Generation of Antitumor Invariant Natural Killer T Cell Lines in Multiple Myeloma and Promotion of Their Functions via Lenalidomide: A Strategy for Immunotherapy

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Abstract

Purpose: CD1d-restricted invariant natural killer T (iNKT) cells are important immunoregulatory cells in antitumor immune responses. However, the quantitative and qualitative defects of iNKT cells in advanced multiple myeloma hamper their antitumor effects. Therefore, the development of functional iNKT cells may provide a novel strategy for the immunotherapy in multiple myeloma.

Experimental Design: We activated and expanded iNKT cells from multiple myeloma patients with a-galactosylceramide (a-GalCer)-pulsed dendritic cells, characterized their antitumor effects by the cytokine production profile and cytotoxicity against multiple myeloma cells, and explored the effects of immunomodulatory drug lenalidomide on these iNKT cells. We also investigated the expression of CD1d by primary multiple myeloma cells and its function to activate iNKT cells.

Results: We established highly purified functional iNKT cell lines from newly diagnosed and advanced multiple myeloma patients. These CD1d-restricted iNKT cell lines produced high level of antitumor Th1 cytokine in response to a-GalCer-pulsed primary multiple myeloma cells, CD1d-transfected MM1S cell line, and dendritic cells. Moreover, iNKT cell lines displayed strong cytotoxicity against a-GalCer-pulsed primary multiple myeloma cells. Importantly, lenalidomide further augmented the Th1 polarization by iNKT cell lines via increased Th1 cytokine production and reduced Th2 cytokine production. We also showed that CD1d was expressed in primary multiple myeloma cells at mRNA and protein levels from the majority of multiple myeloma patients, but not in normal plasma cells and multiple myeloma cell lines, and CD1d+ primary multiple myeloma cells presented antigens to activate iNKT cell lines.

Conclusions: Taken together, our results provide the preclinical evidence for the iNKT cell-mediated immunotherapy and a rationale for their use in combination with lenalidomide in multiple myeloma treatment.

Multiple myeloma is still a fatal hematologic malignancy characterized by the accumulation of terminally differentiated plasma cells in the bone marrow of patients (1). Although high-dose chemotherapy with stem cell transplant has shown some success, the outcome of the majority of patients with multiple myeloma is unsatisfactory (2). Clinical benefits may be obtained from immunotherapy to stabilize or even eradicate minimal residual disease after the conventional treatments for patients with multiple myeloma.

Invariant natural killer T cells (iNKT cells) constitute an innate lymphocyte lineage that has an important role in regulating immune responses, including antitumor responses. iNKT cells display an extremely restricted T-cell antigen receptor (TCR) repertoire in humans consisting of a specific Vα24-Jα18 chain rearrangement preferentially paired with a Vβ11 chain. Unlike conventional T cells that recognize peptide antigens, iNKT cells recognize glycolipid ligands presented by a nonpolymorphic MHC class I-like antigen-presenting molecule CD1d and are characterized by their capacity to rapidly produce large amounts of immunoregulatory cytokines (3). iNKT cells play a physiologic role in tumor immunosurveillance against carcinogen-induced tumors (4) and are required for the antitumor effects of low-dose interleukin (IL)-12 treatment (5). Most importantly, preclinical studies in murine models have shown that, on activation by a-galactosylceramide (a-GalCer), a highly specific ligand...
for CD1d, iNKT cells can stimulate potent antitumor immune responses through the production of Th1 cytokines (6–8). iNKT cells have also shown the directed killing activity against CD1d+ tumor cells (9–11).

In progressive multiple myeloma, however, iNKT cells are functionally defective as evidenced by deficient ligand-dependent IFN-γ production, resulting in a detrimental Th2 cytokine profile (12). Similarly, studies from several groups indicate that iNKT cells are decreased and/or functionally impaired in various cancer patients including prostate cancer, melanoma, and myelodysplastic syndrome (13–16). Therefore, a novel immunotherapeutic strategy may be developed using adoptive transfer of iNKT cells to multiple myeloma patients after in vitro expansion and functional activation.

