

## C5a Receptor Enables Participation of Mast Cells in Immune Complex Arthritis Independently of Fcγ Receptor Modulation

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### **Abstract:**

**Objective:** Mast cells are tissue-resident immune sentinels that are implicated in the pathogenesis of inflammatory joint disease. The aim of this study was to test our hypothesis that complement fragments could be key activators of synovial mast cells in autoimmune arthritis.

**Methods:** In vivo studies used the murine K/BxN arthritis model, a distal symmetric polyarthritis mediated by IgG immune complexes. Expression of C5aR on synovial mast cells was determined by immunohistochemical and functional studies. C5aR<sup>-/-</sup> and control mast cells were engrafted into mast cell-deficient WBB6 F1-Kit<sup>w</sup>/Kit<sup>w-v</sup> (W/W<sup>v</sup>) mice to examine the requirement for this receptor in arthritis. C5aR-dependent activation of mast cells was investigated in C5aR<sup>-/-</sup> animals and in murine and human mast cell cultures.

**Results:** Murine synovial mast cells express functional C5aR. Unlike their wild-type counterparts, C5aR<sup>-/-</sup> mast cells adoptively transferred into W/W<sup>v</sup> mice were not competent to restore arthritis, despite equivalent synovial engraftment. Activation of C5aR<sup>-/-</sup> mast cells by K/BxN serum in vivo remained intact, indicating that C5aR is dispensable for normal IgG-mediated triggering. Consistent with this result, cultured mast cells treated with C5a failed to modulate the expression of Fcγ receptors (FcγR) or to otherwise alter the activation threshold. In human mast cells, C5a promoted the production of the neutrophil chemotaxin interleukin-8, and recruitment of neutrophils at 24 hours after serum administration was impaired in C5aR<sup>-/-</sup> mice, suggesting that enhanced neutrophil chemoattractant production underlies the requirement for C5aR on mast cells in arthritis.

**Conclusion:** Stimulation via C5aR is required to unleash the proinflammatory activity of synovial mast cells in immune complex arthritis, albeit via a mechanism that is distinct from C5a-modulated expression of FcγR.

**Keywords:** mast cells | arthritis | immune complex | C5a

### Article:

The pathogenesis of the idiopathic inflammatory arthritides involves both adaptive and innate immune cells, as well as mesenchymal cell lineages such as the synovial fibroblast. In some of these conditions, including rheumatoid arthritis (RA), strong evidence has accumulated that antibodies play a key role in the translation of impaired immune tolerance to inflammation in the joint. Autoantibodies such as rheumatoid factor and anti-citrullinated peptide antibodies are common in RA, while the joints of seropositive RA patients are encrusted with immune complexes and display marked activation of complement via both classical and alternative pathways (for review, see ref.1).

The effector phase of immune complex-driven arthritis has been modeled in multiple murine systems, including collagen-induced arthritis and K/BxN arthritis. These models share many key features, including a dependence on both IgG Fcγ receptors (FcγR) and complement, as well as effector pathways, such as the proinflammatory cytokine interleukin-1 (IL-1)<sup>(2)</sup>. Multiple innate immune lineages have been implicated in the inflammatory reaction to articular immune complexes in these models, including neutrophils and macrophages<sup>(3,4)</sup>. We and other investigators have demonstrated a role of the synovial mast cell in this process, at least in certain genetic backgrounds<sup>(5-7)</sup>. These hematopoietically derived cells make up ~3% of the cells in the normal synovial sublining, where they take up residence in perivascular and perineural tissues and immediately deep to the synovial lining<sup>(8,9)</sup>. Given their relatively limited numbers, mast cells are thought to act as sentinels, rapidly liberating a broad range of mediators that mobilize adaptive and innate immune responders, including circulating neutrophils. In this way, mast cells have been shown to facilitate resistance to bacterial peritonitis<sup>(10,11)</sup>, and we have hypothesized that they play a similar role in surveillance of the vulnerable synovial space<sup>(9)</sup>.

In immune complex arthritis, this sentinel function becomes maladaptive. In addition to receptors for IgE, mast cells express Fcγ receptors and can be readily activated by IgG immune complexes. K/BxN serum-transfer arthritis is mediated by IgG antibodies that form immune complexes with their autoantigen glucose-6-phosphate isomerase, and several strains of mast cell-deficient mice are resistant to disease induction<sup>(5,6)</sup>. Resistance can be overcome by engraftment with cultured mast cells, confirming that mast cells can play a key role in arthritis susceptibility<sup>(5)</sup>. Recently, we elucidated the mechanisms that underlie this activity, finding that synovial mast cells become activated by immune complex binding to FcγRIII, resulting in the release of IL-1 and potentially of other mediators that “jump start” inflammation within the joint<sup>(12)</sup>.

However, mast cells can express receptors besides Fc $\gamma$ R that are of potential relevance in arthritis. These include receptors for the complement anaphylatoxins C3a and C5a, which are generated through the activation of complement by immune complexes and are readily demonstrable in RA synovial fluid<sup>(1, 13, 14)</sup>. We hypothesized that complement receptors could synergize with Fc $\gamma$ R to promote the proinflammatory activity of the synovial mast cell. Precedent for such an interaction is strong. Rat macrophages activated via both Fc $\gamma$ R and C5a have been shown to produce an expanded repertoire of mediators of inflammation<sup>(15)</sup>. More recently, elegant studies of murine macrophages have shown that C5a can reciprocally modulate the expression of activating (Fc $\gamma$ RIII) and inhibitory (Fc $\gamma$ RII) Fc $\gamma$  receptors, leading to a cellular state of enhanced susceptibility to immune complex activation<sup>(16–19)</sup>. This mechanism has been detailed down to the molecular level, where a 2-bp difference in the promoters for these receptors has been found to account for their differential response to C5aR-initiated intracellular signaling<sup>(20, 21)</sup>.

Modulation of Fc $\gamma$ R expression represents an important mechanism by which the activation of leukocytes that are transiently exposed to immune complexes could be limited<sup>(22)</sup>. However, it remains unclear whether this mechanism is active in all cells that express C5aR and Fc $\gamma$ R. In particular, mast cells acquire a functional phenotype only upon maturation within the tissues and would be expected to have little “incidental” contact with immune complexes. We therefore studied whether complement receptors participate in mast cell effector activity in inflammatory arthritis and whether such participation depends on complement-mediated modulation of the threshold to activation via Fc $\gamma$  receptors.

## **MATERIALS AND METHODS**

### **Mice.**

C5aR<sup>-/-</sup> and C3aR<sup>-/-</sup> mice were derived as described previously<sup>(23, 24)</sup>. WBB6 F1-Kit<sup>w</sup>/Kit<sup>w-v</sup> (W/W<sup>v</sup>), WBB6 littermate controls, C57BL/6J (B6), B6;129S4-Fcgr2b<sup>tm1Ttk</sup>/J (Fc $\gamma$ RII<sup>-/-</sup>), B6.129P2-Fcgr3<sup>tm1Sjv</sup>/J (Fc $\gamma$ RIII<sup>-/-</sup>), and B6;129P2-Fcer1g<sup>tm1Rav</sup>/J (FcR $\gamma$ <sup>-/-</sup>) animals were purchased from The Jackson Laboratory. C57BL/6-Kit<sup>w-sh/w-sh</sup> (Wsh) mice<sup>(25)</sup> were maintained at The Jackson Laboratory. Animals were housed in the specific pathogen-free animal facilities of the Dana-Farber Cancer Institute or Children's Hospital Boston. All procedures were approved by the appropriate animal care and use committees.

