Monosodium Urate Crystals Induce Upregulation of NK1.1-Dependent Killing by Macrophages and Support Tumor-Resident NK1.1⁺ Monocyte/Macrophage Populations in Antitumor Therapy

Stefanie Steiger, Sabine Kuhn, Franca Ronchese and Jacqui L. Harper

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The formation of monosodium urate (MSU) crystals is a well-recognized autoinflammatory danger signal that exhibits potent endogenous adjuvant activities to alter local immune cell phenotypes and boost adaptive immune responses (1–4). Macrophages are among the key cells that rapidly respond to and coordinate the local inflammatory response to MSU crystals (5–10). Cells of the monocyte/macrophage lineage are highly dynamic immune cells that readily alter their functional phenotype in response to the local inflammatory environment and in a stimulus-specific manner (11, 12). MSU crystal stimulation of macrophages is widely known to trigger NLRP3 inflammasome activation and IL-1β production (1, 2). However, there is currently limited information on how MSU crystal stimulation might otherwise alter macrophage functions.

In addition to classical macrophage immune functions such as cytokine production, phagocytosis of pathogens, and bactericidal activity, a number of studies suggest that macrophages may acquire functions commonly associated with NK cells. For example, macrophage precursors can develop NK cell–like cytolytic activity following IL-2 stimulation (13, 14). In vivo treatment with bacterial adjuvants has also been shown to elicit CD4+CD8− macrophages that express NK receptors and are capable of cytotoxic function (15).

A previous study investigating the immune boosting effect of MSU in antitumor therapy indicates a role for NK1.1+ cells in successful tumorcidal activity (16). In this study, we show that MSU crystal–stimulated primary resident macrophages exhibit NK cell–like functions including upregulation of surface NK1.1 expression and development of NK1.1-dependent cytotoxic function via the engagement of perforin/granzyme B killing pathways. We also identify the presence of elevated numbers of NK1.1+ monocytes and macrophages in tumors receiving MSU-mediated tumor therapy.

Materials and Methods

Mice

C57BL/6j and perforin-deficient (Pfp−/−) mice (17) (The Jackson Laboratory, Bar Harbor, ME) were bred and housed at the Malaghan Institute of Medical Research, Wellington, New Zealand. All experiments were approved by the Victoria University Animal Ethics Committee and carried out in accordance with their guidelines for the care of animals. All animals used for the experiments were aged between 6 and 10 wk.

Reagents

Mouse IL-1β ELISA kit was from R&D Systems (Minneapolis, MN). Anti-mouse CD11b, anti-mouse F4/80 mAbs, PE-conjugated Annexin V (AnV), propidium iodide (Pi), GolgiStop, and tissue plasticware were obtained from BD Biosciences (North Ryde, Australia). PE-conjugated anti-mouse IgG2b, κ, CD11b (clone M1/70), Streptavidin PE-Texas Red, FITC-conjugated anti-mouse Ly49A, NK1.1−/−, CD45.2−/−, and purified anti–IL-6 were from eBioscience (San Diego, CA). PerCP–Cy5.5, Ly6G-biotin, and anti-mouse IL-12 (3B11) mAb were from BioLegend. Mouse IL-12 was from BioLegend. Cell culture reagents including RPMI 1640 media, Dulbecco’s PBS, penicillin-streptomycin, glutamax, BSA, and cytchalasin D were obtained from Life Technologies (Auckland, New Zealand). LPS and calcium pyrophosphate dihydrate (CPPD) were obtained from Sigma-Aldrich (Auckland, New Zealand). Caspase-1 inhibitor Ac-YVAD-cmk (YVAD) was from Cayman Chemical. Rasburicase (FASTURTEC) was obtained from Wellington Hospital (Wellington, New Zealand). NK-sensitive T lymphoma cell lines were from Dr. Jacqui L. Harper (Malaghan Institute of Medical Research, Wellington, New Zealand).

Abbreviations used in this article: MSU, monosodium urate; CPPD, calcium pyrophosphate dihydrate; DC, dendritic cell; MHC II, MHC class II; AnV, annexin V; CD45.2−/−, perforin-deficient; PL, propidium iodide.

