Clinical Cancer Research

Platelets Enhance Multiple Myeloma Progression via IL-1β Upregulation

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Abstract

Purpose: Tumor cell–platelet interactions contribute to tumor progression and metastasis in solid tumors. However, the role of platelets in hematological malignancies is not clear. We investigated the association of platelet activation status with clinical stages in multiple myeloma (MM) patients and explored the role of platelets in MM progression.

Experimental Design: Platelets were obtained from healthy donors and MM patients. We examined platelet activation status in MM patients by flow cytometry and transmission electron microscopy. We also observed the enriched pathways that are involved with platelet activation in RNA sequencing of platelets. MM cell lines were used to assess the effect of platelets on MM cell proliferation *in vitro* and their engraftment *in vivo*. RNA sequencing of MM cell lines was performed to explore molecular mechanisms underlying MM cell–platelet interaction and a CRISPR/Cas9 knockout approach was used for validation.

Results: Platelets from MM patients were highly activated with disease progression. RNA sequencing of platelets revealed that genes involved in platelets were enriched in patients with smoldering MM (SMM) or MM. Platelets promoted MM cell proliferation *in vitro* and contributed to tumor engraftment in bone marrow *in vivo*. RNA sequencing revealed that *IL-1β* was upregulated in MM cell lines co-cultured with platelets, whereas *IL-1β* knockout in MM cell lines abrogated the effects of platelets on MM cell proliferation and engraftment *in vivo*.

Conclusions: Platelets from MM patients were highly activated with disease progression. IL-1β is critical to platelet-mediated MM progression and might be a potential target for MM treatment. *Clin Cancer Res; 24(10); 2430–9. ©2018 AACR.*

Introduction

The interplay between tumor cells and their microenvironment has been known to play a critical role in tumor malignancy (1). The tumor microenvironment is a collective term used to identify many cells and non-cellular compartments, including stroma, the different effectors of the immune systems, and platelets (2). Platelets are a key player in physiological and pathological pathways, including hemostasis, inflammation as well as oncological processes (3). The role of platelets in malignancy is best exemplified by the correlation between increased platelet counts and shorter survival time that have been described in the majority of solid tumors (4). Recently, data have suggested that platelets play a role in each step of solid tumor malignancy facilitating metastasis by protecting circulating tumor cells (CTC) from shear stress and immunological assault during their intravascular phase and by supporting extravasation of the CTCs (5, 6). Platelets are thought to orchestrate this through the release of soluble factors, which enhance proliferation and migration of endothelial cells, thereby promoting tumor growth, metastasis, and angiogenesis (7). In fact, platelets serve as a reservoir of more than 30 growth related proteins, such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), TGF-β, and basic fibroblast growth factor (bFGF), unleashing their cargo and byproducts when activated (8, 9). Experimental mouse models reveal that platelets infiltrate into the tumor region (10–12), as tumor vasculature is leakier compared with normal blood vessels (13). Therefore, platelets have been increasingly recognized as essential for promoting tumor growth and metastasis (14); however, these reports have been limited to solid tumors and the importance of platelets in supporting hematologic malignancies is unknown.

Multiple myeloma (MM) is characterized by the clonal proliferation of malignant plasma cells in the bone marrow

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Translational Relevance

Tumor cell–platelet interactions contribute to tumor progression and metastasis in solid tumors. However, the role of platelets in hematological malignancies has not been unexplored. We report here for the first time that platelets in the peripheral blood of MM patients are activated and their activation status correlates with disease progression, assessed by P-selectin expression on the surface of platelets, electron microscopic studies, and RNA sequencing of platelets. Platelets have supportive effect on the proliferation and engraftment of MM cell lines *in vivo*. RNA sequencing identified *IL-1β* as an upregulated gene in MM cell lines co-cultured with platelets, and knockout of the *IL-1β* gene in MM cell lines annulled the platelet-mediated effect on MM cell proliferation and engraftment *in vivo*. The data suggest that *IL-1β* is critical to the platelet-mediated MM progression and might be a relevant therapeutic target for the treatment of patients with MM.

microenvironment, an abnormal monoclonal immunoglobulin in the blood and urine, and organ impairment (15). It is the second most common hematological malignancy in the United States and constitutes 1% of all cancers (16). MM is usually preceded by monoclonal gammopathy of undetermined significance (MGUS) that progresses to smoldering multiple myeloma (SMM) and, finally, to symptomatic MM (17). The response rate and overall survival of MM have significantly improved due to the introduction of novel agents and autologous stem cell transplantation (18); however, MM is still an incurable disease. This could be partially due to the supportive effects of the bone marrow microenvironment in proliferation, survival, drug resistance, and tumor homing of malignant plasma cells (19, 20). It is well known that the BM microenvironment of MM patients differs in its cellular and non-cellular composition from that of healthy individuals (21). However, the role of platelets in tumor progression in MM has not been studied.

Here, we show that MM cell lines activate platelets as evidenced by increased P-selectin on the platelet surface as well as relevant pathways that were enriched via RNA sequencing of platelets from SMM/MM patients. The activated platelets promote MM cell proliferation and tumor engraftment *in vivo*, and *IL-1β* is essential for this platelet-mediated MM cell proliferation and tumor engraftment *in vivo* in multiple mouse models. Thus, our results reveal that platelet-mediated upregulation of *IL-1β* is important in disease progression and may represent a novel target for therapeutic intervention.

Materials and Methods

Isolation of human and murine platelets

Human blood collection was performed in accordance with the Declaration of Helsinki and ethics regulations with the Dana-Farber Cancer Institute Institutional Review Board approval. Platelets were isolated from healthy donors (HD). The donors and MM patients were not receiving known platelet inhibitors, such as aspirin or nonsteroidal anti-inflammatory drugs. Human whole blood was collected in purple-cap BD Vacutainers containing EDTA anti-coagulant for analysis of patients' platelet and collected with sodium citrate solution (0.38%) for *in vitro* experiment. Platelet-rich plasma (PRP) was obtained from supernatant of whole blood by centrifugation at $200 \times g$ for 20 minutes. Platelets were washed and resuspended in the modified Tyrode's buffer (20 mmol/L HEPES, 150 mmol/L NaCl, 2.5 mmol/L KCl, 12 mmol/L NaHCO3, 1 mg/mL of glucose, 1 mmol/L MgCl2, and 1 mg/ml BSA). Murine whole blood was drawn by cardiac puncture from C57BL/KaLwRij mice terminally anesthetized with chloroform and taken with sodium citrate solution (0.38%).

