Chapter 1

IL-23 modulates CD56⁺/CD3⁻ Natural Killer Cell and CD56⁺/CD3⁺ Natural Killer-like T Cell function differentially from IL-12.

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Abstract

Natural killer (NK) and natural killer-like T (NK-like T) cells play an essential role in linking innate and adaptive immunity, through their ability to secrete interferon gamma (IFN-\(\gamma\)). The exact trigger initiating production of IFN-\(\gamma\) is uncertain. Antigen presenting cell (APC)-derived interleukin-12 (IL-12) is thought to be the classical IFN-\(\gamma\) inducing cytokine, but requires an additional stimulus such as IFN-\(\gamma\) itself. Interleukin (IL)-23 and IL-18 are among the first cytokines secreted by APC in response to binding of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS). Thus, early APC-derived IL-23 may be an initial trigger of IFN-\(\gamma\) production in NK and NK-like T cells. Herein, we characterized the effect of IL-23 on IFN-\(\gamma\) secretion by NK and NK-like T cells. Our findings show that IL-23 and IL-18 synergistically elicit IFN-\(\gamma\) production in NK-like T cells but not in NK cells. In contrast, IL-12 together with IL-18 induced secretion of IFN-\(\gamma\) in both populations. The observed synergy between IL-23 and IL-18 in NK-like T cells coincided with IL-23 mediated up-regulation of IL-18R\(\alpha\). Furthermore, IL-23 up-regulated CD56 expression in NK-like T cells and, together with IL-18, induced proliferation of NK and NK-like T cells. We postulate a role for APC-derived IL-23 in the activation of NK and NK-like T cell early in infection and in shaping Th1-differentiation, via induction of IFN-\(\gamma\), which provides the additional stimulus needed for APC to subsequently produce IL-12.
Introduction

Innate immunity plays an essential role in the defense against pathogens. NK cells constitute an important arm of the innate immune system and several murine models have shown NK cell-derived cytokines to be critical in the early response against intracellular pathogens such as *Salmonella typhimurium* (1,2) and *Mycobacterium tuberculosis* (3). Early in infection, NK cells are thought to be the primary source of interferon gamma (IFN-γ) (4-6), shaping the acquired immune response through differentiation of T helper (Th) cells to the Th1 subclass (6,7). At a later stage of infection, Th1 cells become the predominant source of IFN-γ.

Host defense against intracellular bacterial pathogens such as *Salmonellae* and *Mycobacteria* is dependent on the type-1 cytokine pathway (8). Generally thought, this pathway is initiated by bacterial stimulation of pattern recognition receptors on APC, resulting in the production of interleukin-23 (IL-23), IL-12 and IL-18 (9). IL-12 and IL-18 subsequently induce IFN-γ production in NK cells and Th1 cells by binding to their respective receptors (10), while IL-23 is known to induce IFN-γ production in naïve T cells and in memory T cells (11). IFN-γ in turn binds to the IFN-γR on the macrophages and dendritic cells to enhance their bactericidal activity and antigen presentation and increase production of IL-12 (12).

IL-23 and IL-12 are heterogenic cytokines composed of a shared IL-12p40 subunit bound to an IL-23p19 or IL-12p35 subunit, respectively. IL-23 and IL-12 signal through a common IL-12Rβ1 chain complemented by the IL-23R and the IL-12Rβ2 respectively. While IL-12Rβ1 is constitutively expressed in naive CD4+ T cells, CD56+ NK-like T cells and NK cells (13,14), IL-23R and IL-12Rβ2 expression are critical for the ability to respond to IL-23 and IL-12, respectively.

The role of IL-23 in shaping the immune response is poorly defined. IL-23 is produced by APC in response to PAMPs like LPS. Moreover, IL-23 rather than IL-12 is the first type 1 cytokine released by activated pro-inflammatory macrophages (9). To induce production of IL-12 by monocytes and macrophages, IFN-γ signaling is required in addition to a PAMP (9). In dendritic cells both IFN-γ and IL-4 can enhance PAMP induced IL-12 production (15). Since IL-23 is expressed early in infections (16) and capable of inducing IFN-γ (17-19), we hypothesized that IL-23 may serve as a factor important in initiating early Th1 differentiation by inducing the extra signal needed, IFN-γ, for APC to produce IL-12 in response to pathogens or PAMPs. In order to achieve this, IL-23 needs to target cells of the innate immune system and induce IFN-γ production in these cells. NK and NK-like T cells may be candidate cells providing IFN-γ as they are part of the innate immune system and both known to express IL-12Rβ1 mRNA (14,20). Moreover, NK cells are reported to express *IL23R* mRNA (20). To verify this hypothesis, we tested the ability of IL-23 to induce activation and IFN-γ secretion in primary human CD56+/CD3- NK and CD56+/CD3+ NK-like T cells.
Methods

