Optimal Effector Functions in Human Natural Killer Cells Rely upon Autocrine Bone Morphogenetic Protein Signaling

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Abstract

Natural killer (NK) cells are critical for innate tumor immunity due to their specialized ability to recognize and kill neoplastically transformed cells. However, NK cells require a specific set of cytokine-mediated signals to achieve optimal effector function. Th1-associated cytokines promote effector functions that are inhibited by the prototypic Th2 cytokine IL4 and the TGFβ1 superfamily members TGFβ1 and activin-A. Interestingly, the largest subgroup of the TGFβ superfamily are the bone morphogenetic proteins (BMP), but the effects of BMP signaling on NK cell effector functions have not been evaluated. Here, we demonstrate that blood-circulating NK cells express type I and II BMP receptors, BMP-2 and BMP-6 ligands, and phosphorylated isoforms of Smad-1/-5/-8, which mediate BMP family member signaling. In opposition to the inhibitory effects of TGFβ1 or activin-A, autocrine BMP signaling was supportive to NK cell function. Mechanistic investigations in cytokine and TLR-L–activated NK cells revealed that BMP signaling optimized IFNγ and global cytokine and chemokine production, phenotypic activation and proliferation, and autologous dendritic cell activation and target cytotoxicity. Collectively, our findings identify a novel auto-activatory pathway that is essential for optimal NK cell effector function, one that might be therapeutically manipulated to help eradicate tumors.

Introduction

Natural killer (NK) cells are circulating innate immune sentinels specialized to recognize and kill tumor and virus-infected cells (1). Consisting typically of 2% to 5% of peripheral blood mononuclear cells (PBMC), NK cells represent a significant component of the human immune repertoire. Historically, human NK cells have been subdivided into at least two subgroups based on their expression of CD16 and CD56 (2). CD56 intermediate (int)/CD16+ NK cells are credited with more immediate cytotoxic capability, whereas the numerically smaller (in blood) CD56 high (hi)/CD16− subset is associated with more rapid IFNγ release and proliferation upon activation (3, 4). This early IFNγ production can act in an autocrine fashion to stimulate NK cells own effector functions and can also significantly enhance important dendritic cell (DC) functions such as tumor antigen processing, presentation, and cross-presentation for the priming of tumor antigen-specific CD4+ and CD8+ T cells. Nevertheless, uncontrolled proliferation and/or release of NK cell inflammatory cytokines can result in pathology or cellular transformation (5, 6). Thus, the immune system has coordinately developed mechanisms to avoid these potentially life-threatening events through the release of inflammatory mediators (7).

With relation to NK cells, their effector functions are best known to be negatively regulated via engagement of inhibitory receptors with MHC class I proteins or by suppressive cytokines such as IL4 or TGFβ1 (8–10). Although it has been known for some time that exogenous TGFβ1 can suppress NK cells IFNγ production, it is only relatively recently that activin-A (a further TGFβ superfamily member) has also been described to be inhibitory (11). Considering the TGFβ superfamily consists of more than 30 members, our understanding of how members other than TGFβ1 and activin-A may potentially influence NK cells’ effector functions is very poor. Interestingly, the largest subset of the TGFβ superfamily are the BMPs, yet aside from our recent report demonstrating a role for autocrine BMP signaling in human thymic NK cell development (12), the literature is yet to describe the consequences of BMP signaling to NK cells’ effector functions.

The BMPs signal via type I and II serine/threonine kinase receptor heterodimers in complex with the bound ligand. Three type I receptors have been shown to bind BMP ligands, i.e., BMPRIA (or ALK-3), BMPRIB (or ALK-6), and the activin

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receptor ActRIA (or ALK-2). There are also three type II receptors termed BMPRIA, ActRIA, and ActRIBB. Whereas BMPRIA, BMPRIB, and BMPRII specifically bind BMPs, ActRIA, ActRIA, and ActRIBB are also receptors for the activins (13). Activated receptors relay signals to the nucleus predominantly via the canonical Smad-dependant pathway (although noncanonical Smad-independent pathways exist, e.g., p38 MAPK; ref. 14) where phosphorylated (p)-Smads-1/-5/-8 complex with co-Smad-4 and translocate into the nucleus to trigger target gene expression (14, 15).

As their name suggests, the BMPs' originally defined biological function was the capacity to induce bone formation (16). However, the biological actions of the BMPs are now known to be diverse with critical roles in numerous developmental processes (17–21). Indeed, specific BMPs influence the formation of disparate tissues in mammals such as skin, eyes, teeth, heart, kidneys, and testes and dysregulated signaling can result in major muscular and skeletal abnormalities (22–28). These diverse functions are accounted for in part by (i) their significant number; (ii) the existence of numerous antagonists; (iii) their complex interactions with other TGFβ superfamily members; (iv) their promiscuous receptor usage; (v) the presence of corepressors and activators; and (vi) the existence of multiple inhibitors of signaling such as the pseudoreceptor BAMBI, Smads-6 and -7, and the HECT-type E3 ligases Smurf 1 and 2 (13, 29–38).