### **Antibodies and reagents.**

Antibodies used for cytofluorometric analysis of human cells included phycoerythrin (PE)–conjugated CD16, CD32A/B (clone AT10), and CD64 (Caltag); fluorescein isothiocyanate (FITC)–conjugated C5aR and Alexa Fluor 488–conjugated C3aR (AbD Serotec); and Alexa Fluor 488–conjugated anti-Fc $\gamma$ RIIb (2B6; gift of MacroGenics)<sup>(26)</sup>; and matched isotype controls. Reagents for murine cytofluorometry included anti-C5aR (20/70; gift of J. Zwirner [27]), PE-conjugated anti-Fc $\gamma$ RII/III (2.4G2) and PE–Cy7–conjugated anti-CD45 (BD

PharMingen), carboxyfluorescein-conjugated anti-Fc $\gamma$ RIII (clone 275003; R&D Systems), FITC-conjugated anti-CD11b (Caltag), and Alexa Fluor 647-conjugated Gr-1 (Caltag), as well as matched isotype controls. Unlabeled anti-Fc $\gamma$ RIII was used as a blocking reagent prior to staining with PE-conjugated 2.4G2 to assess surface expression of Fc $\gamma$ RII, as described elsewhere<sup>(21)</sup>. Anti-human Fc $\gamma$ RIIa antibodies were purified from clone IV.3 supernatants (American Type Culture Collection). Rabbit anti-murine C5a receptor antiserum for immunohistochemistry was generated as described previously<sup>(28)</sup>. Recombinant human stem cell factor (SCF) was purchased from Research Diagnostics or PeproTech. Recombinant human C5a, recombinant mouse SCF, and enzyme-linked immunosorbent assay (ELISA) reagents were purchased from R&D Systems. Recombinant mouse IL-3 was purchased from Pierce. Flow cytometry was performed using a FACSDiva cytometer (Becton Dickinson).

### **Cell culture and stimulation.**

Murine bone marrow-derived mast cells (BMMCs) were used following at least 4 weeks of culture in 12.5 ng/ml of recombinant mouse SCF and 10 ng/ml of recombinant mouse IL-3, as described elsewhere<sup>(5)</sup>. To accomplish Fc $\gamma$ RIII-mediated stimulation *in vitro*, lipopolysaccharide (LPS)-free 2.4G2 was coated overnight at 4°C onto a high-binding ELISA plate (Costar 9018) at 100  $\mu$ l/well in 0.2N carbonate buffer, pH 8. Plates were washed twice with Dulbecco's modified Eagle's medium (DMEM). Mast cells were resuspended in 200  $\mu$ l of fresh cytokine-free BMMC medium per well and then centrifuged at 900 revolutions per minute (160g) for 1 minute to initiate contact between cells and bound antibody.

Human skin mast cells (SMCs) were cultured from tissue obtained at abdominoplasty in X-Vivo media (BioWhittaker) and 100 ng/ml of recombinant human SCF, as described previously<sup>(29)</sup>. To generate the Fc $\gamma$ RIIa crosslinking reagent, anti-human Fc $\gamma$ RIIa antibody IV.3 was incubated for 45 minutes at 37°C with goat F(ab')<sub>2</sub> fragments directed against murine Fab (Sigma); the concentration of reagents was optimized for each IV.3 batch and varied from 1  $\mu$ g/ml to 20  $\mu$ g/ml for IV.3 and from 0.6  $\mu$ g/ml to 5  $\mu$ g/ml for anti-mouse F(ab')<sub>2</sub>. Warm crosslinking reagent was added at the volume indicated for a final well volume of 200  $\mu$ l. The release of  $\beta$ -hexosaminidase was quantified by colorimetric assay and was expressed as a percentage of total cellular  $\beta$ -hexosaminidase, as determined by ultrasonic lysis of parallel wells. For both human and murine mast cells, the cells were washed into media containing fresh cytokines 1 day prior to experimentation. At initiation of each experiment, the cells were resuspended in warm cytokine-free medium.

### **Analysis of calcium flux.**

Cells were washed into calcium-containing buffer (Hanks' balanced salt solution plus 1 gm/liter of bovine serum albumin, 1 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub>) and incubated for 30 minutes at 37°C with Fura 2 AM (2.5  $\mu$ l/ml; Molecular Probes). Following a further wash, cells were resuspended

at  $0.5\text{--}1 \times 10^6/\text{ml}$  in the same buffer, and calcium-sensitive fluorescence was read using a Hitachi F-4500 fluorescence spectrophotometer.

### **Quantitative polymerase chain reaction (PCR).**

Total RNA and complementary DNA were generated using standard commercial kits (Qiagen). PCR for murine Fc $\gamma$  receptors II and III was performed using published primers<sup>(30)</sup>. Alternate validated primers were used in some experiments, with similar results; these were as follows: for Fc $\gamma$ RII, 5'-AGAAGCTGCCAAAAGTGG-3' (forward) and 5'-CTTCGGGATGCTTGAGAAGTG-3' (reverse); for Fc $\gamma$ RIII 5'-CAGAATGCACACTCTGGAAGC-3' (forward) and 5'-GGGTCCCTTCGCACATCA-3' (reverse); and for GAPDH 5'-AGGTCGGTGTGAACGGATTTG-3' (forward) and 5'-TGTAGACCATGTAGTTGAGGTCA-3' (reverse). GAPDH primers were confirmed to have stable expression under all experimental conditions. Data were analyzed using the  $2^{-\Delta\Delta C_T}$  method.

### **Induction of arthritis and measurement of paw swelling.**

Arthritis was induced by intraperitoneal administration of 150  $\mu\text{l}$  of serum obtained from arthritic K/BxN mice and was scored as described previously<sup>(5)</sup>. Briefly, each paw was scored from 0 (no inflammation) to 3 (maximal inflammation), and the results were summed to arrive at an overall clinical index. Acute paw swelling was determined immediately prior to serum injection. Spring-loaded calipers were used to measure the thickness of each paw: for the forepaw, measurement across the dorsal–ventral axis with the paw in neutral position; for the ankle, measurement across the malleoli with the ankle in full flexion. Measurements were repeated 30 minutes following serum injection to quantitate changes in thickness over this interval (“flare”).

A similar technique was used to quantitate acute edema following periarticular instillation of C5a. In these experiments, 20  $\mu\text{l}$  of a concentrated stock solution of C5a (400 nM in LPS-free phosphate buffered saline [PBS]) were injected into the dorsal wrist or lateral ankle tissue of otherwise unmanipulated animals. A similar volume of PBS alone was instilled into the contralateral wrist and ankle. The solutions were distributed through the tissues by brief massage, and the joints were then measured at baseline and after 10 minutes to assess acute edema.