Cells and culture conditions
CD56\(^+\) cells were isolated from buffy coats from healthy donors (Sanquin, Leiden, The Netherlands) by Ficoll-Amidotrizoate density gradient centrifugation and subsequent selection using anti-CD56 MACS beads (Miltenyi Biotech, Utrecht, The Netherlands). For the proliferation assay, CD56\(^+\) bead isolated cells were labeled with PE-labeled anti-human CD3 (BD PharMingen, Amsterdam, The Netherlands) and CD3\(^-\) cells were sorted with a FACSVantage SE (BD Biosciences, Amsterdam, The Netherlands). Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Bio-Whittaker) supplemented with 20 mM GlutaMAX (Gibco/Invitrogen, Breda, The Netherlands), 10% FCS, 100 U/ml Penicillin, 100 µg/ml Streptomycin (Gibco/Invitrogen, Breda, The Netherlands).

FACS analysis
To assess STAT phosphorylation by FACS analysis, overnight-rested CD56\(^+\) bead isolated cells were stimulated with recombinant human 10 ng/ml IL-23 or 1 ng/ml IL-12 for times indicated. Cells were fixed using 4% formaldehyde and permeabilised with 90% methanol. Cells were labeled directly with anti-phosphorylated STAT1 (pY701)-Alexa 647, anti-phosphorylated STAT3 (pY705)-PE or STAT3 (pY705)-Alexa 647, anti-phosphorylated STAT4 (pY693)-Alexa 647 or anti-phosphorylated STAT5 (pY694)-PE (BD PharMingen). CD56\(^+\) magnetic bead isolated cells were stained in combination with anti-human CD3 and anti-human CD56.

For intracellular staining for IFN-\(\gamma\), CD56\(^+\) isolated cells were seeded 10\(^5\) cells per well in 96-well plates (Greiner Bio-One) and stimulated with IL-23 (R&D Systems, Abingdon, United Kingdom), IL-12 (R&D Systems), IL-18 (MBL, Woburn, USA) or a combination of these cytokines for 48 hours. The last 6 hours of stimulation BD GolgiPlug (BD PharMingen) was added (final concentration 1:1000). Cells were fixed in 4% paraformaldehyde (Sigma, Zwijndrecht, The Netherlands) and permeabilised in 90% methanol. Cells were stained with Alexa 647-labeled anti-human IFN-\(\gamma\) in combination with PE-labeled anti-human CD56 and FITC-labeled anti-human CD3 (BD PharMingen). To assess IL-18R\(\alpha\) expression, overnight rested CD56\(^+\) beads isolated cells were stimulated for 2 days with IL-23 10 ng/ml, IL-12 1 ng/ml, IL-18 100 ng/ml or a combination of these cytokines. Cells were directly labeled with PE-conjugated mouse-anti-human IL-18R\(\alpha\) mAb FAB840P (R&D Systems) in combination with FITC-conjugated anti-human CD3 and Alexa 647 anti-human CD56.

Functional analysis
To determine cytokine production, overnight rested CD56\(^+\) beads isolated cells were seeded 10\(^5\) cells per well and stimulated for 48h with IL-23, IL-12, IL-18 or a combination of these cytokines in a
96-well plate in a final volume of 200 μl. Concentrations are indicated in the figures. The concentration of IFN-γ, IL-10 and IL-17 in the supernatants was determined by cytokine-specific ELISAs (Biosource, Etten-Leur, The Netherlands). For proliferation assays in CD56⁺-isolated cells, cells were carboxyfluorescein (CFSE) (Celltrace, Invitrogen, Breda, The Netherlands) labelled. 10⁶ cells were labelled in 2 ml medium containing 1 μM CFSE for 15 minutes. After labelling cells were washed twice and seeded 10⁵ cells per well in 96 well plate (Costar, Badhoevedorp, The Netherlands). Cells were stimulated with 10 ng/ml IL-23, 1 ng/ml IL-12, 100 ng/ml IL-18 or a combination of these cytokines. Four days after stimulation, cells were directly labelled with PE-labelled anti-human CD3 and Alexa 647-labelled anti-human CD56 and analyzed on a FACS Calibur (BD Bioscience).