The BMPs (as with the TGFβs and activins) are pleiotropic in nature and can both inhibit or promote tumorigenesis. Indeed, BMP signaling can have prometastatic effects on breast cancer cells (39) and antagonism of BMP type I receptors has been reported to decrease growth and induce cell death of lung cancer cell lines (40). In contrast, BMP-2 signaling has been demonstrated to inhibit the development of colon cancers (41), whereas BMP-4 can potently inhibit the tumor-initiating capacity of human glioblastoma precursors (42). Strikingly, this study also demonstrated that prophylactic or therapeutic in vivo delivery of BMP-4 dramatically reduced mortality in mice following intracerebral grafting of human glioblastoma cells.

Given that TGFβ1 and activin-A are potent negative regulators of NK cells’ effector functions, we hypothesized that BMP signaling may also have important consequences. Our studies demonstrate that NK cells express cell surface and intracellular stores of BMP receptors, mRNAs for BMP-2 and -6, and p-Smads-1/-5/-8. In sharp contrast with the suppressive effects of TGFβ1 or activin-A (11), inhibition of autocrine BMP signaling in NK cells reveals a novel autocrine activating pathway that confers optimal IFNγ production, global cytokine and chemokine production, phenotypic maturation, proliferation, autologous DC activation, and most importantly cytotoxicity. Furthermore, we have also identified that NK cells resident in the bone marrow of patients with acute lymphoblastic leukemia (ALL) at high risk of relapse display significantly reduced levels of the high-affinity type I BMPR receptor, BMPRIA, and this correlates with a phenotype indicative of a reduced activating state.

These data have important implications for the development of new methods aimed to enhance NK cells’ capacity to kill tumors directly in vivo or potentially to enhance NK cells’ effector functions before adoptive immune therapy into patients with cancer.

Materials and Methods

Cell culture

PBMCs from buffy coats of healthy donors (Red Cross Blood Bank, Melbourne, Australia and Centro de Transfusión de la Comunidad de Madrid, Spain) were prepared by Ficoll-Paque (GE Healthcare Bio-Sciences) density-gradient centrifugation. NK cells were isolated by negative selection using a NK cell Isolation Kit and MACS (Miltenyi Biotech). In some cases, NK cells were further purified by FACS (MoFlow; Beckman Coulter). Unless otherwise stated, culture media consisted of RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 20 mmol/L HEPES, 60 mg/L penicillin, 12.5 mg/L streptomycin, and 2 mmol/L L-glutamine. NK cells were maintained in culture media alone or culture media supplemented with IL2 at 20 to 50 ng/mL with or without the addition of 10 ng/mL IL12 together with 10 µg/mL of poly IC (InvivoGen). In some cases, NK cells were cultured in 20 to 50 ng/mL IL15 alone. CD1c+ myeloid DCs, CD14+ monocytes, and CD4+ and CD8+ T cells were isolated by positive selection by MACS. To generate monocyte-derived DCs (MoDC), CD14+ monocytes were cultured in culture media containing 10% FCS, 20 ng/mL GM-CSF, and 20 ng/mL IL4 (Invitrogen) for 6 to 7 days. For NK cell and DC cocultures, autologous NK cells and CD1c+ or MoDCs were cocultured at a DC:NK cell ratio of 1:1 or 1:5, respectively, for 24 hours. For the experiments shown in Fig. 5, NK cells were treated under the conditions shown for 12 hours then extensively washed before coculture with autologous MoDCs. To assess the effects of blocking BMP signaling, compound C/dorsomorphin, an inhibitor of BMPRIA, BMPRIB, ActRIA, and AMPK, or the highly selective BMPRIA and ActRIA inhibitor DMH1 (both TOCRIS Bioscience), or recombinant human noggin (R&D Systems) was added to NK cell cultures for 12 to 24 hours in the absence or presence of IL12 with or without the addition of IL12 and poly IC.

Quantitative real-time polymerase chain reaction

RNA was isolated using the RNeasy Mini Kit (Qiagen) and cDNA synthesized. Gene expression was quantified using a Stratagene Mx3005P machine. Primers were designed for the detection of BMPRIA, IB, RII, BMP-2, -4, -6, -7, and -7 and used with probes from a Universal ProbeLibrary (Roche Applied Science). Pre-Developed TaqMan Assay Reagents were obtained from Applied Biosystems and included Smad-1 (Hs00195432_m1), Smad-3 (Hs00969210_m1), Smad-7 (Hs00998193_m1), BAMBI (Hs00180818_m1), and TGFβ1 (Hs00998133_m1). 18S rRNA and GNB2L1 were used for normalization. PCR reactions were set up in 96-well plates and analyzed using SDS program version v1.9. The frequency of target gene expression was calculated using the formula $\frac{(ΔC_{T} \text{GOI})}{(ΔC_{T} \text{GOI}) + (ΔC_{T} \text{target gene})}$, where $E' = \text{efficiency}$. Otherwise, the relative expression was calculated using $2^{(-ΔΔC_{T} \text{GOI})}$, where $ΔΔC_{T} \text{GOI}$ is the expression of $\text{GOI}$ relative to different calibrators or controls.

