IMMUNOBIOLOGY

Lenalidomide augments actin remodelling and lowers NK cell activation thresholds

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Short title: Lenalidomide lowers NK cell activation thresholds

Word counts: Abstract 240; Main text: 3632

References: 40

Figures: 7
**Key Points**

1. Lenalidomide augments nanoscale rearrangements in cortical actin at the human Natural Killer cell immune synapse.

2. Lenalidomide lowers the threshold for NK cell activation; allowing activation by low levels of ligands on tumour cells.

**Abstract**

As multiple myeloma (MM) progresses, Natural Killer (NK) cell responses decline against malignant plasma cells. The immunomodulatory drug lenalidomide is widely used for the treatment of MM but its influence on NK cell biology is unclear. Here, we report that lenalidomide lowers the threshold for NK cell activation, causing a 66% decrease in the EC$_{50}$ for activation through CD16, and a 38% decrease in the EC$_{50}$ for NKG2D-mediated activation, allowing NK cells to respond to lower doses of ligand. In addition, lenalidomide augments NK cell responses, causing a 2-fold increase in the proportion of primary NK cells producing IFN-γ, and a 20-fold increase in the amount of IFN-γ produced per cell. Importantly, lenalidomide did not trigger IFN-γ production in unstimulated NK cells. Thus, lenalidomide enhances the NK cell arm of the immune response, without activating NK cells inappropriately. Of particular clinical importance, lenalidomide also allowed NK cells to be activated by lower doses of rituximab, an anti-CD20 mAb widely used to treat B cell malignancies. This supports the combined use of lenalidomide and rituximab in a clinical setting. Finally, super-resolution Stimulated Emission Depletion (STED) microscopy revealed that lenalidomide increased the periodicity of cortical actin at immune synapses, resulting in an increase in the area of the actin mesh predicted to be penetrable to vesicles containing IFN-γ. This establishes that nanometre-scale rearrangements in cortical actin, a recently discovered step in immune synapse assembly, are a potential new target for therapeutic compounds.
Introduction

NK cells contribute to defence against cancer by lysis of diseased or stressed cells and secretion of inflammatory cytokines including IFN-γ\textsuperscript{1,2}. NK cell responses are triggered through germ-line encoded activating receptors, including Natural Killer Group 2 member D (NKG2D), which recognises stress-inducible ligands such as MHC Class I chain-related protein A (MICA), and the Fc receptor CD16, which mediates antibody-dependent cellular cytotoxicity (ADCC)\textsuperscript{3-8}. Super-resolution microscopy revealed that activating receptor ligation triggers remodelling of cortical actin in specific domains within the NK cell immune synapse where lytic granules and vesicles containing IFN-γ accumulate\textsuperscript{9-13}.

Multiple myeloma (MM) is a haematological malignancy characterised by a clonal proliferation of plasma cells in bone marrow and is associated with progressive dysregulation of the immune system\textsuperscript{14}. NK cells may initially contribute to the control of malignant cells\textsuperscript{15-17} and evidence suggests that NKG2D is involved in NK cell recognition of bone marrow-derived MM cells\textsuperscript{18}. However, NK cell surveillance and cytotoxicity against MM decreases as the disease progresses\textsuperscript{19-23}. There is some evidence that lenalidomide, used for the treatment of MM, can increase NK cell-mediated lysis\textsuperscript{24}. However, studies also report that lenalidomide does not directly affect NK cell effector functions\textsuperscript{25,26} but rather helps via CD4+ T cell activation\textsuperscript{26}.

Here, we establish that lenalidomide augments NK cell responses directly on both a population level and a single cell level. Crucially, lenalidomide lowered the threshold for NK cell activation through both CD16 and NKG2D, including via rituximab, indicating that NK cells could respond to lower densities of activating ligand. Also, super-resolution STED microscopy revealed that lenalidomide works to augment actin remodelling at the NK cell immune synapse.
Methods

Cells

Primary human NK cells were obtained from healthy donor peripheral blood by negative magnetic selection and cultured as previously described\(^2\(^7\). NK cells were then used 6 days later, unless otherwise stated. Daudi and Raji were cultured in RPMI 1640 (Sigma-Aldrich), 10% fetal calf serum (FCS; Gibco), 2 mM L-glutamine (Gibco) and 1 mM penicillin and streptomycin (Sigma-Aldrich). NK cells were treated with lenalidomide (Celgene Corporation; 30 mM stock in DMSO) at a final concentration of 0.001 µM – 10 µM (as indicated) in culture medium. 150 U/ml hrIL-2 was added alongside lenalidomide or vehicle control (DMSO), unless otherwise indicated. Where indicated, cells were also treated with brefeldin A (5 µg/ml; Sigma-Aldrich).