Cell lines

MM.1S, U266, H929, and K562 cell lines were purchased from the ATCC; OPM2 cells were purchased from DMSZ; KMS-11 cells were kindly provided by Kawano Y (Kumamoto University, Japan). Cells were cultured in RPMI-1640 media containing 10% FBS.

Activation of platelets

Platelets were activated in *vitro* by exposure to 25 mmol/L thrombin receptor activating peptide (TRAP, Sigma-Aldrich) or 3×10^6/mL MM.1S for 10 minutes at 37°C. The activation status of platelets was determined by flow cytometry using PE anti-CD62P (P-selectin) antibody (BD Biosciences). Following activation by TRAP, platelet releasates were isolated by centrifugation.

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qPCR) was performed using the Power SYBR Green Master Mix (Applied Biosystems) and the StepOne Real-Time PCR Systems (Applied Biosystems) by the $\Delta\Delta C_t$ method normalized to GAPDH expression. Primer pairs used in qRT-PCR were as follows: human *IL-1β* forward, 5′-AAATGTCGGGAAGGTACTCG-3′; human *IL-1β* reverse, 5′-GCCAGGCAAGTGTTCCAC-3′; human *GAPDH* forward, 5′-CCAACCGCGAGAAGATGA-3′; human *GAPDH* reverse, 5′-CCAGAGGCGTACAGGGATAG-3′. Complementary DNAs were prepared with SuperScript III First-Strand Synthesis SuperMix (Thermofisher) according to the manufacturer's protocols.

Plasmid construction and CRISPR/Cas9 knockout of *IL-1β*

Human *IL-1β* targeting sgRNA was designed by using web tool created by the Broad institute of MIT and Harvard (http://crispr.mit.edu/) as follows: sgRNA targeting human *IL-1β*#1: GGTGGTCGGAGATTCGTAGCTGG, #2: CATGGCCACAACAACTGACGCGG, #3: TCCGACCACCACTACAGCAAGGG, and scramble sequence. Annealed sgRNA was digested by the BsmBI restriction enzyme and cloned into the pLentiCRISPRv2 plasmid (Addgene). Transduction of luciferase gene or CRISPR/Cas9 system targeting human *IL-1β* was performed by lentivirus infection according to the manufacturer's protocol and as described previously (22).

Immunoblot analysis

Cells were lysed in lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 μg/mL leupeptin]. Cell lysates were electrophoresed in sodium dodecyl sulfate-polyacrylamide gel. The proteins were then transferred to nitrocellulose membranes and immunoblotted with anti-human IL-1β (clone: H-153, Santa Cruz Biotechnology), or anti-α-tubulin (clone:

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B-5-1-2, Sigma Aldrich) mAbs. Enhanced chemiluminescence reagent (Thermofisher) and X-ray films were used in detection.

Flow cytometric analysis

Cells or platelets were incubated with APC anti-CD61 antibody (Clone: VI-PL2, eBioscience), FITC anti-CD62P antibody (Clone: AK4, eBioscience), PE anti-CD62P antibody (Clone: AK4, BD Pharmingen) or isotype controls conjugated with each fluorescence dye. Flow cytometric analysis was performed using a BD FACS Canto II or a BD LSRFortessa X-20 (BD Biosciences).

Platelet aggregation assay

Washed platelets were prepared from pellets of PRP by centrifugation at 1,000 × *g* for 10 minutes following washing with modified Tyrode's buffer. Washed platelets were resuspended in the modified Tyrode's buffer containing with 1% plasma and 200 μmol/L $CaCl_2$ (1×10^9 platelets/mL). Platelet suspension (200 μL) was mixed with cell suspension (5×10^4 cells/10 μL) and incubated for 1 hours at 37°C with stirring. Platelet aggregation rate was calculated from the light transmission rate of platelet suspension. The light transmission rate of PBS treated platelet was designated as 100%, and that of the modified Tyrode's buffer alone was designated as 0%. Experiments were performed in triplicate.

***In vitro* proliferation assays**

To assess *in vitro* cell proliferation, the BrdUrd Cell Proliferation Assay Kit (Cell Signaling Technology) was used according to the manufacturer's protocol. Briefly, MM cell lines were seeded in a 96-well plate at a concentration of 1×10^4 cells/100 μL and co-cultured with washed platelets resuspended in 10 μL of the modified Tyrode's buffer containing with 1% plasma and 200 μmol/L $CaCl_2$ for 2 days. Subsequently, the bromodeoxyuridine (BrdUrd) solution was treated to the cells for 24 hours. The amount of incorporated BrdUrd into newly synthetized DNA was detected by anti-BrdUrd antibody.

Cytokine antibody array

Washed platelets resuspended in the modified Tyrode's buffer (2×10^8 platelets/mL) were mixed with OPM-2 cells (2.5×10^4 cells/10 μL) and incubated for 30 minutes at 37°C. Supernatant of the platelet aggregation reactant was used for the cytokine array analysis according to the manufacturer's protocol of Human Cytokine Antibody Array C1000 (RayBio C-Series, #AAH-CYT-1000-4). The platelet alone and MM alone samples were used as negative controls. The activated platelet sample was prepared by the TRAP stimulation as described earlier.

Transmission electron microscopy

For transmission electron microscopy (TEM), washed platelets were fixed with an equal volume of 0.1% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) at 4°C overnight. Preparation of ultrathin section was performed by the Harvard electron microscopy core facility. Samples were observed by the JEOL 1200EX.

Animals

C.B-*Igh*-1b/GbmsTac-*Prkdc*^{scid}-*Lyst*^{bg} N7 (SCID-beige) mice were purchased from Taconic Biosciences. C57BL/KaLwRij (KaLwRij) mice were colonized in our laboratory. All mice were treated, monitored, and sacrificed in accordance with an approved protocol of the Dana-Farber Cancer Institute Animal Care and Use Committee.