Lenalidomide (CC-5013, Revlimid, and IMiD3), which belongs to a class of thalidomide analogues known as the immunomodulatory drugs, was approved for the treatment of multiple myeloma in 2006. Lenalidomide induces apoptosis, decreases the binding of myeloma cells to stromal cells in bone marrow, and inhibits angiogenesis (17). Additionally, lenalidomide increases conventional T-cell costimulation and NK cell cytotoxicity (18, 19), and a report has recently revealed that lenalidomide can also enhance ligand-dependent activation of iNKT cells (20).

In this study, we evaluated the expression and function of CD1d on multiple myeloma tumor cells. We established iNKT cell lines from multiple myeloma patients, characterized their antitumor profile, and further addressed the effects of lenalidomide on these CD1d-restricted iNKT cells. Our research provides the preclinical basis and rationale for the use of iNKT cells in antmyeloma immunotherapy.

Materials and Methods

**Samples.** Healthy donor leukopacks were obtained from Dana-Farber Cancer Institute. Normal bone marrow samples were purchased from AllCells. Multiple myeloma patient blood and bone marrow samples were obtained from Dana-Farber Cancer Institute following informed consent approved by the institutional review boards. Patients were classified as multiple myeloma according to standard diagnostic criteria.

**Generation of dendritic cells.** Dendritic cells were generated from adherent peripheral blood mononuclear cells (PBMC) in the presence of granulocyte-macrophage colony-stimulating factor (1,000 units/mL) and IL-4 (25 ng/mL; both from R&D Systems). On days 5 to 6, the dendritic cells were matured by lipopolysaccharide (100 ng/mL; Sigma-Aldrich) overnight in the presence of 100 ng/mL α-GalCer (KRN7000; Kirin Brewery) and irradiated at 50 Gy immediately before use.

**Expansion of iNKT cells.** Due to the low frequency of iNKT cells in PBMCs, iNKT cells were first enriched by staining with unconjugated anti-TCRVα24 monoclonal antibody (mAb; Immunotech) followed by the immunomagnetic isolation with goat anti-mouse IgG microbeads (Miltenyi Biotec). Subsequently, Vα24+ and α-GalCer-pulsed mature dendritic cells were cocultured in PRMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 15 mmol/L HEPES, 5.5 x 10^{-5} mol/L β-mercaptoethanol, 50 units/mL penicillin, 50 μg/mL streptomycin, and 2 mmol/L L-glutamine. Recombinant human IL-2 (100 units/mL; Chiron) was added to the cultures on day 2 and supplemented every 4 to 6 days. When the cells had proliferated, iNKT cells were further enriched with PE-conjugated anti-TCRVβ11 mAb (Immunotech) followed by immunomagnetic isolation with...
anti-PE microbeads (Miltenyi Biotec). Culture was then gradually expanded and restimulated every 7 to 10 days. The frequencies of iNKT cells pre- or post-expansion were monitored by quantifying \( V_{a24}+V_{h11}+ \) cells using flow cytometric analysis. Considering the low frequency of iNKT cells in PBMCs, at least \( 1 \times 10^5 \) to \( 3 \times 10^5 \) cells were collected for each analysis in the lymphocyte gates.

**Isolation of normal plasma cells and primary multiple myeloma cells.** Normal plasma cells and primary multiple myeloma cells were purified from bone marrow mononuclear cells by positive selection with CD138 microbeads (Miltenyi Biotec) according to the manufacturer’s instructions.

**Microarray analysis.** Gene expression of CD1d was done by microarray analysis. Total RNA from normal plasma cell samples, multiple myeloma cell lines, and primary multiple myeloma cells was isolated using a “RNeasy” kit (Qiagen) and gene expression profile was evaluated using HG-U133 arrays (Affymetrix). GeneChip arrays were scanned on a GeneArray Scanner (Affymetrix). Array normalization, expression value calculation, and clustering analysis were done using the dChip Analyzer.