Cytotoxicity Assay

NK cell cytotoxicity was assessed against Daudi in a standard 5 hour \(^{35}\)S-methionine release assay in triplicate. pNK cells were added to the target cells at an E:T ratio of 10:1 (5 x 10\(^4\) pNK cells per well). After 5 hours, \(^{35}\)S activity in the supernatant was quantified (MicroBeta, Perkin Elmer) and results expressed as percent lysis: (experimental release – spontaneous release)/(maximal release – spontaneous release).

ELISA

NK cells were treated with 0.1–10 µM lenalidomide or DMSO, plus 150 U/ml IL-2 and were plated on antibody- or ligand-coated 96 well plates (100,000 pNK cells per well) and incubated for 24 hours at 37°C (5% CO\(_2\)). Antibodies against CD16 (Clone 3G8 BD Biosciences, 1 µg/ml), NKG2D (Clone 149810, R&D systems, 3 µg/ml), 2B4 (Clone 2-69, BD Biosciences, 3 µg/ml) and murine IgG1 isotype control (BD Biosciences) were used. Recombinant proteins used were MICA-Fc (R&D systems; 2 µg/ml unless otherwise stated), ICAM-1 (R&D systems, 2.5 µg/ml) and human IgG (Sigma-Aldrich, 1–500 µg/ml).
For conjugate ELISAs, pNK cells were co-incubated with Daudi or Raji target cells at an E:T ratio of 10:1 (20,000 target cells and 200,000 pNK cells per condition) for 24 hours in the presence of 0.001–10 µM lenalidomide ± 150 U/ml IL-2.

ELISA plates were coated overnight with anti-IFN-γ capture mAb (clone NIB42, BD Biosciences, 1 µg/ml) in binding buffer (carbonate bicarbonate; Sigma), blocked with PBS with 1% BSA + 0.05% Tween-20, and supernatants were added in triplicate for 1 hour. Following this, plates were incubated with biotinylated IFN-γ detection antibody (clone 4S.B3, BD Biosciences, 1 µg/ml) for 1 hour and streptavidin HRP (BD Biosciences), followed by TMB ELISA substrate (Sigma-Aldrich) and the reaction was halted with 0.5 M H₂SO₄. Absorbance was measured at 450nm.

Quantitative RT-PCR

Cells were treated with 1 µM lenalidomide or DMSO plus 150 U/ml IL-2 and were plated on glass slides coated with 0.01% poly-L-lysine and then mAb or recombinant proteins in PBS for 4 hours at 37°C. Antibodies used were anti-CD16 mAb (Clone 3G8 BD Biosciences; 3 µg/ml) and murine IgG1 isotype control (BD Biosciences; 3 µg/ml). Recombinant proteins used were MICA-Fc (R&D systems; 2 µg/ml) and ICAM-1 (R&D systems; 2.5 µg/ml). Cells were then lysed and RNA extracted using an RNeasy kit (Qiagen). RNA was reverse transcribed, and subjected to qPCR (Applied biosystems, CA, USA) using Sybr Green detection for IFN-γ using the following primer pair: Forward primer - 5'-AAAAATAATGCAGAGCCAAATTG -3'; Reverse primer - 5'- TAGCTGCTGGCGACAGTTCA -3'. Data analysed by δδCT method (Livak and Schmittgen 402-08); gene expression was normalised to the housekeeping gene GAPDH, which was detected using the following primer pair: Forward primer - 5'- GAAGGTGAAGGTCGGAGT -3'; Reverse primer - 5'- CATGGGTGGAATCATATTGGAA -3'.

Preparation of coated slides

Eight-well borosilicate coverglass chambers (Lab-Tek, Nunc) were coated with 0.01% poly-L-lysine and then coated with mAb or recombinant proteins in PBS. Antibodies against the following proteins
were used: CD16 (Clone 3G8, BD Biosciences; 3 µg/ml), murine IgG1 isotype control (BD Biosciences), NKG2D (Clone 149810, R&D systems; 3 µg/ml). Rituximab (Invivogen) was also used (10 µg/ml). Recombinant proteins used were MICA-Fc (R&D systems; 2 µg/ml) and ICAM-1 (R&D systems; 2.5 µg/ml).

**Microscopy**

Cells were incubated as indicated and fixed in 4% paraformaldehyde and permeabilised with 0.1% Triton-X-100. For NKG2D and LFA-1 co-stimulation, cells were incubated for 90 min before fixation, as identified as the optimal for IFN-γ production\(^1\). To visualise F-actin, cells were stained with phalloidin-AlexaFluor-488 (Invitrogen) or phalloidin-Atto-590 (Atto-tec). To visualise IFN-γ, cells were stained with AlexaFluor-488 or AlexaFluor-647-conjugated anti-IFN-γ mAb (clone B27, BD Biosciences). Bright-field, fluorescence and STED images were obtained (Leica TCS SP8 STED CW) using a 100x oil immersion lens (NA 1.4) at room temperature. STED of AlexaFluor-488 was achieved using 592 nm continuous-wave fibre laser. For confocal images, 35-40 optical slices were collected at 0.2 µm intervals.

