European Journal of Immunology

IL-27 stimulates human NK-cell effector functions and primes NK cells for IL-18 responsiveness

Andrea Ziblat[1,2], Carolina I. Domaica[1], Raúl G. Spallanzani[1], Ximena L. Raffo Iraolagoitia[1], Lucas E. Rossi[1], Damián E. Avila[1,3], Nicolás I. Torres[1,4], Mercedes B. Fuertes[1,4] and Norberto W. Zwirner[1,2]

[1] Laboratorio de Fisiopatología de la Inmunidad Innata, Instituto de Biología y Medicina Experimental (IBYME), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina
[2] Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina
[3] Cátedra de Inmunología, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina
[4] Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

IL-27, a member of the IL-12 family of cytokines, is produced by APCs, and displays pro- and anti-inflammatory effects. How IL-27 affects human NK cells still remains unknown. In this study, we observed that mature DCs secreted IL-27 and that blockade of IL-27R (CD130) reduced the amount of IFN-γ produced by NK cells during their coculture, showing the importance of IL-27 during DC–NK-cell crosstalk. Accordingly, human rIL-27 stimulated IFN-γ secretion by NK cells in a STAT1-dependent manner, induced upregulation of CD25 and CD69 on NK cells, and displayed a synergistic effect with IL-18. Preincubation experiments demonstrated that IL-27 primed NK cells for IL-18-induced IFN-γ secretion, which was associated with an IL-27-driven upregulation of T-bet expression. Also, IL-27 triggered NKp46-dependent NK-cell-mediated cytotoxicity against Raji, T-47D, and HCT116 cells, and IL-18 enhanced this cytotoxic response. Such NK-cell-mediated cytotoxicity involved upregulation of perforin, granule exocytosis, and TRAIL-mediated cytotoxicity but not Fas-FasL interaction. Moreover, IL-27 also potentiated Ab-dependent cell-mediated cytotoxicity against mAb-coated target cells. Taken together, IL-27 stimulates NK-cell effector functions, which might be relevant in different physiological and pathological situations.

Keywords: Cytotoxicity · IFN-γ · IL-18 · IL-27 · NK cells

Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

NK cells, through the secretion of IFN-γ and other proinflammatory cytokines and a cytotoxic response, are critical effector cells during immunity against viruses and tumors [1]. Such functions are tightly regulated during their interaction with myeloid DCs during which NK cells promote maturation of DCs and become activated by cell surface receptors and cytokines such as IL-12, IL-15, and IL-18 [2, 3]. NK cells are also activated upon recognition of PAMPs through TLRs [4] or tumor cells through different activating receptors [5, 6].

Correspondence: Dr. Norberto W. Zwirner
e-mail: nzwirner@ibyme.conicet.gov.ar

While IL-12 plays a major role during NK-cell activation, IL-18 primes NK cells for IL-12 responsiveness [7]. IL-12 belongs to a family of cytokines that also includes IL-23, IL-27, and IL-35 [8, 9]. In particular, IL-27 is a heterodimeric cytokine composed by EBI3 and p28, signals through the IL-27R composed by WSX-1 and CD130/gp130 [8, 10], is produced by APCs upon stimulation through TLRs [11, 12], and displays pro- and anti-inflammatory functions due to the activation of STAT1 and STAT3, respectively [9]. Its proinflammatory effects depend on the induction of T-bet and IL-12Rβ2 expression [13–15].

Although the effect of IL-27 on T cells has been characterized [10, 16–20], its effects on NK cells remain ill-defined. In mice, IL-27 stimulates NK-cell-mediated tumor immunity [21–25] but in humans, a single report has been published where it was observed that IL-27 increases viability and inhibits NK-cell proliferation in response to IL-12 plus IL-15 [26]. As IL-27 is produced by DCs and its closely related cytokine IL-12 has a dramatic impact on NK-cell effector functions, we hypothesized that IL-27, alone or in concert with other DC-derived cytokines, may regulate human NK-cell effector functions. Therefore, our aim was to investigate the effect of IL-27 on human NK cells. We observed that mature DCs (mDCs) secreted IL-27, which contributed to NK-cell-mediated IFN-γ secretion. IL-27 directly induced activation of NK cells, promoted IFN-γ secretion, and NK-cell-mediated cytotoxicity through NKp46- and CD16-dependent mechanisms via granule exocytosis and TRAIL-mediated cytotoxicity. Also, IL-27 primed NK cells for IL-18-mediated IFN-γ secretion, which involved upregulated T-bet expression. Therefore, our results indicate that IL-27 directly activates human NK cells and that this cytokine constitutes a novel player of the intricate DC–NK-cell crosstalk.

Results

mDCs produce IL-27 that stimulates NK-cell effector functions

First, we investigated whether IL-27 participates in the crosstalk between DCs and NK cells. We observed that mDCs secreted IL-27 (Fig. 1A) that stimulated IFN-γ secretion by NK cells as coculture with mDCs in the presence of anti-CD130-blocking mAb induced significantly less IFN-γ than the isotype-matched negative control mAb (IC mAb) (Fig. 1B). As CD130 is shared by the IL-6R and IL-27R, we addressed whether NK cells directly respond to IL-27. We observed that human NK cells stimulated with IL-27 secreted increased amounts of IFN-γ, assessed by ELISA (Fig. 1C) but not IL-4, IL-10, IL-17, IL-6, and TNF, assessed by cytokine bead array (CBA) and flow cytometry (FC, data not shown). Pharmacologic inhibition revealed that MEK1/MEK2, JNK, PI3K, mTOR, and NF-κB but not p38 MAPK or Jak2 were involved in IFN-γ production by NK cells stimulated with IL-27 (Fig. 1D). STAT1 was also involved in this response as fludarabine [27] strongly reduced the amount of IFN-γ secreted by NK cells stimulated with IL-27 (Fig. 1E).

IL-27 promoted NK-cell activation as it induced upregulated expression of CD25 (Fig. 1F and G) and CD69 (Fig. 1H and I). In addition, NK cells stimulated with IL-27 secreted higher amounts of IFN-γ than resting NK cells when restimulated with IL-2, suggesting that the upregulated expression of CD25 driven by IL-27 has functional consequences (Fig. 1J). IL-27 also triggered NK-cell-mediated cytotoxicity against NK-cell-resistant Raji (Fig. 2A), T-47D (Fig. 2B), and HCT116 (Fig. 2C) cells, which was associated with a significant upregulation of NKp46 (Fig. 2D and E). Conversely, IL-27 did not affect the expression of CD16, NKG2D, NKp30, DNAM-1, NKG2A, CD158a,h (KIR2DL1/KIR2DS1), and CD158b1,b2 (KIR2DL2/KIR2DL3) (data not shown). Accordingly, such increased cytotoxic response was predominantly NKp46-dependent (Fig. 2F–H, for Raji, T-47D, and HCT116 cells, respectively). Also, IL-27-driven upregulation of NKp46 and heightened NK-cell-mediated cytotoxicity required functional mTOR and NF-κB but not MEK1/MEK2, JNK, p38 MAP kinase, Jak2, or PI3K as they were inhibited only by rapamycin and BAY11–7082 (Fig. 2I and J).

Therapeutic efficacy of humanized mAbs against tumors relies partially on Ab-dependent-cell-mediated cytotoxicity (ADCC). Remarkably, rituximab (RTX) coated Raji cells, trastuzumab (TRZ) coated T-47D cells, and cetuximab (CTX) coated HCT116 cells were susceptible to NK-cell-mediated cytotoxicity and IL-27 further increased such susceptibility in a statistically significant manner (Fig. 3A–C). The background cytotoxicity of NK cells previously stimulated with IL-27 detected in the presence of the IC mAb was due to NKp46-mediated lysis of target cells as ADCC performed with RTX-coated Raji cells in the presence of a blocking anti-NKp46 mAb reduced such background cytotoxicity but barely affected the specific cytotoxicity induced by RXT (Fig. 3D).

When we investigated the mechanisms responsible for the IL-27-driven NK-cell-mediated cytotoxicity, we observed that IL-27 increased the percentage of degranulating NK cells, assessed as CD107a expression (Fig. 4A and B), promoted upregulation of perforin (pfp) (Fig. 4C and D), and a rise in NK cells expressing cell surface TRAIL (Fig. 4E and F). Moreover, concanamycin A (CCA, [28]) inhibited cytotoxicity of NK cells stimulated with IL-27 against Raji (Fig. 4G) and T-47D (Fig. 4H) cells, confirming the involvement of the secretory pathway. Also, blockade of TRAIL inhibited the cytotoxicity of NK cells stimulated with IL-27 against Raji (Fig. 4I) but not T-47D (Fig. 4J) cells, while blockade of FasL had no effect on cytotoxicity triggered by NK cells (stimulated or not with IL-27) against Raji or T-47D cells (Fig. 4K and L, respectively). These results suggest that IL-27 enhanced the ability of NK cells to trigger cytotoxicity through the secretory pathway and TRAIL.