**Preparation of CD1d-transfected MM1S cell line (MM1S.CD1d).** Multiple myeloma cell line MM1S (kindly provided by Dr. Steven Rosen, Northwestern University) was transfected with a CD1d cDNA in the pSRα-neo expression vector (21) or empty vector using Nucleofector Kit V (Amaxa Biosystems) according to the manufacturer’s instructions. CD1d+ MM1S cells were further isolated with magnetic microbeads and followed by selection with G418 for generating the cell line stably expressing CD1d. Mock cell line was only selected by G418. CD1d expression was determined by immunofluorescence using anti-CD1d-PE mAb. The expression of CD138 on transfected MM1S cells was detected by anti-PE-CD138-PE mAb to monitor the change of MM1S cell line. CD1d- and mock-transfected MM1S cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 50 units/mL penicillin, 50 \( \mu \)g/mL streptomycin, and 2 mmol/L L-glutamine.

**Cytokine production of iNKT cells in response to multiple myeloma cell lines and dendritic cells.** Mock/CD1d-transfected MM1S cells and matured dendritic cells were preloaded with vehicle or \( \alpha \)-GalCer (100 ng/mL) overnight. The cells were then washed to remove the supernatant before use. Resting iNKT cells (\( 1 \times 10^5 \)) from multiple myeloma patients were added into 96-well plate with above mock/CD1d-transfected MM1S cells or dendritic cells at ratio of 2:1 and 4:1.

### Table 1. Phenotype analysis of iNKT cell lines from multiple myeloma

<table>
<thead>
<tr>
<th>Percentage of positive cells</th>
<th>iNKT cell lines</th>
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<tbody>
<tr>
<td>CD4+</td>
<td>CD8+</td>
</tr>
<tr>
<td>A</td>
<td>21</td>
</tr>
<tr>
<td>B</td>
<td>53</td>
</tr>
<tr>
<td>C</td>
<td>77</td>
</tr>
<tr>
<td>D</td>
<td>44</td>
</tr>
</tbody>
</table>

NOTE: Surface phenotype was detected by direct immunofluorescence. Data are obtained from four representative iNKT cell lines from multiple myeloma patients (A-D).
respectively. Supernatants were collected at 48 h for IL-2 and IL-4 and 72 h for IFN-γ measurements, respectively.

**Function of CD1d+ primary multiple myeloma cells on iNKT cells.** Resting iNKT cells (1 × 10^5) were plated in 96-well plates with either medium alone or 5 × 10^4 primary multiple myeloma cells. α-GalCer (100 ng/mL) was added as indicated. IL-2 was included in the culture medium at a final concentration of 10 units/mL. The supernatants were collected for the cytokine detection at 48 h. Meanwhile, the cells were harvested and stained by anti-TCRVα24-FITC and anti-CD25-PC5 mAbs.

**Effects of lenalidomide on iNKT cells.** Lenalidomide (Celgene) was dissolved in DMSO and stored at -20°C. Drug was diluted in culture medium with <0.1% DMSO immediately before use. The following systems were set up: iNKT cells alone and iNKT cells cultured with MM1S.CD1d in the absence or presence of α-GalCer (100 ng/mL) with or without the treatment with lenalidomide (2 μmol/L). The supernatant was collected at 72 h and subject to ELISA detection.

**Cytokine assays.** Released cytokine levels were determined by ELISA. IFN-γ, IL-2, and IL-4 were quantified by the Quantikine immunoassay (R&D Systems) according to the manufacturer’s instruction.

