Aberrant mast cell activation promotes chronic recurrent multifocal osteomyelitis

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Short title: Mast cells promote sterile inflammation

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Abstract

Objective Chronic recurrent multifocal osteomyelitis (CRMO) is an autoinflammatory bone disease that is studied using chronic multifocal osteomyelitis (CMO) mice that develop IL-1β-driven sterile inflammation. The goal of this study was to evaluate the involvement of mast cells in the CMO model and CRMO disease samples.

Methods Histologic and immunostaining of mast cells was performed in CMO and CRMO disease tissues. Genetic ablation of connective tissue mast cells was performed to study their role in CMO disease onset and severity. Immune infiltrates were analyzed using both genetic and pharmacological methods targeting mast cells.

Results Mast cell density was increased in CMO lesions compared to control mice, and genetic ablation of connective tissue mast cells led to significant protection from developing inflammatory bone lesions. Elevated mast cell degranulation and activation was observed in primary cultures of CMO mast cells, and within diseased CMO mice. Treatment of CMO mice with either a mast cell agonist (compound 48/80) or an antagonist (cromolyn) led to altered disease onset and severity. Profiling of human samples from CRMO patients revealed elevated mast cell density in bone biopsies and serum levels of Chymase compared to healthy controls.

Conclusion Together, our results implicate mast cell mediators in promoting sterile inflammation, and rationale for pursuing mast cell-targeted therapies in autoinflammatory diseases.

Keywords chronic recurrent multifocal osteomyelitis, mast cells, sterile inflammation, immune regulation

INTRODUCTION

Chronic recurrent multifocal osteomyelitis (CRMO) is an autoinflammatory disease characterized by sterile osteomyelitis.[1-3] CRMO partially resembles other syndromic autoinflammatory bone diseases, such as deficiency of the interleukin (IL)-1 receptor antagonist (DIRA) or Majeed syndrome.[4 5] CRMO patients with active disease are characterized by elevated pro-inflammatory cytokines (Tumor necrosis factor (TNF)-α, IL-1β, IL-6 and IL-8) in the serum, [6 7] and reduced IL-10 production in peripheral blood mononuclear cells.[8] Reduced IL-10 expression correlates with increased inflammasome activation, IL-1β expression, and release.[9]

Several key features of CRMO are resembled in chronic multifocal osteomyelitis (CMO) mice.[2 10] Although the exact molecular pathophysiology of CMO disease remains unclear, a skewed inflammatory microbiome has been reported to influence the production of IL-1β.[11] CMO mice carry destabilizing homozygous L98P mutations in proline-serine-threonine phosphatase interacting protein 2 (*Pstpip2*).[2,10] Expression of PSTPIP2 is restricted to hematopoietic progenitors, and innate immune cells.[10 12-14] PSTPIP2 was recently shown to suppress differentiation and effector function of macrophages,[10 15 16] neutrophils,[11 13 14 17] and osteoclasts.[18] This may be explained by PSTPIP2 promoting plasma membrane recruitment of negative regulators of immune cell activation, [19 20] including PEST protein-tyrosine phosphatases, [18 21 22] C-terminal Src kinase (Csk),[22] and phosphatidylinositol-3, 4, 5-trisphosphate 5-phosphatase (SHIP-1).[22] These interactions are enhanced in response to immune cell activation by a variety of receptors. [15 22 23] PSTPIP2-deficient neutrophils show elevated responses to inflammatory stimuli (lipopolysaccharide (LPS), FcR capping, crystalline silica (CS)), [13 22] likely due to increased Src/Ras/Erk,[24 25] and PI3K/Akt signaling.[26 27] However,

PSTPIP2 is also highly expressed in other cells, including mast cells,[10] but no functional studies have been reported to date.

Mast cells are specialized innate immune cells residing near potential portals of entry for pathogens.[28] Mast cell degranulation results in the release of mast cell proteases (Chymase, Tryptase), cytokines/chemokines (TNF-α, CXCL1/KC), and histamine that all promote tissue inflammation, leukocyte recruitment, and blood vessel permeability.[29 30] Mast cell activation also leads to rapid production and release of lipid mediators implicated in leukocyte recruitment and tissue inflammation.[31 32] Studies using transgenic models of connective tissue mast cell (CTMC) ablation implicated key roles of mast cells in allergic skin inflammation and arthritis.[33-35] Hence, mast cells and their mediators may also contribute to inflammatory disorders driven by aberrantly activated innate immune cells, such as CMO/CRMO.

In this study, we demonstrate that mast cells accumulate in inflammatory bone lesions in CMO mice, and that genetic ablation of CTMCs protects from disease. We show that mast cells cultured from CMO mice are aberrantly activated, and that mast cell targeting drugs alter CMO disease onset and severity. Furthermore, mast cells infiltrate inflamed bone tissue of CRMO patients, and mast cell-derived mediators are elevated in the serum of CRMO patients, providing new evidence for mast cell involvement in this autoinflammatory disease.

MATERIALS AND METHODS

Mice

The constitutive CTMC ablation model (Mcpt5-Cre:Rosa-Stop$^{fl/fl}$-Dta)[33] was crossed for 10 generations with *Pstpip2*$^{cmo/cmo}$ (CMO) mice[18] to achieve a uniform BALB/cAnPt background for all (CMO/MC-) mice. All animals were housed at Queen's University Animal Care Services under specific pathogen-free conditions. All procedures were approved by the Queen's University Animal Care committee in accordance with Canadian Council on Animal Care guidelines.

Disease scoring, pharmacological treatments, and preparation of tissue homogenates

Wild-type (WT), CMO and CMO/MC- mice were housed in separate cages and initial appearance of a tail kink or paw deformity was recorded as the age of disease onset. To test the effects of mast cell activation or stabilization on CMO disease, CMO mice were subjected to intraperitoneal injections (100 µl/mouse) every 2-3 days with saline, Compound 48/80 (C48/80, 4 mg/kg, Sigma) or Cromolyn salt (25 mg/kg, Sigma) starting at 4 weeks of age. Male mice were treated for 2 months, and females for 3 months. At endpoint, popliteal lymph nodes and disease tissues (paw, tail) were collected. For tissue cytokine measurements, equal weights of distal tail segments and hind paws were snap frozen, homogenized, and ELISA performed for IL-1β (e-bioscience), TNF-α (e-bioscience), IL-6 (e-bioscience) and CXCL1/KC (R&D Systems).

