Lenalidomide Enhances Natural Killer Cell and Monocyte-Mediated Antibody-Dependent Cellular Cytotoxicity of Rituximab-Treated CD20$^+$ Tumor Cells

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Abstract

Purpose: Lenalidomide has significant activity in myelodysplastic syndromes, multiple myeloma, and non-Hodgkin's lymphoma (NHL). In previous studies, natural killer (NK) cell expansion by lenalidomide was shown to enhance the cytotoxic effect of rituximab. This study assessed the ability of lenalidomide to enhance antibody-dependent cellular cytotoxicity (ADCC) in rituximab-treated NHL cell lines and primary tumor cells from patients with B-cell chronic lymphocytic leukemia (B-CLL) *in vitro*.

Experimental Design: An *in vitro* ADCC system was used to assess the ability of lenalidomide to enhance human NK cell and monocyte function in response to rituximab.

Results: Lenalidomide directly enhanced IFN-γ production via Fc-γ receptor-mediated signaling in response to IgG. It was also a potent enhancer of NK cell-mediated and monocyte-mediated tumor cell ADCC for a variety of rituximab-treated NHL cell lines *in vitro*, an effect that was dependent on the presence of antibody and either interleukin-2 or interleukin-12. Lenalidomide also enhanced the ability of NK cells to kill primary tumor cells derived from three patients with B-CLL who have been treated previously with fludarabine plus cyclophosphamide. Enhanced NK cell ADCC was associated with enhanced granzyme B and Fas ligand expression and could be inhibited by a granzyme B inhibitor and partially inhibited by antibody to FasL. Enhanced NK cell Fc-γ receptor signaling is associated with enhanced phosphorylated extracellular signal-related kinase levels leading to enhanced effector function.

Conclusions: These findings suggest that lenalidomide has the potential to enhance the rituximab-induced killing of NHL cell lines and primary B-cell chronic lymphocytic leukemia cells via a NK cell-mediated and monocyte-mediated ADCC mechanism *in vitro*, providing a strong rationale for the combination of lenalidomide with IgG1 antibodies to target tumor-specific antigens in patients with cancer.

Monoclonal antibodies (mAb) are important weapons in the chemotherapeutic arsenal, targeting specific receptors on cancer cells (1). Rituximab is a chimeric anti-CD20 mAb that has significant clinical activity when used alone or in combination with chemotherapy in patients with relapsed or refractory non-Hodgkin's lymphoma (NHL; ref. 2). In patients with NHL and B-cell chronic lymphocytic leukemia (B-CLL), rituximab enhances antibody-dependent cellular cytotoxicity (ADCC) as a major mechanism of action (2). However, the relevant cell populations and effector mechanisms leading to tumor cell death during ADCC are the subject of ongoing research.

Experiments using a novel flow cytometric assay have shown that an increased number of natural killer (NK) cells degranulate in response to CD20$^+$ lymphoma cells in rituximab-treated patients with NHL (3). In ADCC, immunoglobulins complexed on a cell surface activate Fc-γ receptors on both host NK cells and monocytes. This triggers a cytolytic response mediated by perforins and granzymes as well as the induction of apoptosis through Fas ligand (FasL) and oxidative mechanisms (4–7).

Lenalidomide (Revlimid; Celgene) has been shown to have significant activity in the treatment of myelodysplastic syndromes in patients with the 5q deletion (8). Administration of 10 mg lenalidomide, given either daily (continuous treatment) or for 21 days every 4 weeks, reduced the requirement for transfusion and also decreased cytologic and cytogenetic abnormalities in these patients. Lenalidomide (25 mg given for 21 days every 4 weeks) in combination with dexamethasone was also highly effective in patients with multiple myeloma (9, 10). Other studies suggest that lenalidomide monotherapy may have clinical efficacy in relapsed or refractory, indolent or aggressive NHL (11).

Potential mechanisms of action of lenalidomide include immunomodulatory and nonimmunomodulatory activities.

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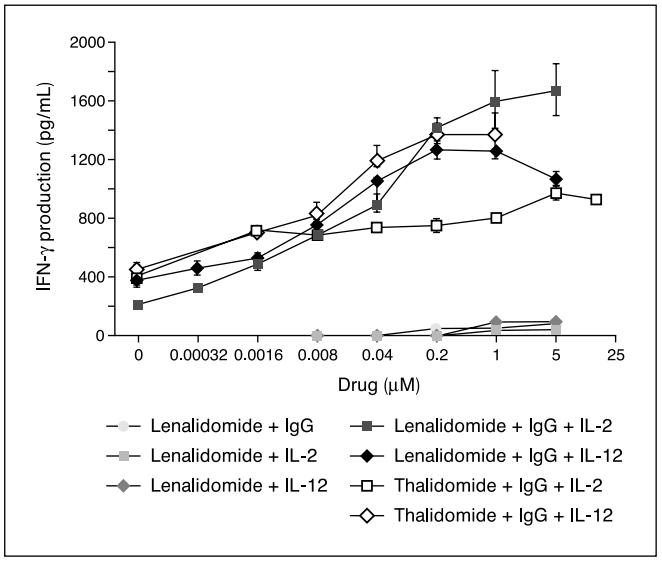

Fig. 1. *In vitro* production of NK cell IFN-γ in response to lenalidomide or thalidomide incubated for 48 h with IgG and IL-2 or IL-12. Mean ± SE.

