Primining of Human Resting NK Cells by Autologous M1 Macrophages via the Engagement of IL-1β, IFN-β, and IL-15 Pathways

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The cross talk between NK cells and macrophages is emerging as a major line of defense against microbial infections and tumors. This study reveals a complex network of soluble mediators and cell-to-cell interactions allowing human classically activated (M1) macrophages, but not resting (M0) or alternatively activated (M2) macrophages, to prime resting autologous NK cells. In this article, we show that M1 increase NK cell cytotoxicity by IL-23 and IFN-γ–dependent upregulation of NKG2D, IL-1β–dependent upregulation of NKP44, and trans-presentation of IL-15. Moreover, both IFN-γ–dependent cis-presentation of IL-15 on NK cells and engagement of the 2B4-CD48 pathway are used by M1 to trigger NK cell production of IFN-γ. The disclosure of these synergic cellular mechanisms regulating the M1–NK cell cross talk provides novel insights to better understand the role of innate immune responses in the physiopathology of tumor biology and microbial infections. The Journal of Immunology, 2015, 195: 2818–2828.

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during the course of viral infections (20). However, the mechanism(s) adopted by activated human macrophages to regulate autologous resting NK cell functions are largely unknown. A proper activation of NK cells can be achieved with different proinflammatory cytokines, such as IL-2, IL-12, IL-15, IL-18, and type I IFNs (1, 21, 22). In particular, IL-15 has been shown to play an important role in the context of NK development, homeostasis, and functions. After its binding to the high-affinity IL-15R α-chain (IL-15Rα) expressed by DCs and monocytes/macrophages, IL-15 is trans-presented to surrounding cells bearing the lower-affinity IL-15R βγ-chains (22, 23). The membrane-associated IL-15–IL-15R complex can be also cleaved from cell surface and released in biologic fluids, such as plasma (24, 25). More recently, it emerged that IL-15 can also be presented by IL-15Rβγ-chains expressed on the same cell, thus allowing a mechanism of IL-15 cis-presentation that triggers NK cell effector functions (26). Another potent proinflammatory cytokine produced by M1 is IL-1β, which has been reported to modulate homeostasis and activation of innate lymphoid cells (ILCs) in secondary lymphoid tissues (27–29). ILCs have been recently identified as an additional lymphopoietic population that could be divided in different subsets (ILC1, ILC2, ILC3) (30). Because NK cells differ from ILC1 by the expression of comesodermin during their differentiation (30), they are considered as a distinct immune cell population. However, whether NK cells are responsive to IL-1β is still being debated in both human and murine settings (26–28, 31, 32).

This study investigates on the cross talk between human resting NK cells and autologous polarized macrophages, and discloses the selective ability of M1 to prime NK cells through a complex network of interactions that comprises mechanisms associated with both the production of soluble mediators and cell-to-cell contacts.

Materials and Methods

NK cell and macrophage isolation and culture

Human PBMCs were isolated from buffy coats of healthy donors obtained in accordance with clinical protocols approved by the Institutional Review Board of Desio Hospital, Milan, Italy. After centrifugation on a Ficoll density gradient (GE Healthcare Biosciences), NK cells and monocytes were isolated by negative magnetic cell purification (STEMCELL Technologies) (33, 34). The purity of enriched resting NK cells was ≥95%, and the frequency of other contaminating cells was ≤5% for NK/T cells, ≤2% for CD56−CD127−ILCs, ≤0.5% for CD109+ B cells, ≤0.2% for CD14+ monocytes, and ≤0.02% for M-DC8+ slan-DCs (Supplemental Fig. 1A, 1B). Freshly purified and enriched NK cells were frozen in FBS supplemented with 10% DMSO (Sigma) immediately after their isolation, thawed and autologous resting or polarized macrophages were ready, and cultured for 7 d with RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, 1% pen/strept, 100 ng/ml rhIFN-α, and 0.4% for CD19+ B cells.

Incubation of NK cells with macrophage-conditioned media or autologous macrophages

NK cells were incubated for 24 h at 1.25–2.5 × 10^5 cells/ml with 30% conditioned media taken from macrophage (M0/M1/M2) cultures, 200 U/ml rhL-2, 20 ng/ml rhIL-15 (Peprotech), 1 ng/ml rhIL-1b (Milteny), 100 ng/ml rhIFN-α, or 200 U/ml rhIFN-β (R&D Systems). For direct NK cell–macrophage coculture, 1 × 10^5 NK cells were grown for 24 h at a 1:1 cell ratio with resting or polarized macrophages, which were extensively washed to eliminate all soluble factors accumulated during the polarization period before analysis.

