Published in final edited form as:

Cell Immunol. 2012 ; 278(0): 103–112. doi:10.1016/j.cellimm.2012.07.005.

***In vivo* imaging implicates CCR2⁺ monocytes as regulators of neutrophil recruitment during arthritis**

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Abstract

The infiltration of neutrophils and monocytes is a prominent feature of inflammatory diseases including human rheumatoid arthritis. Understanding how neutrophil recruitment is regulated during pathogenesis is crucial for developing anti-inflammatory therapies. We optimized the K/B × N serum-induced mouse arthritis model to study neutrophil trafficking dynamics *in vivo* using two-photon microscopy. Arthritogenic serum was injected subcutaneously into one hind footpad to induce a local arthritis with robust neutrophil recruitment. Using this approach, we showed that the depletion of monocytes with clodronate liposomes impaired neutrophil recruitment specifically at the transendothelial migration step. The depletion of CCR2⁺ monocytes with the monoclonal antibody MC-21 reproduced these effects, implicating CCR2⁺ monocytes as key regulators of neutrophil extravasation during arthritis initiation. However, monocyte depletion did not prevent neutrophil extravasation in response to bacterial challenge. These findings suggest that anti-inflammatory therapies targeting monocytes may act in part through antagonizing neutrophil extravasation at sites of aseptic inflammation.

Keywords

Arthritis; Neutrophil; Monocyte; Two-photon; microscopy; K/B×N serum

1. Introduction

Rheumatoid arthritis (RA) is a debilitating autoimmune disease that affects millions of people globally [1]. RA is characterized by progressive inflammation with extensive infiltration of neutrophils and monocytes into the synovial cavity and articular tissues [1,2]. The recruitment of neutrophils from the circulation requires the sequential actions of various

adhesion molecules and chemokine signals [3]. Once neutrophils enter the tissue parenchyma, lipid mediators, cytokines and different chemokine receptors play non-redundant and necessary roles in effective neutrophil recruitment to the joint [4]. In preclinical models of RA, neutrophils within articular tissues contribute to disease by releasing pro-inflammatory mediators and tissue-degrading proteases [5] that augment inflammation and induce joint damage [6–8]. Moreover, neutrophils have been shown to be essential for disease development and persistence in the K/B×N serum-induced arthritis mouse model [9] and the blockade of neutrophil recruitment by a small molecule antagonist of CXCR2 reduced disease severity significantly in both an adjuvant arthritis rat model and a methylated BSA-induced arthritis mouse model [6,7]. Therefore, identifying the mechanisms that regulate neutrophil recruitment during arthritis has substantial clinical relevance. In addition, monocytes have been implicated in RA pathogenesis [10,11] and are generally presumed to give rise to effector macrophages in the inflamed tissues. However, the specific contribution of CCR2⁺CX₃CR1⁺Ly6C^{hi}CD43⁺ (CCR2⁺) and CCR2⁺CX₃CR1⁺⁺Ly6C^{lo}CD43⁺⁺ (CX₃CR1^{hi}) monocyte subsets remains unclear [12,13] and their role in regulating neutrophil trafficking has not been well characterized [14–16].

To study neutrophil trafficking to the joints in real-time, we developed an accelerated K/B×N serum transfer arthritis model (aSTA) optimized for two-photon (2P) imaging. In our model, arthritogenic serum is injected subcutaneously (*s.c.*) into one hind footpad to produce a local arthritis with synchronized neutrophil recruitment. This approach provided a detailed spatiotemporal view of neutrophil trafficking and revealed that CCR2⁺ monocytes are required for efficient neutrophil transendothelial migration (TEM) during arthritis induction. In contrast, neutrophil recruitment to bacterial challenge was monocyte-independent, indicating that different recruitment mechanisms may be involved during infection. These findings suggest that anti-inflammatory therapies targeting monocytes may act in part through antagonizing neutrophil extravasation at sites of aseptic inflammation.