Antibody-mediated platelet depletion in mice

SCID-beige or KaLwRij mice were intraperitoneally injected 1 mg/kg anti-mouse CD41 mAb (clone: MWReg30, BioLegend). The mechanism of anti-CD41 Ab-mediated platelet depletion has been reported as Fc-dependent (23). Nieswandt and colleagues found that this anti-CD41 Ab does not induce platelet activation *in vitro* and that the injection of $F(ab)'_2$ fragments of this anti-CD41 Ab does not significantly affect platelet counts in mice models. Moreover, decreased platelet count in mice recover in 48 to 72 hours after the antibody injection. Suggesting that the anti-CD41 antibody does not deplete megakaryocytes in the bone marrow. Peripheral blood collected from the submandibular vein was used for platelet count analysis using a Hemavet 950 FS hematology after 24 hours of antibody injection. Subsequently, MM.1S or 5TGM1 cells (5×10^6 cells/100 μL) suspended in Hanks' Balanced Salt Solutions (HBSS) were intravenously injected into mice at the same time of blood sampling. Bio luminescence images (BLI) was taken by the Xenogen imager 3 to 4 minutes after intraperitoneal injection of luciferin. Exposure time: 5 minutes. Tumor size was calculated from the ROI values of BLIs.

RNA-sequencing for platelets and MM cell lines

RNA sequencing was performed as previously described (24). Total RNA from HD and MM patient platelets was extracted using the TRizol (Invitrogen) and total RNA from MM cell lines was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Whole RNA was subject to library preparation with NEBNext Ultra RNA Library prep for Illumina Kit (New England BioLabs). A single unique index was assigned to each sample. Quality control of the libraries was evaluated by the Agilent 2100 Bioanalyzer and Agilent's High Sensitivity DNA Kit (Agilent). Sequencing was performed on a HiSeq 2500 (Illumina) by 2 × 50bp paired-end reads.

RNA-sequencing analysis

We used Picard and STAR to process and align the RNA-seq reads with human reference genome (GRCh37) and to compute a series of quality control metrics. RNA-seq transcript abundances were estimated using RSEM (25). Genes with low expression (RSEM expected counts < 1) were filtered out and differential gene expression between samples was determined using DESeq2 (26). Differential expression was selected based on fold changes >2.0 and an FDR—adjusted *P* value threshold of 0.05. Gene set enrichment analysis (GSEA) was used to identify significantly enriched pathways (27), with FDR <0.25 and *P* value < 0.05. Gene sets were downloaded from the Broad Institute's MSigDB (http://www.broadinstitute.org/gsea/index.jsp).

Statistical analysis

The Mann–Whitney *U* test was performed to determine the statistical significance of the results of the patient-derived platelet analysis and the mice analysis. Survival was analyzed using the Kaplan–Meier method and the LogRank test. Some results were compared using the Student *t* test. Significant *P* values are shown as *, $P < 0.05$; **, $P < 0.01$. All statistical tests were two-sided.

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Results

MM cell lines activate platelets and the activation status of patient-derived platelets correlates with disease progression from MGUS to SMM/MM

To investigate the relevance of platelet activation status and clinical stages in MM patients, the level of platelet activation in peripheral blood of patients with MGUS, SMM/MM was measured by detecting the P-selectin expression levels on platelet surface. Although the platelet activation status in HDs ($N = 5$) was lower than 10%, the platelet status in patients with MGUS ($N = 10$) and SMM/MM ($N = 11$) was significantly activated ($P < 0.01$) compared with HDs. Indeed, there was significant activation of platelets in SMM/MM samples compared with MGUS samples ($P < 0.05$, Fig. 1A). Next, we performed morphological studies by using TEM on washed platelets from patient samples and HDs. As shown in Fig. 1B, HD-derived platelets were in the resting state as demonstrated by their discoid shape as well as the presence of α-granules, the major storage compartments in platelets and minimal presence of the open canalicular systems (OCS). On the other hand, in patient-derived platelets as well as in the TRAP stimulated HD-derived platelets, the platelets demonstrate markers of activation, including absence of α-granules, developing extension of OCSs, and protruding filopodia on their plasma membrane. These morphological changes in patient-derived platelets were more pronounced in MM than MGUS, corresponding with the results of platelet activation status in patients with MGUS and SMM/MM observed in Fig. 1A. In addition, we performed RNA sequencing of platelets from patients with SMM or MM as well as HDs. Twenty-two hundred genes were upregulated and 804 genes were downregulated in platelets from SMM/MM compared to HDs and various platelet activation related gene sets, including response to elevated platelet cytosolic CA2 gene sets and platelet activation signaling and aggregation gene sets, were enriched in platelets from SMM/MM patients compared with HDs (Fig. 1C and D; Supplementary Table S1). This further confirms that activation status of platelets in SMM/MM patients compared to HDs. Taken together, these results indicate that platelets in the peripheral blood of MM patients are activated and their activation status correlates with the disease progression.

To define the effect of MM cells on platelet activation, platelets derived from HDs were incubated with MM.1S cells for 10 minutes, and expression of P-selectin on the platelet surface was measured by flow cytometry. Platelets not exposed to MM cell lines showed less than 10% activation, whereas the activation of those incubated with MM.1S cells was significantly increased by approximately 50% ($P < 0.05$, Fig. 1E). Furthermore, we measured