Lenalidomide has been shown to enhance T cells and NK cell activation markers in patients with advanced cancers (12). NK cell expansion by lenalidomide has also been associated with an increase in the cytotoxic effect of rituximab in a severe combined immunodeficient mouse lymphoma model *in vivo* (13). In other studies, lenalidomide was shown to enhance the cytotoxicity of an anti-CD40 mAb (SGN-40) in CD40-expressing human multiple myeloma cell lines (14). The action of lenalidomide in these studies was attributed to direct antiproliferative and apoptotic effects as well as to increased sensitivity of multiple myeloma cells to ADCC by functional cytotoxic NK effector cells.

The present study was undertaken to assess the ability of lenalidomide to enhance ADCC of rituximab-treated NHL cell lines and primary B-CLL cells *in vitro* in an attempt to better elucidate the mechanism of action of lenalidomide.

Materials and Methods

NK cell and monocyte preparation. NK cells and monocytes were isolated from fresh, buffy-coated, whole blood by 30-min incubation with RosetteSep cocktail (Stem Cell Technologies) followed by Ficoll-Hypaque density gradient centrifugation. $CD56^+$ NK cells were isolated to ~85% purity and $CD14^+$ monocytes were isolated to ~80% purity as determined by flow cytometry.

NK IgG-induced cytokine/chemokine assays. Flat-bottomed plates were coated with 100 μg/mL human IgG (Sigma-Aldrich) overnight at 4°C; unbound IgG was washed away. NK cells were plated at 2×10^5 per well into 96-well plates, and 10 ng/mL either interleukin (IL)-2 or IL-12 (R&D Systems) was added. Lenalidomide was added to the plate wells in the concentration range of 0.00032 to 5 μmol/L. After 48-h incubation, the supernatants were harvested and analyzed for levels of IFN-γ (R&D Systems). In some of the experiments, the supernatants were also analyzed for the levels of granzyme B or perforin (Cell Sciences) and analyzed for the level of various cytokines [granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-6] and chemokines [IL-8, inducible protein-10 (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES] using ELISA (R&D Systems) or Luminex. Statistical analysis was done to determine the significance of the addition of lenalidomide to IL-2 plus IgG or to IL-2 alone using the unpaired *t* test algorithm from GraphPad Prism. All donors tested were used in the analysis.

ADCC assay. Purified NK cells (1×10^5) were seeded in 96-well U-bottomed plates in 100 μL RPMI 1640 supplemented with 2% human AB serum. The cells were treated with lenalidomide or thalidomide (0.001-10 μmol/L) plus 10 ng/mL IL-2 or IL-12. The cells were then incubated at 37°C overnight.

Various NHL cell lines (Namalwa, Raji, Farage, and Jeko-1) as well as primary B-CLL cells, which were obtained from three patients, and a non-CD20-expressing control cell line (Jurkat) were treated with 20 μg/mL rituximab (Rituxan; Genentech), 100 μg/mL trastuzumab (Herceptin; Genentech), or a mouse IgG1 antibody control for 30 min at 37°C. For evaluating the ADCC effect in primary CLL cells, peripheral blood mononuclear cells from three patients with CLL were purchased (ProteoGenex). Two of the CLL patients had relapsed after prior treatment with fludarabine and cyclophosphane.

After washing off unbound antibody, target cells (1×10^4 per 100 μL/well) were added to the pretreated effector NK cells at a ratio of 10:1. The cells were then coincubated for 4 h at 37°C. Control groups included NK and tumor cells treated with medium alone, rituximab only, trastuzumab only, mouse IgG1 antibody only, or IL-2/IL-12 alone.

NK cell cytotoxicity against the tumor cell lines was analyzed using a standard lactate dehydrogenase release assay (CytoTox 96 Nonradioactive Cytotoxicity Assay; Promega) to measure ADCC in a 50 μL aliquot of supernatant. The experimental release was corrected by subtraction of the spontaneous release of effector cells at the corresponding dilution. Spontaneous release from target cells alone was <15% of the maximum release as determined with target cells lysed in 1% Triton X-100.

Specific lysis for each effector-to-target (E:T) cell ratio was calculated with the following formula: % specific lysis = [(experimental release - spontaneous release) / (maximum release - spontaneous release)] × 100.

Similar methods were used to evaluate monocyte ADCC. However, the incubation time was increased to 16 h, and IL-2 and IL-12 were omitted.

Flow cytometry. Fluorochrome-conjugated antibodies, unconjugated antibodies, and isotype control mAb were obtained (PharMingen). Cytometry was undertaken on 50 μL samples containing 1×10^6 cells/mL in washing buffer and 2% fetal bovine serum with 0.1% NaN_3 in PBS. The cells were stained with anti-CD56-PE, anti-FasL-FITC, or isotype control mAb (1 μg/10^6 cells) and analyzed by flow cytometry as per manufacturer's instructions.

