Neutrophil activation and survival are modulated by interaction with NK cells

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Abstract

It is increasingly evident that neutrophils are able to cross-talk with other leukocytes to shape ongoing inflammatory and immune responses. In this study, we analyzed whether human NK cells may influence the survival and activation of neutrophils under co-culture conditions. We report that NK cells exposed to either IL-15 or IL-18 alone strongly protect the survival of neutrophils via the release of IFNγ and granulocyte macrophage colony-stimulating factor (GM-CSF) plus IFNγ, respectively, and cause a slight up-regulation of neutrophil CD64 and CD11b expression. In comparison, NK cells exposed to both IL-15 and IL-18 show a lesser ability to increase the survival of neutrophils but can more potently up-regulate CD64 and CD11b expression, as well as induce the de novo surface expression of CD69, in neutrophils. Analysis of the events occurring in neutrophil/NK co-cultures exposed to IL-15 plus IL-18 revealed that (i) neutrophil survival is positively affected by NK-derived GM-CSF but negatively influenced by a CD18-dependent neutrophil/NK contact, (ii) NK-derived IFNγ is almost entirely responsible for the induction of CD64, (iii) both soluble factors (primarily GM-CSF) and direct cell–cell contact up-regulate CD11b and CD69 and (iv) NK-derived GM-CSF induces the expression of biologically active heparin-binding EGF-like growth factor (HB-EGF) in neutrophils. Finally, we demonstrate that NK cells can also express HB-EGF when stimulated with either IL-2 or IL-15, yet independently of endogenous GM-CSF. Altogether, our results define a novel interaction within the innate immune system whereby NK cells, by directly modulating neutrophil functions, might contribute to the pathogenesis of inflammatory diseases.

Keywords: apoptosis, cytokines, HB-EGF, inflammation

Introduction

Polymorphonuclear neutrophil leukocytes (PMN) are commonly known as ‘professional’ phagocytic cells of the innate immune system. However, it is now clear that the activities of PMN go far beyond phagocytosis and pathogen killing. Indeed, PMN may perform sophisticated regulatory functions, having implications not only in the inflammatory and immune responses but also in hematopoiesis, wound healing and antiviral defense (1, 2). In addition, PMN are fine-tuned by exposure to the environment, as well as by the interaction with other leukocyte types, to optimally execute their functions. For instance, neutrophils can be activated by direct contact with dendritic cells (DC) (3) or T cells (4), as well as by locally produced cytokines, such as in the case of T Follicular helper cells (Tfh), which have been recently shown to recruit neutrophils in a CXCL8-dependent manner and, in turn, increase their survival and expression of activation markers via the release of granulocyte macrophage colony-stimulating factor (GM-CSF), IFNγ and tumor necrosis factor (TNF) (5).

NK cells were originally identified and named as cells displaying an innate ability to recognize and destroy malignant cells. However, NK cells can also produce a variety of cytokines, displaying important functional consequences for the evolution of the immune response (6). NK cells are also engaged in cross-talks with accessory cells, such as DC, monocytes and macrophages, which, besides being fundamental for NK cell activation in response to most pathogens (7), also contribute to shape the subsequent immune response (8). For example, activated NK cells induce the maturation of DC via the release of IFNγ and TNFα and cell–cell contact-dependent mechanisms (9). On the other hand, activated NK cells can kill autologous immature myeloid DC, a process that is particularly relevant for non-immunogenic...
Materials and methods

Isolation and co-culture of neutrophils and NK cells
Granulocytes and PBMC were isolated under endotoxin-free conditions from buffy coats of healthy donors. Highly purified neutrophils (>99.7% pure) were obtained as previously described (5). NK cells were isolated from PBMC by the Easy-Sep® NK Cell Enrichment kit (StemCell Technologies, Vancouver, Canada) (>96% pure) (13). Cell purity was determined by either double staining (neutrophils: CD66b–FITC/CD3–PE and CD16–FITC/CCR3–PE) or triple staining (NK cells: CD66b–FITC/CD56–PE–CD3–PerCP). All antibodies were purchased from BioLegend (San Diego, CA, USA), with the exception of CD69–PE that was purchased from Treestar, Inc., San Carlos, CA, USA. Immediately after purification, cells were suspended in RPMI 1640 medium supplemented with 10% low endotoxin fetal bovine serum (<0.5 EU ml⁻¹), 10 ng ml⁻¹ GM-CSF (goat IgG, Solgent, Korea) (14), 10 ng ml⁻¹ IFNγ (clone B154.2, 10²), IFNα (clone B133.3, 10 μg ml⁻¹) (15) and αGM-CSF (goat IgG, 10 μg ml⁻¹, R&D). In selected experiments, the following antibodies were used: αTNFα (clone B154.2, 10 μg ml⁻¹) (14), αIFNγ (clone B133.3, 10 μg ml⁻¹) (15) and αGM-CSF (goat IgG, 10 μg ml⁻¹, R&D). In other experiments, 10⁻⁷ M highly purified diphtheria toxin (DT) (kindly provided by Dr A. Rigo, Department of Clinical and Experimental Medicine, Verona, Italy) was used. All reagents used were of the highest available grade and were dissolved in pyrogen-free water for clinical use.

