Synovial Fibroblasts Promote the Expression and Granule Accumulation of Tryptase via Interleukin-33 and Its Receptor ST-2 (IL1RL1)*§

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A characteristic feature of tissue resident human mast cells (MCs) is their hTryptase-β-rich cytoplasmic granules. Mouse MC protease-6 (mMCP-6) is the ortholog of hTryptase-β, and we have shown that this tetramer-forming tryptase has beneficial roles in innate immunity but adverse roles in inflammatory disorders like experimental arthritis. Because the key tissue factors that control tryptase expression in MCs have not been identified, we investigated the mechanisms by which fibroblasts mediate the expression and granule accumulation of mMCP-6. Immature mouse bone marrow-derived MCs (mBMMCs) co-cultured with fibroblast-like synoviocytes (FLS) or mouse 3T3 fibroblasts markedly increased their levels of mMCP-6. This effect was caused by an undefined soluble factor whose levels could be increased by exposing FLS to tumor necrosis factor-α or interleukin (IL)-1β. Gene expression profiling of mBMMCs and FLS for receptor-ligand pairs of potential relevance raised the possibility that IL-33 was a sought after fibroblast-derived factor that promotes tryptase expression and granule maturation via its receptor IL1RL1/ST2. MCs lacking IL1RL1 exhibited defective fibroblast-driven tryptase accumulation, whereas recombinant IL-33 induced mMCP-6 mRNA and protein accumulation in wild-type mBMMCs. In agreement with these data, synovial MCs from IL1RL1-null mice exhibited a marked reduction in mMCP-6 expression. IL-33 is the first factor shown to modulate tryptase expression in MCs at the mRNA and protein levels. We therefore identified a novel pathway by which mesenchymal cells exposed to inflammatory cytokines modulate the phenotype of local MCs to shape their immune responses.

Mast cells (MCs) are granulated cells of the myeloid lineage that reside within connective tissues (1). Although it has been known for some time that MCs complete their differentiation and granule maturation after they exit the bone marrow (2–4), the factors and mechanisms governing the final stages of their development remain poorly understood at the molecular level. Although it was originally proposed that the phenotype of a mature MC was irreversibly determined before its progenitor exits the bone marrow, it is now known that human and mouse MCs exhibit substantial plasticity in their development and that MCs can quickly alter the expression of their granule mediators in a cytokine-dependent manner (2, 5–9).

All human MCs contain abundant amounts of hTryptase-β (10–12), which is a tetramer-forming serine protease with tryptic-like substrate specificity (13). The ortholog of hTryptase-β is mouse MC protease (mMCP)-6 (14, 15). No human has been identified who lacks MCs, in part, because their tryptase-serglycin proteoglycan complexes are essential for combating bacterial and helminthic infections efficiently (16–18).

In the context of inflammatory arthritis, the number of MCs often increases >10-fold in the chronically inflamed joint (19). Their prominent roles in experimental arthritis and other MC-dependent inflammatory disorders have heightened interest in hTryptase-β and mMCP-6 (20, 21). Neutrophil accumulation and loss of aggrecan proteoglycans from cartilage are markedly reduced in mMCP-6−/− mice relative to (WT) B6 mice in two inflammatory arthritis models (20, 21). These in vivo studies provided the first direct evidence for a prominent involvement of MC-restricted tryptases in arthritis.

Given the growing evidence documenting the functional consequences of MC-restricted tryptases in health and disease, the factors and mechanisms that control the expression and granule accumulation of these neutral proteases need to be identified. Exposure of mouse bone marrow-derived MCs (mBMMCs) to IL-3 results in the transient expression of mMCP-6 mRNA (15, 22). Nevertheless, the amount of enzymatically active mMCP-6 protein in these nontransformed IL-3−/− developed cells is paltry relative to that of the mature MCs of the synovium, skin, and other connective tissues. The key factor that controls tryptase accumulation in MCs apparently does not originate from T cells because tryptase levels are not increased by exposing FLS to interleukin; IL1RL1, IL-1 receptor-like 1; Kitl, kit ligand; mMCP, mouse MC protease; PBS, phosphate-buffered saline; RT-qPCR, real time-quantitative PCR; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; WT, wild type; mBMMC, mouse bone marrow-derived MC.
diminished in the synovial MCs of lymphocyte-deficient mice (23). Although the development of all MCs in vivo is highly dependent on mesenchymal cell-derived kit ligand (Kitl)/stem cell factor (1, 24, 25), exposure of mMBCs to recombinant Kitl does not result in a significant increase in mMCP-6 mRNA and/or protein levels (26). Thus, the most important factor that regulates tryptase expression in tissue MCs remains obscure.