Lymph node immune profiling

Popliteal lymph nodes from each mouse were collected, weighed, and manually homogenized in PBS-EDTA solution. Cells were counted, resuspended in PBS with sodium azide (0.1%) and BSA (0.5%), and stained with: FITC anti- CD117 (c-KIT, Biolegend), PE anti- FcεRI (Biolegend), FITC anti-I-Ek/RT1-D (MHC II, BioLegend), PE anti-CD8a (BioLegend), PE/Cy5 anti-CD4 (BioLegend), FITC anti-Ly6C (BD Biosciences), PE anti-Ly6G (BD Biosciences), and PE/Cy7 anti-CD11c (BioLegend). Samples were analyzed using a cytomics Fc500 (Beckman Coulter), and FlowJo software (Tree Star), with gating set to exclude dead cells (low forward and side scatter; isotype control antibodies were used to set threshold values).

Human CRMO patient serum and bone biopsy analyses

The cohort of CRMO patient serum samples taken at initial diagnosis was described previously.[7] Chymase levels were measured by ELISA (CUSABIO) at University Hospital Carl Custav Carus in sera from 20 CRMO patients and 21 matched controls. Patients and controls had no records of allergies in their patient records, and all patients and/or their legal guardians gave written informed consent (ethics committees at University of Würzburg and University of Technology Dresden).[7] Formalin-fixed, decalcified, and paraffin-embedded bone biopsy specimen from patients with bacterial osteomyelititis (OM; n=3), early CRMO lesions (n=4), chronic phase CRMO lesions (n=4), or healthy control osteotomies (n=2) were stained with a human Tryptase antibody (Abcam, ab2378, dilution: 1:500) using standard techniques. Nuclei were stained using Hämatoxylin, and Bluing Reagent on the BenchMarkXT staining system (Ventana Medical Systems/Roche). Slides were scanned on the AxioScan Z1 (Zeiss, Germany), and quantified using Zen Blue software (Zeiss, Germany) in 15-25 fields of view (32188.774 μm^2).

Statistical analysis

When comparing three genotypes, nonparametric Kruskal-Wallis one-way ANOVA, followed by a Dunn's multiple comparison test was used. When comparing two genotypes, the Student's t test was used (GraphPad Software, La Jolla, Calif).

RESULTS

CTMCs promote chronic multifocal osteomyelitis

To test the relevance of CTMCs to CMO, we collected tail tissues from wild-type (WT) and CMO mice at 5 months of age, when severe multifocal osteomyelitis develops.[2 13 36] Compared to WT animals, a striking increase in Alcian blue-stained cells were observed in the tail tissue of CMO mice (Fig 1A/B). To test for mast cell activation, plasma was collected and levels of a mouse mast cell Chymase (MCPT1) was measured by ELISA. MCPT1 levels were significantly elevated in plasma from CMO mice as compared to WT animals (Fig 1C). To test the contribution of CTMCs to the development of CMO disease, we employed a transgenic model that results in constitutive ablation of CTMCs (Mcpt5-Cre:Rosa26-Stop$^{fl/fl}$-Diptheria toxin-α).[33] Mice were crossed with *Pstpip2*$^{cmo/cmo}$ (hereafter referred to as CMO),[2] to produce CTMC-deficient CMO animals (hereafter referred to as CMO/MC-; backcrossed to maintain a Balb/c background). The extent of CTMC ablation in skin tissue from CMO/MC- mice was nearly complete, and correlated with reduced edema and inflammation when compared to CMO mice (Supplementary Fig S1). In addition, immunostaining of KIT-positive cells in tail tissue sections confirmed that CTMCs were ablated in CMO/MC- mice, and provided evidence of CTMC activation, based on expression of Transglutaminase-2 (KIT^{+}TGM2^{+} cells)[37] in CMO mice (Supplementary Fig S2). These results suggest a potential role for CTMC activation in CMO disease, and demonstrate the efficacy of mast cell ablation in the CMO/MC- model.

To determine the role of CTMCs in CMO, cohorts of CMO and CMO/MC- littermates were monitored for early symptoms of CMO, including tail kinks or deformities in hind paws. The onset of CMO disease was significantly delayed in CMO/MC- mice, (no disease observed in WT mice) (Fig 1D/E). Further analysis of disease onset times within our cohorts revealed a

difference between sexes, with male CMO mice developing overt disease significantly earlier than female CMO mice (Fig 1F). However, both male and female CMO/MC- cohorts showed a significant delay in disease onset compared to sex-matched CMO mice (Fig 1F).

CTMCs deficiency protects against bone damage in CMO mice

To test the role of CTMCs in CMO disease progression, tail and paw tissues were collected from cohorts of male WT, CMO and CMO/MC- mice at 5 months of age. At the gross tissue level, fewer deformities in the tails and paws were observed in CMO/MC- mice as compared to CMO mice (Fig 2A). Quantification of bone density from the microcomputed tomography (µCT) scans revealed that CMO/MC- mice had significantly fewer and less severe bone lesions compared to CMO mice (Fig 2A/B). This also correlated with fewer TRAP-positive osteoclasts in CMO/MC- compared to CMO tissues (Supplementary Fig S3). These results indicate that CTMCs promote bone inflammation and resulting damage, hallmarks of this autoinflammatory disease.

CTMCs promote inflammatory cytokine production and immune cell infiltration in CMO mice

To characterize the potential contribution of CTMCs to the production of inflammatory cytokines and chemokines in tissues from CMO mice, homogenates were prepared and ELISAs were performed to test for several cytokines and chemokines (IL-1β, TNFα, KC, and IL-6). Consistent with previous studies,[13 36] CMO tissues exhibited increased levels of inflammatory cytokines. Of note, with the exception of IL-6, pro-inflammatory cytokine and chemokine levels

were reduced to normal levels (IL-1β, TNFα) or lower (CXCL1) in CMO/MC- as compared to CMO mice (Supplementary Fig S4A/B). Specifically, CMO/MC- tail tissues had less of the mature form of IL-1β, as measured by immunoblot (Supplementary Fig S4C). Overall, these results implicate a role of CTMCs in tissue inflammation in CMO disease.

Prior studies correlated the severity of CMO with the size of popliteal lymph nodes that "drain" the tail and hind paws.[10 14] Indeed, popliteal lymph node mass was elevated in CMO mice as compared to WT animals, but this expansion was not observed in CMO/MC- mice (Fig 3A). Flow cytometric analyses of immune cell markers in lymph nodes revealed significant reductions in mast cells (FcεRI$^+$/KIT$^+$), inflammatory macrophages (Ly6C$^+$), neutrophils (Ly6G$^+$), dendritic cells (MHCII$^+$/CD11c$^+$), CD4$^+$ and CD8$^+$ T cells in CMO/MC- mice when compared to CMO animals (Fig 3B/C, Supplementary Fig S5). Importantly, immune staining of tail tissue sections with Ly6C and Ly6G markers show similar differences in the bone of CMO/MC- and CMO animals (Supplementary Fig S6). Together, these results strongly suggest a role of CTMCs in the recruitment of inflammatory cells in CMO mice.

