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Research Article

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Phenotypic and Functional Activation of Hyporesponsive KIR^{neg}NKG2A^{neg} Human NK-Cell Precursors Requires IL12p70 Provided by Poly(I:C)-Matured Monocyte-Derived Dendritic Cells

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Abstract

A functionally responsive natural killer (NK)–cell repertoire requires the acquisition of inhibitory NKG2A and killer immunoglobulin-like receptors (KIR) through pathways that remain undefined. Functional donor NK cells expressing KIRs for non-self class I MHC ligands contribute to a positive outcome after allogeneic hematopoietic stem cell transplantation (alloHSCT) by targeting HLA-matched recipient leukemic cells. Insofar as circulating donor conventional dendritic cells (DC) reconstitute with comparable kinetics with donor NK cells after alloHSCT, we used hyporesponsive KIR^{neg}NKG2A^{neg} precursor cells to evaluate how specific DC subtypes generate a functionally active NK-cell repertoire. Both monocyte-derived DCs (moDC) and Langerhans-type DCs (LC) induce KIR^{neg}NKG2A^{neg} precursor cells to express the inhibitory receptors NKG2A and KIR, without requiring cell proliferation. Poly(I:C)-matured moDCs significantly augmented the expression of NKG2A, but not KIR, in an IL12p70-dependent manner. Although all DC-stimulated KIR^{neg}NKG2A^{neg} cells were able to acquire cytolytic activity against class I MHC-negative targets, the ability to secrete IFNγ was restricted to cells that were stimulated by IL12p70-producing, poly(I:C)-matured moDCs. This critical ability of poly(I:C)-matured moDCs to provide IL12p70 to developing KIR^{neg}NKG2A^{neg} precursors results in a dominant, multifunctional, NKG2A^{pos} NK-cell population that is capable of both cytolysis and IFNγ production. Poly(I:C)-matured moDCs are, therefore, the most effective conventional DC subtype for generating a functionally competent NK-cell repertoire by an IL12p70-dependent mechanism. *Cancer Immunol Res; 2(10); 1000–10. ©2014 AACR.*

Introduction

Dendritic cells (DC) are the primary orchestrators of the quality and magnitude of the immune response (1–3), with distinct DC subsets playing central roles in natural killer (NK)–cell biology (4, 5). Monocyte-derived DCs (moDC), generated *in vitro* and corresponding to inflammatory DCs *in vivo* (1), are critical for activating resting mature NK cells (5–7), whereas Langerhans-type DCs (LC) are essential to sustaining activated NK-cell viability through their provision of IL15 (5, 8). In contrast to prior studies emphasizing bulk NK cells, the DC-based mechanisms for the development of a functionally responsive NK-cell repertoire from hyporesponsive KIR^{neg}NKG2A^{neg} precursors, which could in turn be manipulated for more effective immunotherapy, remain important unknowns. We therefore hypothesized that distinct human moDC and LC subtypes would make specific testable contributions to the stepwise development of mature, activated NK cells.

A functionally responsive NK-cell repertoire involves the acquisition and engagement of the inhibitory receptors NKG2A and killer immunoglobulin-like receptors (KIR) with their respective cognate ligands, HLA-E (9), and groups of HLA-A, HLA-B, and HLA-C alleles (10, 11). These receptor–ligand complexes render NK cells responsive to activating signals and capable of target-cell lysis and cytokine secretion (12–15). This capability is especially important because NK cells, and at least circulating conventional DCs, are among the first wave of cells to repopulate after allogeneic hematopoietic stem cell transplantation (alloHSCT), and functional NK cells are critical to promoting bone marrow engraftment and a graft-versus-tumor effect, especially against myeloid leukemias (16–19). Therefore, an understanding of how distinct DC subsets and their secreted cytokines mediate the induction of NKG2A and/or KIRs and the functional maturation of NK cells is essential to influencing

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a positive outcome after alloHSCT (19, 20). This knowledge would also help ensure optimal activation of NK cells that are less likely to undergo rapid apoptosis when administered as adoptive immunotherapy after alloHSCT or for the immunotherapy of a variety of cancers (21, 22).

Starting with a subpopulation of hyporesponsive NK cells, which lack both KIRs and NKG2A ($KIR^{neg}NKG2A^{neg}$; refs. 23, 24), we examined the ability of LCs and moDCs to induce both phenotypic and functional maturation and activation of these cells. By exposing moDCs and LCs (5, 8, 25) to a variety of maturation stimuli, including a combination of inflammatory cytokines (general inflammation; ref. 25), lipopolysaccharide (LPS; bacterial TLR4 ligand), or poly(I:C) (viral TLR3 ligand), we recapitulated the inflammatory scenarios in which other groups have reported functional NK maturation (12, 19, 24, 26–30), thereby ascertaining whether and how activated conventional DC subtypes support the generation of a functional NK-cell repertoire. Our findings have important implications for generating functional NK cells for immunotherapy, in which activation by exogenous cytokines alone has not proved optimally effective and the resulting activation-induced cell death has compromised NK-cell expansion after administration *in vivo*.

Materials and Methods

Media and cell lines

Complete RPMI-1640 medium [Memorial Sloan Kettering Cancer Center (MSKCC; New York, NY) Media Prep Core Facility] was supplemented with 10 mmol/L HEPES (Sigma-Aldrich), 2 mmol/L L-glutamine (CellGro; Mediatech), 50 μmol/L 2-ME (Gibco; Invitrogen), 1% penicillin/streptomycin, and heat-inactivated pooled human serum (PHS) from healthy donors (Gemini Bio-Products), as indicated. X-VIVO 15 media (BioWhittaker) were used without supplementation. The class I MHC–negative, NK cell–sensitive cell line LCL 721.221 (ATCC) was maintained in complete RPMI-1640–10% FCS (Gemini Bio-Products) and tested to be *Mycoplasma* free and class I MHC–negative using a FITC-conjugated anti–HLA-ABC monoclonal antibody (mAb; clone G46-2.6; BD Pharmingen). No additional authentication assay was performed. All media and reagents were endotoxin free.

Primary human cells

All collection and use of human specimens adhered to protocols reviewed and approved by the Institutional Review and Privacy Board of MSKCC. Leukocyte concentrates ("buffy coats") from healthy donors were obtained from the Greater New York Blood Center, American Red Cross, or the MSKCC Blood Donor Services. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation over Ficoll-Paque PLUS (GE Healthcare Bio-Sciences Corp.). NK cells were isolated from normal PBMCs using an EasySep negative selection enrichment kit (STEMCELL Technologies) without additional manipulation. Enriched NK-cell purity was routinely >95% $CD3^{neg}CD56^{pos}$. Bulk NK cells were then sorted on either a MoFlo (DakoCytomation) or a FACSAria (Becton Dickinson) flow cytometer for the presence or absence of KIR and NKG2A receptors using the following phycoerythrin (PE)-conjugated mouse anti-human mAbs: anti-CD158a,h (clone EB6.B; anti-KIR2DL1, 2DS1), anti-CD158b1/b2,j (clone GL183; anti-KIR2DL2, 2DL3, 2DS2), anti-NKG2A (clone Z199; Beckman Coulter), and anti-NKB1 (clone DX9; anti-KIR3DL1; BD Pharmingen). Purity after sorting was routinely >98%.