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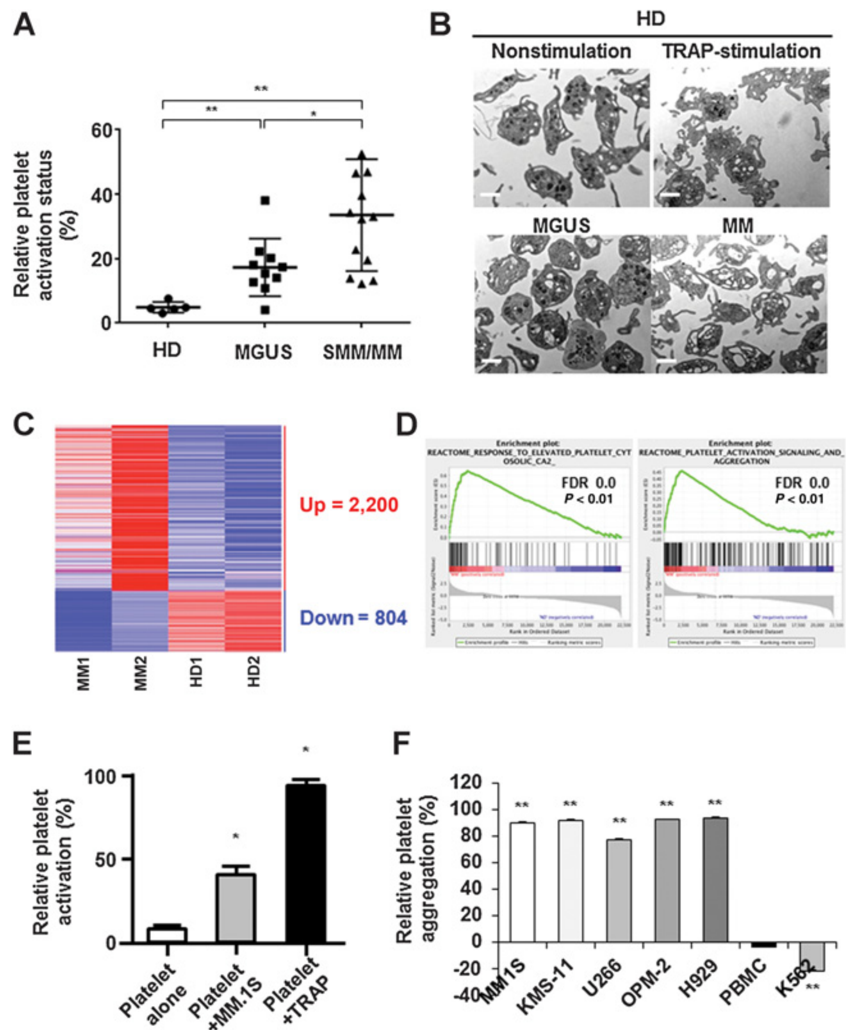

Figure 1.

Platelets from MM patients are activated with disease progression and MM cell lines activate platelets. **A,** Platelet activation status in the peripheral blood of patients with MGUS or SMM/MM assessed by P-selectin expression levels on platelet surface. Platelets isolated from HDs were used as negative controls; *, $P < 0.05$; **, $P < 0.01$. **B,** TEM images of platelets isolated from HDs, MGUS, and MM. Stimulated HD-derived platelets by TRAP were imaged as positive controls; bars, 1 μm. **C,** A gene-expression heatmap which shows up- or downregulated genes in platelets from SMM/MM patients or HDs. **D,** Genes that are involved in response to elevated platelet cytosolic CA2 and platelet signaling were enriched in patients' platelets compared with HDs' platelets from Gene Set Enrichment Analysis (GSEA). **E,** Platelet activation was induced by MM.1S cells. Platelets isolated from HDs were co-cultured with MM.1S cells for 10 minutes and P-selectin expression on the platelet surface was measured by FACS. Stimulated HD-derived platelets by TRAP were used as positive controls. **F,** Platelet aggregation, inducing abilities of human myeloma cell lines, MM1.S, KMS-11, U266, OPM-2, and H929. Chronic myelogenous leukemia cells, K562, and PBMC were used as negative controls; *, $P < 0.05$; **, $P < 0.01$.

the platelet aggregation inducing abilities of five human MM cell lines (MM.1S, KMS-11, U266, OPM-2, and H929), one leukemia cell line (K562) and peripheral blood mononuclear cells (PBMC) from HDs. Interestingly, all MM cell lines, but not K562 or PBMC, demonstrated high platelet aggregation-inducing ability ($P <$ 0.01, Fig. 1F). These results indicate that MM cell lines exert high platelet activation and aggregation properties. We next investigated whether platelets co-localize in the bone marrow niches with MM cells in the syngeneic 5TGM1 mouse model. As shown in Supplementary Fig. S1, we observed the presence of megakaryocytes and platelet microthrombi-like anuclear spots in the tumor region of 5TGM1 cell–injected mice femur.

Platelets and/or their releasate enhance MM cell proliferation *in vitro*

Activated platelets have been well known to release several growth factors and cytokines from their storage granules (8, 9). Therefore, we next investigated the effects of platelets on MM cell lines *in vitro*. Human MM cell lines were co-cultured with human platelets and the proliferation rate was measured by the BrdUrd proliferation assay. Proliferation rate of MM cell lines co-cultured with platelets was increased in a platelet count-dependent manner (Fig. 2A). We confirmed that BrdUrd was not incorporated into platelets and this platelet-dependent proliferation in human MM cell lines was also observed in murine myeloma 5TGM1 cell lines co-cultured with murine platelets (Supplementary Fig. S2). To investigate the effects of soluble factors released from activated platelets on the proliferation of MM cell lines, we collected the releasate of TRAP-activated platelets and treated MM cell lines with the releasate directly. Consistent with the platelet treatment, platelet releasate significantly increased the proliferation of 4 out of 5 MM cell lines (Fig. 2B). These results indicate that platelets and/or their releasate enhance the MM cell proliferation *in vitro*. We further investigate the releasate content from platelets that were co-cultured with MM cell lines using a cytokine array for well-known growth factors and cytokines. Control platelets activated with TRAP or OPM-2 cells cultured alone were used as controls. As shown in Fig. 2C; Supplementary Fig. S3, there was a significant increase in 15 cytokines, including many that are essential for growth and proliferation as well as regulation of *IL-1β* signaling, including EGF, PDGF, GRO, MIP-1 beta, MIF, and G-CSF.