### **Engraftment of cultured mast cells.**

BMMCs were engrafted into mast cell-deficient W/W<sup>v</sup> mice by injection into the tail vein ( $10^7$  cells per recipient), as described previously<sup>(5)</sup>. Experiments were initiated 10 weeks after cell transfer. Engraftment was quantified by microscopic examination of three 20 $\times$  high-power fields in each ankle, as determined by an examiner (PAN) who was blinded to treatment group<sup>(12)</sup>.

### **Microscopy.**

For immunohistochemical staining, histology sections were deparaffinized and subjected to antigen retrieval in sodium citrate buffer, pH 6.0, for 15 minutes at 86°C. After blocking with normal goat serum, samples were incubated overnight at 4°C with 10 µg/ml of primary antibody, for 30 minutes with biotinylated secondary antibody (Vector), for 30 minutes with avidin–biotin–alkaline phosphatase ABC reagent (Vector), and finally, for 20 minutes with 1 mg/ml of fast red substrate (Sigma). Tissues were counterstained with Gill's hematoxylin no. 2. To assess degranulation, ankles were stained with toluidine blue and examined in a blinded manner to identify mast cells demonstrating granule exocytosis, as described elsewhere<sup>(5)</sup>. Neutrophil infiltration was assessed by staining with the antineutrophil antibody NIMP-R14 after antigen retrieval with proteinase K, as described previously<sup>(31)</sup>, followed by microscopic analysis to enumerate the number of infiltrating NIMP-R14+ cells in the 3 richest 40× fields.

### **Neutrophil recruitment assay.**

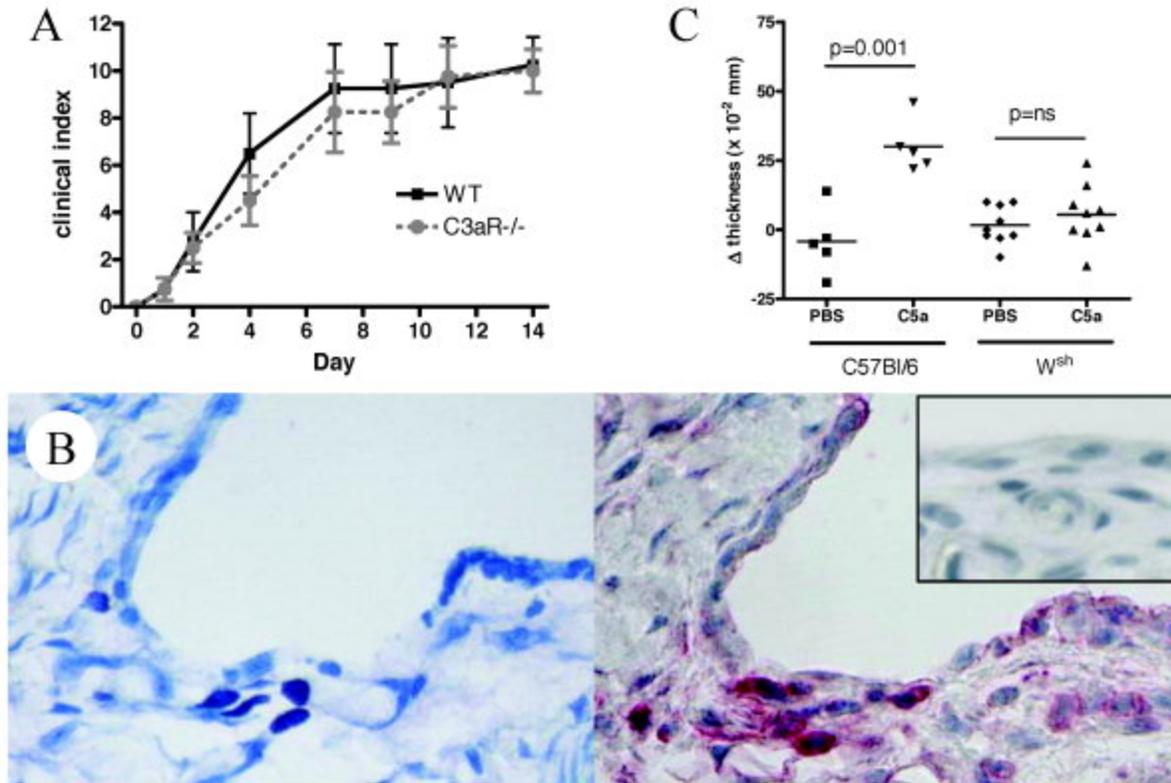
For functional assessment of a C5aR-dependent, BMDC-derived neutrophil chemoattractant, 250,000 FcγRII<sup>-/-</sup> BMDCs in 1 ml of 2% fetal bovine serum (FBS)/BMDC media per well, in the presence or absence of 50 nM C5a, were spun onto a 12-well plate (Costar 3402) that had been coated overnight with carbonate buffer with or without 10 µg/ml of 2.4G2, as described above. After 20 minutes, polycarbonate Transwells with 3-µm pores were inserted into the wells, and whole bone marrow from a C5aR<sup>-/-</sup> mouse that had been resuspended in 0.1% FBS/DMEM was divided into the upper chambers. After 4 hours, the Transwells were removed, and the migrated neutrophils (defined as CD45+CD11b<sup>high</sup>Gr-1<sup>high</sup>) were enumerated by flow cytometry using added counting beads as a standard (catalog no. 18328; Polysciences).

### **Statistical analysis.**

Results were compared using the Student's *t*-test. *P* values ≤ 0.05 were considered significant. Error bars reflect SEM.