Aberrant activation of PSTPIP2-deficient mast cells

Because of the aforementioned increased CTMC density and activation in inflammatory bone lesions of CMO mice *in vivo* (Fig 1A-C, Supplementary Fig S2), we tested for cell autonomous defects in PSTPIP2-deficient mast cells *ex vivo*. Bone marrow-derived mast cell (BMMC) were cultured using IL-3 and SCF, and maturation was measured by surface expression of FcεRI and KIT.[38] After 4 weeks in culture, both WT and CMO BMMCs exhibited similar maturation profiles (Fig 4A). To study mast cell responses to inflammatory stimuli, we analyzed

degranulation of WT and CMO BMMCs treated with calcium ionophore A23187, CS or IL-33. Cells from both genotypes degranulated to similar extent with A23187 treatment, but increased degranulation was observed for CMO BMMCs treated with CS or IL-33 (Fig 4B). To test whether PSTPIP2 deficiency affects the activation of CTMCs, we co-cultured the BMMCs with fibroblasts, and observed elevated degranulation of CMO CTMCs in response to C48/80, CS, and IL-33 compared to WT CTMCs (Fig 4C). To extend these studies to *de novo* mediators, we also treated WT and CMO BMMCs for 18 hours with LPS, CS, or IL-33 and measured release of TNF-α, IL-1β. Interestingly, CMO BMMCs released significantly more TNF-α following CS or IL-33 treatment (Fig 4D). Together, these results provide evidence that PSTPIP2 acts as a previously not known suppressor of CTMC activation.

Pharmacological targeting of mast cells alters bone inflammation in CMO mice

To complement our findings that functionally implicated CTMCs in CMO disease, we tested whether systemic pharmacological suppression of CTMC activation alters disease progression. We selected C48/80 as a CTMC agonist that promotes degranulation, and Cromolyn, a mast cell stabilizer that dampens mediator production. Compared to the saline controls, treatment with C48/80 led to early onset of disease, while Cromolyn treatment led to delayed disease onset in both male and female CMO mice (Fig 5A/B). Furthermore, immune cell infiltrates in the popliteal lymph nodes were increased in the C48/80 group and decreased in the Cromolyn treatment group (Fig 5B/C). Examination of the effects of C48/80 and Cromolyn on osteoclast density in tail tissue sections revealed increased numbers of $TRAP^{+}$ osteoclasts in C48/80 treated, and reduced osteoclasts counts in Cromolyn treated CMO mice (Fig 5D). Together, these findings suggest that mast cell-targeted therapies may reduce sterile bone inflammation.

Mast cell activation in CRMO patients

To test the potential involvement of mast cells in human CRMO, we screened serum from a previously reported cohort of treatment naïve, newly diagnosed CRMO patients, and healthy controls.[7] We tested for mast cell Chymase by ELISA, and detected very low levels of Chymase in 4 of 21 healthy controls, whereas the vast majority of CRMO patients (17 of 20) exhibited detectable serum Chymase levels (Fig 6A). It is worth noting, that no patient in these cohorts had reported allergies. Of note, a comparable increase in serum Chymase levels was observed in oligo juvenile idiopathic arthritis (Oligo JIA) patients (Fig 6A), which is consistent with a recent study implicating mast cells in arthritis disease models.[35] To assess mast cell infiltration to inflamed bone tissue, we performed immunohistochemistry staining of Tryptase-positive mast cells in tissue sections from bone biopsies taken from healthy controls (osteotomies), CRMO patients, or bacterial osteomyelitis (OM) patients. While no mast cells were detected in bone biopsies from healthy individuals, we detected mast cells in CRMO lesions, including early CRMO lesions marked by innate immune infiltrates (Fig 6B/C). Particularly high mast cell counts were detected in chronic CRMO lesions marked by coexisting infiltrates of innate immune cells and lymphocytes (Fig 6B/C). Mast cell counts were also increased in bacterial OM bone biopsies when compared to controls, but failed to reach statistical significance (Fig 6B/C). Together with aforementioned data from CMO mice, these results suggest an involvement of mast cells in human CRMO (Fig 6D).

DISCUSSION

Studies in CMO and related mouse models helped to gain insights into the pathophysiology of human CRMO. This includes the identification of a skewed microbiome, increased IL-1β production, and aberrantly activated innate immune cells.[10 11 13 18 36] In the study presented here, we show that mast cells accumulate in CMO lesions, contribute to bone inflammation, and that mast cell-directed therapies can alter disease courses. By crossing CMO mice with CTMC deficient animals,[33] we provide evidence for CTMCs promoting CMO disease onset and severity. Furthermore, we identify PSTPIP2 as a suppressor of mast cell mediator release in response to sterile inflammatory stimuli. In fact, the extent of CMO disease can be altered by mast cell agonists or antagonists. Lastly, we translate our observations in CMO mice to human CRMO, by providing evidence for the presence of mast cells in bone biopsies from CRMO patients that coincide with elevated levels of mast cell chymase in the serum. Together, these findings implicate mast cells in promoting bone inflammation in CMO mice, and suggest a role for mast cells in the pathophysiology of CRMO in humans.

We also report that female mice show a significant delay in CMO disease onset. This may be due to the anti-inflammatory effects of estrogen in mice,[39] that has also been reported in women after menopause.[40] In a recent study of cross-talk between mast cells and osteoclasts, estradiol treatment of mast cells suppressed degranulation and osteoclast activation.[41] In fact, our study provides further *in vivo* evidence that mast cell activation alters the density of osteoclasts and the extent of bone loss in the CMO model. While beyond the scope of this project, future studies are warranted to fully dissect the potential cross-talk between mast cells, osteoclasts and other relevant cell types in CRMO and related diseases.

Although our study provides first evidence for an involvement of mast cells in CMO/CRMO, future studies will be needed to identity the particular mast cell mediators that are most relevant. Since CMO mice are protected on either IL-1R-null or IL-1β-null backgrounds,[13 36] mast cells could be one of the sources of IL-1β. Alternatively, reduced IL-1β levels in CMO/MC- mice may also be due to impaired recruitment of IL-1β-producing immune cells. Indeed, draining lymph nodes from CMO/MC- mice exhibited significant reduction in the numbers of inflammatory macrophages and neutrophils when compared to CMO mice. Mast cell proteases released in response to activation may also enhance cleavage of pro-IL-1β in inflamed tissues, based on studies in other models.[42 43] Interestingly, Chymase is elevated in serum from CMO mice and CRMO patients at diagnosis. Thus, Chymase may indeed participate in CMO and CRMO pathophysiology.

A previous study identified a skewed microbiome in CMO mice that may serve as a trigger for disease by priming neutrophils to express IL-1β.[11] Based on the selective loss of CTMCs in our model, with no effect on mucosal mast cells,[33 44] we have not yet tested the mast cell involvement in this skewed microbiome interaction. However, future studies using Cpa3^{Cre} mice, lacking both mucosal and connective tissue mast cells,[45] would be an improved model to test the overall contribution of mast cells to this disease.