Results

IL-23 synergizes with IL-18 in inducing IFN-γ production in primary human CD56⁺ cells.

IL-23 is one of the first cytokines produced by APC in response to PAMPs, while NK and NK-like T cells may be the first innate immune system cells to respond to IL-23. Therefore, we determined whether IL-23 was able to induce IFN-γ production in CD56⁺/CD3⁻ NK and CD56⁺/CD3⁺ NK-like T cells. As IL-18 has been reported to stimulate Th1 responses synergistically with IL-12 by enhancing NK activity and IFN-γ production (21), we also tested the effect of IL-18 in combination with IL-23 on IFN-γ production. Isolated human CD56⁺ cells were analyzed by FACS for CD56 and CD3 expression. More than 90% of the cells were CD56⁺ and of these, 30-70% were CD3⁺. To determine the kinetics of the IFN-γ production, CD56⁺ cells were stimulated for 4, 8, 24 and 48 hours with IL-12 or IL-23 with or without IL-18. IL-23 or IL-18 alone did not induce IFN-γ production at any time point (Fig. 1A and table 1). IL-12 alone induced IFN-γ production 48 hours after stimulation. Four hours after stimulation, small amounts of IFN-γ were detected in supernatants from cells stimulated with IL-12 plus IL-18 (Fig. 1A). IL-23 plus IL-18 induced IFN-γ with slower kinetics and IFN-γ was not detected until 8 hours of stimulation. Highest amounts of IFN-γ were detected after 48 hours of stimulation, in response to IL-12 plus IL-18 or IL-23 plus IL-18 (Fig. 1A).

To determine the response to various concentrations of IL-23 and IL-12 in combination with various concentrations of IL-18, CD56⁺ cells were stimulated for 48 hours. Again, IL-23 or IL-18 alone did not induce IFN-γ production in CD56⁺ cells (Fig. 1B). However, when IL-23 was combined with IL-18, we observed synergistic effects on IFN-γ secretion (Fig. 1A and 1B). IL-12 alone induced minimal IFN-γ production. As expected, a synergistic effect on IFN-γ production was observed when IL-12 and IL-18 were combined (Fig. 1A and 1B). Both IL-23 and IL-12 showed strongest synergy with the highest concentrations of IL-18 (Fig. 1B). Furthermore, as IL-15 is known to enhance IL-12 induced IFN-γ production, we tested IL-15 in combination with IL-23. IL-15 synergized with IL-23 in
inducing IFN-γ production by CD56⁺ cells; however, this synergy was not as strong as the synergy observed between IL-18 and IL-23 (data not shown). We also stimulated CD56⁺ cells with IL-18 in combination with IL-12 plus IL-23. IL-23 slightly inhibited the effect of IL-12 (data not shown). As IL-23 is reported to play a role in the induction of IL-17, we tested IL-17 production in these supernatants as well. However, no IL-17 was detected (data not shown). In antiCD2/antiCD28 activated CD56⁺ cells, IL-23 inhibited the production of the Th2 cytokines IL-4 and IL-13 (data not shown).

Figure 1. IL-23, in combination with IL-18, induces IFN-γ in primary human CD56⁺ cells. A Anti-CD56 MACS bead isolated cells were rested overnight and then left unstimulated or stimulated with indicated concentrations of IL-23 or IL-12 plus or minus IL-18. Supernatants were collected 4, 8, 24 and 48 hours after stimulation and IFN-γ concentration was measured by ELISA. Both IL-23 and IL-12 synergize with IL-18 in the induction of IFN-γ. B Overnight rested CD56⁺ cells were left unstimulated or stimulated with indicated concentrations of IL-23 or IL-12 in combination with various concentrations of IL-18 for 48 hours. IFN-γ concentrations were determined by ELISA. One representative of experiments with cells from 3 donors.
**Effect of IL-23 on NK and NK-like T cells**

*IL-23 induces STAT phosphorylation in CD56+/CD3+ NK-like T, but not in CD56+/CD3- NK cells.*

