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MOLECULAR TARGETS FOR THERAPY

PVR (CD155) epigenetic status mediates immunotherapy response in multiple myeloma

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TO THE EDITOR:

The immune system is tightly regulated but plastic in humans. It has several lines of control, and its imbalance has severe consequences for our health. Epigenetics encompasses heritable biochemical changes of the chromatin that do not affect the DNA sequence [1]. Epigenetics is constituted of various levels of control, from structural changes (such as 3D chromatin arrangement) to small biochemical changes (such as DNA methylation) which affect gene expression[1]. In the context of immunity, epigenetics has been described to control important events for the system, such as cytotoxic cell activation or exhaustion [2]. Cytotoxic cell activation is critical for tumor clearance. To activate cytotoxicity, several interactions need to occur between the target cell and the immune cell. This group of interactions are commonly known as immune checkpoint (IC) events and determine the outcome of the synapse [3]. IC signals can be co-stimulatory or co-inhibitory, and depending on the amount of signals the immune cells receive, they will determine if the system activates or not. Several rounds of inhibitory signals may induce a senescent state or exhaustion phenotype on the cytotoxic cells [3]. One of these inhibitory markers is the poliovirus receptor (PVR, also known as CD155), which interacts mainly with the T cell immunoreceptor with Ig and ITIM domains (TIGIT) in cytotoxic cells [4]. Tumoral cells can use DNA methylation to regulate inhibition of co-stimulatory or overexpression of co-inhibitory markers [3]. Hematological malignancies present aberrant promoter methylation in several immune checkpoint genes and this dysregulation supports their tumorigenesis [5–7]. Multiple myeloma (MM) is a hematological malignancy characterized by the abnormal accumulation of plasma cells in the bone marrow. MM is notorious for its incurable nature and tendency for relapse, often becoming refractory to treatment [8]. This poses significant challenges, highlighting the urgent need for innovative therapies to enhance the prognosis of affected patients. This work aims to elucidate the epigenetic regulation in PVR, an immune checkpoint marker, and its relation to cytotoxic activation and immunotherapy sensitivity in the context of MM cells.

Distinct expression levels of PVR in primary samples from multiple myeloma (MM) patients [9] suggest that gene expression deregulation could be mediated by alterations in regulatory mechanisms, ranging from epigenetic changes to post-transcriptional modifications. By focusing on DNA methylation, we first observed PVR promoter hypermethylation in a CpG island at the 5′-end of the transcription start site in 14.6% (15 of 104) of primary MM samples available at the European Genome-phenome Archive (EGA) (accession number EGAS00001000841) [10] (see Supplementary Fig. S1). Pyrosequencing analyses (Supplementary Methods) validated the presence of PVR hypermethylation in 2 of 7 additional primary MM samples (Supplementary Table S1). Then, using a comprehensive DNA methylation microarray platform [11] (Supplementary Methods), we observed promoter hypermethylation in a similar 15.4% (2 of 13) of MM cell lines (Fig. 1A). The studied CpG island was found unmethylated in B-Plasma cells (CD138 +) from three healthy donors (Fig. 1A), suggesting that the gain of methylation was a cancer-specific event. The CpG island methylation status of the hypermethylated MM cell lines (AMO1 and KMS-12-BM) and of three unmethylated ones (RPMI-8226, JJN-3 and EJM) was further validated by bisulfite genomic