Analysis of cytokine production
Cytokine levels in cell-free supernatants were measured by specific ELISA kits for human: IFNγ (2 pg ml⁻¹ detection limit, Immunotools), TNFα (8 pg ml⁻¹, R&D) and GM-CSF (2 pg ml⁻¹, BioLegend).

Determination of apoptosis
Cytofluorimetric analysis of single-cell populations was performed after electronic separation of neutrophils on one side and NK cells on the other side on the basis of their physical characteristics, that is, forward scatter (FSC) and side scatter (SSC). For analysis of apoptosis, either Annexin V–propidium iodide (Milenyi) or Vybrant® FAM Caspase-3- and -7 Assay Kit (Molecular Probes, Invitrogen, Eugene, OR, USA) were used, according to the manufacturer’s instructions. Alternatively, neutrophils were analyzed for apoptotic (hypodiploid) nuclei, as previously described (16). Cells were acquired with either a FACScan or a FACSCalibur flow cytometers (BD Biosciences) and analysis performed by FlowJo software (Treestar, Inc., San Carlos, CA, USA).

Determination of antigen expression
For analysis of antigen expression, samples were stained with CD64–FITC/CD69–PE/CD16–PerCP–Cy5.5/CD56–PE–Cy7/CD11b–APC/CD45–APC–Cy7 (all from BD Biosciences, with the exception of CD69–PE that was purchased from BioLegend) or with isotype-matched, irrelevant mAbs and acquired by a FACSScanto flow cytometer (BD Biosciences). Analysis was performed on live cells by FlowJo software. For antigen expression, histograms were normalized using FlowJo and depict the percentage of maximum, i.e. the maximum number of events is set at 100%.

Superoxide anion production
O₂⁻ release was estimated by the cytochrome C reduction assay (17). Briefly, after cell culture, an equal volume of HBSS, pH 7.4, containing 160 μM ferricytochrome C (Sigma) with or without superoxide dismutase and either 200 mM fMLF (Sigma) or 40 μg ml⁻¹ β-glucan (Sigma) was added to each well. The plate was then placed in an automated ELX 808 microplate reader (Bio-Tec Instruments), pre-warmed at 37°C, and absorbance at 550 nm and 468 nm recorded at intervals of 5 min. O₂⁻ production in nanomoles was calculated using an extinction coefficient of 24.5 mM. Each condition was performed in triplicate.

Quantitative real-time reverse transcription–PCR
Real-time reverse transcription–PCR was performed exactly as described (18) using gene-specific primer pairs (purchased from Invitrogen) available in the public database RTPRimerDB (http://medgen.ugent.be/rtprimerdb/) under the following entry codes: GAPDH (3539), HB-EGF (8165) and GM-CSF (8166).

Statistical analysis
Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).
Results

