The inhibitory receptor PVRIG is dominantly expressed in the bone marrow of patients with multiple myeloma and its blockade enhances T-cell engager's immune activation

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Therapeutic advances in treating patients with multiple myeloma (MM), including novel immunotherapies, have improved the disease control, but it remains incurable. Although traditional immune check point inhibitors have shown limited clinical benefit, targeting alternative immune-inhibitory pathways may offer a novel way to address relapsed disease. Blockade of the immune regulator TIGIT was shown to enhance antitumor immunity in preclinical MM models. Beyond TIGIT, the DNAM-1 axis includes the novel inhibitory receptor PVR related immunoglobulin (PVRIG). In this study we evaluated the expression of DNAM-1 axis receptors and the function of PVRIG in bone marrow of individuals with MM, specifically highlighting PVRIG blockade as a potential therapeutic opportunity in combination with bispecific T-cell engager (BiTE).

HIGHLIGHTS

- PVRIG is highly expressed in various T-cell populations, including natural killer (NK) and natural killer T (NKT) cells.
- PVRIG blockade might contribute to a positive clinical outcome in patients with multiple myeloma (MM).
- PVRIG blockade, in combination with bispecific T-cell engager (BiTE), enhances T-cell activation in MM bone marrow.

Multiple myeloma (MM) is a hematologic malignancy characterized by uncontrolled clonal proliferation of plasma cells in the bone marrow (BM) [1]. There has been a significant advancement in the development of treatment approaches, including proteasome inhibitors (PIs), immunomodulatory drugs (IMIDs), monoclonal antibodies, and chimeric antigen receptor (CAR) T-cell therapies. Additionally, an emerging promising therapeutic strategy for MM is the use of bispecific T-cell engagers (BiTEs), engineered to activate T cells toward myeloma cells killing, through cross-linking CD3 on their surface, while targeting a specific antigen on the tumor cell [2]. Despite all these advances, MM still remains an overwhelmingly incurable disease [3].

Immunologic escape of tumor cells has been described as a resistance mechanism influencing myeloma progression. $CD8^+$ T cells from patients with MM exhibit an exhausted phenotype, as evidenced by expression of various immune checkpoint receptors, including PD-1, CTLA-4, TIM-3, LAG-3, B7-H3, and TIGIT [3,4]. Notably, TIGIT was shown to be the most upregulated immune-inhibitory receptor on $CD8^+$ T cells in MM patients' BM compared with other checkpoints in both preclinical models and patients [5,6]. Nonetheless, to date, immune checkpoint blockade in MM has not yielded significant clinical results [7].

The DNAM-1 pathway, which includes TIGIT, is essential for regulating both innate and adaptive immunity, controlling the cytotoxic activity of T and natural killer (NK) cells against tumors [8]. Targeting this axis has shown promise in preclinical studies, with ongoing clinical trials evaluating its effectiveness in combination with other treatments such as PD-1 inhibitors [8]. In addition to TIGIT, the DNAM-1 pathway includes PVRIG, a recently discovered inhibitory receptor expressed on T and NK cells [8,9,10]. PVRIG competes with the coactivating receptor DNAM-1 for binding to their shared ligand PVRL2. Recently we showed that PVRIG is expressed on early differentiated stem-like memory T cells (Tscm), which retain proliferative potential and can differentiate into potent antitumor effectors [11]. In preclinical models, coblockade of TIGIT and PVRIG synergistically enhanced T-cell antitumor activity [9]. Moreover, the triple combination blockade of PVRIG, TIGIT, and PD-1 is being evaluated in clinical trials in patients with solid indications (NCT04570839).

In this study, we analyzed the expression of PD-1 and of DNAM-1 axis receptors in BM from patients with MM with newly diagnosed and relapsed disease. Subsequently, we evaluated the effect of PVRIG blockade in combination with a BiTE, on T-cell activation in freshly isolated BM of patients with MM.