**Cytotoxicity assay.** Cytotoxicity was assessed using a 4-h calcein-AM release assay as described previously (22). MM1S.CD1d cells or primary multiple myeloma cells were labeled with calcein-AM (5 μg/mL; Molecular Probes) and used as target cells at 5,000 per well in the presence or absence of α-GalCer (100 ng/mL). iNKT cell lines from healthy donors and multiple myeloma patients were used as effector cells. The E:T ratio was 20:1. Calculation of cytotoxicity was done using the following equation: % cytotoxicity = 100 × (experimental release - spontaneous release) / (maximal release - spontaneous release).

**Antibodies and flow cytometric analysis.** The following antibodies were used for the flow cytometric analysis: FITC- or PE-conjugated anti-TCRVα24, anti-TCRVβ11, anti-CD16, and anti-CD161 mAbs (Immunotech); FITC-, PE-, or PC5-conjugated anti-CD1d, anti-CD4, anti-CD8, anti-CD25, anti-CD56, and anti-CD94 mAbs (BD PharMingen); and PE-conjugated anti-CD138 mAb (Miltenyi Biotec). Isotype-matched mAbs were used as controls. Flow cytometry was done with a Coulter EPICS Elite flow cytometer (Beckman-Coulter).

**Statistical analysis.** Statistics analyses were done by Student’s t test. P < 0.05 was considered significant.

**Results**

**Establishment of highly purified iNKT cell lines.** To develop an immunotherapy approach using iNKT cells, we first determined the frequency of iNKT cells in the PBMCs from healthy donors and multiple myeloma patients. Consistent with published data in other types of cancer, the frequency of iNKT cells was reduced in patients with advanced multiple myeloma (0.01 ± 0.008%; n = 7) compared with healthy controls (0.064 ± 0.030%; n = 7; P < 0.001). We established primary iNKT cell lines from healthy donors and newly diagnosed and advanced multiple myeloma patients. Healthy donors exhibited over 90% success rate for iNKT cell line generation, whereas multiple myeloma patients exhibited a 50% success rate (5 of 10 patients). Flow cytometry showed >97% purity of Vα24+Vβ11+ cells (Fig. 1). The phenotype analysis on multiple myeloma iNKT cell lines showed majority cells were CD4+ or CD4-CD8-. The expression of CD161 was variable, whereas no iNKT cell lines expressed CD16 or CD94. A certain level of CD56 expression was also observed (Table 1). No significant phenotypic difference was observed between established iNKT cell lines from healthy donors and multiple myeloma patients. Generally, >10^8 iNKT cells could be harvested in 6 to 8 weeks.
**CD1d expression by primary multiple myeloma cells.** To evaluate potential in vivo interaction between iNKT cells and myeloma cells, we studied the profile of CD1d expression on primary multiple myeloma cells as well as multiple myeloma cell lines by gene expression profiling. A total of 15 CD138+ primary multiple myeloma cell samples and 6 multiple myeloma cell lines (MM1S, ARD, ARK, ARP, PRM18226, and U266) were compared with normal plasma cells; the majority of primary multiple myeloma cells expressed higher levels of CD1d (11 of 15). In contrast, all 6 multiple myeloma cell lines tested showed no expression of CD1d (Fig. 2A). Flow cytometric analysis using anti-CD1d-PE mAb further confirmed the expression of CD1d on primary multiple myeloma cells but lack expression on 12 different multiple myeloma cell lines (MM1S, MM1R, ARD, ARK, ARP, PRM18226, U266, OPN1, OPN2, CAG, 12PE, and 28PE; Fig. 2B; data not shown).

**Establishment of CD1d-transfected multiple myeloma cell line.** Because CD1d is expressed by primary myeloma cells but not by multiple myeloma cell lines, we therefore established a stable CD1d-transfected MM1S cell line (MM1S.CD1d) for the feasibility of the functional study (Fig. 2C). Flow cytometry showed that 100% of the transfected cells expressed CD138. No phenotypic change or growth characteristic difference was observed in the CD1d-transfected MM1S cells compared with the parental cell line (data not shown).