To translate findings from our study on the involvement of mast cells in murine CMO to CRMO in humans, we stained bone samples from CRMO patients and detected increased mast cell populations in the chronic CRMO patients. Profiling of CRMO serum biomarkers delivered a number of cytokines and chemokines that were elevated at diagnosis, [6 7] many/some of which can be produced by human mast cells.[46-48] Furthermore, the here reported elevated levels of mast cell Chymase in sera from most CRMO patients provide further evidence for mast cell

activation. This was also true for Oligo JIA patient serum, suggesting a common immune mechanism involving mast cells. Of note, Chymase can directly promote IL-1β production,[49] and other mast cell mediators may contribute to IL-1β-driven disease in CRMO patients.[7 9] Taken together, mast cells may amplify inflammation in CMO mice and CRMO in humans, which makes them a promising candidate in the search for new biomarkers and treatment targets.

In conclusion, we demonstrate a key role for CTMCs in the progression of an autoinflammatory bone disease using mouse models and human CRMO patient samples. We show that aberrant mast cell activation enhances the IL-1β-driven sterile inflammation (Fig 6D). This study provides new insights and promises potential efficacy of mast cell-targeted therapies in autoinflammatory bone disease.

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FIGURE LEGENDS

Figure 1 CTMCs promote CMO disease onset. (**A)** Representative staining of mast cells with Alcian blue in decalcified tail tissue sections from WT and CMO male mice at 5 months of age (20x). Black arrows indicate mast cells (40x). (**B)** Quantitative image analyses of male WT and CMO diseased tail sections; total mast cells/field and percentage of degranulated mast cells. (**C)** Levels of MCPT1 were profiled in plasma from WT (N=3) and CMO (N=6) male mice at 5 months of age by ELISA. (**D)** Kaplan-Meier disease-free survival curve is shown for WT (N=50), CMO (N=172), and CMO/MC- (N=42) mice. (**E)** Graph depicts the average age of disease onset in CMO and CMO/MC- mice. (**F)** Graph depicts the average age of disease onset comparison between males (WT, N=25; CMO, N=16; CMO/MC-, N=17) and females (WT, N=25; CMO, N=24; CMO/MC-, N=26). (*P<0.05, **P<0.01, ***P<0.001)

Figure 2 CTMCs promote bone lesion development in CMO mice. (**A)** Representative images of tails and hind paw tissues, and microCT images of underlying bone lesions in male 5 month old WT, CMO and CMO/MC- male mice are shown. (**B)** Graphs depict quantification of bone volume to total volume ratios from microCT image stacks for tails and hind paws for each cohort (N=6 per group; *P<0.05, **P<0.01).

Figure 3 Impaired immune cell recruitment to popliteal lymph nodes in CTMC-deficient CMO mice. **(A)** Graph depicts the mean mass of popliteal lymph nodes for WT, CMO, and CMO/MC- male mice (N=6/group). (**B)** Graph depicts the numbers of mast cells ($Fc\varepsilon RI^{+}/KIT^{+}$) in popliteal lymph nodes from WT, CMO and CMO/MC- male mice based on flow cytometry (N=6/group).

(**C)** Graphs depict the numbers of MHCII+, CD4+, CD8a+, Ly6G+, Ly6C+, MHCII+CD11c+ cells in popliteal lymph nodes from WT, CMO and CMO/MC- male mice based on by flow cytometry (N=6-21/group; results pooled from repeated experiments). (*P<0.05, **P<0.01, ***P<0.001).

Figure 4 PSTPIP2 deficiency promotes mast cell maturation, degranulation and inflammatory mediator release. **(A)** Representative flow cytometry histograms are shown for WT and CMO BMMC cultures following staining for surface markers FcεRI and KIT after 4 weeks in culture with SCF and IL-3. (**B)** Degranulation was measured using β-hexosaminidase release assay for 3 independent WT and CMO cultures treated with or without calcium ionophore A23187 (Ca Ion; 5 μM), crystalline silica (CS; 100 μg/cm^2), or IL-33 (10 ng/ml). (**C)** Mature BMMCs were co-cultured with Swiss 3T3 cell line for 2 weeks to induce differentiation to connective tissue mast cells. Degranulation was measured using β-hexosaminidase release assay for 4 independent WT (N=4) and CMO (N=4) cultures treated with or without calcium ionophore A23187 (Ca Ion; 5 μM), crystalline silica (CS; 100 μg/cm^2), IL-33 (10 ng/ml), or C48/80 (30 μg/ml). (**D)** WT and CMO BMMCs (N =5/group) were treated with or without LPS (1 μM), CS (100 μg/cm^2), or IL-33 (10 ng/ml) for 18 hours, and conditioned media subjected to ELISA for TNFα. (n.s.=not significant; *P<0.05, **P<0.01, ***P<0.001)

Figure 5. Mast cell targeted drug intervention can alter CMO disease progression. **(A)** Young CMO mice (4 weeks) were subjected to intraperitoneal injections with saline, C48/80 (5mg/kg), or Cromolyn (25mg/kg) every 2-3 days. The graphs show the disease onset times for male CMO

mice and female CMO mice. (**B)** Graphs depict the mean mass of popliteal lymph nodes from CMO mice post injection protocol. (**C)** Graphs depict the numbers of Ly6G+, Ly6C+, cells in popliteal lymph nodes from CMO mice post injection protocol based on by flow cytometry. (**D)** Representative histological images are shown for decalcified tail tissue sections stained with TRAP and graph depicts quantification of osteoclast populations in CMO male mice post injection protocol based on by flow cytometry. (*P<0.05, **P<0.01, ***P<0.001)

Figure 6. Detection of mast cells and mast cell mediators in CRMO patient samples. (**A)** Serum samples from human patients with CRMO (N=20), Oligo JIA patients (N=20), or from healthy controls (N=21) were tested for the levels of mast cell chymase by ELISA. Graph depicts individual values with median and interquartile range overlayed. (**B)** Representative images of tryptase staining of bone from healthy controlscontrol, early and chronic CRMO patients and infectious osteomyelitis patients. (**C)** Graph depicts the percentage of tryptase-positive mast cells relative to total nucleated cells in the field of view for bone sections from healthy controls, early and chronic lesions from CRMO patients, and bacterial OM patients. (**D)** Graphical Abstract: Model for cross-talk of mast cells with other aberrantly activated immune cells that drive excess IL-1β driven sterile inflammation by osteoclasts (OC), macrophages (MØ), neutrophils (PMN), dendritic cells (DC) in CMO/CRMO. (*P<0.05, n.s.=not significant)

