Abstract

Purpose: Immune checkpoint inhibitors have recently revolutionized cancer immunotherapy. On the basis of data showing KIR-ligand mismatched natural killer (NK) cells reduce the risk of leukemia and multiple myeloma relapse following allogeneic hematopoietic stem cell transplantation, investigators have developed a checkpoint inhibition antibody that blocks KIR on NK cells. Although in vitro studies suggest the KIR2D-specific antibody IPH2101 induces KIR-ligand mismatched tumor killing by NK cells, our single-arm phase II clinical trial in patients with smoldering multiple myeloma was prematurely terminated due to lack of clinical efficacy. This study aimed at unveiling the underlying mechanisms behind the lack of clinical efficacy.

Experimental Design: Treatment-naive patients received an intravenous infusion of 1 mg/kg IPH2101 every other month for up to a year. Peripheral blood was collected at baseline and 24 hours after first infusion, followed by weekly samples for the first month and monthly samples thereafter. NK cell phenotype and function was analyzed using high-resolution flow cytometry.

Results: Unexpectedly, infusion of IPH2101 resulted in rapid reduction in both NK cell responsiveness and KIR2D expression on the NK cell surface. In vitro assays revealed KIR2D molecules are removed from the surface of IPH2101-treated NK cells by trogocytosis, with reductions in NK cell function directly correlating with loss of free KIR2D surface molecules. Although IPH2101 marginally augmented the antmyeloma cytotoxicity of remaining KIR2Ddim patient NK cells, the overall response was diminished by significant contraction and reduced function of KIR2D-expressing NK cells.

Conclusions: These data raise concerns that the unexpected biological events reported in this study could compromise antibody-based strategies designed at augmenting NK cell tumor killing via checkpoint inhibition. Clin Cancer Res; 22(21); 5211–22.

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See related commentary by Felices and Miller, p. 5161

Introduction

Natural killer (NK) cells play a significant role in the defense against cancer. Early studies identified the lack of MHC class-I expression on target cells as the common denominator for NK cell cytotoxicity and formed the basis for the "missing-self" hypothesis (1, 2). Subsequent research has further revealed that NK cells undergo a functional maturation process referred to as "education" to become highly responsive to cells that lose self-MHC class-I expression (3, 4). The response potential of NK cells is maintained through constant tuning by MHC class-I molecules in the microenvironment (5). Importantly, not all MHC class-I-binding receptors are involved in this process. In humans, signaling through the receptors CD94/NKG2A and killer cell immunoglobulin-like receptors (KIR), but not leukocyte Ig-like receptor (Lir)-1, are reported to tune NK cell responsiveness to targets devoid of HLA class-I (6).

Clinically, NK cells have been shown to mediate antitumor responses in the context of KIR-ligand mismatched adoptive NK cell transfer and allogeneic hematopoietic stem cell transplantation (HSCT; refs. 7–9). In both these settings, donor NK cells are present that can kill patient tumor cells lacking HLA class-I molecules specific for donor KIR ("missing-self"). However, allogeneic HSCT is associated with a significant risk of morbidity and mortality and the HLA types of the patient and the donor may preclude a "missing-self" scenario. Theoretically, these limitations could be overcome by inducing "missing-self" in the autologous setting by antibody-mediated masking of NK cell inhibitory KIRs. Given the recent success of checkpoint inhibitor antibodies, such as anti-CTLA4 and anti-PD1 (10, 11), investigators have now developed antibodies against both KIR and NKG2A receptors to disrupt their signaling through pathways that inhibit NK cell function.

IPH2101 is a clinical-grade fully human antibody that binds to KIR2D molecules. In contrast to tumor-targeting antibodies, the IPH2101 antibody contains an IgG4 constant fragment (Fc) with low affinity for C1q and most Fc receptors (12, 13), which minimizes the risk for both complement-dependent cytotoxicity...
Translational Relevance

Despite solid preclinical in vitro and animal data, most drug candidates fail to reach final approval due to unexpected adverse events or lack of efficacy when evaluated in early clinical trials. This report provides the first insights into the immunologic mechanisms that may compromise the efficacy of a checkpoint inhibitor antibody developed to induce "missing-self" tumor targeting by natural killer (NK) cells in cancer patients. The data in this paper highlight unforeseen negative biological consequences of trogocytosis triggered by therapeutic antibodies when used in patients. Furthermore, this article establishes that NK-cell responsiveness can be detuned in humans by agents that interfere with the homeostatic process of NK-cell education. As the KIR2D-specific checkpoint inhibition antibody IPH2101 is currently being evaluated in other malignancies and similar antibodies targeting other NK-cell receptors (e.g., NKG2A) are under development, our data have high translational relevance and it is important that these unanticipated findings are published in a timely fashion.