IL-23 has been reported to induce STAT1, STAT3, STAT4 and STAT5 tyrosine phosphorylation in the human T cell line Kit225 (20). Primary human CD56+ can be divided into CD56+/CD3- NK cells and CD56+/CD3+ NK-like T cells. To test in which of these CD56+ populations IL-23 induces STAT phosphorylation, we stimulated CD56+ cells for 30-90 minutes with IL-23 and determined STAT phosphorylation by FACS analysis, using antibodies specifically directed against phosphorylated STAT1, STAT3, STAT4 or STAT5, in combination with anti-CD3 and anti-CD56. IL-23 induced both STAT3 and STAT4 phosphorylation in CD56+/CD3+ NK-like T cells, but not in CD56+/CD3- NK cells (Fig. 2). We did not observe any STAT1 or STAT5 phosphorylation in response to IL-23 (Fig. 2). These results suggest a direct effect of IL-23 on CD56+/CD3+ NK-like T cells.

**Figure 2.** IL-23 induces STAT phosphorylation in CD56+/CD3+ NK-like T but not in CD56+/CD3- NK cells. Anti-CD56 MACS bead isolated cells were rested overnight, and subsequently stimulated with 10 ng/ml IL-23 or 1 ng/ml IL-12 for indicated times. Cells were labelled with anti-human pSTAT1, pSTAT3 or pSTAT4-Alexa 647 in combination with anti-human CD3-FITC and anti-human CD56-PE and analyzed by FACS. Graph shows fold increase of the mean fluorescence intensity (mfi) as compared to medium stimulated cells. IL-23 induces phosphorylation of STAT3 and STAT4, but not STAT1 and STAT5, in CD56+/CD3+ NK-like T cells, but not in CD56+/CD3- NK cells. IL-12 induces STAT4 phosphorylation in both populations. One representative of experiments with cells from 3 donors.

For control purposes, cells were also stimulated with IL-12 and IL-2. IL-12 induced STAT4 phosphorylation in both NK and NK-like T cells (Fig. 2). IL-2 induced STAT5 phosphorylation in both CD3+ and CD3- populations (data not shown). The fact that only CD3+ NK-like T-cells and not in CD3- NK cells are responsive to IL-23 may indicate that only NK-like T cells express the IL-23R. To
date, no antibody is available for the detection of the IL-23 on the cell membrane (22) instead, we analyzed IL23R mRNA expression in both CD3+ NK-like T and in CD3- NK cells by real time PCR and observed a 5-6 fold higher expression of IL23R mRNA in CD3+ NK-like T cells, compared to CD3- NK cells (data not shown).

**Figure 3. IL-23 in combination with IL-18 induces IFN-γ production in CD56+/CD3+ NK-like T cell but not in CD56-/CD3- NK cells.** Anti-CD56 MACS bead isolated cells were rested overnight and then left unstimulated (A) or stimulated with 1 ng/ml IL-12 (B), 10 ng/ml IL-23 (C), 100 ng/ml IL-18 (D), 100 ng/ml IL-18 plus 1 ng/ml IL-12 (E), 100 ng/ml IL-18 plus 10 ng/ml IL-23 (F), for 48 hours. Cells were fixed and permeabilised and labeled with anti-human CD3-PE, anti-human CD56-FITC and anti-human IFN-γ-Alexa 647. Unstimulated cells and cells stimulated with IL-12, IL-23 or IL-18 do not produce IFN-γ. IL-12 plus IL-18 induce IFN-γ production in CD3- NK and CD3+ NK-like T cells. IL-23 in concert with IL-18 induced IFN-γ production in CD3+ NK-like T cells, but not in CD3- NK cells. One representative of experiments with cells obtained from 6 donors.

**IL-23, in concert with IL-18, induces IFN-γ in CD56+/CD3+ NK-like T, but not in CD56+/CD3- NK cells.** In the STAT phosphorylation assay we observed a specific effect of IL-23 on CD56+/CD3+ NK-like T cells. We next wanted to determine whether these IL-23 responsive CD56+/CD3+ NK-like T cells were responsible for the observed IFN-γ production by CD56+ cells. CD56+ cells were stimulated with IL-23, IL-12, IL-18, combinations of these cytokines, or left unstimulated two days. Intracellular IFN-γ production, as well as CD56 and CD3 expression were assessed by FACS to compare IFN-γ production by CD56+/CD3+ NK cells and CD56+/CD3+ NK-like T cells. Unstimulated cells did not produce IFN-γ (Fig. 3A and Table 1). In response to IL-12 or IL-23 or IL-18 alone no IFN-γ production could be detected (Fig. 3B-D and Table 1). IL-12 in combination with IL-18 induced IFN-γ production in both NK cells and NK-like T cells (Fig. 3E and Table 1). In contrast, after stimulation with IL-23 in combination with IL-18, NK-like T cells produced IFN-γ, whereas NK cells did not (Fig. 3F).
Moreover, IL-23 plus IL-18 induced IFN-γ production only in CD56 bright NK-like T cells, whereas IL-12 plus IL-18 induced IFN-γ production in CD56 bright and CD56 dim cells (data not shown).