## **RESULTS**

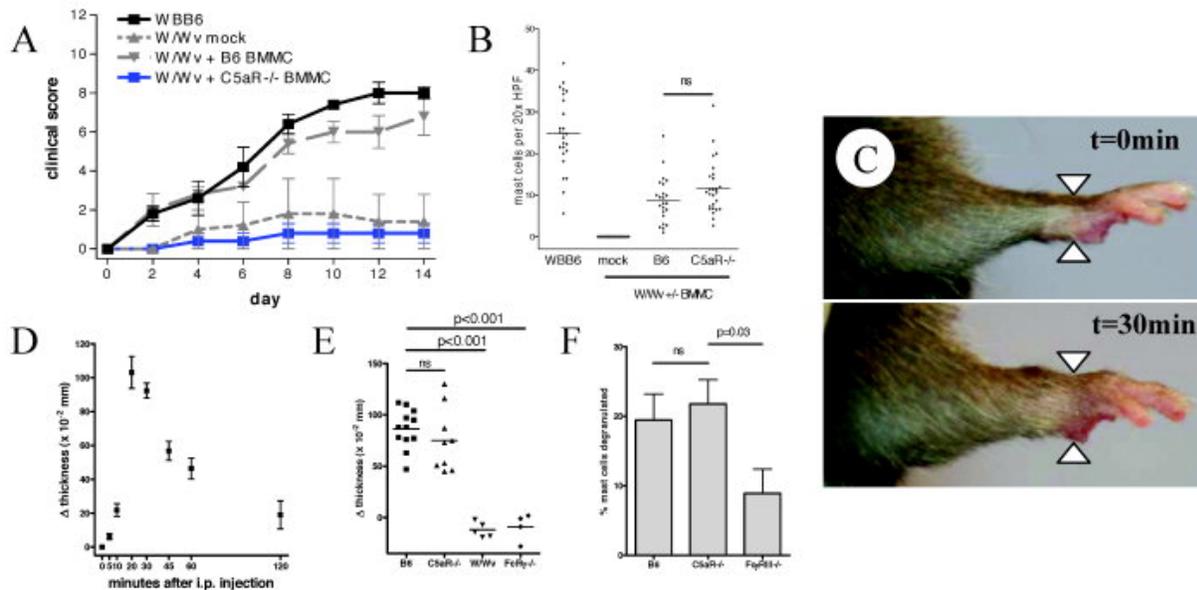
In K/BxN arthritis, C5aR<sup>-/-</sup> animals are densely resistant to arthritis, a resistance that depends in part on a requirement for C5aR in neutrophils but could include other lineages<sup>(32, 33)</sup>. However, the role of C3aR has not been examined. We therefore initiated arthritis by transfer of K/BxN mouse serum into C3aR<sup>-/-</sup> mice and found them to be normally susceptible to disease, as has previously been shown in studies of collagen antibody–induced arthritis<sup>(34)</sup> (Figure 1A). We therefore focused further study on the role of C5aR in synovial mast cells.



**Figure 1.** **A**, C3aR<sup>-/-</sup> mice and matched wild-type (WT) controls were injected with K/BxN mouse serum on days 0 and 2. Clinical outcomes in the 2 groups were indistinguishable. Results are representative of 2 independent experiments. Values are the mean  $\pm$  SEM of 4 mice per group. **B**, Photomicrographs of ankle joint sections show mast cells lying just deep to the synovial lining in the tibiotalar joint, as indicated by toluidine blue staining (left), and bright staining for C5aR in an adjacent section (right). Staining with isotype control is shown in the **inset**. Results are representative of 4 experiments. (Original magnification  $\times$  400.) **C**, Instillation of C5a into the periarticular tissues of the wrist and ankle joints resulted in acute edema, as indicated by the change in paw thickness, in the C57BL/6 mice, but not the mast cell-deficient W<sup>sh</sup> mice, after treatment with phosphate buffered saline (PBS) or C5a. Each data point represents a single mouse; horizontal lines show the mean. Mice were pooled from 2 experiments. NS = not significant.

While expression of C5aR by mast cells has been well documented, the characteristics of an individual mast cell population depend strongly on the developmental stage and the tissue microenvironment<sup>(35,36)</sup>. Specifically, some murine mast cell populations do not express C5aR until they are activated by another signal<sup>(37)</sup>. It was therefore important to demonstrate that C5aR was expressed and functional on synovial mast cells. Using immunohistochemical techniques, we demonstrated strong expression of C5aR on mast cells in the synovial sublining (Figure 1B). The function of this receptor was confirmed by instillation of C5a into periarticular tissues, resulting in measurable edema within 10 minutes in a mast cell-dependent manner (Figure 1C). We concluded that C5aR is expressed and functional on normal synovial mast cells and is therefore a potential participant in the activation of mast cells by immune complexes.

To examine the role of C5aR on mast cells in isolation from other cell types, we used a genetic approach. BMMCs were cultured from wild-type and C5aR<sup>-/-</sup> animals and engrafted into mast cell-deficient animals using a standard technique<sup>(5, 12, 38)</sup>. After 10 weeks, animals were injected with K/BxN serum, and arthritis susceptibility was monitored by clinical examination. Unlike wild-type mast cells, C5aR<sup>-/-</sup> BMMCs failed to restore arthritis susceptibility to W/Wv mice (Figure 2A). To ensure that this phenomenon did not reflect a failure of engraftment, ankle tissue from recipient animals was examined for mast cell density, which was found to be equivalent (Figure 2B). These data indicate that C5aR is dispensable for the migration of mast cells into synovial tissue but is required for the arthritogenic activity of these cells.