Figure 1

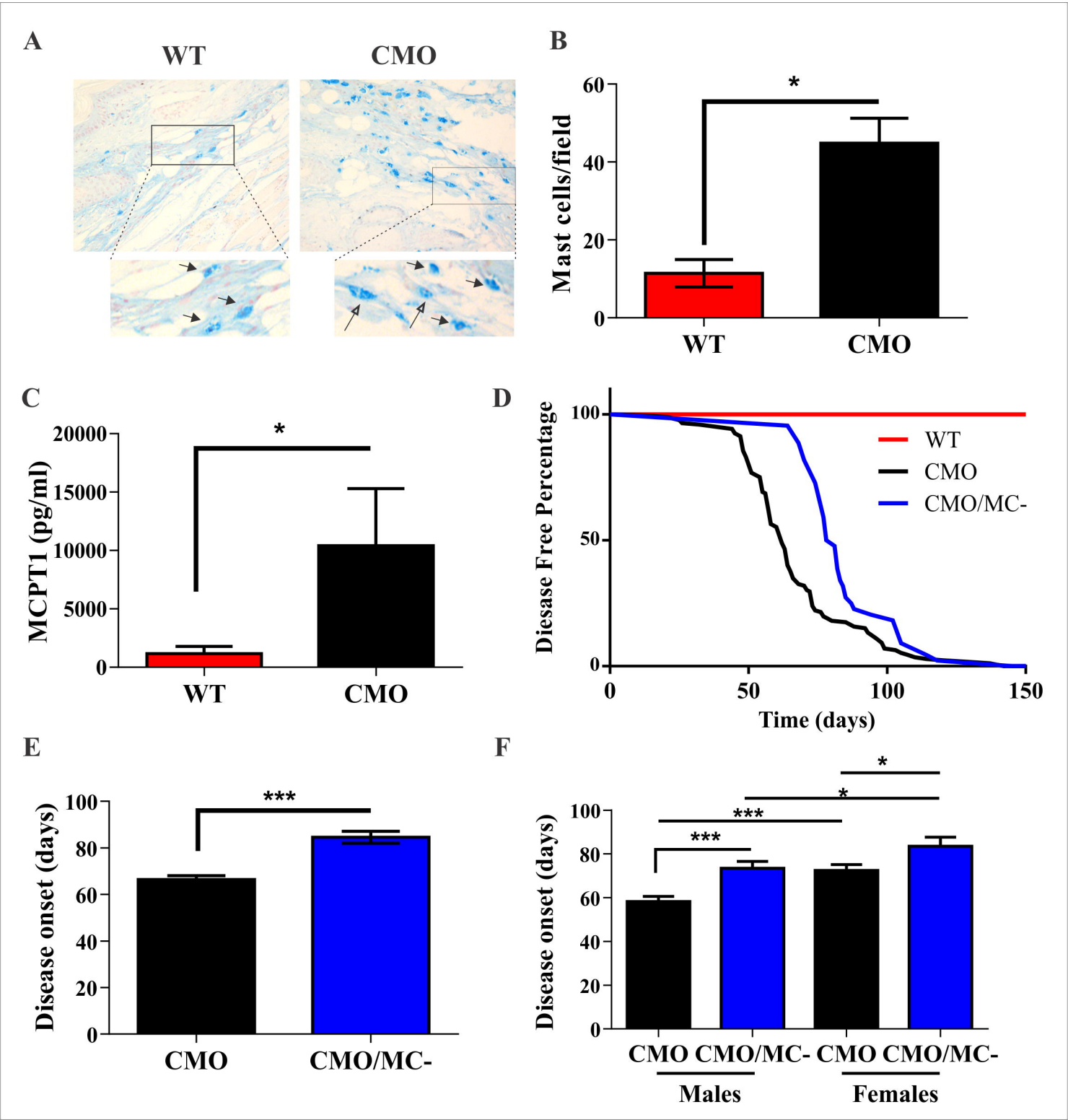

A
WT
CMO
B
Mast cells/field
60
40
20
0
*
WT
CMO
C
MCPT1 (pg/ml)
20000
15000
10000
5000
0
*
WT
CMO
D
Diesase Free Percentage
100
50
0
0
50
100
150
Time (days)
WT
CMO
CMO/MC-
E
Disease onset (days)
100
80
60
40
20
0
***
CMO
CMO/MC-
F
Disease onset (days)
100
80
60
40
20
0
***
***
*
*
CMO
CMO/MC-
CMO
CMO/MC-
Males
Females