Table 1. Percentages of IFN-γ positive NK and NK-like T cells from 6 donors

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<tr>
<th></th>
<th>medium</th>
<th>IL-12</th>
<th>IL-23</th>
<th>IL-18</th>
<th>IL-18+IL-12</th>
<th>IL-18+IL-23</th>
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<tr>
<td>IFN-γ+ve NK cells</td>
<td>0.24 (0.22)</td>
<td>0.55 (0.57)</td>
<td>0.16 (0.13)</td>
<td>0.23 (0.22)</td>
<td>33.6 (11.8)*</td>
<td>2.05 (1.38)</td>
</tr>
<tr>
<td>IFN-γ+ve NK-like T cells</td>
<td>0.53 (0.38)</td>
<td>1.06 (0.88)</td>
<td>0.56 (0.30)</td>
<td>0.70 (0.53)</td>
<td>26.57 (9.11)*</td>
<td>10.76 (3.56)**</td>
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Isolated CD56 cells were stimulated and analysed exactly as in Figure 3. Average percentages and standard deviation (SD) of IFN-γ positive cells are shown of 6 donors. Two-tailed paired t-tests were performed of stimulated versus unstimulated cells. * indicates a p-value <0.01, ** indicates a p-value <0.02.

**IL-23 in combination with IL-18 induces proliferation of CD56+/CD3− NK and CD56+/CD3+ NK-like T cells**

Because the above observed expansion of the CD56 bright CD3− NK cells could be due to upregulation of CD56, to enhanced survival, or to proliferation of CD56 bright cells we analyzed proliferation in response to cytokines. CD56+ cells were CFSE labeled and stimulated with IL-23, IL-12, IL-18, combinations of these cytokines, or left unstimulated. Proliferation was measured by FACS six days after stimulation. Cells were anti-CD56 and anti-CD3 labeled. Unstimulated cells did not proliferate (Fig. 4A and G and Table 2). IL-12 induced proliferation of CD56+/CD3− NK cells (Fig. 4B and table 2), predominantly of CD56 bright cells (Fig. 4H and Table 2). In contrast to IL-12, IL-23 alone did not induce proliferation of NK or NK-like T cells (Fig. 4C and I and Table 2). IL-18 alone induced proliferation of a small population of CD56+/CD3− NK cells (Fig. 4D and Table 2), these cells were mostly CD56 bright (Fig. 4J). IL-12 in combination with IL-18 (Fig 4E and Table 2) induced proliferation of CD56+/CD3− NK cells and, to a lesser extent, of CD56+/CD3+ NK-like T cells. Both CD56 bright and CD56 dim cells proliferated in response to IL-12 plus IL-18 (Fig 4K). IL-23 in combination with IL-18 induced proliferation of CD56+/CD3− NK cells (Fig. 4F and Table 2). Both IL-12 and IL-23 in combination with IL-18 induced proliferation of CD56+/CD3+ NK-like T cells, but less compared to CD56+/CD3− NK cells (Fig. 4E and F). Because in these experiments CD56+/CD3− NK and CD56+/CD3+ NK-like T cells are co-cultured, the effects observed in CD3− NK cells could have been induced directly in CD3− NK cells or induced indirectly via IL-23 activated CD3+ NK-like T cells. However, when CD3+ NK-like T cells were depleted from the CD56+ cells using FACS sort, proliferation in response to IL-23 plus IL-18 was still observed in the CD56+/CD3− NK cells (data not shown). The purity of the sorted CD56+/CD3− NK cells was >99%.

