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RUNNING HEAD: GM-CSF drives MSU inflammatory macrophages

TITLE:

GM-CSF drives MSU crystal-induced inflammatory macrophage differentiation and NLRP3 inflammasome upregulation *in vivo*.

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ABSTRACT

Objective: The aim of this study was to determine the role of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the differentiation of inflammatory macrophages in an *in vivo* model of monosodium urate (MSU) crystal-induced inflammation.

Methods: C57Bl/6J mice were treated with either clodronate liposomes to deplete peritoneal macrophages or GM-CSF antibody, and challenged by intraperitoneal injection of MSU crystals. Peritoneal lavages were collected and cellular infiltration determined by flow cytometry. Purified resident and MSU crystal-recruited monocyte/macrophages were stimulated *ex vivo* with MSU crystals. The interleukin (IL)-1 β levels in lavage fluids and *ex vivo* assay supernatants were measured. GM-CSF- and macrophage colony-stimulating factor (M-CSF)-derived macrophages were generated *in vitro* from bone marrow cells. Protein expression of IL-1 β , caspase-1, NOD-like receptor protein (NLRP)-3 and apoptosis-associated speck-like protein containing a CARD (ASC) by *in vitro* and *in vivo*-generated monocyte/macrophages was analyzed by western blot.

Results: Depletion of resident macrophages lowered MSU crystal-induced IL-1 β and GM-CSF levels *in vivo*, and IL-1 β production by MSU crystal-recruited monocytes stimulated *ex vivo*. GM-CSF neutralization *in vivo* decreased MSU crystal-induced IL-1 β levels and neutrophil infiltration. MSU crystal-recruited monocyte/macrophages from GM-CSF-neutralized mice expressed lower levels of the macrophage marker cluster of differentiation (CD)115 and produced less IL-1 β following *ex vivo* stimulation. These monocytes exhibited decreased expression of NLRP-3, pro/active-IL-1 β and pro/active caspase-1. *In vitro*-derived GM-CSF-differentiated macrophages expressed higher levels of NLRP-3, pro/active-IL-1 β and pro/active caspase-1 compared to M-CSF-differentiated macrophages.

Conclusions: GM-CSF plays a key role in the differentiation of MSU crystal-recruited monocytes into pro-inflammatory macrophages. GM-CSF production may therefore contribute towards exacerbating inflammation in gout.

INTRODUCTION

The inflammatory microenvironment plays a significant role in directing the differentiation of recruited monocytes during host defence and inflammation (1-3). This environment varies with the inflammatory trigger resulting in stimulus-dependent development of different inflammatory macrophage phenotypes (4-6). During an acute gout attack, the deposition of monosodium urate (MSU) crystals induces monocyte infiltration into the joint where they are exposed to a highly inflammatory local environment. How this environment contributes to the differentiation of the MSU crystal-recruited inflammatory macrophage phenotype *in vivo* has not been investigated.

Classification of macrophage populations can be difficult due to the innate heterogeneity of the macrophage phenotype. The terms M1 and M2 are applied broadly to describe macrophages with pro- (M1) or anti- (M2) inflammatory phenotypes (6). Experimentally, monocytes/macrophages generated in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) or macrophage colony-stimulating factor (M-CSF) *ex vivo* are skewed towards a M1 and M2 phenotypes respectively (7). Although not yet shown in gout, there is evidence for a role of GM-CSF in joint inflammation in patients with rheumatoid arthritis and research points towards both hemopoietic cells and resident tissue cells as sources of GM-CSF (8, 9).

We have shown previously that MSU crystal-recruited monocytes can differentiate into inflammatory M1-like macrophages *in vivo* that may prolong and exacerbate a gout attack (10). In this study we investigated the impact of GM-CSF on the development of MSU crystal-induced inflammatory macrophages, as well as NOD-like receptor protein (NLRP)-3 inflammasome expression and activation, during MSU crystal-induced inflammation *in vivo*.

MATERIALS AND METHODS

Mice. C57Bl/6J mice were bred and housed at the Malaghan Institute of Medical Research, Wellington, New Zealand. All experimental procedures 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 experiments were male, aged between 8-11 weeks.