Figure 2

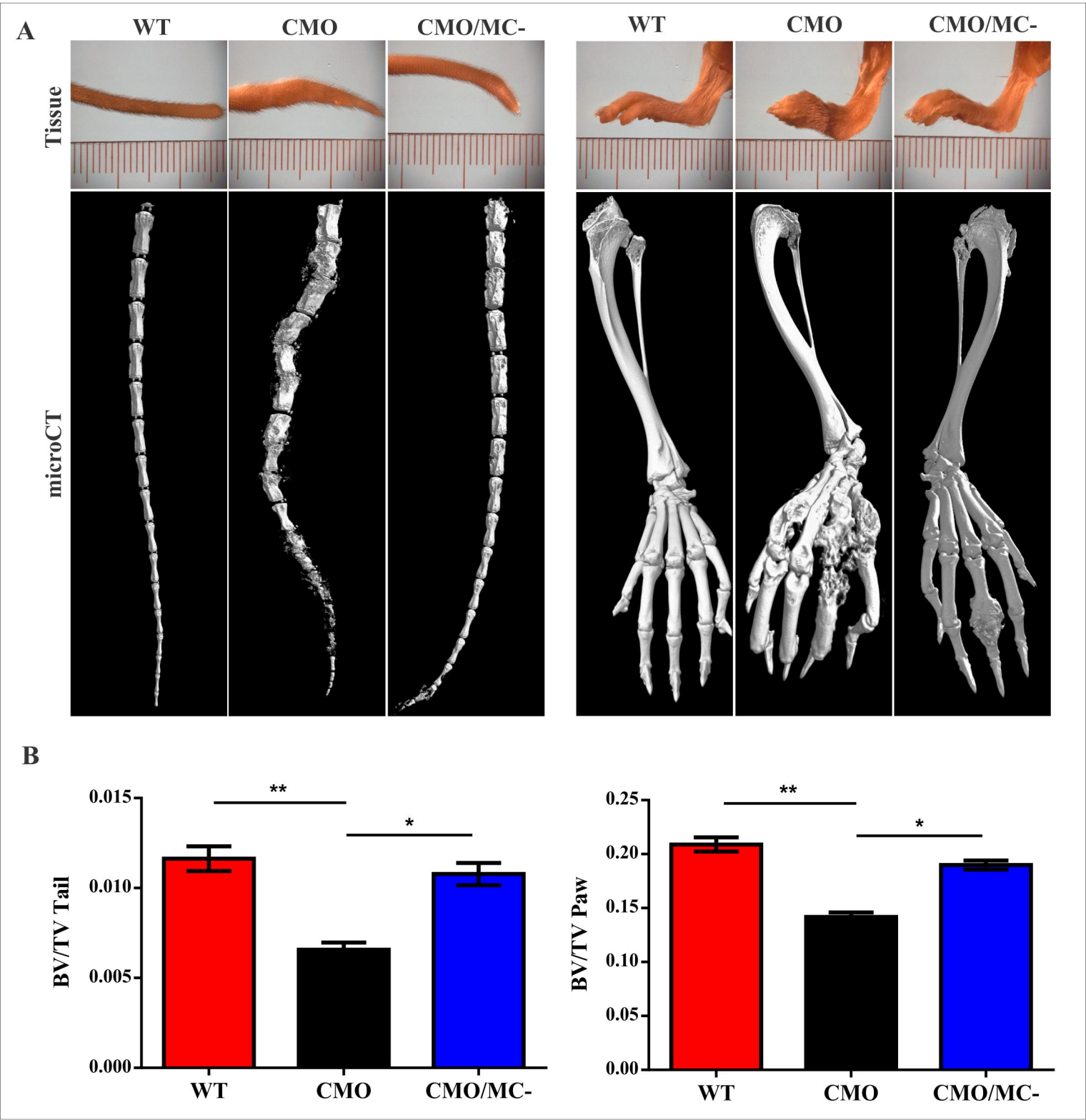

A
WT
CMO
CMO/MC-
WT
CMO
CMO/MC-
Tissue
microCT
B
0.015
0.010
0.005
0.000
BV/TV Tail
**
*
WT
CMO
CMO/MC-
0.25
0.20
0.15
0.10
0.05
0.00
BV/TV Paw
**
*
WT
CMO
CMO/MC-

Figure 3

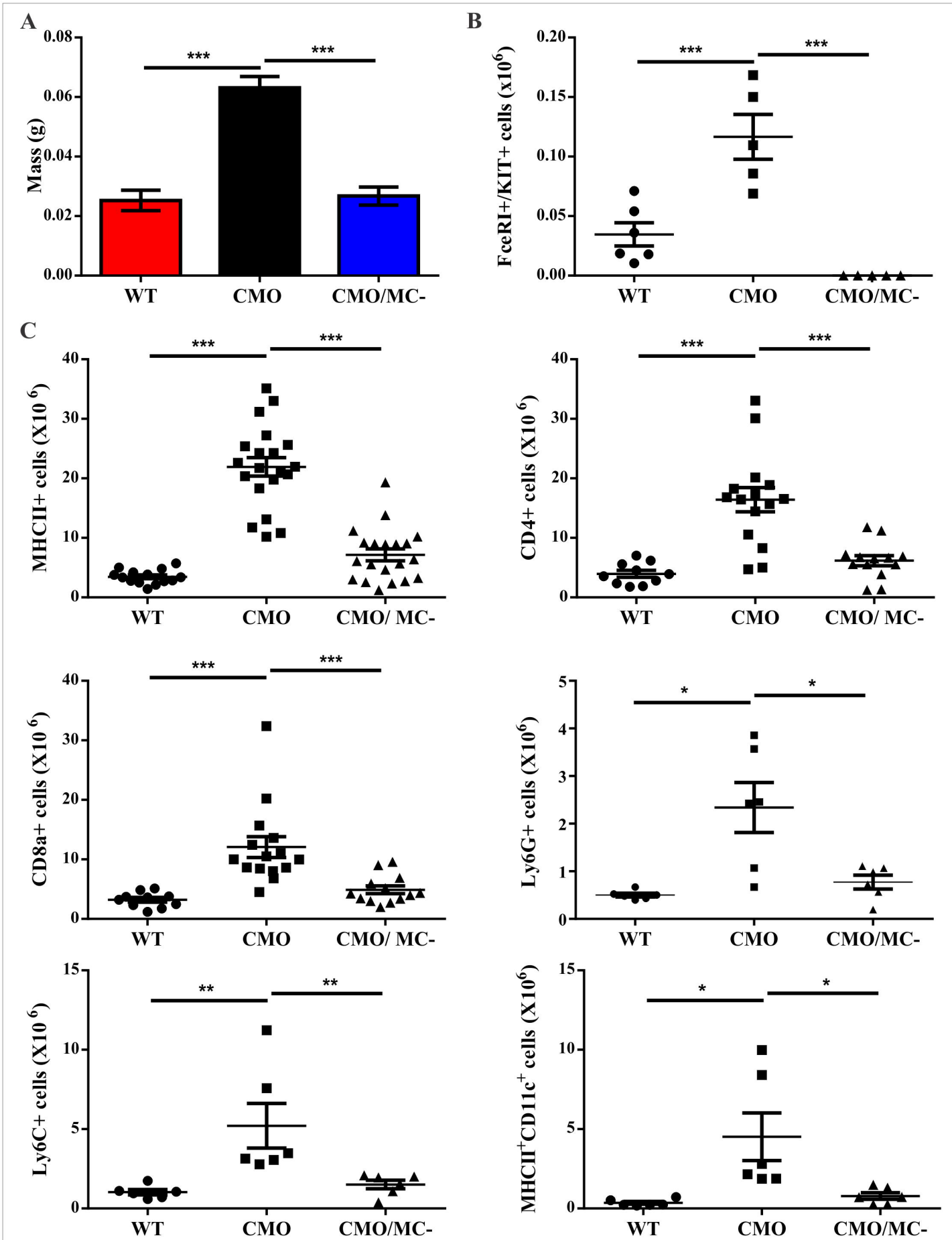

A
Mass (g)
0.08
0.06
0.04
0.02
0.00
***
***
WT
CMO
CMO/MC-
B
FceRI+/KIT+ cells (x10^6)
0.20
0.15
0.10
0.05
0.00
***
***
WT
CMO
CMO/MC-
C
MHCII+ cells (X10 6)
40
30
20
10
0
***
***
WT
CMO
CMO/ MC-
CD4+ cells (X10 6)
40
30
20
10
0
***
***
WT
CMO
CMO/ MC-
CD8a+ cells (X10 6)
40
30
20
10
0
***
***
WT
CMO
CMO/ MC-
Ly6G+ cells (X10^6)
5
4
3
2
1
0
*
*
WT
CMO
CMO/MC-
Ly6C+ cells (X10 6)
15
10
5
0
**
**
WT
CMO
CMO/MC-
MHCII+CD11c+ cells (X10^6)
15
10
5
0
*
*
WT
CMO
CMO/MC-