Western blot. For analysis of intracellular protein phosphorylation, NK cells were seeded at 2×10^6 per 3 mL medium in six-well plates. Cells were serum starved and incubated for 1 h with lenalidomide (0.01-10 μmol/L) and then stimulated with IL-12 and IgG for 30 min. Lysates were made and run on 10% Tris-glycine gels. Proteins were transferred to nitrocellulose membranes and probed with the antibodies of interest. Antibodies tested include phosphospecific phospho-ERK (T183; Promega) and total ERK (Cell Signaling). Statistical analysis was done on the Western blots after being quantified using ImageQuant software on the Storm Molecular Imager. Phosphorylation levels of the NK cells treated with IL-12 and IgG or treated with IL-12 alone were compared with treatment with lenalidomide (0.1-10 μmol/L) using the unpaired *t* test algorithm from GraphPad Prism. All donors tested were used in the analysis.

Results

Enhancement of IFN-γ production by NK cells. The addition of lenalidomide (0.000382-5 μmol/L) to human IgG incubated with either IL-12 or IL-2 enhanced IFN-γ production by ~3- to 8-fold, respectively, in comparison with lenalidomide plus IgG, IL-12, or IL-2 (Fig. 1). The enhancing effect of lenalidomide was

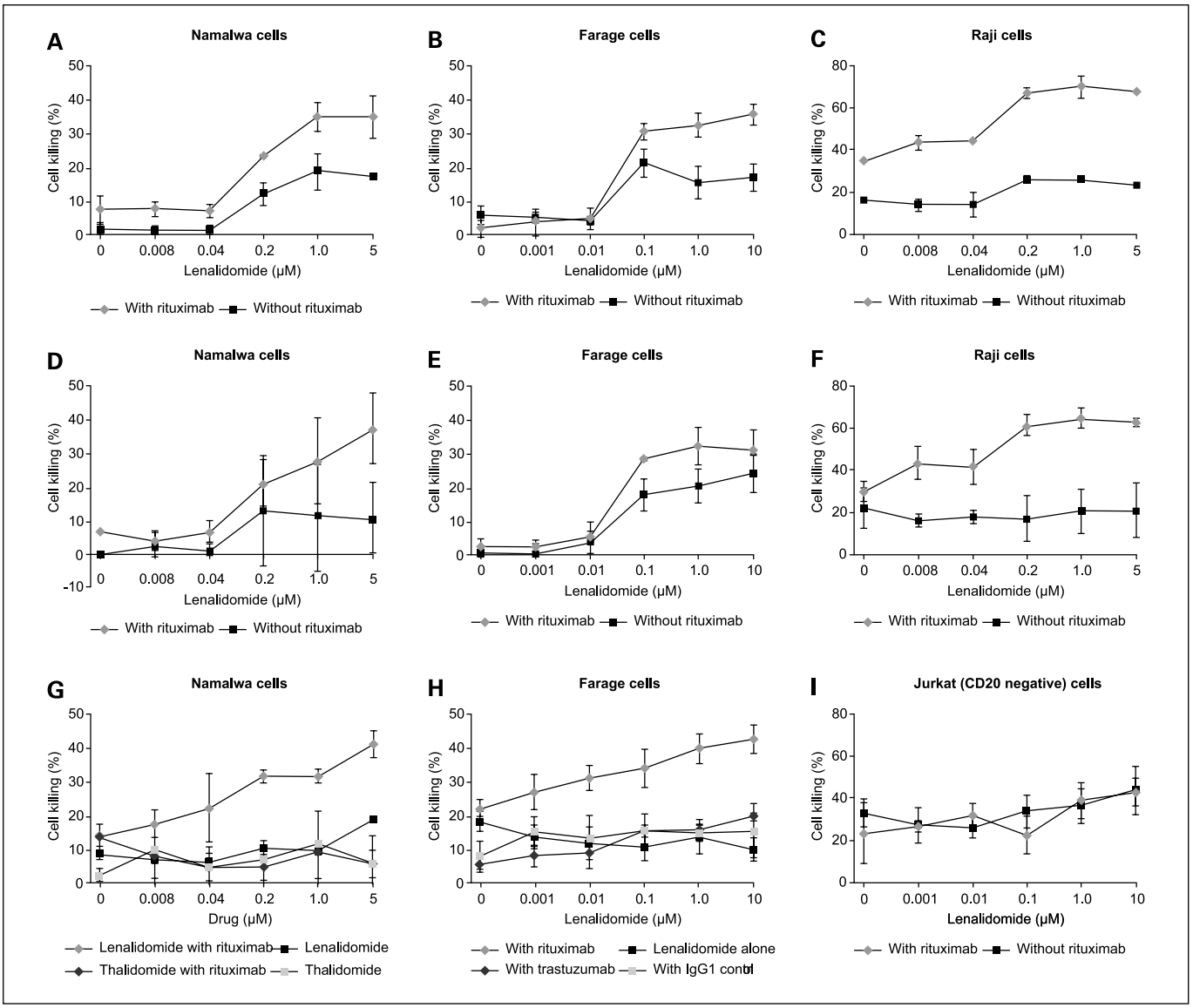

Fig. 2. *In vitro* enhancement by lenalidomide of NK cell cytotoxicity against rituximab-coated NHL cell lines Namalwa, Farage, and Raji by ADCC in the presence of IL-2 (*A-C*) or IL-12 (*D-F*). Thalidomide is unable to enhance ADCC (*G*). Control experiments indicate no effect of lenalidomide on trastuzumab-coated or mouse IgG1-coated Farage cells (*H*) or on rituximab-coated Jurkat cells (*I*) in the presence of IL-2. Mean ± SD of two donors.

dose dependent for both IL-2 and IL-12. Similar results were observed with thalidomide, which enhanced IFN-γ production by >3-fold in the presence of IL-12 and ~2-fold in the presence of IL-2 (Fig. 1). There was no IFN-γ production in response to lenalidomide alone, immobilized IgG alone, or lenalidomide plus IgG. Either IL-2 or IL-12 plus immobilized IgG was required for IFN-γ production (Fig. 1).