Platelets contribute to tumor engraftment of MM cell lines *in vivo*

We next investigated the effects of platelets on the tumor engraftment of MM cell lines *in vivo*. SCID-beige mice treated with the platelet-depletion antibody (CD41 Ab) were intravenously injected with MM.1S cells and the tumor engraftment rate was measured. We confirmed that treatment of CD41 Ab significantly reduced the platelet count in SCID-beige mice compared with the control IgG group ($P < 0.01$, Supplementary Fig. S4A). As shown in Fig. 3A and B, the tumor engraftment rate of MM.1S cells was significantly lower in the CD41 Ab treated group ($P < 0.05$). Consistently, the survival of CD41 Ab treated group was longer than that of control IgG group ($P = 0.005$, Fig. 3C). These effects were not only observed in xenograft MM models but also in the syngeneic MM models using KaLwRij mice and murine 5TGM1 cells (Supplementary Fig. S4B and Fig. 3D). These results suggest that platelets contribute to the tumor cell engraftment of MM cell lines *in vivo*.

MM cell lines exposed to platelets demonstrated a higher rate of tumor cell engraftment *in vivo*

Considering that exposure to platelets enhanced the proliferation of MM cell lines *in vitro* co-culture experiment, we hypothesized that pre-exposure to platelets would affect MM cells and enhance the tumor cell engraftment *in vitro*. To that end, we used OPM-2 cells with limited engraftment and dissemination properties in *in vivo* MM models. We pre-cultured platelets with OPM-2 cells for 2 days, washed the co-cultured OPM-2 cells to remove the tumor associated-platelets (Supplementary Fig. S5), and then intravenously injected them into SCID-beige mice. Control mice were injected with OPM-2 cells treated with Tyrode's buffer. As shown in Fig. 4A and B, OPM-2 cells did not engraft into mice in the control group. On the other hand, mice that were injected with the OPM-2 cells pre-exposed to platelets (pre-cultured group) demonstrated tumor derived luminescent signals. Consistent with a higher rate of tumor cell engraftment, the survival rate of the pre-cultured group was significantly lower than the control group (Fig. 4C).

***IL-1β* expression is increased in MM cell lines after co-culture with platelets**

To identify genes whose expression levels were significantly changed in the MM cells co-cultured with platelet, we performed RNA sequencing studies using platelet-interacting OPM-2 cells. Three hundred thirty-six genes were upregulated and 441 genes were downregulated in OPM-2 cells after co-culture with platelets (Fig. 5A). The platelet-interacting OPM-2 cells demonstrated enrichment for interleukin signal pathways as well as inflammatory response pathways (Fig. 5B). Expression of *IL-1β* as well as *AIM2*, which is a part of the inflammasome and regulates the processing of IL-1β protein (28), was upregulated in platelet-interacting OPM-2 cells compared with cells alone. We also confirmed that *IL-1β* mRNA level was increased in three MM cell lines after co-culture with platelets by qPCR (Supplementary Fig. S6). In addition, IL-1β protein level was increased after co-culture with platelets in OPM-2 cells and the releasate of activated platelets contained minimal IL-1β protein (Fig. 5C), suggesting that IL-1β is generated in MM cell lines by exposure of platelets, but is not transferred from the platelets.

IL-1β* is essential for platelets-mediated MM cell proliferation and engraftment *in vivo

To investigate the role of IL-1β on MM cell proliferation and engraftment, we generated *IL-1β* knockout (KO) OPM-2 cells (OPM-2/IL1b#1 to #3) by using the CRISPR/Cas9 system and confirmed by immunoblotting (Supplementary Fig. S6A). *IL-1β* KO did not affect the growth rate of OPM-2 cells (Supplementary Fig. S6B); however, platelet-mediated proliferation was suppressed in *IL-1β* KO OPM-2 cells *in vitro* (Fig. 6A). This result indicated that IL-1β is essential for platelets-mediated MM cell proliferation. Furthermore in *in vivo* studies, engraftment and progression of MM cell mediated by pre-exposure to platelets was decreased in *IL-1β* KO OPM-2 cells (Fig. 6B). Consistently, the survival rate of mice injected with OPM-2/IL1b#1 cells was much higher than mice injected with OPM-2/scramble cells even though cells were pre-cultured with platelets (Fig. 6C). These results indicated that MM cell-platelet interaction enhances MM cell proliferation and engraftment *in vivo* by inducing the upregulation of *IL-1β* gene expression in MM cell lines.

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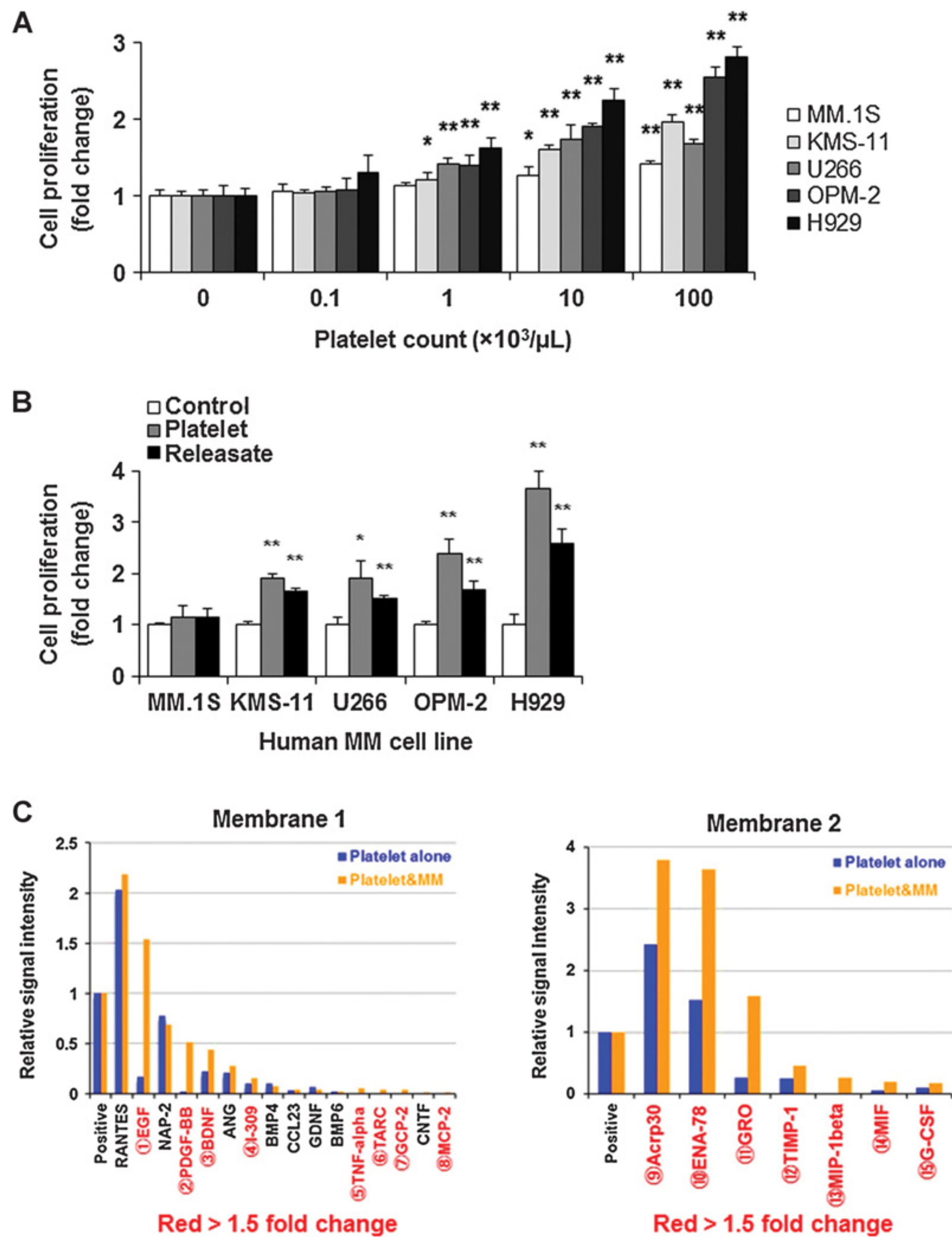