Flow cytometry

NK cells or MoDCs were costained with combinations of fluorochrome-labeled antibodies against human CD1c, CD3, CD14, CD16, CD56, CD69, CD80, CD83, CD94, LAMPI/CD107a, BMPRIA, BMPRIB, BMPRII, NKp30, NKp46, KIR2DL2/L3, and NKp44. NK cells were further stained with a combination of fluoro-488 anti-NKp46 and 7-AAD for viability studies. NKp3 and NKp4 expression was analyzed on an FACSCalibur (BD Biosciences) and data were analyzed with FlowJo software (TreeStar). The geometric mean fluorescence intensity (GmFI) was used to determine the proportion of CD56 bright and CD16 bright NK cells. Also, the proportion of NK cells expressing NKp3 and NKp4 was determined. Statistical comparisons were made using the Mann-Whitney test.
NKG2A, and D (BD Biosciences, BioLegend, and R&D Systems). Intracellular staining was performed using anti-human BMPRIA (R&D Systems) and anti-human p-Smad-1/-5/-8 antibodies (Ser463/Ser465; Santa Cruz Biotechnology). The proportion of apoptotic NK cells was determined by Annexin V (BioLegend) staining and flow cytometry and the data were analyzed using the FlowJo software (version 3.4).

Assessment of NK cell proliferation

Purified NK cells were labeled with 1 μmol/L CFSE (Molecular Probes) at 37°C for 10 minutes. After washing, the cells were cultured in IL2 or IL15 alone or IL2 in combination with IL12 and poly I:C with or without the addition of 100 ng/mL of noggins or 20 μmol/L DMH1 for 5 or 6 days.

Cytokine ELISA and multiplex cytokine and chemokine arrays

Cytokine ELISA kits were used to quantify IFNγ, IL12p70 and TNFα (BD Biosciences and BioLegend). Cytokine and chemokine bead arrays (LINCoplex) were used to quantify IFNγ, IL6, -10, -13, TNFα, GM-CSF, CXCL8, CXCL10, CCL3, and CCL4 and samples analyzed on a Luminex instrument (all Millipore). IL6, -10, GM-CSF, CCL5, CXCL10, and CCL2 were also measured using a Cytometric Bead Array Flex Set system (BD Biosciences).

Assessment of NK cell cytotoxicity

Purified NK cells were cultured with or without increasing concentrations of DMH1 before being washed and then cocultured with the erythroleukemic K562 target cell line in 96-well plates for 4 hours at the effector-to-target ratios indicated. In some cases, DMH1 was added during the 4-hour cytotoxicity assay. Specific lysis was determined using a nonradioactive Cytotoxicity Detection Kit (LDH; Roche Diagnostics) by measuring lactate dehydrogenase activity in culture supernatants. In some experiments, LAMP-1/CDF107a expression by NK cells was assessed by flow cytometry after 4 hours of culture at an NK cell/K562 ratio of 10:1.

Bone marrow samples

Samples were obtained from bone marrow aspirates at diagnosis (n = 12) or at relapse (n = 5) from children with ALL at Hospital Niño Jesús (Madrid, Spain). Patients were treated under the PETHEMA protocol (43). The patients were classified according to this protocol into high risk (n = 5) or low-/intermediate risk (n = 7). Expression levels of different surface markers on NK cells were determined by multiparameter flow cytometry on bone marrow samples. A CD45-positive CD3-negative CD56-positive gate was used for each sample to analyze the NK cell population. Samples were acquired on a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo software.

Statistical analysis

Unless indicated, results are expressed as the mean ± SD of 3 or more donors. Data were analyzed using the Student t test, and P < 0.01 (†) was considered significant.

Results

BMP receptor and ligand gene expression in human NK cells

To assess the relevance of BMP signaling to NK cells, we first investigated BMP receptor and ligand expression at the mRNA level. To do this, we highly purified NK cells (Fig. 1A) and autologous CD4+ and CD8+ T cells from human blood and analyzed the expression of mRNAs for BMP-2, -4, -6, and -7 ligands and the BMP type I (BMPRIA and BMPRII) and type II (BMPRII) receptors by quantitative real-time polymerase chain reaction (qRT-PCR). We found that NK cells exclusively expressed BMP-2, but that each cell type commonly expressed BMP-6 message (Fig 1B). Furthermore, although highly expressed on skeletal muscle controls (not shown), BMP-4 and -7 mRNAs were absent from each of the lymphocyte populations tested (Fig 1B). The analysis further demonstrated that BMPRIA and BMPRII mRNAs are expressed by each of these lymphocyte populations but that NK cells expressed significantly higher levels of BMPRII mRNA than either CD4+ or CD8+ T cells (Fig. 1C). Interestingly, BMPRIB expression was absent from each cell type, suggesting that NK cells and CD4+ and CD8+ T cells are likely to use a BMPRIA–BMPRII receptor pair for BMP ligand binding and signal transduction.