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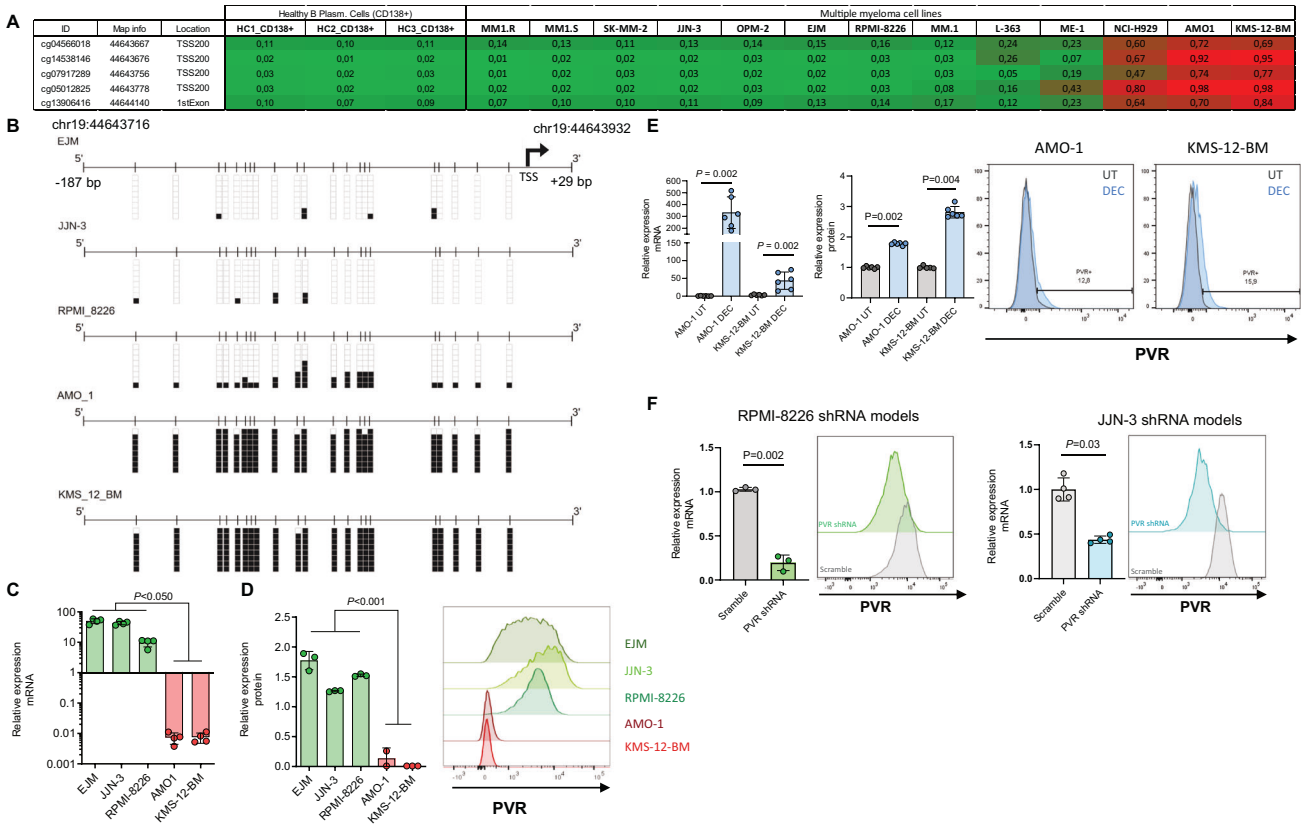
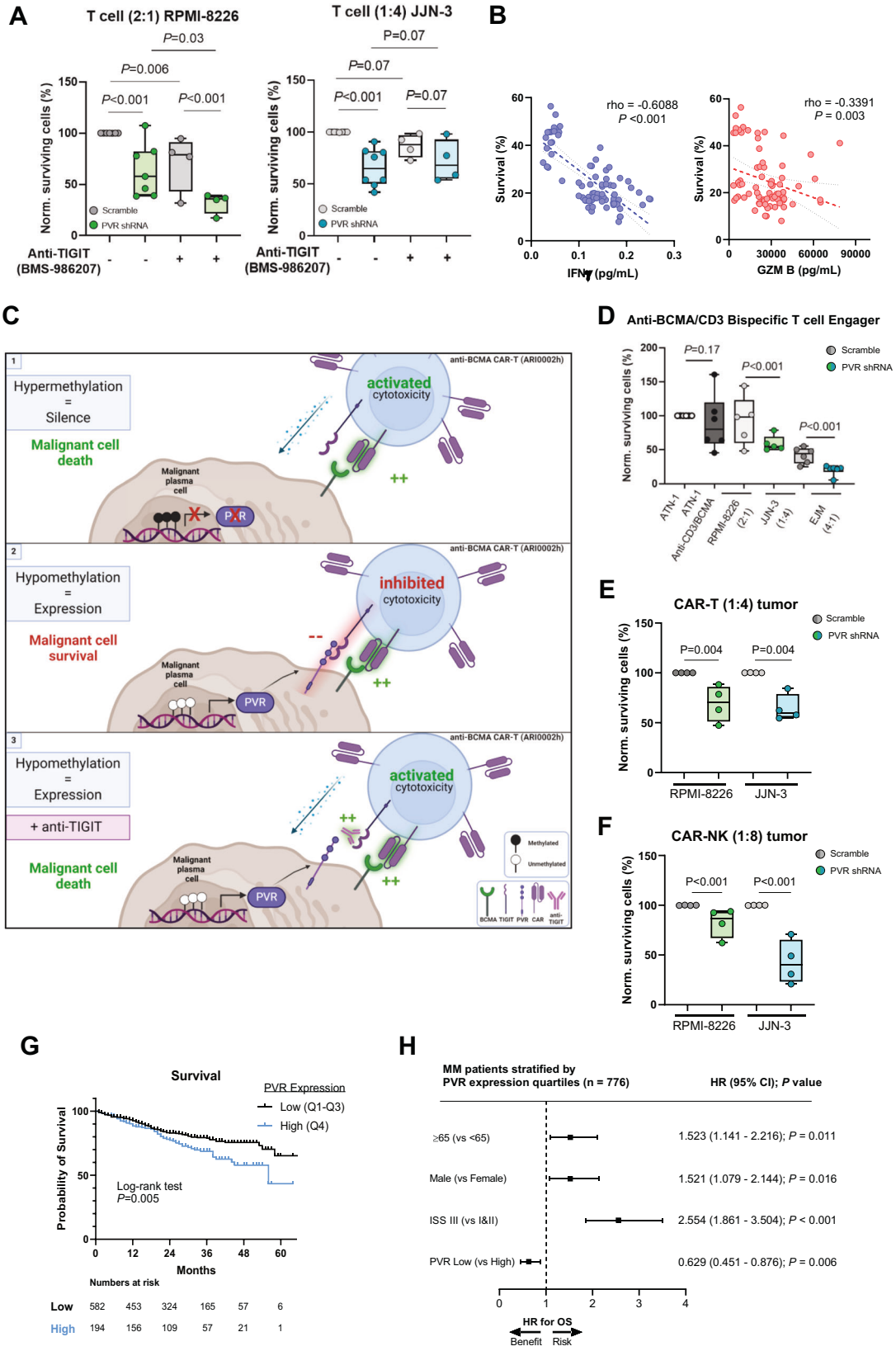