Sympathetic tryptase+ MCs reside in close proximity to fibroblast-like synoviocytes (FLS) (27). Likewise, the tryptase+ MCs in connective tissues are often in direct contact with fibroblasts and other mesenchymal cells. These observations prompted us to reexamine the interactions between MCs and mouse FLS and 3T3 fibroblasts vis-à-vis tryptase expression. We previously noted that in vivo-differentiated rat peritoneal and human lung MCs (as well as in vitro-differentiated mMBCs) tightly adhere to mouse 3T3 fibroblasts (28–30) and rat chondrocytes (31). During co-culture with fibroblasts, immature mMBCs develop a histochemical phenotype that is more similar to that of the mature MCs in the arthritic joint, due in part to increased expression of heparin-containing serglycin proteoglycans (30). The co-cultured mMBCs also undergo granule maturation as evidenced by their marked increased granule accumulation of histamine and the exopeptidase carboxypeptidase A3 (32, 33). In support of these data, the cell granules become electron-dense at the ultrastructural level (34). The accumulated data led us to hypothesize that the FLS in synovial tissue elaborate a factor other than Kitl that is essential for inducing MC granule maturation into tryptase+ cells that resemble those in many connective tissues.

Here, we report that mouse FLS and 3T3 fibroblasts induce cultured mMBCs to markedly increase their accumulation of enzymatically active mMCP-6. Although these mesenchymal cells constitutively produce the unknown MC regulatory factor, we discovered that its levels are markedly increased when these cells encounter cytokines that participate prominently in arthritis and other inflammatory disorders. Unexpectedly, we discovered that IL-33, a recently identified cytokine (35, 36), that induces MCs to exocytose a spectrum of inflammatory cytokines and chemokines (37), is an important fibroblast-derived factor in our in vitro system that induces tryptase accumulation. Finally, we show that the relevant receptor on the surface of the mMBCs that recognizes mouse FLS- and 3T3 fibroblast-derived IL-33 is “IL-1 receptor-like 1” (IL1RL1; also known as ST2) (38). Consistent with our in vitro observations, mMCP-6 mRNA levels were significantly reduced in the MCs that reside in the joint tissues of IL1RL1-null mice.

EXPERIMENTAL PROCEDURES

Mice—WT B6 mice were obtained from The Jackson Laboratory. IL1RL1−/− (39) and mMCP-6−null (17, 21) B6 mice have been previously described. Experiments were conducted using animal protocols approved by the Animal Care and Use Committee of the Dana Farber Cancer Institute and Brigham and Women’s Hospital.

mMBC-FLS and mMBC-3T3 Fibroblast Co-culture Systems—IL-3-dependent B6 mMBCs and FLS were generated as described previously (40–42). B6 mMBCs were chosen for these studies because the MCs in B6 mice cannot express the related tetramer-forming tryptase mMCP-7 due to a splice-site mutation in its gene (43, 44), thereby allowing easier interpretation of our enzymatic data. FLS were chosen for our co-culture studies because these cells represent a more physiologic population of nontransformed mesenchymal cells obtained from the ankle joint where mMCP-6 MCs reside. Mouse 3T3 fibroblasts (line TIB 68, American Type Culture Collection, Manassas, VA) also were chosen because we have shown that this cell line induces WT mMBCs to undergo granule maturation and because 3T3 fibroblasts are readily available, thereby allowing others to reproduce and extend our findings.

For 2-week co-culture experiments with FLS, 2 × 10⁴ FLS were seeded into each 24-well plate. Forty-eight h later, 1 × 10⁵ IL-3/Kitl-generated WT mMBCs that had been in culture for more than 8 weeks were seeded into the plates and allowed to directly contact the FLS monolayer. Alternatively, the mMBCs were physically separated from the FLS during the co-culture by placing the mMBCs into the upper chamber of a transwell culture dish with a membrane that contains 0.2-μm pores (Nalge Nunc International, Roskilde, Denmark). Half of the medium was changed every 4 days in these mMBC-FLS co-cultures. Cells were enumerated by cytofluorometric staining, and microbead quantification after adherence to one another and/or the extracellular matrix was disrupted using trypsin or 10 mM EDTA (45). mMBCs were identified by their surface expression of Kit and their unique morphologic features (e.g. presence of intracellular granules). Viability was determined by trypan blue exclusion.

For 3-week co-culture experiments with mouse 3T3 fibroblasts, 5 × 10⁴ 4-week IL-3-generated WT and mMCP-6−/− B6 mMBCs were seeded into 35-mm culture dishes that in each instance contained a confluent monolayer of fibroblasts, as described previously (30). The resulting mMBC-3T3 fibroblast co-cultures were maintained for 3 weeks in the presence of 50% WEHI-3 cell conditioned medium as a source of IL-3. Kitl was not added to the culture medium in these experiments. Because the entire conditioned medium was replaced every other day, only those mMBCs that physically contacted the 3T3 fibroblast cell line were studied at the end of the co-culture in the experiments carried out with these cells.