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METHODS

Single Cell Analysis

For single cell RNA sequencing (scRNA-seq) data, unique molecular identifiers (UMIs) were quantified and mapped to reference transcriptome GRCh38 (GENCODE v32/Ensembl 98) using Cellranger 7.0 (10x Genomics). To allow detection of PVRIG in 10x Chromium-derived data, reads were mapped to a corrected gene-reference as previously described [12]. Subsequent analyses were performed using Scanpy 1.9, applying standard quality control (QC) and preprocessing steps. Cells having total counts of more than 6,000 or a percentage of mitochondrial genes above 15% were filtered out. Top principal components were used to construct uniform manifold approximation and projection (UMAP) embedding and K-nearest neighbor (KNN) graph was calculated with $n = 15$ followed by Leiden clustering. Clusters were annotated based on conventional marker expressions.

Preparation of MM BM Mononuclear Cells

Investigators from the Institute for Myeloma & Bone Cancer Research, received informed written consent from all participants before inclusion in the study, which was conducted in agreement with the institutional review board (IRB) approval (Protocol 101). Fresh BM aspirates were collected from 29 patients with MM which characteristics ranged from newly diagnosed to relapsed refractory (Supplementary Tables 1 and 2). BM mononuclear cells (MCs) were isolated using density gradient centrifugation with Histopaque-1077 (Sigma, St. Louis) according to standard protocol. Among the samples, 20 were used for expression, and 9 for ex vivo assays as outlined below.

Fluorescence-Activated Cell Sorting Analysis

Single cell suspensions were blocked using 5% fetal bovine serum (FBS) for preventing non-specific binding. The cells were incubated with antibodies for cell surface markers (human anti-PVRIG, anti-TIGIT, anti-DNAM, anti-CD3, anti-CD4, anti-CD8, anti-CD56, and their matched isotype controls as detailed in Supplementary Table 3) and then fixed using 2% paraformaldehyde for 30 minutes on ice. Flow cytometric analyses were performed using a Becton Dickinson FACSymphony S6. Analysis was completed using FlowJo (TreeStar LLC) and gated on specific populations. Gating lineages for human tumor panels are described in Supplementary Table 4.

Immunohistochemical Staining

BM biopsies from 6 patients with MM (Tissue Micro-Array T291, USBiomax) were stained for PVRL2 (clone 181H3L2, Abcam, rabbit mAb). Antigen retrieval was performed using citrate-based pH 6.2. Formalin-Fixed Paraffin-Embedded (FFPE) slides (4 mm sections) were stained on the Biocare intelliPATH automated platform using the manufacturer's recommended settings.

Ex Vivo MM BM Functional Assays

Fresh BM cells from 9 patients with MM with progressive disease (PD) were treated for 48 hours with 3 concentrations of B-cell maturation antigen (BCMA) BiTE antibody (1 nM, 2.5 nM, and 5 nM; Creative Biolabs, catalog number SCIGG-H200), and antihuman PVRIG blocking antibodies (10 μg/mL; Compugen, Ltd.) or hIgG4 isotype control. Supernatants were collected from each experimental group, and interferon (IFN)γ (R&D Systems, catalog number DIF50C) and Granzyme B (R&D Systems, catalog number DGZB00) were measured according to the manufacturer's protocol.