Fig. 1 Epigenetic characterization of PVR shows the correlation between promoter DNA methylation and expression. A DNA methylation profile of the *PVR* promoter CpG island analyzed by the Infinium EPIC DNA methylation array. Single CpG absolute methylation *B*-values are shown (0 to 1). Red, methylated; Green, unmethylated. Thirteen cell lines from multiple myeloma are shown in the figure together with three samples from normal plasmatic B-cells. **B** Bisulfite genomic sequencing of *PVR* promoter CpG island in multiple myeloma cell lines. CpG dinucleotides are represented as short vertical lines; the TSS is indicated with a black arrow. Single clones are shown for each sample. Black squares represent methylated CpG dinucleotides, whereas white squares denote unmethylated positions. **C**,**D** PVR expression levels in multiple myeloma cell lines determined by qRT-PCR and flow cytometry protein quantification. The bar plots represent the mean and SD of at least three biological replicates. The results were compared using One-way ANOVA. Representative flow cytometry histograms of PVR are shown for each cell line. **E** Recovery of *PVR* transcript and PVR protein expression after 1 μM DEC treatment for 120 h. The graphics represent the mean and SD of at least three biological replicates. The results were compared using unpaired two-tailed Student's t test. Representative flow cytometry histograms show the recovery at protein level in AMO-1 and KMS-12-BM cell line. The number indicates the percentages of positive cells for PVR. **F** Efficient shRNA-mediated depletion of PVR at transcript level determined by qRT-PCR and flow cytometry in the unmethylated cell lines RPMI-8226 and JJN-3 from multiple myeloma. The graph represents the mean and SD of at least three biological replicates from three different shRNA models. The results were compared using unpaired two-tailed Student's t test.