IL-27 primes NK cells for IL-18-induced IFN-γ production

As mDCs produce IL-27, which regulates NK-cell effector functions, and also produce IL-18 which, in mice, primes NK cells for IL-12 responsiveness [7], we investigated whether IL-27 cooperates with IL-18 for NK-cell stimulation. We observed that simultaneous stimulation of NK cells with both cytokines induced

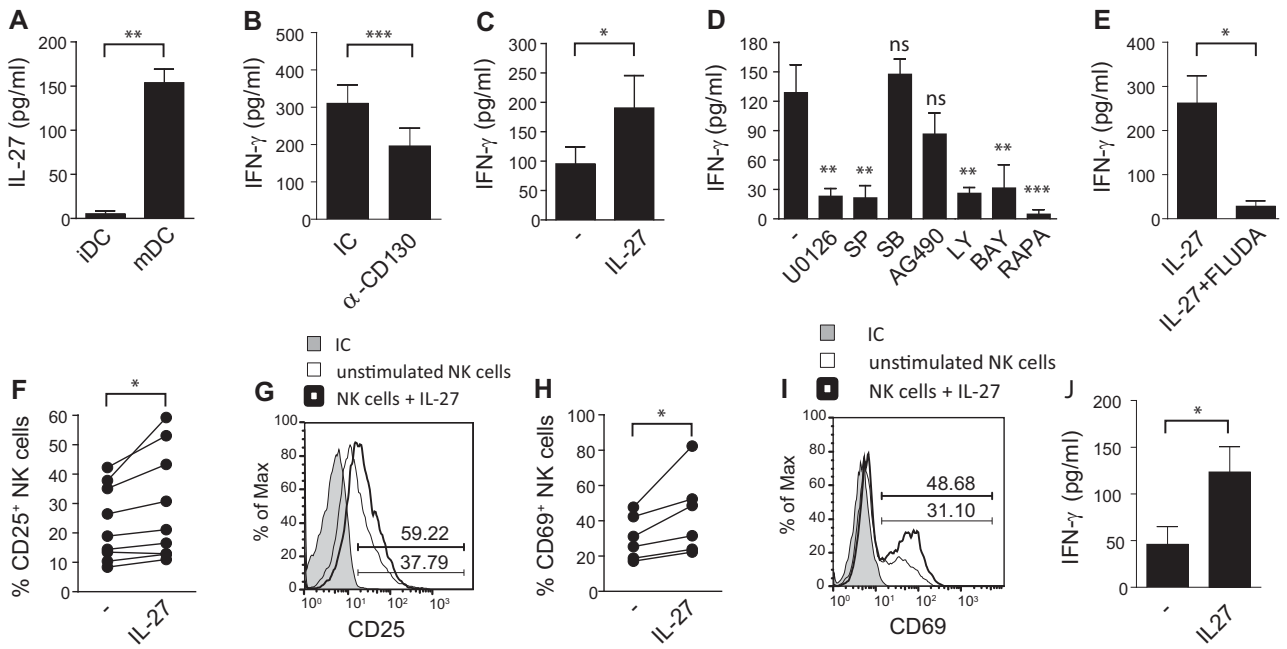

Figure 1. DC-derived IL-27 activates NK cells and stimulates IFN-γ secretion. (A) IL-27 secretion by human monocyte-derived iDCs and mDCs. (B) IFN-γ secretion by NK cells upon coculture for 24 h with mDCs in the presence of an IC mAb or a blocking mAb against CD130 (α-CD130). (A, B) $N = 6$ donors. (C) IFN-γ secretion by NK cells cultured for 24 h in the absence (–) or in the presence of 10 ng/mL of IL-27. $N = 13$ donors. (D) IFN-γ secretion by NK cells stimulated for 24 h with 10 ng/mL of IL-27 in the absence (–) or in the presence of U0126, SP600125 (SP), SB202190 (SB), AG490, Ly294002 (Ly), BAY11–7082 (BAY), or rapamycin (RAPA). $N = 3$ donors. (E) IFN-γ secretion by NK cells stimulated for 24 h with 10 ng/mL of IL-27 in the absence (–) or in the presence of fludarabine (FLUDA). $N = 4$ donors. (F–I) Percentage of NK cells expressing (F, G) CD25 or (H, I) CD69 after 5 or 3 days of culture in the absence (–) or in the presence of 10 ng/mL of IL-27, respectively, determined by FC. (F) $N = 9$ donors, (H) $N = 6$ donors. (G, I) Representative histograms. Gray: IC. Thin line: unstimulated NK cells. Thick line: NK cells stimulated with IL-27. Numbers within histograms: percentage of positive cells for each marker in each condition. (J) IFN-γ secretion by NK cells cultured for 5 days in the absence (–) or in the presence of 10 ng/mL of IL-27 and thereafter restimulated 24 h with 8 ng/mL of IL-2. $N = 6$ donors. (A–E and J) IL-27 and IFN-γ levels were determined by ELISA. Data are shown as mean + SEM, and are cumulative of at least three independent experiments performed with cells from at least three different donors. ns, no significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; paired *t*-test (A–C, E, F, H, and J); one-way ANOVA test with Dunnett's post-hoc test (D).

a significant and synergistic increase in the amounts of IFN-γ secreted by NK cells when compared with IL-18 or IL-27 alone (Fig. 5A). The amounts of IFN-γ detected upon stimulation with IL-18 (a cytokine that when acting alone does not trigger IFN-γ secretion by NK cells) might be due to a cooperative effect with the low amounts of IL-15 added to the cultures as survival factor and required to preactivate NK cells. Nevertheless, the synergistic effect of IL-27 and IL-18 was due to priming of NK cells by IL-27 for IL-18 responsiveness as prestimulation of NK cells with IL-18 induced a minor increase in IFN-γ secretion when NK cells were thereafter stimulated with IL-27, while prestimulation of NK cells with IL-27 and subsequent stimulation with IL-18 induced a significant increase in IFN-γ secretion compared with NK cells that were not pretreated with IL-27 (Fig. 5B). Heightened IFN-γ production was not due to NK-cell proliferation, as IL-27 either in the absence or presence of IL-18 exerted a statistically significant inhibition of NK-cell proliferation triggered by IL-15 (Fig. 5C) without affecting the percentage of terminally differentiated NK cells ($CD57^+$ and $NKG2A^-$ NK cells) or NK-cell viability (data not shown).

Remarkably, IL-27 (alone or in combination with IL-18) induced a significant upregulation of T-bet in NK cells (Fig. 5D and E), suggesting that such upregulation might be involved in the priming of NK cells by IL-27.

IL-27 cooperates with IL-18 to stimulate NK-cell-mediated cytotoxicity

The cooperative effects of IL-27 and IL-18 also impact NK-cell-mediated cytotoxicity (Fig. 6A), as NK cells stimulated with IL-27 and IL-18 exhibited a significant increased cytotoxicity compared with NK cells stimulated individually with each cytokine. As before, cytotoxicity was inhibited by blockade of NKp46 (Fig. 6B), CCA (Fig. 6C), and an anti-TRAIL mAb (Fig. 6D) but not by an anti-FasL mAb (Fig. 6E), indicating the involvement of the secretory pathway and TRAIL but not FasL. Moreover, cytotoxicity of NK cells stimulated with IL-27 and IL-18 was also significantly inhibited upon neutralization of IFN-γ (Fig. 6F), which was associated with a significant reduction in the percentage of $ICAM\text{-}1^+$ Raji (target) cells (Fig. 6G and H) but not in the percentage of $ICAM\text{-}1^+$ NK cells (data not shown). As ICAM-1 is involved in the synapse between target and effector cells, we assessed the percentage of NK cells stimulated with IL-27 and IL-18 forming conjugates with target cells (Fig. 6I). We observed that such values were significantly reduced by the anti-IFN-γ-blocking mAb and by an anti-ICAM-1-blocking mAb, suggesting that both actively contribute to the formation of conjugates between target and effector cells. Thus, NK-cell-derived IFN-γ triggers upregulation of ICAM-1