Activated NK cells protect the survival of human neutrophils

Highly purified human neutrophils (5) were isolated from the peripheral blood of healthy donors and co-cultured with autologous NK cells (13) at a 1:1 ratio in the presence of IL-15 and/or IL-18. To evaluate whether NK cells could directly modulate neutrophil survival and activation, IL-15 and/or IL-18 were deliberately used at concentrations (10 ng ml⁻¹) that are known to specifically stimulate NK cells only (19, 20) but not neutrophils (at least for the parameters under evaluation) (21–25). Accordingly, IL-15 and IL-18, used singly or in combination, neither influenced the spontaneous apoptotic process that neutrophils undergo upon a prolonged in vitro incubation, as measured by Annexin V staining (Fig. 1A and B) or caspase-3 and -7 activation assays (data not shown), nor modified the expression of classical activation markers such as CD64, CD69 and CD11b (Fig. 2) (21, 26). On the other hand, IL-15 and/or IL-18 significantly induced the expression of CD69 in NK cells; yet, they did not significantly influence their survival (data not shown), as expected after such short-term cultures (27). Nonetheless, the survival of neutrophils was significantly increased when they were co-cultured with NK cells in the presence of IL-15 and/or IL-18 (Fig. 1A and B), suggesting that activated NK cells were responsible for such pro-survival effects. Notably, NK cells resulted more effective in promoting neutrophil survival when IL-15 or IL-18 was used alone rather than in combination (Fig. 1A and B). Interestingly, such positive modulatory effects remained fully evident in a transwell co-culture system, in which neutrophils were separated from NK cells by a 0.4-μm porous membrane (Fig. 1B), indicating that, at least under IL-15 or IL-18 treatment, they were entirely mediated by soluble factors. In fact, since the survival of neutrophils co-cultured with NK cells in the presence of IL-15 plus IL-18 proved to be further increased in transwell (Fig. 1B), the possibility exists that, under IL-15 plus IL-18 treatment, the viability of neutrophils is negatively influenced by their direct contact with NK cells. Since CD18 has been implicated in mediating the intercellular contacts that neutrophils (28, 29), as well as NK cells (30, 31), may establish with other leukocytes, we incubated IL-15 plus IL-18-treated neutrophil/NK cell co-cultures in the presence of neutralizing mAbs against CD18, specifically 7E4 (Fig. 1C) and IB4 F(ab⁻¹)₂ (data not shown). As a result, the survival of neutrophils was enhanced at levels comparable to those observed in the presence of transwell membranes (Fig. 1B and C). Finally, in the presence of 200 U ml⁻¹ IL-2, which did not directly affect neutrophil viability, in agreement with published data (22), the survival of neutrophils was significantly increased by their co-culture with NK cells, whether they were cultured in direct contact or separated by a transwell (Fig. 1B). Altogether, these data show that, upon activation, NK cells release soluble factors that increase the survival of neutrophils. However, at least under specific stimulatory conditions, this NK-mediated pro-survival effect on neutrophils might be partially prevented by direct contact-dependent mechanisms.

Role of NK-derived IFNγ and GM-CSF as neutrophil pro-survival factors

Preliminary experiments confirmed that conditioned medium harvested from NK cells incubated with IL-15 and/or IL-18 produced similar protective effects on neutrophil apoptosis (data not shown). To identify the NK-derived soluble factors preserving the survival of neutrophils in our co-cultures, we initially measured the levels of cytokines known to delay neutrophil apoptosis, such as IFNγ (32), GM-CSF (33) and TNFα (34), in cell-free supernatants harvested from activated NK cells. As shown in Table 1, while the IL-15 plus IL-18 combination was the strongest stimulatory condition for the production of IFNγ, GM-CSF and TNFα by NK cells, modest amounts of IFNγ and GM-CSF, but not TNFα, were also detectable in supernatants from IL-2-, IL-15- or IL-18-activated NK cells. Based on these findings, we subsequently incubated our neutrophil/NK cell co-cultures in the presence of specific neutralizing antibodies toward IFNγ, GM-CSF and TNFα as well as related isotype-matched controls. As a result, we observed that the NK-mediated action on neutrophil survival was completely suppressed by the use of αIFNγ antibodies only, when co-cultures were carried out in the presence of either IL-2 or IL-15 (Fig. 1D), and by the combined use of αIFNγ and αGM-CSF antibodies, when co-cultures were incubated with IL-18 (Fig. 1D). The combination of αIFNγ and αGM-CSF antibodies also displayed a strong, but not complete, inhibitory effect on the NK cell-mediated protection of neutrophil survival in co-cultures exposed to IL-15 plus IL-18 (Fig. 1D), indicating that other soluble factors may also be involved. The latter did not likely include TNFα, as αTNFα mAbs did not inhibit the pro-survival effect of activated NK cells in any of the co-culture conditions employed, either when used alone or in combination with the αIFNγ and αGM-CSF antibodies (data not shown). Isotype controls were without effect in all the stimulatory conditions employed (data not shown). Taken together, our results show that IFNγ and GM-CSF almost entirely mediate the protective effects of IL-2-, IL-15- and IL-18-activated NK cells on neutrophil survival.