Stefanie Steiger, Sabine Kuhn, Franca Ronchese, and Jacquie L. Harper

Macrophages display phenotypic and functional heterogeneity dependent on the changing inflammatory microenvironment. Under some conditions, macrophages can acquire effector functions commonly associated with NK cells. In the current study, we investigated how the endogenous danger signal monosodium urate (MSU) crystals can alter macrophage functions. We report that naive, primary peritoneal macrophages rapidly upregulate the expression of the NK cell-surface marker NK1.1 in response to MSU crystals but not in response to LPS or other urate crystals. NK1.1 upregulation by macrophages was associated with mechanisms including phagocytosis of crystals, NLRP3 inflammasome activation, and autocrine proinflammatory cytokine signaling. Further analysis demonstrated that MSU crystal–activated macrophages exhibited NK cell–like cytotoxic activity against target cells in a perforin/granzyme B–dependent manner. Furthermore, analysis of tumor hemopoietic cell populations showed that effective, MSU-mediated antitumor activity required coadministration with Mycobacterium smegmatis to induce IL-1β production and significant accumulation of monocytes and macrophages (but not granulocytes or dendritic cells) expressing elevated levels of NK1.1. Our findings provide evidence that MSU crystal–activated macrophages have the potential to develop tumoricidal NK cell–like functions that may be exploited to boost antitumor activity in vivo.

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Malaghan Institute of Medical Research, Wellington 6242, New Zealand

ORCID: 0000-0002-5990-494X (S.S.).

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Address correspondence and reprint requests to Dr. Jacqui L. Harper, Malaghan Institute of Medical Research, P.O. Box 7060, Wellington South 6242, New Zealand. E-mail address: jharper@malaghan.org.nz

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Abbreviations used in this article: MSU, monosodium urate; CPPD, calcium pyrophosphate dihydrate; DC, dendritic cell; MHC II, MHC class II; MSU, monosodium urate; NKDC, NK dendritic cell; Pfp−/−, perforin-deficient; PI, propidium iodide.

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and cells (murine YAC-1 cells) were kindly provided by Alexander McLellan (University of Otago, Dunedin, New Zealand). Liberase TL and DNase I were from Roche, MgU, CaU, KU, and NH4U crystals were kindly provided by Henry Hudson (Malaghan Institute of Medical Research). YAC-1 cells were provided by Alexander McLellan.

Preparation of MSU crystals
MSU crystals were prepared and characterized as previously described (5). The resulting MSU crystals were needle shaped (5–20 μm in length) and showed optical birefringence under polarized light. The MSU crystals were endotoxin free as determined by Limulus amebocyte cell lystate assay (<0.01 EU/10 μg).

In vitro macrophage stimulation assays
Peritoneal exudate cells from naive C57BL/6J mice were harvested by peritoneal lavage (3 ml Dulbecco’s PBS containing 25 U/ml heparin). Peritoneal macrophages (2 × 10^5 cells/ml, RPMI 1640, and 10% FBS) were cultured in vitro in the absence or presence of MSU, CPPD, CaU, MgU, and KU crystals (200 μg/ml) or LPS (100 ng/ml). At different time points, cells were harvested for flow cytometry using a FACSCalibur (BD Biosciences). In assays investigating intracellular expression of granzyme B, macrophages were cultured with GolgiStop (1:1000) to block granzyme B secretion. Macrophage purity was >95%.

Blockade of cytokines
Macrophages were cultured in the absence or presence of anti–IL-1β (5 μg/ml), the caspase-1 inhibitor YVAD (10 μg/ml), anti–IL-6 (5 μg/ml), or anti–TNF-α (5 μg/ml). After 1 h, macrophages were stimulated with 200 μg/ml MSU crystals or left untreated. At different time points, supernatants were collected for IL-1β, IL-6, and TNF-α ELISA and cells harvested for flow cytometry.

Macrophage phagocytosis assay
Macrophage phagocytosis was blocked using cytochalasin D (10 μg/ml). After 24 h, supernatants were collected for IL-1β ELISA and cells harvested and analyzed by flow cytometry.