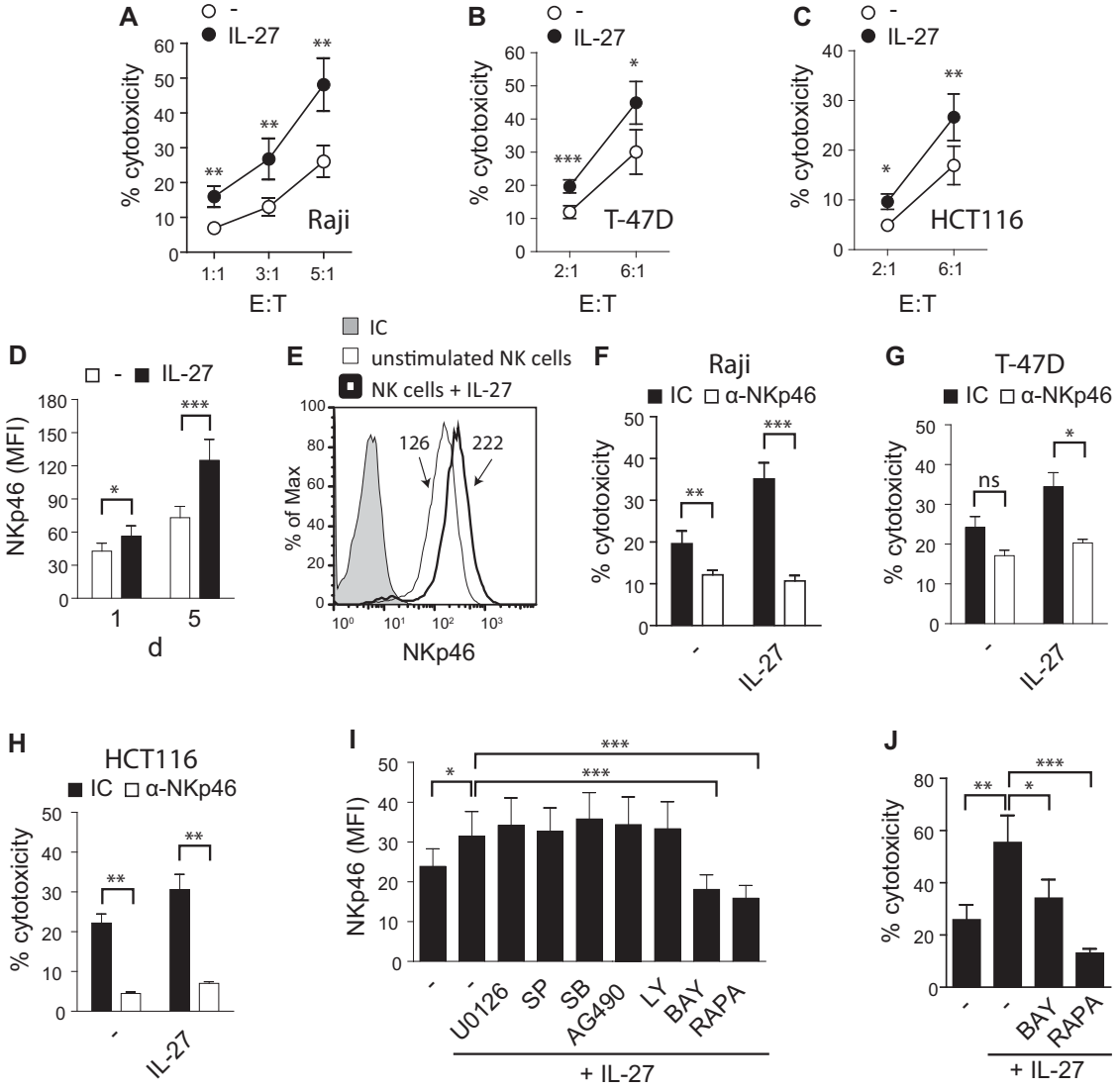

Figure 2. IL-27 stimulates NKp46-dependent NK-cell-mediated cytotoxicity. (A–C) Cytotoxic activity of NK cells previously cultured overnight in the absence (○) or in the presence of 10 ng/mL of IL-27 (●) against (A) Raji, (B) T-47D, and (C) HCT116 cells at different E:T. $N \geq 6$ donors. Cytotoxicity determined by FC as explained in the Materials and methods. (D) Expression of NKp46 on NK cells cultured in the absence or in the presence of 10 ng/mL of IL-27 for 1 or 5 days, determined by FC. $N = 8$ donors. (E) Representative histograms of NKp46 expression at day 5. Gray: IC. Thin line: unstimulated NK cells. Thick line: NK cells stimulated with IL-27. Numbers within histograms represent the MFI of each condition. (F–H) Cytotoxic activity of NK cells cultured overnight in the absence (–) or in the presence of 10 ng/mL of IL-27 against (F) Raji, (G) T-47D, or (H) HCT116 cells in the presence of a blocking mAb against NKp46 (white bars) or an IC mAb (black bars). E:T ratio: (F) 5:1 and (G, H) 6:1. (I) Expression of NKp46 on NK cells cultured for 24 h in the absence (–) or in the presence of 10 ng/mL of IL-27, in the absence (–) or in the presence of U0126, SP600125 (SP), SB202190 (SB), AG490, Ly294002 (Ly), BAY11–7082 (BAY), or rapamycin (RAPA). (J) Cytotoxicity of NK cells stimulated for 24 h with 10 ng/mL of IL-27 against Raji cells, in the absence (–) or in the presence of BAY11–7082 (BAY) or rapamycin (RAPA). E:T: 5:1. $N = 4$ donors. (F–J). Data are shown as mean ± SEM, and are cumulative of at least three independent experiments performed with cells from at least three different donors. ns, no significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; paired *t*-test for each E:T ratio (A–C); two-way ANOVA with repeated measures matched by both factors and Bonferroni post-hoc test (D, F–H); one-way ANOVA test with Dunnett's post-hoc test (I and J).

and contributes to the formation of an increased percentage of conjugates between target and effector cells, and to the cytotoxic response, as has been reported [29]. Overall, the enhanced NK-cell-mediated cytotoxicity triggered by the cooperative action of IL-27 and IL-18 involves the secretory pathway and TRAIL, and the induction of an IFN-γ-dependent increase in the percentage of ICAM-1^{+} target cells.

Discussion

IL-27, a cytokine that belongs to the IL-12 family of cytokines, displays pro- and anti-inflammatory functions [8, 9, 30–33]. It is mostly produced by APCs upon activation stimuli [11, 12, 34, 35] and it has been suggested that while IL-27 induced by TLR agonists may contribute to the acute inflammatory response against

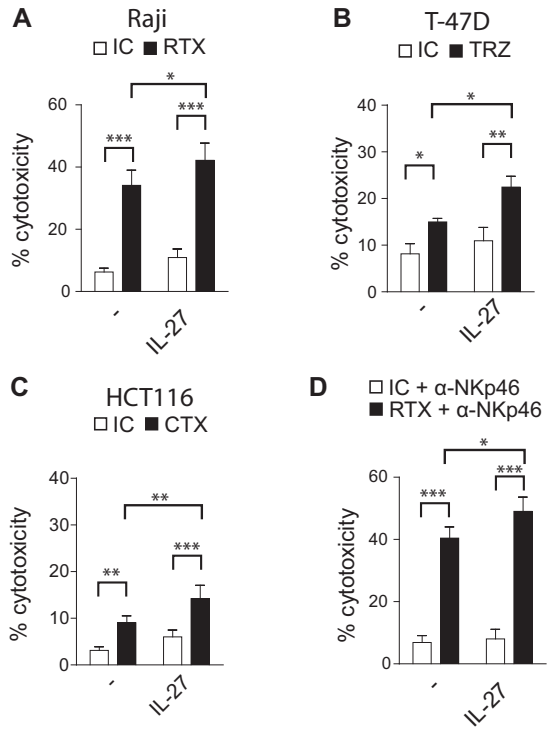

Figure 3. IL-27 stimulates ADCC by NK cells. (A–C) ADCC of NK cells (−) or NK cells previously stimulated overnight with 10 ng/mL of IL-27 against (A) Raji cells incubated with an IC mAb (white bars) or against RTX-coated Raji cells (black bars), (B) T-47D cells incubated with an IC mAb (white bars) or against TRZ-coated T-47D cells (black bars), or (C) HCT116 cells incubated with an IC mAb (white bars) or against CTX-coated HCT116 cells (black bars). (D) ADCC of NK cells against Raji cells incubated with an IC mAb (white bars) or against RTX-coated Raji cells (black bars) using NK cells preincubated with an anti-NKp46-blocking mAb to prevent NKp46-mediated cytotoxicity. E:T: 1:1. Percentage of cytotoxicity was determined by FC as explained in the Materials and methods. Data are shown as mean ± SEM of $N = 7$ (A, D), $N = 3$ (B), and $N = 5$ (C) donors and are cumulative of at least three independent experiments performed with cells from at least three different donors. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; two-way ANOVA with repeated measures matched by both factors and Bonferroni post-hoc test.

pathogens, at later stages, IL-27 may display anti-inflammatory functions on macrophages that may contribute to the attenuation of the inflammatory response [36]. Notably, some chronic inflammatory conditions in humans are accompanied by increased concentration of IL-27 [37] or the presence of high amounts of IL-27 transcripts [38], indicating a possible pathophysiological role. Accordingly, IL-27 has been shown to display proinflammatory effects on human DCs and macrophages [39]. Also, IL-27 has been shown to contribute to tumor immunity [40]. In mice, IL-27 stimulates survival of tumor-specific $CD8^+$ T cells and tumor rejection [41], shapes the tumor microenvironment and indirectly stimulates NK and NKT cells via stimulation of IL-12 secretion by DCs [25], and promotes increased NK-cell-mediated cytotoxicity without involving IFN-γ [21, 23]. Nonetheless, the effects of IL-27 on human NK cells remain poorly defined.

In this work, we demonstrated that DCs secreted IL-27 in response to TLR4 stimulation and that blockade of CD130 reduced the amount of IFN-γ secreted by NK cells cocultured with mDCs. The partial inhibition of IFN-γ secretion in this setting could be due to the secretion of other NK-cell-stimulating cytokines such as IL-12, IL-15, and/or IL-18 by mDCs, together with the engagement of NK-cell-activating receptors such as NKp30 [42] and DNAM-1 [43]. Accordingly, IL-27 directly triggered IFN-γ secretion without affecting the production of other cytokines such as IL-4, IL-10, IL-17, IL-6, and TNF in NK cells. Of note, we added IL-15 (1 ng/mL) to the cultures as survival factor and due to the fact that IL-15 is also necessary to preactivate NK cells [44]. It was reported that IL-27 enhanced the transcription of IFN-γ mRNA in human NK cells stimulated with IL-12 plus high concentrations of IL-15 but not by IL-27 alone [26]. Conversely, we observed that IL-27 in the presence of suboptimal amounts of IL-15 stimulated the secretion of IFN-γ, which required functional MEK1/MEK2, JNK, PI3K, mTOR, and NF-κB. Some of them have been involved in the development of NK-cell effector functions [4, 45–47]. Moreover, IL-27 triggers activation of Jak2, STAT1, and STAT3 in some immune cells [13–15, 48], but we observed that STAT1 but not STAT3 (a downstream mediator of Jak2) was involved in IFN-γ production by NK cells stimulated with IL-27.