Figure 4

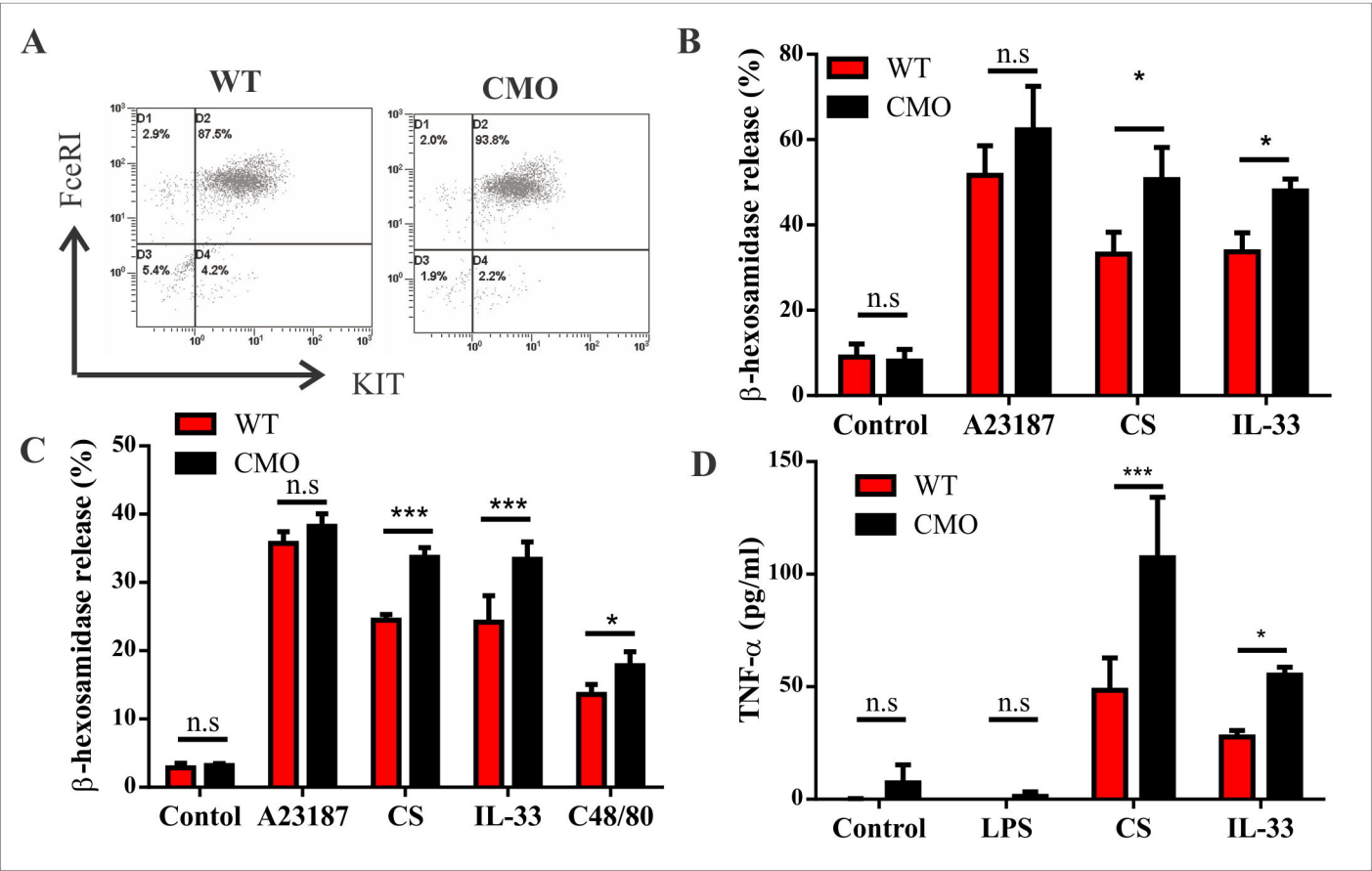

A
WT
CMO
FcεRI
KIT
D1 2.9%
D2 87.5%
D3 5.4%
D4 4.2%
D1 2.0%
D2 93.8%
D3 1.9%
D4 2.2%
B
β-hexosamidase release (%)
WT
CMO
n.s
*
*
n.s
Control
A23187
CS
IL-33
C
β-hexosamidase release (%)
WT
CMO
n.s
***
***
*
n.s
Contol
A23187
CS
IL-33
C48/80
D
TNF-α (pg/ml)
WT
CMO
***
*
n.s
n.s
Control
LPS
CS
IL-33