2. Materials and methods

2.1. Mice

C57BL/6 and Cx₃cr1^{gfp/+} mice were obtained from The Jackson Laboratory (Bar Harbor, ME). LysM-GFP mice were a gift of Klaus Ley (La Jolla Institute for Allergy and Immunology, CA) who back-crossed the original knockin mouse from T. Graf (Albert Einstein College of Medicine, NY) onto the B6 background. Female mice 6–10 wk old were used throughout the study. All mice were maintained and bred under specific pathogen-free conditions in the animal facility at Washington University School of Medicine.

2.2. Induction of arthritis

Mice were injected either with 50 μ l *s.c.* or with 200 μ l intravenously (*i.v.*) of serum from K/B×N mice. Ankle thickness was measured with a caliper daily as described previously [17]. The change of ankle thickness was quantified by subtracting the day 0 measurement, taken at the time of injection, from each subsequent measurement.

2.3. Histology

The entire hind paw was removed on day 6 after K/B×N serum transfer and fixed in 4% phosphate-buffered formaldehyde (Sigma, HT501128), decalcified in 14% EDTA, and embedded in paraffin. Serial sections (5 μ m) were cut and stained with hematoxylin and eosin and evaluated by a board-certified veterinary pathologist (TPL).

2.4. Cell isolation and flow cytometry

Blood was collected from the retroorbital plexus of anesthetized mice and stained as described below. Joint tissues were obtained as described previously [18,19]. Briefly, tissue was harvested and minced, then incubated with 28 mU/ml Liberase (Liberase TM, Roche Diagnostics, IN) and 10 μ g/ml of DNase I (Sigma) in HBSS for 1 h at 37 °C with rocking. The cell suspension was passed through a 70 μ m-mesh sieve and analyzed by flow cytometry. Cells were stained with antibody to CD115 (clone AFS98), Ly6C (clone AL-21), CD11b (clone M1/70), Ly6G (clone 1A8) and appropriate isotype control antibodies (BD PharMingen, San Diego, CA). Samples were analyzed on a FACSCalibur equipped for four-color flow cytometry (BD Bioscience, San Jose, CA) with FlowJo software (Tree Star).

2.5. 2P microscopy

Imaging of mouse paws was performed as described previously [20–22]. Briefly, LysM-GFP mice were injected either with 200 μ l *i.v.* or with 50 μ l *s.c.* of serum from arthritic K/B×N mice. In some experiments, neutrophil recruitment was induced by injecting *s.c.* 1×10^6 *Listeria Monocytogenes* (EGD strain) resuspended in 10 μ l of PBS. Blood vessels were visualized by injection of 20 μ l *i.v.* of 655-nm nontargeted Q-dots diluted in 150 μ l PBS. Mice were anesthetized, placed on a warming pad and their rear paw was secured to the imaging chamber using VetBond (3M). Time-lapse imaging was performed using a custom-built dual-laser video-rate 2P microscope [23]. GFP-labeled neutrophils and Q-dot labeled blood vessels were excited by a Chameleon XR Ti:Sapphire laser (Coherent) tuned to 890 nm. Fluorescence emission was passed through 480 and 560 nm dichroic mirrors placed in series and detected as red (>560 nm), green (480–560 nm), and blue (<480 nm) channels by three head-on multi-alkali photomultiplier tubes. Each plane represents an image of 220 μ m (x) by 240 μ m (y) (at 2 pixels/ μ m). Z-stacks were acquired by taking 21 sequential steps at 2.5 μ m spacing. Image reconstruction and multidimensional rendering was performed with Imaris (Bitplane), and cell tracking and analysis of cell motility were performed with Volocity (Improvision).