Reagents. Uric acid, lipopolysaccharide (LPS), anti- β -actin and all chemicals, unless otherwise stated, were from Sigma (Auckland, New Zealand). Cytokine Bead Array kit and allophycocyanin (APC)-conjugated anti-Gr-1 were from BD Biosciences (North Ryde, New South Wales, Australia). Phycoerythrin (PE)-conjugated anti-mouse F4/80, anti-cluster of differentiation (CD)206 and fluorescein isothiocyanate (FITC)-conjugated 7/4 antibodies were from Serotec (Oxford, UK). PerCP-Cy5.5 anti-CD14, FITC anti-major histocompatibility complex (MHC)II, Pacific blue anti-Gr-1, PE-Cy-7 anti-CD11c, APC-Cy7 anti-F4/80, Alexafluor700 anti-Ly6C and APC-anti-CD11b antibodies were from BioLegend (San Diego, CA, USA). Bio-Plex multiplex arrays, DC Lowry protein assay kit and polyvinylidene fluoride (PVDF) membranes were from Bio-Rad (Hercules, CA, USA). Anti-apoptosis-associated speck-like protein containing a CARD (ASC)/TMB1 was from Novus Biologicals, (Littleton, CO, USA), interleukin (IL)-1 β antibody was from BioVision Inc. (San Francisco, CA, USA), NLRP-3 antibody was from AbCam (Cambridge, UK) and caspase-1/ICE antibody was from Life Technologies (Auckland, New Zealand). Horseradish peroxidase (HRP) -conjugated anti-mouse and anti-rabbit IgG antibodies were from SantaCruz Biotechnology (Dallas, TX, USA). Enhanced chemiluminescence (ECL) detection kits were from Perkin Elmer (Waltham, MA, USA). Protease inhibitor cocktail (EDTA-free) was from Roche (Basel, Switzerland). Murine IL-1 β and GM-CSF enzyme-linked immunosorbent assay (ELISA) kits were from R&D Systems (Minneapolis, MN, USA). *Limulus* amoebocyte cell lysate (LAL) assay kit was from Associates of Cape Cod (East Falmouth, MA, USA). Heparin was from Mayne

Pharma (Melbourne, Vic, Australia). Lympholyte[®]-M cell separation media was from Cedarlane Laboratories Ltd. (Hornby, Canada). Recombinant murine GM-CSF and M-CSF were from PeproTech (Rocky Hill, CT, USA). GM-CSF neutralizing monoclonal antibody (22E9) was from DNAX, (Palo Alto, CA, USA). Unless otherwise stated, all other products were from Life Technologies (Auckland, New Zealand).

Preparation of MSU crystals. MSU crystals were prepared as previously described (11). MSU crystals were needle-like, 5-20 μm , displayed optical birefringence under polarized light and were endotoxin-free (<0.01 EU/10mg, LAL assay).

Murine model of MSU crystal-induced peritonitis. C57Bl/6J mice received an intraperitoneal (i.p) injection of MSU crystals (3 mg, 0.5 ml Dulbecco's Phosphate Buffered Saline-D-PBS). At different time-points, mice were euthanased (CO_2 asphyxiation) and the peritoneal cells harvested by lavage (3 ml D-PBS, 25units/ml heparin). Total cell numbers were counted, peritoneal lavage fluid was centrifuged and the supernatants stored at -80°C . The peritoneal cells were retained for further analysis.

Clodronate liposome depletion of peritoneal resident macrophages. Clodronate liposomes were prepared as previously described (12). To deplete resident peritoneal macrophages, mice were injected i.p. with clodronate liposomes (200 μl , PBS). Macrophage depletion ($>95\%$) was confirmed by flow cytometry at day 3-post treatment as previously described (11, 13).

Purification of peritoneal monocyte/macrophages. Peritoneal lavage cells were pooled by treatment group and washed twice in D-PBS. Cell suspensions were enriched for monocyte/macrophages using Lympholyte[®]-M cell separation media as per manufacturer's instructions. Cell suspensions were $>95\%$ monocytes as determined by flow cytometry.

***Ex vivo* restimulation assay** (11). Purified peritoneal monocyte/macrophages (5×10^5 cells/ml, Roswell Park Memorial Institute medium 1640, 10% fetal calf serum) were stimulated with MSU crystals (500 $\mu\text{g/ml}$, 8 hours, 37°C). Supernatants were collected and stored at -80°C .

● Monocyte/macrophages were recovered for protein extraction.

Generation of bone marrow-derived GM-CSF and M-CSF macrophages. Bone marrow cells were flushed (Dulbecco Modified Eagle's Medium - DMEM) from the hind femur of naïve C57Bl/6J mice. Cells were cultured overnight (1×10^6 cells/ml, DMEM, 10% heat-inactivated fetal bovine serum, 2 mM glutamax, 100 units/ml penicillin-streptomycin). The non-adherent cells were cultured (1×10^6 cells/ml, DMEM) for six days in the presence of either 1000 units/ml GM-CSF or 5000 units/ml M-CSF, media was refreshed on days three and five. On day seven, the adherent macrophages were harvested and processed for protein extraction.