Killing assay
Peritoneal macrophages were harvested from C57BL/6J or Pfp−/− mice and cultured in vitro with MSU crystals (200 μg/ml), LPS (100 ng/ml), or CPPD crystals (200 μg/ml) for 24 h. MSU crystals were dissolved using rasingurcase (75 μg/ml) for 40 min. The macrophages were then washed and replenished with complete RPMI 1640. YAC-1 cells were labeled with the fluorescent dye CFSE and cocultured with untreated or stimulated macrophages (100,000 cells) at different cell ratios (E:T cells: 0:1; 8:1; 4:1; 2:1; and 1:1) for 4 h. Cells were collected and the percentages of apoptotic (AnV+/PI+) and necrotic (AnV−/PI+) YAC-1 (CFSE+/F4/80−) cells determined by flow cytometry.

Tumor cell line and tumor challenge
The B16.OVA cell line (18) was maintained in complete IMDM as described (19) and extended in vitro passaging was avoided. Mice were injected with 10^6 B16 melanoma cells s.c. into the flank. On day 13, tumors were excised, digested using Liberase TL and DNase I, and passed through a 70-μm cell strainer to give a single-cell suspension (20).

Peritumoral treatments
PBS (100 μl) or MSU plus Mycobacterium smegmatis (250 μg MSU crystals and 2 × 10^6 CFU live M. smegmatis mc^2 155 in a total volume of 100 μl PBS) was injected in the area immediately adjacent to the tumor on days 7, 9, and 11.

Flow cytometry
Cells were washed, blocked with anti-mouse CD16/32 (2.4G2), and resuspended in wash buffer (0.1% BSA, 0.01% sodium azide in PBS or 10 mmol EDTA, 2% FBS, and 0.01% azide in PBS [pH 7.4]). Cells were stained with fluorescent Abs for the surface markers F4/80, CD11b, Ly6C, Ly6G, CD11c, CD45, MHC II, and NK1.1, washed, and then resuspended in fresh wash buffer. For biotinylated flow Abs, cells were stained with streptavidin–Texas Red. For intracellular staining, cells were permeabilized using saponin buffer (PBS, 0.1% saponin azide, 1% FBS, and 0.1% sodium azide), stained for granzyme B, and then resuspended in wash buffer.

To measure cell death by flow cytometry, cells were washed in AnV binding buffer (PBS, 10 mmol HEPES, 140 mmol NaCl, and 2.5 mmol CaCl_2 [pH 7.4]) and stained with AnV-PE and PI.

Singlet, live CD45+ cell populations were identified as: resident peritoneal macrophages (CD45.2+/F4/80+/CD11b−); tumor macrophages (CD45.2+/CD11c+/F4/80+/CD11b−); tumor monocytes (CD45.2+/CD11c+/CD11b−/MHC II−/Ly6C^hi); tumor granulocytes (CD45.2+/CD11c+/CD11b−/Ly6G^hi); and dendritic cells (DCs; CD45.2+/CD11c^−/MHC II^hi).
**Statistical analysis**

Statistical analysis was carried out using Student two-tailed t test, Mann–Whitney U test, one-way ANOVA with Tukey post hoc test, or two-way ANOVA with Bonferroni multiple comparison (as indicated) using GraphPad Prism5 Software (GraphPad). Significance was considered to be attained at a p value <0.05.

**Results**

**Upregulation of NK1.1 expression by MSU crystal–stimulated macrophages**

Resident peritoneal macrophages (F4/80^+^CD11b^+^) were stimulated with MSU crystals or LPS in vitro over 24 h. Flow cytometry analysis showed increased expression of the NK cell-surface marker NK1.1 compared with unstimulated cultured macrophages that was not observed in the presence of unlabeled NK1.1 Ab (Fig. 1A). To investigate whether changes in urate crystal composition could affect NK1.1 expression by macrophages, peritoneal macrophages were also cultured in vitro in the presence of urate crystals prepared with different counterions (Ca^{2+}, NH_4^+, K^+, and Mg^{2+}) or CPPD crystals, the causative agent for pseudogout (21). Only MSU crystal–stimulated macrophages exhibited a significant increase in both the percentage of NK1.1^+^ macrophages and the level of expression of NK1.1 on NK1.1^+^ cells (Fig. 1B, 1C).

To investigate whether macrophages have the ability to express the specific NK cell receptor Ly49A (killer inhibitory receptor), peritoneal macrophages were stimulated with MSU crystals or left untreated for 24 h. The expression of Ly49A increased in control macrophages as well as MSU crystal–treated macrophages, regardless of whether they expressed NK1.1.