Activated NK cells modulate the expression of activation markers in neutrophils

To verify whether additional functional parameters of neutrophils could be modulated by NK cells under our co-culture conditions, we subsequently analyzed the expression levels of surface antigens that are typically modulated upon neutrophil activation, such as IFNγ and GM-CSF, as neutrophil pro-survival factors. As shown in Fig. 2, the expression of CD64 and CD11b was significantly up-modulated in neutrophils, under co-culture with NK cells, in the presence of IL-18 or IL-15 plus IL-18 (Fig. 2A and B). Although a modest up-modulation of CD64 and CD11b expression in neutrophils was also observed in co-cultures exposed to IL-2 and IL-15, it did not reach statistical significance (Fig. 2A and B). In addition, NK cells induced the expression of CD69 in neutrophils only in the presence of IL-15 plus IL-18 (Fig. 2C). Since the three neutrophil markers were optimally up-modulated by IL-15 plus IL-18-treated NK cells, we explored the underlying mechanisms under the latter experimental conditions only.
Neutrophils were thus first co-cultured with NK cells in trans-well to assess the eventual contribution of soluble factors; the latter were then identified by the use of specific \( \alpha \)IFN, \( \alpha \)GM-CSF or \( \alpha \)TNF\( \alpha \) antibodies. These experiments revealed that different mechanisms regulate the NK-mediated effects on neutrophil expression of CD64, CD11b and CD69. Accordingly, the induction of CD64 expression was found to be entirely dependent on NK-derived soluble factors, as it was almost totally inhibited by the \( \alpha \)IFN\( \gamma \) (Fig. 3A) but not \( \alpha \)GM-CSF (Fig. 3A) or \( \alpha \)TNF\( \alpha \) (data not shown) antibodies.

**Fig. 1.** NK cells exposed to IL-2, IL-15, IL-18 or IL-15 plus IL-18 promote neutrophil survival. (A–D) Neutrophils (0.5 \( \times \) 10\(^6\) ml\(^{-1}\)) were cultured alone or with NK cells (ratio 1:1), either in direct contact or separated by a transwell membrane (tw) (B), and stimulated with IL-2 (200 U ml\(^{-1}\)), IL-15 (10 ng ml\(^{-1}\)), IL-18 (10 ng ml\(^{-1}\)) or IL-15 plus IL-18 in the absence (A and B) or the presence of \( \alpha \)CD18 (C), \( \alpha \)IFN\( \gamma \) and/or \( \alpha \)GM-CSF antibodies (D). After 18 h, cells were harvested and analyzed for their viability by Annexin V/propidium iodide staining as described in Materials and methods. (A) A representative experiment is shown comparing the vitality of neutrophils cultured alone or with NK cells in the presence of the indicated stimuli. (B) Bars display Annexin V-negative cells and are represented as percentage of mean \( \pm \) SEM of three to six experiments. Symbols stand for \( P < 0.001 \) (*** by two-way analysis of variance of paired samples. (C and D) Bars indicate the inhibition of the effect of NK cells on neutrophil survival by the indicated neutralizing antibodies and are represented as percentage of mean \( \pm \) SEM of three independent experiments.
By contrast, both cell–cell contact-dependent mechanisms and soluble factors were found to be responsible for the NK cell-mediated actions on neutrophil CD11b and CD69 expression, as this response was partially inhibited by transwell cultures (Fig. 3B and C) as well as by the GM-CSF, but not IFNγ or TNFα, antibodies (Fig. 3B and C and data not shown). However, if neutrophils were cultured in a transwell system in the presence of GM-CSF and IFNγ antibodies, then the up-regulation of CD11b (Fig. 3B) and CD69 (Fig. 3C) was strongly or completely inhibited, respectively. All in all, these data indicate that, in addition to their effects on neutrophil survival, NK cells exposed to IL-15 and/or IL-18 are also able to up-regulate neutrophil antigen expression. The latter effects occur via NK-derived soluble factors acting in combination with cell–cell contact-dependent mechanisms.

Both recombinant IFNγ and GM-CSF mimic, at very low doses, the effects of activated NK cells on neutrophils

Previous results suggest that very low concentrations of IFNγ and GM-CSF (Table 1) promote the survival of neutrophils. The latter was confirmed by the experiments shown in Fig. 4(A), which illustrates that GM-CSF and IFNγ strongly, and at similar degree, promote the survival of neutrophils over a wide range of concentrations (from 10 ng ml⁻¹ to either 1 or 10 pg ml⁻¹, respectively). In addition, IFNγ on the one hand and GM-CSF on the other hand induce neutrophil CD64 and CD69 expression, respectively, at concentrations higher than 10 pg ml⁻¹ (Fig. 4B), while, although both cytokines up-regulate CD11b surface levels, GM-CSF is more potent than IFNγ (Fig. 4B). Taken together, these data indicate that extremely low concentrations of recombinant IFNγ and GM-CSF perfectly mimic the effects of activated NK cells on the survival and expression of activation markers by neutrophils.

Activated NK cells prime neutrophils for superoxide anion production in response to fMLF or β-glucan

To more extensively characterize the effect of NK cells on the functional activation of neutrophils, we evaluated the production of superoxide anion. Therefore, neutrophils and NK cells were cultured separately or together in the presence of IL-2, IL-15, IL-18 or IL-15 plus IL-18. After 18 h, extracellular GM-CSF, IFNγ and TNFα were measured by ELISA (n = 4). n.d., not detected.