(CDC) and antibody-dependent cellular cytotoxicity (ADCC). In vitro studies show that IPH2101 (formerly 1-7F9) augments NK cell-mediated lysis of KIR-ligand matched tumor cells (14, 15) and enhances NK cell–mediated ADCC (15) against antibody-bound tumors without having a deleterious effect on NK cell responsiveness against MHC class-I-deficient targets (16). Moreover, the therapeutic potential of antibody-mediated KIR blockade with IPH2101 has been demonstrated in preclinical mouse models (14–17), forming the basis for trials evaluating IPH2101 in humans with cancer.

In this article, the outcomes of 9 patients with smoldering multiple myeloma following treatment with IPH2101 are reported. Remarkably, during treatment and follow-up, there was no evidence that antibody therapy triggered regression of smoldering multiple myeloma or prevented or delayed progression to multiple myeloma. A correlative analysis on this clinical cohort showed patients who received IPH2101 had an unexpected contraction and reduced cytotoxic function in their circulating KIR2D+ NK cells. As a result, NK cells isolated from these patients after IPH2101 treatment showed only marginally augmented antilymphoma cytotoxicity when cocultured with KIR-ligand matched multiple myeloma cells. In vitro assays revealed that IPH2101 not only blocked KIR2D molecules but also led to a reduction in the number of KIR2D molecules on the NK cell surface by triggering trogocytosis. Furthermore, IPH2101-induced reductions in KIR2D resulted in NK cells becoming hypo-responsive to K562 cells, with declines in NK cell function directly correlating with reductions in the number of free IPH2101-unbound KIR2D molecules on their surface. This report is the first to describe contraction and detuning of NK cells in vivo following KIR blockade with IPH2101 in humans and raises concerns that these effects could compromise the therapeutic efficacy of this agent when used as single therapy.

Materials and Methods

Study design

Treatment-naive smoldering multiple myeloma patients were enrolled on NIH Institutional Review Board (IRB)-approved study NCT01248455 after signed informed consent. The study was an open-label single-arm two-stage phase II clinical trial performed at the NIH (Bethesda, MD) between December 2010 and July 2012 (Supplementary Fig. S1A). Demographics, clinical characteristics, outcomes, and follow-up data on these patients are presented in Supplementary Table S1 and by Korde and colleagues (21). Patients were treated with six intravenous infusions of 1 mg/kg of IPH2101 given every 2 months for one year (Supplementary Fig. S1B), with response rate at one year (response criteria: >50% decline in M-protein compared with baseline) being the primary objective. Secondary objectives included evaluating the biological effects of IPH2101 on the NK cells. Nine patients were enrolled on the first stage according to protocol, but a second stage was not initiated due to lack of patients meeting the study-defined criteria in the first stage (response in 3 or more patients of 9 required for study continuation). Peripheral blood was collected throughout the study as outlined in Supplementary Fig. S1B. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient (MP Biomedicals) and frozen in FBS supplemented with 10% DMSO (both Sigma Aldrich). Healthy-donor PBMCs were processed as described above. Following the completion of protocol NCT01248455, patients who continued to have smoldering multiple myeloma without signs of progression, who remained in an observation mode without going on to subsequent treatment, were enrolled on NIH IRB-approved natural history study NCT01109407 so that follow-up data could be obtained.

Cell lines

The K562 and Raji cell lines were obtained from ATCC in 1999, and the OPM1, Apr1-CAG, and EJM cell lines were obtained from Walter Reed National Military Medical Center (Bethesda, MD) in 2010. All cells were propagated in complete medium [RPMI1640 with 2 mmol/L-glutamine (Life Technologies) supplemented with 10% heat-inactivated FBS]. The cell lines have not been authenticated by the authors.

Antibodies and reagents

The following antibodies and reagents were used: anti-CD56 (B159), anti-CD56 (NCAM-1), anti-CD3 (UCHT1), anti-CD16 (3G8), anti-CD64 (10.1), and IgG1 (MOPC21) from Becton Dickinson; anti-KIR2DL1/DS1 (E6b), anti-KIR2DL2/3/DS2 (GL183), and anti-NKG2A (Z199) from Beckman Coulter; anti-CD107a (H4A3) from BioLegend; anti-Lir-1 (HPF1) from...
IPH2101 Contracts and Detunes NK Cells In Vivo