Cytofluorometry—Cytofluorometric staining was performed as described previously (46). In brief, samples were washed with phosphate-buffered saline (PBS) supplemented with 2% fetal calf serum and then stained with appropriate antibodies and isotype controls. Cells were preincubated with an anti-CD16/CD32 antibody to avoid Fc-receptor-mediated staining. Intracellular staining was performed after fixation and permeabilization with Perm/Cytoperm solution (BD Biosciences), following the manufacturer’s instructions. Cytofluorometric analyses were performed using an FACSDiva cytometer (BD Biosciences). Data were analyzed utilizing the Flow-Jo software package (Tree Star, Ashland, OR). The antibodies used in these experiments were CD117-Alexa Fluor 647 (Caltag, Carlsbad, CA), rat IgG-Alexa Fluor 647 (Caltag), IL1RL1-FITC (MD Bioscience, St. Paul, MN), affinity-purified rabbit anti-mMCP-6 antibody (47), and anti-rabbit-IgG-FITC (Jackson ImmunoResearch, West Grove, PA).
IL-33–IL1RL1-dependent Expression of Tryptase in Mast Cells

Immunochemical Staining of mBMMCs with Anti-mMCP-6 Antibody—The 3-week WT mBMMC fibroblast cocultures were washed with serum-free RPMI 1640 medium and then exposed to trypsin for ~5 min. The detached and separated cells were then placed on glass slides using a standard cytocentrifuge approach (5 min of centrifugation, 500 rpm). Immunochemical staining was performed as described previously (48).

SDS-PAGE Immunoblot Analysis—Mouse FLS were grown to confluence in 6-well plates. In some instances, the resulting cells were stimulated with 5 ng/ml mouse recombinant TNF-α and/or IL-1β (PeproTech, Rocky Hill, NJ) for 24 h before the cytokine-treated cells were placed in lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 5 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture (Sigma)). Lysates were clarified by centrifugation at 9,500 × g for 10 min at 4 °C and then boiled in Laemmli sample buffer for 5 min at 95 °C. The proteins in the lysates were separated by SDS-PAGE using 15% acrylamide gels. After transfer to polyvinylidene difluoride membranes, the resulting protein bands were blocked for 30 min at room temperature with 5% milk proteins in PBS, washed in PBS, and incubated with anti-IL-33 antibody (1:5,000 dilution; MBL, Nagoya, Japan) or anti-β-actin antibody (1:5,000 dilution; BioLegend, San Diego) for 2 h at room temperature. The treated blots were washed three times for 5 min each in PBS and then incubated for 1 h with horseradish peroxidase-conjugated donkey anti-rabbit antibody (1:1,000 dilution; Jackson ImmunoResearch). After another three washes with PBS, the blots were developed using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Waltham, MA). Densitometric analyses were performed by using NIH ImageJ. Each signal was evaluated in comparison with that of β-actin.

Using a similar approach, lysates of WT and mMCP-6-null B6 mBMMCs before and after 3 weeks of co-cultured with mouse 3T3 fibroblasts were subject to SDS-PAGE immunoblot analysis by probing the protein blots with affinity-purified rabbit anti-mMCP-6 antibody directed against residues 160–178 in this tryptase (47).

Tryptase Biochemical Assay—Lysates were prepared from mBMMCs by sonication after normalization of lysate buffer volume based on cell numbers. All culture conditions contained growth factors for mBMMCs with >95% viability based on trypan blue exclusion. Tryptase enzymatic activity was quantified using the chromogenic tryptase/trypsin substrate S-2288 (DiaPharma, Westchester, OH), measuring absorbance at 405 nm after a 0.5–6-h incubation at room temperature. Tryptase activity was expressed as the amount of substrate cleaved relative to a standard curve performed with known amounts of recombinant hTryptase-β (R & D Systems, Minneapolis, MN) or pancreatic trypsin (Sigma).

Microarray Analyses of mBMMCs and 3T3 Fibroblasts—Total RNA was isolated from IL-3-differentiated mMCP-6-null B6 mBMMCs, mMCP-6-null B6 BMMCs, and mMCP-6-null B6 BMMCs (17) were used in these microarray analyses rather than WT B6 mBMMCs because we concluded that the former cells probably would optimally express those surface receptors that control the expression of the tryptase to try to correct for their mMCP-6 deficiency created by our homologous recombination approach. Total RNA also was isolated from 3T3 fibroblasts. In all instances, the extracted RNA was purified using the RNeasy kit (Qiagen, Valencia, CA). The resulting RNA samples were further processed using the recommended protocols, hybridized to mouse 430_2.0 GeneChips™ and read on a GeneChip scanner (Affymetrix, Santa Clara, CA) using GenePix Pro 4.1 software by the Arthritis Microarray Core Facility, Brigham and Women’s Hospital and Harvard Medical School, Boston. Data filtering, transformation, and normalization were performed according to established protocols. Differential expression analyses were performed using GenePattern software from the Broad Institute.