Table 1. Release of GM-CSF, IFNγ and TNFα by NK cells exposed to IL-2, IL-15, IL-18 or IL-18

<table>
<thead>
<tr>
<th>NK cells incubated with</th>
<th>Medium</th>
<th>IL-2</th>
<th>IL-15</th>
<th>IL-18</th>
<th>IL-15 plus IL-18</th>
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<td>Range</td>
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<tr>
<td>GM-CSF (pg ml⁻¹)</td>
<td>n.d.</td>
<td>2–3.2</td>
<td>3.5–6.8</td>
<td>2–4.4</td>
<td>50–121</td>
</tr>
<tr>
<td>IFNγ (pg ml⁻¹)</td>
<td>n.d.</td>
<td>2–12</td>
<td>5.5–11.1</td>
<td>3–6.5</td>
<td>38–114</td>
</tr>
<tr>
<td>TNFα (pg ml⁻¹)</td>
<td>n.d.</td>
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NK cells were stimulated or not with IL-2 (200 U ml⁻¹), IL-15 (10 ng ml⁻¹), IL-18 (10 ng ml⁻¹) or IL-15 plus IL-18. After 18 h, extracellular GM-CSF, IFNγ and TNFα were measured by ELISA (n = 4). n.d., not detected.

Fig. 2. NK cells exposed to IL-2, IL-15, IL-18 or IL-15 plus IL-18 up-regulate the expression of CD64, CD11b and CD69 by neutrophils. (A–C) Neutrophils were cultured alone or with NK cells (ratio 1:1) and stimulated with IL-2, IL-15, IL-18 or IL-15 plus IL-18. After 18 h, cells were harvested and neutrophils were analyzed for the expression of CD64 (A), CD11b (B) and CD69 (C) as described in Materials and methods. (A–C) Bars display the mean fluorescence intensity of the indicated antigen after subtraction of the corresponding isotype control and are represented as percentage of mean ± SEM of four to seven independent experiments. Symbols stand for P < 0.001 (**), P < 0.01 (**) and P < 0.05 (*) by two-way analysis of variance of paired samples.
co-cultures proved more effective than IL-18-treated co-cultures in priming the production of superoxide anion by agonist-stimulated neutrophils, whereas the IL-15 plus IL-18 combination produced an effect comparable to that of IL-15 alone. By contrast, NK cells cultured alone did not produce O$_2^\cdot$ under any stimulatory condition (data not shown). Taken together, these data demonstrate that activated NK cells prime neutrophils for an enhanced O$_2^\cdot$ production, in line with their effect on the survival of, and expression of activation markers by, neutrophils.

**IL-15 plus IL-18-activated NK cells stimulate the expression of biologically active HB-EGF by neutrophils**

HB-EGF is a fibrogenic cytokine (35) that, among various biological activities, also functions as a receptor for DT, enabling its entry into susceptible cells and consequently triggering its ability to induce apoptosis (36). Recently, we showed that biologically active HB-EGF can be specifically induced by GM-CSF in neutrophils (16). Thus, the fact that IL-15 plus IL-18-activated NK cells produced elevated
amounts of GM-CSF (Table 1) prompted us to evaluate whether neutrophils, upon co-culture with NK cells, could express HB-EGF and, if so, whether this occurs in a GM-CSF-dependent manner. As shown in Fig. 6(A), HB-EGF mRNA was clearly induced in neutrophils co-cultured with NK cells in a transwell system under IL-15 plus IL-18 treatment. No induction of HB-EGF transcripts was observed in the absence of NK cells (data not shown), thus ruling out a direct effect of IL-15 plus IL-18 on neutrophils and indicating that NK-derived soluble factors were responsible for such HB-EGF induction. Accordingly, conditioned medium harvested from NK cells incubated for 18 h with IL-15 plus IL-18 induced a marked neutrophil expression of HB-EGF mRNA, which was completely abrogated by αGM-CSF antibodies (Fig. 6B). Furthermore, the same conditioned medium, similar to recombinant GM-CSF, both prolonged neutrophil survival (data not shown) and rendered neutrophils sensitive to DT-induced apoptosis (Fig. 6C), consistent with the surface expression of biologically active HB-EGF (16). Taken together, our results demonstrate that the levels of GM-CSF produced by IL-15 plus IL-18-activated NK cells are sufficient to induce the expression of functional HB-EGF in neutrophils.