NK cell degranulation and cytokine production assay
PBMCs were thawed one day prior to the assay and maintained overnight in complete medium with and without 1,000 IU/mL IL2. Both PBMCs and target cells were preincubated with 10 µg of human IgG per 10^6 cells to block Fc receptors prior to assay. IPH2101 was added at a final concentration of 1 µg/mL to cocultures of PBMCs and target cells at a ratio of 1:1 in a final volume of 200 µL in 96-well plates at 37°C and 5% CO2. GolgiPlug (BD) was added one hour into the coculture. After 6 hours of coculture, cells were stained with cell surface mAbs and the LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) for 15 minutes on ice, followed by washing in FACS buffer and permeabilization with Cytoperm/Fix (BD). Staining for intracellular epitopes was conducted after washing in Perm/Wash buffer (BD). Cells were acquired on a BD LSR II Fortessa following washing in Perm/Wash buffer and a final fixation in 1% PFA (MP Biomedicals). In the analysis, NK cells were defined by CD56, CD3, LIVE/DEAD negative lymphocytes.

Trogocytosis assays
Monocytes and NK cells were isolated from PBMCs using the Monocyte Isolation Kit and NK Cell Isolation Kit, respectively (Miltenyi Biotec). Neutrophils were isolated from HetaSep-treated PBMC using Neutrophil Isolation Kit (both from Stem Cell Technology). PKH26 (Sigma Aldrich) was used according to the protocol to label cell membranes when assessing membrane transfer between cells. NK cells were mixed at a 1:1 ratio with either monocytes or neutrophils, and IPH2101 was added at 1 µg/mL. Where indicated, IVIG or anti-FcRI antibodies were added at a final concentration of 10 µg/10^6 cells prior to the assay to block FcRs. At the end of assay, cells were stained with appropriate antibodies and acquired on a BD LSR II Fortessa.

Statistical analysis
Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc.).

Results
Assessment of patients during the active treatment phase of the study, as well as at the 1-year follow-up time point and during long-term follow-up showed no evidence for single-agent IPH2101 having activity for smoldering multiple myeloma. Subsequently, this open-label single-arm two-stage phase II clinical trial was terminated after the first stage given the protocol requirement that enrollment into a second stage would only occur if at least 3 of the first 9 enrolled subjects had a disease response (Supplementary Fig. S1A; Supplementary Table S1; ref. 21). In addition to one patient that progressed to multiple myeloma while actively being treated with IPH2101, two additional patients progressed to multiple myeloma during follow-up (Supplementary Table S1). Of the three patients who developed symptomatic multiple myeloma, one patient (patient 1; Supplementary Table S1) subsequently received treatment with carfilzomib, lenalidomide, and dexamethasone (KRd) and eventually required an autologous bone marrow transplant. The other two patients who developed symptomatic multiple myeloma as well as two patients who continued to have smoldering multiple myeloma were subsequently enrolled and treated on clinical trials evaluating the KRd regimen. Although the sample size of the study was small, the annual progression rate observed in the trial is in line with previously reported progression rates of about 10% per year for untreated smoldering multiple myeloma patients (22). Taken altogether, there was no evidence that single-agent IPH2101 triggered regression of smoldering multiple myeloma or prevented or delayed progression to multiple myeloma.

Administration of IPH2101 results in an immediate and selective decrease in KIR2D expression on circulating NK cells
To gain insight into why IPH2101 treatment was ineffective in our clinical trial, in contrast to preclinical animal models where significant activity was observed (14–17), we first assessed the effects of IPH2101 treatment on NK cells isolated from the blood of patients with smoldering multiple myeloma. With the exception of a small transient decline in week one, IPH2101 infusions did not significantly alter the overall absolute numbers of circulating NK cells (Supplementary Fig. S2). However, a 50% reduction in the percentage of circulating NK cells that expressed KIR2D^+ was observed immediately after administration (Fig. 1A). Furthermore, the NK cell fraction that remained KIR2D^+ had a reduced intensity of KIR2D cell-surface expression (Fig. 1B). Importantly, we used a direct staining method that allowed us to detect both IPH2101-bound (“KIR occupied”) and free KIR2D molecules on a single-cell level. This approach allowed us to determine that reductions in KIR2D occurred as a consequence of this molecule being lost from the surface of NK cells rather than merely being “occupied” by IPH2101. Each IPH2101 cycle triggered a drop followed by a partial recovery in both the proportion and intensity of KIR2D expression, with full recovery to baseline levels not occurring until 5 months after IPH2101 treatment had been discontinued (Fig. 1A and B). Loss of KIR2D expression was observed in all KIR2D^+ NK cell subsets, including NK cells that expressed KIR2D receptors as their only major IHLA class-I-binding receptor (referred to as KIR2D^+; Fig. 1C), and was reciprocal to an increase of NK cells that lacked expression of KIR, NKG2A, and LIR-1 (Fig. 1D). We also observed a similar reduction of KIR2D expression on KIR2D^+ T cells (Fig. 1E). Prior studies have shown that IPH2101 treatment blocks the KIR2D receptor. However, the data provided here show for the first time that IPH2101 treatment also leads to a reduction in the number of KIR2D molecules expressed on the surface of both NK cells and T cells.