Generation and maturation of DCs

LCs and moDCs (collectively termed DCs, except when the subtypes are specified) were generated respectively from either tissue culture plastic-adherent peripheral blood monocytes or $CD34^{pos}$ hematopoietic progenitor cells, as published previously (8, 25). Both immature moDCs and LCs were terminally matured in 6-well tissue culture plates (Costar) at a concentration of 1×10^6 cells/3 mL/well, by exposure for 2 days to either a combination of inflammatory cytokines [IL1β (2 ng/mL), IL6 (1,000 IU/mL), and TNFα (10 ng/mL), and prostaglandin E2 (5 μmol/L; ref. 25)], termed LC_{cyto} or $moDC_{cyto}$, TLR3 ligand [25 μg/mL poly(I:C); Invivogen], termed LC_{poly} or $moDC_{poly}$, or TLR4 ligand (10 ng/mL LPS; Sigma-Aldrich), termed LC_{LPS} or $moDC_{LPS}$. A $CD14^{neg}HLA\text{-}DR^{bright}CD83^{pos}CD86^{bright}$ flow cytometric phenotype confirmed maturation of moDCs and LCs (25).

NK:DC coculture

Sorted KIR^{pos} and/or $NKG2A^{pos}$ or $KIR^{neg}NKG2A^{neg}$ NK cells were cocultured with either terminally matured allogeneic moDCs or LCs in 96-well round-bottomed plates (Costar) in complete RPMI-1640–5% PHS at a ratio of 10:1 (NK:moDC or NK:LC) for 6 days. No additional exogenous cytokines were added to these cultures.

Flow cytometric analysis

Cells were incubated with fluorochrome-conjugated mAbs and analyzed on either an FC 500 (Beckman Coulter) or an LSRFortessa (Becton Dickinson) flow cytometer with quadrants set to score ≥99% of fluorochrome-conjugated mouse immunoglobulin (Ig) isotype controls (BD Pharmingen and DakoCytomation) as negative. FITC-, PE-, ECD-, APC-, PE-Cy5–, PE-Cy7–, PerCP-Cy5.5–, and AF700-conjugated mouse anti-human mAbs included anti-CD16 (clone 3G8), anti-NKG2D (clone 1D11), anti-CD117, anti-CD127, anti-CD14, anti–HLA-DR, anti-CD86 (BD Pharmingen), anti-CD3, anti-CD56, anti-NKp46, anti-CD83 (Beckman Coulter), and anti-NKB1 (clone DX9; BioLegend). mAbs for sorting were specified above. DAPI (Invitrogen) was used to exclude dead cells. Flow cytometric data were analyzed with FlowJo 9.5 software (TreeStar).

DC cytokine production assay

LCs and moDCs were matured as described above, and supernatants were collected after 48 hours and frozen immediately at −80°C until assayed. The Human Inflammation Cytometric Bead Array Kit (BD Biosciences) measured cytokine production by paired immature and mature moDCs and LCs from the same donors. All experimental sample and standard curve data were acquired in duplicate during the

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same experimental run according to the manufacturer's instructions.

Blocking experiments

In selected NK:DC coculture experiments, soluble IL12p70 was blocked using a neutralizing anti-IL12p70 mAb (clone 24910; R&D Systems). DCs were opsonized before coculture with 5 μg/mL of neutralizing anti-IL12p70 mAb or IgG1 control for 30 minutes on ice. Anti-IL12p70 mAb or IgG1 control was also added at 5 μg/mL to NK:DC cocultures at days 0, +3, and +5.

Proliferation assay

Sorted $KIR^{neg}NKG2A^{neg}$ cells were labeled using the CellTrace Violet (CTV) Cell Proliferation Kit (Invitrogen), according to the manufacturer's instructions. These labeled NK cells were cocultured either alone or with $moDC_{poly}$ in 96-well round-bottomed plates (Costar) in complete RPMI-1640–5%PHS at a ratio of 10:1 (NK:moDC) for 6 days. Dilution of CTV fluorescent intensity among dividing NK cells quantified their proliferation.

CD107a (LAMP-1) mobilization assay

Steady-state or 6-day (d+6) DC-stimulated NK cells (50,000) were added to class I MHC–negative LCL 721.221 cell targets at a 1:1 ratio in complete RPMI-1640–5%PHS, with either FITC- or APC-H7–conjugated anti-CD107a mAb (BD Pharmingen) in a 96-well round-bottomed tissue culture plate (Costar). After 1 hour, monensin (GolgiStop; BD Biosciences) was added at the manufacturer's recommended concentration, and the coculture was continued for an additional 3 hours. Cells activated for granule-mediated cytotoxicity were identified as $CD107a^{pos}$ (31).

Intracellular cytokine production assay

NK cells were activated as described for the CD107a mobilization assay. After 1 hour, brefeldin-A (GolgiPlug; BD Biosciences) was added at the manufacturer's recommended concentration, and the coculture was continued for an additional 5 hours. After surface staining, cells were fixed and permeabilized using a BD Biosciences kit and stained with FITC-conjugated anti-IFNγ mAb or isotype-matched control IgG1 (BD Pharmingen).

Statistical analysis

Comparison of multiple samples was done by one-way ANOVA, followed by either a Dunnett (comparing all populations against a control population) or Tukey (intercomparing all populations) test. Otherwise, statistical significance was calculated using *t* tests. When multiple sample comparisons included paired samples (e.g., $moDC_{poly}$ + anti-IL12 vs. $moDC_{poly}$+IgG1 control), a paired *t* test was first used to compare these samples. If the paired *t* test results for the samples were not significantly different, then each sample pair was averaged and the mean values were used in a one-way ANOVA and the subsequent post-test analysis versus other unpaired (e.g., $moDC_{cyto}$, $moDC_{LPS}$, or LC_{cyto}) sample populations. If the *t* test results for the paired sample populations were significantly different, separate comparisons were conducted between each paired sample and the additional unpaired sample populations using the one-way ANOVA and an appropriate post-test. All statistical analyses were calculated using the Prism 5.0 application program (GraphPad).