RESULTS

To evaluate the expression of the immune checkpoints TIGIT, PD-1, DNAM-1, and PVRIG on T cells, we analyzed scRNA sequencing data obtained from 18 patients with MM [13]. DNAM-1 is highly expressed in NK, NKT, and $CD8^+$ and $CD4^+$ T cells, as seen in Figure 1A. TIGIT shows a high expression pattern across T and NK cell populations. Similarly to TIGIT, PVRIG is highly expressed on $CD8^+$ T and NK cells as well as in $CD4^+$ T cells. Specifically, among T-cell populations, its expression is highest in Tscm (Figure 1B), as previously shown in solid tumors [11]. Interestingly, the expression of the immune checkpoint PD-1 was relatively low among all the lymphoid populations. To evaluate protein expression of these receptors, BM from patients with MM (majority of patients were with relapsed/refractory disease and four were newly diagnosed) were collected and analyzed by fluorescence-activated cell sorting (FACS). PVRIG demonstrated the highest protein expression on NK, NKT, and $CD8^+$ T cells. Its expression was significantly higher than PD-1 and TIGIT on all evaluated cell populations (Figure 1C). TIGIT showed substantially higher expression on NK and $CD8^+$ T cells compared with PD-1. All tested cell populations showed increased levels of DNAM-1 (>50%). Coexpression of TIGIT and PVRIG was detected in 50% and 60% of $CD8^+$ T and NK cells, respectively (Supplementary Figure 1A). PVRIG was not only expressed in significant cell fractions of T, NK, and NKT cells, but its expression level on these populations was prominent (Supplementary Figure 1B). Notably, PVRIG is not expressed in BM of healthy donors (Supplementary Figure 2).

PVRL2 was previously shown to be expressed on $CD14^+$ and plasma cells [14]. To validate PVRL2 expression on MM plasma cells, we performed Immunohistochemistry (IHC) analysis on tissue microarrays (TMAs) of MM (6 cases/24 cores). PVRL2 staining was seen on both endothelial and malignant plasma cells (Supplementary Figure 3A). FACS analysis further supported PVRL2 expression on plasma cells in BM of patients with MM, with 17/20 (85%) of patients having PVRL2 expression (3% to 87%) on $CD138^+$ myeloma cells (Supplementary Figure 3B and C). Looking at RNA sequencing data of several MM cell lines, PVRL2 was clearly expressed, mostly induced following treatment with different therapeutic agents (Supplementary Figure 3D).

Given that TIGIT and PVRIG share their ligands with the costimulatory receptor DNAM-1 and their blockade enhances DNAM-1 engagement and costimulation [8], we assessed the coexpression of PVRIG, TIGIT, and PD-1 with DNAM-1 on $CD8^+$ T cells. $CD8^+$ T cells, negative for DNAM-1 ($DNAM\text{-}1^{neg}$), demonstrated higher expression of TIGIT (81%) and PD-1 (57%), than $DNAM\text{-}1^{pos}$ $CD8^+$ T cells, indicative of accumulation of an exhausted ($PD\text{-}1^+TIGIT^+DNAM1^-$) $CD8^+$ T-cell population in the MM tumor microenvironment (Supplementary Figure 4A) as previously reported [3]. Accordingly, when exploring DNAM-1 expression on $CD8^+$ T cells in BM of patients with MM, we detected a trend for higher DNAM-1 expression among patients in complete remission (CR) than patients with PD, at the time of sample collection, suggesting the contribution of DNAM-1 expression to a positive clinical outcome (Supplementary Figure 4B). Interestingly, PVRIG showed