Figure 2.
Platelets enhance proliferation of MM cell lines *in vitro*. **A,** Proliferation rate of human MM cell lines co-cultured with human platelets for 3 days. The proliferation rate of MM cell lines was measured by the BrdUrd proliferation assay. **B,** Proliferation rate of human MM cell lines which were treated with soluble factors released from activated platelets. Cells were treated with Tyrode's buffer (Control), platelets (Platelet), or releasates of activated platelets (Releasate) for 3 days. Proliferation rate was measured by BrdUrd proliferation assay; *, $P < 0.05$; **, $P < 0.01$. **C,** A cytokine array of the releasate from platelets alone or platelets co-cultured with OPM-2 cells. HD-derived platelets activated with TRAP or OPM-2 cells cultured alone were used as controls. The relative signal intensity was normalized by that of positive control spots from Human Cytokine Antibody Array C1000 (RayBio C-Series, #AAH-CYT-1000-4). Each membrane 1 and 2 include different growth factors and cytokines.

Discussion

Platelets aid cancer cells in seeding metastatic sites in a variety of ways, including coating tumor cells to help them evade the immune system, shielding tumor cells from high shear forces, aggregating tumor cells to embolize to new extravasation sites, and facilitating the adhesion of tumor cells to the vascular endothelium (29, 30). Furthermore, tumor cells can activate

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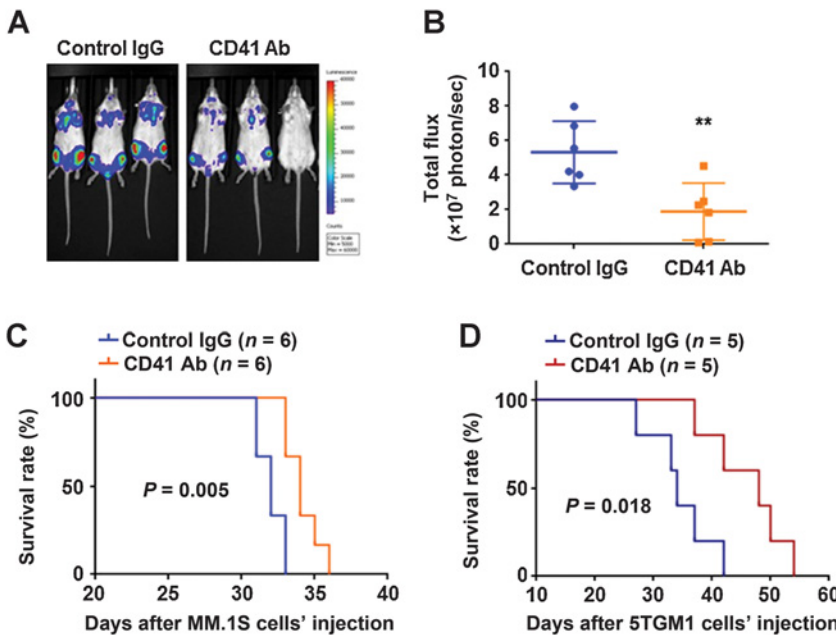

Figure 3.
Platelets contribute to the tumor engraftment of MM cell lines *in vivo*. **A–C,** Mice were intravenously injected with MM.1S cells 24 hours before intraperitoneal injection of a normal rat IgG (Control IgG, $n = 6$) or an anti-CD41 antibody (CD41 Ab, $n = 6$). **A,** BLIs of antibody injected mice were taken at 3 weeks after tumor injection, and **(B)** the total flux of them were calculated; **, $P < 0.01$. **C,** Survival rate of mice was shown as a Kaplan–Meier plot. **D,** Effects of platelet depletion on 5TGM1 cell engraftment and tumor growth in KaLwRij mice. Mice were intravenously injected with 5TGM1 cells 24 hours before intraperitoneal injection of a rat Control IgG ($n = 5$) or CD41 Ab ($n = 5$). Survival rate of mice was shown as a Kaplan–Meier plot.

platelets and increased platelet activation has been demonstrated in malignancy (31, 32). It has also been demonstrated that many human and animal cancer cells are able to aggregate platelets, and this ability correlates with the tumor's metastatic potential (33–36). Most convincing of the essential role platelets in metastasis are studies demonstrating that mice that are rendered thrombocytopenic (low platelet counts) no longer demonstrate metastasis formation. In addition, numerous studies have shown that either defective platelet function, such as occurs with anti-platelet agents, or reduced platelet counts associated with decreased metastasis in transgenic mouse models (37–40). Whether similar associations between platelets and tumor cells is relevant in nonsolid malignancies is unknown. In this article, we establish a role for platelets in MM thus demonstrating that the platelet–tumor cell interaction extends beyond solid tumors to hematologic malignancies.