Enhancement of NK cell cytotoxicity in rituximab-coated NHL cell lines. The killing of Namalwa cells by lenalidomide (1 μmol/L), rituximab, and lenalidomide (1 μmol/L) plus rituximab was 18.8%, 7.4%, and 35.1%, respectively, in the presence of IL-2 (Fig. 2A). In addition, in the presence of IL-2, lenalidomide dose-dependently increased the NK cell (E:T = 10:1) specific lysis of rituximab-coated Farage cells (Fig. 2B) and Raji cells (Fig. 2C). For Farage cells, cell killing by lenalidomide (1 μmol/L), rituximab, and both was 15.4%, 1.8%, and 32.6%, respectively, in the presence of IL-2. For Raji cells, cell killing by lenalidomide (1 μmol/L), rituximab, and both was 26.6%, 34.5%, and 70.1%, respectively, in the presence of IL-2. In the presence of IL-12, the killing of Namalwa cells by lenalidomide (1 μmol/L), rituximab, and lenalidomide (1 μmol/L) plus rituximab was 11.5%, 7.1%, and 28.0%, respectively (Fig. 2D). Lenalidomide also dose-dependently increased the NK cell (E:T = 10:1) specific lysis of rituximab-coated Farage cells (Fig. 2E) and Raji cells (Fig. 2F), in the presence of IL-12. For Farage cells, cell killing by lenalidomide (1 μmol/L), rituximab, and both was 20.5%, 2.0%, and 32.5%, respectively. For Raji cells, cell killing by lenalidomide (1 μmol/L), rituximab, and both was 16.5%, 29.9%, and 65%, respectively, in the presence of IL-12. Enhancement of NK cell-mediated killing against rituximab-coated Namalwa cells was not observed with thalidomide

(Fig. 2G). Additionally, lenalidomide did not enhance NK cell-mediated killing of trastuzumab (HER-2/*neu*, IgG1) or mouse IgG1 coated Farage cells (Fig. 2H). There was also no killing of Jurkat (T cells, $CD20^-$) cells in the presence of rituximab (Fig. 2I).

Additionally, lenalidomide (1 μmol/L) with or without rituximab also enhanced the ability of NK cells to kill primary tumor cells derived from three patients with B-CLL who have been treated with fludarabine plus cyclophosphamide, two of whom had relapsed after treatment (Fig. 3A). In further experiments, lenalidomide (1 μmol/L) in the presence of IL-2 increased the NK cell cytotoxicity of rituximab-coated $CD20^+$ Jeko-1 (mantle cell lymphoma) cells from 70% to 95% at an E:T ratio of 50:1, from 60% to 85% at an E:T ratio of 25:1, and from 40% to 55% at an E:T ratio of 10:1 (Fig. 3B). Lenalidomide alone had a negligible (<5%) effect on tumor cell killing relative to the control (background killing = 40%). Rituximab-only treatment increased tumor cell killing by ~30% above the background. Therefore, the combined killing effects of lenalidomide and rituximab acted synergistically, enhancing the NK cell-mediated killing of tumor cells by 55% above background.

Enhancement of monocyte-mediated lysis of NHL cells. Mononocyte-mediated lysis of rituximab-coated Farage cells showed very good killing activity, from 5% up to 20% at a 50:1 ratio, in the presence of lenalidomide (10 μmol/L; Fig. 3C). Unlike the NK cell assay, the addition of IL-2 or IL-12 was not required to show ADCC. However, the specific lysis of rituximab-coated Farage cells by lenalidomide-pretreated monocytes was completely blocked by neutralizing anti-IL-12 antibody.

Lenalidomide also enhanced monocyte-mediated lysis of rituximab-coated Raji cells from ~30% killing with rituximab alone to >60% at an E:T ratio of 50:1 (data not shown).

Enhancement of NK cell cytokine/chemokine production by lenalidomide. Enhancement of ADCC by lenalidomide was associated with increased NK cell expression of IL-8, MCP-1,