Protein extraction, gel electrophoresis and western blot analysis. Macrophages were lysed in protein lysis buffer (10 mM hydroxyethyl piperazine ethanesulfonic acid, 1.5 mM MgCl_2 , 10 mM KCl, 10mM Na_2MoO_4 and protease inhibitor cocktail) for 20 minutes on ice. The cell lysate was centrifuged and the supernatants collected, protein concentration was quantified (Bio-Rad DC Lowry protein assay kit) then stored at -80°C for western blot analysis. Protein samples were run on NuPAGE 4-12% Bis-Tris gel and transferred onto a PVDF membrane. Non-specific protein binding was blocked using 2% non-fat milk powder in PBS with 0.1% Tween20. Membranes were incubated with primary antibodies (overnight, 4°C) then incubated with HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (1 hour, room temperature). HRP activity was measured using chemiluminescence signal captured with Carestream GelLogic 4000 PRO imaging system (Rochester NY, USA).

Flow cytometry. Peritoneal cells were washed and resuspended in staining buffer (0.1% bovine serum albumin, 0.01% sodium azide, PBS, pH 7.4). Cells were stained with fluorescent antibodies for the surface markers for CD115, Gr-1, F4/80, clone 7/4, CD14, MHCII, CD11c, Ly6C and CD11b then analyzed by flow cytometry (BD LSRII flow cytometer). Cell populations were identified as neutrophils (CD11b+/Gr-1hi/Ly6Clo/clone7/4hi), resident macrophages (CD11b+/Ly6G-/Ly6Clo/F4/80hi/CD14+), monocytes (CD11b+/Ly6G-/Ly6Chi/F4/80lo/CD14lo) or dendritic cells (CD11b+/CD11c+/MHCII+).

Cytokine analysis. Cytokine levels in peritoneal wash and culture supernatants were measured using Cytokine Bead Array, Bio-Plex multiplex array or ELISA as per manufacturer's instructions.

Statistical analysis. Data was analyzed by either one-way analysis of variance (ANOVA) with Tukey's post-hoc test, two-way ANOVA with Bonferoni post-hoc test or Two-tailed student t-test using Prism v5 software (GraphPad, San Diego, CA).

RESULTS

Effect of resident macrophage depletion on inflammatory function of MSU crystal-recruited monocytes. Analysis of peritoneal lavage fluids following MSU crystal administration showed a significant decrease in the levels of IL-1 β (Figure 1A) and IL-6 (data not shown) in macrophage-depleted compared with non-depleted mice. Purified monocytes from macrophage-depleted mice also produced significantly less IL-1 β following MSU crystal stimulation *ex vivo* (Figure 1B). Western blot analysis of MSU crystal-recruited monocytes confirmed that MSU crystal-recruited monocytes from macrophage-depleted mice expressed lower levels of intracellular pro- and active-IL-1 β (Figure 1C). Macrophage depletion resulted in elevated M-CSF (Figure 1D) in naïve mice consistent with reduced turnover of M-CSF by macrophages (14) but there were no significant differences in M-CSF levels between the naïve and CloLip-treated mice following MSU crystal challenge. There was a reduction in GM-CSF levels in the peritoneal fluid following MSU crystal administration *in vivo* (Figure 1E). These data indicated that, in macrophage-depleted mice, MSU crystal-recruited monocytes differentiate into a less inflammatory macrophage phenotype, possibly due to the reduction in GM-CSF.

Flow cytometry analysis of MSU crystal-recruited cells showed no significant levels of GM-CSF in monocytes, T cells or dendritic cells (data not shown). However, *ex vivo* MSU crystal stimulation of excised peritoneal membrane from naïve mice resulted in increased GM-CSF secretion (Figure 1E). Resident peritoneal macrophages did not exhibit GM-CSF secretion.