Results

NK cells lacking both KIR and NKG2A receptors comprise a distinct, phenotypically mature, but functionally hyporesponsive, population among circulating bulk NK cells

We first characterized the double-negative NK cells lacking both inhibitory KIR and NKG2A receptors ($KIR^{neg}NKG2A^{neg}$), as all subsequent studies would focus on their DC-stimulated activation. A combination of mAbs comprising anti-CD158a,h, anti-CD158b1/b2,j, and anti-NKB1 identified KIR expression. These KIR mAbs could not discriminate, however, between the inhibitory and activating KIRs. The $KIR^{neg}NKG2A^{neg}$ subset accounted for 16.8% ± 3.8% SD ($n = 30$) of steady-state circulating NK cells, with the remainder expressing either KIR, NKG2A, or both (KIR^{pos} and/or $NKG2A^{pos}$). Because NK cells may retain certain KIR molecules intracellularly (32, 33), we stained these cells for intracellular KIR and NKG2A (Supplementary Fig. S1A). As expected, KIR^{pos} and/or $NKG2A^{pos}$ cells expressed KIR and NKG2A intracellularly. In contrast, $KIR^{neg}NKG2A^{neg}$ cells did not express either intracellular or surface NKG2A or KIR, thereby excluding the possibility that $KIR^{neg}NKG2A^{neg}$ cells had simply downregulated the receptors. Despite the differential expression of KIR and NKG2A between KIR^{pos} and/or $NKG2A^{pos}$ and $KIR^{neg}NKG2A^{neg}$ cells, we did not find a significant difference in the surface expression of a variety of other receptors associated with phenotypic maturity between the two NK-cell subsets, consistent with previous reports (Supplementary Fig. S1B; ref. 23). $KIR^{neg}NKG2A^{neg}$ NK cells also demonstrated significantly diminished cytolytic activity ($P = 0.0042$; Supplementary Fig. S1C) and IFNγ secretion ($P = 0.001$; Supplementary Fig. S1D) in response to class I MHC–negative target cells, compared with KIR^{pos} and/or $NKG2A^{pos}$ NK cells isolated from the same healthy donors. Overall, these data confirmed that resting $KIR^{neg}NKG2A^{neg}$ NK cells constituted a functionally hyporesponsive, but phenotypically mature, subpopulation among the total circulating NK cells in the steady state (19, 23, 24).

MoDCs matured with the TLR3 ligand, poly(I:C), efficiently induce NKG2A expression on $KIR^{neg}NKG2A^{neg}$ NK cells in an IL12p70-dependent manner

Despite the known ability of DCs to activate resting bulk NK cells (5–7), how specific DC subsets stimulate the development of hyporesponsive $KIR^{neg}NKG2A^{neg}$ NK cells into NK cells expressing NKG2A and/or KIR family receptors with the capacity for cytolytic degranulation or IFNγ secretion has remained an unanswered question. Because of the critical role that bioactive IL12p70 plays in both NK-cell activation and function (5, 34, 35) and NKG2A induction (36), we first compared

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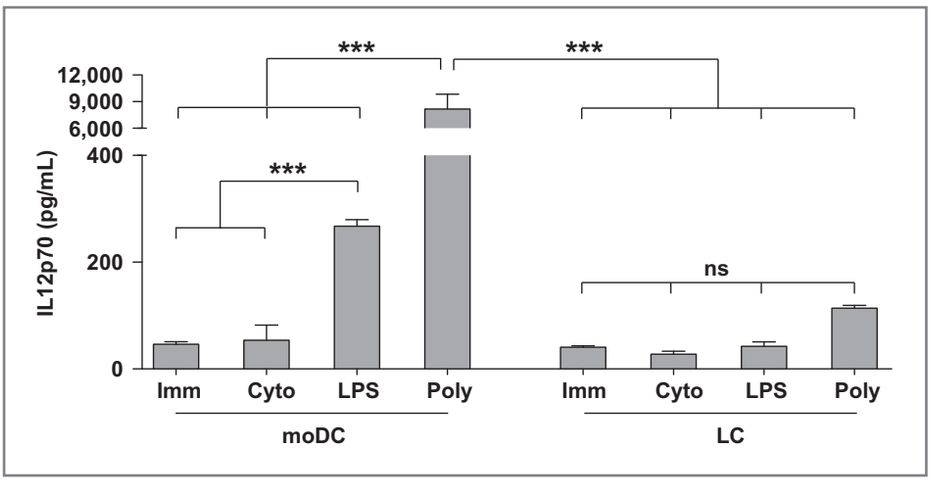

Figure 1. Poly(I:C)-matured moDCs are optimal producers of IL12p70. Soluble bioactive IL12p70 was measured in the supernatants of immature and mature moDCs and LCs (mean ± SD; $n = 3$). Maturation was compared between the following stimuli: inflammatory cytokine cocktail (cyto; ref. 25), TLR4 ligand (LPS), or TLR3 ligand, poly(I:C) (poly). ***, $P < 0.001$; ns, not statistically significant.

moDCs and LCs under different maturation conditions for their capacity to secrete IL12p70. These two DC subsets were therefore evaluated as both immature (imm) and mature (mat) cells. Maturation required 48-hour exposure to an inflammatory cytokine cocktail (25), TLR3 ligand poly(I:C), or TLR4 ligand LPS (Fig. 1). MoDCs stimulated by poly(I:C) ($moDC_{poly}$) produced significantly more soluble IL12p70 than the immature moDCs, the moDCs stimulated by LPS ($moDC_{LPS}$), or the moDCs stimulated by the cytokine cocktail ($moDC_{cyto}$; $P < 0.001$). Even though $moDC_{LPS}$ also produced significant amounts of IL12p70 compared with immature moDCs or $moDC_{cyto}$, the level was still 30-fold less than that produced by $moDC_{poly}$. In contrast, none of the maturation stimuli induced secretion of notable amounts of IL12p70 by LCs. In fact, despite LC expression of TLR3 (37), $moDC_{poly}$ still secreted 70-fold higher amounts of IL12p70. Hence, biologically relevant secretion of IL12p70 after TLR3 signaling by poly(I:C) proved unique to moDCs. For all subsequent experiments, LCs were therefore matured only with the inflammatory cytokine cocktail (LC_{cyto}) for phenotypic and functional comparisons with moDCs.

Because both autologous and allogeneic stimulator DC populations elicit comparable levels of NK-cell activation (5, 6), we focused on the ability of allogeneic LC_{cyto}, $moDC_{cyto}$, $moDC_{LPS}$, or $moDC_{poly}$ to induce NKG2A expression on sorted $KIR^{neg}NKG2A^{neg}$ NK cells (Fig. 2A and B). All matured DC subtypes induced significantly higher NKG2A expression than unstimulated $KIR^{neg}NKG2A^{neg}$ cells cultured in medium alone ($P < 0.001$). Consistent with the capacity of IL12p70 to augment NKG2A expression (36), $moDC_{poly}$ stimulation of $KIR^{neg}NKG2A^{neg}$ cells resulted in nearly 2-fold more NKG2A expression, compared with LC_{cyto}, $moDC_{cyto}$, or $moDC_{LPS}$ stimulation ($P < 0.001$), and this occurred progressively over the 6-day coculture period (Supplementary Fig. S2A). The addition of neutralizing anti-IL12p70 mAb to $moDC_{poly}$:NK-cell cocultures significantly decreased NKG2A expression ($P < 0.001$) to levels comparable with those achieved by the exposure of $KIR^{neg}NKG2A^{neg}$ cells to $moDC_{cyto}$, $moDC_{LPS}$, or LC_{cyto} ($P =$ ns; Fig. 2B). Hence, IL12p70 secreted by $moDC_{poly}$ significantly enhanced NKG2A expression over that mediated by the other conventional DC subtypes, which provided less or negligible IL12p70. Furthermore, when data were restricted to conditions in which the same donor provided moDCs matured by each of the three stimuli [cytokine cocktail vs. LPS vs. poly(I:C)], $moDC_{poly}$ again induced the highest NKG2A expression (data not shown). Collectively, these data demonstrate that whereas all conventional DC subtypes can induce NKG2A on initially $KIR^{neg}NKG2A^{neg}$ NK cells, $moDC_{poly}$ exert superior NKG2A upregulation, mediated by their significantly greater production of bioactive IL12p70.