sequencing of multiple clones (Fig. 1B) (Supplementary Methods). Most importantly, we observed by quantitative RT-PCR (Fig. 1C) and flow cytometry (Fig. 1D) (Supplementary Methods) that both at the RNA and protein levels, respectively, the presence of CpG island hypermethylation was associated with the loss of PVR expression. The link between DNA methylation and transcriptional silencing was further strengthened using the DNA methylation inhibitor 5-aza-2′-deoxycytidine (DEC) that restored PVR expression in the hypermethylated MM cell lines (Fig. 1E).

To study the role of PVR expression levels in engaging immune response in MM, we successfully created stable models of PVR depletion by shRNA interference in the expressing and unmethylated MM cell lines RPMI-8226 and JJN-3 (Fig. 1F) (Supplementary Methods). To assess the effect of PVR downregulation in T cell cytotoxicity, we developed a co-culture system where T cells from healthy individuals were cultured for 48 h in the presence of the MM cell line models (Supplementary Methods). We considered biological variability by performing all the experiments with T cells from at least four different donors. We observed that shRNA-mediated depletion of PVR induced an increase in the cytotoxicity of T cells on both MM cell lines in comparison with shRNA-scrambled cells (Fig. 2A). To define further the mechanisms by which PVR downregulation increases T cell cytotoxicity, we performed the same co-culture experiments in the presence of the anti-TIGIT antibody BMS-986207, since PVR is a ligand of this T cell immunoreceptor that restrains immune activation [4]. We found that the increased susceptibility to T cell cytotoxicity mediated by PVR knockdown can be augmented in some cases using the anti-TIGIT antibody (Fig. 2A). The enhancement of cell death was accompanied with the augment of interferon gamma and granzyme b (Fig. 2B).

The above results prompted us to assess the impact of PVR expression levels on different immunotherapy approaches designed against MM: an anti-CD3/BCMA bispecific T cell engager, an anti-BCMA CAR-T and an anti-BCMA CAR-NK (Fig. 2C). For the initial strategy, following the demonstration that the antibody had no effect on a T-ALL cell line (that does not express BCMA), we found that the shRNA-mediated depletion of PVR in MM cells increased T cell toxicity upon the used of the bispecific antibody (Fig. 2D). For adoptive cell therapies, we used ARI0002h, an academic second-generation humanized 4-1BB-based CAR transduced on autologous T, for which promising results in MM have been recently published [12]. Since one of the main suspects for CAR-T treatment failure is T cell exhaustion [13], we evaluated how



our MM models for PVR levels related to the presence of CAR-T cells. We observed overall an enhanced cytotoxicity of PVR-depleted MM cells upon exposure to CAR-T cells (Fig. 2E). Finally, we explored the potential use of CAR-NK cells in our models, since they lack several of the complications associated with CAR-Ts (i.e. ICANS or CRS [14, 15]) and share some regulatory mechanisms with T cells, including the expression of TIGIT [15]. In these experiments, for the two MM cell lines, we found that anti-BCMA