In addition, IL-27 also induced the expression of CD25 and CD69, confirming that this cytokine activates NK cells. However, IL-27 also exerted an antiproliferative effect on NK cells that was not accompanied by an accumulation of terminally differentiated NK cells or changes in NK-cell viability. The upregulated expression of CD25 was accompanied by an increased secretion of IFN-γ when these cells were subsequently stimulated with IL-2, indicating that IL-27-driven activation of NK cells has functional consequences.

IL-27 also promoted upregulation of NKp46 and NKp46-dependent NK-cell-mediated cytotoxicity against different target cells including Raji cells, which are naturally resistant due to HLA class I expression and KIR engagement [49]. Therefore, through upregulation of NKp46, IL-27 can overcome resistance to NK-cell-mediated cytotoxicity. It has been observed that NK-cell-mediated cytotoxicity requires mTOR [50] and NF-κB [51]. Accordingly, we observed that the cytotoxic response of NK cells triggered by IL-27 against Raji cells required functional mTOR and NF-κB at least in part because they regulate IL-27-induced upregulation of NKp46. In addition, we also established that the cytotoxic response of NK cells exposed to IL-27 involves the secretory pathway and TRAIL, which was associated with an upregulated expression of pfp and TRAIL. We did not observe redundancy in the cytotoxicity experiments against Raji cells performed with CCA and the anti-TRAIL-blocking mAb because CCA inhibits the exocytic pathway, which includes pfp release and mobilization of TRAIL to the cell surface as TRAIL is stored in granules in NK cells [52]. Moreover, susceptibility of T-47D cells (which are resistant to TRAIL-mediated apoptosis [53]) to cytotoxicity of NK cells stimulated with IL-27 and its inhibition by CCA might indicate that IL-27 triggers pfp release and cell surface mobilization of TRAIL, and that apoptosis induction by one, the other, or both mechanisms depends on the particular susceptibility of the target cell. Accordingly, Raji cells

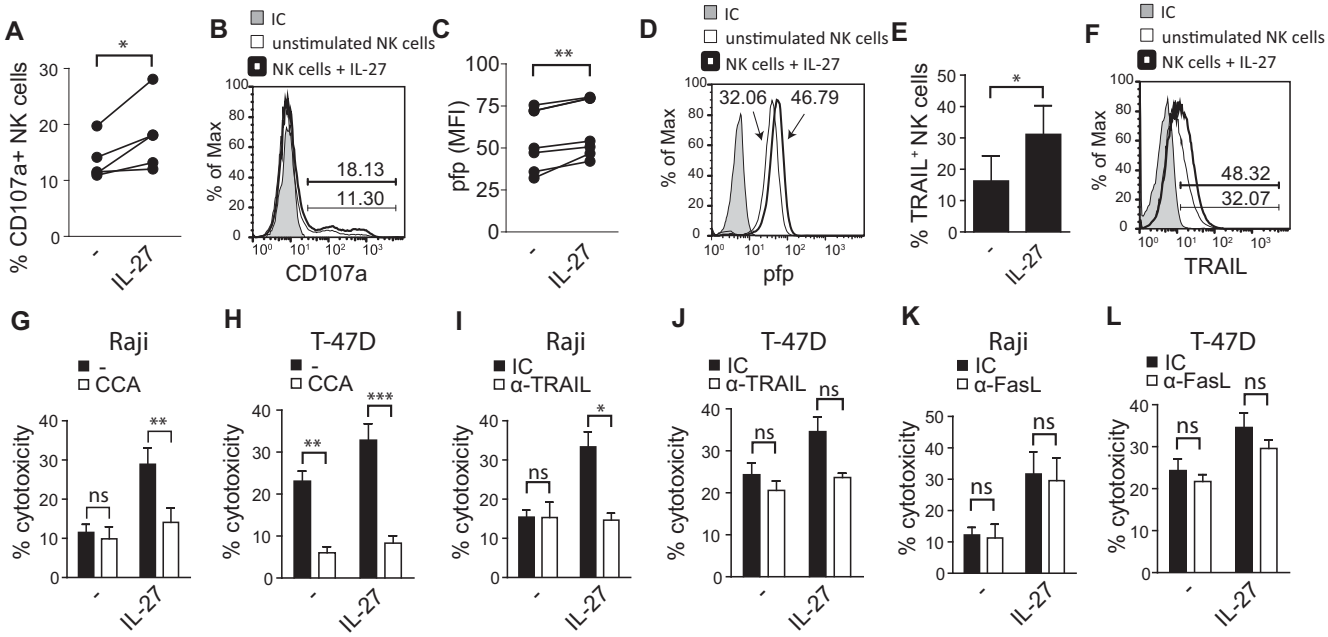

Figure 4. IL-27-driven cytotoxicity in NK cells involves the secretory pathway and TRAIL. (A) Degranulation of NK cells previously cultured overnight in the absence (–) or in the presence of 10 ng/mL of IL-27, after coculture with Raji cells, determined by FC of CD107a. E:T: 1:3. $N = 5$ donors. (B) Representative histograms. Gray: IC. Thin line: unstimulated NK cells. Thick line: NK cells stimulated with IL-27. Numbers within histograms: percentage of CD107a$^+$ NK cells in each condition. (C) pfp expression in NK cells cultured overnight in the absence (–) or in the presence of 10 ng/mL of IL-27, determined by FC. $N = 6$ donors. (D) Representative histograms. Gray: IC. Thin line: unstimulated NK cells. Thick line: NK cells stimulated with IL-27. Numbers within histograms: MFI for pfp expression in each condition. (E) Percentage of TRAIL$^+$ NK cells after an overnight culture in the absence (–) or in the presence of 10 ng/mL of IL-27, determined by FC. $N = 3$ donors. (F) Representative histograms. Gray: IC. Thin line: unstimulated NK cells. Thick line: NK cells stimulated with IL-27. Numbers within histograms: percentage of TRAIL$^+$ NK cells in each condition. (G, H) Cytotoxic activity of NK cells previously cultured overnight in the absence (–) or in the presence of 10 ng/mL of IL-27 against (G) Raji (E:T: 5:1, $N = 6$ donors) or (H) T-47D (E:T: 6:1, $N = 5$ donors) cells, in the absence (black bars) or in the presence of CCA (white bars). (I–L) Cytotoxic activity of NK cells cultured overnight in the absence (–) or in the presence of 10 ng/mL of IL-27 against (I, K) Raji (E:T: 5:1, $N = 5$ donors and $N = 4$ donors, respectively) or against (J, L) T-47D (E:T: 6:1, $N = 5$ donors) cells, in the presence of an IC mAb (black bars) or in the presence of an anti-TRAIL-blocking mAb (white bars, I and J) or an anti-FasL-blocking mAb (white bars, K and L). (G–L) Percentage of cytotoxicity was determined by FC as explained in the Materials and methods. Data are shown as mean ± SEM, and are cumulative of at least three independent experiments performed with cells from at least three different donors. ns, no significant; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; paired *t*-test (A, C, and E); two-way ANOVA with repeated measures matched by both factors and Bonferroni post-hoc test (G–L).

would be killed mainly via TRAIL (as cytotoxicity was inhibited by CCA and by the anti-TRAIL mAb), while T-47D would be killed mainly via pfp (as cytotoxicity was inhibited by CCA but not by the anti-TRAIL mAb).

Moreover, IL-27 potentiated ADCC induced by RTX, TRZ, and CTX against Raji, T-47D, and HCT116 cells, respectively, suggesting that IL-27 may be helpful as adjuvant during therapy with humanized mAb.

The described stimulatory effects of IL-27 may occur when APCs secrete this cytokine during their crosstalk with NK cells. As IL-18 is secreted at the synaptic cleft between NK cells and DCs [54] and primes NK cells for IL-12 [7], we addressed whether IL-18 and IL-27 cooperate for NK-cell stimulation. We observed that both cytokines exerted a cooperative effect on IFN-γ production and NK-cell-mediated cytotoxicity. Indeed, IL-27 primed NK cells for IL-18-induced IFN-γ secretion, and the underlying mechanism likely involves upregulation of T-bet, a critical transcription factor that regulates IFN-γ production in T and NK cells [55, 56] because it stimulates IFN-γ gene transcription [57]. Accordingly, the proinflammatory effects of IL-27 have been associated to its capacity to induce T-bet expression [13–15].