Reduced KIR2D expression on patient NK cells can be mediated by IPH2101-induced trogocytosis via FcγRI-expressing monocytes and neutrophils
To better understand the mechanism through which IPH2101 reduces the proportion of circulating KIR2D^+ NK cells in vivo, we studied the KIR2D expression on NK cells in PBMCs and on purified NK cells isolated from healthy donors following exposure to IPH2101 in vitro. KIR2D surface
expression levels did not change when IPH2101 was added to a purified population of NK cells. In contrast, exposure of PBMCs to IPH2101 induced a significant reduction in the proportion of KIR2D⁺ NK cells (Fig. 2A and B). Pan-FcγR blockade of PBMCs with human IgG (IVIG) prior to the addition of IPH2101 almost completely abrogated the loss of KIR2D expression on NK cells (Fig. 2A and B).

Monovalent IgG4 antibodies are known to primarily bind high-affinity FcγRI receptors expressed by mono-myelocytic cells, including monocytes and activated neutrophils (23, 24). Therefore, we next examined whether these cells could mediate reductions in KIR2D expression on NK cells exposed to IPH2101. Coculturing resting neutrophils expressing low levels of FcγRI with NK cells in the presence of IPH2101 did not reduce NK cell KIR2D expression. In contrast, a marked reduction in NK cell KIR2D expression was observed when IFNγ-activated neutrophils with upregulated FcγRI expression were cocultured with NK cells in the presence of IPH2101 (Fig. 2C).

**Figure 1.** Sustained decrease of KIR2D⁺ NK cells in peripheral blood following infusion of IPH2101 in vivo. **A,** the proportion and relative intensity of expression (**B**) of major HLA class-I-binding receptors on NK cells in peripheral blood before and after infusion of IPH2101. GMFI, geometric mean fluorescence intensity. The first data points in both figures defined on day 0 represent results on NK cells obtained from patients prior to receiving antibody treatment. **C,** the relative expression of KIR2D on NK cells expressing KIR2D alone (KIR2D⁺) or on NK cells coexpressing KIR2D with other major HLA class-I-binding receptors (KIR2D⁺). **D,** relative changes in the proportion of NK cell subsets in patients following IPH2101 administration. **E,** the relative reduction of KIR2D expression on NK cells and T cells following IPH2101 administration. C1–C6, cycles 1–6; FU, follow-up. Each symbol represents the mean value (n = 9). Error bars, SEM. Wilcoxon signed-rank test (*, P < 0.05; **, P < 0.01).
Figure 2.
Reduced cell surface expression of KIR2D on NK cells following coculture with FcγRI-expressing cells in the presence of IPH2101. A, representative flow cytometry plots showing the gating strategy for evaluating KIR2D expression on NK cells in PBMCs following exposure to IPH2101 for 4 hours. B, IPH2101 was added to PBMCs on ice after 4 hours of co-culture to define baseline KIR2D expression without affecting its expression. C, KIR2D expression on NK cells (puriﬁed, white columns; among PBMCs, black columns) following exposure to IPH2101 with or without IVIG-mediated FcγR blockade (n = 10). D, KIR2D expression on NK cells exposed to IPH2101 with or without IVIG-mediated FcγR blockade when incubated with resting (white columns) and IFNγ-activated (black columns) neutrophils or resting (white columns) and IFNγ-activated (black columns) monocytes (D, n = 3). E, levels of FcγR expression on resting (thin line) and IFNγ-activated neutrophils (thick line) compared with isotype (thin tinted line and dashed line, respectively). F, levels of FcγR expression on resting (thin line) and IFNγ-activated monocytes (thick line) compared with isotype (thin tinted line and dashed line, respectively). G, KIR2D expression on NK cells in PBMCs exposed to IPH2101 without or with either IVIG-mediated FcγR blockade or FcγR blockade with a speciﬁc FcγRI antibody (n = 7). One of three independent experiments are shown for C, D, E, and F. Columns, mean; error bars, SEM. Wilcoxon signed-rank test (*, P < 0.05; ***, P < 0.01).
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(12, 13), IPH2101 did not induce apoptosis in KIR2D this process (Fig. 2G). Consistent with being an IgG4 antibody g IVIG, selective blockade of Fcᵢ in PBMCs, de abolishes IPH2101-mediated KIR2D reduction on NK cells. Similar to broad-spectrum blockade of FcᵢRs using IVIG, selective blockade of FcᵢRs with a mAb completely abolished IPH2101-mediated KIR2D reduction on NK cells. Determining whether KIR2DSP NK cells via ADCC or CDC (Supplementary Fig. S3).

