visca” (Buenos Aires, Argentina). Monocytes (CD14+ cells) were isolated by MACS (Miltenyi); NK cells were isolated using RosetteSep (StemCell) and Ficoll-Paque™ Plus (GE Life Sciences) centrifugation. Purity of isolated cells was always above 90%, as assessed by flow cytometry (FC; CD14+ cells or CD3−CD56+). Monocytes (1 × 10^6) were incubated for 24 h with LPS (1 µg/ml), then, NK cells (1 × 10^5) were added for another 24 h in the presence of an IC mAb or a neutralizing anti-IL-23p19 mAb (10 µg/ml) and IFN-γ was evaluated in the supernatants. Also, cell culture supernatants of monocytes incubated for 48 h with LPS were used for analysis of IL-23 production. Monocytes were cultured for 6 days with GM-CSF and IL-4 to obtain immature DC (iDC) characterized as CD11c+MHC-II+CD83−flow CD86−low. DCs (1 × 10^5) were cultured for 18 h with previously stimulated and washed NK cells (1 × 10^5) plus LPS (0.1 µg/ml) in the absence or in the presence of an IC mAb or a neutralizing anti-IFN-γ mAb (10 µg/ml). Cells were cultured in RPMI 1640 (Gibco) supplemented with 10% inactivated fetal bovine serum (Gibco), sodium pyruvate, glutamine, and gentamicin (Sigma). Cell culture supernatants were collected and used for analysis of IL-12 production and cells were used to assess CD86 expression. Also, NK cells (1 × 10^6/ml) were cultured for 24 h or 5 days in the presence of IL-15 (4 ng/ml) and in the absence or in the presence of IL-18 (10 ng/ml), IL-23 (10 ng/ml), or their combination. In some experiments, NK cells stimulated with IL-23 were thereafter
stimulated with IL-2 (8 ng/ml) for 24 h. For dose–response experiments, IL-23 was also used at 1 ng/ml. Cells were used for phenotypic analysis, cytokine production, proliferation, and cell death evaluation. For cytotoxicity assessment and NK cell “helper” function evaluation, NK cells (2 × 10^6/ml) were incubated overnight. To investigate the signaling pathways involved in the IFN-γ response, NK cells were incubated with pharmacologic inhibitors for 45–60 min and then stimulated with the different cytokines for another 23 h. For priming experiments, NK cells (1 × 10^6/ml) were cultured overnight in the absence or in the presence of IL-18 or IL-23, extensively washed and further cultured (1 × 10^6/ml) for 24 h in the absence or in the presence of IL-23 or IL-18, as indicated in the figure. Studies have been approved by the institutional review committee and informed consent of participating subjects was obtained.

**NK Cell Proliferation**

Natural killer cells cultured for 5 days as described, were pulsed with 1 μCi/well of methyl-3H-thymidine (3H-Thy; New England Nuclear Life Science) during the last 18 h of cell culture, harvested on glass-fiber filters, and incorporated radioactivity was measured in a scintillation counter. Results are expressed as mean counts per minute (cpm) of triplicate wells ± SEM.

**Flow Cytometry and Cell Sorting**

Expression of cell surface receptors on NK cells or DC was analyzed by FC as previously described (43). Expression of IFN-γ, Eomes and T-bet was analyzed by intracellular FC using Cytotox/ Cytoperm (BD). For assessment of IFN-γ production, cells were cultured in the presence of Golgi-Plug® and Golgi-Stop® reagents during the last 4 h. For CD40L analysis, cells were cultured in the presence of the specific mAb and Golgi-Stop® during the last 6 h, as described (44) with slight modifications. Human CD40L-transfected fibroblasts were used as positive controls. Samples were acquired in a FACScanto II-plus flow cytometer (BD) or MACSQuant Analyser 10 (Miltenyi Biotec). Data were analyzed using FlowJo software (Tree Star). Results were expressed as percentage of positive cells or MFI. For cell sorting, NK cells were isolated using RosetteSep (StemCell), stained with mAb against CD56 and CD16 and then CD56^bright (CD56^brightCD16^-low) and CD56^dim (CD56^dimCD16^high) NK cells were sorted in a FACSAria II-plus cell sorter (BD Biosciences).