The cooperative effect of IL-27 and IL-18 on NK-cell-mediated cytotoxicity not only involved the secretory pathway and TRAIL, as IL-27 alone, but also IFN-γ. Secretion of this cytokine during effector target cell contact increased the percentage of ICAM-1$^+$ target cells and in this way, IFN-γ facilitated the formation of conjugates between NK cells and target cells, and delivery of the cytotoxic hit as has been demonstrated previously [29].

In summary, we demonstrated that IL-27, a cytokine of the IL-12 family that is secreted by mDCs, directly activates NK cells, triggers IFN-γ secretion, primes them for enhanced IL-18-driven IFN-γ secretion, promotes NK-cell-mediated cytotoxicity through NKp46, and increases ADCC. Therefore, IL-27 produced by APCs stimulates NK-cell effector functions, which might be relevant in different physiological and pathological situations. In addition, IL-27 may emerge as a cytokine that may result useful as adjuvant therapy to elicit improved NK-cell-mediated immunity against tumors or viral infections.

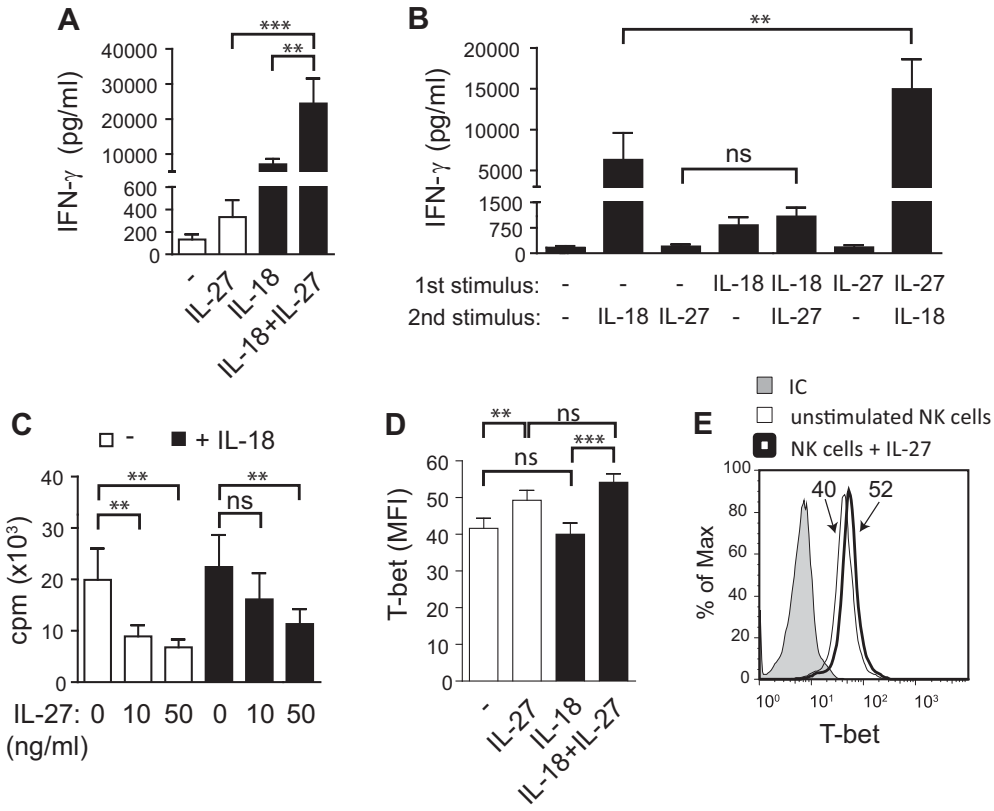

Figure 5. IL-27 primes NK cells for IL-18-driven IFN-γ secretion upregulating T-bet expression. (A) IFN-γ secretion by NK cells cultured for 24 h in the absence (–) or in the presence of 10 ng/mL of IL-27, 10 ng/mL of IL-18, or 10 ng/mL of IL-27 and 10 ng/mL of IL-18 (IL-18 + IL-27). $N = 14$ donors. (B) IFN-γ secretion by NK cells cultured overnight in the absence (–) or in the presence of 10 ng/mL of IL-27 or 10 ng/mL of IL-18 (first stimulus), washed, and thereafter incubated for 24 h in the absence (–) or in the presence of 10 ng/mL of IL-27 or 10 ng/mL of IL-18 (second stimulus). $N = 7$ donors. IFN-γ levels were determined by ELISA. (C) Proliferation of NK cells incubated with 1 ng/mL of IL-15 and the indicated doses of IL-27 in the absence (white bars) or in the presence of 10 ng/mL of IL-18 (black bars) for 5 days. $N = 6$ donors, as determined by ^{3}H-thymidine incorporation. (D) Expression of T-bet in NK cells cultured overnight in the absence (–) or in the presence of 10 ng/mL of IL-27, 10 ng/mL of IL-18, or 10 ng/mL of IL-27 and 10 ng/mL of IL-18 (IL-18 + IL-27), determined by FC. $N = 4$ donors. (E) Representative histograms (only data of IL-27 are shown). Gray: IC. Thin line: unstimulated NK cells. Thick line: NK cells stimulated with IL-27. Numbers within histograms represent the MFI of T-bet in each condition. (A–D) Data are shown as mean ± SEM, and are cumulative of at least three independent experiments performed with cells from at least three different donors. ns, no significant; $^{**}p < 0.01$; $^{***}p < 0.001$; one-way ANOVA with repeated measures and Bonferroni post-hoc test (A–D).

Materials and methods

Antibodies and reagents

Human rIL-2 and rIL-15 (PeproTech), rIL-18 (MBL International), rIL-27 (eBioscience), rGM-CSF (Sigma), and rIL-4 (R&D) were used. The following mAb against human molecules were used: anti-NKp30 (AZ20) and anti-NKp46 (BAB281 and KL247) (kindly provided by Dr. Alessandro Moretta, University of Genoa, Italy); PE-anti-NKG2D (1D11), PE-anti-CD25 (BC96), PE-anti-CD54 (ICAM-1, HA58), PE-anti-T-bet (4B10), PE anti-CD1a (HI149), and unlabeled anti-CD253 (TRAIL, RIK-2) were from eBioscience; PE-anti-NKp46 (9E2), PE-anti-CD3 (UCHT-1), Alexa488-anti-pfp (dG9), PE-anti-IFN-γ (4S.B3), FITC-anti-CD57 (HCD57), unlabeled anti-CD178 (FasL), FITC anti-CD14 (HCD14), FITC anti-HLA-DR (L243), PE anti-CD86 (IT2.2), FITC anti-CD83 (HB15e), unlabeled anti-IFN-γ (NIB42), fluorochrome-labeled and unlabeled IC were from Biolegend; FITC-anti-CD226 (DNAM-1, DX11) and FITC-anti-CD69 (FN50) were from BD; allophycocyanin- or PE/Cy7-anti-CD56 (N901), PE-anti-CD158a,h (EB6.B), and anti-CD158b1,b2 (GL183) were from Beckman Coulter; unlabeled anti-CD130 (gp130, B-R3) and anti-CD54 (1A29) were from Abcam. RTX and TRZ (Roche) were used at 10 μg/mL, CTX (Merck) was used at 1 μg/mL, and normal human polyclonal IgG (IgG2500, Purissimus, Argentina) was used at 1 or 10 μg/mL. CCA (Sigma) was used at 100 nM; the inhibitor of JNK SP600125 (Calbiochem) was used at 20 μM; the Jak2 inhibitor AG490 (Calbiochem) was used at 25 μM; the p38 MAPK inhibitor SB202190 (Calbiochem) was used at 10 μM; the inhibitor of PI3K Ly294002 (Sigma) was used at 2 μM; the inhibitor of MEK1/MEK2 kinases (MAPKs) U0126 (Sigma) was used at 5 μM; the inhibitor of cytokine-induced IκBα phosphorylation BAY11–7082 (Sigma) was used at 1 μM; the inhibitor of the mammalian target of rapamycin (mTOR) rapamycin (Sigma) was used at 5 nM; fludarabine (Fludara®, Schering) was used at 0.1 μg/mL. LPS (*Escherichia coli* 0111:B4 strain) was from Sigma.