We first demonstrate that platelets derived from MM patients are highly activated and correlated with the disease status from MGUS to MM, and that MM cell lines induce activation of normal platelets. These results were further confirmed using RNA sequencing of platelets showing that genes involved in platelet activation, signaling, and aggregation were upregulated in SMM/MM patients' platelets compared with HDs' platelets. These results indicate that MM cell lines have an ability to activate platelets

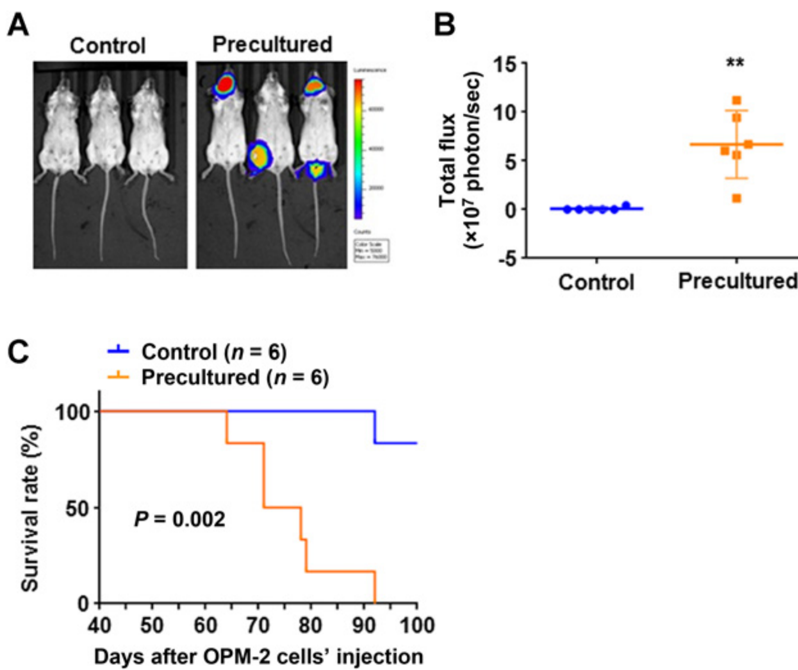

Figure 4.
Pre-culture of MM cell lines with platelets enhances MM cell engraftment and tumor growth *in vivo*. OPM-2 cells were pre-cultured with Tyrode's buffer (Control) or human platelets (Pre-cultured) for 2 days, washed with large amount of PBS to remove tumor surface-associated platelets completely, and then injected into SCID-beige mice ($n = 6$ per each group). **A,** BLIs of mice were taken 7 weeks after tumor injection, and **(B)** the total flux of them were calculated; **, $P < 0.01$. **C,** Survival rate of mice was shown as a Kaplan–Meier plot.

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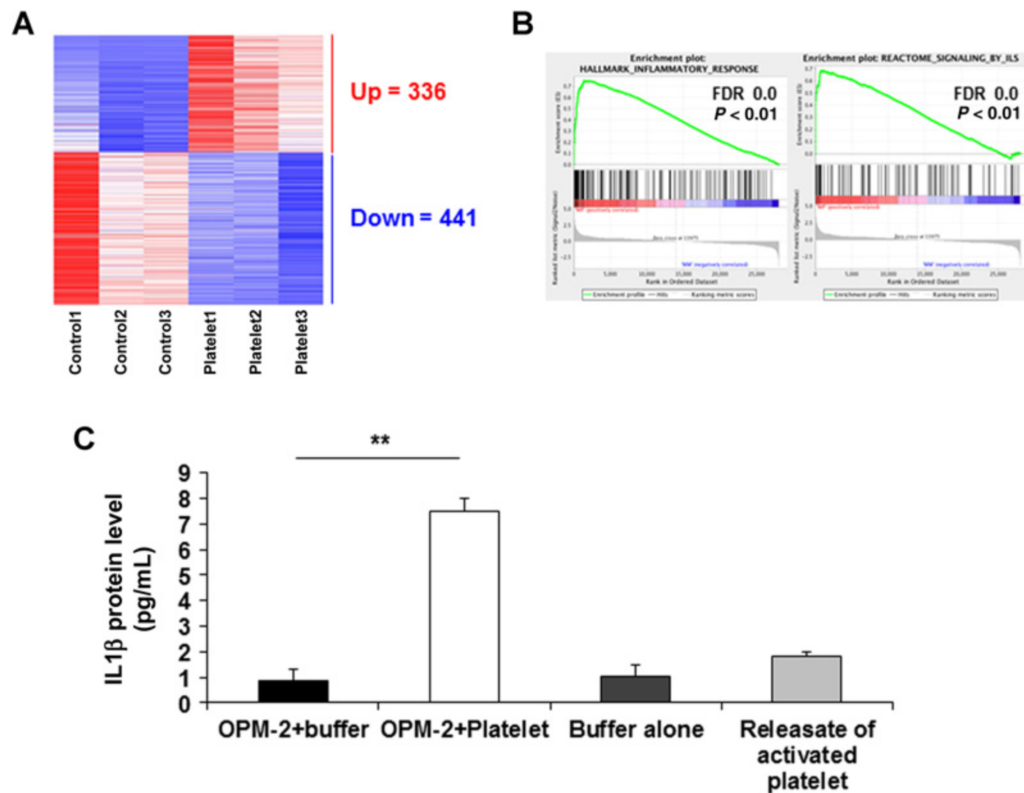

Figure 5.

IL-1β expression is increased in MM cell lines by the co-culture with platelets *in vitro*. **A,** A gene expression heat map which shows up- or downregulated genes in OPM-2 cells co-cultured with platelets. OPM-2 cells were treated with Tyrode's buffer (Control, $n = 3$) or platelet (Platelet, $n = 3$). **B,** Increased interleukin–related gene enrichment and inflammatory response related gene enrichment from RNA-seq datasets comparing differential RNA expression between Control and Platelet. **C,** IL-1β protein level in the supernatant of co-cultured OPM-2 cells and the releasate of activated platelets were detected by the ELISA. Cells were co-cultured with platelets for 2 days. Platelet activation was induced by the TRAP treatment; **, $P < 0.01$.

establishing the importance of the platelet-tumor cell interaction not only in solid tumors but also hematologic malignancies.