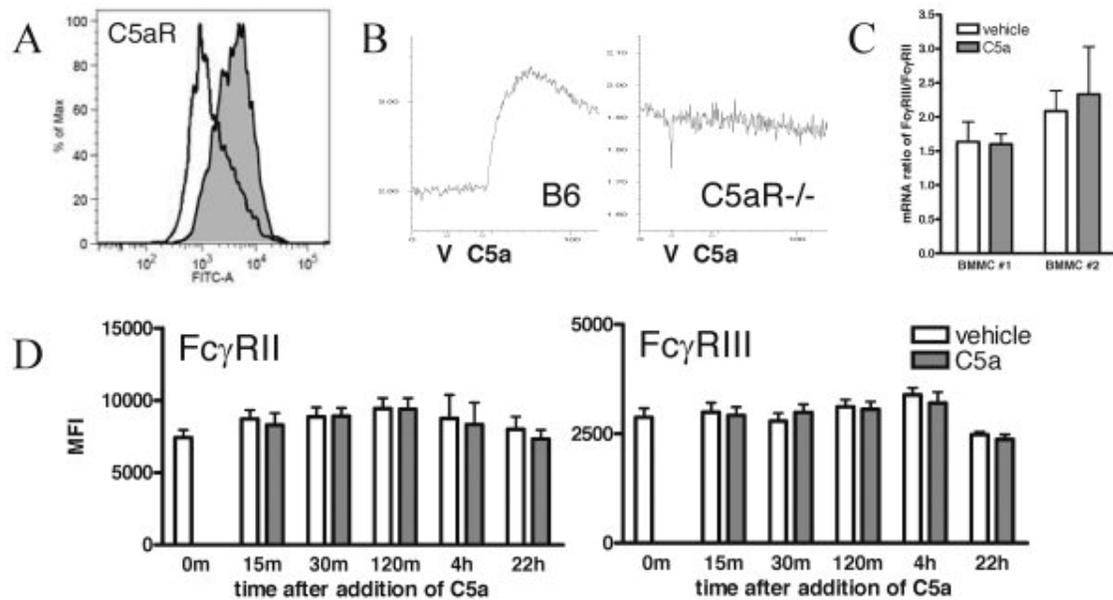


**Figure 2.** A, W/Wv mice were engrafted for 10 weeks with bone marrow-derived mast cells (BMMCs) from B6 or C5aR<sup>-/-</sup> mice, followed by initiation of K/BxN arthritis. Results are representative of 5 independent experiments. Values are the mean ± SEM of 5 mice per group. B, Engrafted mast cells in the ankle joints of the mice in A were quantified, and the results are shown. Each data point represents a single mouse (n = 23–26 per group); horizontal lines show the mean. NS = not significant; hpf = high-power field. C, Paw edema (flare) before (top) and 30 minutes after (bottom) intraperitoneal injection of 150 μl of K/BxN serum into a B6 mouse. D, Time course of paw edema (flare) in B6 mice following intraperitoneal (IP) injection of K/BxN serum. Results are representative of 4 experiments. Values are the mean ± SEM of 5 mice per group. E, Assessment of flare at 30 minutes after injection of K/BxN serum in B6, C5aR<sup>-/-</sup>, and mast cell-deficient (W/Wv) mice and in Fcγ<sup>-/-</sup> mice lacking Fcγ receptor III. Five of the B6 mice and 4 of the C5aR<sup>-/-</sup> mice received a second serum injection on day 2; their mean ± SEM clinical scores on day 7 were 11.6 ± 0.4 and 0 ± 0, respectively (P < 0.0001). Each data point represents a single mouse; horizontal lines show the mean. Mice were pooled from 3 experiments. F, Degranulation of synovial mast cells was assessed histologically 24 hours after administration of K/BxN serum in B6, C5aR<sup>-/-</sup>, and FcγRIII<sup>-/-</sup> mice. Values are the mean and SEM.

The impact of C5aR on macrophage FcγR expression is well established. We therefore anticipated that C5aR on synovial mast cells would play a key permissive role in immune complex-mediated activation that is otherwise constrained by the presence of the inhibitory receptor FcγRII<sup>(39)</sup>. We examined this hypothesis in 2 ways. First, we used a functional approach. Mice injected intravenously with K/BxN serum develop an acute, but transient, increase in paw microvascular permeability, which can be visualized with the technically demanding technique of intravital microscopy in anesthetized mice given high molecular weight intravascular dye<sup>(40)</sup>. This vascular leak is dependent on mast cells<sup>(40)</sup>. We observed that this phenomenon could be quantified in animals receiving serum via the usual intraperitoneal route by measuring paw thickness before and 30 minutes after the intraperitoneal injection (Figures 2C and D). We therefore injected K/BxN serum into C5aR<sup>-/-</sup> and control B6 animals, as well as negative control mast cell-deficient W/W<sup>v</sup> and FcRγ<sup>-/-</sup> mice lacking FcγRIII. Despite profound arthritis resistance in C5aR<sup>-/-</sup> animals, we found that vascular leak remained intact in these animals (Figure 2E). This finding is consistent with the published observation that C5aR<sup>-/-</sup> mice exhibit typical dye extravasation after intravenous administration of serum in the more involved assay<sup>(40)</sup>.

Our second approach to the assessment of mast cell activation was by direct histologic examination for granule exocytosis. We examined sections of ankles harvested from wild-type, C5aR<sup>-/-</sup>, and negative control FcγRIII<sup>-/-</sup> animals 24 hours after administration of K/BxN serum, a time point at which degranulation is readily apparent<sup>(5)</sup>. Consistent with the vascular leak result, degranulation of C5aR<sup>-/-</sup> mast cells was indistinguishable from that of mast cells from B6 controls and greater than that of mast cells lacking the relevant activating FcγR (Figure 2F). These studies confirm that IgG-dependent activation of synovial mast cells in K/BxN serum-transfer arthritis does not require costimulation via C5a and suggest that C5aR-mediated changes in FcγR expression may be less important in mast cells than in macrophages.

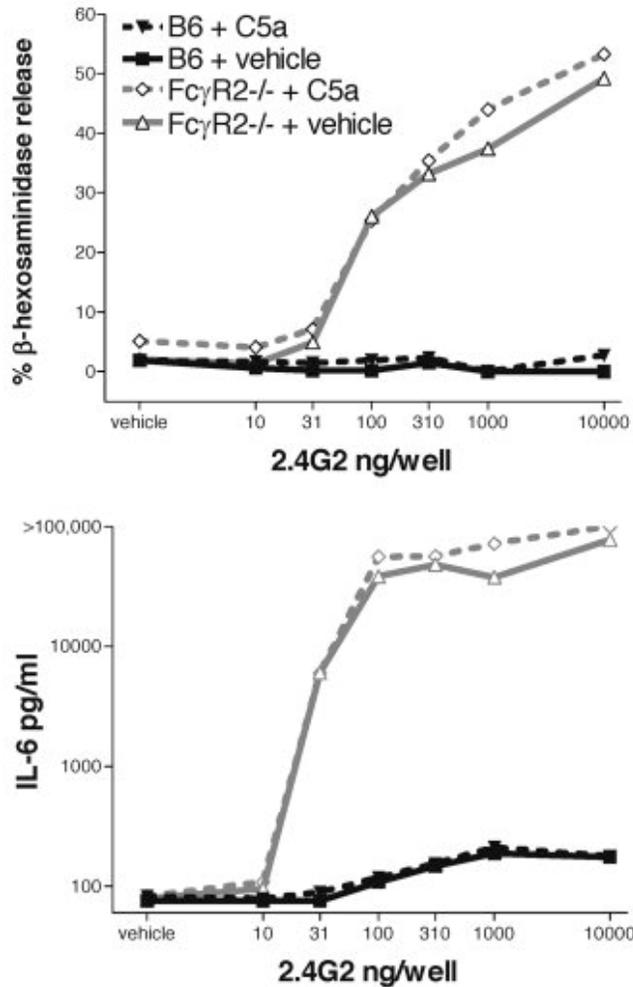
To corroborate this observation, we investigated C5aR-mediated FcγR expression and activation in cultured mast cells. Murine BMMCs express both the inhibitory receptor FcγRII and the activating receptor FcγRIII<sup>(39)</sup>. Expression of C5aR on these cells was confirmed by flow cytometry (Figure 3A). Function was confirmed by a calcium flux assay, in which C5a uniformly induced a brisk signal in B6 BMMCs, as assessed within 1 day of media change, that was absent in C5aR<sup>-/-</sup> BMMCs (Figure 3B). Contrary to our expectations, stimulation with C5a did not alter FcγRII or FcγRIII expression, as assessed by quantitative PCR as well as by specific surface staining<sup>(16,17)</sup> (Figures 3C and D).



**Figure 3.** **A**, Bone marrow–derived mast cells (BMMCs) were stained with 20/70 antibody (rat anti-mouse C5aR) (shaded histogram) or isotype control (open histogram), followed by a fluorescein isothiocyanate (FITC)–conjugated anti-rat antibody. FITC-A = fluorescence intensity. **B**, BMMCs from B6, but not C5aR<sup>-/-</sup>, mice exhibited calcium flux. Vehicle (V) and C5a (100 nM) were added at the indicated time points (seconds). Calcium flux was absent in BMMCs from “spent” media (data not shown), so refreshed cells were used for all experiments. Values are arbitrary units. **C**, B6 mouse BMMCs exposed to 100 nM C5a or vehicle were harvested at 2 hours for measurement of Fcγ receptor (FcγR) mRNA by quantitative polymerase chain reaction analysis. Shown are the results from 2 separate BMMC cultures, each of which was stimulated in triplicate. Data are expressed as the ratio of FcγRIII to FcγRII and are representative of 3 experiments at 2 hours as well as 3 experiments at 4 hours (data not shown). Values are the mean and SEM. **D**, B6 mouse BMMCs stimulated with 50 nM C5a or vehicle were harvested at the indicated times and tested for FcγR expression by flow cytometry. No effect of stimulation was observed. Values are the mean and SEM of pooled data from 2 experiments, each of which included 2 separate BMMC cultures. MFI = mean fluorescence intensity.