Real Time-Quantitative-PCR (RT-qPCR) Assays—For RT-qPCR assays, total RNA was isolated from mBMMCs, mouse FLS, and ankle joints using the RNeasy minikit (Qiagen). FLS were stimulated with 5 ng/ml mouse recombinant TNF-α and/or IL-1β (PeproTech) for 24 h. To extract total RNA from mouse ankle joints, the skin around the ankle joints was removed, and tissue from the distal tibia to the mid paw was carefully collected to avoid bone marrow contamination. Harvested ankles were immersed in RNAlater (Qiagen) to minimize degradation of RNA. The samples were treated with proteinase K (55 °C, 15 min), and cells were disrupted with Buffer RLT lysis buffer (Qiagen). For the follow-up RT-qPCR assays, purified RNA was converted in each instance into cDNA using Quantitect reverse transcription kit (Qiagen). RT-qPCRs were then performed with SYBR Green Mastermix (SABiosciences) on an Mx3000p PCR machine (Stratagene, La Jolla, CA). Relative expression was calculated using the comparative threshold cycle method. mMCP-6 and IL-33 mRNA levels were then normalized to that of the GAPDH or Kit transcript.

Histomorphometric Enumeration of Synovial MCs—Histomorphometric enumeration of MCs was performed in a blinded fashion as described previously (23). Briefly, synovial MCs were enumerated by counting the number of toluidine blue stained growth factors for mBMMCs with a blinded fashion as described previously (23). Briefly, synovial MCs were enumerated by counting the number of toluidine blue + cells in synovial tissue surrounding ankle and tarsal joints in mid-sagittal hind paw sections. An eyepiece reticle (Leica Microsystems, Wetzlar, Germany) was used to define a unit of 0.04 mm² restricted to within 200 μm of the synovial lining layer.

Statistical Analysis—p values were calculated using Student’s t test in Prism software package 4.00 (GraphPad Software, San Diego). p values smaller than 0.05 were considered significant.

RESULTS

A Soluble FLS-derived Factor Induces Immature mBMMCs to Increase Their Expression of mMCP-6—Using WT B6 mBMMCs (40), we examined whether FLS induced this non-transformed immature population of MCs to undergo granule maturation by measuring their levels of mMCP-6. After 2 weeks of co-culture with primary FLS, the granule content of mMCP-6 protein was quantified by intracellular cytofluorometry. In these studies, co-cultured mBMMCs exhibited a 2.5-fold increase in mMCP-6 protein relative to that of mBMMCs...
Mast cells in fibroblast co-cultures express more mMCP-6 than monocultured cells. A, intracellular cytometric staining of Kit−/− mBMMCs with anti-mMCP-6 antibody. B6 mBMMCs were collected after 14 days of co-culture with FLS (continuous line). Monoculture mBMMCs were maintained in parallel in medium supplemented with IL-3 and Kit (dotted line). Gray shading shows control staining with anti-mMCP-6 antibody in tryptase-null (mMCP-6−/−/mMCP-7−/−) mBMMCs. B, tryptase enzymatic activities in mBMMCs co-cultured with FLS compared with control WT or mMCP-6−/− mBMMCs. As control, tryptase activities in FLS alone are also shown. IL-3 and Kit (10 ng/ml, PeproTech) were added to all culture conditions. Results shown are means ± S.E. of data pooled from three independent experiments. *, p < 0.001 MCs + FLS co-culture versus MC monoculture condition. C, mMCP-6 protein levels in 4-week mBMMCs before and after an additional 3 weeks of co-culture with mouse 3T3 fibroblasts. Lysates were prepared from WT (lane 1) and mMCP-6−/− (lane 2) mBMMCs fibroblast co-cultures and from WT monoculture mBMMCs maintained in parallel (lanes 3–5). Lanes 1 and 2 contain the protein content from −13,500 co-cultured mBMMCs in each instance. Lanes 3–5 contain the protein content from −15,000, 45,000, and 150,000 monocultured mBMMCs, respectively. As can be seen by comparing the data noted in lane 1 with that in lanes 3–5, co-cultured WT mBMMCs contain at least 10-fold more mMCP-6 protein on a per cell basis than non-co-cultured WT mBMMCs. The lack of an immunoreactive band in lane 2 documents the specificity of the anti-mMCP-6 antibody used in the SDS-PAGE immunoblot assay. The heterogeneous nature of the immunoreactive protein recognized by the anti-mMCP-6 antibody in this experiment is likely due to known variable glycosylation of the tryptase (75). Data are representative of two independent experiments. D, immunohistochemical staining with anti-mMCP-6 antibody of WT mBMMCs before (left panel) and after (right panel) co-culture with 3T3 fibroblasts. The alkaline phosphatase detection system confers a red color at sites of antibody binding. The slides were counterstained with hematoxylin. Fibroblasts (arrowheads) and MCs (arrows) in the right panel are labeled for clarity. Original magnification, ×630.

