NKT cell defects in multiple myeloma and the impact of lenalidomide therapy

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Running title: NKT cells and multiple myeloma.
Summary

The causes of multiple myeloma (MM) remain obscure and there are few known risk factors, however NKT cell abnormalities have been reported in patients with MM, and therapeutic targeting of NKT cells is promoted as a potential treatment. We characterized NKT cell defects in treated and untreated patients with MM and determined the impact of lenalidomide therapy on the NKT cell pool. Lenalidomide is an immunomodulatory drug with co-stimulatory effects on NKT cells in vitro and is an approved treatment for MM, although its mode of action in that context is not well defined. We find that patients with relapsed/progressive MM had a marked deficiency in NKT cell numbers. In contrast, newly diagnosed patients had relatively normal NKT cell frequency and function prior to treatment, although a specific NKT cell deficiency emerged after high dose Melphalan and autologous stem cell transplantation (ASCT) regime. This also impacted NK cells and conventional T cells, but the recovery of NKT cells was considerably delayed, resulting in a prolonged, treatment-induced NKT cell deficit. Longitudinal analysis of individual patients revealed that lenalidomide therapy had no in vivo impact on NKT cell numbers or cytokine production either as induction therapy or as maintenance therapy following ASCT, indicating that its clinical benefits are independent of NKT cell modulation in this setting.

Key words: multiple myeloma, NKT cells, lenalidomide.
Introduction

NKT cells are CD1d-restricted T cells that recognize lipid-based antigens. They are capable of rapidly releasing cytokines that affect a wide range of innate and adaptive immune responses, including against cancer [1-3]. Several subpopulations of NKT cells have been identified, but the most widely studied are Type 1 NKT cell, which express an evolutionarily conserved semi-invariant TCR [1] [4]. Defects in Type 1 NKT cells have been associated with multiple myeloma (MM) and are the focus of this study, so for simplicity, we will refer to them as NKT cells from the point onwards.

Defects in the NKT cell pool are associated with autoimmune diseases [5] [6], allergies [7] and many forms of cancer [8-14], but there is an emerging focus on NKT cells in the context of multiple myeloma (MM), where immune dysfunction has been identified as potentially important in disease predisposition and progression {Dhodapkar, 2003 #806}[12, 13, 15, 16]. Defective IFN-γ production by NKT cells and a trend toward lower NKT cell frequencies reportedly affect patient groups with MM and are also observed in animal models of MM [8, 12, 17]. As a result, many studies have nominated NKT cells as a potential new target for treatment of MM in humans [18] [12, 13, 16, 19, 20].

The mechanism by which NKT cells may protect against MM development is not defined, although several studies link these cells to protective anti-myeloma responses. For example, NKT cell lines isolated from patients with MM respond to primary MM cells pulsed with α-GalCer (a potent NKT cell agonist), resulting in cytokine release and lysis of the myeloma cells [19]. The CD1d antigen-presenting molecule recognized by NKT cells is highly expressed in premalignant and early myeloma plasma cells, and progression of MM is associated with reduced CD1d
expression by myeloma cells [21]. Engagement of CD1d (NKT ligand) also kills tumour cells in vitro [21] and NKT cells specific for an inflammation-associated lipid antigen, lysophosphatidylcholine, have been detected in patients with advanced myeloma [22]. Collectively, these findings indicate why a defective NKT cell pool may be associated with MM onset and relapse and support the concept that therapeutic targeting of NKT cells may be a useful strategy in treatment of MM in humans [12, 19] [13] [16, 17, 23].

The immunomodulatory drug lenalidomide (Revlimid®, Celgene Corp., Summit, NJ. USA.) has been approved as a treatment for MM [24, 25], although its exact mode of action is not well understood. Lenalidomide may potentially impact on MM through several mechanisms, including inhibition of angiogenesis, modulation of plasma cell proliferation, and promoting immune cell adhesion and activation [25, 26]. Lenalidomide promotes T cell and NK cell activation [27], but it also has specific stimulatory effects on NKT cells that are independent of T cells [12, 28]. These include enhancing NKT cell proliferation and cytokine production, and promoting a Th1 cytokine profile [18, 19, 29-31].