Consistent with this result, C5a did not promote FcγRIII-mediated activation of B6 or FcγRII<sup>-/-</sup> BMMCs, as assessed by the β-hexosaminidase assay for degranulation or by production of the cytokine IL-6 (Figure 4). Note that C5a alone was not a potent activator of BMMCs, inducing degranulation of 0–5% of cells and no appreciable production of IL-6 in unstimulated cells (Figure 4, vehicle data). Multiple experiments failed to identify conditions in which C5a could modulate BMMC activation as assessed by these parameters, including dose ranging (1–500 nM C5a), time course (stimulation with C5a for up to 24 hours before or 4 hours after FcγR ligation), and conditioning experiments as well as costimulation with other potentially relevant ligands, including C3a (0–100 nM), LPS (0–10 μg/ml), IL-1β (10 ng/ml), IL-4 (10 ng/ml), transforming growth factor β (10 ng/ml), IL-33 (10 ng/ml), and IL-3–deficient and/or high levels of SCF (250 ng/ml) culture conditions (data not shown). Similarly, stimulation of the phenotypically more mature peritoneal-derived mast cells<sup>(41)</sup> with C5a together with IgG

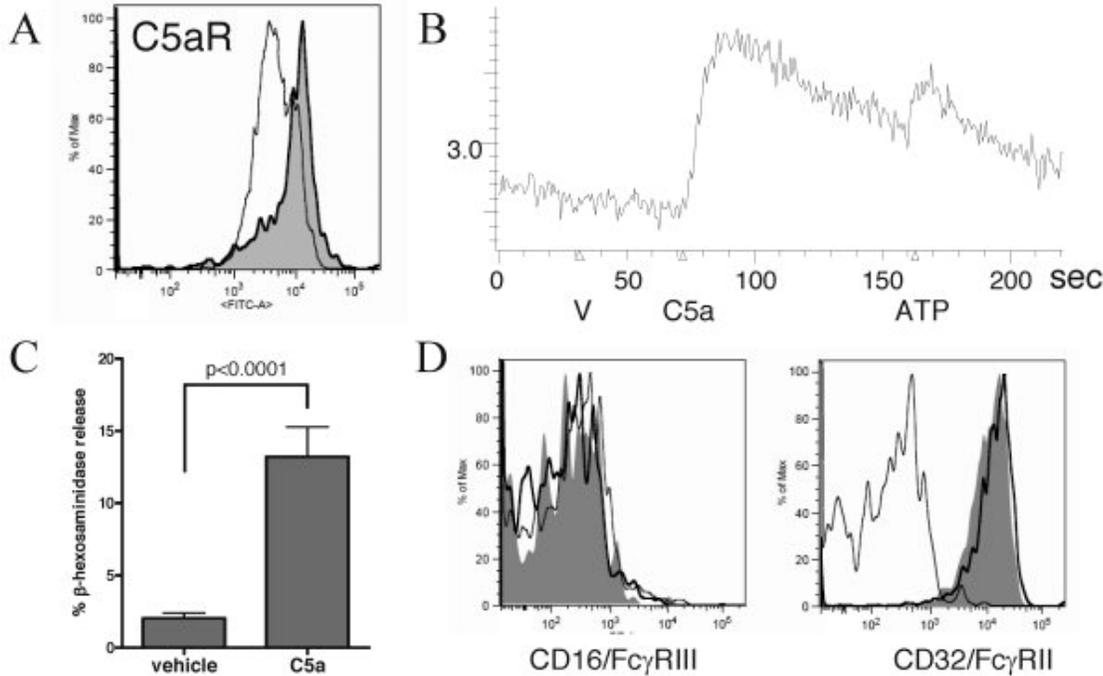
immune complexes failed to demonstrate a change in activation parameters, although the expression of functional C5aR could not be established conclusively in this model cell (data not shown).



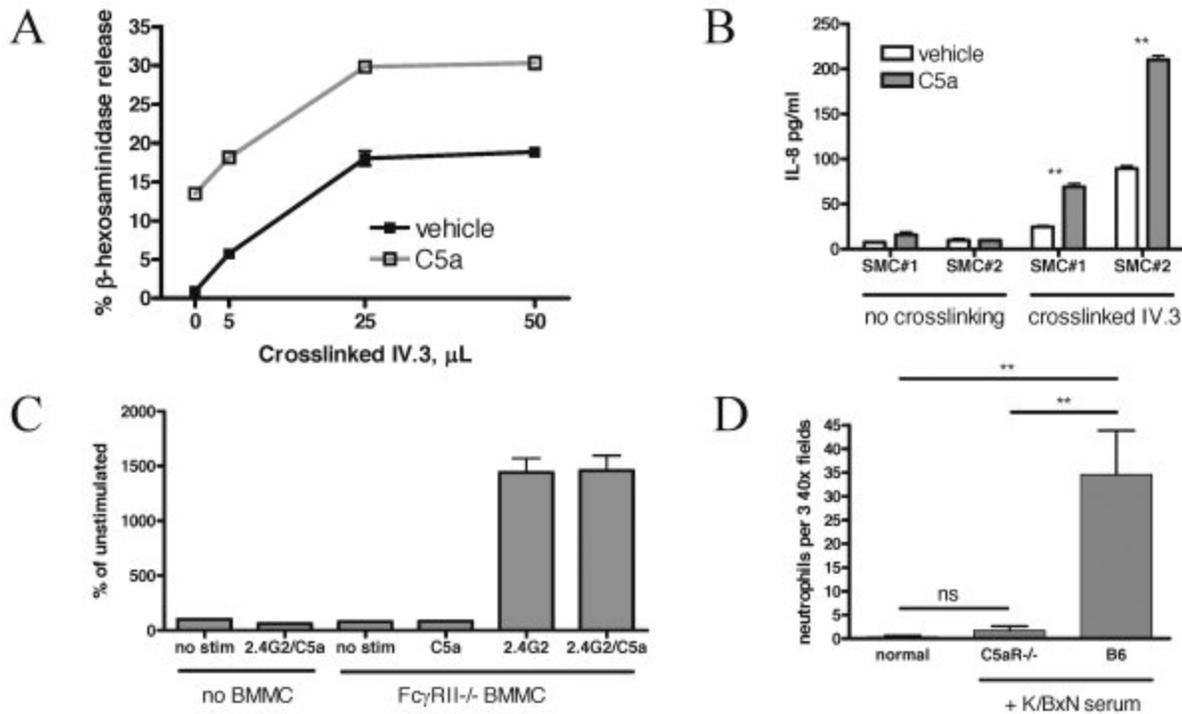
**Figure 4.** Bone marrow–derived mast cells (BMMCs) from B6 or FcγRII<sup>-/-</sup> mice were spun onto a plate coated with the anti-FcγRII/III antibody 2.4G2, in the presence of vehicle or 100 nM C5a, and after overnight incubation, supernatants from duplicate wells were harvested. The percentage release of β-hexosaminidase (top) and the amount of interleukin-6 (IL-6) (bottom) in the supernatants were determined. Note the logarithmic scale for the IL-6 data. Degranulation remained suppressed in BMMCs from B6 mice, although low levels of IL-6 production could be detected in some experiments, as shown here. Results are representative of at least 3 separate experiments.