Finally, NK cells express TLR-3, and the TLR-3 ligand poly I:C is currently being evaluated in experimental clinical settings to enhance NK cell–mediated immunity against cancers. Furthermore, we previously have found that the addition of poly I:C to IL2- and IL12-stimulated NK cells significantly enhances their proinflammatory cytokine and chemokine production, proliferation and killing functions (11). We therefore tested whether a brief exposure to IL2 alone or IL2 in combination with IL12, with or without addition of poly I:C could influence NK cells’ mRNA expression of BMP ligands. We found that the expression of BMP-6 mRNAs by NK cells was unaffected by the addition of cytokines or poly I:C, but culture in a low concentration of IL12 to NK cell cultures did not synergize with IL2 to enhance BMP-2 expression, but the addition of poly I:C increased BMP-2 mRNA levels significantly above that induced by IL2 alone (Fig 1D). BMP-4 or BMP-7 mRNA expression, however, was not induced under any of the conditions tested. Furthermore, NK cells cultured in IL2, IL12, and poly I:C significantly upregulated mRNA for BMPRIA and BMP signaling–specific Smad-1, but conversely, simultaneously downregulated mRNA for inhibitory Smad-7, TGFβ1, Smad-3, and BAMBI (Supplementary Fig. S1). To better understand the relationship between NK cells’ BMP receptor mRNA expression and cell surface receptor expression, we screened purified NK cells by flow cytometry for BMPRIA and BMPRII expression at the protein level ex vivo and after exposure to cytokines with or without addition of poly I:C. Immediately following purification from blood, NK cells’ BMPRIA expression ranged between donors from barely detectable (Fig. 2A) to low expression (Fig. 2D). Culture in IL2 induced some upregulation of BMPRIA on NK cells, but this was unaffected by the further addition of IL12.
Figure 1. NK cells express mRNAs for BMP receptors and ligands. A–C, NK cells and autologous CD4<sup>+</sup> and CD8<sup>+</sup> T cells were purified from buffy coats of healthy donors by MACS separation and FACS cell sorting. Highly purified (>99%) CD56<sup>+</sup> CD3<sup>−</sup> cells were then lysed and assessed for expression of BMP-2, -4, -6, and -7 ligands (B) and BMPRIA, IB, and BMPRII (C) by qRT-PCR. D, NK cells were purified by MACS (purity typically >95%) from three donors, then lysed after 4 hours of culture in media without cytokines or in culture media supplemented with IL2 alone, or with IL2 and IL12 with or without the addition of poly I:C. RNA was then extracted and qRT-PCR performed. RNA isolated from skeletal muscle served as positive controls (data not shown). Data are shown as the mean ± SD of three separate donors. *P < 0.01 versus media only and †P < 0.01 versus media only, IL2 alone, or IL2 with IL12.

and poly I:C over the 20-hour culture period (Fig. 2A). These NK cells did however significantly upregulate their expression of the activation marker CD69 when exposed to IL12 and poly I:C (not shown). BMPRIA expression differed and was relatively abundant on CD56<sup>hi</sup> NK cells with levels approaching 50% for some donors (Fig. 2B). As with NK cells’ BMPRIA expression, culture in IL2, IL12, and poly I:C had no significant impact on their expression of BMPRII (Fig. 2B).

NK cells’ interactions with DCs or target cells can significantly influence their phenotype. To test if this was the case for NK cells’ expression of BMP receptors, we cocultured NK cells with activated autologous CD1c<sup>+</sup> myeloid DCs or with K562 target cells and measured BMP receptor expression by flow cytometry. The results demonstrated that neither of these stimulatory conditions had any effect on NK cells’ expression of either BMPRIA or BMPRII (not shown). Thus, stimuli typically associated with the induction of NK cell activation did not affect NK cells’ BMP receptor expression.

To confirm the mRNA and protein expression of BMP receptors and ligands’ facilitated autocrine BMP signaling, we next measured the NK cells’ intracellular p-Smad-1/-5/-8 levels by flow cytometry. Immediately after isolation, a significant proportion of NK cells stained p-Smad-1/-5/-8 positive, and these levels increased through culture in IL2 alone and further increased through addition of IL12 and poly I:C (Fig. 2C). Although these results are in agreement with those showing increased expression of mRNAs encoding BMPRIA and Smad-1 by NK cells cultured under the same conditions (Supplementary Fig. S1), they were not fully reconcilable with our data showing more limited cell surface expression of BMPRIA and differential expression of BMPRII, i.e. between CD56<sup>int</sup> and CD56<sup>hi</sup> NK cells (Fig. 2A and B). To understand this more fully, we explored the possibility that NK cells may have an alternative mechanism to facilitate BMP signaling. Others have shown in transfected cell lines that BMPRIA can be present intracellularly, and to test if this was relevant to NK cells, we compared cell surface and intracellular BMPRIA staining immediately after isolation and following culture in IL2, IL12, and poly I:C. Consistent with our previous data (see Fig. 2A), BMPRIA was upregulated on the surface of a minority (range 2%–15%) of NK cells after stimulation (Fig. 2D). However, in complete contrast with this, the intracellular staining revealed that the majority of NK cells stained BMPRIA positive ex vivo and that these levels increased upon in vitro stimulation with IL2, IL12, and poly I:C (Fig. 2E). The analysis also demonstrated that both the CD56<sup>int</sup> and CD56<sup>hi</sup> NK cell subsets each expressed similar levels of intracellular BMPRIA ex vivo and that each subset increased their expression of intracellular BMPRIA and p-Smads-1/-5/-8 upon stimulation with IL2, IL12, and poly I:C (Supplementary Fig. S2A and S2B).