**ELISA**

Secretion of IFN-γ (Biolegend), IL-12 (ELISA MAX Standard kit, Biolegend), and IL-23 (DuoSet, R&D) was analyzed by ELISA as described (13).

**Cytokine Bead Array**

Secretion of IL-4, IL-10, IL-17, IL-6, TNF, and IFN-γ by cytokine-stimulated NK cells was analyzed by the CBA (BD) as indicated by the manufacturer.

**NK Cell-Mediated Cytotoxicity**

Natural killer cells were cultured overnight with IL-15 in the absence or in the presence of IL-18, IL-23, or the combination of both, washed, and co-cultured for 5 h with CFSE-labeled Raji cells (Burkitt’s lymphoma, ATCC) at different E:T ratios. Cells were thereafter labeled with 7-AAD and analyzed by FC. Percentage of cytotoxicity was calculated as 100 x percentage of CFSE+ cells/percentage of CFSE+ cells. Percentage of spontaneous dead cells (without effecter NK cells) was always below 5%. For ADCC, CFSE-labeled Raji cells previously incubated with RTX or normal human IgG for 2 h were used as target at a 1:1 E:T ratio.

**Statistical Analysis**

Paired t-test or Wilcoxon matched paired test (when data did not pass the normality test) were used when two experimental groups were compared. A one-way ANOVA test with Bonferroni post hoc test was used when three or more experimental groups were compared. A one-way ANOVA test with Dunn’s post hoc test was used in pharmacologic inhibition experiments. When data did not pass normality test, Friedman test with Dunn’s post hoc test was used instead. A two-way ANOVA with repeated measures and Bonferroni post hoc test was used for IFN-γ production by sorted NK cells and by IL-23 plus IL-18-stimulated NK cells, for cytotoxicity and for DC-NK cell co-cultures in the presence of neutralizing mAb experiments. The interaction effect in the 2 × 2 factorial ANOVA was performed for the definition of synergism, and P values for it were reported in the legends of the figures (45). Data were analyzed using GraphPad Prism 6.0 software.

**RESULTS**

**Monocytes Produce IL-23 That Stimulates NK Cell IFN-γ Production**

To assess whether IL-23 participates in the crosstalk between monocytes and NK cells, we first evaluated the capacity of monocytes to produce this cytokine. Accordingly, we observed that they secreted IL-23 upon stimulation with LPS (Figure 1A). Moreover, IL-23 blockade led to a significant reduction in the amounts of IFN-γ secreted by NK cells during their coculture with LPS-stimulated monocytes (Figure 1B). Next, we stimulated isolated NK cells with recombinant human IL-23 and confirmed that this cytokine-induced IFN-γ production not only by resting (Figure 1C) but also by NK cells previously activated with a combination of IL-12, IL-15, and IL-18 (data not shown). Conversely, IL-23 did not promote the secretion of IL-4, IL-10, IL-17, IL-6, and TNF by NK cells, assessed by Cytokine Bead Array (CBA) and FC (data not shown). Pharmacologic inhibition revealed that MEK1/MEK2, JNK, PI3K, and NF-κB not p38 MAPK, Jak2 (Figure 1D), or STAT1 (data not shown) were involved in the IL-23-driven IFN-γ response. Besides, IL-23 did not affect NK cell-mediated cytotoxicity (Figure 1E). Accordingly, this cytokine did not affect the expression of the activating receptors CD335 (Nkp46), CD336 (Nkp44), CD337 (Nkp30), and Nkp80 (KLRF1), CD226 (DNAM-1), NKG2C nor the expression of TRAIL and CD178/FasL (data not shown). Therapeutic efficacy of humanized monoclonal antibodies directed against tumor cell surface-expressed molecules relies partially on ADCC. We observed that RTX-coated Raji cells were susceptible to NK cell-mediated cytotoxicity and that IL-23 further increased such...
susceptibility in a statistically significant manner (Figure 1F). Of note, IL-23 did not alter the expression levels of CD16 on NK cells (data not shown).