The well documented in vitro impact of lenalidomide treatment on NKT cells is consistent with a report showing increased frequency and cytokine responsiveness of NKT cells from patients treated with lenalidomide [32]. Although only 2 patients with MM were examined in that study, a more recent study of patients with asymptomatic myeloma treated with lenalidomide in combination with α-galactosylceramide (αGalCer) - loaded monocyte-derived dendritic cells (DCs) identified activated NKT cells and reduced serum paraprotein [20]. Although it was difficult to isolate the impact of the lenalidomide from the transferred DCs in that
study, the study speculated that combination therapies targeting NKT cells could help to prevent disease progression in humans.

We and others have argued that although these data are promising, more knowledge is required about whether NKT cell defects contribute to MM in humans, and whether NKT cell agonists (possibly including lenalidomide) are viable approaches to anti-MM treatment. In this study, we present findings from a longitudinal analysis of NKT cells from a clinical trial exploring the efficacy of lenalidomide therapy in newly diagnosed patients with untreated MM. These results were compared to patients enrolled in a lenalidomide clinical trial for MM, which had relapsed, or was refractory to prior anti-MM therapy; and to a control group of healthy donors. We characterised the frequency and functional defects of NKT cells patients with MM, and determined their NKT cell response to lenalidomide therapy.
Materials and Methods

Trial and study design

Anti-coagulated whole blood from healthy donors was obtained from the Australian Red Cross Blood Bank Service (Southbank, Melbourne, Australia). Patient samples were obtained from 2 clinical trials; 1. The Revlite trial (RL NCT00482261) in patients with either relapsed/refractory MM evaluating the effects of low dose lenalidomide (Revlimid; Celgene) (15mg D1-21) with high dose dexamethasone (20mg d1-4, 9-11 and 17-21) (these patients had been previously treated with chemotherapy and ASCT (for most)); and 2. The LitVacc trial (ACTRN12613000344796) in patients with newly diagnosed MM undergoing 4 cycles of induction with low dose lenalidomide (15mg D1-21), low dose dexamethasone (20mg weekly) followed by high dose cyclophosphamide and GCSF stem cell mobilisation and high dose melphalan (200mg/m²) AuSCT/ followed by lenalidomide maintenance commencing on D21-35 post transplant (25mg d1-21/ 28 day cycle) and DC vaccination with autologous DC loaded with primary MM cell lysate. All enrolled patients in either trial had active MM requiring treatment. Patients with MGUS or smouldering MM were not included). The trials were approved by the Peter MacCallum Centre Human Research Ethics committee and are registered on ClinicalTrials.gov.

Sample processing and storage

Serial blood samples were obtained as per study protocol. Samples from patients in the RL trial were taken at enrolment only and samples from patients in the LV trial were taken at enrolment and on day 1 of lenalidomide induction cycle (C) 2 and 3, at the
end of the induction (EOI) immediately prior to ASCT, 21 days post ASCT and on day 1 of maintenance (M) cycles 2, 4, and 6 (Supplementary Figure 1).

Peripheral blood mononuclear cells (PBMCs) were isolated from whole anticoagulated blood by gradient centrifugation by Histopaque (density 1.077g/mL; Sigma-Aldrich, MO, USA) and then cryopreserved in 10% dimethyl sulfoxide and 90% fetal bovine serum at -80°C prior to storage in liquid nitrogen for later batch analysis.

**Antibodies and flow cytometry**

Fluorochrome-labelled antibodies used for flow cytometry (FITC-conjugated anti-CD25, anti-IgG1 and anti-IFNγ (4S.B3), PE-Cy7-conjugated anti-CD3 (SK7), APC-H7-conjugated anti-CD8 (SK1), APC-conjugated anti-IL-4 (MP4-25D2) and anti-IgG1, AlexaFluor 700-conjugated anti-CD56 and anti-TNF (MAb11) and Pacific Blue-conjugated anti-CD4 (RPA-T4),) were purchased from BD Biosciences (San Diego, CA, USA). APC-conjugated anti-CD161 (191B8) was purchased from Miltenyi Biotech. PE-conjugated αGalCer-loaded mouse CD1d tetramer was produced in-house by Konstantinos Kyparissoudis (University of Melbourne) using a construct originally provided by Mitchell Kronenberg, La Jolla institute for Allergy and Immunology. Flow cytometry data was acquired on a LSRII (BD Biosciences) and analysed using FlowJo software (Treestar Inc., OR, USA). Autofluorescent cells, doublets, non-specifically stained cells and dead cells were excluded using 7-aminoactinomycin D (Invitrogen Life Technologies, CA, USA) and vehicle-loaded CD1d tetramer.