**Autocrine BMP signaling confers optimal NK cell effector functions**

To address our hypothesis that BMP signaling in NK cells may alter their effector functions, we first used compound C/ dorsomorphin, a chemical known to inhibit BMP signaling by binding to BMPRIA, BMPRIIB, and ActRIB (44). In summary,
Figure 2. NK cells express BMPRIA and BMPRII on their surface. MACS-purified NK cells (purity >95%) were stained with monoclonal antibodies against CD3, CD56, BMPRIA, BMPRII, or p-Smads-1/-5/-8 either immediately after purification or after 12 to 48 hours of culture in 96-well plates at 1 × 10^6 cells per well in media supplemented with IL2 with or without the addition of IL12 and poly I:C. The dot plots show CD56^hi and CD56^int CD3^- NK cells with the exclusion of a small (<5%) population of CD56^- cells. NK cells' surface expression of BMPRIA is shown in A and D, intracellular BMPRIA expression in E, BMPRII expression in B, and intracellular p-Smads-1/-5/-8 expression in C. Data are representative of three to six donors.
the addition of compound C/dorsomorphin to IL2- and IL12-stimulated NK cells resulted in their reduced expression of NKG2D, and in a dose-dependent manner, inhibition of IFNγ release (Supplementary Fig. S3A and S3B). Furthermore, the addition of compound C/dorsomorphin to IL2-, IL12-, and poly I:C-stimulated NK cells resulted in the near complete inhibition of their capacity to kill K562 target cells (Supplementary Fig. S3C).

Given the potentially confounding effects of compound C/dorsomorphin on other kinases, we reassessed our findings by using the well-documented natural BMP antagonist, noggin (45). We first assessed the effects of the addition of noggin on NK cells’ proliferation, as this is an important mechanism to amplify antitumor defenses. To do this, we CFSE labeled NK cells and cultured them for 5 days in IL2, IL12, and poly I:C, with or without noggin. The results demonstrate that this stimulatory combination of cytokines, together with a TLR-3 agonist, was effective at inducing NK cell proliferation (especially in the CD56hi subpopulation; data not shown) but most importantly, inhibition of BMP signaling via the addition of noggin significantly inhibited the proliferation globally (Fig. 3A). Furthermore, in agreement with our findings using dorsomorphin/compound C (Supplementary Fig. S3B), the addition of noggin (even at low concentrations) significantly inhibited IFNγ release by IL2-, IL12-, and poly I:C-stimulated NK cells (Fig. 3B). To extend our analysis, additional donor NK cells were

![Figure 3](https://example.com/figure3.png)

