Immunoregulatory roles of versican proteolysis in the myeloma microenvironment

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KEY POINTS

1. Interplay between myeloma niche stromal cells and myeloid cells generates versikine, a novel damage-associated-molecular pattern (DAMP).

2. Versikine may promote antigen-presenting cell maturation and CD8+ T cell activation/recruitment to the tumor bed.
ABSTRACT

Myeloma immunosurveillance remains incompletely understood. We have demonstrated proteolytic processing of the matrix proteoglycan, versican, in myeloma tumors. Whereas intact versican exerts tolerogenic activities through Toll-like receptor (TLR)-2 binding, the immunoregulatory consequences of versican proteolysis remain unknown. Here we show that human myeloma tumors displaying CD8+ infiltration/aggregates underwent versican proteolysis at a site predicted to generate a glycosaminoglycan-bereft N-terminal fragment, versikine. Myeloma-associated macrophages (MAM), but not tumor cells, produced V1-versican, the precursor to versikine, whereas stromal cell-derived ADAMTS1 was the most robustly expressed versican-degrading protease. Purified versikine induced early expression of inflammatory cytokines IL1β and IL6 by human myeloma marrow-derived MAM. We show that versikine signals through pathways both dependent and independent of Tpl2 kinase, a key regulator of NFκB1-mediated MAPK activation in macrophages. Unlike intact versican, versikine-induced IL6 production was partially independent of Tlr2. In a model of macrophage-myeloma cell crosstalk, versikine induced components of “T-cell-inflammation”, including IRF8-dependent type I-interferon (IFN)-transcriptional signatures and T-cell-chemoattractant CCL2. Thus the interplay between stromal cells and myeloid cells in the myeloma microenvironment generates versikine, a novel bioactive damage-associated-molecular-pattern (DAMP) that may facilitate immune sensing of myeloma tumors and modulate the
tolerogenic consequences of intact versican accumulation. Therapeutic versikine administration may potentiate T-cell-activating immunotherapies.
INTRODUCTION

Myeloma is a tumor of plasma cells which are antibody-producing, terminally differentiated B lymphocytes. Myeloma plasma cells typically live within the bone marrow microenvironment (“canonical” myeloma niche) but can often thrive in extramedullary sites and soft tissues (“non-canonical” niches).

We have hypothesized that infiltrating myeloid cells may exert crucial trophic and immunoregulatory functions in both “canonical” and “non-canonical” niches, in part through their regulation of extracellular matrix composition and remodeling. We and others have previously demonstrated that versican, a large, chondroitin-sulfate matrix proteoglycan, accumulates in myeloma lesions and have hypothesized that versican may contribute to myeloma niche immunoregulation. Versican has crucial, non-redundant significance in embryonic development and emerging roles in cancer inflammation and metastasis. Versican promotes tolerogenic polarization of antigen-presenting cells through TLR2. Versican is proteolytically cleaved by ADAMTS-type proteases in a highly–regulated manner. A cleavage product generated by proteolysis of the Glu^{441}- Ala^{442} bond within the versican V1 isoform, has been termed versikine. Versikine has been shown to be bioactive (proapoptotic) during interdigital web regression in the mouse embryo; however, the roles of versican proteolysis and/or versikine in tumor immunomodulation remain unknown.
METHODS.

Versikine production, purification and analysis. Methods for expression and purification of recombinant versikine from mammalian cells have been published \textsuperscript{12,13}. We excluded endotoxin and hyaluronan contamination as detailed in Supplementary Methods.

Primary sample processing and cell culture. Bone marrow aspirates were collected with informed consent under a University of Wisconsin IRB-approved protocol (HO07403) and processed as detailed in Supplementary Methods. Cell culture methods are detailed in Supplementary Methods.

Animal experiments. Animal experiments were carried out under an IACUC-approved protocol (M2395).

RNA analyses. Cells were lysed in Trizol reagent and RNA extracted per standard methods. RT-PCR and RNA-seq were performed as detailed in Supplementary Methods.

Immunohistochemistry. The University of Wisconsin myeloma tissue microarray (TMA) has been previously reported \textsuperscript{3}. A second myeloma TMA was purchased from US Biomax (Rockville, MD, USA)(catalog no. T291b). Antibodies are listed in Supplementary Methods.