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Figure 4. IL-23 in combination with IL-18 induces proliferation of CD56+/CD3- NK cells and in CD56+/CD3- NK-like T cells. Anti-CD56 MACS bead isolated cells were rested overnight and then CFSE labeled. These labeled cells were left unstimulated (A and G) or stimulated with 1 ng/ml IL-12 (B and H), 10 ng/ml IL-23 (C and I), 100 ng/ml IL-18 (D and J), 100 ng/ml IL-18 plus 1 ng/ml IL-12 (E and K), 100 ng/ml IL-18 plus 10 ng/ml IL-23 (F and L). Six days after stimulation cells were labeled with anti-human CD3-PE and CD56 alexa 647 and analyzed by FACS. IL-23 and IL-18 alone do not induce or induce only little proliferation. IL-23 and IL-18 together induce proliferation of CD3- NK cells. IL-12 alone induces proliferation of CD3- NK cells. IL-18 enhances the proliferative effect of IL-12. CD3- NK-like T cells proliferate less compared to CD3- NK cells in response to all stimuli. Experiments were performed with cells obtained from 6 donors. Representative graphs from one donor are shown.
Table 2. Percentages of proliferating NK and NK-like T cells from 6 donors

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<tr>
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<th>(-)</th>
<th>IL-12</th>
<th>IL-23</th>
<th>IL-18</th>
<th>IL-18+IL-12</th>
<th>IL-18+IL-23</th>
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<tr>
<td>Proliferating NK cells</td>
<td>1.53 (0.32)</td>
<td>7.08 (2.38)*</td>
<td>1.39 (0.43)</td>
<td>2.54 (0.83)</td>
<td>22.17 (2.04)*</td>
<td>9.47 (3.76)*</td>
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<td>Proliferating NK-like T cells</td>
<td>0.28 (0.09)</td>
<td>0.99 (0.74)*</td>
<td>1.16 (2.40)</td>
<td>0.29 (0.27)</td>
<td>3.83 (1.53)**</td>
<td>2.16 (0.69)*</td>
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Isolated CD56⁺ cells were stimulated and analysed exactly as in Figure 4. Average percentages of proliferating NK and NK-like T cells obtained from 6 different donors. Two-tailed paired t-tests were performed of stimulated cells versus unstimulated cells. * indicates a p-value <0.05, ** indicates a p-value <0.01

Figure 5. IL-23 up-regulates IL-18Rα expression in CD56⁺/CD3⁺ NK-like T, but not in CD56⁺/CD3⁺ NK cells. Anti-CD56 MACS bead isolated cells were left unstimulated (A) or stimulated with 1 ng/ml IL-12 (B), 10 ng/ml IL-23 (C), 100 ng/ml IL-18 (D), 1 ng/ml IL-12 plus 100 ng/ml IL-18 (E) or 10 ng/ml IL-23 plus 100 ng/ml IL-18 (F). 48 hours after stimulation cells were labelled with anti-human CD56-Alexa 647, anti-human CD3-FITC and anti-human IL-18Rα-PE and analyzed by FACS. Unstimulated CD3⁺ NK-like T cells express slightly more IL-18Rα as compared to CD3⁺ NK cells. IL-12 up-regulates IL-18Rα in NK and NK-like T cells. IL-23 up-regulates IL-18Rα expression mainly in NK-like T cells. IL-18 alone slightly enhances IL-18Rα in NK cells. IL-18 synergizes with IL-12 and IL-23 in the up-regulation of IL-18Rα expression. One representative of experiments with cells from 6 donors.
IL-23 enhances IL-18Rα expression in CD56+/CD3+ NK-like T but not in CD56+/CD3- NK cells.

We have shown that IL-23 induces IFN-γ production in CD56+/CD3+ NK-like T cells in synergy with IL-18. We have also shown that IL-23 in synergy with IL-18 induced enhanced CD56 expression in NK-like T cells and that IL-23 plus IL-18 induced proliferation in CD56+/CD3+ NK cells. The mechanism underlying the synergy between IL-18 and IL-12 involves IL-12 induced IL-18Rα expression (23). To determine whether the synergy between IL-23 and IL-18 is similarly dependent on the up-regulation of IL-18Rα, CD56 isolated cells were stimulated for two days with IL-23, IL-12, IL-18, IL-23 plus IL-18 or IL-12 plus IL-18. Cells were then analyzed for IL-18Rα, CD3 and CD56 expression. Unstimulated NK and NK-like T cells expressed low amounts of IL-18Rα (Fig. 5A and Table 3). IL-12 enhanced IL-18Rα expression in both NK and NK-like T cells (Fig. 5B and Table 3). IL-23 up-regulated the IL-18Rα expression in NK-like T cells, but only marginally in NK cells (Fig. 5C and Table 3). IL-18 alone enhanced the expression of the IL-18Rα only marginally in both populations (Fig. 5D and Table 3), but synergized with the effect of IL-12 on the expression of IL-18Rα in both NK and NK-like T cells (Fig. 5E and Table 3). IL-18 synergized with IL-23 in the up-regulation of the IL-18Rα in NK-like T cells (Fig. 5F and Table 3). In NK cells a slight upregulation of the IL-18Rα was observed in response to IL-23 plus IL-18 (Fig. 5F and Table 3). Regardless of the stimulation, cells with high expression of the IL-18Rα were CD56bright (data not shown).