**Multiple myeloma iNKT cell lines exhibit Th1 cytokine profile.** To confirm the function and CD1d reactivity of α-GalCer-expanded iNKT cells from multiple myeloma, we evaluated their cytokine profile using mock/CD1d-transfected MM1S cells and dendritic cells. iNKT cell lines stimulated by mock-transfected MM1S cells produced very low to undetectable levels of IL-2, IFN-γ, and IL-4 with or without α-GalCer (data not shown). When cultured with MM1S.CD1d cells alone, iNKT cells secreted low levels of these cytokines. Notably, when stimulated with α-GalCer-pulsed MM1S.CD1d cells, iNKT cells produced high levels of IFN-γ as well as IL-2 and a low amount of IL-4 (Fig. 3). These data were confirmed using α-GalCer-pulsed dendritic cells, which resulted in a more pronounced Th1 immune response (Fig. 3). In addition, we observed IL-10 production was at the low level when iNKT cell lines responded to MM1S.CD1d cells in the presence or absence of α-GalCer. Together, our results indicate that α-GalCer-reactive CD1d-restricted iNKT cell lines can be obtained from multiple myeloma patients and exhibited a Th1 antitumor cytokine profile.

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**Fig. 4.** CD1d+ primary multiple myeloma cells present antigens to activate iNKT cell lines. Resting iNKT cells (1 × 10⁵) were cultured alone or with 5 × 10⁴ CD1d+ primary multiple myeloma cells for 48 h. α-GalCer (100 ng/mL) was added. The activation of iNKT cells was evaluated by CD25 expression and functional cytokine production. A, representative flow cytometric analysis shows the activation of one multiple myeloma iNKT cell line in the cultures. The sample was stained with anti-Vα2-4-FITC and anti-CD25-PC5 mAbs. B, percentage (mean ± SD) of CD25+ iNKT cells in the cultures by 5 iNKT cell lines in response to 5 different primary multiple myeloma cell samples. Statistical analysis was done by Student’s t test. C, measurement of IFN-γ and IL-4 production in the cultures by ELISA. Mean ± SD of triplicate assays. Data represent one of three independent experiments that provided similar results.
CD1d+ primary multiple myeloma cells present antigens to activate iNKT cell lines. We further addressed whether iNKT cell line activation could be mediated by CD1d+ primary multiple myeloma cells. As shown in Fig. 4, CD25 expression and IFN-γ production by iNKT cells were dramatically increased in the presence of α-GalCer-pulsed primary multiple myeloma cells, showing the efficiency of CD1d-mediated antigen presentation by multiple myeloma tumor cells. Moreover, the functional cytokine profile evaluated by IFN-γ and IL-4 production further confirmed the Th1 antitumor iNKT cell lines obtained. In addition, significant increases in CD25 expression and IFN-γ production also observed when iNKT cells cocultured with primary multiple myeloma cells in the absence of α-GalCer.

Lenalidomide polarizes and augments Th1-type immune responses. To further augment iNKT cell immune responses, we evaluated the effect of lenalidomide, an immunomodulatory drug, on iNKT cell lines. Lenalidomide did not directly stimulate iNKT cells (data not shown) but significantly increased IFN-γ and IL-2 production and significantly decreased IL-4 production when iNKT cells were activated using α-GalCer-pulsed MM1S.CD1d cells (Fig. 5) and CD1d-transfected C1R cells (data not shown). Furthermore, lenalidomide also increased IFN-γ and IL-2 production when iNKT cells were cultured with MM1S.CD1d cells in the absence of α-GalCer (Fig. 5).

iNKT cell lines display strong cytotoxicity against multiple myeloma cells. We also investigated whether the expanded iNKT cell lines had the direct killing activity against multiple myeloma cells. MM1S.CD1d cells and CD1d+ primary multiple myeloma cells were used as target cells. The iNKT cell lines from both healthy donors and multiple myeloma patients showed strong cytotoxicity against α-GalCer-loaded target cells. Meanwhile, a low level of cytotoxicity was found in the absence of α-GalCer (Fig. 6). No cytotoxicity observed by iNKT cell lines against CD1d-negative primary multiple myeloma cells or MM1S mock cells in the absence or presence of α-GalCer (data not shown).