Immunoblot, cytokine detection and cell cycle analysis. Antibodies and reagents are listed in Supplementary Methods.

Statistical Methods. All \textit{in vitro} experiments were performed in triplicate and statistical significance was assessed using two-tailed Student's \textit{t} test or two-way ANOVA analysis where appropriate. Multiple comparisons were made using the
Tukey method. A p-value of less than 0.05 was considered statistically significant.
RESULTS AND DISCUSSION

To determine whether versikine acts as an endogenous DAMP, we exposed freshly-explanted primary myeloma CD14+ cells to recombinant human versikine (1μM). Versikine rapidly induced inflammatory cytokines IL1β and IL6 (Figure 1A/B). However, recombinant versikine had no discernible effects on cell cycle progression of MM1.S myeloma cells (Supplementary Figure 1) or primary CD138+ myeloma cells (not shown).

Versican, the precursor to versikine, has been variably reported to be expressed by carcinoma tumor cells or components of the carcinoma microenvironment, however its cellular origin in the myeloma niche is unknown. We carried out RT-PCR using versican isoform-specific primers in paired myeloma CD138+ plasma cells, CD14+ monocytes/macrophages and CD14/CD138-double negative fractions from 3 newly-diagnosed patients. All four versican isoforms were predominantly expressed by CD14+ monocytes/macrophages (Supplementary Figure 2A). Known versicanases include ADAMTS-1, -4, -5, -9, -15 and -20. We readily detected ADAMTS1, ADAMTS4 and ADAMTS5 mRNA expression in bone marrow mononuclear cell lysate whereas the rest were undetectable (data not shown). ADAMTS expression has been reported in marrow-derived mesenchymal stromal cells (BM-MSC). We found that ADAMTS1 was expressed at higher levels by myeloma-derived BM-MSC compared to normal donor BM-MSC (Supplementary Figure 2B). BM-MSC expressed much higher levels of ADAMTS1 message than either CD138+ plasma cells or CD14+ monocytes/macrophages (Supplementary Figure 2C).
Inflammatory signaling in macrophages engages the MAP3K Tpl2 (Cot, MAP3K8) \(^\text{3,15}\). Tpl2 loss in murine bone-marrow-derived macrophages (BMDM) abrogated IL1β production in response to versikine but had little impact on IL6 production (Figure 1C). Interestingly, versikine induced IL12p40 but not IL10 (Figure 1D and 1E). Tpl2 negatively regulated IL12p40 production in response to versikine (Figure 1D), in a manner analogous to TLR agonists \(^\text{16}\). Whereas versikine induced IL12p40 in isolation, concurrent Fcγ receptor ligation through addition of ovalbumin (OVA)/anti-OVA immune complexes promoted IL10 production and skewed macrophage polarization towards an immunoregulatory M2b phenotype (IL12\(^{\text{lo}}\)-IL10\(^{\text{hi}}\)) \(^\text{17}\) (Figure 1E).

Intact versican is thought to signal through TLR2 \(^\text{10}\). However, Tlr2\(^{-/-}\) BMDM showed only a partial IL6 production defect (approximately 50%) in response to recombinant versikine (Figure 1F).

Versikine stimulation of wild-type BMDM rapidly induced JNK, p38-MAPK and AKT phosphorylation but Tpl2 loss only affected p38-MAPK phosphorylation (Figure 1G).

Human THP-1 monocytic cells can be induced to generate macrophages that provide a functional model to study macrophage regulation, including polarization \(^\text{18}\). THP-1 macrophages transduced with versikine-plasmid or empty-vector control, were co-cultured with human myeloma MM1.S cells for 48 hours and RNA-seq analysis was performed on each cell type. Remarkably, only 23 genes were differentially expressed in MM1.S cells exposed to versikine-secreting
macrophages and all were overexpressed: among these, 13 genes defined a type I-interferon signature (Figure 1H). Interestingly, VCAN transcription was itself induced, suggesting a positive auto-regulatory loop responding to cleaved versican. THP-1 macrophages expressing versikine demonstrated differential regulation (> 2-fold) of 39 genes (4 downregulated, 35 upregulated): 13 upregulated genes defined a type-I-interferon signature (Supplementary Table 1). Recombinant versikine induced upregulation of interferon-stimulated-genes (ISGs) in THP-1 macrophages as well as in co-cultured MM1.S myeloma cells (Figure 1I and 1J). IRF8 was dramatically induced at 18 and 48 hours in MM1.S cells but induction of other interferon-regulatory factors (IRFs) was minimal (Figure 2A and 2B). Interestingly, IRF8 was upregulated in MM1.S cells only in the presence of versikine-producing macrophages. Knockdown of IRF8 expression impaired ISG induction in THP-1 macrophages (Figure 2C). Irf8 expression in transplanted tumor cells has been shown to be inducible through an Il27-dependent mechanism19. We observed upregulation of the IL27 subunit, EBI3, in tumor cells and in primary MAM treated with versikine (Figures 2D and 2E).

