

RAPID CCL2 RELEASE BY MEMBRANE STROMAL CELLS INITIATES MONOSODIUM URATE CRYSTAL-INDUCED MONOCYTE RECRUITMENT IN A PERITONEAL MODEL OF GOUTY INFLAMMATION

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Received November 29, 2011 – Accepted may 2, 2012

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Monocyte recruitment is a characteristic feature of the inflammatory response to monosodium urate (MSU) crystals in gout, however the specific cell population(s) responsible for initiating this event is unclear. We therefore investigated the contribution of resident and stromal cell populations to the initiation of MSU crystal-induced inflammatory cytokine and chemokine production in a peritoneal murine model of gout. Depletion of resident macrophages decreased neutrophil infiltration but did not affect MSU crystal-induced monocyte recruitment *in vivo* despite a significant decrease in overall CCL2 production. Activation of isolated resident peritoneal cells and peritoneal membrane with MSU crystals *in vitro* indicated that resident peritoneal cells, more specifically resident macrophages, were primarily responsible for the production of the neutrophil chemokine CXCL1, whereas CCL2 was exclusively produced in membrane cultures. Primary culture of membrane mesothelial cells followed by MSU crystal stimulation resulted in CD14-independent CCL2 release from intracellular stores. These findings confirm that MSU crystal-induced neutrophil recruitment is dependent on CXCL1 production by resident macrophages. Conversely, monocyte infiltration may be primarily initiated by the release of low level CCL2 by stromal cells in the surrounding tissue. As such, the synovial tissue in the joint may play a direct role in regulating inflammation in gout.

The onset of acute inflammation in gouty arthritis is triggered by the formation of monosodium urate (MSU) crystals in the joint and surrounding tissues. In addition to the production of inflammatory cytokines, including IL-1 β , IL-6 and TNF α , joint inflammation is associated with the production of the chemokines IL-8 (CXCL8) and MCP-1 (CCL2) primarily responsible for the recruitment of neutrophils and monocytes, respectively (1, 2).

Neutrophil infiltration and activation has long been

viewed as a defining characteristic of inflammation in acute gout. However, there is strong evidence to support a pivotal role for resident macrophages and monocyte responses in both the inflammatory and resolution phase of MSU crystal-induced inflammation (3-6). Understanding the pathways involved in the recruitment of both neutrophils and monocytes could provide key information for the development of multicellular-targeted therapies for gout treatment.

Key words: CCL2, monocyte trafficking, MSU crystals, inflammation, mesothelial cells

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Studies into acute inflammation indicate that monocyte recruitment may be driven by neutrophil-derived factors such as IL-6 augmenting CCL2 production at the site of inflammation (7-9). However, recent *in vivo* data show that monocyte recruitment can occur in the absence of neutrophils (10). The depletion of the resident peritoneal macrophages has also been shown to abrogate MSU crystal-induced IL-1 β production and neutrophil infiltration leaving monocyte recruitment unaltered (11). These results indicate that monocyte recruitment does not immediately depend on either macrophage activation or neutrophil infiltration, and may in fact be orchestrated by other resident cells at the site of inflammation.

Recent mRNA analysis of dissected membranes from the murine air pouch model of MSU crystal-induced inflammation has identified significant transcription of proinflammatory molecules *in situ* (12). Cells in the synovial membrane are also reported to produce cytokines and chemokines, including the monocyte chemokine CCL2, in response to MSU crystals in rabbits (13). Human synovial stromal cells from arthritis patients have been reported to produce CCL2 in culture (14, 15) and synoviocytes have also been shown to release stores of CCL2 following MSU crystal treatment *in vitro* (16). Recently, CCL2 production and monocyte recruitment *in vivo* was also found to decrease following antagonism of the bradykinin 2 receptor (17), a receptor that is ubiquitously expressed on synovial membranes (18).

Taken together, these results provide evidence that stromal cells in the surrounding tissues are involved in pro-inflammatory chemokine production and therefore cell recruitment in response to MSU crystal stimulation.

The aim of this study is to extend our understanding of the contribution of resident cells versus stromal tissue to MSU crystal-induced production of the leukocyte chemokines CCL2 and CXCL1 (murine neutrophil chemokine KC), and monocyte and neutrophil recruitment *in vivo*.