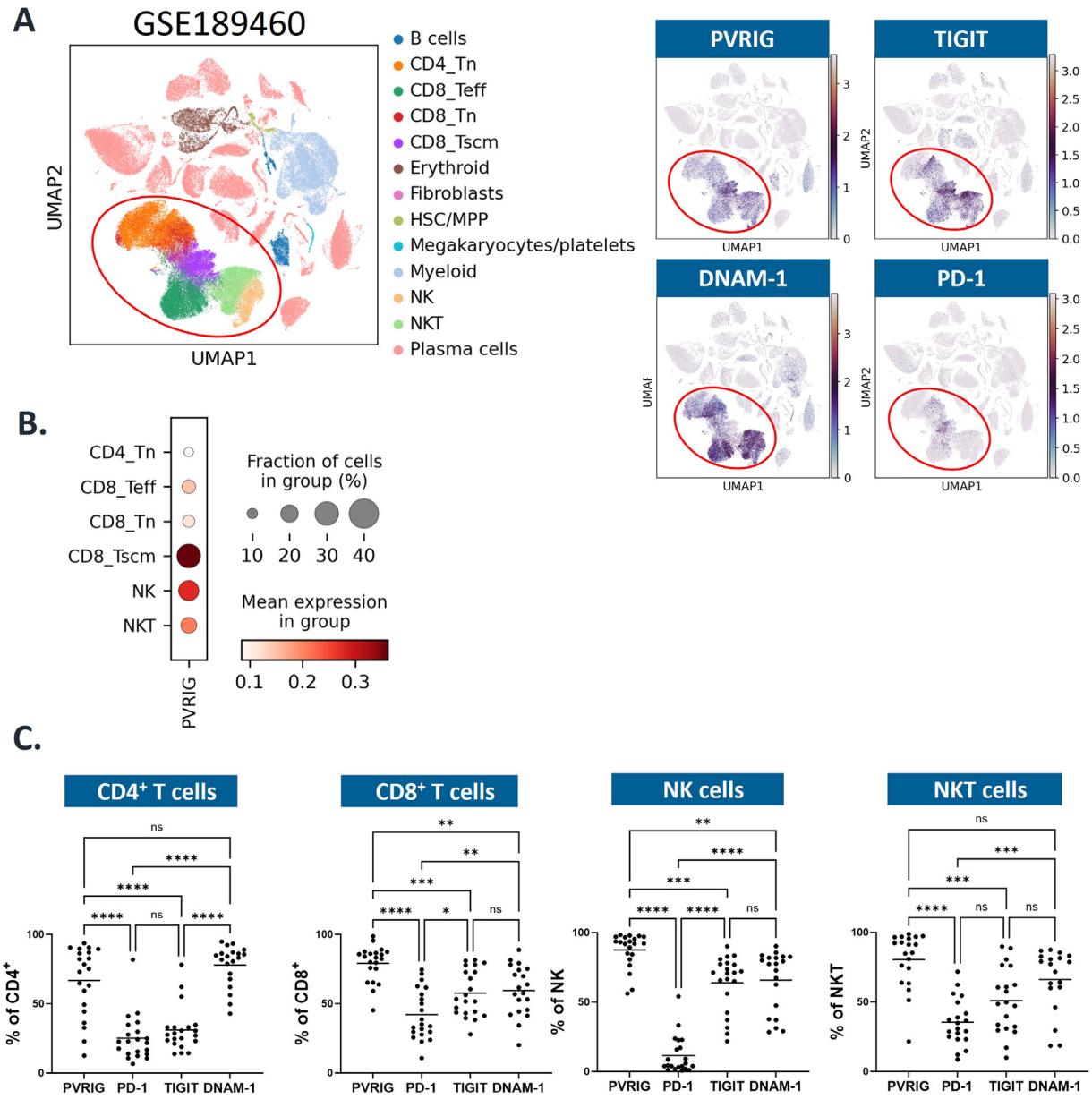

Figure 1 Dominant DNAM-1 axis receptor expression in MM BM aspirates. (**A**) Left: UMAP visualization of scRNA-Seq derived from mononuclear cells isolated from BM aspirates (15 patients) color-coded for the indicated cell types. Right: UMAP of the same data, showing the expression of indicated genes. Red circle defines T and NK cells. (**B**) Dot plot depicting the expression of PVRIG across different T-cell populations. (**C**) BM mononuclear cells from BM aspirates from 20 patients with MM were isolated using density gradient centrifugation. Single cells were analyzed for protein expression by FACS. Two-way ANOVA test was used to compare between PVRIG/PD-1/TIGIT and DNAM-1 expression among cell populations.

significantly higher coexpression with DNAM-1 on $CD8^+$ T cells, compared with TIGIT and PD-1, supporting the potential of PVRIG blockade to enhance DNAM-1 signaling and subsequent higher $CD8^+$ T-cell activation (Supplementary Figure 4C).