We stained myeloma bone marrow biopsy sections with antibodies against a versican neoepitope (DPEAAE<sup>441</sup>) generated by Glu<sup>441</sup>-Ala<sup>442</sup> cleavage of V1-versican<sup>5</sup>. DPEAAE<sup>441</sup> constitutes the C-terminus of versikine. Consecutive sections were stained for T cell marker CD8. We observed four staining patterns in 19 core biopsies (Figure 2F). Myeloma tumors displaying CD8+
infiltration/aggregates\textsuperscript{20} (n=5 out of 19) demonstrated intense/moderate versican proteolysis, as detected by the anti-DPEAAE antibody.

We hypothesized that the regulated degradation of versican by ADAMTS-type versicanases may modulate its tolerogenic potential by controlling versican bioavailability, disrupting its extracellular matrix networks and/or by generating novel bioactive fragments. We report that versikine, a product of versican proteolysis, possesses immunoregulatory activities that may promote innate myeloma sensing and “T-cell-inflammation” through induction of type-I-IFN signatures, macrophage activation (IL1\(\beta\), IL6), T-cell chemotactic mediators (CCL2)\textsuperscript{21,22} and IRF8 activation, the latter known to be critical for dendritic and myeloid-derived suppressor cell maturation and homeostasis, respectively\textsuperscript{23,24} as well as macrophage inflammatory responses\textsuperscript{25} (Figure 2G). Taken together, our results suggest that versikine may antagonize the tolerogenic actions of intact versican and thus, may provide a novel anti-tumor strategy. The findings also suggest that, in addition to small leucine-rich proteoglycans, previously shown to act as DAMPs\textsuperscript{26}, fragments of large aggregating proteoglycans may have the capacity to stimulate innate immunity and provide a bridge to adaptive immunity.
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AUTHOR CONTRIBUTIONS

CH, SF, JJ, SXC, JLJ, SP and FA performed experiments. CL, IM, NC, SM and PH provided reagents and/or advice on study design. SSA provided crucial
expertise, advice and reagents throughout the study. FA was overall responsible for study design. CH and FA wrote the manuscript. All co-authors reviewed, edited and approved the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST

CH and FA are listed as inventors in U.S. Provisional Patent Application No. 62/305,761 (3/9/16).
REFERENCES


FIGURE LEGENDS

Figure 1. Versikine, a novel DAMP with immunoregulatory roles in the myeloma microenvironment.

A. and B. Freshly-explanted MAM from the human myeloma marrow cases indicated, were exposed to 1μM versikine (Vkine) for 12 hours. Relative expression of IL1β and IL6 transcripts is shown. Black bars, vehicle; grey bars, versikine-treated. Veh, vehicle.

C. and D. WT and Tpl2/- murine bone-marrow-derived macrophages (BMDM) were treated with 1 μM versikine and cytokine concentrations were measured in the culture supernatant at 12 hours post-exposure. C., IL1β (left), IL6 (right); Prior to IL1β assay, cells were treated with 5mM ATP for 20 mins. D., IL10 (left); IL12p40 (right).

E. Versikine modulates macrophage polarization. BMDM were exposed to vehicle, versikine alone or versikine + ovalbumin (OVA)/anti-OVA immune complexes (IC), as previously described 17. Versikine exposure resulted in M1-like phenotype (IL12\textsuperscript{hi}, IL10\textsuperscript{lo}) in the absence of concurrent Fcγ ligation. Versikine + IC promoted macrophage polarization towards an M2b-like, immunoregulatory phenotype (IL12\textsuperscript{lo}, IL10\textsuperscript{hi}).