MATERIALS AND METHODS

Mice

C57Bl6/J mice were bred and housed at the Malaghan Institute of Medical Research, Wellington, New Zealand.

All experimental animals were male, aged between 8-12 weeks. Experimental procedures were approved by the Victoria University of Wellington Animal Ethics Committee.

Reagents

Uric acid and lipopolysaccharide (LPS) were from Sigma (Auckland, NZ). Mouse KC (CXCL1), IL-6, TNF α and MCP-1(CCL2) Bio-Plex multiplex arrays were from Bio-Rad (Hercules, CA, USA). F4/80-PE and Clone7/4-FITC were from Serotec (Oxford, UK). 2.4G2 Fc antibody was produced from HB-197 hybridoma cell line (ATCC, Manassas, VA). Unconjugated murine CD14 antibody and CD14-PerCP-Cy5.5 were from BioLegend. Gr-1-APC was from BD Biosciences (North Ryde, NSW, Australia). GolgiStop was from BD Biosciences, (Mississauga, Ontario, Canada). MCP-1(CCL2)-FITC was from eBioscience. Cytokeratin antibody was from Abcam (San Francisco, CA, USA). Hydrocortisone was from Sigma-Aldrich (Auckland, NZ). Epidermal growth factor (EGF) was from Roche Diagnostics (Auckland, NZ). Mouse IL-1 β ELISA kit was from R&D Systems (Minneapolis, MN, USA). The Limulus amoebocyte cell lysate assay kit was from Associates of Cape Cod (East Falmouth, MA, USA). Heparin was from Mayne Pharma (Melbourne, Victoria, Australia). Diff-Quik was from Dade Behring Diagnostics (Newark, DE, USA). Anti-mouse CCL2 neutralising antibody was from eBioscience (San Diego, CA). Unless otherwise stated, all other products were from Invitrogen (Auckland, NZ).

Preparation of MSU crystals

MSU crystals were prepared as previously described (11, 19). MSU crystals were needle-shaped, between 5-20 μ m and displayed optical birefringence under polarized light microscopy. MSU crystals were confirmed as endotoxin-free (<0.01 EU/10mg) by LAL assay.

Isolation and purification of resident peritoneal cell populations

C57Bl6/J mice were sacrificed (CO₂ asphyxiation) and the peritoneal cavities lavaged (3 ml PBS, 25 units ml⁻¹ heparin). The lavaged peritoneal cells were pooled and used directly in stimulation assays. To determine macrophage and mast cell content, peritoneal wash cells were fixed onto slides using a cytospin and stained with the Diff-Quick Staining Kit. The percentages of macrophages and mast cells were then determined microscopically using standard histological criteria. % cells \pm SEM: Macrophages 85 \pm 3%, Mast cells 3.0 \pm 0.5%.

Alternatively, peritoneal cells were supplemented with 10% fetal calf serum and DNaseI (10 units ml⁻¹) then enriched for macrophages or mast cells.

Enrichment of naïve peritoneal macrophages. Peritoneal macrophages were enriched using a modified method of DaMatta et al.(20) Peritoneal cells were resuspended in 70% (v/v) Percoll solution containing DNaseI (10 units ml⁻¹). The cells were separated on a Percoll gradient (10 ml of 51% Percoll solution, 3 ml 70% Percoll-cell preparation, 2 ml DNaseI solution -10 units ml⁻¹ in Medium 199) by centrifugation (18 min, 680 x g, 4°C). Macrophages were recovered from the 8-15 ml band and washed with 35 ml DNaseI solution (10 units ml⁻¹ in HBSS). Final purity >80%.

Enrichment of naïve peritoneal mast cells. Naïve peritoneal cells were centrifuged (5 min, 500 x g) and resuspended in 8 ml of 70% Percoll solution (6.3 ml Percoll Plus, 3.7 ml 2.5x PBS, pH 7.2) and overlaid with 2 ml of RPMI-1640 (5% FCS). The gradient was centrifuged (15 min, 580 x g, room temperature), the Percoll solution was removed and the mast cell pellet washed in cRPMI-1640. Final purity>90%.