In addition, IL-23 induced a higher percentage of CD69+ (Figures 2A,B) and CD25+ (Figures 2C,D) NK cells indicating that this cytokine promoted NK cell activation. Furthermore, NK cells first incubated with IL-23 secreted higher amounts of IFN-γ than non-stimulated NK cells when they were re-stimulated with IL-2, suggesting that the effect of IL-23 on CD25 expression had functional consequences (Figure 2E).

Next, we analyzed intracellular IFN-γ production by CD56bright and CD56dim NK cells in response to IL-23. Although we did not detect differences in the percentage of IFN-γ+ NK cells in these subpopulations, we observed higher amounts of IFN-γ expression in CD56bright NK cells stimulated with IL-23 compared to unstimulated cells (data not shown). To confirm this result, we FACS-sorted CD56bright and CD56dim NK cells and observed a statistically significant increase in IFN-γ secretion by CD56bright but not CD56dim NK cells upon IL-23 stimulation (Figure 3A). Accordingly, CD56bright NK cells expressed higher amounts of IL-23 receptor (IL-23R) than CD56dim NK cells (Figure 3B).

**IL-23 Primes NK Cells for IL-18-Induced IFN-γ Production**

Previous reports demonstrated that IL-12 (46) and IL-27 (32) display a cooperative effect with IL-18 on the activation of NK cell effector functions. Therefore, we investigated whether IL-23 cooperates with IL-18 for NK cell stimulation. First, we performed a dose–response curve and observed a statistically significant increase in the secretion of IFN-γ when NK cells were stimulated with two different concentrations of IL-23 plus IL-18 in comparison with IL-18 alone (Figure 4A). We confirmed the existence of a cooperative effect with a larger number of donors and using resting (Figure 4B) and activated NK cells (data not shown). Statistical analysis based on testing the interaction effect in a two-way ANOVA (45) demonstrated the existence of a synergistic effect between IL-23 and IL-18. Moreover, in opposition to the effect of IL-23 alone, we detected that this synergistic effect was noticeable both on CD56bright (Figures 4C,D) and on CD56dim (Figures 4E,F) NK cells. As IL-23 only exerted an effect on IFN-γ production by CD56dim NK cells when combined with IL-18, we explored whether such combination also affected NK cell-mediated cytotoxicity. However, even in combination with IL-18, IL-23 did not regulate NK cell-mediated cytotoxic activity (Figure 4G).

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**FIGURE 1** Monocyte-derived interleukin (IL-23) promotes IFN-γ secretion by human natural killer (NK) cells while cytotoxicity remains unaltered after rIL-23 stimulation. (A) IL-23 secretion by monocytes cultured for 48 h in the absence (−) or in the presence of LPS; n = 8. (B) IFN-γ secretion by NK cells upon coculture for 24 h with monocytes pre-stimulated for 24 h with LPS, in the presence of an IC mAb (IC) or a blocking mAb against IL-23 (α-IL-23); n = 9. (C) IFN-γ secretion by resting NK cells cultured for 24 h in the absence (−) or in the presence of 10 ng/ml of IL-23; n = 23. (D) IFN-γ secretion by NK cells stimulated for 24 h with 10 ng/ml of IL-23 in the absence (−) or in the presence of U0126, SP600125 (SP), SB202190 (SB), AG490, Ly294002 (Ly), BAY11-7082 (BAY), or rapamycin (RAPA); n = 6. (E) Cytotoxic activity of NK cells previously cultured overnight in the absence (○) or in the presence (●) of IL-23 against Raji cells at different E:T ratios; n ≥ 6. (F) ADCC of NK cells previously cultured in the absence (−) or in the presence of IL-23 against Raji cells incubated with an IC mAb (white bars) or against RTX-coated Raji cells (black bars) at 1:1 E:T ratio; n = 6. Mean ± SEM are shown. ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; paired t-test (A), Wilcoxon test (B,C), one-way ANOVA with Dunnett’s post hoc test (D), and two-way ANOVA with Bonferroni’s post hoc test (E,F).
pre-stimulation of NK cells with IL-18 and subsequent stimulation with IL-23 induced a minor increase in IFN-γ secretion. Conversely, pre-stimulation of NK cells with IL-23 and subsequent stimulation with IL-18 induced a significant increased IFN-γ secretion compared to NK cells not pretreated with IL-23 (Figure 5A). Therefore, the synergistic effect was due to a priming of NK cells by IL-23 for IL-18 responsiveness.