2.6. Clodronate liposomes, CX₃CR1 blockade and CCR2⁺ monocyte- depletion

Clodronate liposomes (CL) were prepared as described previously [15]. Mice were given CL *i.v.* for four consecutive days (initial dose of 200 μ l followed by 100 μ l) starting 48 h before *s.c.* injection of serum or *L. monocytogenes* [20]. The clodronate liposome protocol depletes >90% of CD115⁺ cells from the blood as assessed by flow cytometry. Mice were given 1.5 mg/kg of anti-CX₃CR1 polyclonal rabbit *i.p.* to block CX₃CR1 at 24 and 6 h before *s.c.* serum transfer [24] or control antibody (gift of Pfizer). To deplete CCR2⁺ monocytes, mice were given 0.5 mg/kg of anti-CCR2 rat monoclonal antibody MC-21 *i.p.* [10] for five consecutive days, starting 24 and 6 h before *s.c.* serum transfer or control antibody (Rat IgG2b, Clone RTK4530, BioLegend).

2.7. Statistical analysis

Data are represented as the mean $\pm$ standard deviation. The change of ankle thickness was compared using a Student's *t*-test (two-tailed). The meandering index and median track velocity were compared using a Mann-Whitney test. A difference is considered significant if $p < 0.05$. In all figures, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. Results

3.1. Leukocyte recruitment kinetics in the classic serum transfer arthritis model

In vivo imaging models have contributed greatly to our understanding of the cellular and molecular mechanisms that regulate leukocyte recruitment [14,25,26]. However, the single-

cell dynamics of neutrophil recruitment during arthritis have not been characterized. We analyzed real-time neutrophil trafficking in the widely used systemic K/B×N serum transfer arthritis model using *in vivo* 2P microscopy [17]. This model recapitulates several features of human RA including the infiltration of monocytes and neutrophils into the joint synovium. Neutrophils were imaged using LysM-GFP mice, in which GFP expression is high in neutrophils and lower in monocytes and tissue macrophages [15]. In our images, neutrophils are readily distinguishable from monocytes and macrophages based on fluorescence intensity (Supplemental Fig. 1) and distinct morphology and only the brightest GFP⁺ cells, which were ≈95% neutrophils, were included in our analysis. Arthritis was induced by injecting 200 μl of K/B×N serum *i.v.* and the rear paw imaged longitudinally as previously described [20,21]. Neutrophil trafficking displayed three prominent stages. In the first 0.5–12 h after serum transfer, neutrophils were found rolling and arresting in blood vessels located adjacent to the interphalangeal joints (Fig. 1A and Supplementary movie 1, upper left panel). During this time window, a small number of neutrophils extravasated and entered the tissue parenchyma (Supplementary movie 1, upper right panel). Between 12–24 h after arthritis induction, the majority of neutrophils were extravascular and motile; migrating with a median velocity of $7.11 \pm 2.13 \mu\text{m min}^{-1}$ and a weak directional bias (meandering index = 0.61 ± 0.12) (Fig. 1B and Supplementary movie 1, lower left panel). At 48–72 h after arthritis induction, neutrophils accumulated near the dorsal edges of the interphalangeal joints and formed large aggregates (Fig. 1C and Supplementary movie 1, lower right panel). While many neutrophils arrested near the joint, others formed dynamic clusters.

3.2. The accelerated serum transfer arthritis model for *in vivo* imaging

Although it was possible to image neutrophil trafficking in the *i.v.* serum transfer model, cell tracking and quantitative analysis were difficult because trafficking steps were poorly synchronized and recruitment was spread out over several days. Moreover, the systemic administration of a high dose of arthritogenic serum initiated inflammation at multiple sites and therefore the total number of cells that could be imaged at any one site was limited. To address these issues, we developed an accelerated serum transfer arthritis model (aSTA) optimized for 2P imaging that consists of injecting 50 μl of K/B×N serum *s.c.* into one hind footpad. This approach produced a more localized arthritis with a shorter disease course. Erythema and edema were visible 24 h after injection, peaked at day 7 and fully resolved around two weeks (Fig. 1D). Control injections of PBS or KRN serum were absorbed rapidly and did not lead to sustained swelling or inflammation. Histological evaluation of the paw confirmed that the aSTA model produced a mild arthritis, similar to, but less severe than the standard *i.v.* serum transfer model in terms of cellular infiltration, cartilage damage, bone resorption and pannus invasion (Fig. 1E and F).