Induction of KIR on DC-stimulated $KIR^{neg}NKG2A^{neg}$ NK cells is independent of soluble IL12p70

We next determined whether $moDC_{poly}$-secreted IL12p70 would similarly promote KIR acquisition (Fig. 2C and D). All DC subtypes and maturation conditions induced comparable KIR expression when compared with unstimulated $KIR^{neg}NKG2A^{neg}$ NK cells ($P < 0.001$), and this developed progressively over the 6-day coculture period (Supplementary Fig. S2B). Moreover, the addition of neutralizing anti-IL12p70 mAb to $moDC_{poly}$:NK cell cocultures had no effect on KIR induction ($P =$ ns), indicating the independence of KIR expression from IL12p70. Restricting data to each single-donor source, maturation-matched moDC:NK cell cocultures revealed a similar pattern of KIR acquisition.

Induction of NKG2A and KIR on DC-stimulated $KIR^{neg}NKG2A^{neg}$ NK cells does not require cell proliferation

Because moDCs can induce NK-cell proliferation (5), we investigated whether cell proliferation was necessary for the induction of NKG2A and KIR on $KIR^{neg}NKG2A^{neg}$ cells (Fig. 2E and F). Using poly(I:C)-matured moDCs as stimulators, we observed that proliferation was not a prerequisite for the induction of NKG2A or KIR on DC-stimulated $KIR^{neg}NKG2A^{neg}$ NK cells. When proliferative ability could be associated with either NKG2A or KIR induction, only $NKG2A^{pos}$ cells proliferated, consistent with our current understanding of NK-cell differentiation, whereby differentiated KIR^{pos} NK cells have reduced proliferative capacity.

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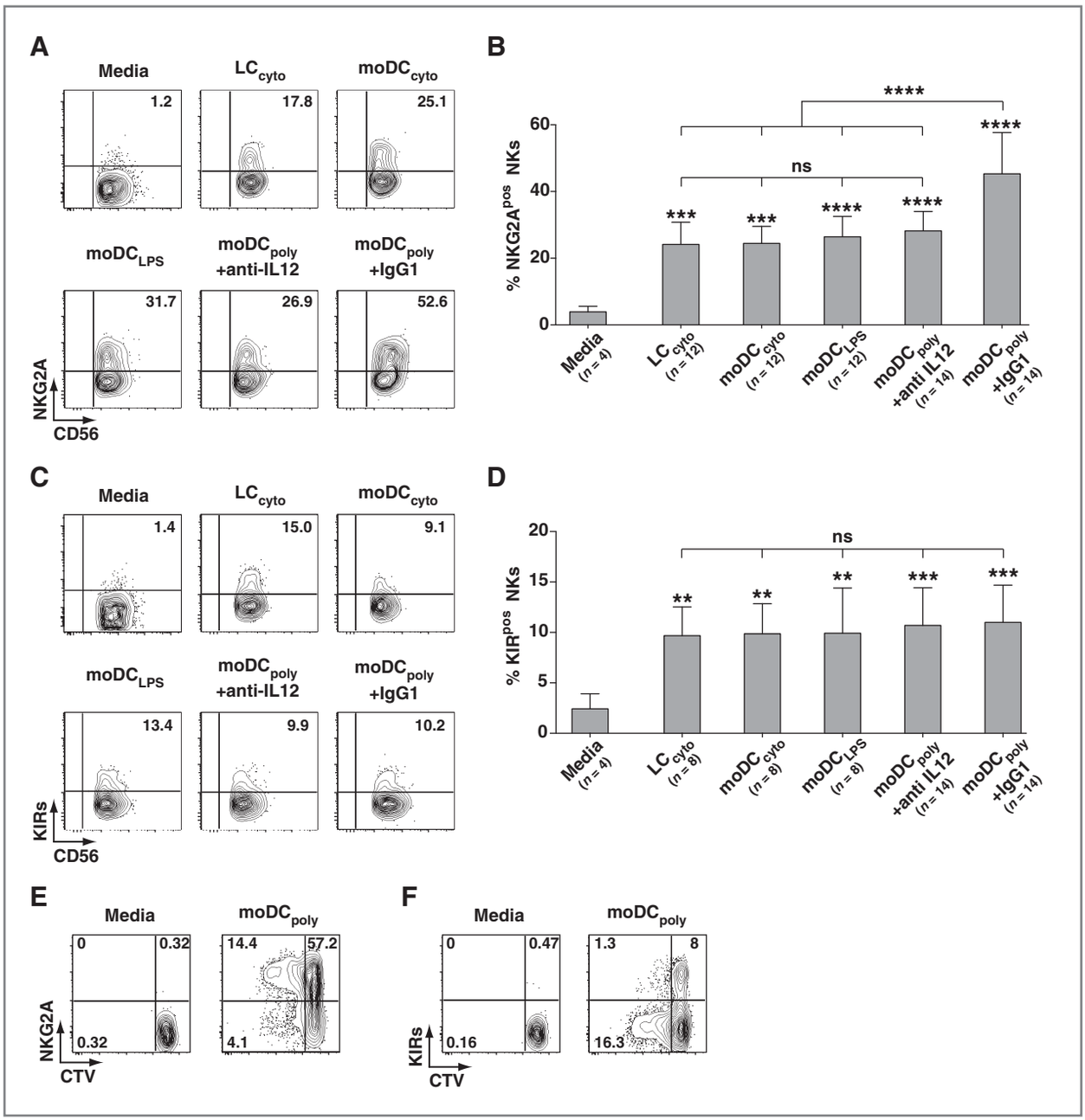

Figure 2. Soluble IL12p70 from poly(I:C)-matured moDC preferentially induces NKG2A but not KIR on KIR^{neg}NKG2A^{neg} NK cells. A–D, freshly isolated NK cells were sorted for the absence of KIR and NKG2A receptors (KIR^{neg}NKG2A^{neg}), then cocultured for 6 days either alone (media) or with LC$_{cyto}$, moDC$_{cyto}$, moDC$_{LPS}$, or moDC$_{poly}$, but without any additional cytokines in culture. A neutralizing antibody to soluble IL12p70 (+anti-IL12) or its isotype control (+IgG1) was added to moDC$_{poly}$ cocultures. After 6 days of stimulation, these KIR^{neg}NKG2A^{neg} NK cells were assayed for NKG2A (A and B) or KIR (C and D) expression, with representative flow cytometric contour plots from the same experimental sample shown. Bar graphs depict pooled phenotypic data from all experiments (mean ± SD). Asterisks above each error bar refer to the statistical difference between that condition and the unstimulated d+6 KIR^{neg}NKG2A^{neg} NK cells (media), whereas statistical notations above the brackets refer to the indicated comparisons between conditions. E and F, CTV-labeled KIR^{neg}NKG2A^{neg} cells were cocultured for 6 days either alone (media) or with moDC$_{poly}$ to correlate proliferation with NKG2A (E) or KIR (F) induction. Proliferation was measured by the dilution in fluorescent intensity of CTV. One representative experiment out of three is shown. **, $P = 0.001–0.01$; ***, $P < 0.001$; ****, $P \leq 0.0001$; ns, not statistically significant.