Recently published data have established that monocyties can remove antibody-bound molecules from the surface of cells by trogocytosis (25–27). Therefore, we next explored whether IPH2101-bound KIR2D could be removed from the surface of NK cells via this mechanism. By labeling the NK cell surface with the membrane dye PKH26 prior to coculturing with monocyties in the presence of IPH2101, we observed a time-dependent loss of KIR2D surface expression on NK cells temporally correlating with a concomitant increase in monocyte uptake of PKH26-labeled NK cell surface membrane (Fig. 3A–C). This process was unidirectional in the NK cell to monocyte direction and completely abrogated when the phagocytic capacity of monocyties was inhibited with phenylarsine oxide (Fig. 3D and E). Western blot assays confirmed NK cell response. Upon treatment with IPH2101, KIR2D surface expression decreased after these cells were cocultured with monocyties in the presence of IPH2101 ( Supplementary Fig. S4). Taken altogether, these in vitro results suggest that the decreased KIR2D expression that we observed on circulating NK cells in patients following IPH2101 treatment may have occurred as a consequence of trogocytosis.

IPH2101 induces hyporesponsiveness of KIR2D-blocked NK cells

NK cells are continuously tuned via HLA molecules; therefore, their ability to kill tumor targets could theoretically be abrogated as a consequence of antibody-mediated KIR blockade. Consistent with the above, we observed KIR2DSP NK cells isolated from patients after IPH2101 infusion had reduced degranulation and cytokine production when cocultured with HLA class-I–deficient K562-cells compared with NK cells obtained from patients prior to antibody treatment (Fig. 4A and B).

Concurrent with literature on NK cell education (5), we observed this level of detuning was less in KIR2D⁺ NK cells coeducated via other receptors (i.e., NKG2A, KIR3DL1) compared with KIR2D⁻ NK cells (Fig. 4C). Remarkably, KIR2D⁻ NK cells that are educated solely via KIR2D receptors acquired similar levels of hyporesponsiveness against K562 cells following IPH2101 treatment as was observed at baseline for uneducated NK cells (pan-negative for KIR/NKG2A/Lir-1). In contrast, NK cells that did not express KIR2D, but were educated via NKG2A or KIR3DL1, remained highly responsive to K562 cells following IPH2101 treatment (Fig. 4A and B). As the majority of NK cells are educated via at least one of these non-KIR2D receptors, the overall responsiveness by the whole NK cell population to K562 cells remained largely unchanged in patients following the administration of IPH2101 (Fig. 4C).

In vitro assays revealed that detuning of KIR2D⁺ NK cells by IPH2101 occurred rapidly following KIR2D ligation (Fig. 4D). Furthermore, there was a direct correlation between the proportions of IPH2101-free or unbound KIR2D molecules on the NK cell surface and NK cell responsiveness to K562 cells (Fig. 4E). As these experiments were conducted with purified NK cells, we can conclude that hyporesponsiveness is mediated directly by the antibody without a need for other cells. Of note, this detuning effect was reversible as recognition of K562 cells was restored to baseline several days after the IPH2101 antibody was washed off the NK cells (Fig. 4F). Taken altogether, these data show the IPH2101 antibody detunes the responsiveness of KIR2D⁺ NK cells in vivo and in vitro, with retuning occurring as the proportion of IPH2101-free or unbound KIR2D molecules increases on the NK cell surface.

KIR2DSP NK cells losing KIR2D surface expression following IPH2101-mediated trogocytosis become hyporesponsive KIR/NKG2A/Lir-1⁺ NK cells