Flow cytometry and cell sorting

For direct multicolor flow-cytometry analysis (FACSComp II; BD Biosciences) or cell sorting (FACSARia II; BD Biosciences), NK cells, monocytes, and polarized macrophages were stained with HBSS (Lonza) supplemented with 2% FBS and incubated for 20 min at 4°C with the following antibodies: 1:00 dilutions of Abs labeled with indicated fluorochromes: CD3/CD56-PE/PC5/FCPC (Beckman Coulter), CD3-FITC (BD Pharmingen), CD14-Brilliant Violet 650 (Biolegend), CD14-FITC (BD Pharmingen), CD14-Brilliant Violet 570 (Biolegend), CD16-PE-Cy7 (BD Pharmingen), CD16-PerCP-Cy5.5 (BD Pharmingen), CD19-allophycocyanin-Cy7 (BD Pharmingen), CD48-PE (BD Pharmingen), CD56-Brilliant Violet 421 (Biolegend), CD69-PE-Cy7 (BD Pharmingen), CD80-PE (BD Pharmingen), CD117-PE-Cy7 (eBioscience), CD206-FITC (BD Pharmingen), CD209-PE (BD Pharmingen), M-DC8-allophycocyanin (Milteny), NKp46-PE (Beckman Coulter), NKp44-PE (Beckman Coulter), NKp30-PE (Beckman Coulter), NGK2D-PE (Beckman Coulter), DNAM-1-PE (Beckman Coulter), 2B4-PE (R&D Systems), IL-15Rα-PE (Biolegend), IL-1RI-PE (R&D Systems), and their appropriate isotype controls. For IL-1RI indirect staining, NK cells were incubated for 45 min at room temperature (RT) with purified goat anti-human IL-1RI (R&D Systems) and its polyclonal IgG control, and for 30 min at RT with mouse anti-Goat Alexa Fluor 647–labeled secondary Ab (Invitrogen).

Real-time PCR

Cells were lysed with QIAzol Reagent (Qiagen), and total RNA was extracted using miRNeasy mini kit (Qiagen). RNA was converted in cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and quantification of the following transcripts was performed following the recommended protocols for SYBR Green Master Mix (Applied Biosystems) or TaqMan Fast Advanced Master Mix (Applied Biosystems), and quantification of the following transcripts was performed following the recommended protocols for SYBR Green Master Mix (Applied Biosystems) or TaqMan Fast Advanced Master Mix (Applied Biosystems).

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rorophagy analysis. Data were analyzed by SDS 2.4 software with a 7900HT Fast Real Time PCR System (Applied Biosystems) and Opticon Monitor 3 with a PCT-200 PCR (MJ Research).

Masking experiments
Saturating concentrations of the following neutralizing Abs were added to the NK cell culture: NKG2D (1 μg/ml; Biolegend), IL-1β (5 μg/ml; BD Pharmingen), IL-15 (0.5 μg/ml; R&D Systems), IL-18 (5 μg/ml; MBL International Corporation), IL-23p19 (0.8 μg/ml; R&D Systems), IFN-β (0.2 μg/ml; R&D Systems), IL-1RI (2 μg/ml; R&D Systems), and IL-13Rα2 (5 μg/ml; R&D Systems). Anti-Nkp30 (clone F252), anti-Nkp44 (clone KS538), anti-Nkp46 (clone KL247), anti-2B4 (clone C054), and anti-DNAM-1 (clone DX11) mAbs were kindly provided by Prof. Alessandro Moretta (University of Genova, Italy). Macrophage-conditioned media were incubated for 45 min at RT with blocking Abs against cytokines before macrophage-conditioned media treatment and left until harvest before NK cell stimulation. IL-1RI on NK cells was blocked at RT for 45 min were incubated for 45 min at RT with blocking Abs against cytokines before they were incubated with target cells and left until harvest of the cells. Anti–IL-15Rα was given at the beginning of the treatment with macrophage-conditioned media or NK cell–macrophage coculture and left until the conclusion of the experiment. Blocking Abs to activating receptors were incubated for 45 min at RT with macrophage-primed NK cells before they were incubated with target cells and left until harvest of the cells and on resting NK cells before they were cocultured with polarized macrophages and left for all the period of the coculture. Each neutralizing condition was compared with a matched isotype control.