DC-derived IL12p70 does not influence NK-cell expression of FcγRIII (CD16)

Functional maturation and differentiation of NK cells correspond to the stepwise acquisition of NKG2A and KIR, together with a concomitant increase in the surface expression of FcγRIII (CD16), with terminally differentiated cells having an NKG2A^{neg}KIR^{pos}CD16^{high} phenotype (12, 13, 38). As DC-derived IL12p70 had contrasting effects on KIR and NKG2A

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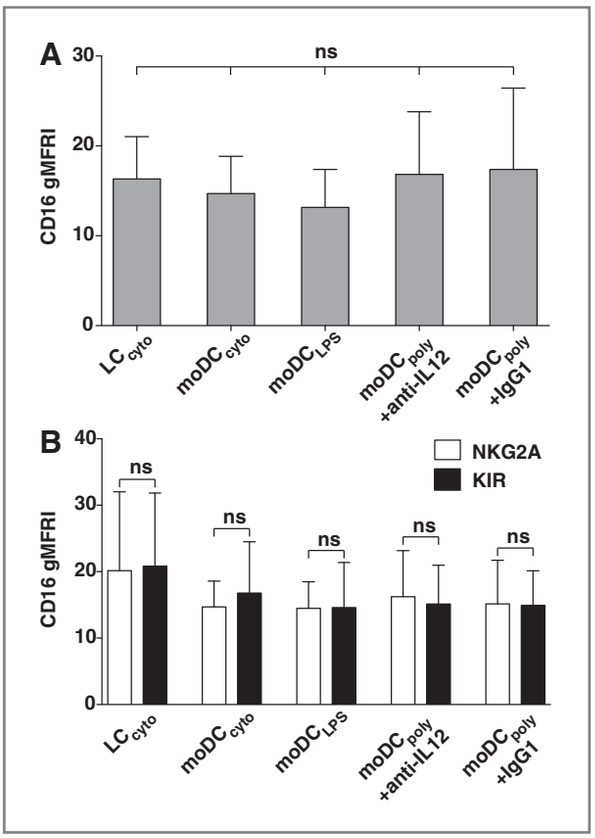

Figure 3. All DC-stimulated KIR^{neg}NKG2A^{neg} NK cells have comparable CD16 expression. Pooled data illustrating CD16 surface expression of (A) stimulated KIR^{neg}NKG2A^{neg} NK cells (mean ± SD; $n = 6$) and (B) NKG2A- or KIR-expressing NK cells (mean ± SD; $n = 4$) following 6 days of DC coculture. Geometric mean fluorescence ratio intensity (gMFRI) relates receptor expression to paired unstained cells. ns, not statistically significant.

induction, and because we could not fluorescently stain stimulated NK cells to distinguish cells induced to express only NKG2A or KIR versus those that expressed both, CD16 served as a proxy marker for phenotypic maturation (Fig. 3). All DC subtypes induced comparable levels of CD16 expression on stimulated NK cells, and blocking soluble IL12p70 in moDC$_{poly}$-stimulated cultures did not alter CD16 induction (Fig. 3A). There was no difference in CD16 expression among initially KIR^{neg}NKG2A^{neg} NK cells that were stimulated by moDC$_{poly}$ or by any other DC subtype to express either NKG2A or KIR (Fig. 3B).

All DC subtypes support cytolytic proficiency of initially KIR^{neg}NKG2A^{neg} hyporesponsive NK cells

Because DC-stimulated KIR^{neg}NKG2A^{neg} NK cells exhibited phenotypic evidence of maturation, albeit with differential responsiveness to IL12p70, we next addressed the corresponding functional competency of cytolytic degranulation after exposure to an NK cell–sensitive, class I MHC–negative cell line. All DC-stimulated KIR^{neg}NKG2A^{neg} cells achieved significantly higher CD107a expression compared with both steady-state KIR^{neg}NKG2A^{neg} NK cells (Fig. 4A) and unstimulated KIR^{neg}NKG2A^{neg} cells cultured for 6 days in medium alone (Fig. 4B). This acquired capacity for cytotoxic response to the class I MHC–negative target cells was comparable with that of steady-state NK cells already expressing KIR and/or NKG2A. MoDC$_{poly}$-stimulated KIR^{neg}NKG2A^{neg} precursors, however,

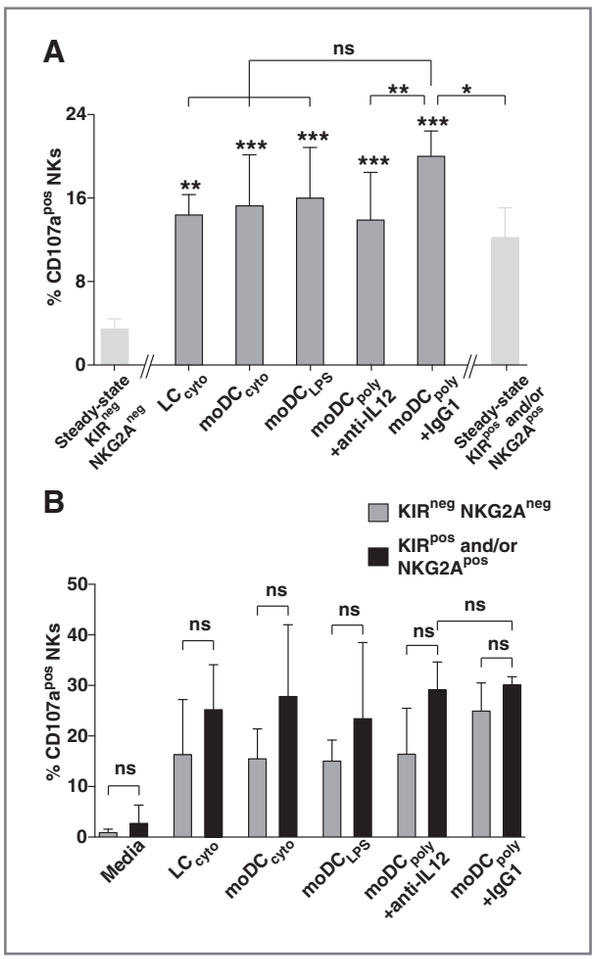

Figure 4. DC-stimulated KIR^{neg}NKG2A^{neg} NK cells display increased cytolytic ability over steady-state NK cells. Sorted, resting KIR^{neg} NKG2A^{neg} compared with KIR^{pos} and/or NKG2A^{pos} NK cells from the same donor, cocultured in the absence of exogenous cytokines for 6 days either alone (media) or with LC$_{cyto}$, moDC$_{cyto}$, moDC$_{LPS}$, or moDC$_{poly}$ supplemented with neutralizing anti-IL12p70 antibody (moDC$_{poly}$ + anti-IL12) or isotype control (moDC$_{poly}$+IgG1), were analyzed for CD107a response to LCL 721.221 target cells. A, pooled data documenting total CD107a response of KIR^{neg}NKG2A^{neg} NK cells after stimulation by indicated DC subtypes (percentage of total NK cells expressing CD107a; mean ± SD; $n = 4$). Cytolytic activity of steady-state KIR^{neg}NKG2A^{neg} and KIR^{pos} and/or NKG2A^{pos} NK cells is depicted in light shading and separated from stimulated data by parallel lines intersecting the *x*-axis. Asterisks above each error bar indicate statistical differences between that condition and steady-state KIR^{neg}NKG2A^{neg} NK cells, whereas notations above the brackets refer to the indicated comparisons between conditions. B, pooled data documenting total cytolytic activity of paired KIR^{neg}NKG2A^{neg} and KIR^{pos} and/or NKG2A^{pos} NK cells from the same donor following 6 days of coculture with the same stimulator DC subtype (percentage of total NK cells expressing CD107a; mean ± SD; $n = 3$). *, $P = 0.01$–0.05; **, $P = 0.001$–0.01; ***, $P \leq 0.001$; ns, not statistically significant.