Our data show that KIR2D blockade with IPH2101 rapidly induces hyporesponsiveness of KIR2DSP NK cells to K562 cells. We next investigated whether NK cells that had complete loss of KIR2D expression as a consequence of trogocytosis likewise became hyporesponsive. We observed PBMC from healthy donors incubated with IPH2101 for 24 hours in vitro had a substantial loss of KIR2D expression, with the disappearance of KIR2DSP NK cells being associated with a reciprocal increase in the number of NK cells that lacked KIR, NKG2A, and Lir-1 expression (Fig. 5A). To determine whether KIR2DSP NK cells became hyporesponsive as a consequence of KIR2D loss, we next compared the capacity of KIR2DSP and KIR/NKG2A/Lir-1⁺ NK cells with target K562-cells before and after exposure to IPH2101 for 24 hours in vitro. As shown in Fig. 5B, although the size of the KIR/NKG2A/Lir-1⁺ NK cell subset following IPH2101 exposure increased significantly, as it now contained a large fraction of formerly educated KIR2DSP NK cells (all of which now completely lacked KIR2D expression), this subset remained functionally hyporesponsive to K562 cells. As previously shown, the small fraction of NK cells treated with IPH2101 that remained KIR2DSP (whereby KIR2D was bound by IPH2101 antibodies) developed hyporesponsiveness to K562 cells compared with KIR2DSP⁺ NK cells unexposed to IPH2101. An analysis of patient-derived NK cells prior to and 24 hours after IPH2101 administration revealed similar changes in NK cell phenotype and function (Fig. 5C and D). Taken altogether, these data show that complete removal of KIR2D molecules from the surface of KIR2DSP NK cells by trogocytosis induced hyporesponsiveness to KIR2DSP⁺ NK cells similar to that observed in NK cells that continued to express KIR2D molecules following IPH2101 treatment, where KIR2DSP "blockade" had occurred as a consequence of these molecules being bound by IPH2101.

IPH2101 only marginally augments NK cell killing of KIR-ligand matched multiple myeloma cells

As summarized in Fig. 6A, data presented in this article show that administration of IPH2101 induces contraction and detuned responsiveness of KIR2DSP NK cells in vivo. To evaluate whether IPH2101 would still have the capacity to bolster NK cell killing of KIR-ligand matched multiple myeloma cells, we next cocultured NK cells collected from patients before and
multiple time points after IPH2101 administration with KIR-ligand matched multiple myeloma cells in the presence of IPH2101. Although activation of KIR2DSp patient NK cells ex vivo with IL2 significantly boosted their cytotoxic function against K562 cells (Supplementary Fig. S5), degranulation levels observed with IL2-activated KIR2DSp patient NK cells against multiple myeloma cells were low and increased only modestly following KIR2D blockade with IPH2101 (Fig. 6B). Similar data were obtained when assessing NK cell–mediated tumor targeting based on IFNγ production (data not shown). Collectively, these data show that KIR2D blockade with IPH2101 resulted in a relatively small increase in the antitumor activity by the remaining KIR2D dull NK cell subset.

