Enhancement of ligand dependent activation of human Natural Killer T cells by Lenalidomide: Therapeutic Implications

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Abstract

Natural-Killer T (NKT) cells are CD1d restricted glycolipid reactive innate lymphocytes that play an important role in protection from pathogens and tumors. Pharmacologic approaches to enhance NKT cell function will facilitate specific NKT targeting in the clinic. Here we show that lenalidomide (LEN), a novel thalidomide (Thal) analogue, enhances antigen-specific expansion of NKT cells in response to NKT ligand, α-galactosyl-ceramide (α-GalCer) in both healthy donors and myeloma patients. NKT cells activated in the presence of LEN have greater ability to secrete interferon-γ. Antigen-dependent activation of NKT cells was greater in the presence of dexamethasone (DEX) plus LEN, than with DEX alone. Therapy with LEN / Thal also led to an increase in NKT cells in vivo in patients with myeloma and del5q myelodysplastic syndrome. Together these data demonstrate that LEN and its analogues enhance CD1d mediated presentation of glycolipid antigens and support combining these agents with NKT targeted approaches for protection against tumors.
NKT cells are T lymphocytes bearing NK markers that recognize glycolipid ligands in the context of CD1d molecules. NKT cells play an important role in immune regulation and protection against tumors and pathogens. These cells mediate anti-tumor effects by several mechanisms, including direct effects on tumor cells, strong cytokine production, anti-angiogenesis and activation of other cells, particularly NK cells and dendritic cells.

Thalidomide and its analogue Lenalidomide have shown clinical activity in myeloma and myelodysplastic syndrome (MDS). However the mechanism of their observed anti-tumor effects remain unclear. Haslett et al. demonstrated that thalidomide can costimulate human T cells which led to the development of Lenalidomide (LEN) as an immune-modulatory derivative. Initial studies of thalidomide in myeloma described an effect on NK cells. However subsequent studies have suggested that the observed effects on NK cells were indirect. We hypothesized that innate CD1d restricted NKT cells may be a proximal cellular target of LEN. Here we show that LEN greatly enhances ligand dependent activation, proliferation and cytokine production by human NKT cells, as well as enhance NKT cells in vivo.
Materials and Methods

**Cells and Materials**

Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated from buffy coats purchased from New York Blood Center, or from patients with multiple myeloma, following informed consent approved by the institutional review board. Lenalidomide (LEN; Celgene Corporation, Warren, NJ) was dissolved in dimethyl sulfoxide (DMSO) (Sigma) at 1 mM. NKT ligand α-galactosyl-ceramide (α-GalCer) was kindly provided by Kirin Breweries, Japan.

**Dendritic Cell mediated NKT expansion**

CD14+ monocytes were isolated from PBMC using immunomagnetic bead selection with CD14 microbeads and cultured in the presence of GM-CSF and IL-4 to generate DCs, as described 11,12. After day 5-6 of culture, DCs were matured by adding an inflammatory cytokine cocktail as described12. To stimulate NKT cells, DCs were pulsed with α-GalCer (100ng/ml), and cultured with CD14 negative cells at DC: responder ratio of 1:10-20 with or without LEN (Celgene) at 1 µM, or otherwise indicated 12,13. For some experiments, DEX (0.5 nM) was also added as indicated at the beginning of expansion. NKT expansion was monitored by flow cytometry based on the expression of invariant T cell receptor (Vα24/Vβ11) and/or binding to CD1d-dimer loaded with α-GalCer, as described 12.
Intracellular cytokine Staining (ICS)

The ability of NKT cells to secrete cytokines following stimulation with NKT ligand (α-GalCer) was tested using both intracellular cytokine staining and TaqMan assays, as described\textsuperscript{12,14}. Freshly isolated PBMC were stimulated with α-GalCer, with or without LEN (1 µM). Stimulation with PMA and ionomycin was used as a positive control. For the detection of cytokine production by expanded NKT cells, these cells were restimulated overnight with unpulsed or α-GalCer-pulsed DCs (DC/responder ratio 1:10-20), prior to ICS assay.

TaqMan RT-PCR quantitation of cytokine mRNA

For the TaqMan analysis, PBMCs were cultured with α-GalCer as described in ICS. Stimulated cells were pelleted and total RNA was isolated using the QIAGEN RNeasy kit. The primers and probes for IFN-γ, IL4, IL10, and IL13 were purchased from Applied Biosystems /Perkin Elmer. RTPCR reactions were performed in duplicate samples as described previously \textsuperscript{12}. mRNA levels for a housekeeping gene GAPDH were used to normalize gene expression from each sample.