**Table 3.** IL-18Rα expression in NK and NK-like T cells from 6 donors

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<th>(-)</th>
<th>IL-12</th>
<th>IL-23</th>
<th>IL-18</th>
<th>IL-18+IL-12</th>
<th>IL-18+IL-23</th>
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<tr>
<td>CD3- NK cells upper half</td>
<td>0.19 (0.08)</td>
<td>10.30 (5.48)**</td>
<td>0.85 (0.48)**</td>
<td>1.11 (1.60)</td>
<td>34.99 (21.24)*</td>
<td>4.17 (1.23)</td>
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<td>CD3+ NK-like T cells upper half</td>
<td>0.36 (0.26)</td>
<td>11.84 (10.4)</td>
<td>3.98 (4.85)</td>
<td>0.65 (0.49)</td>
<td>50.98 (24.12)*</td>
<td>36.34 (2.92)*</td>
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Isolated CD56 cells were stimulated and analysed exactly as in Figure 5. Average percentages and standard deviation of data obtained from 6 different donors are shown. Two-tailed paired t-tests were performed of stimulated cells versus unstimulated cells. * indicates a p-value <0.01, ** indicates a p-value <0.02.

**Discussion**

The main finding of this study is that IL-23, an APC-derived cytokine, in combination with IL-18, another cytokine elicited in APCs early after binding of pathogen-associated molecular patterns, can elicit the production of IFN-γ by NK-like T cells as well as proliferation and activation of human NK and NK-like T cells. We hypothesize that in this way, APCs become primed to subsequently produce large amounts of IL-12 and thus amplify the production of IFN-γ. This
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conclusion is supported by the following findings. Firstly, IL-23 and IL-18 synergistically increase IFN-γ production in CD56⁺/CD3⁺ NK-like T cells. This synergy between IL-23 and IL-18 in NK-like T cells coincided with IL-23 mediated up-regulation of IL-18Rα. Secondly, IL-23 plus IL-18 induced proliferation of CD56⁺/CD3⁻ NK cells and CD56⁺/CD3⁺ NK-like T cells.

We observed a strong synergy between IL-23 and IL-18 to induce cytokine production and induce cell proliferation. In this respect, the combination of IL-23 and IL-18 on NK and NK-like T cells differed from that induced by IL-12 in combination with IL-18: IL-23 plus IL-18 induced IFN-γ production in NK-like T cells only, whereas IL-12 plus IL-18 induced IFN-γ in both NK as well as NK-like T cells. Previously it has been described that IFN-γ production by NK-like T cells can be triggered by IL-12/IL-18 (14) stimulation or TCR stimulation. We show that in NK-like T cells, IL-23 plus IL-18 can also induce IFN-γ production, in absence of TCR ligation. In CD56⁺/CD3⁻ NK cells and to a lesser extent in CD56⁺/CD3⁺ NK-like T cells, IL-23 and IL-18 synergized in eliciting cell proliferation.

In CD56⁺/CD3⁺ NK-like T cells IL-18Rα expression was up-regulated by IL-23. The synergy between IL-23 and IL-18 in inducing IFN-γ production in these cells is likely to be dependent on this up-regulation of IL-18Rα expression. In CD56⁺/CD3⁻ NK cells the IL-18Rα expression was not enhanced by IL-23, suggesting that in these cells the synergistic effect of IL-23 and IL-18 on proliferation is achieved via another mechanism. The proliferation observed in NK cells could be indirectly induced via NK-like T cells. However, IL-23 in combination with IL-18 also induced proliferation in FACS sorted CD56⁺/CD3⁻ NK cells that were cultured without CD56⁺/CD3⁺ NK-like T cells (>99% pure). This result suggests a direct effect of these cytokines on NK cells.