NK cells express newly synthesized HB-EGF upon incubation with either IL-2 or IL-15

While investigating HB-EGF expression in neutrophil/NK cell co-cultures, we unexpectedly observed that, in our transwell system, NK cells also expressed low levels of HB-EGF mRNA under IL-15 plus IL-18 incubation (Fig. 6A). Therefore, we decided to perform ad hoc experiments to better evaluate whether also NK cells express biologically active HB-EGF. Interestingly, we were able to detect a remarkable, but transient, accumulation of HB-EGF transcripts in human
(Fig. 7B). Notably, the effect of IL-2, IL-15 (Fig. 7A) or IL-15 plus IL-18 (Fig. 7B) was not influenced by the presence of αGM-CSF antibodies, indicating that it was independent from endogenous GM-CSF. In addition, IL-18-activated NK cells clearly expressed GM-CSF mRNA, similar to IL-15 plus IL-18-activated NK cells (Fig. 7B), indicating a lack of correlation between the expression of GM-CSF and the induction of HB-EGF. In line with the latter observations, higher concentrations of recombinant GM-CSF induced little, if any, HB-EGF mRNA in NK cells (Fig. 7A).

The ability of NK cells to inducibly express the HB-EGF gene also resulted in the production of biologically active HB-EGF, since an incubation for 18 h with IL-15 or IL-15 plus IL-18, but not IL-18, rendered NK cells sensitive to a pro-apoptotic action of DT (Fig. 8A). Again, this was not blocked by αGM-CSF antibodies (Fig. 8B and data not shown). Finally, incubation with GM-CSF did not induce any sensitivity to DT-mediated apoptosis (Fig. 8A). Taken together, these data demonstrate that human NK cells must be added to the list of leukocytes able to express HB-EGF; however, unlike neutrophils (16), NK cells do not require GM-CSF to produce the fibrogenic cytokine.

**Discussion**

The results presented in this work provide novel insights into the ability of NK cells to engage cross-talks with accessory cells. Our experiments have indeed revealed that, under coculture conditions, NK cells exposed to IL-15 and/or IL-18 on the one hand and to IL-2 on the other hand (to mimic, respectively, an ‘innate immune’ and an ‘adaptive immune’ scenario) are able to prolong the survival and to modulate functional parameters of human neutrophils. We also show that such NK cell-mediated effects require both direct cell–cell interactions and NK-derived soluble factors. More specifically, we report that (i) NK cells strongly promote the survival of highly purified neutrophils in the presence of IL-2, IL-15, IL-18 or IL-15 plus IL-18; (ii) NK cells exposed to IL-2, IL-15, IL-18 or IL-15 plus IL-18 up-modulate the expression of the activation markers CD64, CD11b and CD69 by neutrophils; (iii) NK cells activated with IL-2, IL-15, IL-18 or IL-15 plus IL-18 prime neutrophils for an enhanced production of O

\[ \text{O}_2^- \] in response to fMLF or β-glucan and (iv) neutrophils express biologically active HB-EGF upon co-culture with, or incubation with conditioned medium from, IL-15 plus IL-18-activated NK cells. It is important to remark that both IL-15 and IL-18 were used at concentrations that, either alone or in combination, did not directly activate neutrophils in terms of survival and activation marker expression. Indeed, the biological activity of the IL-15 used in the present study (10 U ml\(^{-1}\)) was at least 200-fold lower than the one reported to delay the apoptosis of human neutrophils (22). Similarly, confirming published data, IL-18 alone neither modulated neutrophil apoptosis (23) nor induced the expression of CD69 (21), under our experimental conditions. By contrast, IL-15 and IL-18 efficiently stimulated NK cells, as revealed by the induction of CD69 expression (data not shown) and cytokine production. In this latter regard, we found that both GM-CSF and IFN\(_\gamma\) were released by NK cells stimulated with either IL-15 or, at much higher levels, IL-15 plus IL-18-activated NK cells. It is important to remark that both IL-15 and IL-18 were used at concentrations that, either alone or in combination, did not directly activate neutrophils in terms of survival and activation marker expression. Indeed, the biological activity of the IL-15 used in the present study (10 U ml\(^{-1}\)) was at least 200-fold lower than the one reported to delay the apoptosis of human neutrophils (22). Similarly, confirming published data, IL-18 alone neither modulated neutrophil apoptosis (23) nor induced the expression of CD69 (21), under our experimental conditions. By contrast, IL-15 and IL-18 efficiently stimulated NK cells, as revealed by the induction of CD69 expression (data not shown) and cytokine production. In this latter regard, we found that both GM-CSF and IFN\(_\gamma\) were released by NK cells stimulated with either IL-15 or, at much higher levels, IL-15 plus IL-18, in agreement with published reports (37, 38). We could also measure low levels of both GM-CSF and IFN\(_\gamma\) in supernatants from IL-18-activated NK cells, as already reported in the literature, yet with some exceptions (37–41). Differences in the experimental conditions and/or in the detection limits of the ELISA kits that were used might explain the latter discrepancies. Nonetheless, we could also detect a marked and rapid induction of GM-CSF and IFN\(_\gamma\) mRNAs in IL-18-stimulated NK cells (Fig. 7B and data not shown), consistent with the extracellular detection of the related cytokines. Finally, we were able to detect small amounts of TNF\(_\alpha\) but only in supernatants from IL-15 plus IL-18-activated NK cells,
confirming the notion that monokine-activated NK cells produce very modest levels of TNFα (38).