Preparation of cultured mesothelial cells

Mesothelial cells were cultured as previously described (21, 22). Briefly, naïve C57BL6/J mice were sacrificed and peritoneal cells were removed by peritoneal lavage (2x 10 ml PBS). Trypsin/EDTA (0.25%, 10 ml) was injected i.p. After 15 mins the Trypsin/EDTA lavage was recovered and the cells washed with cRPMI-1640. The cells were cultured in supplemented RPMI-1640 (20ng/ml EGF, 5 µM hydrocortisone, collagen-coated flasks). After three passages, cells were harvested for *in vitro* testing.

Cell viability. Cell viability was >95% for all cell preparations as determined by trypan blue exclusion.

Preparation of whole peritoneal membrane. Naïve C57BL6/J mice were sacrificed and the peritoneal membrane (~2 x 3cm squares) was harvested under sterile conditions, placed in RPMI-1640 and stored on ice.

In vitro activation of cells

Mixed peritoneal leukocytes (10⁶/ml), macrophages (10⁶/ml), mast cells (10⁵/ml) or mesothelial cells (0.4x10⁶/ml) were plated out in cRPMI-1640 (5% FCS) and stimulated with MSU crystals (200 µg/ml in PBS) or with LPS (100 ng/ml). For whole peritoneal tissue culture, cRPMI-1640 containing MSU, LPS or PBS control was added to 96-well plates and the peritoneal tissue placed across the wells, with the visceral side facing down. The plate lid was sealed over the peritoneal tissue and the plate inverted -exposed membrane surface area of 0.5cm² per well. Tissue was cultured for 4 hours (37°C, 5% CO₂). Supernatants were collected and stored at -20°C for subsequent analysis.

For immunofluorescent microscopy, mesothelial cells (200 µL, 1x10⁵ cell/ml) were transferred into 8 chamber

tissue culture glass slides (Falcon BD Labware, Franklin Lakes, NJ, USA) incubated for 1 h then stimulated for four hours with 500µg/ml MSU crystals in the presence of golgistop.

MSU crystal-induced peritonitis

Naïve C57BL6/J mice were treated i.p. with MSU crystals (3 mg, 0.5 ml Dulbecco's Phosphate Buffered Saline). Mice were sacrificed and the peritoneal cells harvested by lavage (3 ml PBS, 25 units/ml heparin). Total cell numbers were counted then the peritoneal lavage fluid was collected and stored at -80°C. The peritoneal cells were analysed by flow cytometry to identify macrophages (F4/80^{hi}/Gr1⁻/Clone 7/4⁻), monocytes (F4/80^{lo}/Gr1⁺/Clone 7/4⁺) and neutrophils (F4/80⁻/Gr1^{hi}/Clone 7/4⁺) as previously described (2, 11).

In vivo depletion of peritoneal resident macrophages

Clodronate liposomes were prepared as previously described (23). To deplete resident peritoneal macrophages, mice were injected i.p. with 200 µl of clodronate liposomes (11).

CCL2 neutralisation in vivo

CCL2 neutralising antibody (50 µg/mouse in 200 µl PBS) was administered i.p. 2 h before MSU crystal administration.

Flow cytometry

Cells were suspended in FACS buffer (0.1% BSA, 0.01% sodium azide in PBS, pH 7.4), stained with fluorescent antibodies for the surface markers F4/80, Gr-1, Clone 7/4 then washed and resuspended in FACS buffer. Cells were analysed using a BD FACSCalibur (BD Biosciences, New Zealand).

Immunofluorescent microscopy

Immunofluorescent staining of mesothelial cells was carried out as previously described (24). Briefly, non-specific antibody binding sites were blocked with 2.4G2 antibody then the cells were incubated with unconjugated cytokeratin antibody. Cells were washed and stained with anti-rabbit-FITC and CD14-Alexa555. For CCL2 intracellular staining, cells were permeabilized with 200 µL ice-cold methanol. ProLong Gold anti-fade with DAPI was applied to the slide. Slides were examined using an Olympus BX51 fluorescent microscope (Olympus, Central Valley, PA, USA) using AnalySIS Life Imaging Software (Olympus Soft Imaging Solutions, Munster, Germany).

Cytokine analysis

Murine cell and peritoneal membrane culture

supernatants were analysed for IL-1 β , CXCL1 (KC), IL-6, TNF α and CCL2 (MCP-1) by Bioplex multiplex bead ELISA kits. Human serum samples were analysed for CCL2 by Lincoplex bead array.

Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Tukey, or Bonferroni, post-hoc multiple comparisons test if overall $p < 0.05$.

RESULTS

Resident macrophage depletion abrogates MSU crystal-induced neutrophil but not monocyte recruitment *in vivo*

We have previously reported that resident macrophage depletion attenuates neutrophil but not monocyte recruitment during the first 8 hours following MSU crystal administration *in vivo* (11). To determine whether this differential response was present beyond 8 hours, the MSU crystal response was monitored out to 7 days. As reported previously, resident peritoneal macrophages were depleted by i.p. injection of clodronate liposomes (23, 25) and over 99% of resident F4/80⁺ macrophages were depleted after three days (Fig. 1A). Resident macrophage depletion resulted in a significant decrease in neutrophil recruitment following MSU administration up to 18 hours (Fig. 1B). As expected, MSU-induced monocyte recruitment was unaffected (Fig. 1C).

Resident peritoneal cells and peritoneal stroma exhibit discreet proinflammatory profiles in response to MSU crystal stimulation

To identify sources of MSU crystal-induced proinflammatory cytokine production in the naïve peritoneal resident cells and tissues during the early phase of the inflammatory response, isolated naïve peritoneal cells and membrane were stimulated separately with MSU crystals *in vitro* for 8 hours - consistent with the time of peak inflammatory cytokine production *in vivo* (11). MSU crystal-activated peritoneal cells produced increased levels of IL-1 β , IL-6 and TNF α and the murine neutrophil chemokine CXCL1 *in vitro* whereas MSU crystal-activated membrane preparations produced only the monocyte chemokine CCL2 (Fig. 2A). In stark contrast, LPS treatment induced cytokine and

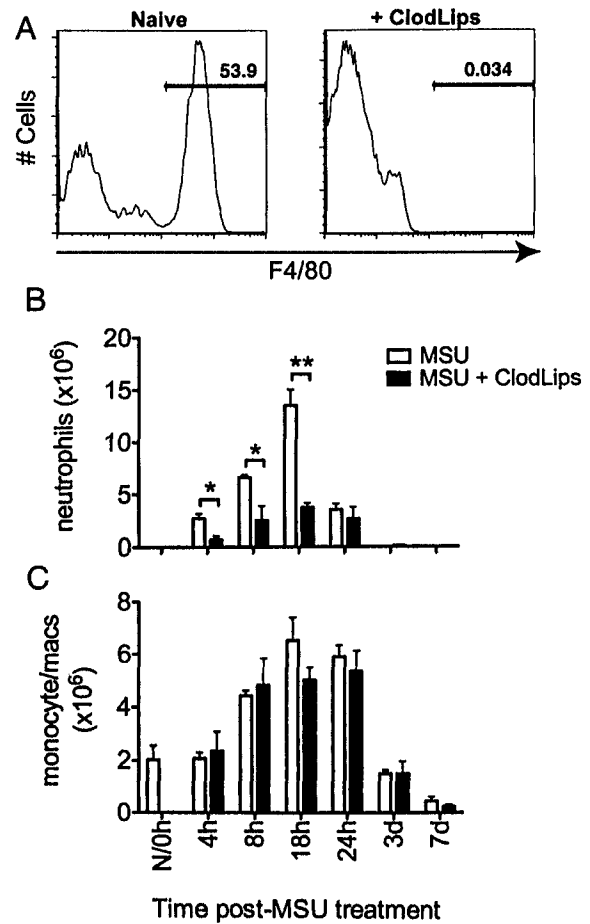


Fig. 1. Depletion of peritoneal macrophages abrogates MSU crystal-induced neutrophil recruitment but not monocyte recruitment. **A)** Mice were depleted of peritoneal resident macrophages using clodronate liposomes administered i.p. 3 days before MSU crystals (3mg, 200 μ l PBS, i.p.). The peritoneal lavages were collected and analysed by flow cytometry to determine **(B)** neutrophil (Gr1hi/Clone7/4+/F4/80⁻) and **(C)** monocyte (Gr1int/Clone7/4+/F4/80^{int}) infiltration. Data represents the mean \pm SEM ($n = 4$ per group) of replicate experiments. * $p < 0.05$; ** $p < 0.005$.

chemokine production in both peritoneal cells and membrane preparations (Fig. 2B).