Figure 3. The natural BMP antagonist noggin inhibits NK cells’ proliferation and cytokine and chemokine production. A, MACS-purified NK cells (purity ≥95%) were CFSE labeled, then cultured at 2 × 10⁶ cells per well in 96-well round-bottomed plates in media supplemented with IL2, IL12, and poly I:C in the absence or presence of noggin (100 ng/mL) for 5 days. NK cells’ proliferation was assessed through dilution of CFSE intensity by flow cytometry. B, purified NK cells were cultured at 1 × 10⁵ cells per well in 96-well round-bottomed plates in media supplemented with IL2, IL12, and poly I:C with or without the indicated concentrations of noggin for 2 to 24 hours and IFNγ levels in the NK cells’ supernatants were measured by ELISA. C-L, purified NK cells were cultured in IL2 alone with or without addition of IL12 or poly I:C or noggin (100 ng/mL) for 20 hours and the indicated cytokines and chemokines were measured by Luminex. One representative donor of three is shown in A, whereas in B–L, the data are the mean ± SD of three separate donors. *P < 0.01 versus IL2-, IL12-, and poly I:C-activated NK cells without addition of noggin.
cultured in IL2 alone or together with IL12 with or without the addition of poly I:C or noggin before the supernatants were interrogated for cytokine and chemokine content by LumineX. First, the results demonstrate that the addition of poly I:C (together with IL2 and IL12) induced significant increases in NK cells' release of the majority of cytokines and chemokines tested, i.e., above that induced by IL2 and IL12 stimulation alone (Fig. 3 C–I). This method confirmed and extended our ELISA data by again demonstrating that inhibition of autocrine BMP signaling through the addition of noggin to IL2-, IL12-, and poly I:C-activated NK cells significantly reduced their IFNγ production (Fig. 3C), but also revealed that the addition of noggin suppressed NK cells' production of Th1-associated IL6, TNFα, and GM-CSF (Fig. 3D–F). Th2-associated IL10 and IL13 (Fig. 3G and H), and the chemokines CXCL8, CXCL10, CCL3, and CCL4 (Fig. 3I–L). Noggin neutralizes a number of BMPs (including BMP-2, -4, -5, -6, and -7), but its binding affinity varies, e.g., very high affinity for BMP-2 but moderate affinity for BMP-7. Therefore, to further substantiate our findings, we modified our experimental method by incorporating the use of DMH1, a second-generation compound C/dorsomorphin homolog known to be a highly specific inhibitor of BMPRIA (and also ActRIA). We first tested whether the addition of DMH1 to IL2- and IL12- and poly I:C increased p-Smad-1/-5/-8 induction in each instance (Supplementary Fig. S4A). Finally, further analysis also confirmed that addition of DMH1 to IL2-, IL12-, and poly I:C in the presence of DMH1 had reduced expression of the cytotoxic granule marker LAMP1/CD107a upon subsequent coculture with K562 target cells (Fig. 6B), which most importantly, the addition of DMH1 effectively inhibited p-Smad-1/-5/-8 induction in each instance (Fig. 4A).

Next, to test whether inhibition of NK cells' BMP signaling via DMH1 supported our findings using noggin, we compared the two reagents side by side. The results demonstrated that addition of DMH1 to IL2-, IL12-, and poly I:C-stimulated NK cells inhibited their IFNγ release, and importantly, this was comparable with the inhibition induced by noggin (Fig. 4B), was dose dependent (Fig. 4C), and was not superficially due to inhibitor toxicity (Fig. 4D). Intracellular IFNγ staining also revealed that both CD56dim and CD56bright NK subsets contained IFNγ+ cells upon stimulation with IL2, IL12, and poly I:C, and that the proportion of each diminished upon DMH1 treatment (Supplementary Fig. S4A). Also, similar to noggin's inhibitory effects on NK cell proliferation, blockading autocrine BMP signaling through the addition of DMH1 to either IL2- or IL15-stimulated NK cells also resulted in inhibition of proliferation (Supplementary Fig. S4B and S4C). Finally, further analysis also confirmed that addition of DMH1 to IL2-, IL12-, and poly I:C-stimulated NK cells resulted in a very similar pattern of cytokine (IL6, IL10, and GM-CSF) and chemokine (CXCL10, CXCL10, and CCL2) inhibition as that induced by BMP neutralization through addition of noggin (compare Fig. 3C–L with Fig. 4E). Thus, inhibition of BMP signaling through addition of a naturally occurring antagonist or prevention of BMP signaling through chemically mediated receptor blockade each resulted in a decreased capacity of NK cells to produce inflammatory cytokines and chemokines. Given that each method of BMP signaling inhibition resulted in the same functional outcome, and taking into consideration that small-molecule inhibitors have several advantages over their endogenous counterparts (including lower cost and more consistent activity), we concluded our studies using DMH1.

As mentioned previously, the importance of bidirectional communication between NK cells and DCs for Th1-mediated antitumor immunity is well documented. Therefore, we wished to identify if inhibition of BMP signaling in NK cells resulted in their altered capacity to activate autologous DCs. To do this, we cultured NK cells in the presence or absence of IL2, IL12, and poly I:C with or without the addition of DMH1 for 12 hours before the NK cells were washed thoroughly and then cocultured with autologous MoDCs. After 24 hours, we then assessed the phenotype of the MoDCs and measured cytokine production. In agreement with the literature, activated NK cells had an enhanced capacity to induce CD80 and CD83 expression by MoDCs, but importantly, DMH1-treated NK cells were compromised in this regard (Fig. 5A). In line with these findings, DMH1-treated IL2, IL12 and poly I:C activated NK cells completely failed to induce IL12p70 production by MoDC, and DMH1 treatment inhibited global TNFα production (Fig. 5B). Taken together, these results show that the auto-activatory effects of BMP signaling in NK cells are important not only for their own capacity to produce inflammatory cytokines and chemokines but also for their paracrine capacity to activate DCs.