**PBMC stimulation**
Cells were cultured in 12-well plates in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (JRH), 100U penicillin (Invitrogen Life Technologies), 100µg/mL streptomycin (Invitrogen Life Technologies), 2mM Glutamax (Invitrogen Life Technologies), 1mM sodium pyruvate (Invitrogen Life Technologies), 15mM HEPES (Invitrogen Life Technologies), 0.1mM non-essential amino acids (Invitrogen Life Technologies) and 50µM 2-mercaptoethanol (Sigma-Aldrich) at 37°C and 5% CO₂. For in vitro stimulation, 10ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 1µg/mL ionomycin (Sigma-Aldrich) for 4h with 2µM monensin (Golgistop; BD Biosciences). After stimulation, cells were prepared for intracellular analysis of IFNγ, TNF and IL-4 using the Cytofix/Cytoperm staining kit (BD Biosciences).

Statistical analyses

Statistical analyses were performed with GraphPad Prism V5.0 software (GraphPad Software Inc., CA, USA) using the Mann-Whitney test or Kruskal-Wallis test with Dunn’s Multiple Comparisons post-test as appropriate.
Results

Relapsed/refractory MM patients (RMM) have lower NKT cell frequencies than healthy donors

We analysed T and NKT cells from peripheral blood of 24 patients with relapsed/refractory MM (RMM) and compared them to samples from 47 healthy blood donors, some of whom were also part of an earlier study [29]. RMM patients had a normal distribution of CD4+, CD4- and CD8+ subsets, but a statistically significant NKT cell deficiency (Fig 1). The conventional T cell pool of RMM patients was also moderately deficient, but NKT cells remained selectively deficient even when expressed relative to T cells (rather than as a % of PBMCs), which indicates a specific NKT cell deficiency in RMM patients.

NKT cell frequency in patients with newly diagnosed MM prior to treatment

Given the significant NKT cell deficit in RMM patients, it was important to investigate whether this was directly related to MM. We therefore compared the RMM group to newly diagnosed MM patients who had not commenced treatment and to healthy donors (Fig 2). Analysis of the T cell and NKT cell pools showed that newly diagnosed patients had a normal NKT cell frequency. The overall frequency of conventional T cell levels in untreated patients with MM and the ratio between CD4+ and CD8+ T cells was comparable to that of healthy subjects (Fig 2B-C). Interestingly, there was a trend toward an abnormal ratio between CD4+ and CD4- NKT cell subsets in untreated patients with MM, reflecting a comparative deficit of the CD4-NKT cell subset that is regarded as the most important for anti-cancer activities [33] [34] [4, 35, 36], however, this was variable and not statistically significant.
**NKT cell frequency after lenalidomide and ASCT**

Our data indicated that NKT cell numbers were relatively normal in newly diagnosed patients with MM, suggesting that the deficit we observed in the group of relapsed or progressive patients with MM may have been due to the treatment of the disease rather than MM itself. To directly examine this and to measure the impact of standard therapy with lenalidomide, we conducted longitudinal analysis of T cells, NK cells and NKT cells in 11 MM patients throughout treatment as part of the LitVacc trial (Fig 3). Blood samples were taken prior to treatment and throughout a treatment course of lenalidomide and dexamethasone induction therapy, ASCT and subsequent lenalidomide - DC vaccination maintenance therapy (DC vaccination was given to 10/11 patients).