Resident macrophages are required for MSU crystal-induced CXCL1 production

To identify which cells within the naïve resident cell population were primarily responsible for the production of the pro-inflammatory cytokines

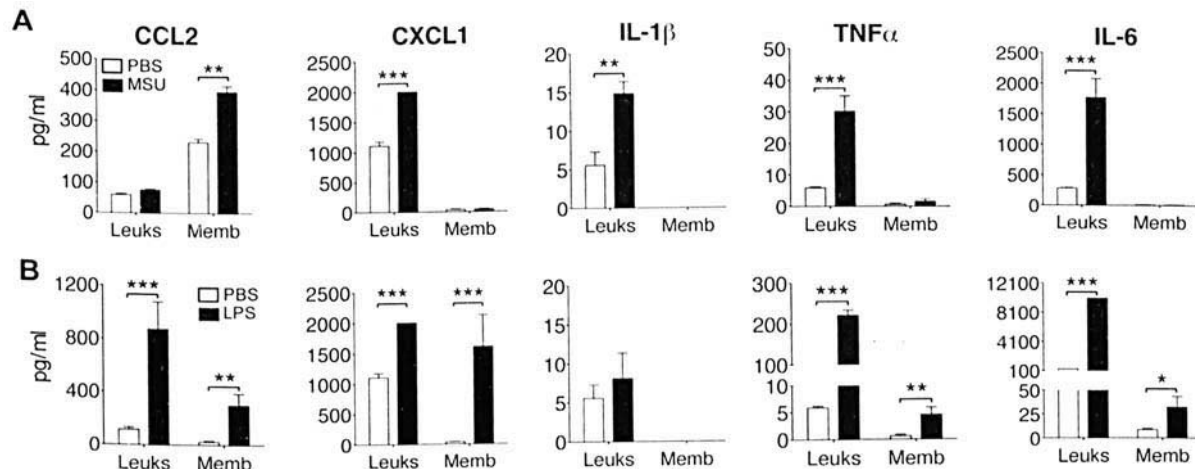


Fig. 2. MSU crystal and LPS induced cytokine and chemokine production by peritoneal cells and membrane. Peritoneal wash cells (PWC) and membrane were isolated from naïve C57Bl6/J mice and treated with (A) MSU crystals (200 μ g/ml) or (B) LPS (100 ng/ml) for 8 hours *ex vivo*. Supernatants were collected and analysed for cytokine and chemokine production. Data represents the mean \pm SEM ($n = 4-5$ per group) of replicate experiments. ** $p < 0.01$ *** $p < 0.001$.