To explore the mechanisms involved in the priming effect, we addressed the expression of T-bet, Eomes, IL-18Rα, and IL-18Rβ in NK cells stimulated with IL-23. While T-bet and IL-18Rβ expression remain unchanged, IL-23 induced a decrease in Eomes expression (data not shown) and a statistically significant upregulation of the expression of IL-18Rα in CD56<sup>bright</sup> but not CD56<sup>dim</sup> NK cells (Figure 5B). Therefore, upregulated expression of IL-18Rα induced by IL-23 may explain the increased responsiveness of NK cells to IL-18.

As IL-23 promotes the proliferation of memory T cells and the increased levels of IFN-γ observed could also be a result of an increase in the total number of NK cells, we investigated the effect of IL-23 on NK cells proliferation. Surprisingly, IL-23 exerted a statistically significant inhibition of NK cell proliferation triggered by IL-15 and IL-15 plus IL-18 (Figure 5C) without affecting NK cell viability (data not shown).

To further interrogate the cause of the synergistic effect induced by IL-23 and IL-18 on IFN-γ production by NK cells, we performed sequential stimulations. We observed that

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**FIGURE 2** | Interleukin (IL)-23 activates natural killer (NK) cells. (A–D) Percentage of NK cells expressing CD69 (A,B) or CD25 (C,D) after 5 days of culture in the absence (−) or in the presence of 10 ng/ml of IL-23; n = 9 (A) and n = 10 (C). Representative histograms are shown in (B,D). Gray: IC. Thin line: unstimulated NK cells. Thick line: NK cells stimulated with IL-23. Numbers within histograms: percentage of positive cells for each marker in each condition. (E) IFN-γ secretion by NK cells cultured for 5 days in the absence (−) or in the presence of 10 ng/ml of IL-23 and thereafter re-stimulated 24 h with 8 ng/ml of IL-2; n = 7. Mean ± SEM are shown in (E). ns, not significant; *p < 0.05; **p < 0.01; paired t-test (A,C,E).

**FIGURE 3** | CD56<sup>bright</sup> natural killer (NK) cells secrete IFN-γ in response to interleukin (IL)-23 and express higher amounts of IL-23R than CD56<sup>dim</sup> NK cells. (A) IFN-γ secretion by FACS-sorted CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells cultured for 24 h in the absence (○) or in the presence (●) of IL-23; n = 5. (B) IL-23 receptor (IL-23R) expression on CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells; n = 5. ns, not significant; **p < 0.01; ***p < 0.001; two-way ANOVA with Bonferroni’s post hoc test (A) and paired t-test (B).
IL-23 Cooperates with IL-18 in the Promotion of DC Activation

As NK cell–DC crosstalk shapes adaptive immunity, we examined whether NK cells co-stimulated with IL-23 and IL-18 affect DC maturation. NK cells were stimulated overnight in the absence or in the presence of IL-23, IL-18, or both, and then cocultured with DC pulsed with LPS. We observed a statistically significant increase in CD86 expression (Figures 6A,B) but not in CD83 or HLA-DR expression (data not shown) on DC when NK cells were pre-stimulated with IL-23 and IL-18 compared to DC cocultured with NK cells stimulated with each cytokine alone. Moreover, we observed an increased secretion of IL-12 (Figure 6C) by DC when NK cells were pre-stimulated with both cytokines. To investigate the underlying mechanisms, we stimulated NK cells with IL-23, IL-18, or IL-23 and IL-18, and we evaluated the expression of CD40L, DNAM-1, NKG2D, NKG2C, NKp30, NKp46, TIGIT, and ILT2 on NK cells. However, we did not observe differences that could suggest the involvement of any of these receptors in the potentiation of DC activation when they were cocultured with NK cells pre-stimulated with IL-23 and IL-18 (data not shown).
Conversely, neutralization of IFN-γ during the cocultures of DC and NK cells pre-stimulated with the combination of the cytokines abrogated both effects, CD86 expression (Figures 6D,E), and IL-12 secretion (Figure 6F). Therefore, IL-23 in concert with IL-18 promotes NK cell activation that drives DC maturation mainly by IFN-γ produced by NK cells. 