To investigate the functional significance of PVRIG expression observed on $CD8^+$ T cells in MM, we tested the effect of PVRIG blockade on T-cell activation in fresh bone marrow mononuclear cells (BMMCs), directly ex vivo. For this aim, a BCMA BiTE was combined with either anti-PVRIG antibody or hIgG4 isotype, and granzyme B and IFNγ secretion was assessed (Figure 2A). Although a limited cohort of only 9 patients tested, the combination of PVRIG blockade with BCMA BiTE induced the secretion of granzyme B and IFNγ indicative of increased T-cell activation across all evaluated BM cultures (Figure 2B and C).

DISCUSSION

MM is a hematologic malignancy with a poor outcome despite the rapid advances in treatment approaches, which exhibit limited

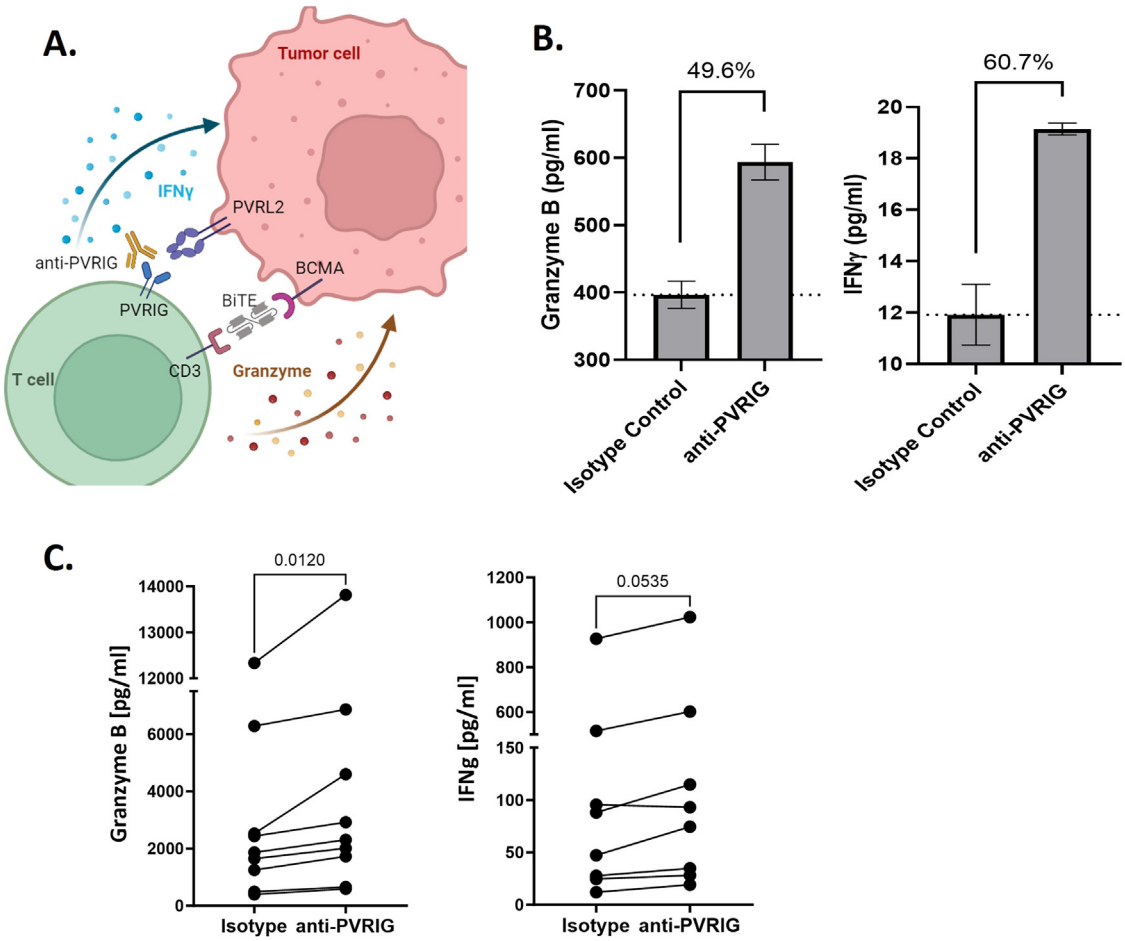

Figure 2 Blocking of PVRIG in combination with BCMA/CD3 BiTE treatment induces T-cell activation. (**A**) Schematic representation of T cell and MM tumor cell with the combination of BiTE and anti-PVRIG antibody will potentially induce T-cell activation followed by IFNγ and granzyme B secretion. Created with BioRender.com. (**B**) Granzyme B and IFNγ secretion induced by a treatment with 5 nM BCMA/CD3 and 10 μg/mL hIgG4 isotype or anti-PVRIG antibodies for 48 hours in BM culture from a representative patient with MM with PD. Levels of Granzyme B and IFNγ in supernatants were analyzed by ELISA. (**C**) Summary of BM from 8 patients with PD and 1 patient with CR MM showing an increase in both granzyme B and IFNγ secretion following treatment with 1 nM, 2.5 nM, or 5 nM BCMA/CD3 and 10 μg/mL hIgG4 isotype or anti-PVRIG antibodies for 48 hours. Samples were pooled from different concentrations of BiTE based on their functional activity. Paired t-test was used to compare between isotype and anti-PVRIG groups.

efficacy and associated toxicity. Hence, there is a necessity for the advancement of novel targeted therapies employing diverse mechanisms of action to attain profound and durable responses. Immune checkpoint inhibitors, particularly PD-1 blockers, have shown promise in preclinical studies but failed to yield significant clinical benefits in patients with MM [15].