Co-culture of MM cell lines with platelets dramatically changed the character of MM cells from low to high engraftment rate in SCID-beige mice model. This was confirmed in several other MM *in vivo* mouse models. Using RNA sequencing of MM cell lines co-cultured with platelets, we identified the critical role of *IL-1β* in the regulation of tumor proliferation *in vivo* and *in vitro*.

Interestingly, Lacy MQ and colleagues reported that greater than 95% of MM patients but less than 25% of MGUS patients have malignant plasma cells with IL-1β positive and no IL-1β expression was detected in normal plasma cells by *in situ* hybridization (41). This report suggests that IL-1β and its role in platelet activation could be a central cytokine involved in the progression of MGUS to MM. IL-1β has been reported as a potent inducer of IL-6 that is a major tumoral growth and survival factor for MM cells. Costes and colleagues (42) reported that an inhibitor of IL-1, IL-1RA, reduced PGE2 synthesis and IL-6 production could be reversed by adding synthetic PGE2 in MM cells isolated from patients samples, suggesting that platelet-mediated MM cell proliferation may be caused by the IL-1β–IL-6 loop in MM cells.

Torcia and colleagues (43) has revealed a critical role of IL-1β in the pathogenesis of MM bone disease by showing that the osteoclast activating abilities in MM patients was related to IL-1β. In human MM cells, overexpression of IL-1β increased the expression level of adhesion molecules such as Integrin α4β1 and CD44 (44, 45). These reports suggest that increased IL-1β expression promotes MM cell engraftment *in vivo* by upregulating the adhesion molecules in MM and affecting to host bone microenvironment.

A protective role for aspirin for improving survival and decreasing metastatic spread in a variety of solid tumor malignancies has been suggested in recent studies (46–48). *In vivo* data demonstrating an inhibitory role for aspirin in metastasis formation was initially reported by Gasic (49), and our recent work points to a platelet-mediated mechanism of action (10). Although the exact mechanism by which aspirin improves cancer outcomes is unknown, aspirin has recently been demonstrated to attenuate the release of proteins from platelets (49).

Our preclinical data indicate that early interception of platelet activation and interaction with MM cells can possibly delay disease progression in the early stages of the disease such as in MGUS or smoldering MM. The answer to this critical question can only be performed in large clinical trials to define the role of platelet-regulating agents in preventing disease progression. A possible indication that indeed inhibition of platelets function can have a positive impact in patients with early prevention of progression of MM has been shown in the large prospective cohort

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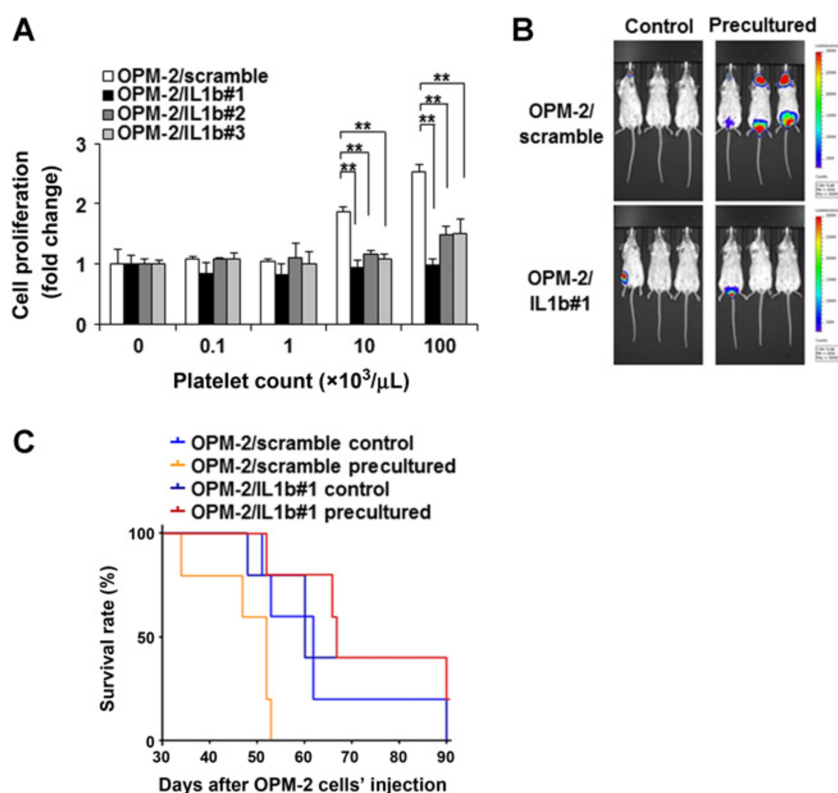

Figure 6.

IL-1β is essential for the platelets-mediated MM cell proliferation and engraftment *in vivo*. **A,** Proliferation rate of OPM-2/IL1b#1 to #3 and OPM-2/scramble cells co-cultured with platelets. Cells were co-cultured with indicated number of platelets for 3 days. Proliferation rate was measured by BrdUrd proliferation assay; **, $P < 0.01$. **B,** BLIs of SCID-beige mice which were intravenously injected with OPM-2/scramble or OPM-2/IL1b#1 pre-cultured with Tyrode's buffer (Control) or platelets (Pre-cultured) for 2 days. Images were taken 5 weeks after tumor injection. **C,** Survival rate of mice was shown as a Kaplan–Meier plot.

studies, including the Nurses' Health Study (1980–2010) and Health Professionals Follow-up Study (1986–2012) that followed up 135,965 health care professionals and showed that the use of aspirin was associated with a significant reduction of risk for overall cancers (50) and specifically the risk of MM (51). However, the use of aspirin for chemoprevention in MM requires further studies.

In conclusion, our data indicate that platelets play a crucial role in the regulation of disease progression in the bone marrow microenvironment of MM and suggest a potential new therapeutic strategy targeting the platelet-cancer interaction in MM and IL-1β as potential targets for future early therapeutic interventions or chemoprevention in MM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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