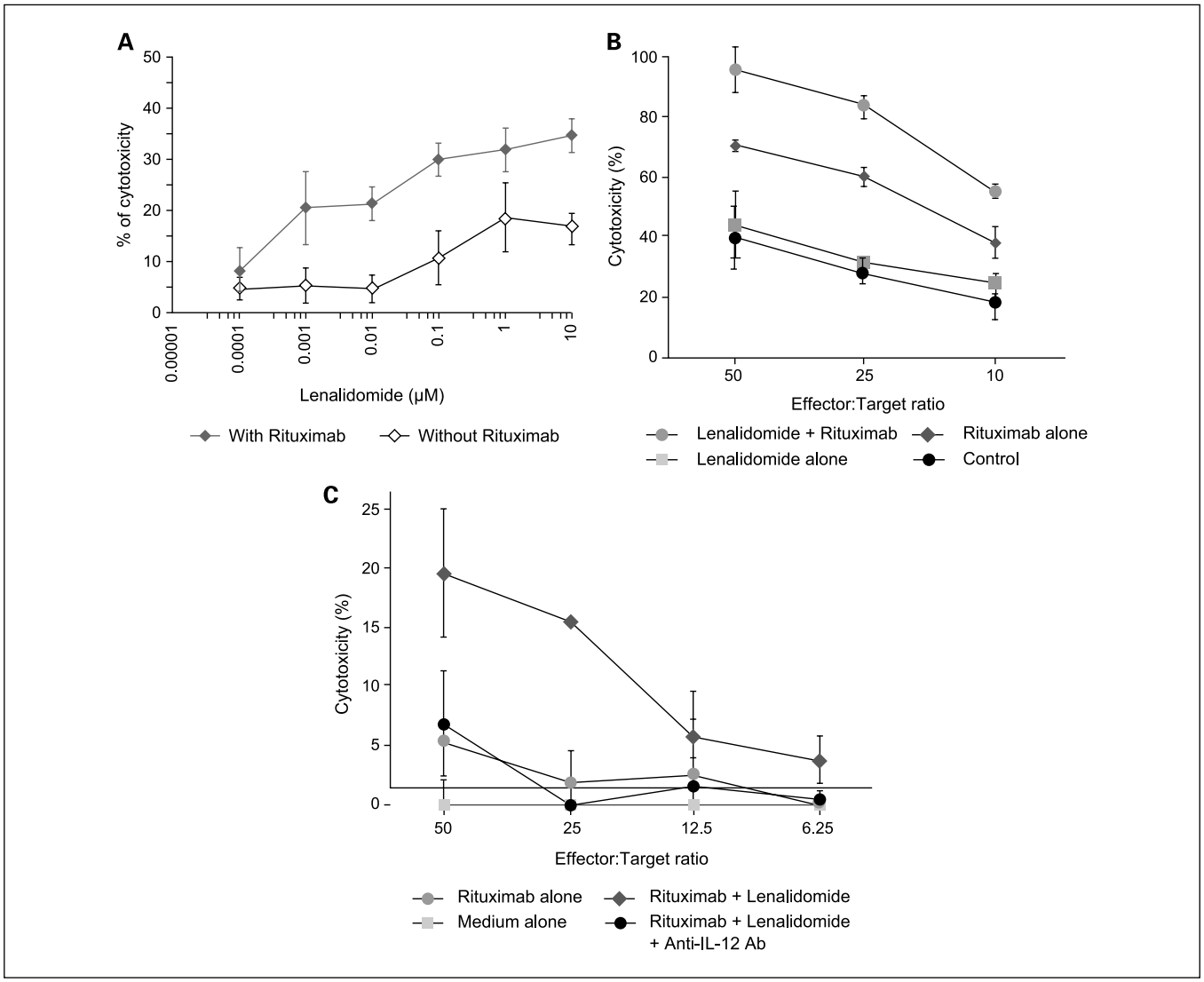

Fig. 3. *A,* lenalidomide-enhanced the ability of NK cells to kill primary tumor cells from patients with B-CLL who have been treated with fludarabine plus cyclophosphamide. Mean ± SD of three donors. *In vitro* enhancement by lenalidomide of (*B*) NK cell cytotoxicity of rituximab-coated $CD20^+$ Jeko-1 (mantle cell lymphoma) cells and (*C*) monocyte-mediated lysis of Farage (human B-cell lymphoma) cells by lenalidomide. Mean ± SD of two donors.