CD107a degranulation assay and cytokine secretion
We used CD107a degranulation assay as indicative of NK cell cytotoxicity, as previously demonstrated (35). NK cells treated with macrophage-conditioned media were incubated with K562 at 2:1 E:T ratio or HEK-293T at 5:1 E:T ratio for 4 h in the presence of PE-labeled CD107a Ab (BD Pharmingen) or its isotype control. K562, JAI3, MOLT-4, Raji, or 221 (E:T ratio 2:1) were incubated for 4 h directly into NK cell–macrophage cocultures, together with PE-labeled CD107a Ab or its isotype control. Thereafter, cells were washed, stained with CD56 and CD3 Abs to allow gating on NK cells, and analyzed by flow cytometry. IFN-γ secretion by NK cells was dosed by commercial ELISA kit (Duoset; R&D Systems). NK cells were washed extensively after treatment with autologous macrophage-conditioned media to eliminate any cytokine carryover. NK cells were stimulated with K562 cells (E:T ratio 2:1) for 4 h in both experimental approaches (indirect or direct culture). Macrophage secretion of IL-1β, IL-12p70, IL-15, and IL-15/IL-15Ra were dosed by commercial ELISA kit (Duoset; R&D Systems). Data were analyzed by SoftMaxPro 5.3 software.

Statistical analysis
Results were expressed as mean ± SEM from multiple independent experiments. Two-tailed Student t test or one-sample two-tailed t tests for comparison against fixed values (% of max, set to 100%) were performed using Prism (GraphPad 4) and/or Excel (Microsoft) software: n.s., not significant; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Results
Priming of resting NK cells requires M1 polarization
To study the ability of either resting or polarized macrophages to prime autologous resting NK cells, we set up two distinct experimental approaches. In the first one, the potential effects of macrophage-derived soluble mediators was assessed by incubating NK cells with 30% macrophage-conditioned media taken from cultures of M0 (M0-primed NK), M1 (M1-primed NK), or M2 (M2-primed NK) (34). Compared with controls (i.e., untreated resting NK cells), we found a significant increase in IFN-γ secretion, CD107a degranulation, and CD69 expression selectively in M1- but not in M0- or M2-primed NK cells (Fig. 1A–C). In the second experimental approach, we analyzed the effects of cellular interactions by directly coculturing M0, M1, or M2 with autologous resting NK cells. Macrophages were previously washed to eliminate soluble factors accumulated during the polarization period. Our data showed that M1, but not M0 and M2, induced a significant induction of IFN-γ secretion, CD107a degranulation, and CD69 surface expression compared with resting NK cells (Fig. 1D–F). In both experimental settings, M1 increased NK cytotoxicity against different target cells, as assessed by CD107a

**FIGURE 1.** Priming of resting NK cells by macrophage-conditioned media or macrophages. Secretion of IFN-γ (A–D), percentages of CD107a<sup>+</sup> (B–E) (gated on CD3<sup>–</sup>CD56<sup>+</sup> cells) in the presence of K562 target cell line, and expression of CD69 presented as mean fluorescence intensity (MFI) (C–F) (gated on CD3<sup>–</sup>CD56<sup>+</sup> cells) after incubation of resting NK cells with either M0-, M1-, or M2-conditioned media from autologous macrophages (M0-primed, M1-primed, M2-primed NK cells) (A–C, black bars) or with autologous M0, M1, and M2 (D–F, black bars) compared with negative controls (i.e., untreated resting NK cells in white bars). Mean ± SEM; n = 11, 17, 7, 10, 15, and 3 for (A)–(F), respectively. *p < 0.05, **p < 0.01, ***p < 0.001.
degranulation assay (35, 36) (Supplemental Fig. 3A, 3C). Taken together, these results demonstrate that proinflammatory M1 prime resting NK cells by both secreting soluble mediators and establishing cell-to-cell interactions. In contrast, neither M0 nor M2 influence NK cell activation and effector functions under these experimental settings.