**Image analysis**

For the single cell IFN-γ analysis, confocal images were imported into Imaris software (Bitplane). Individual cells were segmented based on cell body staining (Atto-590 phalloidin) and nucleus staining (NucBlue). IFN-γ vesicles were identified based on Alexa488-IFN-γ mAb staining within each cell. STED images were deconvolved (Huygens; Scientific Volume Imaging), exported in tagged image file format and analysed using MATLAB (Mathworks) as previously described\(^9\).

**Statistical analyses**

Mean values and SEM are shown. Data was analysed by one-way ANOVA and Tukey’s post-test (Prism version 6; GraphPad). For threshold analysis, IFN-γ production was normalised to the
maximum value seen with each receptor and EC\textsubscript{50} was determined by nonlinear regression (Prism version 6; GraphPad).
Results

Lenalidomide directly affects NK cell IFN-γ secretion after activation through different receptors

To test whether lenalidomide affects NK cells directly, human pNK cells were isolated and co-incubated with Daudi cells (a B cell lymphoma line susceptible to NK cell cytotoxicity) in the presence of increasing concentrations of lenalidomide (0.001–10 µM). Lysis of Daudi target cells was significantly enhanced with 0.1–10 µM lenalidomide in the presence of IL-2 (Fig. 1A). The addition of 10 µM lenalidomide, increased the specific lysis of target cells from 13 ± 2% to 30 ± 1% (Fig. 1A).

Secretion of IFN-γ is another important NK cell effector function and here, production of IFN-γ by pNK cells in conjugate with Daudi increased 3-fold in the presence of 1 µM lenalidomide (215 ± 30 pg/ml without lenalidomide; 640 ± 50 pg/ml with 1 µM lenalidomide; Fig. 1B). Similarly, IFN-γ secretion by pNK cells in conjugates with the EBV-transformed B cell line Raji increased 2.5-fold in the presence of 1 µM lenalidomide (from 245 ± 40 pg/ml without lenalidomide to 650 ± 50 pg/ml with 1 µM lenalidomide) (Fig. 1C). These results clarify that treatment of pNK cells with lenalidomide, in the presence of IL-2, has a direct enhancing effect on NK cell cytotoxicity and IFN-γ secretion.

To test whether the lenalidomide-induced increase in IFN-γ secretion occurs when pNK cells are activated via different receptors, NK cells were stimulated on surfaces coated with anti-CD16 monoclonal antibody (mAb), anti-NKG2D mAb, or recombinant MICA, in the presence of 0.1-10 µM lenalidomide. NK cells treated with lenalidomide in uncoated wells or wells coated with isotype-matched control mAb did not secrete IFN-γ (Fig. 1D). In contrast, lenalidomide resulted in a 2-fold increase in the amount of IFN-γ secreted by pNK cells after ligation of CD16 (3700 ± 420 pg/ml without lenalidomide; 8320 ± 370 pg/ml with 1.0 µM lenalidomide) (Fig. 1D). In addition, lenalidomide significantly increased IFN-γ secretion from NK cells co-stimulated through NKG2D and the integrin LFA-1 (via anti-NKG2D mAb or MICA, and ICAM-1 respectively) (Fig. 1E and F). However,
no significant enhancement was observed when NK cells were treated in the presence of anti-NKG2D mAb, MICA or ICAM-1 alone (Fig. 1E and F). This establishes that lenalidomide increases IFN-γ secretion irrespective of which activating receptor is ligated.

To test the effect of lenalidomide on synergistic NK cell activation, pNK cells were treated with 0.1-10 µM lenalidomide in the presence of immobilised anti-NKG2D and anti-2B4 mAbs. IFN-γ secretion was negligible when either receptor was ligated separately (Fig. 1G) while co-ligation of both receptors led to a 2-fold greater release of IFN-γ compared to co-ligation of NKG2D and LFA-1. IFN-γ release was further increased in the presence of lenalidomide (6610 ± 620 pg/ml without lenalidomide; 10740 ± 380 pg/ml with 1.0 µM lenalidomide) (Fig. 1G). Although, stimulatory conditions elicited a strong induction of IFN-γ mRNA, no differences were observed in the presence of lenalidomide (Fig. 1H and I). Thus, lenalidomide augments IFN-γ secretion, but not IFN-γ transcription, through multiple triggers for NK cell activation. The fact that lenalidomide does not elicit NK cell effector functions in the absence of activating receptor ligation indicates that NK cells retain their specificity and would not activate without appropriate triggers whilst in the presence of the drug.