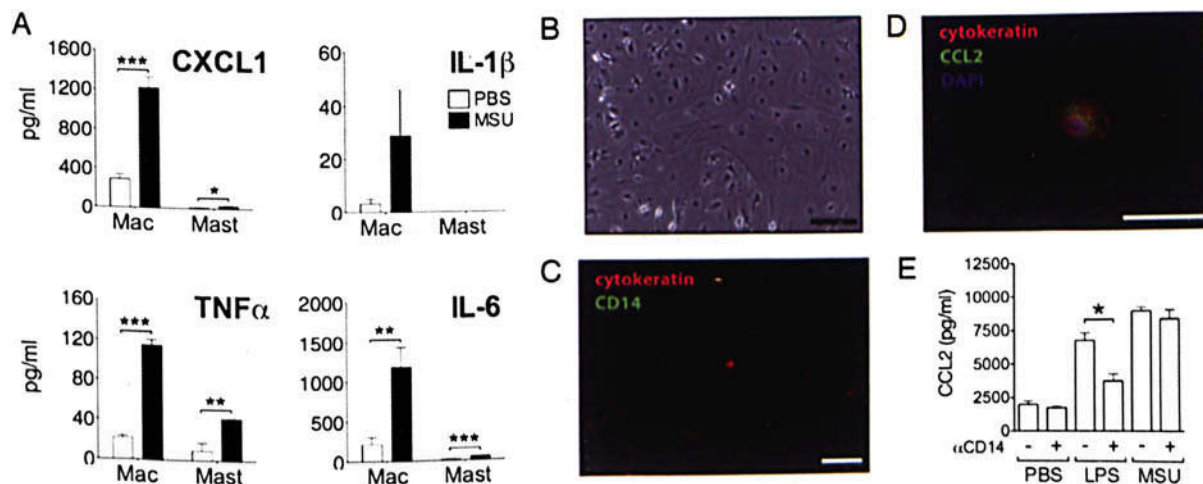


Fig. 3. MSU-induced cytokine and chemokine production by naïve resident cell populations and cultured stromal cells. (A) Purified naïve peritoneal macrophage (Mac) and mast cell (Mast) populations were treated with MSU crystals (200 μ g/ml, 8 hours). Supernatants were analysed for CXCL1, IL-1 β , TNF α , IL-6. (B) Primary mesothelial cells expressed (C) cytokeratin and CD14. (D) Mesothelial cells stimulated with MSU crystals (500 μ g/ml, 8 hours) stained for cytokeratin, CCL2 and DAPI. (E) CCL2 in supernatants from cultured mesothelial cells treated with LPS (100 ng/ml) or MSU crystals \pm CD14 antibody. Data represents the mean \pm SEM ($n = 4-5$ per group) of replicate experiments. * $p < 0.05$, *** $p < 0.001$. B, C, D Scale bar represents 100 μ m.

IL-6, IL-1 β , CXCL1 and TNF α , purified naïve macrophages and mast cells were stimulated with MSU crystals *in vitro*. MSU crystals induced significant IL-6, IL-1 β and CXCL1 and TNF α production by resident macrophages with minimal cytokine production by mast cells (Fig. 3A), despite

evidence of degranulation (data not shown).

Membrane mesothelial cells are a key source of CCL2

Human synoviocytes have been reported to produce CCL2 following direct MSU crystal stimulation (16). To determine potential sources

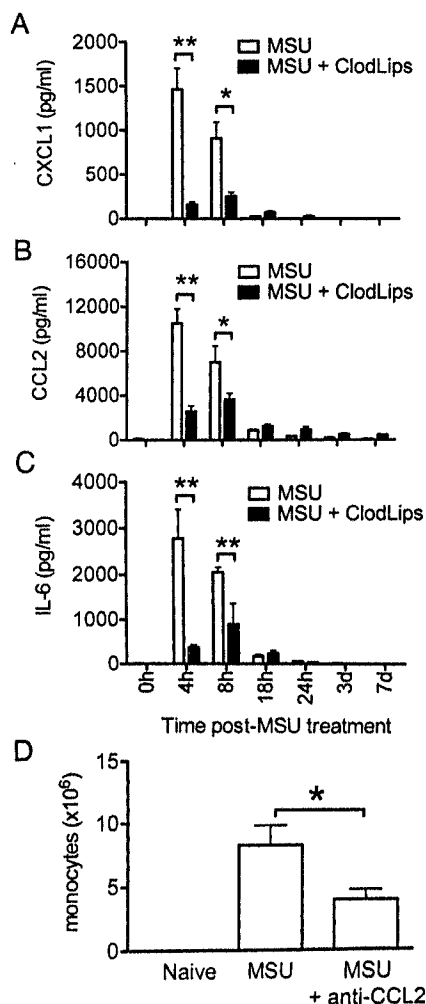


Fig. 4. CCL2 is required for MSU crystal-induced monocyte recruitment. Naïve C57Bl/6J mice were depleted of peritoneal resident macrophages using clodronate liposomes administered i.p. 3 days before MSU crystals (3mg, 200µl PBS). The peritoneal lavage fluid was analysed for (A) CXCL1, (B) CCL2 and (C) IL-6. (D) Naïve mice were treated with CCL2 neutralizing antibody (50 µg/mouse, i.p.) 2 hours before administration of MSU crystals. At 18 hours, the peritoneal lavages were collected and analysed by flow cytometry to determine monocyte infiltration. Data represents the mean \pm SEM ($n = 4-5$ per group) of replicate experiments. * $p < 0.05$; ** $p < 0.005$

of CCL2 production in the stromal tissue, primary peritoneal mesothelial cells were cultured and treated with MSU crystals *in vitro*. Cultured mesothelial cells exhibited classical cobblestone morphology (Fig. 3B). Immunofluorescent microscopy confirmed

that the cells expressed cytokeratin and CD14 (Fig. 3C) and produced CCL2 following MSU crystal stimulation (Fig. 3D). Mesothelial cells produced CCL2 following both MSU crystal and LPS stimulation (Figure 3E). CD14 antibody treatment inhibited LPS but not MSU crystal-induced CCL2 production.