These data showed that lenalidomide induction therapy did not result in a significant increase in the frequency or absolute number of NKT cells (Fig 3B-C). At the end of induction (EoI), patients underwent ASCT, which caused an expected decline in T cell frequency of most patients (Fig 3B and C). A selective reduction in frequencies and absolute numbers of circulating CD4+ T cells was also observed after ASCT (Fig 3B-C), coinciding with a proportional increase in CD8 T cells (Fig 3B-C). Proportions and absolute numbers of NK cells did not change significantly post ASCT (Fig 3B-C) in keeping with the typically rapid recovery of this lymphocyte population post-ASCT.

In contrast to conventional T cells, the NKT cell pool underwent striking post-ASCT changes (Fig 3B-C). The proportions and absolute numbers of NKT cells were greatly decreased, did not recover to pre-ASCT during lenalidomide maintenance therapy and were more severely decreased than for conventional CD4+ and CD8+ T cells and NK cells (Fig 3B-C).
**Longitudinal analysis of NKT cell subsets**

Having identified a severe and prolonged NKT cell deficiency in patients with MM after ASCT-therapy, we separately examined the impact on functionally distinct NKT cell subsets. We analysed NKT cell subsets defined by expression of CD4 and CD8, NK markers such as CD161 and CD56 and the CD25 activation marker in patients with MM during induction therapy, after ASCT and during maintenance therapy (Figs 3C and 4A) [4, 33, 34] [37]. Induction therapy did not significantly alter the composition of the NKT cell compartment and the proportions and numbers of different NKT cell subsets remained relatively consistent (Fig 4B-C). Analysis of 5 patients post--ASCT revealed a decline in numbers across all NKT cell subsets and none fully recovered during maintenance therapy (Fig 4C). CD25 expression was uniformly low on all groups and at all time points, indicating NKT cells were not activated in these patients (Fig 4A and data not shown).

**Longitudinal analysis of NKT cell function**

A previous study reported that NKT cells from patients with progressive MM lacked the ability to produce IFNγ after stimulation [8]. The regulatory functions of NKT cells rely on rapid release of cytokines in response to stimulation, and lenalidomide has been proposed as a means of boosting NKT cell function in patients with MM because several studies have demonstrated improved cytokine production by NKT cells in the presence of lenalidomide [18, 20, 30].

We have previously reported that lenalidomide treatment of patients with MDS did not significantly impact NKT cell frequency or function [29]. To establish a more complete understanding of the nature and kinetics of the NKT cell cytokine

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defect in patients with MM, and the impact of lenalidomide treatment on this important function, we assessed NKT cell cytokine responses from 4 MM patients throughout a 6-8 month course of lenalidomide and dexamethasone induction therapy, ASCT and lenalidomide and DC vaccination maintenance therapy (Fig. 5A-B). We stimulated PBMCs in vitro with PMA and ionomycin and assayed the T cells and NKT cells from these cultures for intracellular IFNγ, TNF and IL-4 using flow cytometry (Fig 5A). NKT cell numbers of two patients did not recover sufficiently for cytokine analysis post ASCT, but in the others assessed, there was NKT cell production of IFNγ, TNF and IL-4 for 6 months after ASCT (Fig 5B). The small blood volumes available from these patients coupled with the low frequency of NKT cells meant that we were unable to conduct functional assays for all patients. Despite this, our data shows that NKT cells from patients with MM remained capable of significant cytokine production of IFNγ, IL-4 and TNF at each timepoint prior to and after treatment.
Discussion

We report on a longitudinal study of NKT cells in patients with MM undergoing treatment with lenalidomide. Earlier reports had indicated that NKT cell defects may be important in the development of MM [12, 13, 18] [8] [17] and that NKT cells could be a new target for immune-based treatment of MM patients [20, 23]. To investigate the therapeutic significance of NKT cells in MM, we analysed NKT cell frequency and function in longitudinal studies of newly diagnosed patients with MM throughout several stages of therapy, with an emphasis on identifying and defining any NKT cell defects and determining the impact of lenalidomide treatment on NKT cell frequency and function. The latter objective followed reports that lenalidomide, which is already an approved treatment of MM, could act to increase NKT cell numbers and cytokine expression [18, 20].