**Lenalidomide increases the proportion of cells secreting IFN-γ as well as the amount of cytokine produced per cell**

The enhancement in IFN-γ secretion with lenalidomide treatment could involve an increase in the number of NK cells secreting IFN-γ and/or an increase IFN-γ production from individual cells. To test this, pNK cells were incubated on glass slides coated with anti-CD16 mAb in the presence of 0.1-10 µM lenalidomide. Cells were fixed and then imaged by confocal microscopy (Fig. 2A). In the absence of lenalidomide, expression of IFN-γ protein could be detected within 30 min in a small proportion of primary NK cells (2.5 ± 1.5%) and peaked after 120 min stimulation, when 22 ± 1% pNK
cells expressed IFN-γ (Fig. 2B). The percentage of pNK cells expressing IFN-γ decreased by 240 min suggesting that the majority of IFN-γ had been secreted by this time (Fig. 2B, white bars). The effect of 1 µM lenalidomide also peaked 120 min after stimulation, almost doubling the proportion of NK cells expressing IFN-γ from 22 ± 1% to 41 ± 3% (Fig. 2B, striped bars). In a separate experiment, to control for lenalidomide treatment time, cells were treated at the start of the experiment and added to anti-CD16 mAb coated surfaces in reverse order for the time course. This meant, for example, that cells undergoing 30 min stimulation had 210 min incubation with lenalidomide prior to plating (Fig. 2C). This confirmed that the effect of lenalidomide on pNK cell IFN-γ production occurs after 90 min of stimulation.

1.0 µM or 10 µM lenalidomide increased the proportion of cells expressing IFN-γ from 19 ± 2% to 35 ± 1% or 33 ± 1% respectively (Fig. 2D). In addition, the total fluorescence intensity per cell increased with lenalidomide treatment 20-fold (1930 ± 310 (AU) without lenalidomide; 39100 ± 5900 with 10 µM lenalidomide; Fig. 2E). These data establish that lenalidomide treatment (i) influences the NK cell population – almost doubling the proportion of NK cells expressing IFN-γ – and (ii) affects NK cells on a single cell level, with a 20-fold increase in the amount of cytokine produced per cell.

**Lenalidomide lowers the threshold for NK cell activation**

Functionally, a threshold concentration of MICA is required for NKG2D-mediated cytolysis of target cells. To test whether lenalidomide influenced the threshold density of MICA proteins needed to activate pNK cells, cells were incubated on surfaces coated with increasing concentrations of MICA (0.1-10 µg/ml) and ICAM-1 (2.5 µg/ml). We then determined the effective concentration of activating receptor that elicited half-maximal responses (EC₅₀) (Fig. 3A). Lenalidomide treatment resulted in 38% decrease in the MICA EC₅₀, from 2.1 µg/ml without lenalidomide to 1.3 µg/ml with lenalidomide (Fig. 3A). A threshold level of CD16 ligation needed to activate NK cells has also been
Lenalidomide caused a 66% decrease in the EC$_{50}$ of immobilised human IgG (hIgG; the cognate ligand for CD16), from 62 µg/ml without lenalidomide to 21 µg/ml with lenalidomide (Fig. 3B). Together, these data establish that lenalidomide lowers the threshold for NK cell activation through different activating receptors.

To observe the effect of activating ligand concentration on IFN-γ production at the single cell level, pNK cells were imaged by confocal microscopy at 1-5 µg/ml MICA (Fig. 3C). Lenalidomide treatment significantly increased the proportion of cells secreting IFN-γ when stimulated by 2.5 µg/ml MICA (33 ± 1.7% without lenalidomide; 55 ± 2.6% with lenalidomide), but not for 1 µg/ml MICA (Fig. 3D). At 5 µg/ml MICA both conditions showed a similar proportion of cells with IFN-γ protein expression (Fig. 3D). This shows that there is a threshold concentration of MICA above which lenalidomide treatment increases the proportion of cells expressing IFN-γ. Interestingly, lenalidomide treatment resulted in a significant increase in the amount of IFN-γ expression per cell in all concentrations of MICA tested (Fig. 3E). This establishes that lenalidomide has two effects on cells: at any level of NK cell stimulation, lenalidomide increases the amount of IFN-γ produced per cell and, in addition, lenalidomide lowers the threshold for activation so that a greater proportion of NK cells are involved in the response.