Having established that a transient arthritis develops in the aSTA model, we then used 2P microscopy to analyze neutrophil trafficking dynamics *in vivo*. Within minutes of *s.c.* serum injection, neutrophils were observed rolling and arresting in small to medium sized vessels (11.43–21.85 μm in diameter) in the plantar tissues of the hindpaw digits (Fig. 1G; Supplementary movie 2, upper left panel). At this time, some neutrophils displayed distinctive intraluminal crawling, presumably searching for extravasation sites [27] (Supplementary movie 3). It is noteworthy that neutrophils arrested on discrete regions of the endothelium, before they elongated and underwent TEM, which took several minutes to complete (Fig. 1G and H and Supplementary movie 2, upper right and lower left panels). In the aSTA model, interstitial trafficking was markedly accelerated; once neutrophils became extravascular, they migrated quickly away from extravasation sites and congregated in dynamic clusters adjacent to joints over 4–6 h (Fig. 1I and Supplementary movie 2, lower right panel), similar to neutrophil behaviors observed in the *i.v.* model at 48–72 h.

Neutrophils migrated with a strong directional bias (Supplementary movie 4, upper panel) and moved in relatively straight paths (Fig. 2A and B) consistent with chemotaxis. Furthermore, neutrophil tracks displayed a high median meandering index (0.85 ± 0.15 , Fig. 2C) and median track velocity ($12.67 \pm 2.39 \mu\text{m min}^{-1}$, Fig. 2D), similar to behavior reported in other models [21,28,29]. In control experiments, the injection of PBS or KRN serum induced only transient cell rolling on the endothelium (Supplementary movie 4, lower left and right panels). Flow cytometry of paw tissue confirmed that large numbers of both neutrophils (Fig. 2E) and monocytes (Fig. 2F) were recruited within 2hrs of serum injection. Collectively, these results show that the aSTA model resembles the *i.v.* K/BxN serum transfer arthritis model in terms of pathology, but has accelerated disease kinetics, which facilitates the quantitative analysis of leukocyte trafficking by 2P microscopy.

3.3. Monocyte depletion impairs neutrophil extravasation during arthritis induction

Previous studies suggested that monocytes can regulate neutrophil extravasation in the murine lung [15,16]. In our movies, the sites of neutrophil extravasation were often associated with the arrest of Q-dot positive cells from the circulation (Fig. 3A), which were shown to be CD115⁺ by flow cytometry (Fig. 3B). We assessed the role of monocytes in neutrophil recruitment in the aSTA model by depleting monocytes with clodronate liposomes [30]. Consistent with published results [15,31], CL-treatment in our hands depleted $\approx 92\%$ of circulating monocytes (CD115⁺ cells) in mice, whereas PBS liposomes (PL)-treated mice had normal numbers (Fig. 3C). Furthermore, CL-treatment did not affect the number of circulating neutrophils (data not shown), in agreement with published studies that showed CL-treatment is not directly toxic to neutrophils [31]. CL-treatment given before arthritis induction completely blocked ankle swelling in the aSTA model (Fig. 3D), in agreement with results obtained in the *i.v.* serum transfer model [32]. Moreover, flow cytometry showed that neutrophil recruitment was sharply attenuated in the paws of CL-treated mice compared to PL-treated controls (Fig. 3E). We assessed the precise impact of monocyte depletion on neutrophil trafficking steps using *in vivo* 2P microscopy. Within minutes after serum injection, neutrophils rolled and arrested along the vascular endothelium in CL-treated mice, similar to PL-treated controls, indicating that neutrophil adhesion was not inhibited (Fig. 4A and Supplementary movie 5, left panel). However, time-lapse imaging revealed that the majority of neutrophils in CL-treated mice ($90.89 \pm 5.45\%$) remained arrested along the endothelium for up to 2 h, in marked contrast to PL-treated mice in which neutrophils extravasated extensively during this same time period (Fig. 4B and movie 5). Although daily *i.v.* CL-treatment significantly reduced neutrophil TEM (Fig. 4A), some neutrophils managed to extravasate. We tracked the extravasated population of neutrophils to determine if guidance cues that direct cells from sites of extravasation to articular tissues were still intact. Neutrophils in CL-treated mice migrated with less directional bias (Fig. 4C and D, meandering index: 0.59 ± 0.17) and displayed significantly decreased velocity (Fig. 4E, median velocity: $9.49 \pm 2.53 \mu\text{m min}^{-1}$) compared to neutrophils in the PL-treated control group. Importantly, the depletion of resident macrophages with *s.c.* CL did not inhibit neutrophil extravasation nor did a single *i.v.* CL-treatment (data not shown). As a control for untoward effects of CL-treatment on neutrophil trafficking, we challenged monocyte-depleted mice with *L. monocytogenes* and then imaged neutrophil recruitment. In response to bacterial challenge, neutrophils extravasated efficiently and displayed chemotaxis towards sites of infection (Fig. 4F and Supplementary movie 6) with a median meandering index of 0.80 ± 0.12 (Fig. 4G) and a median track velocity of $11.41 \pm 2.57 \mu\text{m min}^{-1}$ (Fig. 4H). These results indicate that the signals that regulate neutrophil extravasation during infection are more robust or possibly distinct from those that mediate recruitment during aseptic inflammation.