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achieved significantly higher CD107a mobilization against the LCL 721.221 target cells after activation and maturation, which was IL12p70 dependent (Fig. 4A).

To have a more biologically relevant measure of the ability of DCs to induce functional competency on hyporesponsive KIR^{neg}NKG2A^{neg} NK cells, we compared CD107a expression between KIR^{pos} and/or NKG2A^{pos} NK cells and KIR^{neg}NKG2A^{neg} NK cells isolated from the same donor after 6 days of DC stimulation *in vitro* (Fig. 4B). All DC-stimulated KIR^{pos} and/or NKG2A^{pos} and KIR^{neg}NKG2A^{neg} NK cells achieved significantly higher CD107a expression than unstimulated KIR^{pos} and/or NKG2A^{pos} and KIR^{neg}NKG2A^{neg} NK cells cultured for 6 days in medium alone (Fig. 4B). Although there was a trend toward higher CD107a expression on DC-stimulated KIR^{pos} and/or NKG2A^{pos} NK cells, the differences did not achieve statistical significance, and IL12p70 did not alter CD107a expression by DC-stimulated KIR^{pos} and/or NKG2A^{pos} NK cells (Fig. 4B). These data demonstrate that moDC-derived IL12p70 provides an additional stimulus, but is not essential for conferring cytolytic potential on initially hyporesponsive KIR^{neg}NKG2A^{neg} NK-cell precursors. Furthermore, it confers no additional functional capacity on NK cells already expressing KIR and/or NKG2A.

MoDC-derived IL12p70 is critical in inducing KIR^{neg} NKG2A^{neg} hyporesponsive NK cells to secrete IFNγ

In contrast to CD107a expression, KIR^{neg}NKG2A^{neg} NK cells increased IFNγ secretion in response to LCL 721.221 target cells only after stimulation by moDC$_{poly}$, when compared with the response by resting steady-state KIR^{neg}NKG2A^{neg} NK cells (Fig. 5A). MoDC$_{poly}$-derived IL12p70 also induced higher IFNγ production, compared with other DC stimulatory conditions, achieving IFNγ levels comparable with levels secreted by KIR^{pos} and/or NKG2A^{pos} NK cells in the steady state (Fig. 5A). Blocking IL12p70 with a neutralizing antibody completely abrogated this induced IFNγ response (Fig. 5A). Production of IL12p70 by moDC$_{poly}$ was therefore critical for conferring IFNγ responsiveness on the otherwise hyporesponsive KIR^{neg} NKG2A^{neg} NK-cell population. The more functionally responsive steady-state KIR^{pos} and/or NKG2A^{pos} NK cells, however, secreted significantly more IFNγ after stimulation by each of the moDC conditions and LC$_{cyto}$, compared with paired KIR^{neg} NKG2A^{neg} NK cells from the same donors. Hence, these more developed NK cells proved less dependent on moDC$_{poly}$-secreted IL12p70, even though the observed moDC$_{poly}$ effect was mediated, in large part, by IL12p70 based on the inhibition exerted by the neutralizing anti-IL12p70 mAb (Fig. 5B).

NKG2A expression characterizes cytolytic and IFNγ-secreting NK cells stimulated by IL12p70-secreting moDC$_{poly}$

The above data demonstrated that IL12p70, secreted most abundantly by moDC$_{poly}$, induced the greatest expression of NKG2A. It was also critical to IFNγ secretion, but only marginally contributed to the development of cytolytic capacity by moDC$_{poly}$-stimulated KIR^{neg}NKG2A^{neg} NK-cell precursors. To dissect the role of inhibitory receptor expression in more detail with respect to acquisition of functional competence, we

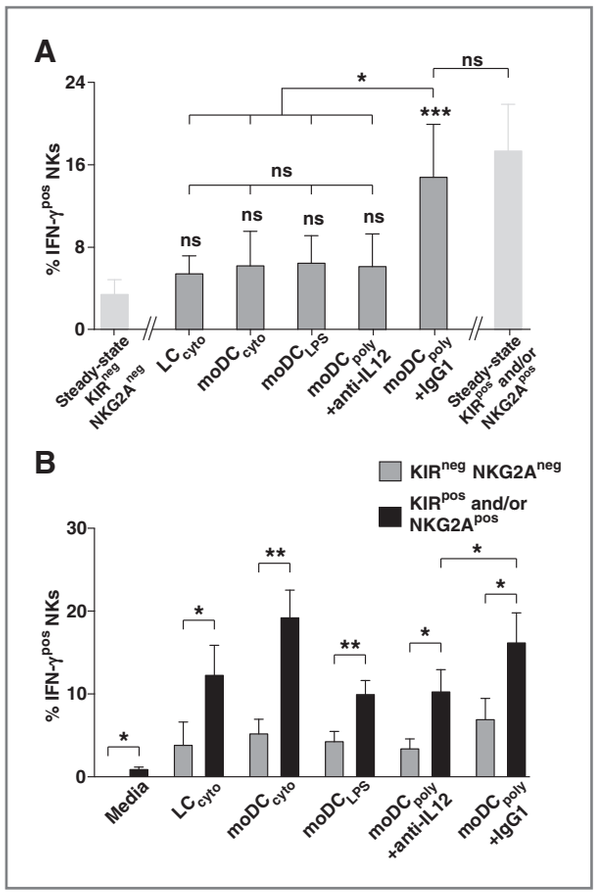

Figure 5. IFNγ production by DC-stimulated KIR^{neg}NKG2A^{neg} NK cells is dependent on IL12p70. Sorted, resting KIR^{neg}NKG2A^{neg} compared with KIR^{pos} and/or NKG2A^{pos} NK cells from the same donor, cocultured in the absence of exogenous cytokines for 6 days either alone (media) or with LC$_{cyto}$, moDC$_{cyto}$, moDC$_{LPS}$, or moDC$_{poly}$ supplemented with neutralizing anti-IL12p70 antibody (moDC$_{poly}$ + anti-IL12) or isotype control (moDC$_{poly}$+IgG1), were analyzed for secretion of IFNγ. A, pooled data documenting total IFNγ secretion by KIR^{neg}NKG2A^{neg} NK cells after stimulation by indicated DC subtypes (percentage of total NK cells expressing IFNγ; mean ± SD; $n = 5$). IFNγ production by steady-state KIR^{neg}NKG2A^{neg} and KIR^{pos} and/or NKG2A^{pos} NK cells is depicted in light shading and separated from stimulated data by parallel lines intersecting the *x*-axis. Notations above each error bar indicate statistical differences between that condition and the steady-state KIR^{neg}NKG2A^{neg} NK cells, whereas notations above the brackets refer to the indicated comparisons between conditions. B, pooled data documenting total IFNγ secretion by paired KIR^{neg}NKG2A^{neg} and KIR^{pos} and/or NKG2A^{pos} NK cells from the same donor following 6 days of coculture with the same stimulator DC subtype (percentage of total NK cells expressing IFNγ; mean ± SD; $n = 3$). *, $P = 0.01$–0.05; **, $P = 0.001$–0.01; ***, $P \leq 0.001$; ns, not statistically significant.