Effect of GM-CSF on MSU crystal-recruited monocyte differentiation and NLRP3 inflammasome activity *in vivo*. *In vivo* neutralization of GM-CSF using 22E9 antibody (15) caused a significant reduction in the levels of IL-1 β , the neutrophil chemokine, chemokine (C-X-C motif) ligand (CXCL)-1 and the infiltration of MSU crystal-recruited neutrophils whereas monocyte infiltration was unaffected (Figures 2A-D, Supplementary figure 1). However, MSU crystal-

recruited monocyte/macrophages from GM-CSF-neutralized mice exhibited decreased expression of macrophage surface markers, M-CSF receptor (CD115) and CD14 (Figures 2 E and F) when there was no change in M-CSF levels (data not shown). After *ex vivo* stimulation MSU crystal-recruited monocytes from GM-CSF-depleted mice produced less IL-1 β (Figure 3A). Western blotting showed that monocytes from GM-CSF-neutralized mice had decreased expression of pro/active-IL-1 β and pro/active caspase-1 (Figure 3B - *in vivo*). We also compared the expression of components of the NLRP3 inflammasome and IL-1 β production in macrophages from bone marrow cells differentiated in the presence of GM-CSF or M-CSF. The expression of pro/active-IL-1 β and pro/active caspase-1 was higher in GM-CSF-derived compared to the M-CSF-derived macrophages (Figure 3B - *in vitro*). GM-CSF derived macrophages produced elevated levels of IL-1 β in response to MSU stimulation compared to M-CSF derived macrophages (Figure 3C).

DISCUSSION

Depending on the inflammatory stimulus, recruited monocytes can differentiate into a variety of pro- or anti-inflammatory macrophage phenotypes (4-6). We now show that the development of the pro-inflammatory macrophage phenotype during MSU crystal inflammation is dependent on local production of GM-CSF leading to the upregulation of the NLRP3 inflammasome. This increased NLRP3 inflammasome activity in the MSU-recruited inflammatory macrophage likely contributes to the heightened IL-1 β response observed following MSU crystal rechallenge *in vivo* (10).

Here the peritoneal cavity membrane also appeared to be a source of GM-CSF that could be triggered, like chemokine (C-C motif) ligand (CCL)-2 (13), by direct membrane:crystal interaction. Synoviocytes have been identified as a potential source of local GM-CSF production (8, 9) indicating that different cell populations within the membrane could be sources of GM-CSF. In the peritoneal membrane this may also include both fibroblast and mast cell populations. We also found evidence that GM-CSF was necessary for the *in vitro* amplification of IL-1 β production by recruited monocytes in response to MSU crystals. This is consistent with other studies where GM-CSF and IL-1 β have been shown to positively regulate each other to drive inflammation (8, 9). Although recruitment of neutrophils to the site of inflammation was adversely affected by GM-CSF neutralization, monocyte recruitment was not significantly impaired. This indicates that, as in other studies (13, 15), GM-CSF and IL-1 β production may not play a central role in the early regulation of acute monocyte chemotaxis.

In this study, GM-CSF was shown to be necessary for the subsequent development of the pro-inflammatory macrophage phenotype induced by MSU crystals *in vivo*. After MSU crystal challenge recruited monocytes were exposed to comparable M-CSF profiles regardless of liposome treatment with the only differences being in the levels of GM-CSF. Furthermore, neutralisation of GM-CSF resulted in the differentiation of less inflammatory macrophages following MSU crystal

treatment. In both cases, this is consistent with the local bias towards M2-like, M-CSF-driven macrophage differentiation. These data illustrate a requirement for GM-CSF in the development of pro-inflammatory M1-like macrophages and highlight the importance of the balance between M-CSF and GM-CSF in local tissues with respect to macrophage differentiation during inflammation.

● This would be of particular interest when profiling inflamed joint in patients with gout where a high GM-CSF:M-CSF ratio could support the differentiation of inflammatory macrophages that exacerbate a gout attack.

It has been reported previously that the MSU-recruited macrophage phenotype is significantly more inflammatory and expresses higher levels of inflammasome components than the resident macrophage population (10). Our data now identify GM-CSF, rather than M-CSF, as a principal driver for upregulation of NLRP3, pro-IL1 β and pro-caspase-1 protein transcription and expression by the inflammatory macrophage phenotype *in vivo*. One way in which this might be controlled is through GM-CSF-driven upregulation of CD14 expression, the engagement of which can signal (through toll-like receptors) for pro-IL-1 β transcription and inflammatory “priming” of macrophages.

Together the results of this study identify GM-CSF as a key mediator of the ongoing inflammatory response to MSU crystals where GM-CSF drives recruited monocytes towards an inflammatory macrophage phenotype (Figure 3D), the stimulation of which likely underpins ongoing exacerbation of inflammation in gout attacks due to elevated IL-1 β production. Targeting GM-CSF therefore has the potential to subvert the development of the inflammatory macrophage phenotypes (10) and exacerbation of recurrent gout attacks.