cultured in the absence of FLS (Fig. 1A). The tryptase activity of each sample was then quantified using the trypsin-susceptible chromogenic substrate S-2288. Consistent with the cytofluorometry results (Fig. 1A), 8-week mBMMCs co-cultured with FLS for an additional 2 weeks contained significantly more enzymatically active tryptase than mBMMCs maintained in the absence of FLS (Fig. 1B). In support of these data, the levels of mMCP-6 protein (Fig. 1C) and tryptase enzymatic activity (data not shown) increased over 10-fold (range 10–25-fold, n = 2) when 4-week WT mBMMCs were co-cultured with 3T3 fibroblasts for an additional 3 weeks in the absence of Kitil. In addition, mBMMCs before or after co-culture with fibroblasts were evaluated immunohistochemically for their mMCP-6 content. Consistent with the SDS-PAGE/trypsin biochemical assay data, mBMMCs in co-culture exhibited higher levels of mMCP-6 protein in their cytoplasmic granules than monon cultured cells (Fig. 1D). Although the major tryptase present in B6 mBMMCs is mMCP-6, these cells also express mPrss31/tryptase-γ/transmembrane tryptase (22). To confirm that our tryptase functional measurements were not confounded by mPrss31, we quantified tryptase activity in mMCP-6−/−/mPrss31+/− B6 mBMMCs co-cultured for 3 weeks with 3T3 fibroblasts; we found this activity was below our limit of detection (data not shown). Thus, mMCP-6 is the major tryptase in the secretory granules of the co-cultured B6 mBMMCs.

We extended these studies to assess whether or not the FLS-derived cytokine-like activity that promotes mMCP-6 expression requires direct cell-cell contact. For these studies, WT mBMMCs were allowed to physically contact FLS in the cultures. Alternatively, the two cell types were separated from one another using a porous membrane. Interestingly, mBMMCs that were prevented from contacting FLS using the transwell approach also demonstrated an elevation in tryptase enzymatic activity and mMCP-6 mRNA (Fig. 2, A and B).

Identification of Candidate Fibroblast-derived Soluble Mediators Using Microarray Approaches—It is well known that fibroblasts express Kitil and that this cytokine promotes the development of MCs in vivo via its interaction with the tyrosine kinase receptor Kitil on the surface of the MC-committed progenitor. Based on this example, we hypothesized that a similar cytokine/cytokine receptor mechanism probably promotes the FLS- and 3T3 fibroblast-dependent induction of mMCP-6 expression in mBMMCs. A high throughput microarray approach was therefore used to identify transcripts in mouse 3T3 fibroblasts that encode nearly every known protein, including 229 cytokines, chemokines, hormones, and growth and differentiation factors. We found that the transcripts that encode 59 of these 229 candidate proteins were constitutively expressed in mouse 3T3 fibroblasts at a level that exceeded 1% of the GAPDH and β-actin transcripts (Table 1 and...
A microarray analysis of mRNA from mouse 3T3 fibroblasts maintained in basal medium was carried out using an Affymetrix 430 2.0 GeneChip. The mRNA data for essentially every known protein can be found in supplemental Table 1. Noted below are the relative levels of the 58 most abundant transcripts that encode IL-33 and other candidate biologically active proteins that potentially could induce mBMMCs to increase their expression of mMCP-6. For reference, the arbitrary levels of the GAPDH and β-actin transcripts in these fibroblasts were 11,246–12,601 and 12,118–13,980 units, respectively. The levels of the noted candidate transcripts exceeded an arbitrary selected threshold of 1% of the arbitrary levels of the transcripts that encode GAPDH and β-actin.