**NK1.1 expression by MSU crystal–stimulated macrophages is partially dependent on IL-1β and TNF-α**

The activation of macrophages by MSU crystals induces the production of the proinflammatory cytokine IL-1β (5). As shown

**FIGURE 2.** The upregulation of NK1.1 by macrophages is partially dependent on IL-1β and TNF-α. Peritoneal macrophages (F4/80^+^CD11b^+^) from C57BL/6J mice were stimulated with MSU crystals (200 μg/ml) in the presence of anti–IL-1β Ab (5 μg/ml) (A and E), caspase-1 inhibitor YVAD (5 μg/ml) (B and F), anti–IL-6 Ab (5 μg/ml) (C and G), or anti–TNF-α Ab (5 μg/ml) (D and H). NK1.1 expression was determined by flow cytometry. Values represent the mean ± SEM of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (two-way ANOVA with Bonferroni post hoc analysis).
in Fig. 2A and 2E, neutralization of IL-1β reduced the upregulation of NK1.1 expression and resulted in a decrease in the percentage of NK1.1+ MSU crystal–activated macrophages. The processing and secretion of IL-1β is initiated via stimulation of the NLRP3 inflammasome that requires the activation of the inflammasome component caspase-1 to release active IL-1β (1, 2).

**FIGURE 3.** Macrophage NK1.1 upregulation is dependent on phagocytosis of MSU crystals. Peritoneal macrophages (F4/80+CD11b+) from C57BL/6J mice were stimulated with MSU crystals (200 μg/ml, 24 h) in the presence of the phagocytosis inhibitor cytochalasin D (Cyto D; 10 μg/ml). (A) Uptake of MSU crystals was identified by flow cytometry as an increase in the macrophage side-scatter high (SSC-H). (B) IL-1β levels in culture supernatants were measured by ELISA. (C and D) NK1.1+ expression was determined by flow cytometry. Values represent the mean ± SEM of two independent experiments. **p < 0.01, ****p < 0.0001 (one-way ANOVA with Tukey post hoc test).

**FIGURE 4.** MSU crystal–activated macrophages induce cell death in YAC-1 cells. Peritoneal macrophages (F4/80+CD11b+) from C57BL/6J mice were stimulated with MSU crystals (200 μg/ml), LPS (100 ng/ml), or CPPD crystals (200 μg/ml) for 24 h. Macrophages were cultured with CFSE-labeled YAC-1 cells for 4 h. CFSE+YAC-1 cells were analyzed by flow cytometry to determine the percentages of AnV−/PI− (A and C) and AnV+/PI− (B and D) cells. Values represent the mean ± SEM of two independent experiments. ***p < 0.001, ****p < 0.0001 (one-ANOVA with Tukey post hoc test or two-way ANOVA with Bonferroni post hoc test).
Blockade of caspase-1 using the caspase-1 inhibitor YVAD also decreased NK1.1 upregulation in MSU crystal–treated macrophages (Fig. 2B, 2F).

The effect of neutralization of MSU crystal–induced IL-6 and TNF-α was also investigated. As shown in Fig. 2C and 2F, neutralization of IL-6 did not alter overall NK1.1 expression triggered by MSU crystal stimulation. However, neutralization of TNF-α reduced the upregulation of MSU crystal–induced NK1.1 and resulted in significantly fewer NK1.1+ cells (Fig. 2D, 2H). The biomodal pattern of NK1.1 staining (Fig. 2D) indicates that TNF-α may contribute to NK1.1 expression on a subpopulation of the macrophages rather than NK1.1 expression on the entire population, as observed with blockade of IL-1β (Fig. 2A). Nevertheless, together, the data indicate that NK1.1 upregulation by MSU crystal–stimulated macrophages is partially dependent on NLRP3 activation and IL-1β and TNF-α production.

**Macrophage NK1.1 upregulation is dependent on phagocytosis of MSU crystals**

It has been shown previously that phagocytosis of MSU crystals by macrophages is necessary to initiate MSU crystal–induced inflammation (22). As shown in Fig. 3A, macrophages treated with the phagocytosis inhibitor cytochalasin D exhibited less MSU crystal uptake [side-scatter high (23)] compared with control macrophages. Annexin/PI staining confirmed that the increase of side-scatter high cells was not due to cell death (Supplemental Fig. 1). Cytokine analysis showed that inhibition of MSU crystal phagocytosis resulted in decreased IL-1β production (Fig. 3B) and in a significant drop in both the percentage of NK1.1+ cells and the levels of NK1.1 expression by macrophages (Fig. 3C, 3D).