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Figure 1. Depletion of peritoneal resident macrophages abrogates inflammatory macrophage differentiation *in vivo*. Mice were pre-treated with clodronate liposomes (200 μ l, intraperitoneal - i.p.) 3 days prior to monosodium urate (MSU) crystal administration (3 mg, i.p.). **A.** Interleukin (IL)-1 β levels in peritoneal lavage fluid. Purified MSU crystal-recruited monocytes/macrophages were **B.** stimulated *ex vivo* (MSU, 200 μ g/ml, 18 hours) and IL-1 β levels in supernatants measured by enzyme-linked immunosorbent assay (ELISA), **C.** analyzed for pro- and active IL-1 β expression by western blot. **D.** Macrophage colony-stimulating factor (M-CSF) and **E.** Granulocyte-macrophage colony-stimulating factor (GM-CSF) levels in peritoneal fluid from MSU crystal challenged mice were measured by ELISA. * $p < 0.05$; ** $p < 0.005$, **** $p < 0.0001$ two-way analysis of variance (ANOVA) with Bonferroni post-hoc analysis ($n = 4$ per group). **F.** Peritoneal macrophages and membranes from naïve mice were stimulated *ex vivo* (MSU crystals, 200 μ g/ml). GM-CSF levels in culture supernatants were measured by ELISA. ** $p < 0.01$, **** $p < 0.0001$, two-way ANOVA with Bonferroni post-hoc analysis. Results are representative of two independent experiments.

Figure 2. GM-CSF neutralization inhibits MSU crystal-recruited inflammatory macrophage differentiation. Mice were challenged with MSU crystals (3 mg, i.p.) with or without treatment with GM-CSF neutralizing antibody (22E9, 100 μ g) and peritoneal lavage fluid collected for analysis of **A,B** IL-1 β and CXCL1 levels at 4h by ELISA. **C,D** neutrophil and monocyte infiltration **E,F** MSU crystal-recruited monocyte expression of cluster of differentiation (CD)14 and CD115 by flow cytometry at 18h. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA with Tukey's post-hoc analysis ($n = 10$ per group, combined data from two independent experiments). See Figure 1 for other definitions.

Figure 3. GM-CSF drives pro-inflammatory macrophage differentiation and enhances NLRP3 inflammasome upregulation and activation. Mice were challenged with MSU crystals (3 mg, i.p.) in the presence of GM-CSF neutralizing antibody (22E9, 100 µg). Peritoneal monocyte/macrophages were isolated and stimulated *ex vivo* with MSU crystals (200 µg/ml, 18 hours) **A.** IL-1β levels in supernatant were measured by ELISA. **B.** Immunoblots of cell lysates from MSU crystal-recruited monocytes (**in vivo**) and GM-CSF (GMM) and M-CSF (MM) bone marrow-derived macrophages (**in vitro**). Results are representative of two independent experiments. **C.** GMM and MM bone marrow-derived macrophages were stimulated *in vitro* by MSU crystals (200 µg/ml, 18 hours) and IL-1β levels in supernatant were measured by ELISA. **A/C.** *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA with Bonferroni post-hoc analysis. **D.** GM-CSF drives differentiation of MSU crystal-recruited monocytes into pro-inflammatory macrophages via upregulation of components of the NLRP3 inflammasome. See Figure 1 for other definitions.

Supplementary Figure 1. Antibody controls do not affect MSU crystal-induced inflammation or the effect of GM-CSF antibody neutralisation *in vivo*.