Thal/Len mediated effects in vivo

To analyze the effects of LEN on NKT cells in vivo, we obtained peripheral blood samples after IRB approved informed consent from MDS patients (n=5) treated with single agent LEN (10 mg/d in 28 day cycles), at baseline and at 3-6 cycles of therapy. Number of NKT cells was monitored by flow cytometry. Similarly, we also measured...
NKT cells in blood of myeloma patients (n=2) before and 1 month after 50-100 mg/d thalidomide.
Results and Discussion:

Human monocyte derived DCs loaded with NKT ligand α-GalCer are efficient at both activation and expansion of NKT cells in culture\textsuperscript{13}. However, the addition of LEN to these co-cultures greatly increased NKT expansion, both in healthy donors and in myeloma patients (Figure 1a and 1b, supplemental table 1). Enhancement of number of NKT cells by LEN was ligand dependent and seen only in the α-GalCer stimulated cultures. In prior studies, we have observed that mature DCs are more effective than immature DCs at mediating NKT expansion, presumably due to higher expression of co-stimulatory molecules \textsuperscript{13}. However, LEN mediated enhancement of NKT cells was observed with both immature and mature DCs (Figure 1c). As LEN and Thal are sometimes used with DEX in the clinic, we tested whether the effect of LEN was maintained in the presence of DEX, which is thought to have a differential effect on T versus NKT cell lines\textsuperscript{15}. DEX alone did not lead to an increase in GalCer mediated NKT expansion. Addition of LEN to DEX enhanced NKT expansion in response to α-GalCer loaded DCs, compared to DEX alone (Figure 1d). Therefore LEN boosts ligand dependent expansion of NKT cells in vitro.

We have recently shown that injection of α-GalCer loaded DCs leads to expansion of NKT cells in vivo in patients with advanced cancer\textsuperscript{12}. We treated two of these patients with low dose (50-100 mg/d) thalidomide, a parent analogue of LEN, after NKT cells had returned back to baseline. In both patients, thalidomide led to an increase in circulating
NKT cells at one month post therapy (Figure 1e). We also analyzed the number of NKT cells in MDS patients treated with LEN, at baseline and at 3rd - 6th cycles of therapy. Increase in NKT cells was observed in 3 of 5 patients, which included all 3 of the patients with del5q MDS (Figure 1f).

Anti-tumor properties of NKT cells are linked to their ability to secrete interferon-γ\(^2\). NKT cells expanded in the presence of LEN had greater ability to secrete IFN-γ (Figure 2a). Similar data were observed by real-time RT-PCR (TaqMan) analysis of these cells (Figure 2b). LEN associated increase in interferon-γ production by NKT cells was detectable even in the presence of DEX, which blunted the cytokine response (Figure 2c). LEN also led to an increase in ligand dependent induction of interferon-γ production by freshly isolated NKT cells in human PBMCs (Figure 2d). Therefore, LEN also leads to an increase in ligand reactive interferon-γ secretion by human NKT cells in vitro.

In summary, these data show that LEN has a significant effect on the function of human CD1d restricted NKT cells. LEN may therefore help improve clinical NKT targeting. NKT activation may also contribute to NK activation in vivo\(^{16-18}\), and serve as an adjuvant for improving T cell based vaccines\(^19\). These finding also have implications for understanding the mechanism of action of LEN in human studies. A major fraction of T cells in the human bone marrow are CD1d restricted\(^20\). CD1d restricted T cells in the bone marrow (including those lacking invariant T cell receptor) may therefore be one of the proximal targets of LEN in both myeloma and MDS. It is of interest that recent studies have identified selective deficiency of NKT cells in MDS patients\(^{21,22}\). NKT cells
may also play a role in regulating hematopoiesis in vivo. Combining NKT ligands with LEN may provide a simple rational approach to enhance the efficacy of either therapy against human cancer.
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Figure legends

Figure 1. LEN / Thalidomide mediated enhancement of NKT cells *in vitro* and *in vivo*.

Figure 1a. LEN boosts expansion of NKT cells from healthy donors by DCs pulsed with α-GalCer. T cells were expanded with DCs loaded with α-GalCer (or unpulsed DCs) in the presence or absence of LEN / DMSO control. After 2 weeks of culture, the presence of Vα24+Vβ11+ or CD1d-aGC dimer+ NKT cells was monitored by flow cytometry.