The engagement of activatory and inhibitory receptors allows NK cells to discriminate between healthy cells and those requiring execution. Therefore, we next examined the significance of autocrine BMP signaling in this character-defining function. These studies first identified that autocrine BMP signaling in NK cells is required for their optimal expression of the activation markers CD69 and Nkp46 (Fig. 6A). Moreover, NK cells cocultured with K562 target cells in the presence of DMH1 completely failed to release IFNγ (Supplementary Fig. S5A). Finally, NK cells activated with IL2, IL12, and poly I:C in the presence of DMH1 had reduced expression of the cytotoxic granule marker LAMP1/CD107a upon subsequent coculture with K562 target cells (Fig. 6B), which most importantly, directly correlated with a dramatic reduction in killing capacity (Fig. 6C). However, the addition of DMH1 during the 4-hour cytotoxicity assay had no inhibitory effect on NK cells' cytolytic capacity (Supplementary Fig. S5B). Taken together, our data define autocrine BMP signaling as an inherent pathway underpinning NK cells' most defining property, killing.

In our concluding analyses, we wished to understand if NK cells from patients with cancer displayed some changed phenotypic expression within the BMP signaling system and whether this correlated with other important markers of NK cell activation. To this end, we collected bone marrow samples from patients with ALL who were assessed as being at low/intermediate or high risk of relapse, or those having already relapsed. The analysis demonstrated that NK cells from relapsed patients had significantly reduced expression of the activation marker CD69 as well as the natural cytotoxicity receptors Nkp30 and Nkp46 (Fig. 7). Conversely, high risk and relapsed patients coexpressed increased levels of the inhibitory receptors NKG2A and KIR2DL1/3, respectively. Most
importantly, the analysis demonstrated that bone marrow NK cells from high-risk patients had significantly reduced ($P < 0.05$) expression of the high-affinity BMP binding receptor BMPRIA and relapsed patients had reduced ($P = 0.06$) expression (Fig. 7). Thus, lower levels of BMPRIA expression correlate with an NK cell phenotype representing reduced activatory marker and increased inhibitory marker expression.

**Discussion**

A major distinguishing feature separating the BMPs from the TGFβs and activins is that the BMPs predominantly signal via a complex of p-Smads-1/-5/-8, whereas the TGFβs and activins share the use of Smads-2 and -3 (14). It was these differences in Smad usage that led to the synthesis of our hypothesis that BMP signaling might influence NK cells’
effector functions differently to the inhibitory signals provided by TGFβ1 (10,46) or activin-A (11). Our first PCR-based screen aimed to identify whether blood-circulating human NK cells (and CD4+ and CD8+ T cells) express a genetic signature indicative of a capacity to respond to and/or produce specific BMP ligands. These results identified that NK cells express mRNAs for BMPRIA (the principal BMP ligand binding receptor) as well as mRNAs for BMPRII (the exclusive BMP type II receptor; ref. 14). It is interesting that neither NK cells nor CD4+ or CD8+ T cells expressed mRNAs for BMPRIB. These data are therefore the first to demonstrate that NK cells have the genetic makeup to express the prototypic BMP type I–II receptor pair. Furthermore, the analysis revealed the novel findings that these same NK cells contained mRNAs for distinct BMP ligands, i.e. BMP-2 and -6 but not BMP-4 or -7. In comparison with CD4+ or CD8+ T cells, NK cells were exclusive in their expression of BMP-2 mRNAs, but interestingly, each lymphocyte population expressed BMP-6 mRNAs. Taken together, this information formed the foundation of our investigations, which led to our demonstration that inhibition of

Figure 5. DMH1-treated NK cells display a reduced capacity to induce DC maturation. NK cells were cultured for 12 hours in culture media or IL2 (20 ng/mL) or IL2, IL12, and poly I:C with or without the addition of DMH1 (20 μmol/L). NK cells were then washed three times before additional culture alone or together with immature in vitro derived MoDCs at a ratio of 1:5. After 18 hours of culture, the levels of CD80 and CD83 expressed by MoDCs (A) was determined by flow cytometry and in B, the levels of IL12p70 and TNFα in the culture supernatants were determined by ELISA. One representative donor of three is shown in A and the data are the mean ± SD of three separate donors in B. *: P < 0.01 versus IL2-, IL12-, and poly I:C-activated NK cells without DMH1 treatment. B, the addition of LPS to MoDC served as a positive control.
autocrine BMP signaling in cytokine and TLR-3–stimulated NK cells confers optimal (i) IFNγ and global cytokine and chemokine production; (ii) phenotypic activation and proliferation; (iii) autologous DC activation; and (iv) cytotoxicity.

To date, the literature describing roles for TGFβ superfamily members in the mediation of NK cells effector functions has almost exclusively focused on TGFβ1. However, we recently demonstrated that activin-A displays some similarities in function to TGFβ1 in that it inhibits NK cells proliferation and cytokine and chemokine production, but unlike TGFβ1, does not directly affect their cytolytic capacity (11). This is particularly relevant considering we have found that activated human CD1c⁺ myeloid DCs and MoDCs produce large amounts of activin-A yet questionable levels of TGFβ1 (47). These findings are intriguing and raise the possibility that it is DC-derived activin-A that is the most relevant negative regulator of NK cells’ functions during bidirectional DC/NK cross-talk.