**Lenalidomide alters the periodicity of the actin mesh at the NK cell ADCC synapse**

A nanoscale re-organisation of synaptic actin has been observed prior to IFN-γ secretion$^{13}$. To test the effect of lenalidomide on actin remodelling, pNK cells were treated with 1 µM lenalidomide and stimulated on surfaces coated with anti-CD16 or an isotype-matched control mAb for 120 min, and F-actin imaged by STED microscopy (Fig. 4A). Areas between individual actin filaments within the central region of the synapse, “holes”$^{9}$, were calculated and false colour heat maps were created to display the variation (Fig. 4A). The “penetrable area” that would allow passage
of vesicles of 200–500 nm diameter – the size of the majority of vesicles containing IFN-γ (Fig. 4B) – was also calculated and displayed as a false colour heat map (Fig. 4A).

For control-treated cells, the mean area of holes between actin filaments was 0.055 ± 0.004 µm², and did not change significantly in the presence of lenalidomide (Fig. 4C). The penetrable area also did not significantly change with lenalidomide (Fig. 4D), consistent with the results from Fig. 1, where lenalidomide only has an effect when accompanied by activating receptor ligation. When cells were stimulated by anti-CD16 mAb, the mean hole area significantly increased (Fig. 4C) and lenalidomide caused a small, but not significant, further increase, from 0.74 ± 0.004 µm² to 0.89 ± 0.005 µm² (Fig. 4C). However, lenalidomide increased the penetrable area of the synapse nearly two-fold for a wide range of vesicle sizes (Fig. 4D). This indicates that, after lenalidomide treatment, a higher proportion of the actin mesh has opened up to become permissive to IFN-γ vesicles. Lenalidomide increased the penetrable area within the actin mesh, irrespective of whether or not the NK cells stained for IFN-γ (in the presence of brefeldin A to block IFN-γ release; Fig. 4E), and, unlike the enhancement of IFN-γ production after lenalidomide treatment, the increase in cortical actin remodelling by lenalidomide was independent of IL-2 (Fig. 4F). Overall, these data establish that lenalidomide influences the nanoscale organisation of cortical actin at the NK cell synapse.

For pNK cells stimulated on anti-CD16 mAb for only 6 minutes, the proportion of the synapse predicted to be penetrable by lytic granules (of at least 250 nm diameter; as defined previously³) was 1.5 ± 0.15% in DMSO-treated cells and increased to 1.9 ± 0.2% in lenalidomide treated cells (Fig. 5A and 5B). The penetrable area of the cortical actin mesh increased significantly after lenalidomide treatment in cells that were activated (determined by a dense ring of F-actin at the synapse periphery; Fig. 5C). Thus, lenalidomide treatment also augments cortical actin remodelling at an early time point, relevant for lytic granule secretion, although to a lesser extent than observed later, at the time point relevant for cytokine secretion.
Lenalidomide alters the periodicity of the actin mesh after NKG2D and LFA-1 co-ligation

NKG2D is a major NK cell receptor responsible for the recognition of MM cells. We therefore tested the effect of lenalidomide treatment following NKG2D ligation (Fig. 6A). When pNK cells were in contact with slides coated with ICAM-1 alone, the mean hole area both with and without lenalidomide treatment was <0.053 µm^2 (Fig. 6B) and the percentage area of the synapse predicted to be penetrable by IFN-γ vesicles was <0.7% (Fig. 6C). Ligation of NKG2D resulted in an opening of the cortical actin mesh (Fig. 6B and C) but crucially, lenalidomide enhanced actin remodelling only when NKG2D and LFA-1 were co-ligated (Fig. 6C). NKG2D engagement does not lead to NK cell effector functions without LFA-1 co-ligation and hence this is further evidence that lenalidomide augments rather than triggers cellular activation.

Lenalidomide augments NK cell activation after rituximab ligation

Lenalidomide in combination with anti-CD20 mAb rituximab is currently in clinical trials for some lymphomas. To test whether lenalidomide influenced NK cell activation by rituximab, lenalidomide-treated pNK cells were incubated on surfaces coated with increasing concentrations of rituximab (0.0001-100 µg/ml). A distinct threshold for NK cell activation was observed and there was a 74% decrease in the EC50 in lenalidomide-treated cells (1.7 µg/ml to 0.4 µg/ml; Fig. 7A). This indicates that lenalidomide lowers the minimum concentration of rituximab required to activate NK cells.