3.4. The role of monocyte subsets in regulating neutrophil recruitment

Systemic CL-treatment depletes circulating monocytes broadly [33], thus it cannot be used to assess the role of different monocyte subsets in arthritis. We investigated whether CX₃CR1^{hi} monocytes play a role in regulating neutrophil recruitment in the aSTA model (Fig. 5). Previously it was shown that CX₃CR1^{hi} monocytes extravasate rapidly in response to tissue injury and mediate early inflammatory responses [14]. Therefore, we characterized the recruitment and trafficking behavior of CX₃CR1^{hi} monocytes during arthritis using Cx₃cr1-gfp knockin mice [12,14]. In heterozygous Cx₃cr1^{gfp/+} mice, a small number of CX₃CR1-GFP cells were occasionally observed patrolling the blood vessels in the phalanges in the steady-state (Fig. 5A and Supplementary movie 7, upper left panel). Surprisingly, the intraluminal patrolling of CX₃CR1-GFP cells was equally rare during the first few hours after *s.c.* K/B×N serum injection (Fig. 5B and Supplementary movie 7, upper right panel). Moreover, we did not detect CX₃CR1-GFP cell extravasation 1–2 h after serum injection, despite the fact that many neutrophils had extravasated over this same time period. On day 5 after serum transfer, CX₃CR1-GFP cells were found clustered in the articular tissues (Fig. 5C and Supplementary movie 7, lower left panel), indicating that although CX₃CR1^{hi} monocyte recruitment occurs in the aSTA model, it is markedly delayed relative to that of neutrophils.

The chemokine receptor CX₃CR1 is required for monocyte recruitment and cytokine production in other models [14,34], therefore we examined whether CX₃CR1 was required for arthritis development and monocyte trafficking in the aSTA model. CX₃CR1-deficient, Cx₃cr1^{gfp/gfp} mice developed ankle swelling equivalent to that of control mice (Fig. 5D), suggesting that CX₃CR1 is dispensable for arthritis development. Furthermore, CX₃CR1-GFP cells in Cx₃cr1^{gfp/gfp} mice appeared to patrol normally (Supplementary movie 7, lower right panel) and accumulate near the joint on day 5 (data not shown) similar to Cx₃cr1^{gfp/+} mice. Moreover, in complementary experiments we found that blocking antibodies to CX₃CR1 [24] did not inhibit arthritis (Fig. 2E) nor did they impair neutrophil recruitment in LysM-GFP mice as compared to treatment with isotype control antibody (Fig. 5F and G).