Our analysis of relapsed patients with MM identified a significant NKT cell deficiency, but the frequency and cytokine production of NKT cells was normal in newly diagnosed patients. Longitudinal analysis of newly diagnosed MM patients demonstrated that a profound and extended deficiency of NKT cells developed after high dose chemotherapy with ASCT. We observed that NKT cells were capable of producing IFN-γ, TNF and IL-4 at all time points in our study, including prior to treatment, which contrasts with earlier reports of severely impaired IFN-γ production by NKT cells in patients with MM.

Our data suggests that NKT cell defects are not present at the time of MM diagnosis and therefore would appear not to predispose to the onset of MM. Rather, NKT cells are markedly depleted by ASCT and/or associated prior chemotherapy and recover far more slowly than conventional T cells or NK cells. The impact of NKT cell deficiency in patients with MM after intensive prior therapy (regardless of cause)
is not known, but is worthy of further investigation, given the significance of NKT cell deficiency in other patient groups. For example, we do not exclude the possibility that such a defect could still potentially impact on duration of remission from MM and/or their response to subsequent treatments that affect, or rely on, NKT cells.

Our longitudinal analysis of individuals also allowed us to investigate the effect of lenalidomide on NKT cells. This followed several in vitro studies (including our own [29]) [19, 31], and one in vivo study [18], that showed lenalidomide could promote NKT cell proliferation and cytokine production, suggesting that lenalidomide’s mode of action in MM might involve direct effects on NKT cells [12, 20, 23]. However, NKT cell frequency and function were normal in patients with MM prior to treatment and subsequent induction therapy with lenalidomide had no significant impact on the number or cytokine responsiveness of NKT cells. The results suggest that the clinical benefit of lenalidomide treatment of patients with MM does not rely on counteracting a pre-existing NKT cell defect, or on stimulating residual NKT cells.

Our finding that lenalidomide had no measurable effect on NKT cells in patients with MM, even after an extended treatment regimen, is consistent with our recent analysis of NKT cells from patients with myelodysplastic syndromes (MDS) treated with lenalidomide [29]. We cannot formally exclude that lenalidomide promoted a moderate NKT cell expansion masked by similar NKT cell losses through death or selective egress from the blood, however, we contend that is extremely unlikely because our longitudinal analysis showed no major changes in NKT cell frequency or function at any stage after lenalidomide treatment. The more likely explanation is that NKT cells were not meaningfully affected by lenalidomide in vivo and are unlikely to have contributed significantly to clinical improvements.
The similar frequencies and cytokine production of NKT cells from MM patients and healthy donors means that we should not discount the possibility that NKT cells could be targeted for therapeutic advantage in patients with MM. A recent clinical trial reported that a combination therapy approach of α-galactosylceramide (α-GalCer)-loaded monocyte-derived dendritic cells and low dose lenalidomide to treat patients with MM, led to NKT cell activation and tumor regression, with the authors concluding that direct NKT cell activation (and the synergistic activation of innate cells), perhaps in combination with the costimulatory activities of lenalidomide, may benefit anti-cancer treatment in humans [20]. The stimulation of NKT cells in that study was partly attributed to the effects of lenalidomide on NKT cells, however we question whether it is possible to fully delineate the respective roles of NKT cells versus lenalidomide because lenalidomide and the α-GalCer–loaded DCs were administered together, and because NKT cells, NK cells and other innate cells activated in the study can reciprocally stimulate one another. Furthermore, the clinical benefit of any immunotherapy that acts via NKT cell stimulation is likely to be maximized in the setting of normal NKT cell populations. This indicates that such therapies should be employed early in the treatment schedule, when NKT cells are preserved, and not be delayed to a time were NKT cells have been functionally depleted or incorporate mechanisms aimed at restoring NKT cell numbers such as glycolipid-based vaccines [39].

An issue worthy of further investigation is the distribution of NKT cell subsets in patients with MM. Prior to treatment, the average CD4- to CD4+ ratio of NKT cell subsets was >2-fold higher among patients with MM, however, there was high variability of this parameter and the difference between groups was not significant. The question of whether the different means is indicative of a true defect in patients
with MM is important to resolve because CD4- NKT cells have potent anti-tumor activities and the CD4- subset deficiency implied by our study could conceivably contribute to tumor predisposition, even when overall NKT cell numbers are normal.