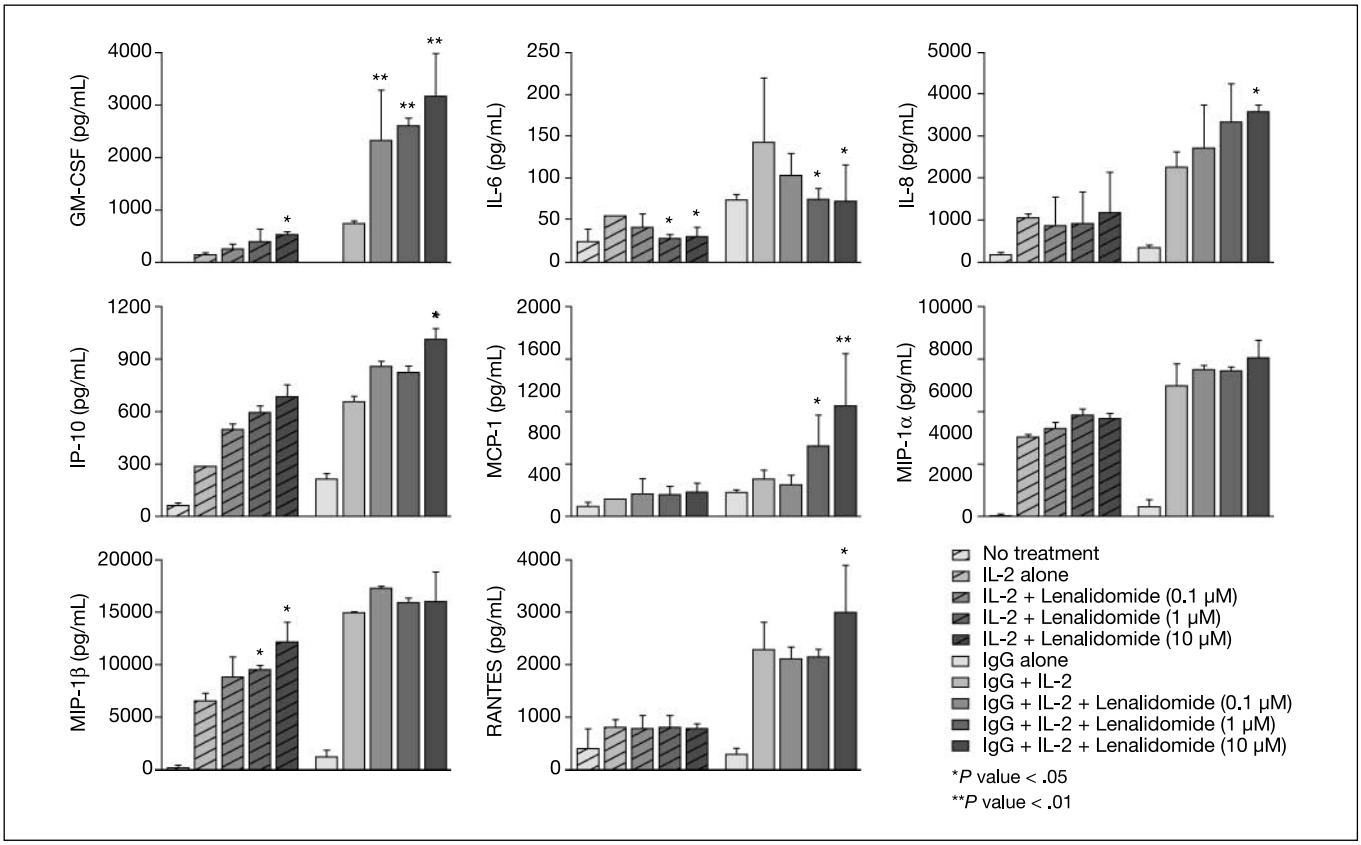

Fig. 4. *In vitro* enhancement of ADCC by lenalidomide and increased NK cell expression of a variety of chemokines. *Striped columns, P* values, significance versus IL-2 alone; *solid columns, P* values, significance versus IgG + IL-2. Mean ± SE.

RANTES, IP-10, and GM-CSF but decreased expression of IL-6 (Fig. 4). For IL-8, IP-10, and RANTES, enhancement was only statistically significant with immobilized IgG at the highest lenalidomide dose (10 μmol/L). In the case of GM-CSF, IP-10, MIP-1β, and IL-6, marked effects were also observed with lenalidomide plus IL-2 but in the absence of the antibody. No significant change was observed with MIP-1α production.

Mechanism of enhancement of ADCC by lenalidomide. Lenalidomide (10 μmol/L) together with immobilized IgG increased the percentage of $CD56^+$ NK cells expressing FasL from ~5% (at a lenalidomide concentration of 0.1 μmol/L) to ~10% (Fig. 5A). IL-2 (10 ng/mL) treatment with immobilized IgG also increased the NK cell FasL expression comparable with lenalidomide treatment. Moreover, the combination of lenalidomide (0.1-10 μmol/L), IL-2 (10 ng/mL), and immobilized IgG enhanced the NK cell FasL expression to a level greater than that achieved by either agent alone, showing partially additive responses at 0.1 and 10 μmol/L lenalidomide (Fig. 5A). Notably, lenalidomide (1 μmol/L) in conjunction with IL-2 and immobilized IgG showed a clear synergistic enhancement of NK cell FasL expression [IgG alone, ~5%; IL-2 + IgG, ~8%; lenalidomide (1 μmol/L) + IgG, ~5%; and lenalidomide (1 μmol/L) + IgG + IL-2, ~25%].

The addition of lenalidomide was shown to enhance granzyme B production over that observed with IL-2 alone in a dose-dependent manner, but it had no effect on perforin production (Fig. 5B and C), although IL-2 alone had a strong enhancing effect on both. Addition of the granzyme B inhibitor II (40 μmol/mL; Calbiochem) to the NK cells 1 h before lenalidomide and IL-2 treatment abrogated the effect of IL-2 on granzyme B production. As expected, the granzyme B inhibitor II was inactive against the IL-2-stimulated perforin production. ADCC was totally prevented in the presence of a granzyme inhibitor (Fig. 5D) and partially prevented by the addition of anti-FasL (Fig. 5E).

Analysis by Western blot showed that lenalidomide increased levels of pERK with a clear dose-dependent response (Fig. 6A and B).

Discussion

Treatment options for patients with malignant lymphoma have been substantially enriched by the development of mAbs. In particular, the chimeric anti-CD20 antibody rituximab has direct antilymphoma activity and is highly active in indolent and aggressive lymphoma in combination with chemotherapy (15). ADCC is suggested as one of the *in vivo* mechanisms of action of rituximab, although the exact pathways involved are not fully understood (16).

In this study, we found that both lenalidomide and thalidomide enhanced *in vitro* NK cell production of IFN-γ induced by interaction of IgG with the Fc-γ receptor but only in the presence of IL-2 or IL-12. We then investigated the ability of lenalidomide and thalidomide to enhance the ADCC of rituximab. When multiple NHL cell lines were precoated with rituximab and exposed to NK cells pretreated with

lenalidomide, there was strongly enhanced NK cell-mediated cytotoxicity. The specificity of this effect was confirmed by the lack of tumor cell killing by NK cells pretreated with lenalidomide in the absence of either concurrent cytokine signaling (via IL-2 or IL-12 receptors) or an antibody directed against the relevant tumor surface antigen. Thus, enhancement of NK cell-mediated ADCC of NHL cells *in vitro* appears to require both antibody binding to Fc-γ receptors plus IL-2 or IL-12, as lenalidomide alone does not cause an effect.