With the aim of expanding therapeutic opportunities for patients with MM, we explored and compared the expression pattern of DNAM-1 axis receptors, PVRIG, TIGIT and DNAM-1 as well as PD-1 on lymphoid cell populations in BM of patients diagnosed with MM, mostly with relapsed/refractory disease. It was reported that the blockade of TIGIT, which is expressed by $CD8^+$ T and NK cells, in preclinical models of MM improved the antitumor immune response [5,6]. In this study, to our knowledge, we have shown for the first time that among the evaluated receptors, the expression of the inhibitory receptor PVRIG was most prominent, and comparably higher than the expression of TIGIT and PD-1. Moreover, we have shown that the expression of the costimulatory DNAM-1 is higher among patients in CR than those with PD. Although this observation did not reach statistical significance in our study, it is in line with a previous report in mice showing negative correlation between DNAM-1 expression and myeloma cell numbers in BM [5]. PVRIG was the most coexpressed receptor with DNAM-1 on $CD8^+$ T cells, highlighting its predominant expression on early differentiated T cells that possess greater self-renewal capacity and responsiveness [11,16]. Similar phenomenon was demonstrated for DNAM-1^{pos} $CD8^+$ T cells in solid tumors [11]. This observation implies the potential to further increase T-cell activation and improve patient outcome by PVRIG blockade, which may unleash PVRL2 to activate DNAM-1 and enhance activation of early differentiated T cells and NK cells.

Finally, we showed that in ex vivo assays using patient BM samples, PVRIG blockade in combination with BCMA/CD3 treatment

enhances the BiTE activity as reflected by increased secretion of the effector cytokines granzyme B and IFNγ, essential for antitumor responses. Although these effects are likely mediated by T and NKT cells, we cannot exclude the possibility that they may also result from the direct blockade of PVRIG on NK cells.

Our data highlight the unique dominant PVRIG expression among patients with relapsed/refractory MM and suggests new potential therapeutic strategy of blocking PVRIG in combination with T-cell engagers.

Conflicts of Interest Disclosure

The authors do not have any conflicts of interest to declare in relation to this work.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.exphem.2024.104696.

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