Discussion

Various studies have shown an important role of iNKT cell-derived Th1-type cytokines in initiating antitumor immune responses. Through the production of IFN-γ, iNKT cells can stimulate the activation of downstream effectors including T cells, NK cells, dendritic cells, and macrophages and increase NK and T cell proliferation and cytotoxicity through IL-2.
production (23–28). However, both quantitative and qualitative defects of iNKT cells in advanced multiple myeloma hampered their antitumor effects. In this study, we developed a novel immunotherapeutic strategy directed at the activation and expansion of Th1-polarized iNKT cells from multiple myeloma patients. We report the establishment of CD1d-restricted iNKT cell lines from newly diagnosed and advanced multiple myeloma patients. These iNKT cell lines produced Th1 cytokines, indicating that the suppressive effects of the multiple myeloma microenvironment on iNKT cells have been overcome by in vitro culture. Although multiple myeloma patients have a very low frequency of iNKT cells, combination of antibody purification and selective stimulation with α-GalCer-pulsed dendritic cells allowed us to obtain a high yield of iNKT cell lines, which provided adequate cell numbers for the potential adoptive immunotherapy.

We further showed that CD1d was expressed by tumor cells in majority of multiple myeloma patients. This phenomenon indicates an ideal environment for iNKT cell-mediated immunotherapy in multiple myeloma. First, the CD1d+ multiple myeloma cells provide the direct targets for iNKT cells. Our data have shown the strong killing activity by iNKT cell lines against α-GalCer-pulsed multiple myeloma cells. Moreover, CD1d molecule on patient multiple myeloma cells effectively present α-GalCer to activate iNKT cells, resulting in the Th1-polarized cytokine production. Therefore, another immunotherapeutic strategy may be developed using an autologous tumor cell-based vaccine consisting of irradiated α-GalCer-pulsed CD1d+ myeloma cells to boost iNKT cells activity in vivo. We also observed that iNKT cells displayed a certain level of reactivity when culture with CD1d+ multiple myeloma cells, even in the absence of exogenous α-GalCer, suggesting that multiple myeloma cells may express natural CD1d-bound ligands for iNKT cells. Further identification of these ligands would provide a basis for generation of multiple myeloma-specific iNKT cells.

Importantly, we showed that immunomodulatory drug lenalidomide further augments the antitumor effects of iNKT cell lines via CD1d-restricted stimulation, evidenced by not only enhancing the Th1 cytokine production but also reducing the Th2 cytokine production. Our results provide the rationale for the combination with lenalidomide in iNKT cell-mediated therapy. Currently, the mechanism of lenalidomide on iNKT cells is unclear. For conventional T cells, we have observed that lenalidomide, via the B7-CD28 pathway, costimulates these cells (18). We have detected the CD28 expression on our iNKT cell lines from healthy donors and multiple myeloma patients. It showed that all the iNKT cell lines express CD28 (data not shown). The further characterization of whether CD28 and/or other costimulatory signaling pathways are involved will delineate mechanism of the effect by lenalidomide on CD1d-restricted activation.

In summary, our study shows that functional iNKT cell lines can be generated from multiple myeloma patients with α-GalCer-pulsed dendritic cells and further improved by lenalidomide. These results provide the preclinical feasibility and rationale for iNKT cell-mediated immunotherapy in multiple myeloma.

Disclosure of Potential Conflicts of Interest

Dr. K.C. Anderson and Dr. N.C. Munshi are Consultants for Celgene corporation.

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