**M1 macrophages induce NKp44 and NKG2D upregulation via IL-1β and IFN-β**

To identify the mechanisms involved in the M1-mediated priming of NK cells, we first evaluated the expression of a large panel of aNKRs after activation of NK cells with either M1-conditioned medium or autologous M1. Among all receptors tested on M1-primed NK cells, we detected the selective upregulation of NKp44 and NKG2D to levels comparable with those observed on IL-2–activated NK cells (37). The incubation of resting NK cells with either IFN-γ or LPS did not affect the expression of these two aNKRs, thus ruling out a potential carryover artifact of the in vitro culture. Finally, the stimulation of resting NK cells with the supernatant of autologous M0 activated only with LPS induced an upregulation of both NKp44 and NKG2D similar to that of M1-primed NK cells (Fig. 2, Supplemental 3D–O). Because these findings indicate that the upregulation of these two aNKRs is associated with the production of LPS-dependent cytokines, we then proceeded to selectively block those M1-derived cytokines known to modulate NK cell functions (11, 15, 38–40). We first ruled out IFN-γ, whose masking did not decrease the expression of NKp44 (Fig. 3A), as well as IL-18, IL-15, and IL-15Rα, whose masking did not decrease the expression of NKp44 (Fig. 3A). We then analyzed the potential contribution given by IL-12p70 and IL-23p19, two LPS-induced proinflammatory cytokines (41). We then proceeded to selectively block those M1-derived cytokines known to modulate NK cell functions (11, 15, 38–40). We first ruled out IFN-γ, whose masking did not decrease the expression of NKp44 (Fig. 3A), as well as IL-18, IL-15, and IL-15Rα, whose masking did not decrease the expression of NKp44 (Fig. 3A). We then analyzed the potential contribution given by IL-12p70 and IL-23p19, two LPS-induced proinflammatory cytokines (41). The blocking of IL-1β in the M1-conditioned medium and/or IL-1α on NK cells significantly reduced NKp44 upregulation, whereas an anti–IL-23p19 mAb did not decrease the surface expression of these two aNKRs (Fig. 3A, 3C). Consistent with these latter results and in agreement with previous reports (28, 29, 31, 41), we detected constitutive expression of IL-1RI on resting NK cells. Moreover, our data showed a significant increase of IL-1RI on M1-primed NK cells as compared with M0-primed counterparts. Conversely, we did not detect any expression of the decoy IL-1RII, neither on resting nor on macrophage-primed NK cells (42) (Fig. 4). The key role of this cytokine in inducing NKp44 upregulation was also confirmed by incubating resting FACS-sorted and highly pure NK cells directly with rIL-1β (Supplemental Fig. 1D–F). NKp44 expression on FACS-sorted NK cells was also induced by M1-conditioned media (Supplemental Fig. 1F), thus ruling out the contribution of those cellular contaminants present at very low frequencies after NK cell enrichment (i.e., monocytes or slan-DCs) and able to release IL-1β (43, 44). Finally, we also demonstrated that the NKp44 upregulation on M1-primed NK cells did not occur in the presence of a prior incubation of M1 with the inflammasome inhibitor glibenclamide (Fig. 3E), which is indeed capable of blocking IL-1β secretion by M1 (Supplemental Fig. 4B).

Although the masking of IL-18, IL-15, and IL-15Rα (Fig. 3B), as well as the inflammasome pathway inhibition (Fig. 3F), had no effect on NKG2D expression on M1-primed NK cells, a role was evident for IFN-β (Fig. 3D). These results are also consistent with a recent report describing that IFN-β produced by activated murine macrophages regulates NKG2D expression on NK cells (45). The surface levels of NKG2D on M1-primed NK cells also decreased after the masking of IL-23p19 (Fig. 3B), which is known to share with IL-12 some functional properties (46, 47).

Finally, to assess the functional relevance of NKG2D and NKp44 upregulation on M1-primed NK cells, we evaluated NK cell cytolytic activity against K562 (Fig. 3G) and HEK 293T cell lines (Fig. 3H). We found that K562 are preferentially killed via NKp44, as also previously reported (48), and via NKG2D, but not through the engagement of the NKp44 pathway (Supplemental Fig. 3B). Hence we chose K562 as a target cell line to evaluate the NKG2D-mediated cell killing of M1-primed NK cells. Indeed, the masking of NKp44 did not decrease the degree of degranulation of M1-primed NK cells against K562, whereas the blocking of NKG2D significantly reduced their percentage of CD107a expression (Fig. 3H). We found that K562 are preferentially killed via NKp44, as also previously reported (48), and via NKG2D, but not through the engagement of the NKp44 pathway (Supplemental Fig. 3B). Hence we chose K562 as a target cell line to evaluate the NKG2D-mediated cell killing of M1-primed NK cells. Indeed, the masking of NKp44 did not decrease the degree of degranulation of M1-primed NK cells against K562, whereas the blocking of NKG2D significantly reduced their percentage of CD107a expression (Fig. 3H).