**Discussion**

Clinical data supporting a role for KIR-ligand mismatched tumor killing by NK cells (7–9) and the recent success of immune checkpoint inhibition antibodies for cancer (10, 11) have motivated clinical studies exploring IPH2101-mediated checkpoint inhibition of NK cell KIR2D in patients with hematologic malignancies. We recently conducted an open-label single-arm two-stage phase II clinical trial with IPH2101 in patients with smoldering multiple myeloma (21). Although IPH2101 was well tolerated, the trial was terminated before going into the planned second stage as none of the first nine subjects enrolled showed a therapeutic benefit. Correlative studies presented in this report show that administration of
Figure 4.
IPH2101 rapidly detunes the responsiveness of KIR2D-expressing NK cells to K562 cells with retuning correlating with increasing IPH2101-free KIR2D expression on the NK cell surface. A, CD107a expression and intracellular IFNγ expression (B) in patient-derived NK cell subsets after coculture with K562 cells (n = 9). The first data points in both figures defined on day 0 represent results on NK cells obtained from patients prior to receiving antibody treatment. C, relative degranulation against K562 cells by the denoted patient-derived NK cell populations 24 hours after IPH2101 administration compared with NK cells collected before treatment (n = 9). D, relative degranulation by NKG2A<sup>+</sup> and KIR2D<sup>+</sup> healthy donor-derived NK cells following coculture with K562 cells at multiple time points after addition of IPH2101 (n = 10). E, correlation between the relative degranulation by KIR2D<sup>+</sup> NK cells exposed to K562 cells and the proportion of IPH2101-free KIR2D molecules on the cell surface of NK cells. F, relative degranulation by NKG2A<sup>+</sup> and KIR2D<sup>+</sup> healthy donor-derived NK cells following coculture with K562 cells after multiple time points after IPH2101 washout (n = 10). Symbols and columns, mean; error bars, SEM. Wilcoxon signed-rank test (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
IPH2101 induced contraction and detuning of KIR2D⁺ NK cells \textit{in vivo}, possibly explaining the lack of clinical efficacy observed in our trial. This is the first report to show that infusions of the KIR2D-specific antibody IPH2101 in patients result in a decrease in the number of KIR2D receptors on the NK cell surface. Prior reports used an indirect method to characterize KIR2D blockade levels by IPH2101, where the assumption was made that the number of KIR2D molecules on the NK cell surface was static and a reduction in KIR2D expression relative to pretreatment baseline occurred solely as the consequence of KIR2D blockade levels by IPH2101 (also known as "KIR occupancy"). In our analysis, KIR2D blockade levels were determined using a direct method, in which fluorochrome-labeled anti-IgG4 antibodies were used together with anti-KIR2D antibodies labeled with a different fluorochrome to detect both IPH2101-bound ("KIR occupied") and free KIR2D molecules on a single cell level. This allowed us to establish that the decreased levels of free KIR2D receptor observed following IPH2101 treatment occurred not only from KIR occupancy, but also as a consequence of loss of KIR2D molecules from the surface of NK cells. Subsequent \textit{in vitro} experiments showed that IPH2101-bound NK cells can lose KIR2D expression as a result of trogocytosis mediated by FcγRI-expressing monocytes and activated neutrophils. In contrast to previous \textit{in vitro} studies, where antibody-mediated KIR blockade led to receptor internalization over a 24- to 48-hour time span (28–30), our \textit{in vitro} studies showed KIR2D molecules were shaved off the surface of NK cells within only a few hours following IPH2101 exposure. The distinct difference in mechanisms and kinetics observed between prior reports and our study may be explained by the previous studies being confounded by the use of mouse anti-human antibodies, whereas the IPH2101 antibody has a fully human IgG4 Fc that binds to human FcγRI. As FcγRI expression is upregulated in both monocytes and neutrophils in patients with multiple myeloma compared with healthy controls, it is highly possible that trogocytosis plays a role in the loss of NK cell KIR2D expression following treatment with IPH2101 (31). However, although our \textit{in vitro} experiments support this hypothesis, the data presented in this article can only be used to confirm that IPH2101 can induce trogocytosis of KIR2D molecules but cannot quantitate the degree to which this phenomenon occurs \textit{in vivo}. The poor predictive value of \textit{in vitro} models on trogocytosis was recently highlighted in a study by Lindorfer and colleagues (32). Using a monkey model, they showed that although IgG efficiently prevented antibody-mediated trogocytosis of complement receptor (CR)2 molecules from the surface of monkey B cells \textit{in vitro}, infusion of the same antibody led to loss of CR2 expression on B cells \textit{in vivo} as a consequence of trogocytosis. Tissue analysis 24 hours after antibody infusion revealed that trogocytosis of CR2 molecules had occurred primarily in spleen and liver, and not in peripheral blood. Hence, these data have established that trogocytosis may occur in tissues other than the peripheral blood, even in the presence of physiologic concentrations of IgG, a phenomenon that would not have been predicted to occur based on \textit{in vitro} experiments alone. Given this and other data showing unexpected \textit{in vivo} mechanisms of action for other checkpoint inhibition antibodies compared with those predicted by \textit{in vitro} experiments (33), it is clear that no \textit{in vitro} system is able to perfectly model what occurs \textit{in vivo}. Therefore, additional clinical studies are

\textbf{Figure 5.} KIR2D⁺ NK cells undergoing IPH2101-induced KIR2D trogocytosis of KIR2D become hyporesponsive KIR/NKG2A/Lir-1⁻ NK cells. \textbf{A}, the proportion of KIR2D⁺ (gray) and KIR/NKG2A/Lir-1⁻ (white) NK cells in PBMC of healthy donor before and 24 hours after exposure to IPH2101 \textit{in vitro} (n = 6). \textbf{B}, degranulation (measured by CD107a) by these respective NK cell subsets following cocultures with K562 cells (n = 6), ns, not significant. \textbf{C}, the proportion of KIR2D⁺ (gray) and KIR/NKG2A/Lir-1⁻ (white) NK cells in patient blood at baseline and 24 hours after administration of IPH2101 (n = 9). \textbf{D}, degranulation (CD107a) by these respective NK cell subsets following coculture with K562 cells (n = 9). Bars, mean; error bars, SEM. Wilcoxon signed-rank test (*, P < 0.05; **, P < 0.01).
needed to clarify the degree to which trogocytosis contributes to KIR2D loss on NK cells in vivo following IPH2101 administration.

Our study is the first to report that antibody-mediated KIR blockade with IPH2101 can functionally detune KIR2D⁺ human NK cells in vivo and in vitro, reducing their ability to secrete IFNγ and degranulate in the presence of K562 tumor cells. This finding contrasts the recent report from Benson and colleagues (34); however, the strategies used for NK cell analysis were different between the studies. Benson and colleagues assessed the function of all KIR2D⁺ NK cells, regardless of coexpression of other receptors that are capable of mediating NK cell education. In contrast, the NK cell subset analysis in our study was much more detailed, allowing us to analyze the responsiveness of NK cells that expressed KIR2D receptors as their only major HLA class-I-B cell receptor.