Fig. 2 Depletion of PVR in the multiple myeloma cell lines, functional characterization of models, co-culture experiments and clinical impact. A Percentage of MM surviving cells determined by Annexin V incorporation in co-culture assay for 48 h between healthy donor T cells and PVR-depleted cells. The ratios to control cells were represented as surviving portion of cells. **B** Representative pro-inflammatory cytokines (IFNγ and GZMB) levels measured in the supernatant (at 48 h) of the co-culture systems with JJN-3 determined by ELISA assay. Spearman correlation was used to measure statistical significance. Dotted lines represent 95% confidence interval. **C** Graphical representation of the influence of PVR in the interaction between malignant B plasmatic cells and different immunotherapy approaches. **D** Percentage of surviving cells determined by Annexin V incorporation in co-culture assay for 48 h between healthy donor T cells and PVR-depleted cells in the presence of the Bispecific T cell Engager at 10 ng/ml. ATN-1 is a T-ALL not expressing one of the targets for the Bispecific T cell Engager. **E** Percentage of MM surviving cells determined by Annexin V incorporation in co-culture assay for 24 h between PVR-depleted cells and anti-BCMA CAR-T at 1:4 ratio. **F** Percentage of JJN-3 and RPMI-8226 surviving cells determined by Annexin V incorporation in co-culture assay for 24 h between PVR-depleted and anti-BCMA CAR-NK at 1:8 ratio. Statistics: Each dot represents the mean of each of the donors with at least three biological replicates from four different healthy donors. The results were compared using non-parametric paired Wilcoxon test. **G** Kaplan–Meyer survival curves for $n = 756$ patients of MM dividing by expression levels of PVR. **H** Forest plot of overall survival (OS) in subgroups stratified by clinical factors and PVR expression quartile.

CAR-NK cells showed enhanced cell cytotoxicity in the context of shRNA-mediated depletion of PVR (Fig. 2F). Incorporating anti-TIGIT antibody in all previous experiments, increased scrambled MM cells (unmethylated and expressing PVR) susceptibility to the different immunotherapy strategies (Supplementary Fig. S2).

Finally, we analyzed the highly informative CoMMpass project, which includes 776 cases of newly diagnosed multiple myeloma (MM) [8]. In this dataset, we examined the expression of the PVR transcript in CD138+ cells. Notably, we found that low levels of PVR mRNA were associated with extended overall survival compared to MM patients with high transcript levels (Log-rank test: $P = 0.005$, HR = 0.596; 95% CI = 0.415–0.854) (Fig. 2G). Remarkably, multivariate Cox regression analysis indicated that lower PVR expression was an independent predictor of longer overall survival (HR = 0.629; 95% CI = 0.451–0.876; $P = 0.006$) when adjusted for available clinical parameters (age, gender, and the Multiple Myeloma International Staging System, ISS) (Fig. 2H). Furthermore, in a subset of patients with cytogenetic information, we observed that high PVR expression was associated with high-risk alterations such as t(4;14), gain of 1q21, del(13q14), and del(1p22). Conversely, lower PVR expression was linked to lower-risk alterations such as t(11;14) and t(8;14) (Supplementary Fig. S3).

Altogether, these results suggest that better outcomes in multiple myeloma (MM) cases with low PVR might be associated with enhanced immune system engagement. Consequently, targeting PVR may represent a promising therapeutic strategy to improve clinical outcomes in MM. However, immune checkpoint inhibitors used as monotherapy for MM have not proven very effective and do not show the same promise as bispecific antibodies and cellular therapies for relapsed disease. Nonetheless, there is potential in combination therapies. Cytotoxic cell exhaustion remains a predictor of response to immunotherapy, and our in vitro results support the idea that the direct targeting of PVR on malignant cells can enhance the efficacy of bispecific antibodies and adoptive cell therapies in MM patients.

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AUTHOR CONTRIBUTIONS

Contribution: LMV, PLA, LV, GF and ME conceived and designed the study; LMV performed all experiments; YV, CAGP, ANC and DP analyzed in silico data; YV and ML performed pyrosequencing PCR; AB, IC, NB, FP and XA processed and analyzed primary data; AOC, OC, SVS, AGO, AV, EAR, NCG, CFL, JML and GF provided CAR constructs and primary samples; All authors provided scientific advice and result discussion; and LMV, GF and ME wrote the manuscript with contributions and approval from all authors.

COMPETING INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

ADDITIONAL INFORMATION

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