These findings are consistent with previous NK cell depletion studies, which showed that the antitumor activity of rituximab is enhanced in the presence of lenalidomide (13) and can be attributed to an enhancing effect of lenalidomide on purified human effector cells. Interestingly, thalidomide was inactive in

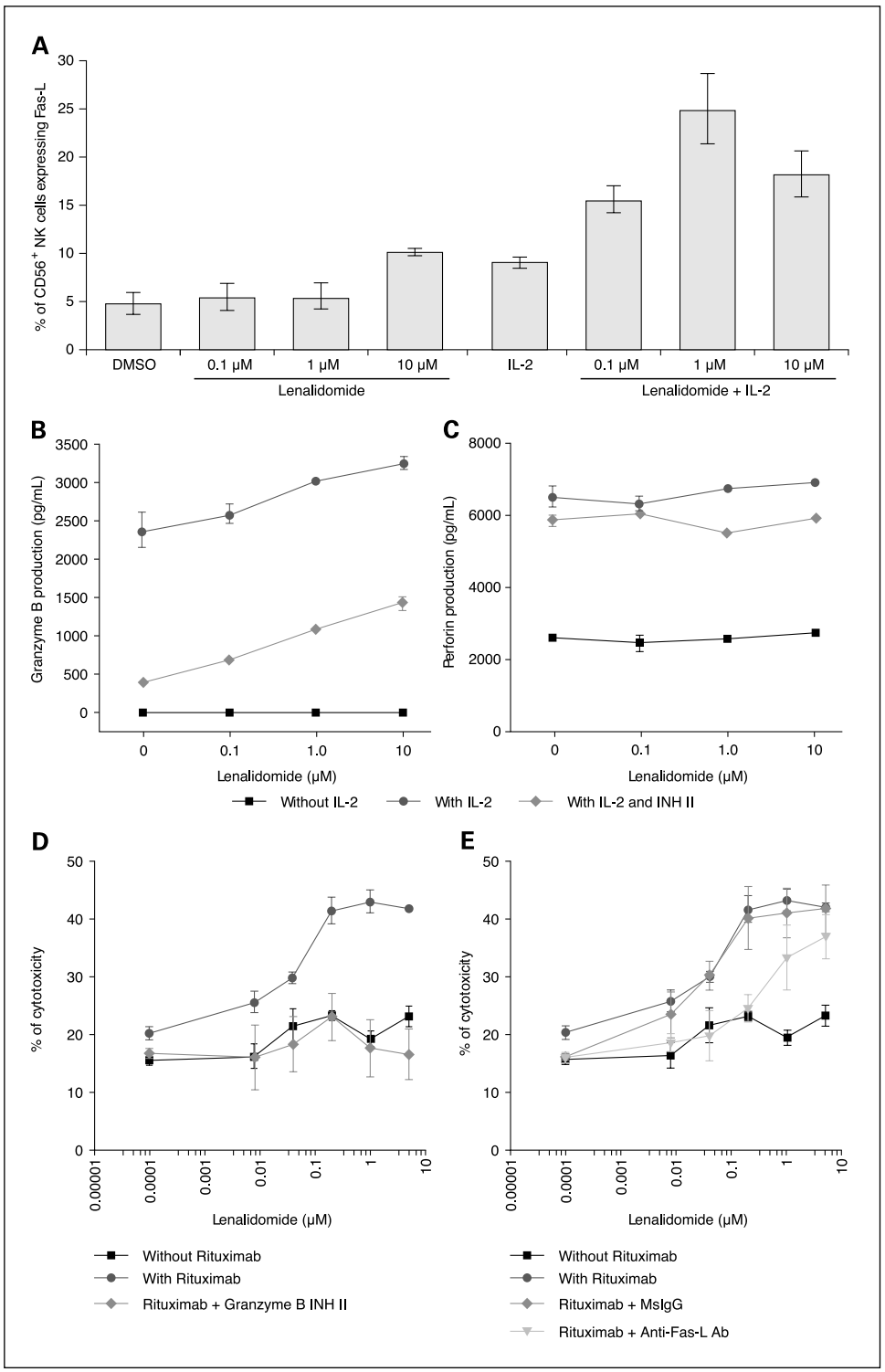

Fig. 5. *In vitro* effect of lenalidomide on NK cell expression of (*A*) FasL, (*B*) granzyme B, and (*C*) perforin production from NK cells in the presence of immobilized IgG and IL-2. Effect of (*D*) granzyme B inhibitor and (*E*) anti-FasL on ADCC. Mean ± SD of two donors. *INH II,* proteinase inhibitor II.