**Figure 6.** IPH2101 marginally augments targeting of KIR-ligand matched myeloma cells by IL2-activated KIR2DSP NK cells. A, schematic model showing the effects of IPH2101 administration on human NK cells. B, degranulation (measured by CD107a) by IL2-activated KIR2DSP patient NK cells against K562 cells (white bars) and KIR-ligand matched multiple myeloma (MM) cell lines in the absence (gray bars) and presence (black bars) of IPH2101 (IPH; n = 9). Bars, mean; error bars, SEM; FU, follow-up; ns, not significant. Wilcoxon signed-rank test (*, P < 0.05; **, P < 0.01).
binding receptor (KIR2DSP NK cells) that are educated and inhibited solely through these receptors. Furthermore, patients in our study received a higher dose of IPH2101 compared with previous clinical trials, resulting in plasma levels of IPH2101 being sufficient to saturate all surface-expressed KIR2D molecules throughout most of the 2-month interval between cycles, thereby increasing the likelihood that NK cell detuning would occur in vivo (16, 34).

The exact mechanisms involved in NK cell education are not yet fully understood. Transfer of allogeneic NK cells across HLA barriers in humans has not been reported to result in hyporesponsiveness of the alloreactive NK cell subset, despite the absence of cognate HLA class-I molecules in the recipient thought necessary to maintain their responsiveness (35, 36). It has been speculated that this may be due to continuous tuning via cis recognition of self-MHC molecules on the same NK cell surface, a phenomenon that has been proven for Ly49 on mouse NK cells (37–39) but has yet to be shown for KIRs on human NK cells. Our data suggest that the signal required for KIR2D+ NK cells to remain fully responsive is lost when this molecule is blocked with IPH2101. We speculate that the antibody causes steric hindrance of the essential KIR2D–HLA-C interactions necessary for NK cell education but cannot exclude the possibility that conformational changes occurring to KIR2D molecules following antibody ligation could contribute to detuned NK cell function. Global NK cell exhaustion following IPH2101-induced activation of KIR2D+ NK cells is less likely to be the mechanism of antibody-induced hyporesponsiveness to K562 cells as our data show that NK cells coexpressing KIR2D with other receptors that mediate education maintained a higher degree of responsiveness than KIR2DSP NK cells following exposure to IPH2101.

Our clinical trial of single-agent IPH2101 therapy involved a cohort of 9 patients with smoldering multiple myeloma. Unfortunately, no patient demonstrated a clinical benefit at the end of treatment or at their last evaluable follow-up time point. One concern with this therapeutic strategy is that the KIR2DSP NK cell subset that would be predicted to gain cytotoxic function following KIR2D blockade may be too small to mediate an antitumor response, especially when further contracted following IPH2101 administration. Strategies to prevent IPH2101-induced loss of NK cells on NK cells may include modifications of the antibody Fc portion to circumvent FcγRI binding (40, 41). An alternative approach would be to develop anti-KIR Fab fragments totally devoid of the Fc portion, a strategy that has been shown successful when blocking the KIR ortholog Ly49 in preclinical mouse models (16, 17, 42). However, our findings not only raise concerns that the therapeutic efficacy of this antibody could be compromised by reduced size of "missing-self" NK cells, but also by IPH2101-induced detuning or anergy of NK cell function. A better understanding of the mechanisms involved in NK cell education may provide the opportunity to explore alternative strategies to disrupt inhibition: KIR signaling to potentiate NK cell tumor killing without inducing NK cell detuning.

In conclusion, although IPH2101 antibody-mediated KIR2D blockade can augment NK cell targeting of KIR-ligand matched tumor cells in vitro, this effect may be limited in vivo due to antibody-induced hyporesponsiveness and contraction of the KIR2DSP NK cell subset. On the basis of these observations and the negative results of our clinical trial in smoldering multiple myeloma, anti-KIR antibody therapy may be of limited therapeutic value for malignancies when utilized as a single agent. Future studies are warranted to establish if antitumor responses can be achieved with this antibody when used in combination with other agents, such as IMIDs (43) or antitumor antibodies, and/or maneuvers that prevent NK cell detuning and loss of antibody-bound KIR molecules from the NK cell surface.

Disclosure of Potential Conflicts of Interest
N. Korde reports receiving speakers bureau honoraria from Medscape. No potential conflicts of interest were disclosed by the other authors.

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Grant Support
Funding for this study was provided by the NHLBI Division of Intramural Research (DIR), the NCI DIR, the Medical Research Scholars Program (MRSP) program at the NIH, and the Swedish Research Council (to M. Carlsten).

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Received May 5, 2016; accepted May 28, 2016; published OnlineFirst June 15, 2016.

References


Clinical Cancer Research

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