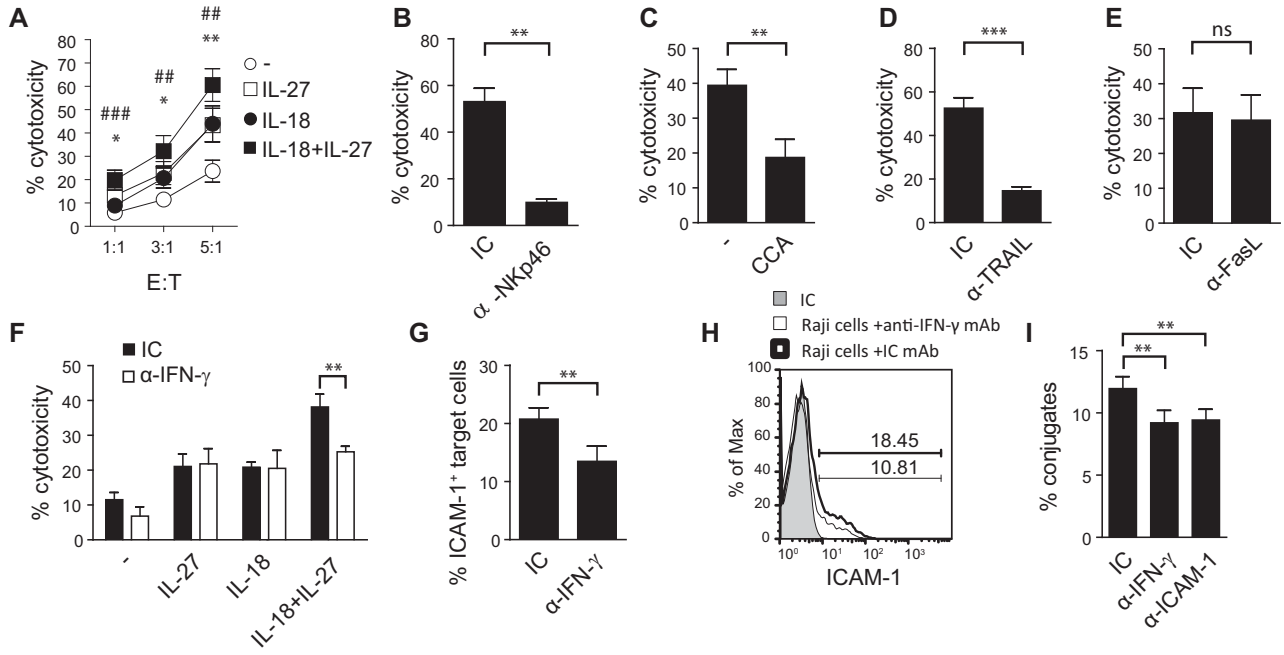

Figure 6. IL-27 cooperates with IL-18 to stimulate NK-cell-mediated cytotoxicity. (A) Cytotoxic activity of NK cells previously cultured overnight in the absence (○) or in the presence of 10 ng/mL of IL-27 (□), 10 ng/mL of IL-18 (●), or 10 ng/mL of IL-27 and 10 ng/mL of IL-18 (■) against Raji cells at different E:T. $N \geq 7$ donors. Asterisk (*) was used to compare cytotoxicity of NK cells treated with IL-18 and IL-27 versus NK cells with IL-27 alone and "#" was used to compare cytotoxicity of NK cells treated with IL-18 and IL-27 versus NK cells treated with IL-18 alone. (B–E) Cytotoxic activity of NK cells previously stimulated overnight with 10 ng/mL of IL-27 and 10 ng/mL of IL-18 against Raji cells (B) in the presence of an IC mAb or an anti-NKp46-blocking mAb, (C) in the absence (–) or in the presence of CCA, (D, E) in the presence of an IC mAb, in the presence of an anti-TRAIL-blocking mAb, (D) or in the presence of an anti-FasL-blocking mAb (E). E:T: 5:1. $N = 4$ donors (B); $N = 6$ donors (C); $N = 5$ donors (D); $N = 4$ donors (E). (F) Cytotoxic activity of NK cells previously cultured overnight in the absence (–) or in the presence of 10 ng/mL of IL-27, 10 ng/mL of IL-18, or 10 ng/mL of IL-27 and 10 ng/mL of IL-18 (IL-18 + IL-27), against Raji cells in the presence of an IC mAb (■) or in the presence of a neutralizing anti-IFN-γ mAb (white bars). E:T: 5:1. $N = 5$ donors. (A–F) Percentage of cytotoxicity was determined by FC as explained in the Materials and methods. (G) Percentage of ICAM-1$^+$ Raji cells after coculture with NK cells previously cultured overnight with 10 ng/mL of IL-27 and 10 ng/mL of IL-18, in the presence of an IC mAb or in the presence of a neutralizing anti-IFN-γ mAb, determined by FC. E:T: 5:1. $N = 3$ donors. (H) Representative histograms. Gray: IC. Thin line: Raji cells incubated in the presence of anti-IFN-γ mAb. Thick line: Raji cells incubated in the presence of IC mAb. Numbers within histograms: percentage of ICAM-1$^+$ Raji cells in each condition. (I) Percentage of NK cells stimulated with IL-27 and IL-18 forming conjugates with Raji (target) cells in the presence of an IC mAb, a neutralizing anti-IFN-γ mAb, or a blocking anti-ICAM-1 mAb. $N = 8$ donors. Percentage of NK cells forming conjugates was determined by FC as explained in the Materials and methods. (A–G and I) Data are shown as mean ± SEM, and are cumulative of at least three independent experiments performed with cells from at least three different donors. ns, no significant; * or #, $p < 0.05$; ** or ##, $p < 0.01$; *** or ###, $p < 0.001$; one-way ANOVA with repeated measures and Bonferroni post-hoc test for each E:T ratio (A), paired *t*-test (B–E and G); two-way ANOVA with repeated measures matched by both factors and Bonferroni post-hoc test (F); one-way ANOVA with repeated measures and Bonferroni post-hoc test (I).

DCs and NK cells

Buffy coats from healthy volunteers were provided by the Blood Bank of the "Carlos Durand" Hospital or by the "Complejo Médico Churruca-Visca" (Buenos Aires, Argentina). Monocytes (CD14$^+$ cells) were isolated by MACS (Miltenyi); NK cells were isolated using RosetteSep (StemCell) and Ficoll-Paque^{TM} Plus (GE Life Sciences) centrifugation. Purity of isolated cells was always above 90%, as assessed by FC (CD14$^+$ cells or CD3$^-$CD56$^+$). Monocytes were cultured for 6 days with GM-CSF and IL-4 to obtain immature DCs (iDCs). To obtain mDCs, iDCs were cultured for 8 h with 1 μg/mL of LPS. iDCs were characterized as CD1a$^+$MHC-II^{low}CD83$^{-/low}$CD86$^{-/low}$, while mDCs were characterized as CD1a$^+$MHC-II^{high}CD83^{high}CD86^{high}. DCs were cultured with NK cells (ratio 1:1) for 24 h in the presence of an IC mAb or the anti-CD130-blocking mAb in RPMI 1640 (Sigma) supplemented with 10% inactivated FBS (Invitrogen), sodium pyruvate, glutamine, and gentamicin (Sigma). Cell culture supernatants were collected and used for analysis of IFN-γ production. Also, NK cells were cultured for 24 h or 5 days in the absence or presence of 10 ng/mL of IL-18, IL-27, or their combinations and 1 ng/mL of IL-15 as survival factor. For cytotoxicity experiments with pharmacologic inhibitors, NK cells were incubated with the drugs, washed, and then incubated with target cells in fresh medium without such inhibitors. Cells were used for phenotypic analysis, cytokine production, proliferation, apoptosis, cytotoxicity, or conjugate formation with target cells. For priming experiments, NK cells were cultured overnight with 1 ng/mL of IL-15 alone or with 1 ng/mL of IL-15 and 10 ng/mL of IL-18 or IL-27, washed and cultured for 24 h with 1 ng/mL of IL-15 alone or with 1 ng/mL of IL-15 and 10 ng/mL of IL-27 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

NK cells cultured for 5 days as described were pulsed with 1 μCi/well of methyl-^{3}H-thymidine (^{3}H-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 liquid scintillation counter. Results are expressed as mean counts per minute of triplicate wells ± SD.

Flow cytometry

Expression of cell surface receptors on NK cells was analyzed by FC as previously described [58]. Expression of IFN-γ, T-bet, and pfp was analyzed by intracellular FC using Cytofix/Cytoperm (BD). For IFN-γ and CD107a, cells were cultured in the presence of Golgi-Plug® and Golgi-Stop® reagents during the last 4 h. Cells were analyzed in a FACSCanto II flow cytometer (BD). Results were expressed as MFI or percentage of positive cells for each marker.

NK-cell viability

Viability of NK cells was assessed by FC using 7-AAD (Biolegend).

ELISA

Secretion of IFN-γ was analyzed by ELISA as described [4]. Secretion of IL-27 was assessed using the human IL-27 DuoSet (R&D).

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

NK cells were cultured overnight with IL-18, IL-27, or both in the presence of 1 ng/mL of IL-15, washed and cocultured for 5 h with CFSE-labeled Raji (*Burkitt's lymphoma*), eFluor Dye 670 (eBioscience)-labeled T-47D (mammary carcinoma), or eFluor Dye 670-labeled HCT116 (colon carcinoma) cells (from ATCC) at different E:T ratios. Raji cells were thereafter labeled with 7-AAD, while T-47D and HCT116 cells were thereafter labeled with Zombie Green (Biolegend) and analyzed by FC. Percentage of cytotoxicity was calculated as 100× percentage of CFSE$^+$7-AAD$^+$ cells/percentage of CFSE$^+$ cells or as 100× percentage of eFluor Dye 670$^+$Zombie Green$^+$ cells/percentage of eFluor Dye 670$^+$ cells. Percentage of spontaneous dead cells (without effector NK cells) was always below 5%. For inhibition of cytotoxic pathways, NK cells were treated with CCA or incubated with anti-TRAIL, anti-FasL mAbs (blockade of death receptors), KL247 hybridoma supernatant (blockade of NKp46), or anti-IFN-γ mAb (blockade of IFN-γ) for 1 h and used as effector cells as described. Cytotoxicity against mAb-coated target cells was performed using fluorescent-tracker-labeled target cells coated with RTX, TRZ, CTX, or normal human IgG for 2 h. In some experiments, NK cells preincubated with anti-NKp46 hybridoma supernatant for 1 h to prevent NKp46-mediated cytotoxicity were used as effector cells.