We then used microscopy to visualise the effect of lenalidomide on rituximab activation at the single cell level. Rituximab-activated pNK cells produced IFN-γ at an earlier time point, 60 mins, than seen when pNK cells were activated with an anti-CD16 mAb (Fig. 7B and 2B respectively). Thus, pNK cells were incubated on glass slides coated with 10 µg/ml rituximab for 60 mins and imaged (Fig. 7C). Lenalidomide treatment led to a significant increase in the percent area of the synapse.
predicted to be penetrable to a vesicle of 200–350 nm diameter (Fig. 7D). This effect was also observed at 120 min (Fig. 7E). Thus, lenalidomide increases the sensitivity of NK cells to rituximab, supporting the combined use of lenalidomide and rituximab in the treatment of B cell malignancies.
Discussion

Lenalidomide has significantly improved overall survival in myeloma patients, but recent studies have focused on the mechanism associated with T cell activation. Here, we establish that lenalidomide augments NK cell effector functions triggered through different activating receptors. This implies that lenalidomide acts downstream of receptor proximal signalling, as the signal pathways for CD16- and NKG2D-mediated activation are distinct and converge later. We show that lenalidomide increased both the proportion of cells producing IFN-γ and the amount of IFN-γ produced per cell but did not activate NK cells without appropriate triggers, indicating that the drug preserves NK cell tolerance to prevent killing of healthy cells. Importantly, lenalidomide lowered the threshold for activation, suggesting that lenalidomide-treated NK cells would respond to lower concentrations of ligand on tumour cells; perhaps important for recognising tumour cells which have partially down-regulated activating ligands as a mechanism of immune evasion.

Recently, nanoscale remodelling of actin at the NK cell immune synapse has been recognised as important for directed secretion. Previously actin remodelling was considered a binary, ‘all-or-nothing’ response but here we found that lenalidomide treatment augments the extent of actin remodelling at the NK cell synapse. Specifically, lenalidomide treatment resulted in a 2-fold increase in the area of the synapse predicted to be penetrable by a vesicle of 200–500 nm diameter. It is possible that the lenalidomide-enhanced actin remodelling in NK cells could be linked to the mechanism by which this drug restores synapse assembly in T cells from Chronic Lymphocytic Leukaemia patients. Importantly, our data also provides the first evidence that synaptic actin remodelling is druggable, indicating that new compounds could be screened for an effect on cortical actin to augment NK cell responses.

Finally, we have shown that lenalidomide also affects NK cell activation after ligation of CD16 with rituximab, which is currently in clinical trials in combination with lenalidomide. Here, we
establish that lenalidomide lowers the threshold for NK cell activation through rituximab, consistent with increased ADCC.\textsuperscript{24,39,40} Overall, our results demonstrate a direct and significant effect of lenalidomide on NK cell effector functions without activating NK cells inappropriately. The fact that lenalidomide lowers the thresholds for activation of NK cells implies that this drug may be useful in other medical conditions where NK cell immune responses are beneficial.
Acknowledgements

We thank K. Stacey for isolation of primary human NK cells. This work was supported by the Biotechnology and Biological Sciences Research Council, the Medical Research Council, the Manchester Collaborative Centre for Inflammation Research and Celgene Corporation.

Authorship

Contributions: K.L., R.C and D.M.D. conceived the project; K.L. and D.M.D designed experiments and wrote the manuscript; K.L. and D.M. performed experiments; K.L. and A.C. analysed the data.

Conflict-of-interest disclosure: R.C. is an employee of Celgene Corporation. The authors have no additional financial interests.
References


Figure legends

Figure 1. Lenalidomide treatment increases IFN-γ secretion from NK cells. (A) Healthy donor pNK cells and Daudi target cells were pre-treated with DMSO or 0.001-10 µM lenalidomide for 24 hours then the Daudi cells were tested for susceptibility to NK cell-mediated lysis. Graph shows mean ± SEM from 3 independent donors. E:T ratio was 10:1. (B and C) pNK cells and (B) Daudi cells or (C) Raji cells were co-cultured for 24 hours with DMSO or 0.001-10 µM lenalidomide (± 150 U/ml IL-2). IFN-γ release was measured by ELISA. E:T ratio was 10:1 in all experiments. Data shows mean ± SEM from 3 donors. Significance compared to DMSO (‘0 µM’) condition. (D-G) Primary NK cells were treated with DMSO or 0.1-10 µM lenalidomide (+ 150 U/ml IL-2) for 24 hours in wells coated with (D) anti-CD16 mAb or IgG1 isotype control mAb, (E) recombinant ICAM-1, anti-NKG2D mAb or both, (F) recombinant ICAM-1, recombinant MICA or both, (G) anti-NKG2D mAb, anti-2B4 or both. IFN-γ release was assessed by ELISA. Data shows mean ± SEM from 3 donors. (H and I) Primary NK cells were treated with DMSO or 1.0 µM lenalidomide (+ 150 U/ml IL-2) for 4 hours in wells coated with (H) anti-CD16 mAb or IgG1 isotype control mAb, or (I) recombinant ICAM-1 or recombinant MICA and ICAM-1. IFN-γ mRNA was assessed by qRT-PCR and is normalised to GAPDH. Data shows triplicate data from (H) 3 donors and (I) 2 donors. Data points for each donor are shaded the same. Red line shows the mean. ns = not significant, * = p <0.05, ** = p <0.01, *** = p <0.001, one way ANOVA with Tukey’s post-test.