While these results mirrored our *in vivo* findings, we wished to replicate our experiments in a model cell relevant to human disease. We therefore tested the effect of C5a on human connective tissue mast cells cultured from skin explants. SMCs display a mature mast cell protease phenotype and can be activated via surface FcγRIIa, although they do not express the inhibitory receptor FcγRIIb<sup>(29, 42)</sup>. Mast cells resident in human skin express C5aR<sup>(43)</sup>, and we confirmed

by cytofluorometric and C5a-mediated calcium flux analyses that this phenotype was maintained in cultured SMCs (Figures 5A and B). Furthermore, C5a was competent to activate SMCs, if only modestly (Figure 5C). However, as with BMMCs, C5a failed to modulate surface expression of FcγR in SMCs (Figure 5D). Stimulation with C5a did not shift the dose-response curve of activation via FcγRIIa, although degranulation effects were additive (Figure 6A).



**Figure 5.** **A**, Human skin mast cells (SMCs) were stained with anti-C5aR antibody (shaded histogram) or with isotype control (open histogram) and assessed by flow cytometry. C3aR was not detectable (data not shown). FITC-A = fluorescein isothiocyanate (fluorescence intensity). **B**, Calcium flux was determined in human SMCs. Vehicle (V), C5a (100 nM), and adenosine triphosphate (ATP; positive control) were added at the indicated time points. Values are arbitrary units. Data shown in **A** and **B** are representative of 2 experiments using distinct SMC cultures. **C**, Degranulation was determined in human SMCs treated for 4–6 hours with 10 nM C5a. Degranulation was observed with as little as 1 nM C5a and did not increase measurably from 10–300 nM (data not shown). Values are the mean and SEM of 14 replicates per group pooled from 7 experiments. **D**, Human SMCs were stained for Fcγ receptor (FcγR) expression 2 hours after incubation with C5a 100 nM (shaded histogram) or vehicle (histogram with thick line) as compared with isotype control (histogram with thin line). Staining for FcγRI (CD64) remained absent, as did 2B6 staining for FcγRIIB (data not shown). Data were confirmed in 3 SMC cultures performed in 2 separate experiments.



**Figure 6.** **A**, Human skin mast cells (SMCs) were stimulated for 4 hours with graded concentrations of Fc $\gamma$  receptor IIa (Fc $\gamma$ RIIa) crosslinker, with or without 10 nM C5a. The Fc $\gamma$ RIIa crosslinking reagent was generated from anti-human Fc $\gamma$ RIIa antibody IV.3 as described in Materials and Methods. C5a contributed to SMC degranulation but did not alter the responsiveness to Fc $\gamma$ RIIa. **B**, Human SMCs were stimulated for 4 hours with 10 nM C5a, 5  $\mu$ l of Fc $\gamma$ RIIa crosslinker, or both, and interleukin-8 (IL-8) levels were determined. Results in **A** and **B** are representative of 5 independent experiments using SMCs from 2 donors. **C**, Bone marrow-derived mast cells (BMMCs) from Fc $\gamma$ RII $^{-/-}$  mice were placed in the bottom of a Transwell system and were left unstimulated (no stim) or were stimulated with 50 nM C5a, 10  $\mu$ g/ml of 2.4G2, or both. The migration of C5aR $^{-/-}$  mouse neutrophils from the upper chamber was determined at 4 hours by flow cytometry. Stimulation with C5a did not result in enhanced migration. Experiments were also performed in the absence of BMMCs. Results are representative of 2 experiments. **D**, The presence of extravasated NIMP-R14 $^{+}$  neutrophils was assessed 24 hours after intraperitoneal instillation of 200  $\mu$ l of K/BxN serum into normal, C5aR $^{-/-}$ , or B6 mice (n = 5 mice [9 ankles] per group). Values in **B–D** are the mean and SEM. \*\* =  $P < 0.01$ . NS = not significant.

If C5aR is not required for IgG-mediated activation of mast cells, what is the contribution of this receptor to the function of mast cells in arthritis? The most likely possibility is that C5a enables the production of a mediator that is vital to the evolution of synovitis, either alone or in conjunction with Fc $\gamma$ R ligation. We have previously shown that mast cells are a key source of IL-1 in early synovitis, and this mediator is elaborated by BMMCs upon activation via Fc $\gamma$ R<sup>(12)</sup>. However, despite extensive study in this model cell, we were unable to demonstrate an effect of C5a, with or without concomitant Fc $\gamma$ R ligation, on the synthesis of IL-1 $\alpha$  or IL-1 $\beta$  protein, the processing of IL-1 $\beta$  from its 31-kd precursor to the active 17-kd cytokine, or the release of active cytokine into the environment (data not shown).

In analogous experiments, we were unable to demonstrate an effect of C5a on BMMC production of arachidonate metabolites (leukotriene B<sub>4</sub>, prostaglandin D<sub>2</sub>) or a large set of candidate cytokines and chemokines, which were assessed via dedicated ELISAs, PCR, or multiplex techniques (Pierce SearchLight, R&D Systems Proteome Profiler Mouse Cytokine Antibody Array) (data not shown). However, in human SMCs stimulated via FcγRIIa, C5a could reproducibly enhance the production of the neutrophil chemoattractant IL-8 (Figure 6B). This effect was specific, in that TNF production remained unaffected; IL-1β could not be detected (data not shown). These results suggest that the interaction between C5a and FcγR in synovial mast cells is likely to be one of synergy in the production of key proinflammatory factors, potentially including one or more neutrophil chemoattractants.

We therefore returned to the murine system to evaluate neutrophil chemoattractants as potential C5a-driven mast cell mediators. IL-8 is not expressed in mice, but is instead represented by a family of related neutrophil chemoattractants that are ligands for the murine counterpart of the human IL-8 receptors CXCR1 and CXCR2, including macrophage inflammatory protein 2 (MIP-2), granulocyte chemotactic protein 2 (GCP-2), neutrophil-activating peptide 2 (NAP-2), keratinocyte chemoattractant (KC), and lipopolysaccharide-induced CXC chemokine (LIX). Individual investigation of each of these mediators in BMMCs stimulated via C5a, with or without FcγR ligation, revealed no consistent changes in protein production (MIP-2 and KC, as well as the CCR5 ligand MIP-1α, which was also tested), or for those without a commercially available ELISA, no consistent changes in gene expression (GCP-2, NAP-2, and LIX) (data not shown).