**DISCUSSION**

Interleukin-23 has been involved in immunity against tumors (29, 47) and acute infections (30, 31) but also contributes to tumor promotion and growth (40, 48) and the development of autoimmune diseases, mainly through the induction of a Th17 response (30, 49). These opposing functions may depend on the context and/or on the immune cells on which IL-23 exerts its effects. In mice, IL-23 triggers pro- (40) and antitumoral (41, 42) effects on NK cells. However, little is known about its effect on human NK cells (50). In this study, we demonstrated that monocytes incubated with LPS secrete IL-23 that stimulates IFN-γ secretion mostly by CD56bright NK cells, which could be due to the observation that CD56bright NK cells express higher amounts of IL-23R than CD56dim NK cells. Of note, we used low doses of IL-15 as survival factor and to prime NK cells as it has been shown that resting NK cells require at least two signals for efficient IFN-γ secretion (9, 12, 21, 51, 52). Also, as the secretion of cytokines is usually restricted to the synaptic cleft between DC and NK cells (53, 54), the concentration of cytokines in sera might be quite different from the concentration that NK cells sense. Nevertheless, the concentration of IL-23 used in our experiments is within the range of values detected in sera from patients with different physiopathological conditions (55, 56). Besides, they are similar to the concentration of IL-23 detected in supernatants from monocyte-derived DCs of multiple sclerosis patients stimulated with LPS (57) or produced by macrophages and DC stimulated with pathogens or TLR ligands (37, 58, 59).

In addition, in this study, we observed that IL-23-driven IFN-γ production requires functional MEK1/MEK2, JNK, PI3K, mTOR, and NF-κB, but not STAT-1, STAT-3 (a downstream mediator of Jak2), nor p38 MAPK. All these signaling pathways have previously been implicated in NK cell effector functions (13, 32, 60, 61).

Interleukin-4, IL-10, IL-17, and TNF were shown to be secreted by NK cells under certain conditions and different immune cells stimulated with IL-23 produce IL-17, IL-6, and TNF (30, 62–65). However, IL-23 did not affect their secretion by NK cells. Nevertheless, we observed a raise in the percentage of CD69+ and CD25+ NK cells upon stimulation with IL-23, confirming that this cytokine activates NK cells. Moreover, IL-23 potentiated ADCC (mediated through CD16) but not NK cell-mediated cytotoxicity through other NK cell activating receptors, suggesting that this cytokine may act as a co-stimulus not only for IFN-γ production but also for CD16-mediated cytotoxicity. Interestingly, potentiation of ADCC is a biological effect that IL-23 shares with IL-12 and IL-27 (32, 60), which suggests that these cytokines of the IL-12 family of cytokines might be suitable candidates as adjuvant therapy during immunotherapy with humanized mAb.

Myeloid cells can produce IL-18 upon recognition of tumors and pathogens, which in combination with other stimuli such as IL-12 or IL-27 activates NK cells (32, 34, 46). Here, we demonstrated that IL-23, like the other members of the same family of cytokines, displays a synergistic effect with IL-18 for NK cell-mediated IFN-γ production by both CD56bright and CD56dim cells. Interestingly, this effect is due to a priming of IL-23 for

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**FIGURE 5 | Interleukin (IL)-23 primes natural killer (NK) cells for IL-18-driven IFN-γ secretion upregulating IL-18Rα and inhibits NK cell proliferation.**

(A) IFN-γ secretion by NK cells cultured overnight in the absence (−) or in the presence of IL-23 or IL-18 ("first stimulus"), washed, and thereafter incubated for 24 h in the absence (−) or in the presence of IL-23 or IL-18 ("second stimulus"); n = 7. (B) IL-18Rα expression on sorted CD56bright and CD56dim NK cells cultured overnight in the absence (−) or in the presence of IL-23; n = 5. (C) Proliferation of NK cells incubated for 5 days with 1 ng/ml of IL-15 and the indicated doses of IL-23 in the absence (white bars) or in the presence of 10 ng/ml of IL-18 (black bars); n = 6. Mean ± SEM are shown. ns, not significant; *p < 0.05; **p < 0.01; one-way ANOVA with repeated measures and Bonferroni’s post hoc test.
IL-18-responsiveness and not to a proliferative effect on NK cells or to changes in their viability. On the contrary, we observed an IL-23-driven inhibition of NK cell proliferation similar to what we have already seen for another member of the IL-12 family of cytokines, IL-27 (32). The upregulation of IL-18Rα expression on CD56bright NK cells observed in our experiments might be responsible for the priming effect that led to an increased responsiveness of NK cells to IL-18 for IFN-γ production.