**TABLE 1**
Constitutively expressed transcripts in mouse 3T3 fibroblasts that encode IL-33 and other candidate proteins that could regulate mMCP-6 expression in IL-3-developed mBMMCs

| Gene Sym. | Name | GeneID | Affymetrix Probe | mRNA Level |
|-----------|------|--------|-----------------|-------------|
| Cxcl12    | chem. (C-X-C motif) ligand 12  | 20315 | 1448823_at | 15,694 |
| Cxcl11    | chem. (C-X-C motif) ligand 1   | 14825 | 14576448_s_at | 12,238 |
| Cxcl2     | chem. (C-X-C motif) ligand 2   | 20296 | 14203860_at | 12,110 |
| Grf1      | interleukin 18                 | 15192 | 14502873_s_at | 3,246 |
| Grf3      | transforming growth factor-β 1 | 21809 | 14174545_at | 2,581 |
| Prf1      | effect of basic factor F         | 18811 | 17277656_at | 2,295 |
| Vegfa     | vascular endothelial growth factor-A | 23340 | 14518030_a_at | 3,932 |
| Il13r1    | interleukin 13 receptor type 1  | 16030 | 14374671_at | 6,214 |
| Il13r2    | interleukin 13 receptor type 2  | 20306 | 14212226_at | 5,293 |
| Il13r3    | interleukin 13 receptor type 3  | 22339 | 14209079_at | 1,609 |
| Il13r4    | interleukin 13 receptor type 4  | 60102 | 14187167_at | 1,359 |
| Il13r5    | interleukin 13 receptor type 5  | 41215 | 14292197_at | 1,159 |
| Il13r6    | interleukin 13 receptor type 6  | 16323 | 14220527_at | 939 |
| Prf2      | effect of basic factor F         | 17311 | 14461177_at | 668 |
| Vegfc     | vascular endothelial growth factor-B | 22340 | 14518030_a_at | 882 |
| Stc1      | stanniocalcin-1                 | 20855 | 14545046_at | 855 |
| Prf3      | effect of basic factor F         | 18812 | 17277656_at | 2,295 |
| Il14      | interleukin-14                  | 16173 | 14179322_at | 744 |
| Tnf        | tumor necrosis factor           | 14560 | 14240076_at | 646 |
| Il13      | interleukin-13                  | 77125 | 14162600_at | 660 |
| Tnf        | tumor necrosis factor           | 57266 | 14184564_a_at | 631 |
| Tnf       | tumor necrosis factor           | 21784 | 14416050_at | 622 |
| Il13      | interleukin-13                  | 16324 | 14288586_at | 605 |
| Tnf        | tumor necrosis factor           | 21943 | 14100603_at | 604 |
| Prf5      | effect of basic factor F         | 18619 | 14270375_at | 560 |
| Tgfb1     | transforming growth factor β-1  | 21608 | 14502621_at | 490 |
| Tgfb2     | transforming growth factor β-2  | 21603 | 14206063_at | 472 |
| Tgfb3     | transforming growth factor β-3  | 46454 | 14221766_at | 406 |
| Ilg5      | interleukin-15                  | 18049 | 14169763_at | 304 |
| Bdnf      | brain-derived neurotrophic factor | 12064 | 14221618_at | 368 |
| Cxcl2     | chem. (C-X-C motif) ligand 2    | 20307 | 14178167_at | 364 |
| Hbega     | heparin-binding EGF             | 15200 | 14183509_at | 325 |
| Cxcl20    | chem. (C-X-C motif) ligand 20   | 20297 | 14222029_at | 318 |
| Cxcl10    | chem. (C-X-C motif) ligand 10   | 19545 | 14186930_at | 296 |
| Edn1      | endothelin 1                    | 13614 | 14592406_s_at | 269 |
| Inha      | inhibin alpha                   | 16322 | 14227275_at | 250 |
| Lei       | leukemia inhibitory factor      | 16878 | 14212018_at | 215 |
| Tnf       | tumor necrosis factor           | 21542 | 14222002_at | 202 |
| Stc2      | stanniocalcin-2                 | 20855 | 14451860_at | 201 |
| Flg       | fibroblast growth factor-9      | 14189 | 14207990_at | 190 |
| Cxcl28    | chem. (C-X-C motif) ligand 28   | 58638 | 14175680_at | 183 |
| Il13      | interleukin-13                  | 16333 | 14244274_at | 148 |
| Agt       | agiotensinogen                  | 11806 | 14233967_at | 148 |

*A* Because the 1427760_s_at probe set on the GeneChip recognizes Prf2c2, Prf2c3, Prf2c4, and Prf2c5, it remains to be determined which prolactin family member is constitutively expressed in the fibroblasts.

**TABLE 2**
Constitutively expressed transcripts in IL-3-developed mBMMCs that encode receptors that recognize the fibroblast-derived factors noted in Table 1

A microarray analysis of IL-3-developed mBMMCs was carried out using an Affymetrix 430 2.0 GeneChip. The mRNA levels in this MC population that encode essentially every mouse protein can be found in supplemental Table 2. Noted below are the mRNA levels of the five most abundant transcripts in mBMMCs that encode the receptors that recognize the fibroblast-derived biologically active proteins/ligands noted in Table 1. For reference, the arbitrary levels of the GAPDH transcript in these cells were 1600–5597 units.