Figure 1b. LEN boosts expansion of NKT cells by DCs pulsed with α-GalCer in both healthy donors and myeloma patients. NKT cells were expanded with DCs loaded with α-GalCer (or unpulsed DCs) in the presence or absence of LEN / DMSO control. After 1-2 weeks of culture, the presence of Vα24+Vβ11+ NKT cells was monitored by flow cytometry. Data shown are fold increase in NKT cells for cultures with α-GalCer loaded DCs. Horizontal bars represent mean NKT expansion.

Figure 1c. Immature versus mature DCs. NKT cells were expanded as in 1a, with immature monocyte derived DCs (iDC), or after DC maturation with inflammatory cytokines (MDC). After 2 weeks of culture, the presence of Vα24+Vβ11+ NKT cells was monitored by flow cytometry. Data are representative of 8 separate experiments.
Figure 1d. Comparison of DEX versus DEX + LEN. NKT cells were expanded as in 1a, in the presence of DEX (5 nM) alone, or with LEN (1 µM). Data shown are % iNKT cells. Horizontal bars represent mean NKT expansion.

Figure 1e. Expansion of NKT cells in myeloma. Circulating NKT cells were monitored by flow cytometry before and 1 month after thalidomide therapy in myeloma patients (n=2).

Figure 1f. NKT expansion in MDS patients (n=5) treated with LEN. iNKT cells were quantified by flow cytometry before and at 3rd and 5th/6th cycle of LEN therapy. Closed symbols represent patients with del5q.

Figure 2. LEN mediated enhancement of NKT effector function.

Figure 2a. NKT cells expanded in the presence or absence of LEN as in Figure 1a, were tested for the ability to secrete interferon-γ in response to unpulsed or α-GalCer pulsed DCs, by intracellular cytokine secretion (ICS) assay. Bottom panel shows data from individual donors (n=7). Horizontal bars represent mean % NKT cells secreting interferon-γ.

Figure 2b. TaqMan analysis showing changes in cytokine gene expression in NKT cells (expanded initially with α-GalCer loaded DCs) and cultured overnight with unpulsed or α-GalCer loaded DCs in the presence of absence of LEN. Data are representative of 2 separate experiments.
Figure 2c. NKT cells expanded in the presence of DEX alone, or DEX+ LEN, as in Figure 1d, were tested for the ability to secrete interferon-γ in response to unpulsed or α-GalCer pulsed DCs, by intracellular cytokine secretion (ICS) assay (n=4).

Figure 2d. NKT cells in freshly isolated PBMCs from healthy donors were tested for interferon-γ secretion in response to vehicle or α-GalCer pulsed DCs, by intracellular cytokine secretion (ICS) assay, as in Figure 2a (n=5).
References:


1a

\begin{align*}
\text{DC(-)} & \quad \text{DC(\alpha GC)} \\
& \quad \text{Len} \\
& \quad \text{Len} \\
\end{align*}

\begin{align*}
V_{\beta 11} & \quad V_{\alpha 24} \\
& \quad 0.47 \\
& \quad 0.19 \\
& \quad 0.50 \\
& \quad 0.24 \\
& \quad 0.90 \\
& \quad 2.92 \\
& \quad 1.08 \\
& \quad 10.3 \\
\end{align*}

CD1d-Dimer

\begin{align*}
& \quad 0.02 \\
& \quad 0.51 \\
& \quad 0.32 \\
& \quad 0.29 \\
& \quad 0.06 \\
& \quad 0.40 \\
& \quad 2.17 \\
& \quad 9.76 \\
\end{align*}

1b

\begin{align*}
\text{Fold Increase NKT Cells} \\
& \quad 0 \\
& \quad 40 \\
& \quad 80 \\
& \quad 120 \\
& \quad 160 \\
& \quad 200 \\
\end{align*}

\begin{align*}
& \quad (-) \\
& \quad \text{Len} \\
& \quad \text{Healthy Donors (N=12)} \\
\end{align*}

\begin{align*}
& \quad (-) \\
& \quad \text{Len} \\
& \quad \text{Myeloma Patients (N=5)} \\
\end{align*}

\begin{align*}
P < 0.001 \\
P = 0.04 \\
\end{align*}
1e

% iNKT

MM1
MM2

Pre Post-Thalidomide

1f

% iNKT

MDS1
MDS2
MDS3
MDS4
MDS5

Del 5q

Pre Cycle 3 Cycle 5/6 Lenalidomide
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% IFNγ-secreting NKT cells / total NKT cells

P = 0.03

2d

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