Figure 5

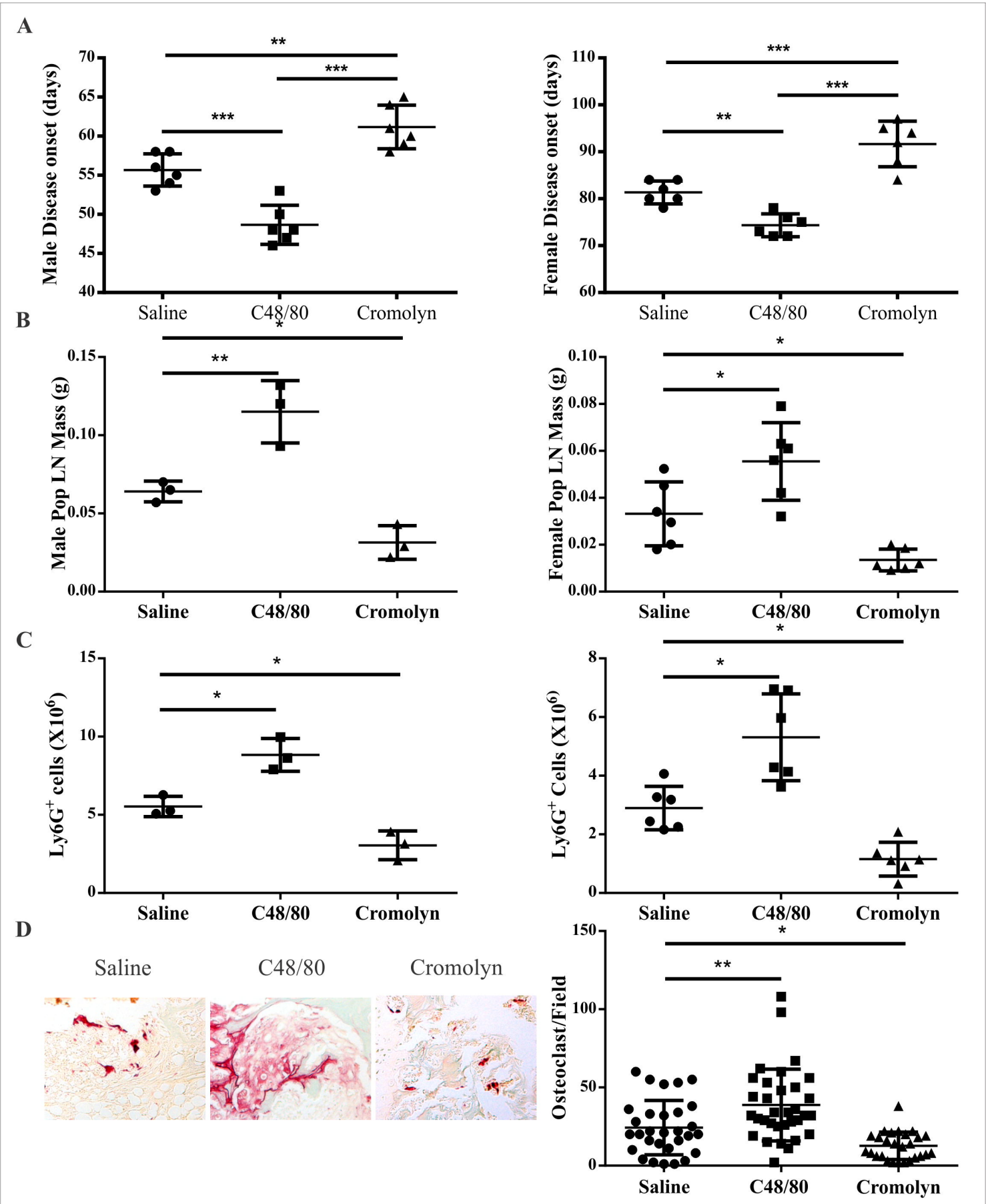
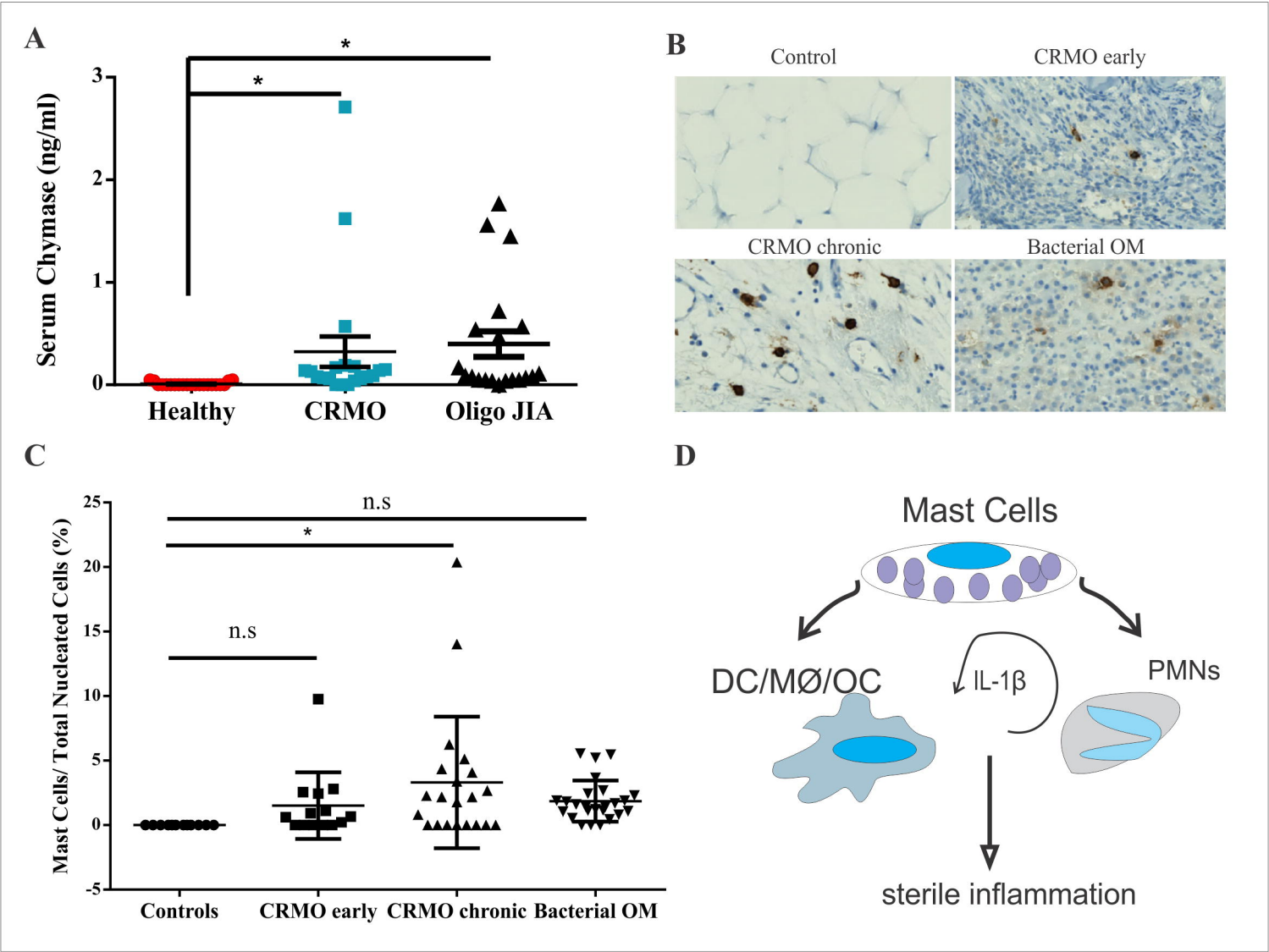

A
Male Disease onset (days)
70
65
60
55
50
45
40
**
***
***
Saline
C48/80
Cromolyn
Female Disease onset (days)
110
100
90
80
70
60
***
***
**
Saline
C48/80
Cromolyn
B
Male Pop LN Mass (g)
0.15
0.10
0.05
0.00
*
**
Saline
C48/80
Cromolyn
Female Pop LN Mass (g)
0.10
0.08
0.06
0.04
0.02
0.00
*
*
Saline
C48/80
Cromolyn
C
Ly6G+ cells (X10^6)
15
10
5
0
*
*
Saline
C48/80
Cromolyn
Ly6G+ Cells (X10^6)
8
6
4
2
0
*
*
Saline
C48/80
Cromolyn
D
Saline
C48/80
Cromolyn
Osteoclast/Field
150
100
50
0
*
**
Saline
C48/80
Cromolyn

Figure 6


A
Serum Chymase (ng/ml)
3
2
1
0
*
*
Healthy
CRMO
Oligo JIA
B
Control
CRMO early
CRMO chronic
Bacterial OM
C
Mast Cells/ Total Nucleated Cells (%)
25
20
15
10
5
0
-5
n.s
*
n.s
Controls
CRMO early
CRMO chronic
Bacterial OM
D
Mast Cells
DC/MØ/OC
IL-1β
PMNs
sterile inflammation