| mBMMC Receptor/Fibroblast Ligand | Receptor mRNA Level (Gene Symbols) |
|-----------------------------------|----------------------------------|
| **IL1RL1/LIL3**                   | 5600                            |
| Kit/Kitl                          | 1525                            |
| Tgfbr1/Tgfbr3                     | 421                             |
| Ccr1/Ccl7                        | 173                             |
| Ccr1/Ccl9                        | 173                             |
| Ccr1/Ccl5                        | 173                             |
| Bmp2r/Bmp10 & Bmp3b              | 139                             |
| Bmp2r/Bmp4                       | 139                             |

**Confirmation That FLS Express IL-33 and That mBMMCs Express IL-1RL1** —Having identified IL-33 as a potential candidate protein by which 3T3 fibroblasts might promote MC expression of mMCP-6, we proceeded to confirm the presence of this cytokine and its receptor in our co-cultured mBMMCs. The IL-33 receptor, IL1RL1, was the first to be identified in this population of MCs actually were greater than the levels of the Kit and GAPDH transcripts. Thus, the IL-33-IL1RL1 cytokine-cytokine receptor pair emerged as a prominent candidate signaling pathway for one of the soluble mediators elaborated by FLS and 3T3 fibroblasts that modulate mMCP-6 expression. Of note, others demonstrated a role for TGF-β-like cytokines in modulating mMCP-6 mRNA levels (49). Indeed, our studies revealed the expression of TGF-β3 and its receptor in FLS and mBMMCs, respectively (Tables 1 and 2). However, given the substantially higher mRNA expression level of the IL1RL1 transcript in mBMMCs, we focused our attention on IL-1RL1 and its receptor.

**IL-33-IL1RL1-dependent Expression of Tryptase in Mast Cells**
were stimulated for 24 h with TNF-α or IL1RL1.  

FLS-derived IL-33 Regulates Tryptase Expression in mBMMCs—We next generated IL-3/Kitl driven mBMMCs from IL1RL1+/− mice and WT mice and examined mMCP-6 expression in these cells after they had been co-cultured with FLS. In contrast to WT mBMMCs, IL1RL1+/− mBMMCs exhibited no substantial change in mMCP-6 mRNA levels when co-cultured with FLS (Fig. 4A, left). In confirmatory studies, we examined the tryptase enzymatic activity in IL1RL1+/− mBMMC using the chromogenic assay. Tryptase activity was significantly increased in WT mBMMCs but not in IL1RL1+/− mBMMC in transwell co-culture with FLS (Fig. 4A, right). We next examined whether mouse recombinant IL-33 can directly induce tryptase expression in mBMMCs. For these studies, WT and IL1RL1-null mBMMCs were maintained in the presence of graded concentrations of recombinant IL-33 for 7 days. In these experiments, both mMCP-6 mRNA and functional tryptase were elevated in the WT mBMMCs by IL-33 in a dose-dependent manner (Fig. 4B). As anticipated, IL1RL1+/− mBMMCs were unresponsive to recombinant IL-33 (Fig. 4C).

From a technical standpoint, peripheral blood basophils are responsive to IL-3 and IL-33 (52, 53). Although mouse basophils express the tryptase family member mPrss34, they do not express mMCP-6 (54, 55). Nevertheless, basophils and basophil-committed progenitors are absent in the cell population obtained after unfractonated bone marrow cells are cultured >3 weeks in IL-3 enriched medium. To avoid potential confounding basophils in our studies, we utilized mBMMCs that had been in culture for at least 4 weeks in IL-3-enriched medium with or without Kitl. These findings established that FLS-derived IL-33 contributes to the regulation of mMCP-6 expression in MCs via the IL-33 receptor IL1RL1.

TNF-α and IL-1β Induce FLS to Increase Their Production of IL-33, Resulting in Augmented Tryptase Expression in Co-cultured mBMMCs—TNF-α and IL-1β are cytokines that participate in arthritis and other inflammatory disorders. mMCP-6 also plays a prominent role in two inflammatory arthritis models, promoting both disease intensity and cartilage injury. Having demonstrated a prominent role for the IL-33-IL1RL1 pathway in mMCP-6 induction in mBMMCs when these cells were co-cultured with FLS or 3T3 fibroblasts under basal conditions, we next examined the ability of TNF-α and IL-1β to modulate FLS regulation of the MC phenotype. Our rationale here was agreement with the microarray data (Table 2 and supplemental Table 2). Indeed, IL1RL1 staining intensity was similar to that of the abundantly expressed surface receptor Kit in this assay (Fig. 3B).