Resident macrophage depletion abrogates MSU crystal-induced chemokine production in vivo

Finally we looked at the production of CXCL1 and CCL2 in macrophage-depleted mice treated with MSU crystals. Resident macrophage depletion resulted in a significant decrease in CXCL1 (Fig. 4A). Both CCL2 and IL-6 levels were significantly lower in macrophage-depleted mice (Fig. 4B, C). To confirm the importance of CCL2 production in MSU crystal-induced monocyte recruitment, mice were treated i.p. with CCL2 neutralising antibody prior to MSU crystal administration. CCL2 neutralisation resulted in a significant decrease in monocyte recruitment in response to MSU crystals *in vivo* (Fig. 4D).

DISCUSSION

The importance of macrophage stimulation in the induction of MSU crystal-induced inflammation, via activation of the NLRP3 inflammasome and IL-1 β production, is widely recognised (5, 26). Consistent with this, it was the resident macrophages that were identified as the primary source of the classical pro-inflammatory cytokines IL-1 β , IL-6 and TNF α following MSU crystal stimulation *in vitro*, and were important for production of IL-1 β and IL-6 production *in vivo*. MSU crystal-activated macrophages were also shown to be a primary source of neutrophil chemoattractant CXCL1 and consequently necessary for MSU crystal-induced neutrophil infiltration *in vivo*. This provides strong evidence that neutrophil infiltration is specifically regulated by macrophage activation and CXCL1 production during an acute gout attack. Mast cell activation by MSU crystals induced minimal inflammatory cytokine or chemokine production. However, this does not preclude mast cell activation and degranulation from playing a part in inflammatory cell recruitment. The release of

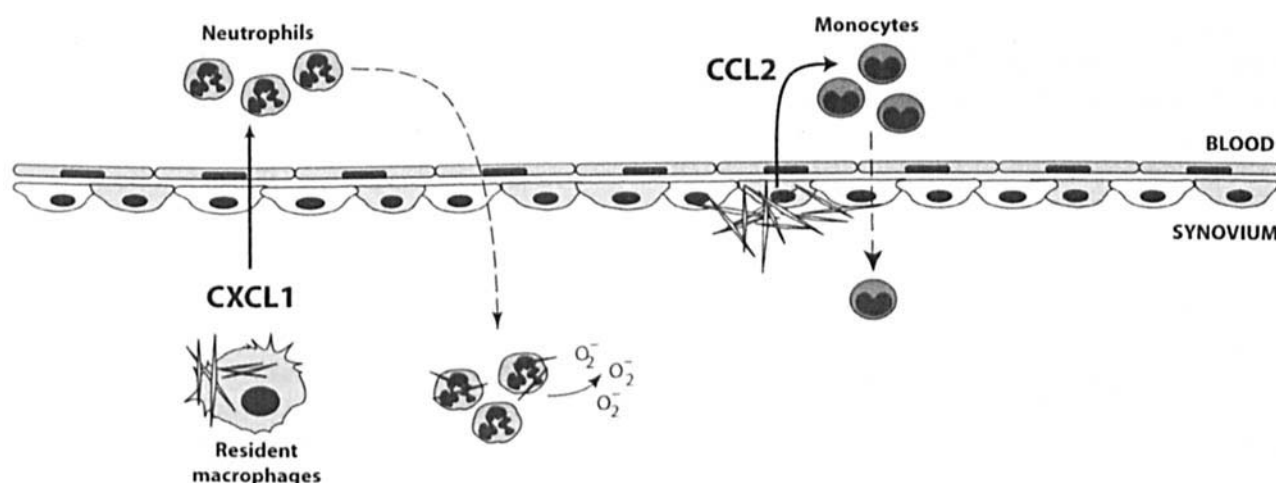


Fig. 5. Resident cells and stromal cells can differentially control leukocyte recruitment in response to MSU crystal deposition.

histamine and serotonin by mast cells is linked with MSU crystal-induced oedema and nociception (27) and may be the primary mast cell-dependent event that facilitates inflammatory cell infiltration (28).