Upon contact with pathogens APCs are unable to release IL-12 in sufficient amounts to recruit and activate T-helper cells and thus initiate a cellular immune response. For substantial IL-12 production by APC, in addition to PAMPs or pathogens, an extra stimulus such as IFN-γ is needed (9, 24). IL-23 on the other hand is produced by APC in response to PAMPs or pathogens without the need of an additional stimulus (9). Because IL-23 and IL-18 are both released soon after first contact between phagocytes and pathogens, we hypothesize that, in vivo, IL-23 plus IL-18 triggers release of IFN-γ by NK-like T cells and that this IFN-γ could provide APCs with the necessary priming to subsequently produce IL-12. The IFN-γ induced by IL-23 plus IL-18 might thus be important to initiate Th1 immunity at early stages of infection. In line with this hypothesis, IL-23 is shown to be critical for the induction of Ag-specific Th1 development in an experimental autoimmune encephalomyelitis mouse model (25). Moreover, IL-23 inhibited the induction of the Th2 cytokines IL-4 and IL-13 in antiCD2/antiCD28 activated CD56⁺ cells. Taken together, the synergy of IL-18 with IL-23 is likely important in initiating Th1 differentiation early in infections, whereas the synergy between IL-18 and IL-12 may be important in further Th1 response in subsequent stages of infection. Host defense against intracellular bacterial pathogens such as
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*Salmonellae* and *Mycobacteria* depends on the IL-23/IL-12/IFN-γ cytokine pathway (12,26). However, the cells that produce IL-23/IL-12 and IFN-γ at different stages of infection in human are not well defined. For instance, the role of CD56⁺ NK-like T cells in *Mycobacteria* and *Salmonellae* infections has not been studied widely. These cells are likely important because of the following observations. First, the number of peripheral blood CD56⁺ T-cells is increased during these infections in humans (27,28). Second, high numbers of CD56⁺ NK-like T cells at diagnosis of pulmonary tuberculosis correlated significantly with negative sputum culture after 8 weeks of treatment (29). Third, in the presence of macrophages infected with live *M. bovis* BCG or *S. typhimurium*, CD56⁺ NK-like T cells, but not CD56⁻ T cells, produce IFN-γ in the absence of TCR stimulation (14). Fourth, in patients with unusual susceptibility to *Mycobacteria* and *Salmonellae* infections due to deficiency of IL-12Rβ1 or IL-12p40, the number of CD56⁺ NK-like T-cells is drastically reduced (14). The reduced numbers of NK-like T cells in these last patients indicate that IL-12 and IL-23 are also needed for the differentiation and/or maintenance of these cells. In addition to these findings, we have shown that IL-23, in combination with IL-18, is able to drive IFN-γ production in CD56⁺ NK-like T cells, in the absence of IL-12 and TCR ligation. This finding indicates that IL-23 could be important in driving IFN-γ production in these cells in early stages of infection, before sufficient IL-12 is produced to drive IFN-γ production. Consistent with this hypothesis, p40⁻/⁻ mice, lacking both IL-12 and IL-23, infected with *S. enteritidis* or *M. tuberculosis* produce lower levels of IFN-γ than p35⁻/⁻ mice, lacking only IL-12 (30,31). Moreover, p35⁻/⁻ mice infected with *S. enteritidis* show higher survival rates or longer survival times than p40⁻/⁻ mice (31). In mycobacterial infection, IL-23 provides protection in the absence of IL-12 (32). Together, this points to a role for IL-23 in protection, independent of IL-12. The relative roles of either IL-12 or IL-23 in driving the IFN-γ response early in infection have not been addressed yet. Monitoring the kinetics of IFN-γ in response to infections with *Salmonellae* and *Mycobacteria* in p19⁻/⁻ and p35⁻/⁻ mice could provide information about the contribution of IL-23 and IL-12 to the induction of IFN-γ.

Taken together, these observations indicate that IL-23 has different effects on NK cells as compared with NK-like T cells. The effects of IL-23 in combination with IL-18 on NK and NK-like T cells differ from the effects induced by IL-12 in combination with IL-18. Moreover, we showed that IL-23, in synergy with IL-18, activates NK-like T cells. This activation was independent of IL-12 and independent of TCR ligation. In conclusion, IL-23 may have an important role in activating NK and NK-like T cells and the initiation of the (Th1) immune response early in an infection.

Acknowledgments

We would like to thank dr. Nigel Savage for carefully reading the manuscript.
References