**NK1.1-expressing MSU crystal–activated macrophages kill YAC-1 cells via perforin**

Studies show that NK cells, NKDCs, and bacterial-activated macrophages can induce NK-sensitive killing of target cells (24, 25). MSU crystal–stimulated macrophages induced a significant increase in the percentage of CFSE+YAC-1 NK-sensitive YAC-1 target cells that were AnV+/PI+ or AnV-/PI-, indicating an increase in cell death and membrane disruption (Fig. 4A, 4B, Supplemental Fig. 2B). CFSE+YAC-1 YAC-1 cells were not killed by either LPS-activated macrophages (Fig. 4A, 4B, Supplemental Fig. 2B) or CPPD crystal–stimulated macrophages (Fig. 4C, 4D, Supplemental Fig. 2C).

NK receptor–expressing cells have been shown to induce cytotoxicity via perforin/granzyme B. Flow cytometry analysis of MSU crystal–stimulated macrophages identified a significant increase in the percentage of granzyme B+ cells compared with untreated macrophages (Fig. 5A). Furthermore, MSU crystal–treated macrophages from Pfp-2 mice induced significantly less apoptotic and necrotic death in YAC-1 cells compared with macrophages from C57 mice (Fig. 5B, 5C). Ab blockade of NK1.1 resulted in a significant decrease in the percentages of both apoptotic (Fig. 6A) and PI+/AnV- CFSE-labeled YAC-1 cells (Fig. 6B), confirming that NK1.1 expression was required for MSU crystal–activated macrophage killing function (Supplemental Fig. 3). Taken together, these results indicated that NK1.1+ MSU crystal–activated macrophages were killing YAC-1 cells in an NK1.1- and perforin-dependent manner.
Infiltration of NK1.1+ monocyte/macrophage populations in effective MSU crystal–mediated antitumor therapy

Previously, we have reported that combined treatment with MSU crystals and *M. smegmatis* elicits effective antitumor activity, whereas treatment with MSU crystals or *M. smegmatis* alone does not. This successful MSU crystal–mediated antitumor therapy is reported to require NK1.1+ cells and associated with the induction of inflammatory cytokines (16). Therefore, we examined NK1.1 expression on CD45+ hematopoietic cells in MSU/*M. smegmatis*–treated tumors. This combination therapy resulted in an overall increase in monocytes and macrophages in the tumor (Fig. 7A), whereas immune cell infiltration was not observed for either MSU crystals or *M. smegmatis* treatments alone (Supplemental Fig. 4). For the combined treatment, there was a significant increase in the percentage of these cells expressing high levels of NK1.1+ (Fig. 7B). In contrast, the percentage of granulocytes and DCs increased only moderately in response to treatment and these cells did not express NK1.1. This illustrated that the MSU crystal/*M. smegmatis*–mediated therapy was capable of selectively inducing NK1.1 expression on monocytes/macrophages in tumors, which may contribute toward MSU crystal–mediated antitumor activity.

Discussion

In this study, we report that MSU crystal–activated macrophages rapidly upregulate NK1.1 expression and NK1.1–specific cytolytic functions and that NK1.1–expressing monocytes and macrophages can be induced in tumors of mice treated with MSU crystal–mediated antitumor therapy.

The upregulation of NK1.1 by MSU crystal–activated macrophages was dependent on a combination of crystal uptake; NLRP3

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**FIGURE 7.** Monocyte and macrophages in tumors treated with MSU + *M. smegmatis* (M.smeg) express NK1.1. C57BL/6d mice bearing established B16. OVA tumors were treated with PBS as a vehicle control or MSU + *M. smegmatis* on days 7, 9, and 11 after tumor challenge. Tumor cell suspensions were analyzed by flow cytometry 2 d after the last treatment. (A) Percentages of macrophages, monocytes, granulocytes, and DCs in the total live tumor cell population. (B and C) Expression of NK1.1 by tumor-infiltrating cell populations. Fluorescence-minus-one (FMO) containing the full staining mix minus NK1.1 Ab is shown as a control. Each symbol corresponds to one mouse. Means ± SEM are shown for scatter plots. *p < 0.05, **p < 0.01 (Mann–Whitney U test). Data are from one of three similar independent experiments.
inflammasome activation, and autocrine proinflammatory cytokine signaling. In the absence of one or more of these events, NK1.1 upregulation was absent or significantly impaired. The lack of NK1.1 upregulation or cytotoxic function following LPS stimulation, or treatment with other urate or CPPD crystals, implies a certain level of specificity in the upregulation of NK1.1 expression by MSU crystal–activated macrophages.