**FIGURE 2.** Upregulation of NKp44 and NKG2D on M1-primed NK cells. Surface expression of NKp44 (A) and NKG2D (B) (gated on CD3−neg CD56+pos cells) on M0-primed, M1-primed, M2-primed NK cells, and on NK cells incubated with conditioned media of LPS-activated macrophage (black bars) compared with untreated NK cells (white bars). Mean ± SEM; n = 7 (for NKp44) and 13 (for NKG2D). *p < 0.05, **p < 0.01, ***p < 0.001, n.s., not significant.

**IFN-β secreted by M1 macrophages induces IL-15 cis-presentation on NK cells and enhances IFN-γ production**

NK cells represent a major source of IFN-γ, an important antiviral and immune-regulatory cytokine that plays a key role in the induction of M1 polarization (11). In line with what has been recently reported (51), we first hypothesized that the engagement of the NK2D pathway could contribute to the increased production of IFN-γ by M1-primed NK cells (Fig. 1A). However, our results ruled out this possibility because the masking of NK2D did not reduce the synthesis and the secretion of IFN-γ in M1-primed NK cells (Fig. 1A). A second potential mechanism enhancing the production of IFN-γ could be associated with the M1 secretion of proinflammatory molecules known to trigger NK cell effector functions (15, 22, 26, 39). Our results showed that blocking of both IL-15 and IL-15Ra significantly reduced IFN-γ secretion in M1-primed NK cells (Fig. 5A). Nevertheless, and in...
line with a previous study (15), we did not detect any level of IL-15 in M1-conditioned medium, neither in a free form nor conjugated to IL-15Rα (Supplemental Fig. 4E, 4F), thus excluding the possible role of IL-15/IL-15Rα soluble complexes (24, 25). Because it has been recently reported that IFN-β produced by DCs induces IL-15/IL-15Rα cis-presentation on murine NK cells (26), we also investigated whether such a mechanism takes place in M1-primed human NK cells, taking into account that M1 polarization is associated with a significant production of IFN-β (52, 53). Indeed, M1-primed NK cells showed significantly higher levels of both IL-15 and IL-15Rα transcripts compared with those of either resting or M0-primed NK cells, albeit at lower levels compared with M1 macrophages (Fig. 5B, 5C). These results also parallel the significantly higher expression of IL-15Rα observed on M1-primed NK cells compared with their resting or M0-primed counterparts (Fig. 5D, 5E).
has been observed with murine DCs (26), IL-15 cis-presentation was IFN-β dependent; as in M1-primed NK cells, its masking reduced both the expression of IL-15Rα (Fig. 5D) and the secretion of IFN-γ (Fig. 5A). Furthermore, rhIFN-β directly induced expression of IL-15Rα selectively on M1-primed NK cells (Fig. 5E). Taken together, these data demonstrate that IFN-β secreted by M1 triggers NK cell production of IL-15 and surface expression of IL-15Rα, allowing a mechanism of cis-presentation that, in turn, increases the secretion of IFN-γ by NK cells.

Mechanisms regulating cell-to-cell-dependent NK cell priming by autologous M1 macrophages

Because M1 polarization was confirmed to be associated with a significant increase in IFN-β production, as well as with an induced expression of IL-15 and IL-15Rα (Supplemental Fig. 2) (39, 52, 53), we next investigated the role of these mediators in autologous resting NK cell priming (Fig. 1D–F). As expected given the pathway normally involved in the NK cell lysis of K562 cell line (Supplemental Fig. 3B), the simultaneous masking of NKp30 and NKG2D significantly reduced the amount of CD107a degranulation by NK cells incubated with either autologous M0 or M1 against this target. Interestingly, the additional blocking of IL-15Rα, but not of IFN-β, significantly further reduced the degree of CD107a degranulation only in NK cells cocultured with M1 and not with M0 (Fig. 6A), thus demonstrating that IL-15/IL-15Rα trans-presentation by M1 to autologous NK cells contributes to enhanced NK cell cytolytic potential. In contrast, this latter mechanism does not play any role in increasing the production of IFN-γ by NK cells cultured with autologous M1 (Fig. 6B).

Finally, we analyzed whether M1 polarization was associated with the induction of ligands for aNKRs, focusing our attention on the 2B4-CD48 pathway, which is known to be involved in regulating the interaction between NK cell and macrophages (15, 16), and contributes to cytokine production by NK cells (16). Our data show that CD48 is highly induced in M1 but not M2 macrophages (Fig. 6C), and demonstrate that the engagement of 2B4 by CD48 is functionally relevant in the context of M1-NK cell cross talk because the masking of 2B4 significantly reduced the levels of IFN-γ produced by NK cells only when they were incubated with M1 (Fig. 6D).