Figure 2. Lenalidomide increases the proportion of pNK cells expressing IFN-γ as well as the amount produced per cell. (A) Representative microscopy images of F-actin (red) and IFN-γ (shown in greyscale in middle column, and then green in merged image) in pNK cells stimulated for 120 min on surfaces coated with IgG1 isotype control mAb or anti-CD16 mAb in the presence of DMSO or 1 µM lenalidomide (+ 150 U/ml IL-2). Scale bars: 10 µm. (B) The proportion of cells expressing IFN-γ after stimulation on anti-CD16 mAb coated-surfaces in the presence of DMSO or 1 µM lenalidomide
(+ 150 U/ml IL-2) for 30-240 mins. Graph shows mean ± SEM, n >200 from 3 donors. (C) The proportion of cells expressing IFN-γ after stimulation on anti-CD16 mAb coated-surfaces in the presence of DMSO or 1 μM lenalidomide (+ 150 U/ml IL-2) for 30-240 mins. All cells treated with DMSO or 1 μM lenalidomide for 240 min total and added to stimulation in reverse order for the time course (for example, for 30 min stimulation, pNK cells were treated with DMSO or 1 μM lenalidomide for 210 min before plating). Graph shows mean ± SEM, n >100 from 2 donors. (D) The proportion of pNK cells expressing IFN-γ after stimulation on anti-CD16 mAb coated surfaces with 0.1-10 μM lenalidomide. Graph shows mean ± SEM, n >100 per donor from 3 donors. (E) Sum IFN-γ fluorescence per cell in pNK cells stimulated as in (C). Each data point represents a single cell and red line shows the mean. n = 75-111 from 3 donors. * = p <0.05, ** = p <0.01, *** = p <0.001, one way ANOVA with Tukey’s post-test.

Figure 3. Lenalidomide lowers the threshold for NK cell activation through NKG2D and CD16. (A and B) pNK cells were treated with DMSO or 1.0 μM lenalidomide (+ 150 U/ml IL-2) for 24 hours in wells coated with (A) 0.1-10 μg/ml MICA plus 2.5 μg/ml ICAM-1 or (B) 1-500 μg/ml human IgG. IFN-γ release was measured by ELISA. Data shows mean ± SEM from 3 donors. Non-linear regression fit was applied to data. EC₅₀ values were calculated to be: 2.09 μg/ml (MICA DMSO), 1.32 μg/ml (MICA 1.0μM), 61.64 μg/ml (hIgG DMSO), 21.41 μg/ml (hIgG 1.0μM Len). (C) Representative microscopy images of F-actin (red) and IFN-γ (shown in greyscale in middle column, and then green in merged image) in pNK cells stimulated for 90 min on surfaces coated with 1 μg/ml or 2.5 μg/ml MICA (+ 2.5 μg/ml ICAM-1) in the presence of DMSO or 1.0 μM lenalidomide (+ 150 U/ml IL-2). Scale bars: 10 μm. (D) The proportion of pNK cells expressing IFN-γ after stimulation with 3 different concentrations of MICA. Graph shows mean ± SEM, n >100 per donor from 3 donors. (E) Sum fluorescence staining for IFN-γ per cell in pNK cells stimulated as in (C). Each data point represents a
Figure 4. Lenalidomide treatment augments opening of cortical actin mesh after CD16 stimulation.