NK-cell–target-cell conjugates

NK cells were cultured overnight with cytokines as described, washed, labeled with CFSE, incubated with blocking mAb for 1 h, and mixed with eFluor Dye 670-labeled Raji cells at an E:T of 1:1. After 5 h, percentage of conjugates was assessed by FC and calculated as 100× percentage of CFSE$^+$ eFluor Dye 670$^+$ cells/percentage of CFSE$^+$ cells.

Statistical analysis

A one-way ANOVA test with Bonferroni post-hoc test was used when three or more experimental groups were compared or with Dunnett's post-hoc test in pharmacologic inhibition experiments; a paired *t*-test was used when two experimental groups were compared; a two-way ANOVA with repeated measures matched by both factors and Bonferroni post-hoc test were used for blocking experiments and for ADCC.

Acknowledgments: This work was supported by grants from the National Agency for Promotion of Science and Technology from Argentina (ANPCYT), the National Research Council of Argentina (CONICET), and the University of Buenos Aires (UBA), all to Norberto W. Zwirner. A.Z., R.G.S., X.L.R.I., D.E.A., and N.I.T. are fellows of CONICET. C.I.D. holds a postdoctoral fellowship from Bunge & Born Foundation. M.B.F. and N.W.Z. are members of the Researcher Career of CONICET. We would like to thank to Dr. Gabriel A. Rabinovich from the Laboratory of Immunopathology of IBYME for his continuous support and for generously providing unlimited access to the Flow Cytometry and Cell Sorting Facility, and to Dr. Alessandro Moretta (Department of Experimental Medicine, University of Genoa, Italy) for providing the anti-NCR mAbs.

Conflict of interest: The authors declare no commercial or financial conflict of interest.

References

1 **Lanier, L. L.**, Evolutionary struggles between NK cells and viruses. *Nat. Rev. Immunol.* 2008. **8**: 259–268.

2 **Ferlazzo, G., Pack, M., Thomas, D., Paludan, C., Schmid, D., Strowig, T., Bougras, G. et al.**, Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. *Proc. Natl. Acad. Sci. USA* 2004. **101**: 16606–16611.

3 **Martin-Fontecha, A., Thomsen, L. L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A. and Sallusto, F.**, Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat. Immunol.* 2004. **5**: 1260–1265.

4 **Girart, M. V., Fuertes, M. B., Domaica, C. I., Rossi, L. E. and Zwirner, N. W.**, Engagement of TLR3, TLR7, and NKG2D regulate IFN-gamma secretion but not NKG2D-mediated cytotoxicity by human NK cells stimulated with suboptimal doses of IL-12. *J. Immunol.* 2007. **179**: 3472–3479.

5 **Lanier, L. L.**, Up on the tightrope: natural killer cell activation and inhibition. *Nat. Immunol.* 2008. **9**: 495–502.

6 **Moretta, A., Marcenaro, E., Parolini, S., Ferlazzo, G. and Moretta, L.**, NK cells at the interface between innate and adaptive immunity. *Cell Death Differ.* 2008. **15**: 226–233.

7 **Chaix, J., Tessmer, M. S., Hoebe, K., Fuseri, N., Ryffel, B., Dalod, M., Alexopoulou, L. et al.**, Priming of NK cells by IL-18. *J. Immunol.* 2008. **181**: 1627–1631.

8 **Kastelein, R. A., Hunter, C. A. and Cua, D. J.**, Discovery and biology of IL-23 and IL-27: related but functionally distinct regulators of inflammation. *Annu. Rev. Immunol.* 2007. **25**: 221–242.

9 **Vignali, D. A. and Kuchroo, V. K.**, IL-12 family cytokines: immunological playmakers. *Nat. Immunol.* 2012. **13**: 722–728.

10 **Pflanz, S., Timans, J. C., Cheung, J., Rosales, R., Kanzler, H., Gilbert, J., Hibbert, L. et al.**, IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. *Immunity* 2002. **16**: 779–790.

11 **de Groot, R., van Beelen, A. J., Bakdash, G., Taanman-Kueter, E. W., de Jong, E. C. and Kapsenberg, M. L.**, Viral dsRNA-activated human dendritic cells produce IL-27, which selectively promotes cytotoxicity in naive $CD8^+$ T cells. *J. Leukoc. Biol.* 2012. **92**: 605–610.

12 **Molle, C., Nguyen, M., Flamand, V., Renneson, J., Trottein, F., De Wit, D., Willems, F. et al.**, IL-27 synthesis induced by TLR ligation critically depends on IFN regulatory factor 3. *J. Immunol.* 2007. **178**: 7607–7615.

13 **Takeda, A., Hamano, S., Yamanaka, A., Hanada, T., Ishibashi, T., Mak, T. W., Yoshimura, A. et al.**, Role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment. *J. Immunol.* 2003. **170**: 4886–4890.

14 **Lucas, S., Ghilardi, N., Li, J. and de Sauvage, F. J.**, IL-27 regulates IL-12 responsiveness of naive CD4+ T cells through Stat1-dependent and -independent mechanisms. *Proc. Natl. Acad. Sci. USA* 2003. **100**: 15047–15052.

15 **Hibbert, L., Pflanz, S., De Waal Malefyt, R. and Kastelein, R. A.**, IL-27 and IFN-alpha signal via Stat1 and Stat3 and induce T-Bet and IL-12Rbeta2 in naive T cells. *J. Interferon Cytokine Res.* 2003. **23**: 513–522.

16 **Owaki, T., Asakawa, M., Morishima, N., Hata, K., Fukai, F., Matsui, M., Mizuguchi, J. et al.**, A role for IL-27 in early regulation of Th1 differentiation. *J. Immunol.* 2005. **175**: 2191–2200.

17 **Cox, J. H., Kljavin, N. M., Ramamoorthi, N., Diehl, L., Batten, M. and Ghilardi, N.**, IL-27 promotes T cell-dependent colitis through multiple mechanisms. *J. Exp. Med.* 2011. **208**: 115–123.

18 **Neufert, C., Becker, C., Wirtz, S., Fantini, M. C., Weigmann, B., Galle, P. R. and Neurath, M. F.**, IL-27 controls the development of inducible regulatory T cells and Th17 cells via differential effects on STAT1. *Eur. J. Immunol.* 2007. **37**: 1809–1816.

19 **Ilarregui, J. M., Croci, D. O., Bianco, G. A., Toscano, M. A., Salatino, M., Vermeulen, M. E., Geffner, J. R. et al.**, Tolerogenic signals delivered by dendritic cells to T cells through a galectin-1-driven immunoregulatory circuit involving interleukin 27 and interleukin 10. *Nat. Immunol.* 2009. **10**: 981–991.

20 **Schneider, R., Yaneva, T., Beauseigle, D., El-Khoury, L. and Arbour, N.**, IL-27 increases the proliferation and effector functions of human naive $CD8^+$ T lymphocytes and promotes their development into Tc1 cells. *Eur. J. Immunol.* 2011. **41**: 47–59.

21 **Hu, P., Hu, H. D., Chen, M., Peng, M. L., Tang, L., Tang, K. F., Matsui, M. et al.**, Expression of interleukins-23 and 27 leads to successful gene therapy of hepatocellular carcinoma. *Mol. Immunol.* 2009. **46**: 1654–1662.

22 **Liu, L., Wang, S., Shan, B., Shao, L., Sato, A., Kawamura, K., Li, Q. et al.**, IL-27-mediated activation of natural killer cells and inflammation produced antitumour effects for human oesophageal carcinoma cells. *Scand. J. Immunol.* 2008. **68**: 22–29.

23 **Matsui, M., Kishida, T., Nakano, H., Yoshimoto, K., Shin-Ya, M., Shimada, T., Nakai, S. et al.**, Interleukin-27 activates natural killer cells and suppresses NK-resistant head and neck squamous cell carcinoma through inducing antibody-dependent cellular cytotoxicity. *Cancer Res.* 2009. **69**: 2523–2530.

24 **Oniki, S., Nagai, H., Horikawa, T., Furukawa, J., Belladonna, M. L., Yoshimoto, T., Hara, I. et al.**, Interleukin-23 and interleukin-27 exert quite different antitumor and vaccine effects on poorly immunogenic melanoma. *Cancer Res.* 2006. **66**: 6395–6404.

25 **Wei, J., Xia, S., Sun, H., Zhang, S., Wang, J., Zhao, H., Wu, X. et al.**, Critical role of dendritic cell-derived IL-27 in antitumor immunity through regulating the recruitment and activation of NK and NKT cells. *J. Immunol.* 2013. **191**: 500–508.

26 **Laroni, A., Gandhi, R., Beynon, V. and Weiner, H. L.**, IL-27 imparts immunoregulatory function to human NK cell subsets. *PLoS One* 2011. **6**: e26173.

27 **Frank, D. A., Mahajan, S. and Ritz, J.**, Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. *Nat. Med.* 1999. **5**: 444–447.