M1-primed NK cells reverse M2 macrophage polarization

Our data demonstrate that M1 macrophages activate NK cells and induce IFN-γ production. Because IFN-γ plays a key role in inducing M1 polarization (7), we asked whether M1-primed NK cells could influence macrophage polarization by reverting an established M2 phenotype. To test this, we assessed expression levels of a panel of M1- and M2-associated genes on M2 macrophages incubated with the supernatant of autologous M1-primed NK cells (M1-p NK). Our results showed that the supernatant of M1-primed NK cells induced a significant upregulation of M1 markers (CD80, IL-15Rα, IL-15, IL-1β) in M2, similar to that detected in M1 (Fig. 7A–D). A significant reduction of M2
markers in already polarized M2 was also observed, although the M2 phenotype was fully reverted for some markers (CD206, ALOX15) and only partially for others (CCL18, CCL22; Fig. 7E–H). Taken together, these results reveal a number of different pathways sustaining the ability of M1 to promote NK cell antitumoral properties (Fig. 8) and suggest that conversely soluble mediators released by M1-primed NK cells have the potential to re-educate the protumoral M2 phenotype.

**Discussion**

Beyond being effector cells able to eliminate virally infected or tumor-transformed target cells, NK cells are also endowed with immune-regulatory functions. Indeed, their ability to interact with other innate immunity cells, such as DCs or neutrophils, is relevant for the development of optimal immune responses against pathogens and tumors (54). The cross talk between NK cells and macrophages has also been recently reported to play a key role

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**FIGURE 5.** IL-15 *cis*-presentation on M1-primed NK cells. (A) Secretion of IFN-γ by M0- and M1-primed NK cells in the presence of neutralizing mAbs against IFN-β (black bars) or IL-15 plus IL-15Rα (hatched bars) compared with isotype controls (white bars). Results are presented as relative percentage referred to the highest level of IFN-γ secretion (M1-primed NK cells, set as 100%) (mean ± SEM; n = 6). (B and C) Transcript levels of IL-15 (B) and IL-15Rα (C) in resting (NK), M0-primed, and M1-primed NK cells and M1 macrophages. Results are presented as 2^{-ΔΔCt} [mean ± SEM; n = 4 for (B) and n = 5 for (C)]. (D) Surface levels of IL-15Rα (gated on CD3 neg CD56pos cells) on untreated (NK), M0-primed, and M1-primed NK cells either in the absence (isotype control, white bars) or in the presence (black bars) of a neutralizing mAb against IFN-β (means ± SEM; n = 7). (E) Fluorescence microscopic images from one representative example of two experiments performed with cells of unrelated healthy donors showing the surface expression of IL-15Rα (green) on untreated (i.e., resting), M0-primed, or M1-primed NK cells and NK cells treated for 3 h with 200 U/ml rIFN-β. Blue staining indicates cell nuclei (DAPI). Original magnification ×60. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**FIGURE 6.** Mechanisms that regulate the cell-to-cell–dependent priming of NK cells by autologous M1 macrophages. (A) Percentages of CD107a pos (gated on CD3 neg CD56pos cells) NK cells cocultured with autologous M0 and M1 either in the absence (isotype controls, white bars) or in the presence of neutralizing mAbs against NKGD2 plus Nkp30 (black bars), NKGD2 plus Nkp30 plus IFN-β (gray bars), NKGD2 plus Nkp30 plus IL-15Rα (hatched bars). Results are presented as relative percentages referred to the highest levels of CD107a degranulation (NK cell cocultured with M1, set as 100%) (mean ± SEM; n = 3). (B) Secretion of IFN-γ by NK cells cocultured with M0 or M1 autologous macrophages either in the absence (isotype controls, white bars) or in the presence of neutralizing mAbs against IFN-β (black bars) or IL-15 plus IL-15Rα (hatched bars). Results are presented as relative percentage referred to the highest level of IFN-γ secretion (NK cells cocultured with M1, set as 100%; mean ± SEM; n = 4). (C) Expression of CD48 on M0, M1, M2, or resting macrophages activated with LPS (M0+LPS) or IFN-γ (M0+IFN-γ; means ± SEM; n = 4). (D) Secretion of IFN-γ by NK cells cocultured with M0 or M1 autologous macrophages either in the absence (isotype control, white bars) or in the presence of a neutralizing mAb against 2B4 (black bars). Results are presented as relative percentages referred to the highest level of IFN-γ secretion (NK cells cocultured with M1; set as 100%; mean ± SEM; n = 4). *p < 0.05, ***p < 0.001, ****p < 0.0001.
in the modulation of innate immunity in the presence of inflammatory insults (15–17), but the underlying mechanisms regulating this interplay are still poorly understood. This study demonstrates that only M1 primed human NK cells, whereas M0 and M2 do not induce NK cell activation and effector functions. This M1-mediated priming increasing NK cell cytolytic potential and IFN-γ production occurs through the engagement of four different pathways working in synergy to ensure the establishment of effective innate immune responses (Fig. 8).