(A) Super-resolution images obtained by STED microscopy of membrane proximal F-actin in pNK cells incubated for 120 min on coverslips coated with isotype control antibody or anti-CD16 mAb (both 3 μg/ml) in the presence of DMSO or 1.0 μM lenalidomide (+ 150 U/ml IL-2). Scale bars: 5 μm. Second column: Holes between actin filaments in the central region of the synapse shown as heat maps, with the smallest holes shown in blue (0.01 μm²) and largest holes shown in red (>3 μm²). Third column: regions are shown within the actin mesh through which a particle (such as an IFN-γ vesicle) of diameter 200 nm (blue) to 800 nm (red) could fit. (B) Histogram of measured vesicle sizes from 10 cells from 2 independent donors. (C) Average size of holes in the actin mesh at the pNK synapse for cells stimulated as in (A). Each data point represents a single cell; red lines shows mean from 3 donors, n = 18-59 per condition. (D) The proportion of the synapse area predicted to be penetrable by a vesicle of 200-500 nm diameter for same cells as in (C). (E) Analysis of STED microscopy of membrane proximal actin in pNK cell stimulated as in (A) plus 5 μg/ml brefeldin A and co-stained with anti-IFN-γ conjugated to Alexa 647. The proportion of the synapse predicted to be penetrable by a particle of 200-500 nm diameter, stratified by whether or not the cells stained positive for IFN-γ. Graph shows mean from 3 donors, n = 50. (F) Analysis of STED microscopy of membrane proximal actin in pNK cells incubated for 120 min on coverslips coated with anti-CD16 (3 μg/ml) without the presence of IL-2. Quantification of proportion of the synapse area predicted to be penetrable by a particle of 200-500 nm diameter. Graph shows mean from 3 donors, n = 37. * = p <0.05, ** = p <0.01, *** = p <0.001, one way ANOVA with Tukey’s post-test.
Figure 5. Lenalidomide treatment enhances opening of cortical actin mesh during initial cell spreading. (A) Super-resolution images obtained by STED microscopy of membrane proximal F-actin in pNK cells incubated for 6 min on coverslips coated with isotype control mAb or anti-CD16 mAb (both 3 µg/ml) in the presence of DMSO or 1.0 µM lenalidomide (+ 150 U/ml IL-2). Scale bars: 5 µm. Second column: holes between actin filaments in the central region of the synapse shown as heat maps, with the smallest holes shown in blue (0.01 µm²) and largest holes shown in red (> 3 µm²). Third column: regions are shown within the actin mesh through which lytic granules of diameter 200 nm (blue) to 800 nm (red) could fit. (B) The proportion of the synapse penetrable by a granule of 250 nm diameter. Each data point represents a single cell; red lines shows the mean from 3 donors, n = 59 per condition. (C) The proportion of the synapse area predicted to be penetrable by a granule of 250 nm diameter with cells separated by presence of peripheral actin ring. ns = not significant, * = p <0.05, **** = p <0.0001, one way ANOVA with Tukey’s post-test.

Figure 6. Lenalidomide treatment augments opening of cortical actin mesh after NKG2D and LFA-1 ligation. (A) Super-resolution images obtained by STED microscopy of membrane proximal F-actin in pNK cells incubated for 120 min on coverslips coated with recombinant human ICAM-1 (2.5 µg/ml), anti-NKG2D mAb (3 µg/ml) or both in the presence of DMSO or 1.0 µM lenalidomide (+ 150 U/ml IL-2). Scale bars: 5 µm. Second column: holes between actin filaments in the central region of the synapse shown as heat maps, with the smallest holes shown in blue (0.01 µm²) and largest holes shown in red (>3 µm²). Third column: regions are shown within the actin mesh through which a particle (such as an IFN-γ vesicle) of diameter 200 nm (blue) to 800 nm (red) could fit. (B) Average size of holes in the actin mesh at the pNK synapse for cells stimulated as in (A). Each data point represents a single cell; red lines shows the mean from 3 donors, n = 18-59 per condition. (C) The proportion of the synapse area predicted to be penetrable by a vesicle of 200-500 nm diameter for same cells as in (B). ** = p <0.01, *** = p <0.001, one way ANOVA with Tukey’s post-test.
Figure 7. Lenalidomide treatment increases NK cell activation through rituximab. (A) pNK cells were treated with DMSO or 1.0 µM lenalidomide (+ 150 U/ml IL-2) for 24 hours in wells coated with 0.0001-100 µg/ml rituximab. IFN-γ release was measured by ELISA. Data shows mean ± SEM from 3 donors. Non-linear regression fit was applied to data. EC50 values calculated to be: 1.63 µg/ml without lenalidomide and 0.44 µg/ml with lenalidomide. (B) Proportion of DMSO treated cells expressing IFN-γ after stimulation on rituximab coated-surfaces in the presence of DMSO or 1 µM lenalidomide for 30-120 mins. Graph shows mean ± SEM, n >200 from 3 donors. (C) Super-resolution images obtained by STED microscopy of membrane proximal F-actin in pNK cells incubated for 60 or 120 min on coverslips coated with rituximab (10 µg/ml) in the presence of DMSO or 1.0 µM lenalidomide (+ 150 U/ml IL-2). Scale bars: 5 µm. Second column: holes between actin filaments shown as heat maps, with the smallest holes shown in blue (0.01 µm2) and largest holes shown in red (>3 µm2). Third column: regions are shown through which a particle (such as an IFN-γ vesicle) of diameter 200 nm (blue) to 800 nm (red) could fit. (D) Proportion of the synapse area predicted to be penetrable by a vesicle of 200-500 nm diameter for cells stimulated on 10 µg/ml rituximab for 60 min. (E) Proportion of the synapse area predicted to be penetrable by a vesicle of 200-500 nm diameter for cells stimulated on 10 µg/ml rituximab for 120 min. * = p <0.05, ** = p <0.01, *** = p <0.001, one way ANOVA with Tukey’s post-test.
Figure 1

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Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

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C

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