A series of attempts to elucidate the potential mechanisms regulating the survival and activation of neutrophils in coculture with NK cells provided us with the following information. First of all, the NK-mediated potentiation of neutrophil survival occurred independently of cell–cell contact, as it was mediated by soluble cytokines produced by NK cells in response to IL-2, IL-15 or IL-18. By using specific neutralizing antibodies, we showed that NK-derived IFNγ and GM-CSF, but not TNFα, were required for the protective effect on neutrophil survival by IL-18-activated NK cells. Similarly, only IFNγ, but not GM-CSF, was responsible for the increased survival of neutrophils observed upon their co-culture with NK cells in the presence of IL-2 and IL-15, even though, under the latter condition, NK cells released more IFNγ and GM-CSF than when stimulated with IL-18. A possible explanation is that additional soluble factors, and/or cell–cell contact-dependent mechanisms contrasting the pro-survival effects of GM-CSF, are concurrently induced in NK cells by IL-15. On the other hand, two mechanisms contribute to the effect of IL-15 plus IL-18-stimulated NK cells on neutrophil survival: one consisting in the release of soluble factors (GM-CSF), which positively affect neutrophil survival, and the other one dependent on a CD18-mediated contact between neutrophils and activated NK cells, which negatively influences neutrophil viability. Interestingly, CD18 has been already involved in the synapse between NK cells and target cells (42) and, more recently, in interactions between NK cells and accessory cells leading to important functional consequences (30, 31). In the case of the NK/neutrophil interactions reported herein, it remains to be determined whether CD18 has a direct role or simply promotes the engagement of other signaling pathways that negatively influence the survival of neutrophils. Second, we could demonstrate that the induction of neutrophil CD64 expression by IL-15 plus IL-18-activated NK cells exclusively relies on endogenous IFNγ, whereas the up-regulation of CD11b depends on both soluble factors (mainly GM-CSF) and cell–cell contact. Third, we show that only NK cells exposed to IL-15 plus IL-18 induced the expression of CD69 by neutrophils and that this requires GM-CSF and cell–cell contact, similar to CD11b. Thus, IFNγ, although potentially able to modulate the surface levels of CD69 (21) and CD11b (26) in neutrophils, does not appear to play any role in the up-modulation of these markers occurring as a result of NK cell activation by IL-15 plus IL-18. One possible explanation might be that the amounts of IFNγ released by NK cells are sufficient to induce CD64 but not CD69 or CD11b, given the high responsiveness of neutrophils to IFNγ in terms of CD64.

Fig. 8. NK cells incubated with IL-15 and IL-15 plus IL-18, but not IL-18 or GM-CSF, are sensitive to DT-mediated apoptosis. (A and B) NK cells were incubated with or without IL-15, IL-18 (A), IL-15 plus IL-18 (A and B) or GM-CSF (A) in the absence (A and B) or the presence (B) of αGM-CSF antibodies. After 18 h, NK cells were incubated with or without 10⁻⁷ M DT for 24 h (A and B). Cells were then harvested and analyzed for Annexin V/propidium iodide staining as described in Materials and methods. (A) A representative experiment is shown out of three performed with similar results; (B) Bars display Annexin V-negative cells and are represented as percentage of mean ± SEM of three experiments.
modulation (26, 43). Finally, extending our previous results on the specific ability of GM-CSF to induce HB-EGF in neutrophils (16), we identified endogenous GM-CSF released by IL-15 plus IL-18-activated NK cells in co-cultures as the responsible molecule for the de novo expression of HB-EGF by neutrophils. All in all, our findings not only uncover that NK cells utilize several strategies to regulate neutrophil survival and activation but also suggest that these actions might contribute to the pathogenesis of inflammatory diseases. For instance, increased percentages of neutrophils and NK cells found in the bronchoalveolar lavage fluids (BALF) from patients with sarcoidosis were associated with a poor disease outcome and a higher probability to receive steroid treatment (10). In addition, BALF neutrophils of sarcoidosis patients show an activated phenotype compared with blood neutrophils, as revealed by an up-regulation of CD11b and down-regulation of CD62L (44). Given the presence of increased levels of IL-15 (45) and IL-18 (46) in BALF from sarcoidosis patients, our data imply that, in BALF, neutrophils might be functionally influenced by the presence of activated NK cells. Similarly, peripheral blood and, at higher levels, synovial fluid neutrophils from RA patients express CD69 (11). In addition, neutrophils from RA synovial fluid, but not peripheral blood, express increased amounts of CD11b when compared with neutrophils from peripheral blood of healthy donors (11). Interestingly, 59 and 95% of analyzed synovial fluids had detectable levels of IFNγ and GM-CSF, respectively (11). Given the established role of IL-15 (47) and IL-18 (48), as well as the presence of activated and cytokine-producing NK cells (12, 19), in the pathogenesis of RA, we would speculate that NK cells might be an important source of these cytokines, in turn influencing neutrophil survival and activation.