Other NK receptors have been shown to be upregulated on macrophages exposed to different stimuli in vitro and in vivo. In particular, increased macrophage NKG2D expression has been linked to regulation of granzyme B/perforin-dependent killing functions (26, 27), similar to that also reported for NK receptor–expressing DCs (28). Consistent with this, we show that MSU crystal–activated NK1.1+ macrophages also expressed granzyme B and exhibited perforin-dependent cytotoxicity against NK-sensitive YAC-1 cells. Although perforin was necessary for killing function by MSU crystal–activated macrophages, NK1.1 Ab treatment did not fully block YAC-1 cell death. It is therefore possible that the perforin–dependent cell death is mediated via more than one cell-surface receptor on MSU crystal–activated macrophages.

Regulation of NK receptor–mediated killer function has been linked to the expression of NK inhibitory and activating receptors (29–31), and Ly49 receptors are reported to be necessary for effective cancer immunosurveillance (32). In this study, upregulation of the inhibitory receptor Ly49A was observed over time, independent of macrophage stimulation or NK1.1 expression. The driver for naive macrophage Ly49A upregulation is yet to be determined, although Ly49A expression has been linked with increased survival in other cell types (33, 34). In the context of the NK1.1 upregulation shown in this study, coexpression of Ly49A allowing recognition of self–MHC class I molecules could serve to regulate NK1.1-mediated killing (35, 36). This would potentially limit inappropriate cytotoxic targeting of healthy cells by MSU crystal–activated macrophages.

MSU crystals are widely recognized as an endogenous adjuvant capable of amplifying both innate and adaptive immunity (1, 2). Our data demonstrate that MSU crystals can also trigger NK cell–like killing function in macrophages and introduces a new dimension to our understanding of the impact of MSU crystals on local immune cell functions. Previously, we have shown that peritumoral MSU crystal administration in combination with M. smegmatis promotes antitumor immune responses that are dependent on NK1.1+ cells and are associated with the induction of proinflammatory cytokines including IL-1β (16). NKDCs that exhibit NK cell–like killing functions have been shown to play a role in enhanced tumor cell death (37). However, our findings indicate that effective MSU crystal treatment in tumor therapy does not induce NKDCs but instead supports the accumulation of NK1.1-expressing monocyte/macrophages with the potential for NK cell–like killing function and tumoricidal activity.

Although M. smegmatis and MSU crystal treatment is reported to induce antitumor activity in vivo (16), interestingly, treatment with either M. smegmatis or MSU alone does not. Individually, these treatments do not induce either IL-1β production or inflammatory cell infiltration in tumors. The need for MSU crystal/ M. smegmatis combination therapy likely reflects the recognized two-stage signaling process associated with release of active IL-1β (38), by which M. smegmatis signals the need to accumulate stores of pro–IL-1β in local monocyte/macrophage populations in preparation to raise the proinflammatory IL-1β response triggered by MSU crystals. In this study, activation of the IL-1β–driven inflammatory signaling cascade would then drive the recruitment and accumulation of NK1.1+ monocyte/macrophages. Resident peritoneal macrophages, however, naturally express pro–IL-1β (6), and therefore, ex vivo, MSU treatment alone is sufficient to trigger the macrophage inflammatory responses resulting in NK1.1 and perforin-dependent killing functions we observe in vitro.

In summary, we report that MSU crystal stimulation of locally primed macrophages induces rapid NK1.1-mediated cytotoxic function that is dependent on the induction of proinflammatory cytokines. The observation that NK1.1+ macrophages exhibit cytotoxicity against target cells and are present in tumors during effective MSU crystal–mediated tumor therapy provides a broader understanding of the arsenal of antitumor strategies induced by MSU crystals and illustrates the far-reaching potential of using MSU crystals for adjuvant immunotherapy in the treatment of cancer.

Disclosures
The authors have no financial conflicts of interest.

References