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the marked increase in IL-33 mRNA levels observed in FLS when these mesenchymal cells encountered TNF-α and/or IL-1β (Fig. 3A). Indeed, exposure of mBMMCs-FLS cocultures to TNF-α or IL-1β resulted in a prominent up-regulation of tryptase enzymatic activity. In contrast, these proinflammatory cytokines did not induce tryptase expression in mBMMCs in the absence of FLS (Fig. 5A). Additionally, the substantial increase of active tryptase enzymatic activity induced by TNF-α and IL-1β was remarkably abrogated in IL1RL1−/− mBMMCs (Fig. 5B). These observations demonstrate that the ability of FLS to impact the tryptase levels in MCs is augmented by stimuli typically derived from the TNF-α/IL-1β-expressing leukocytes that infiltrate tissues in inflammatory responses or potentially from MCs themselves in a local amplification loop (51).

**Decreased mMCP-6 mRNA Expression in IL1RL1−/− Synovial Tissue MCs—**We next assessed the in vivo contribution of IL-33 to tissue MC tryptase levels. Because our previous studies demonstrated that synovial MCs express mMCP-6 (23), we examined the levels of this tryptase in the joint tissues of WT and IL1RL1−/− mice. Consistent with our in vitro data, mMCP-6 mRNA levels in synovial MCs of IL1RL1−/− mice were significantly decreased compared with that of WT mice (Fig. 6A). Because decreased mMCP-6 expression could result from a decreased tissue density of MCs or from less mMCP-6 expression in the MCs, we employed two approaches to control for decreased MCs as a trivial explanation for our findings. In the first method, we normalized our mMCP-6 mRNA levels to Kit mRNA levels because MCs are the major cell type in the joint that express Kit (Fig. 6A). In an independent approach, we quantified the density of MCs in the joints of WT and IL1RL1−/− mice. In these histomorphometric analyses, we found an equivalent density of MCs in both strains of mice (Fig. 6B).

**DISCUSSION**

In contrast to mucosal tissues, where chymase-expressing MCs predominate, the synovial sublining in mice and humans is studded with tryptase-expressing MC (56). This population of MCs increases in number in inflammatory arthritides. Because the phenotype of the MCs in synovial tissue is maintained in the absence of lymphocytes (23), we explored the mechanisms by which the mesenchymal cells in this tissue modulate MC maturation by examining the ability of FLS and a fibroblast cell line to regulate mMCP-6 expression in mBMMCs. Our initial studies revealed that FLSs and 3T3 fibroblasts express IL-33 and demonstrated that FLSs and 3T3 fibroblasts express IL-33 and IL1RL1-null mBMMCs were used in follow-up experimental approaches to show that this cytokine-receptor signaling pathway does indeed control the expression and granule accumulation of mMCP-6 in MCs in vitro (Fig. 4) and in vivo (Fig. 6). Genetic methods confirmed the contribution of IL-33 in modulating MC tryptase expression in vivo. Thus, these studies provide new insights beyond the biology of Kit/Kitl into mechanisms by which mesenchymal lineages in connective tissues regulate MC maturation.

IL-33 is a member of the IL-1 family of cytokines, and this factor was recently identified as a ligand for the orphan receptor IL1RL1 (36). IL-33 is known to be a potent activator of cytokine production in IL1RL1+ MCs (37, 50, 51, 57–59) as well as basophils (52, 53, 60), Th2 lymphocytes (36, 53), eosinophils...
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It deserves mention that the increase in tryptase expression that occurred when WT mBMMCs were co-cultured with FLS was not completely abrogated if IL1RL1−/− mBMMCs was used (Fig. 4). This finding implies the presence of another soluble factor from FLS and fibroblasts that works in synergy with IL-33 to control mMCP-6 levels in MCs. Others have shown that TGF-β can regulate mMCP-6 mRNA levels in mBMMCs (49). As noted in Tables 1 and 2 and supplemental Tables 1 and 2, FLS constitutively express TGF-β3 and mBMMCs constitutively express its receptor Tgfr1. Thus, TGF-β3 and its receptor are attractive candidates for further investigation in our co-culture system.

In summary, we demonstrate that IL-33 produced by mouse FLS and 3T3 fibroblasts promotes tryptase expression in mBMMCs and that synovium-derived IL-33 regulates mMCP-6 expression in the MCs that reside in joint tissues. Previous insights regarding mesenchymal cell-derived factors that regulate the granule phenotype of an MC have focused primarily on Kit-Kitl interactions. Our observations uncover a novel mechanism by which elaboration of IL-33 in the synovium can modulate MC tryptase expression. These observations delineate a means whereby FLS contribute to joint inflammation via enhancing MC tryptase expression. Our data raise the possibility that IL-33 regulation of IL1RL1+ MCs in other connective tissues contributes to beneficial and adverse roles of tryptases at those sites. MC-restricted tryptases have been implicated in numerous diseases and infections (16, 17). Thus, our observations point to further pathways by which tissues can impact and modulate MC-dependent immunity, inflammation, and connective tissue turnover.

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