To ensure that we had not missed a chemoattractant using this candidate approach, we addressed the question functionally. BMMCs stimulated via C5a, FcγR ligation, or both were placed in the bottom of a Transwell chamber with C5aR<sup>-/-</sup> neutrophils (as whole bone marrow) in the upper chamber. Whereas FcγR-stimulated BMMCs elaborated potent chemoattractants, no effect of C5a alone was observed, and no increment was noted with the combination stimulus (Figure 6C). We concluded that we were unable to demonstrate a C5a-dependent neutrophil chemoattractant from our model cell.

Thus, we sought supportive evidence of a role for C5aR in neutrophil infiltration *in vivo*, via immunohistologic staining of ankle tissues with the neutrophil marker NIMP-R14<sup>(31)</sup>. Tissues harvested from B6 and C5aR<sup>-/-</sup> mice 30 minutes after serum administration, which was at the peak of the “flare,” demonstrated no infiltration of neutrophils or other recognizable lineages into ankle tissues of either B6 or C5aR<sup>-/-</sup> mice (data not shown). However, at 24 hours, scattered foci of infiltrating neutrophils could be demonstrated in most WT mice (7 of 9 ankles examined), while these remained absent in C5aR<sup>-/-</sup> animals (Figure 6D). We cannot exclude the possibility that this effect simply represents impaired migration of C5aR-deficient neutrophils. However, we have recently shown that C5aR<sup>-/-</sup> neutrophils can migrate to inflamed joints if drawn by a non-C5a stimulus<sup>(33)</sup>. Thus, these data support the possibility that C5aR is involved broadly in the generation of neutrophil chemoattractants in arthritis. Together with the data from our

engraftment studies, they are consistent with the hypothesis that C5aR on mast cells promotes the formation of one or more neutrophil chemoattractants. The identity of such chemoattractants or other C5aR-dependent mast cell mediators remains undefined.

## DISCUSSION

Innate immune cells are key players in autoimmune synovitis. Among these are mast cells, which populate the normal joint in small numbers but can expand 10-fold as arthritis progresses<sup>(44)</sup>. The contribution of these cells to joint inflammation is likely complex. At initiation of disease, mast cells can serve as first sensors of immune complexes, contributing both to rapid increases in local vasopermeability and to the recruitment of inflammatory cells, an activity we have called the “jump start”<sup>(12,40)</sup>. In established disease, where an expanded population of mast cells is often present, their contribution is as yet largely unexplored. Levels of mast cell-derived mediators, such as heparin and proteases, are elevated in synovial fluid from the joints of both RA and osteoarthritis (OA) patients, and proteases of the tryptase family have been implicated experimentally in cartilage injury<sup>(9,31)</sup>. Other potential functions of mast cells in the diseased joint include recruitment of inflammatory cells, promotion of antigen-directed adaptive immune responses, growth of new blood vessels, development of fibrosis, and degradation of proinflammatory mediators to help terminate inflammation (for review, see ref.9). Indeed, since mast cells from rheumatoid joints are phenotypically diverse (45, 46), different populations of mast cells could exert distinct effects.

The current study focuses on the role of the C5a receptor in murine synovial mast cells at the initiation of arthritis. We found that C5aR expression is required to allow mast cells to accomplish their proinflammatory activity. We found no evidence that C5aR modulates the susceptibility of mast cells to immune complex-mediated activation in the joint or in model cells studied *in vitro*, and we therefore conclude that mast cells can diverge in this respect from murine macrophages. Since the phenotype of mast cells is strongly conditioned by the microenvironment, our results do not exclude the possibility that mast cells exhibit such modulation under other conditions. In the context of the arthritic “jump start” studied here, however, C5aR plays a different role. Most probably, C5aR synergizes with FcγRIII to promote the synthesis of critical mediators, as has been reported in rat alveolar macrophages<sup>(15)</sup>. We could not demonstrate this phenomenon in BMMCs, perhaps reflecting the developmental immaturity of these cells<sup>(35)</sup>. However, synergy between C5a and FcγR on the production of IL-8 by human SMCs confirmed that such a mechanism can occur in mast cells, and histologic data were consistent with the hypothesis that C5aR on mast cells participates in neutrophil recruitment to the joint.

The relevance of mast cell C5aR to human disease remains to be determined. Mast cells isolated from RA synovium, but not those from OA synovium, have been shown to express C5aR and to be capable of mediator release upon activation via C5a<sup>(46)</sup>. Expression of C5aR on mast cells in healthy synovium is unknown. Blockade of C5a/C5aR in established RA has thus far yielded

disappointing results, although effective antagonism at the level of synovial tissue was not confirmed<sup>(47, 48)</sup>. Since C5a is present at physiologically relevant levels in human RA synovial fluid, it would be surprising if this mediator had no effect on C5aR-bearing synovial mast cells<sup>(14)</sup>.

Interestingly, while immune complexes fix complement and are likely a major source of synovial C5a, this anaphylatoxin can be generated in other ways. Hepatic macrophages can elaborate C5, and synthesis of C5 within rheumatoid synovial tissue has been detected in some studies, although its cellular source is unclear<sup>(1, 19)</sup>. Cleavage into C5a can be accomplished by proteases other than C3 convertase, including mast cell tryptase<sup>(49)</sup>. This finding may explain why mice lacking C3 (and therefore incapable of complement-mediated C5 proteolysis) are less densely resistant to K/BxN arthritis than mice lacking C5aR<sup>(32)</sup>. Similar results have been observed in the cutaneous immune complex-mediated Arthus reaction, immune complex alveolitis, and autoimmune hemolytic anemia<sup>(18, 19, 50)</sup>. The implication of these results is that C5aR on mast cells could contribute to joint inflammation even in diseases not mediated by immune complexes.

Together with previous results<sup>(12)</sup>, the current findings demonstrate that the participation of mast cells in immune complex-driven murine inflammatory arthritis is dependent codominantly upon FcγR and C5aR. The need for activation via both pathways does not reside in alterations of FcγR expression or the activation threshold but reflects the contribution of C5a to the elaboration of arthritogenic mediators, including neutrophil chemoattractants. These results define a novel pathway of interaction between complement and FcγR ligation in mast cells that may play an important part in the pathogenesis of immune complex-mediated disease, including inflammatory arthritis.

### **Addendum.**

Since the time this article was accepted for publication, another group of investigators has reported the identification of IL-17A in human synovial mast cells and has shown that this mediator may be elaborated by cultured human mast cells in a C5a-dependent manner<sup>(51)</sup>. While IL-17A was included in our multiplex assays, it was not detected in our study samples, though we cannot exclude that the sensitivity of the assay was inadequate to detect a relevant signal.

### **AUTHOR CONTRIBUTIONS**

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Lee had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Nigrovic, Lee.

**Acquisition of data.** Nigrovic, Malbec, Lu, Markiewski, Kepley, N. Gerard, Lee.

**Analysis and interpretation of data.** Nigrovic, Lu, Markiewski, C. Gerard, Daëron, Lee.

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