Despite resident macrophages producing the majority of proinflammatory molecules investigated in this study, only stromal tissue preparations were shown to produce the monocyte chemokine CCL2 following MSU crystal stimulation *in vitro*. This contrasted significantly with responses to LPS where chemokines and cytokines were induced in both culture systems. Previous reports identifying CCL2 positive human synoviocytes in joint synovia and cultured stromal cells, and release of CCL2 following MSU crystal stimulation (13-16) point towards a contribution from synovial tissues to monocyte infiltration in gouty arthritis. Our results not only provide further evidence that the stromal cells can contribute to monocyte recruitment but in fact, may be principle players controlling the initial phase of MSU crystal-induced monocyte recruitment.

Consistent with previous studies on stromal cells (16), the cultured CD14⁺ mesothelial cells also produced CCL2 following MSU crystal activation. CD14, known to be required for LPS signalling via toll-like receptor 4, has been implicated as playing a role in potentiating MSU crystal inflammation *in vivo* (29) and CXCL1 production in bone marrow-derived macrophages (30). However, blockade of CD14 on mesothelial cells did not inhibit MSU crystal-induced production of CCL2. Therefore,

although CD14 is necessary for LPS-induced CCL2 production by mesothelial cells, CD14 does not appear to make a direct contribution to mesothelial cell-dependent CCL2 production in the context of this study.

It was interesting to note that following resident macrophage depletion, MSU crystal-induced CCL2 levels dropped significantly. Although peripheral blood monocytes produce CCL2 in response to inflammatory stimuli, there is evidence that in the local inflammatory milieu newly MSU crystal-recruited monocytes do not produce CCL2 (31, 32) and are therefore unlikely to be directly contributing to the changes in CCL2 levels observed.

Significant CCL2 production by naïve resident macrophages was not observed in this study however we, and others, have observed MSU crystal-induced CCL2 production with extended MSU crystal stimulation times (2, 16). MSU crystals have been shown to induce upregulation and post-translational stabilisation of CCL2 mRNA *in vitro* (33) whereas synoviocytes and mesothelial cells appear to possess stores of MCP-1 ready for immediate release upon MSU crystal stimulation (13, 16). This differential regulation of CCL2 production may be one reason why only membrane CCL2 production was observed during the first 8 hours of MSU crystal stimulation in this study. As such, the membrane may well be the initial source of CCL2 responsible for monocyte recruitment.

CCL2 is the main chemokine responsible for

monocyte chemotaxis in inflammation (34) and was shown to be important for MSU crystal-induced monocyte recruitment. However, our data showed that efficient monocyte chemotaxis still occurred despite significantly attenuated CCL2 levels. High affinity CCL2 receptors are abundantly expressed on monocytes (35) making these cells incredibly responsive to low levels of CCL2. It is likely that this chemotactic sensitivity drives normal monocyte recruitment during an otherwise abrogated MSU crystal-induced inflammatory response.

One factor that could be contributing to the observed decrease in CCL2 levels is the local proinflammatory cytokine environment. The production of IL-6 has been linked with augmenting CCL2 production (7-9) in acute inflammation. The lower IL-6 levels observed in macrophage-depleted mice following MSU crystal administration could therefore be responsible for lower MSU crystal-induced CCL2 production *in vivo*. Synoviocytes have been reported to produce IL-6 following extended MSU crystal stimulation (36). However, IL-6 production was not observed in our 8 hour membrane cultures indicating that the membrane is not contributing to the peak IL-6 production observed *in vivo*. Based on the present data, the most probable sources of IL-6 are the resident macrophage population and to a lesser extent, mast cells.

In summary (Fig. 5), our findings indicate that, whereas neutrophil recruitment is mediated by resident macrophage activation, membrane CCL2 production plays a pivotal role in the initiation of MSU crystal-induced monocyte recruitment via direct activation of the local stromal cells. As such, membrane activation likely represents an important event in the inflammatory response to MSU crystals in gout.

ACKNOWLEDGEMENTS

This work was funded by the Wellington Medical Research Foundation, New Zealand.

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