We disclosed two distinct mechanisms with impact on NK cell cytotoxicity, evaluated as CD107a degranulation (35). The first relies on soluble mediators and is sustained by the M1 production of IL-23p19, IFN-β, and IL-1β, which, in turn, induces the up-regulation of NKGD2 and NKP44. The binding of these two aNKRs to their putative ligands on target cells enhances NK cell cytotoxicity. The involvement of type I IFN in regulating the expression of NKGD2 receptors has been previously reported (55), and our results showing the role of M1-secreted IFN-β in increasing the surface levels of NKGD2 extend to a human setting a finding already reported for murine NK cells (45). Interestingly, it has been reported that IL-12 is also able to regulate NKG2D expression on NK cells (46) and that STAT3 is involved in the transcriptional regulation of this aNKR (56). Our data showing that human NK cells express detectable levels of IL-1RI and that IL-1β has biological activities on human NK cells (28, 29, 31). In particular, it has been also reported that IL-1β acts as a synergistic cytokine with IL-12, IL-15, and IL-23 for IFN-γ production (28, 31, 32) and, to the best of our knowledge, this study reports for the first time that IL-1β also increases human NK cell cytotoxicity via the induction of NKP44 expression. Indeed, our results demonstrate that: 1) M1-primed NK cells have a significantly higher expression of IL-1RI compared with their resting counterparts; 2) the incubation of NK cells in vitro with rhIL-1 upregulated NKP44; 3) the masking of IL-1 RI in the context of macrophage polarization (39). These discrepancies underline the importance of taking into account species specificity when investigating cellular interactions and ask for caution before transferring to human immunology results obtained in murine experimental settings.

The second mechanism enhancing the cytolytic activity of NK cells requires cell-to-cell interactions and is mediated by IL-15. It is well-known that IL-15 is a potent inducer of NK effector functions (21, 38) and is endowed with the ability to work as a membrane-bound IL-15-IL-15Rα complex trans-presented in a paracrine fashion to cells expressing IL-15Rβ (22, 23). We previously demonstrated that upregulation of IL-15 and IL-15Rα genes is a hallmark of M1 polarization (39), and consistent with this background, we disclose in this article that IL-15Rα expressed...
FIGURE 8. Macrophage–NK cell interactions. Diagram summarizes the cellular pathways that regulate the cross talk between human NK cells and M1 macrophages. First, polarization to M1 induces CD48 expression on macrophages, whose binding to 2B4 triggers NK cell production of IFN-γ (cellular interaction, upper left). Second, M1 secretion of IFN-β induces NK cell production and surface expression of IL-15 and IL-15Rα, respectively. In turn, the IL-15–IL-15Rα membrane-bound complex is cis-presented in an autocrine loop to the same NK cell expressing the IL-15Rα–IL-15Rγ receptor complex, thus enhancing NK cell production of IFN-γ (soluble interaction, upper right). Third, M1 secretion of IL-1β and IFN-β or IL-23 induces the upregulation of NKp44 and NKG2D, respectively, that, in turn, increases NK cell cytotoxicity against target cells expressing their putative ligands (soluble interaction, lower right). Fourth, the IL-15–IL-15Rα membrane-bound complex expressed by M1 is trans-presented in a paracrine loop to surrounding NK cells expressing the IL-15Rα–IL-15Rγ receptor complex and induces NK cell activation and cytotoxicity (cellular interaction, lower left).

by M1 trans-presents IL-15 to surrounding NK cells, thus enhancing their cytotoxicity properties. Different from IFN-β and IL-1β, which selectively upregulate specific aNKRs, this IL-15 trans-presentation pathway might favor NK cell cytotoxicity by increasing intracellular levels of granzyme B (59).