evaluated the proportion of cytolytic, CD107a^{pos} (Fig. 6A) or IFNγ^{pos} (Fig. 6B) NK cells that each expressed NKG2A or KIR after DC stimulation of KIR^{neg}NKG2A^{neg} NK-cell precursors. This was distinct from the prior experiments in which we examined the cytolytic or cytokine secretory capacity of all stimulated NK cells. As shown in Fig. 6A, only moDC$_{poly}$ stimulated significantly greater NKG2A expression among lytic NK cells expressing CD107a after degranulation, an effect

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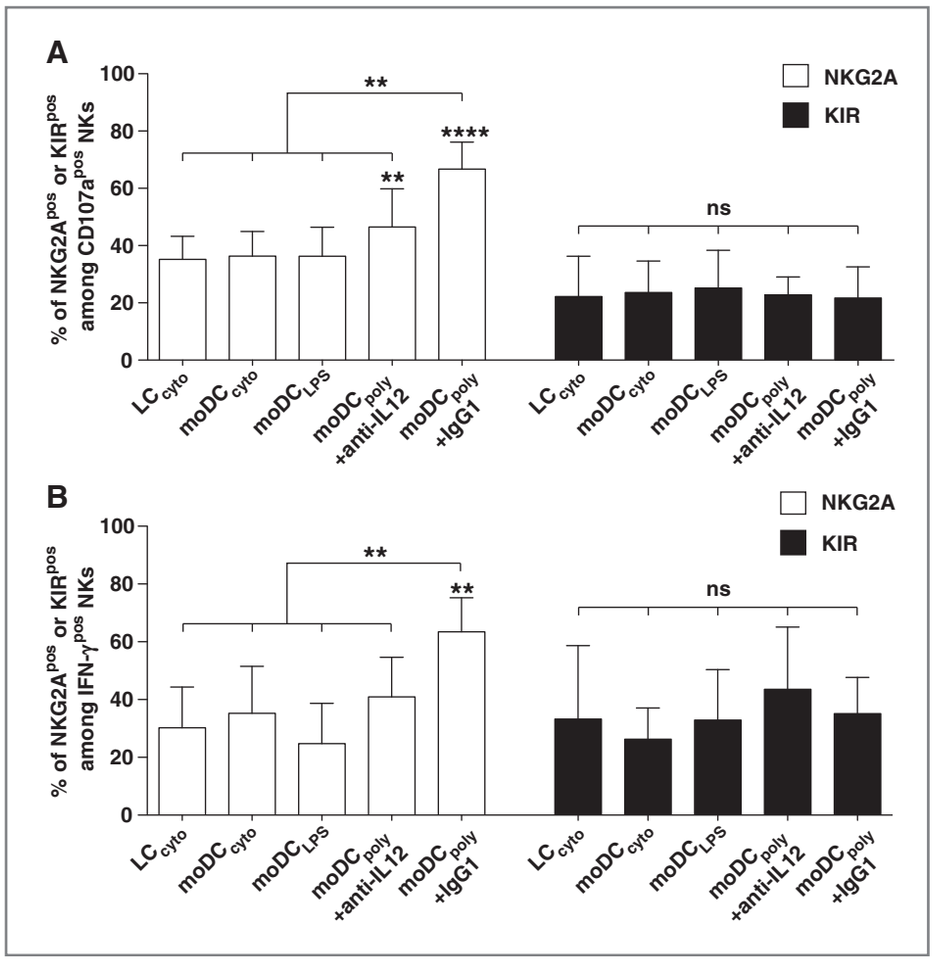

Figure 6. The presence of soluble IL12p70 during moDC$_{poly}$ stimulation of KIR^{neg}NKG2A^{neg} cells results in a dominant population of multifunctional NKG2A^{pos} NK cells. Pooled data illustrating percentage of CD107a^{pos} (A) or IFNγ^{pos} NK cells (B) that express either NKG2A (mean ± SD; $n = 7$) or KIR (mean ± SD; $n = 4$) following 6 days of DC stimulation. Asterisks above error bars indicate statistical differences between NKG2A^{pos} and KIR^{pos} cells for that condition, whereas notations above the brackets refer to the indicated comparisons between conditions. **, $P = 0.001$–0.01; ****, $P \leq 0.0001$; ns, not statistically significant.

largely mediated by IL12p70. All DC stimulatory conditions resulted in comparable KIR expression among the lytic NK cells. Among IFNγ-secreting NK cells, the same pattern emerged (Fig. 6B). Moreover, only the presence of moDC$_{poly}$-derived IL12p70 resulted in significantly more NKG2A^{pos} than KIR^{pos} functional (CD107a^{pos} or IFNγ^{pos}) NK cells, a difference abrogated by the addition of neutralizing anti-IL12p70 mAb to the moDC$_{poly}$:NK cell cocultures. Overall, stimulation of KIR^{neg} NKG2A^{neg} cells by poly(I:C)-matured moDCs generates a population of multifunctional NKG2A^{pos} NK cells, which mediate both degranulation and IFNγ production upon encountering class I MHC–negative NK cell–sensitive target cells.

Discussion

Poly(I:C)-matured moDCs secreting abundant bioactive IL12p70 induced nearly 2-fold higher expression of NKG2A by the initially hyporesponsive KIR^{neg}NKG2A^{neg} NK cells, compared with any other conventional DC subtype or activation condition. IL12p70, in the absence of any exogenous cytokines, also proved critical to the secretion of IFNγ by these moDC$_{poly}$-activated NK cells. All moDCs, regardless of activation condition, as well as activated and matured LCs secreting minimal amounts of IL12p70, induced KIR expression and lytic function in these hyporesponsive KIR^{neg}NKG2A^{neg} NK-cell precursors. IFNγ secretion by the more developed KIR^{pos} and/or NKG2A^{pos} NK cells also exhibited a less stringent requirement for moDC$_{poly}$-derived IL12p70. Evaluation of NKG2A or KIR acquisition in the context of functional capacity demonstrated that NKG2A, but not KIR, expression predominated among both cytolytic and IFNγ-secreting NK cells stimulated by moDC$_{poly}$, which provided critical IL12p70 to developing KIR^{neg} NKG2A^{neg} precursors. Poly(I:C)-matured, IL12p70-secreting moDCs therefore comprise the most effective conventional human DC subtype for generating a functionally competent NK-cell repertoire from a starting population of hyporesponsive KIR^{neg} NKG2A^{neg} NK-cell precursors.