28 **Kataoka, T., Takaku, K., Magae, J., Shinohara, N., Takayama, H., Kondo, S. and Nagai, K.**, Acidification is essential for maintaining the structure and function of lytic granules of CTL. Effect of concanamycin A, an inhibitor of vacuolar type H(+)-ATPase, on CTL-mediated cytotoxicity. *J. Immunol.* 1994. **153**: 3938–3947.

29 **Wang, R., Jaw, J. J., Stutzman, N. C., Zou, Z. and Sun, P. D.**, Natural killer cell-produced IFN-gamma and TNF-alpha induce target cell cytolysis through up-regulation of ICAM-1. *J. Leukoc. Biol.* 2012. **91**: 299–309.

30 **Hunter, C. A. and Kastelein, R.**, Interleukin-27: balancing protective and pathological immunity. *Immunity* 2012. **37**: 960–969.

31 **Yoshida, H., Nakaya, M. and Miyazaki, Y.**, Interleukin 27: a double-edged sword for offense and defense. *J. Leukoc. Biol.* 2009. **86**: 1295–1303.

32 **Bosmann, M. and Ward, P. A.**, Modulation of inflammation by interleukin-27. *J. Leukoc. Biol.* 2013. **94**: 1159–1165.

33 **Murugaiyan, G. and Saha, B.**, IL-27 in tumor immunity and immunotherapy. *Trends Mol. Med.* 2013. **19**: 108–116.

34 **Iyer, S. S., Ghaffari, A. A. and Cheng, G.**, Lipopolysaccharide-mediated IL-10 transcriptional regulation requires sequential induction of type I IFNs and IL-27 in macrophages. *J. Immunol.* 2010. **185**: 6599–6607.

35 **Pirhonen, J., Siren, J., Julkunen, I. and Matikainen, S.**, IFN-alpha regulates Toll-like receptor-mediated IL-27 gene expression in human macrophages. *J. Leukoc. Biol.* 2007. **82**: 1185–1192.

36 **Kalliolias, G. D., Gordon, R. A. and Ivashkiv, L. B.**, Suppression of TNF-alpha and IL-1 signaling identifies a mechanism of homeostatic regulation of macrophages by IL-27. *J. Immunol.* 2010. **185**: 7047–7056.

37 **Tanida, S., Yoshitomi, H., Ishikawa, M., Kasahara, T., Murata, K., Shibuya, H., Ito, H. et al.**, IL-27-producing CD14(+) cells infiltrate inflamed joints of rheumatoid arthritis and regulate inflammation and chemotactic migration. *Cytokine* 2011. **55**: 237–244.

38 **Schmidt, C., Giese, T., Ludwig, B., Mueller-Molaian, I., Marth, T., Zeuzem, S., Meuer, S. C. et al.**, Expression of interleukin-12-related cytokine transcripts in inflammatory bowel disease: elevated interleukin-23p19 and interleukin-27p28 in Crohn's disease but not in ulcerative colitis. *Inflamm. Bowel Dis.* 2005. **11**: 16–23.

39 **Zeitvogel, J., Werfel, T. and Wittmann, M.**, IL-27 acts as a priming signal for IL-23 but not IL-12 production on human antigen-presenting cells. *Exp. Dermatol.* 2012. **21**: 426–430.

40 **Cocco, C., Pistoia, V. and Airoldi, I.**, Anti-leukemic properties of IL-12, IL-23 and IL-27: differences and similarities in the control of pediatric B acute lymphoblastic leukemia. *Crit. Rev. Oncol. Hematol.* 2012. **83**: 310–318.

41 **Liu, Z., Liu, J. Q., Talebian, F., Wu, L. C., Li, S. and Bai, X. F.**, IL-27 enhances the survival of tumor antigen-specific $CD8^+$ T cells and programs them into IL-10-producing, memory precursor-like effector cells. *Eur. J. Immunol.* 2013. **43**: 468–479.

42 **Ferlazzo, G., Tsang, M. L., Moretta, L., Melioli, G., Steinman, R. M. and Munz, C.**, Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *J. Exp. Med.* 2002. **195**: 343–351.

43 **Pende, D., Castriconi, R., Romagnani, P., Spaggiari, G. M., Marcenaro, S., Dondero, A., Lazzeri et al.**, Expression of the DNAM-1 ligands, nectin-2 (CD112) and poliovirus receptor (CD155), on dendritic cells: relevance for natural killer-dendritic cell interaction. *Blood* 2006. **107**: 2030–2036.

44 **Chijioke, O. and Munz, C.**, Dendritic cell derived cytokines in human natural killer cell differentiation and activation. *Front. Immunol.* 2013. **4**: 365.

45 **Ortaldo, J. R., Winkler-Pickett, R., Wigginton, J., Horner, M., Bere, E. W., Mason, A. T., Bhat, N. et al.**, Regulation of ITAM-positive receptors: role of IL-12 and IL-18. *Blood* 2006. **107**: 1468–1475.

46 **Tassi, I., Cella, M., Presti, R., Colucci, A., Gilfillan, S., Littman, D. R. and Colonna, M.**, NK cell-activating receptors require PKC-theta for sustained signaling, transcriptional activation, and IFN-gamma secretion. *Blood* 2008. **112**: 4109–4116.

47 **Kondadasula, S. V., Roda, J. M., Parihar, R., Yu, J., Lehman, A., Caligiuri, M. A., Tridandapani, S. et al.**, Colocalization of the IL-12 receptor and FcgammaRIIa to natural killer cell lipid rafts leads to activation of ERK and enhanced production of interferon-gamma. *Blood* 2008. **111**: 4173–4183.

48 **Charlot-Rabiega, P., Bardel, E., Dietrich, C., Kastelein, R. and Devergne, O.**, Signaling events involved in interleukin 27 (IL-27)-induced proliferation of human naive CD4+ T cells and B cells. *J. Biol. Chem.* 2011. **286**: 27350–27362.

49 **Hasenkamp, J., Borgerding, A., Wulf, G., Uhrberg, M., Jung, W., Dingeldein, S., Truemper, L. et al.**, Resistance against natural killer cell cytotoxicity: analysis of mechanisms. *Scand. J. Immunol.* 2006. **64**: 444–449.

50 **Eissens, D. N., Van Der Meer, A., Van Cranenbroek, B., Preijers, F. W. and Joosten, I.**, Rapamycin and MPA, but not CsA, impair human NK cell cytotoxicity due to differential effects on NK cell phenotype. *Am. J. Transplant.* 2010. **10**: 1981–1990.

51 **Orange, J. S., Brodeur, S. R., Jain, A., Bonilla, F. A., Schneider, L. C., Kretschmer, R., Nurko, S. et al.**, Deficient natural killer cell cytotoxicity in patients with IKK-gamma/NEMO mutations. *J. Clin. Invest.* 2002. **109**: 1501–1509.

52 **Horova, V., Hradilova, N., Jelinkova, I., Koc, M., Svadlenka, J., Brazina, J., Klima, M. et al.**, Inhibition of vacuolar ATPase attenuates the TRAIL-induced activation of caspase-8 and modulates the trafficking of TRAIL receptosomes. *FEBS J.* 2013. **280**: 3436–3450.

53 **Zhang, Y. and Zhang, B.**, TRAIL resistance of breast cancer cells is associated with constitutive endocytosis of death receptors 4 and 5. *Mol. Cancer Res.* 2008. **6**: 1861–1871.

54 **Semino, C., Angelini, G., Poggi, A. and Rubartelli, A.**, NK/iDC interaction results in IL-18 secretion by DCs at the synaptic cleft followed by NK cell activation and release of the DC maturation factor HMGB1. *Blood* 2005. **106**: 609–616.

55 **Szabo, S. J., Sullivan, B. M., Stemmann, C., Satoskar, A. R., Sleckman, B. P. and Glimcher, L. H.**, Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science* 2002. **295**: 338–342.

56 **Yu, J., Wei, M., Boyd, Z., Lehmann, E. B., Trotta, R., Mao, H., Liu, S. et al.**, Transcriptional control of human T-BET expression: the role of Sp1. *Eur. J. Immunol.* 2007. **37**: 2549–2561.

57 **Matsuda, J. L., George, T. C., Hagman, J. and Gapin, L.**, Temporal dissection of T-bet functions. *J. Immunol.* 2007. **178**: 3457–3465.

58 **Rossi, L. E., Avila, D. E., Spallanzani, R. G., Ziblat, A., Fuertes, M. B., Lapyckyj, L., Croci, D. O. et al.**, Histone deacetylase inhibitors impair NK cell viability and effector functions through inhibition of activation and receptor expression. *J. Leukoc. Biol.* 2011. **91**: 321–331.

Abbreviations: **ADCC**: Ab-dependent cell-mediated cytotoxicity · **CBA**: cytokine bead array · **CCA**: concanamycin A · **FC**: flow cytometry · **IC mAb**: isotype-matched negative control mAb · **iDC**: immature DC · **mDC**: mature DC · **pfp**: perforin

Full correspondence: Dr. Norberto W. Zwirner, Laboratory of Physiopathology of Innate Immunity, IBYME-CONICET, Vuelta de Obligado 2490, C1428ADN Ciudad de Buenos Aires, Argentina
Fax: +54-11-4786-2564
e-mail: nzwirner@ibyme.conicet.gov.ar

Received: 28/3/2014
Revised: 10/9/2014
Accepted: 6/10/2014
Accepted article online: 11/10/2014