Our study also provides experimental evidence of two additional pathways involved in the M1-induced NK cell secretion of IFN-γ. The first mechanism relies on the ability of M1-polarized macrophages to synthetize IFN-β (45). We demonstrate that NK cells respond to IFN-β by increasing their surface expression of IL-15Rα and their synthesis of IL-15. This allows autocrine IL-15 cis-presentation on NK cells, which, in turn, enhances their production of IFN-γ. These results are in line with a recent report investigating the DC–NK cell cross talk in the murine setting (26). The second cell contact–dependent mechanism enhancing IFN-γ production by NK cells relies on the activation of the CD48–2B4 pathway (15, 16, 20). We show in this article that this binding occurs only in the presence of M1 polarization. In fact, although NK cells constitutively express high levels of 2B4, M0 and M2 are negative for the expression of its natural ligand CD48. It has been reported that the engagement of the CD48–2B4 pathway induces to the activation of FYNT, which, in turn, induces the NK cell production of IFN-γ (60), thus further supporting our findings showing the importance of the CD48–2B4–IFN-γ axis in the context of NK cell–macrophage cross talk.

The M1-induced NK cell production of IFN-γ likely plays a role in the context of rescuing M2 toward a proinflammatory M1 profile to boost and optimize innate immune responses against infections. Indeed, a defective or aberrant NK cell–M1 cross talk might favor viral or bacterial infections, as reported in HIV-1–infected patients. Indeed, the reported NK cell impairment in the clearance of autologous HIV-1–infected CD4pos T cells, in the killing of cell targets either tumor-transformed or infected by opportunistic pathogens, in the production of antiviral cytokines/chemokines (including IFN-γ), and in the interaction with autologous DCs explain, at least in part, HIV-1 escape from innate immune responses of the host (5). These pathologic NK cell effector functions are directly associated with the expansion of a highly anergic CD56neg/CD16pos (CD56neg) NK cell subset, whose frequency is very low in healthy individuals (35, 37). Moreover, the presence of these aberrant CD56neg NK cells is also paralleled by a preferential expansion of M2 in AIDS patients (61). In contrast, macrophages are susceptible to HIV-1 infection and represent an important reservoir for the virus (61, 62). Hence it is conceivable to hypothesize that the physiopathology of NK cells and macrophages in HIV-1 infection is associated with a dysfunctional M1–NK cell cross talk. This study sheds new light on the mechanisms required for an effective NK cell–M1 interplay, and therefore paves the groundwork to better understanding and breaks this vicious cycle to overcome the defective host immune responses against HIV-1.

Both NK cells and macrophages are also present in tumor stroma and are naturally endowed with antitumoral activities (1, 9). However, under the effect of tumor-derived signals, macrophages acquire a protumoral M2-like functional profile, which not only develops protumoral functions (63) but also, as shown in this study, compromises their priming effects on NK cells. Thus, acting on TAMs, the tumor microenvironment interferes with the antitumoral potential of both cell types. Several approaches have been proposed to target TAMs therapeutically, including blocking their homing to tumors (64) or depleting them in situ (65). As recently demonstrated with CD40 agonists in pancreatic cancer (66), TAM reprogramming to M1-like phenotypes represents a promising approach that not only detracts the tumor from its protumoral activities but also converts them into efficient antitumor
cell mediators (67). Our results indicate that the ability of M1 to engage a positive cross talk with NK cells improves NK cytolytic potential and increases IFN-γ production, which would feedback on macrophages and potentiate their antitumoral activity (Fig. 8). On this important matter, we also demonstrate in this article that M1-primed NK cells are able to downmodulate the expression of protumoral genes (M2 markers) and to increase the expression of antitumoral genes (M1 markers) in M2. Interestingly, the conditioned media from M1-primed NK cells induced in M2 the expression of inflammatory genes (IL-15, IL-15Rα, IL-1β), which, in turn, sustain NK cell activation. This suggests that therapeutic approaches able to re-educate TAM to M1-like macrophages may also take advantage of this positive feed-forward loop with NK cells, which should, in principle, result in setting in motion a powerful antitumoral immune mechanism.

In summary, this study describes a complex network of cellular pathways that regulate, in the context of a human autologous setting, cellular and soluble pathways operated by macrophages to efficiently prime resting NK cells. In particular, we disclose in this article cytokine (IL-15, IL-1β, IFN-β) and membrane receptor pathways (CD48-2B4) working in synergy to ensure appropriate lar pathways that regulate, in the context of a human autologous approaches able to re-educate TAM to M1-like macrophages may

Disclosures

The authors have no financial conflicts of interest.

References