As KIR^{neg}NKG2A^{neg} cells functionally mature, they first develop the ability to kill class I MHC–negative target cells. The capacity to produce inflammatory cytokines follows, in a process characterized by a progressive increase in CD16 surface expression together with the stepwise acquisition of NKG2A (NKG2A^{pos}KIR^{neg}), KIR (NKG2A^{pos}KIR^{pos}), and finally the loss of NKG2A by terminally matured NKG2A^{neg}KIR^{pos} NK cells (12–15, 19, 38, 39). Consistent with this developmental model, our data have demonstrated that all DC subtypes are capable of

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inducing NKG2A and KIR on the initially hyporesponsive NK cells that are devoid of these receptors to a level that is consistent with an intermediate functional/maturation stage, based on a predominance of NKG2A induction together with the ability to degranulate in response to class I MHC–negative target cells. This induction of NKG2A and KIR was not dependent on proliferation, implying a central role for DC intrinsic factors and ruling out the possibility that receptor induction was simply due to proliferation of contaminating $NKG2A^{pos}$ or KIR^{pos} cells. Although the provision of IL12p70 by $moDC_{poly}$ dramatically increased IFNγ production, this did not correspond to a more differentiated stage because neutralizing anti-IL12p70 mAb reduced IFNγ production but had no effect on KIR or CD16 expression. In addition, because induced KIR^{pos} and $NKG2A^{pos}$ NK cells expressed comparable levels of CD16, as well as comparable capacities for lytic degranulation and IFNγ secretion, they likely represent the $NKG2A^{pos}KIR^{pos}$ subset rather than the terminally matured $NKG2A^{neg}KIR^{pos}$ population. Extension of the DC:NK precursor cocultures for an additional 6 days (12 days total), by restimulating the 6-day cocultures with freshly matured DCs from the same donor, in fact decreased NKG2A expression. This concomitantly led to an increase in KIR and CD16 surface expression, reflecting a more terminally differentiated phenotype and excluding receptor regulation solely due to cytokines (Supplementary Fig. S2C and S2D).

A major finding in this study was the crucial role played by $moDC_{poly}$-derived IL12p70, without addition of exogenous cytokines, in conferring functional capacity on the initially hyporesponsive $KIR^{neg}NKG2A^{neg}$ cells. Although $moDC_{poly}$-derived IL12p70 was marginally advantageous for lytic degranulation, it proved critical for functionally activated NK cells to secrete IFNγ. This helps explain why reports of IL12R-β1–deficient patients have a more pronounced reduction in IFNγ secretion than CD107a expression due to the major effect of impaired IL12/23 signaling (40). These results also reinforce observations both in healthy individuals (13) and in patients after alloHSCT (15), in which $NKG2A^{pos}$ cells initially unable to produce cytokines were triggered to produce IFNγ after overnight stimulation with IL12 and IL18. These IL12R-β1–deficient patients also had no defects in receptor phenotype, which is consistent with our finding that the presence of IL12p70 in $moDC_{poly}$ cocultures had no beneficial effect on KIR induction. In addition, we also found no significant difference between the abilities of DC subsets and soluble IL15 (10 ng/mL) to induce KIR expression on $KIR^{neg}NKG2A^{neg}$ cells (unpublished observations), and in fact we observed comparable levels of KIR induction as previously reported by Cichocki and colleagues (41).

Although the cellular and cytokine conditions that transform hyporesponsive NK-cell precursors to active effectors has heretofore eluded investigators (42), these data establish a crucial role for DCs, and especially $moDC_{poly}$-secreted IL12p70. By using distinct DC maturation stimuli that corresponded to bacteria, viruses, or nonspecific inflammation, we recreated the conditions needed for the generation of a functional NK-cell repertoire (12, 19, 24, 26–30), and have demonstrated that DCs represent a common denominator capable of linking all of these studies. The ability of LCs to confer some functional competency on hyporesponsive $KIR^{neg}NKG2A^{neg}$ NK cells, albeit less than that stimulated by $moDC_{poly}$, is surprising in light of our previous report that LCs cannot activate bulk resting NK cells (5). An important distinction, however, is that the studies in this report used phenotypically sorted $KIR^{neg}NKG2A^{neg}$ NK-cell precursors that are functionally hyporesponsive, as opposed to the bulk NK cells used in our previous study. Although LCs and moDCs have comparable levels of class I MHC expression, LCs express nearly 2-fold more of the NKG2A ligand, HLA-E, on their surface (unpublished observations). Because LCs can present substantial amounts of IL15Rα/IL15 complexes to responder lymphocytes to promote the survival and activation of NK cells (5, 8, 43–45), we postulate that increased HLA-E expression might counterbalance an otherwise unchecked activation of NK cells. Although our studies do not exclude roles for other DC subtypes, in particular type I IFN-secreting plasmacytoid DCs, we focused on moDCs and LCs, as these are the principal DC sources of IL12p70 and IL15, respectively, for NK-cell activation and survival (5).

The ability of DCs to generate a functional NK-cell repertoire is perhaps most clinically relevant in the setting of alloHSCT, in which the reconstitution of NK subsets, along with the acquisition of inhibitory receptors and functional maturation, provides critical early effectors of graft-versus-leukemia activity, especially against myeloid malignancies (14, 15, 19, 46, 47). Overall, stimulation of $KIR^{neg}NKG2A^{neg}$ NK cells by poly(I:C)-matured moDCs resulted in an IL12p70-dependent $NKG2A^{pos}$ multifunctional population, which could both degranulate and produce IFNγ upon encountering class I MHC–negative target cells. These data indicate that poly(I:C)-matured moDCs are the most effective DC subtype for stimulating a functionally competent NK-cell repertoire, suggesting that this population may accelerate NK-cell reconstitution after alloHSCT. One of the major challenges to using NK cells for adoptive cellular immunotherapy, however, is the maintenance of their viability and expansion *in vivo* after exogenous activation *in vitro*. The use of moDCs to provide bioactive IL12p70, together with LC-derived IL15, should avoid the dependence on high levels of exogenous cytokines *in vitro*, which renders NK cells more sensitive to apoptosis *in vivo* as cytokine levels become greatly diluted (Supplemental Fig. S2C and S2D; refs. 5, 8, 22). Studies are under way to determine the conditions by which moDCs and LCs can recapitulate the development of donor NK cells reactive against missing ligand in HLA-matched recipients (19).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S.A. Curran, J.W. Young
Development of methodology: S.A. Curran, M.G. Kennedy, J.W. Young
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.A. Curran, E. Romano, M.G. Kennedy, J.W. Young
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.A. Curran, E. Romano, M.G. Kennedy, K.C. Hsu, J.W. Young
Writing, review, and/or revision of the manuscript: S.A. Curran, E. Romano, K.C. Hsu, J.W. Young
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.A. Curran, M.G. Kennedy, J.W. Young
Study supervision: S.A. Curran, J.W. Young

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Cancer Immunology Research

Phenotypic and Functional Activation of Hyporesponsive KIR^{neg} NKG2A^{neg} Human NK-Cell Precursors Requires IL12p70 Provided by Poly(I:C)-Matured Monocyte-Derived Dendritic Cells

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