The fact that CX₃CR1 monocyte recruitment is delayed relative to neutrophil recruitment and that CX₃CR1 deficiency did not impact arthritis development in our STA model, led us to test whether CCR2 monocytes played a role. Previously, Ccr2^{-/-} mice were shown to have exaggerated arthritis [35], and in our hands Ccr2^{-/-} mice are susceptible to aSTA (data not shown), indicating that the CCR2 receptor is not required for arthritis development. However, the depletion of CCR2⁺ monocytes with the monoclonal antibody MC-21 ameliorated collagen induced arthritis [36], illustrating that cell depletion and receptor deficiency are not equivalent. Therefore, we assessed arthritis development and neutrophil recruitment in mice depleted of CCR2⁺ monocytes with MC-21 antibody. MC-21 treatment efficiently depleted circulating monocytes but had no effect on neutrophils (Supplemental Fig. 2A–C). Although MC-21 treatment inhibited early arthritis development (Supplemental Fig. 2D, *p* = 0.009, 0.013, 0.005 and 0.03 from day 2 to 5), the inhibition was short lived, presumably due to the rapid loss of antibody efficacy, as reported previously [36]. Despite the fact that MC-21 mediated depletion was transient, 2P microscopy revealed a striking defect in neutrophil TEM, similar to results obtained with CL-treatment (Fig. 6A and Supplementary movie 8). Moreover, as with CL-treatment, MC-21 inhibited TEM incompletely and a population of neutrophils successfully extravasated into the tissue. The extravasated neutrophil population appeared less responsive to long-range chemotactic cues and often formed clusters near sites of extravasation (Fig. 6A, yellow arrowheads), rather than clustering in articular tissues, as seen in untreated mice. Furthermore, neutrophils in MC-21 treated mice migrated with significantly decreased directional bias (Fig. 6B) and displayed a significantly decreased meandering index (Fig. 6C) and reduced median velocity (Fig. 6D) compared to treatment with isotype-matched control antibody.

To determine if CCR2 monocyte depletion would inhibit neutrophil recruitment in response to other inflammatory signals, we assessed the impact of MC-21 treatment on neutrophil recruitment during *s.c.* bacterial infection. Neutrophil recruitment was examined with 2P microscopy in LysM-GFP mice pre-treated with MC-21 and infected with *L. monocytogenes*. Neutrophil extravasation was highly efficient and cells migrated quickly and with a strong directional bias towards foci of infection (Fig. 6E and F and Supplementary movie 9). Neutrophils in MC-21 treated mice migrated with a high median meandering index of 0.79 ± 0.11 (Fig. 6G) and median track velocity of $11.88 \pm 2.11 \mu\text{m min}^{-1}$ (Fig. 6H) similar to mice given isotype control antibody. The fact that neutrophil recruitment was unaffected MC-21 treatment suggests that neutrophils do not upregulate CCR2 in our acute infection model, in contrast to findings in other models [37]. Importantly, these data suggest that CCR2⁺ monocytes are not required for early neutrophil recruitment in response to bacterial challenge.

4. Discussion

Here, we describe an accelerated mouse arthritis model that is optimized for 2P imaging. The aSTA model resembles the established *i.v.* serum transfer model in terms of histopathology, but has a shorter disease course and more synchronized cell trafficking behavior. In the systemic K/B×N serum transfer arthritis, small soluble immune complexes are formed in the circulation and rapidly enter the distal joints to produce a polyarthritis dependent on both neutrophils and mast cells [38,39]. In the aSTA model, the inflammatory response is more localized because the *s.c.* injection of serum bypasses the initial cell-mediated transport across the endothelium and presumably allows immune complexes to form directly in the injected tissue. Once formed, immune complexes can stimulate mast cell degranulation leading to increased vascular permeability. This may allow arthritogenic serum that was returned to the circulation via lymphatic drainage to re-enter the tissue and deposit immune complexes at the inflamed joint, thus further promoting complement activation and leukocyte recruitment. Despite the fact that aSTA is not an appropriate model of chronic disease, it provides an opportunity to assess distinct neutrophil trafficking steps *in vivo*, and therefore could be useful for screening anti-inflammatory drug candidates and ascertaining their precise mechanism of action.