In summary, we report that patients with newly diagnosed MM were not significantly defective in NKT cell frequency or cytokine production, however NKT cells were specifically depleted after ASCT and remained so even after lenalidomide treatment. This indicated that NKT cell defects do not predispose to MM and that the positive clinical response to lenalidomide in patients with MM does not rely on stimulation of the NKT cell pool.
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Conflict of Interest

The authors declare no conflict of interest.
References


Figure legends

**Figure 1.** NKT cell deficiency in pre-treated multiple myeloma patients. PBMCs isolated from blood collected from relapsed or refractory MM patients (RMM) (n=24) and healthy donors (n=47) were stained with CD3 and αGalCer-loaded CD1d tetramer to detect NKT cells. (A) RMM patients had a significantly lower NKT cell frequency compared to healthy controls, irrespective of whether the frequency was expressed as proportion of overall lymphocytes, or of CD3+ T cells. (B) The distribution of CD4+, DN and CD8+ NKT cell subsets are shown for RMM patients and healthy donors.

**Figure 2.**

T and NKT cell frequency in patients with MM. A) Representative flow cytometry plots of NKT cells from RMM (n=24), untreated patients with MM (n=11) and healthy controls (n=47) are shown. (B) Individual peripheral blood T cell frequencies for RMM, untxMM and healthy donors are shown. (C) The CD4+ T cell: CD8+ T cell ratio was assessed for each group. (D) Individual peripheral blood NKT cell frequencies for RMM, untxMM and healthy donors are shown. The dotted line represents a NKT % below the threshold of detection. (E) The ratio of CD4-NKT:CD4+ NKT cells were also assessed. Means of each group were compared using the Kruskal-Wallis statistical test with Dunn’s multiple comparison post-test.

**Figure 3.**

Poor recovery of NKT cells in untxMM patients after autologous stem cell transplant. PBMCs were isolated from blood collected from untxMM patients prior to treatment (C1), after 2 cycles of LEN induction (C2), after 3 cycles of LEN induction (C3), at the end of induction (EoI), 2 maintenance cycles post auSCT (M2), 4 maintenance
cycles post ASCT (M4) and 6 maintenance cycles post ASCT (M6). (A) Representative FACS plots demonstrate how NKT cell subsets are analysed. (B) The frequency of T cells, NK cells and NKT cells were assessed at each timepoint. CD4+ T cells and CD8+ T cells are expressed as a proportion of T cells. T cells, NK cells and NKT cells are expressed as a proportion of lymphocytes and of T cells as indicated. (C) T cells, NK cells and NKT cell for individual untxMM patients are shown.

**Figure 4.**
The NKT cell compartment is deficient following ASCT. (A) Representative FACS plots demonstrate how NKT cell subsets are analysed. (B) CD4+, CD4-CD8-, CD4-CD8+, CD161+, and CD56+ NKT cell subsets were monitored during a longitudinal study of previously untreated MM patients receiving lenalidomide, ASCT and DC vaccination in MM. (C) Cell numbers for CD4+, CD4-CD8-, CD4-CD8+, CD161+, and CD56+ NKT cell subsets are shown for individual patients.

**Figure 5.**
NKT cells retain functional capacity after ASCT. PBMCS were stimulated ex vivo with PMA/ionomycin for 4 hours. (A) Representative FACS plots from previously untreated patients are shown. (B) The percentage of NKT cells capable of IFNγ, TNF and IL-4 production was assessed for 4 patients with sufficient NKT cells for cytokine analysis.

**Supplementary Figure 1.** Multiple Myeloma clinical trial design. Previously untreated newly diagnosed patients were enrolled onto the Litvacc study (A), where
following len+dex induction the patients received an ASCT followed by myeloma lysate-pulsed DCs + len maintenance or len maintenance alone (in our study, 10/11 patients received myeloma lysate-pulsed DCs + len maintenance). Patients with refractory relapsed disease were enrolled on RevLite study (B), to receive len+dex induction, then repeated cycles of len+dex until disease progression. A comparison between the two trials is provided in table (C) including disease state, drug dose and cycles of therapy.
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