In this study, we also provide a series of evidence proving that not only neutrophils but also NK cells express HB-EGF, yet under specific stimulatory conditions. To our knowledge, no information exists in the literature on the potential ability of primary human or mouse NK cells to express HB-EGF, while published data report a lack of HB-EGF expression in the human NK cell line 2C8 (49). We found that NK cells expressed HB-EGF only and specifically upon stimulation with either IL-2 or IL-15, but not IL-18. Consistently, the ability of IL-2 to induce the expression of HB-EGF has been already reported to occur in other cell types, such as vascular smooth muscle cells and T lymphocytes (50, 51), yet with some exceptions (52). While the latter data would suggest that signaling through the common cytokine receptor γ-chain (γc) pathway may specifically regulate the expression of HB-EGF in NK cells, other γc-dependent cytokines such as IL-4 (53) or IL-21 (54), known to modulate NK cell effector functions, failed to do so (C. Costantini, A. Micheletti and M. A. Cassatella, unpublished observations). Whatever the case is, analysis of the mechanisms involved in the IL-2- and IL-15-dependent induction of HB-EGF expression in NK cells revealed no dependence on endogenous GM-CSF, as indicated by the following observations: (i) addition of GM-CSF to NK cells induced little, if any, HB-EGF mRNA expression; (ii) exposure of IL-2- or IL-15-activated NK cells to αGM-CSF antibodies did not impair the induction of HB-EGF mRNA as well as their sensitivity to DT-induced apoptosis and (iii) HB-EGF mRNA was maximally induced in NK cells by IL-2 or IL-15 as early as after 1 h, a time point in which GM-CSF mRNA was not expressed (C. Costantini, A. Micheletti and M. A. Cassatella, unpublished observations). The role played by NK-derived HB-EGF is not quite clear at this stage, but it is nevertheless of interest, considering the range of biological activities attributed to HB-EGF. For example, HB-EGF is important for reproductive biology, a process in which it appears to regulate the interactions that occur between endometrium and implanting blastocysts (55). Since a population of NK cells with unique properties, named uterine NK cells, are present in the uterine mucosa, prior to and during pregnancy, and whose major function might be to assist in fetal development (56), one could speculate that HB-EGF contributes to the execution of their functions. Indeed, even though IL-2 is absent from the decidua and placenta, the levels of IL-15 rise in the decidua following conception for promoting not only the differentiation of endometrial NK cells toward decidual NK cells (57) but also to influence decidual NK cells proliferation and functions (58). Interestingly, CD9, that physically interacts with HB-EGF to form a receptor complex for DT (59), is also highly expressed by uterine NK cells (60). Another known function of NK cells is the immunosurveillance of tumors (61). In this latter context, HB-EGF, produced by both tumor and stromal cells (62), might positively contribute to angiogenesis and tumor growth (63). Thus, since stimulation of NK cells with IL-2 has already been used in cancer therapy as a strategy to activate either endogenous NK cells in patients or in vitro cultured NK cells as part of an adoptive immunotherapy protocol (64), at the light of the results presented in this paper, it should be taken into account that IL-2-activated NK cells might express HB-EGF with potential adverse effects for the outcome of the therapy.

In summary, the present paper extends our knowledge on the potential regulatory roles of NK cells by showing their ability to modulate the survival and activation of neutrophils, key players of the innate immune system. As such, our results hold potential physiopathological implications, the characterization of which represents an open and promising field of investigation.

Note added in proof

During the revision of the manuscript, a study that substantially confirms the results presented herein has been reported (65).

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