Figure 1

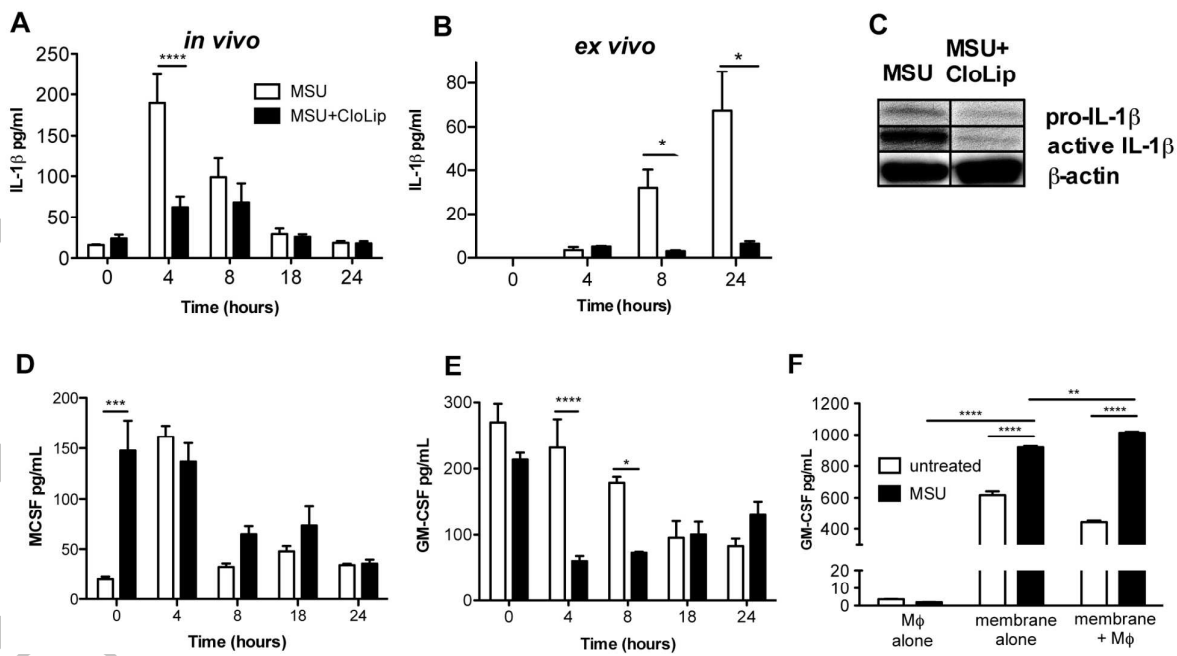


Figure 2

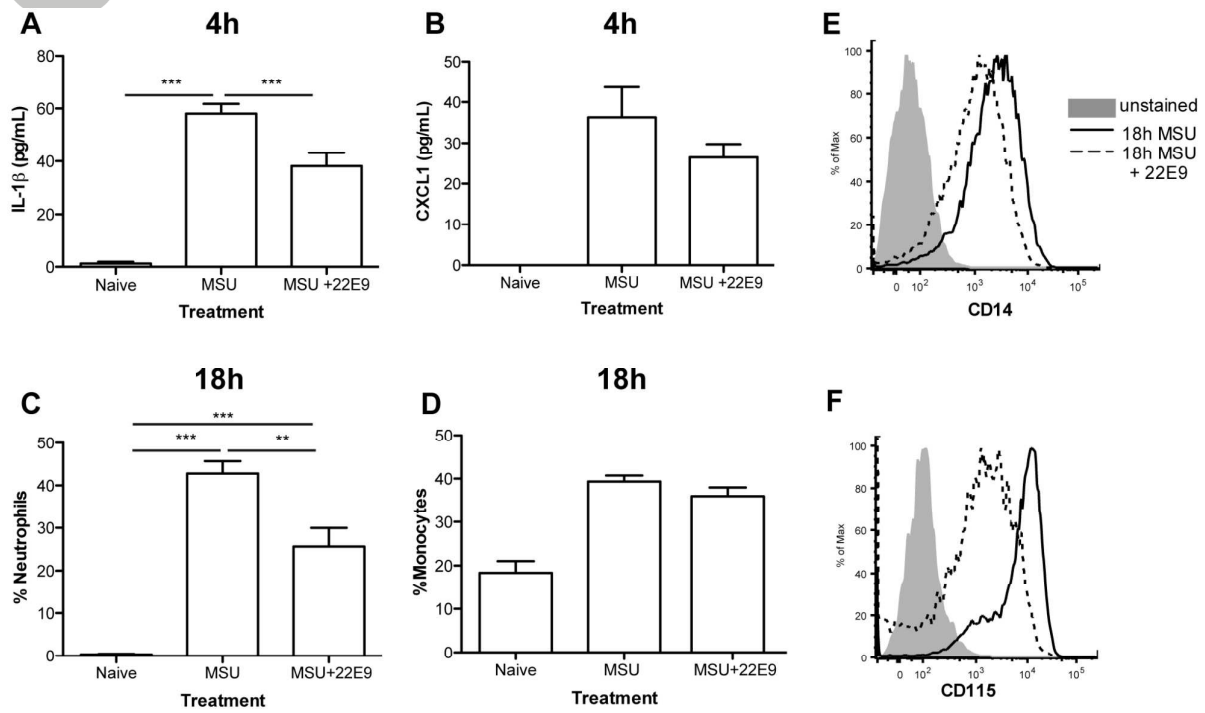


Figure 3