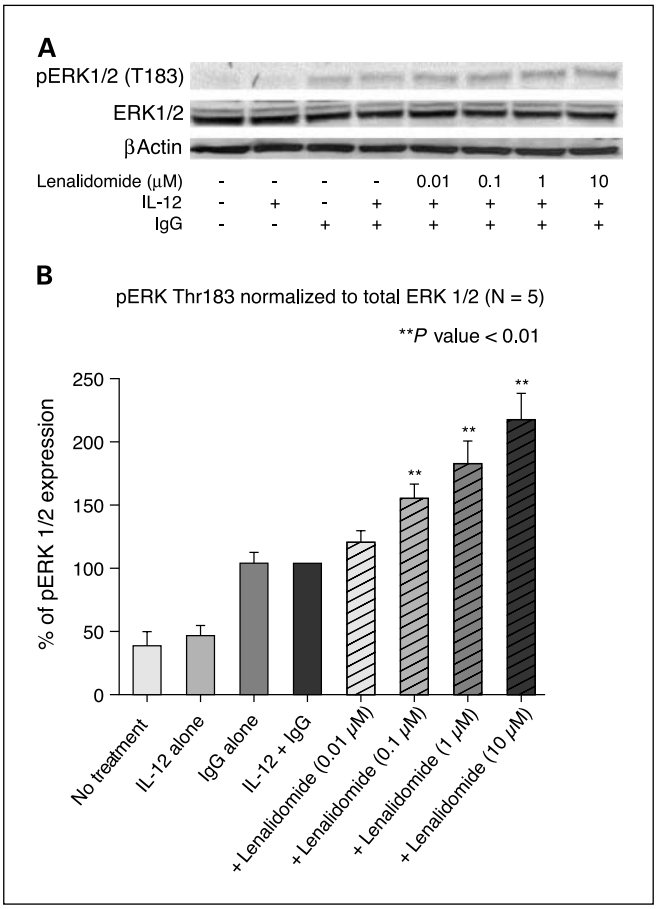

Fig. 6. Western blot (*A*) and analysis of NK cells treated with lenalidomide (*B*). *P* values, significance versus IL-12 + IgG. Mean ± SE.

the ADCC assay despite being a potent enhancer of IFN-γ production by NK cells. This was partly explained by the inability of an IFN-γ mAb to block ADCC, indicating that IFN-γ is not required for ADCC in this system (data not shown). Thalidomide has been shown previously to enhance NK cell expansion and effector function in patients with multiple myeloma who respond to treatment (17) and this may be linked to its ability to enhance NK cell IFN-γ. We also found that lenalidomide enhanced the ability of monocytes to initiate ADCC of rituximab-coated Farage tumor cells, an effect that did not require exogenous cytokine but was completely blocked by a neutralizing anti-IL-12 mAb.

To elucidate the mechanism of action of lenalidomide, we analyzed the effect of lenalidomide on the expression of pERK, which has been implicated as having a role in NK cell-mediated ADCC (18). ERK2 is activated in primary NK cells in humans by stimulation of the low-affinity receptor for the Fc fragment of IgG (Fc-γRIIIA), and this in turn regulates NK cell cytotoxicity (18).

Based on the results presented here, it may be postulated that the NK cell stimulatory effect of lenalidomide is due to enhanced Fc-γ receptor signaling, which is likely to elevate pERK, leading to enhanced expression of granzyme B and FasL. Notably, lenalidomide in combination with IL-2 and immobilized IgG resulted in the enhancement of NK cell FasL expression. The enhanced expression of granzyme B is thought to be associated with activation of caspase-dependent pathways of apoptosis as well as with direct effects on mitochondria and DNA degradation (4). Blocking granzyme B function by a specific soluble inhibitor appeared to totally block the ability of lenalidomide to enhance ADCC, whereas anti-FasL was able to only partially block ADCC. Perforin was enhanced by IL-2 alone and was not further enhanced by lenalidomide.

Our findings support previous reports that NK cells produce T-cell-recruiting chemokines in response to antibody-coated tumor cells (19). Further enhancement of chemokine production by IgG-stimulated NK cells during ADCC in the presence of lenalidomide is suggestive of concomitant promotion of chemoattraction and potential infiltration of tumor-specific T cells. Specifically, in the presence of lenalidomide and antibody (IgG), we observed large increases in NK cell production of MCP-1 and GM-CSF, smaller increases in IL-8, IP-10, and RANTES, and decreased production of IL-6. In particular, it could be postulated that the enhanced production of MCP-1 by NK cells within the tumor site may also attract effector T cells (20), whereas increased GM-CSF may enhance dendritic cell function and antitumor immunity. Also, increased levels of serum GM-CSF have been observed in solid tumor patients treated with lenalidomide (21).

Furthermore, lenalidomide also enhanced the ability of NK cells to kill primary tumor cells derived from three patients with B-CLL who have been treated previously with fludarabine plus cyclophosphamide. This suggests that lenalidomide could be used as salvage therapy in patients who relapse after other treatment regimens, and clinical trials using lenalidomide in this setting are currently under way.

In conclusion, the observation that lenalidomide enhances the rituximab-induced killing of NHL cell lines and primary B-CLL cells via a NK cell-mediated and monocyte-mediated ADCC mechanism *in vitro* provides a strong rationale for the combination of these drugs in patients with NHL and CLL.

Disclosure of Potential Conflicts of Interest

Authors are employees of Celgene and hold stick options in Celgene.

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Clinical Cancer Research

Lenalidomide Enhances Natural Killer Cell and Monocyte-Mediated Antibody-Dependent Cellular Cytotoxicity of Rituximab-Treated CD20$^+$ Tumor Cells

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