Remarkably, CD56dim NK cells only produced IFN-γ in response to the simultaneous stimulation with both cytokines but not in response to IL-23 alone, as did CD56bright NK cells. However, NK cell-mediated cytotoxicity was not affected when NK cells were stimulated with IL-23 or with IL-23 and IL-18. Therefore, IL-23 might display a predominant immunoregulatory but not a cytotoxicity-inducing effect on NK cells, which is in contrast with the effect of other members of the same family of cytokines such as IL-12 and IL-27 (28, 29, 32). These results further support the notion that both effector functions are differentially regulated in NK cells (13, 66). Also, the lack of effect of IL-23 on NK cell cytotoxicity could be due to the fact that CD56dim NK cells express less IL-23R than CD56bright NK cells.

Nonetheless, IL-23 induced IFN-γ secretion by CD56dim NK cells in combination with IL-18 and enhanced ADCC, which indicates that it may act as co-stimulatory cytokine when acting in concert with another primary stimulus for NK cells.

Immature DC are mainly localized in peripheral tissues and migrate to secondary lymphoid organs upon maturation induced by PAMP or tumors to induce an adaptive immune response. NK cells activated upon target cell recognition or by cytokines such as IL-2 or IL-12 induce CD86 expression and IL-12 production by DCs (15, 21, 24, 67). Therefore, we hypothesized that IL-23 and IL-18 secreted by myeloid cells during the onset of an immune response may activate NK cells that in turn might affect maturation of DC that already sensed PAMP, which in turn may affect T cell priming. To mimic this situation in vitro with human cells, we used LPS during DC–NK cell cocultures to activate DC. We observed that NK cells pre-stimulated with IL-18 induced heightened secretion of IL-12 and increased expression of CD86 on DC activated with LPS, and that this effect is further potentiated by IL-23. This effect could be related to the "helper" function acquired by NK cells exposed to IL-18 that leads to the stimulation of IL-12 production by DC.
activated through CD40 and the development of a Th1 response (51). In our experimental setting, we did not observe differences in the expression of CD40L as other authors did (68, 69), or other activating or inhibitory receptors on NK cells that could suggest the involvement of surface molecules in the potentiation of DC activation. Conversely, we observed that IL-23 enhanced IL-18-driven NK cell “helper” function mainly through IFN-γ production.

Our results show that the effects of IL-23 on NK cells are mostly immunoregulatory and that this cytokine cooperates with IL-18, which suggest that IL-23 may actually functions as a co-stimulatory cytokine that principally potentiates the effect of IL-18 in NK cells. These observations also explain the pro- or antitumoral effects described for IL-23 on NK cells (29, 40–42, 47, 48), which may critically depend on the composition of the tumor microenvironment, in particular, the presence of other cytokines that regulate NK cell effector functions.

Overall, we conclude that IL-23 produced by activated myeloid cells induces NK cell activation and IFN-γ secretion, which enhances IL-18-driven NK cell “helper” activity that fosters DC maturation and IL-12 secretion. Therefore, our results unravel the stimulatory functions of IL-23 on NK cells, and their possible impact on the Th1- and CTL-mediated adaptive immune response against tumors and pathogens.

ETHICS STATEMENT

Studies have been approved by the institutional review committee from IBYME.

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AUTHOR CONTRIBUTIONS

AZ, participated in the design of the study, performed and analyzed the experiments, prepared the figures, and wrote and corrected the manuscript. SN, XI, RS, FS, NT, JS, CD, and MF assisted in performing experiments and analyzing results. NZ designed the study, analyzed the experiments, and corrected the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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