F. WT and Tlr2/- BMDM were stimulated by versikine for 12 hours and IL6 protein was measured in the supernatant.

G. Signaling mediators induced by versikine stimulation of wild-type and Tpl2/- BMDM. BMDM were collected following stimulation with versikine at designed
timepoints (each number reflects minutes) and subjected to immunoblot analysis with the antibodies shown.

H. RNA-seq analysis of MM1.S myeloma cells exposed to versikine-transduced macrophages for 48h. Only 23 genes were differentially expressed and all were upregulated (log^2 fold change > or equal to 1). 13 of 23 upregulated genes were interferon-stimulated-genes (ISG, highlighted in grey). VCAN gene transcription changes are underlined.

I and J. Myeloma cell-macrophage co-cultures were exposed to 0.5 μM purified versikine for 4, 18 or 48 hours. Representative ISG transcription is shown for THP-1 (I) and MM1.S cells (J). Relative mRNA transcription is normalized to vehicle-only control at each timepoint.

P-values: *<0.05; ** <0.01; ***< 0.001; ****<0.0001.

**Figure 2. Versikine acts through IRF8 to promote transcription of interferon-stimulated-genes (ISG).**

A. Interferon regulatory factor (IRF) transcription in MM1.S cells, following 48h co-culture with THP-1 macrophages in the presence of 0.5 μM versikine (Vkine) or vehicle (Veh). Expression is normalized to Veh-only levels at 4 h.

B. IRF9 mRNA levels in MM1.S cells co-cultured with THP-1 macrophages in the presence of 0.5 μM versikine (grey bars) or vehicle (black bars) for indicated time-lengths.

C. THP-1 cells expressing versikine (Vkine) or an empty-vector (EV) control were transduced with control lentivirus (NT) or lentivirus expressing shRNA targeting
IRF8 (shIRF8) (see Supplementary Figure 3 for validation of IRF8 knockdown at the protein level). Versikine-mediated induction of three ISG’s shown was measured in the presence and absence of IRF8.

D. *EBI3* transcription in MM1.S cells co-cultured with THP-1 macrophages and treated with 0.5 μM versikine (grey bars) or vehicle (black bars) for indicated time-lengths.

E. RT-PCR analysis for *EBI3* transcripts in patient-derived, freshly-explanted MAM treated with 1 μM versikine for 12 hours. Relative expression is normalized to vehicle-only control (=1).

F. Staining of human myeloma bone marrow core biopsy consecutive sections with antibodies against neoepitope DPEAAE generated by V1-versican cleavage at Glu$^{441}$-Ala$^{442}$ and T-cell marker CD8. DPEAAE constitutes the C-terminus of versikine. Four patterns of staining were observed in 19 informative punches:

Pattern (a): intense/moderate versican proteolysis- CD8 infiltration/aggregates (> 5 CD8+ cells in cluster).

Pattern (b): intense/moderate versican proteolysis- CD8 poor (single/doublet CD8+ cells sparsely distributed within tumor).

Pattern (c): weak/focal versican proteolysis- CD8 poor (single/doublet CD8+ cells sparsely distributed within tumor).

Pattern (d): absent versican proteolysis- CD8 poor (single/doublet CD8+ cells sparsely distributed within tumor).

G. Proposed immunomodulatory roles of versican proteolysis in the myeloma microenvironment. Whereas intact versican is thought to exert tolerogenic
activities through TLR2 binding on antigen-presenting cells, its proteolytic product, versikine, may promote immunosurveillance through IRF8-mediated effects on antigen-presenting cells and tumor cells. Currently untested hypotheses are represented by broken lines.

P-values: *<0.05; ** <0.01; ***< 0.001; ****<0.0001.
**FIGURE 2**

A. Graph showing relative mRNA expression of IRF9.

B. Graph showing relative mRNA expression of IRF9 with different treatments.

C. Graph showing relative mRNA expression of IRF9 with different treatments.

D. Graph showing relative mRNA expression of EBI3.

E. Graph showing relative mRNA expression of EBI3 with different treatments.

F. Images of Neo-epitope DPEAAE and CD8 staining patterns.

G. Diagram illustrating the interaction between ADAMTS Proteases, Versican, and Myeloma tumor cell.
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