Most interestingly, our current findings open the possibility that individual TGFβ superfamily members may play very distinct (as yet unrealized) roles in NK cell biology. Contextually, our current studies complement and extend our recent report that shows that the subpopulation of early human intrathymic CD34⁺CD1a⁻/CD0⁺ progenitor cells that express BMPRIA contains a large population of NK cell lineage committed precursor cells (12). Indeed, in the presence of exogenous IL15 autocrine BMP signaling promotes human intrathymic progenitor differentiation in vitro into an immature CD56low/CD161low population that remains predominantly BMPRIA⁺ and then a major mature CD56int/CD161hi population that has mostly lost BMPRIA expression (12). Likewise, our analysis of human lymph node resident NK cells demonstrated that BMPRIA expression...
receptor exocytosis thus making the receptor available to bind (data not shown). It is certainly possible that BMPs produced BMPRIA expression was the addition of recombinant BMP-2 factor we have tested that results in altered (increased) of important receptors on NK cells, including IL12p70 and are well known to induce or reduce the expression of a number nor their net expression of BMPRII. Considering such stimuli IL12 and poly I:C or co-culture with K562 targets or activated activatory signals to NK cells such as IL2 in combination with moreover, it is noteworthy that the addition of potent activatory signals to NK cells such as IL2 in combination with IL12 and poly I:C or co-culture with K562 targets or activated DCs had no effect on NK cells surface expression of BMPRIA nor their net expression of BMPRII. Considering such stimuli are well known to induce or reduce the expression of a number of important receptors on NK cells, including IL12p70 and TGFβ receptors (respectively; ref. 11), it stands out that the only factor we have tested that results in altered (increased) BMPRIA expression was the addition of recombinant BMP-2 (data not shown). It is certainly possible that BMPs produced by NK cells or other immune or stromal cells could trigger receptor exocytosis thus making the receptor available to bind exogenous ligand. Alternatively, a mechanism may be in place whereby BMPRIA containing "compartments" could fuse with ligand and BMPRII containing endosomes (or other forms of vesicles) to facilitate signaling. In this regard Shi and colleagues described that the FYVE-domain protein endofin, which largely localizes in early endosomes, binds Smad-1 preferentially and enhances Smad-1 phosphorylation and nuclear localization upon BMP stimulation (49). This result suggests that BMP signaling could well be initiated in an early endocytic compartment. Similarly, two other FYVE-domain proteins (SARA and Hgs) also localize in early endosomes and mediate the initiation of TGFβ/activin signaling favoring receptor-induced activation of Smads-2 and -3 (50, 51).

Our initial findings that Compound C/dorsomorphin profoundly inhibited NK cells capacity to kill the K562 human leukemic tumor cell line and inhibited their production of IFNγ significantly raised our awareness of the potential importance of this pathway to NK cells. Indeed, it was this data that prompted our more exhaustive investigations of the effects of BMP signaling inhibition via complementary usage of its natural antagonist noggin and the BMPRIA and ActRIIA inhibitor DMH1. Our data demonstrates that autocrine BMP signal conveys a number of character defining functions to NK cells thus supporting our original hypothesis i.e. that BMP signaling in NK cells may result in functionally distinct outcomes to those induced by activin-A or TGFβ. Indeed, in the absence of autocrine BMP signaling, NK cells' capacity to proliferate was severely compromised, as was their capacity to (i) produce inflammatory cytokines and chemokines, (ii) express phenotypic markers of activation that are known to play important coactivatory roles, (iii) bidirectionally activate autologous DCs, and finally and most importantly, (iv) kill.

The sum of this inhibition could be devastating and life threatening. For example, an NK cell encountering a BMP antagonist-producing tumor or oncogenic virus-infected cell in vivo may become incapacitated and thus functionally defunct. An outcome such as this could clearly have substantial "knock-on" effects, potentially resulting in significantly compromised immunity. This raises the very important question as to the potential production of various BMPs by distinct immune cell populations and to the potential production of BMP antagonists by tumor cells. Indeed, in the context of tumorigenesis, our findings are intriguing and clearly point to the value of a detailed analysis of human immune cell and tumor cell-specific expression of BMP receptors, ligands, and antagonists.

This is especially relevant considering our finding that NK cells resident in the bone marrow of high risk of relapse ALL patients have reduced expression of ligand binding BMPRIA. This raises the possibility that NK cells from patients with some forms of cancer may have a reduced capacity to bind BMP ligands, thus leading to a reduced capacity to receive the activatory signal provided by p-Smad-1/-5/-8 signaling. Clearly, we need to extend this analysis and assess the expression of BMP receptors (cell surface and intracellular) and levels of p-Smad-1/-5/-8 signaling in NK cell populations from patients with other types of cancer. Finally, given inhibition of BMP signaling can so potently inhibit NK cells' effector functions, deliberate activation of this pathway is a logical next step to
understand whether this approach may generate hyperactivated NK cells that are more potent in the killing of tumor cells for immunotherapy. Taken together, our findings have identified a major new piece in the complex puzzle that is the biology of NK cells.

Disclosure of Potential Conflicts of Interest
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