Macrophage infiltration is a salient feature of human RA. In mice, the depletion of monocytes/macrophages by *i.p.* CL-treatment has been shown to prevent arthritis [32]. Generally, monocytes are presumed to function as precursor cells giving rise to tissue macrophages, which produce pro-inflammatory cytokines and contribute to joint destruction. Our findings suggest that during arthritis induction, monocytes also play a key role in regulating neutrophil recruitment, specifically by enhancing TEM efficiency. Although a single dose of *i.v.* CL will deplete phagocytes through out the body [31], we found it necessary to administer CL daily to inhibit neutrophil recruitment. Moreover, the depletion of local macrophages with *s.c.* CL did not inhibit neutrophil extravasation. These observations suggests that monocytes, not tissue resident macrophages, are the source of signals that regulate neutrophil extravasation in our aSTA model.

The finding that monocytes are required for efficient neutrophil extravasation at sites of aseptic inflammation indicates the existence of substantial cross-talk between these two leukocyte subsets. The interdependence of monocyte and neutrophil recruitment has been examined in a variety of animal models, but these studies have reached different conclusions [16,40,41]. Henderson et al. found that monocytes were recruited simultaneously with neutrophils and the recruitment of monocytes occurred independently of neutrophils in a thioglycollate-induced peritonitis model [40]. In contrast, neutrophils facilitated monocyte extravasation following platelet activation factor-induced inflammation [41]. In another

study, Maus et al. used a bone marrow chimera approach to demonstrate that CCR2⁺ monocytes facilitated neutrophil emigration during acute lung inflammation induced with CCL2 and LPS [16]. The different conclusions reached in various models suggests that leukocyte recruitment mechanisms are likely tissue and inflammation dependent [25,42]. Our findings in the aSTA model have uncovered a role for CCR2⁺ monocytes in facilitating neutrophil recruitment during arthritis. Furthermore, our study extends on previous work by showing that CCR2⁺ monocytes increase the efficiency of neutrophil TEM.

The targeting monocytes and their chemokine signals to inhibit arthritis has been explored extensively in animal models. Treatment with anti-fractalkine antibody was shown to ameliorate disease in a collagen-induced arthritis model [11]. However, we found that arthritis induction was normal in both CX₃CR1^{gfp/gfp} mice and mice treated with neutralizing doses of anti-CX₃CR1 blocking antibody. Furthermore, the deficiency of CX₃CR1 didn't affect CX₃CR1-GFP cell recruitment, suggesting that the receptor itself is dispensable for certain aspects of monocyte trafficking. CX₃CR1^{hi} monocytes have been reported to patrol blood vessels and extravasate rapidly in response to tissue damage or infection and contribute to the early inflammatory response through the secretion of TNF-α [14]. In contrast, the recruitment kinetics of CX₃CR1-GFP cells appears to be delayed in our model. We observed CX₃CR1-GFP cells patrolling vessels occasionally in the steady-state and shortly after *s.c.* serum transfer, but these cells did not appear to arrest or extravasate in our imaging experiments (1–2 h after serum transfer). However, a large number of CX₃CR1-GFP cells were observed on day 5 after serum injection, and therefore it is possible that they contribute to pathology at later stages.

In contrast to results with CX₃CR1 inhibition, the depletion of CCR2⁺ monocytes inhibited neutrophil extravasation, albeit less strongly than CL-treatment. Previous studies have shown that CCR2⁺ monocytes are common in the synovial fluid and synovial tissue of patients with different forms of arthritis [43]. In addition, small molecule inhibitors of CCR2 have been efficacious in several rodent models of arthritis [44]. Despite these promising preclinical studies, human clinical trials have yielded disappointing results; small molecule inhibitors and blocking antibodies to CCR2 failed to show meaningful clinical improvement in RA, despite the fact that CCR2⁺ monocytes were unable to respond to chemokine gradients generated within inflamed joints [45]. It is also noteworthy that Ccr2^{-/-} mice display accelerated and severe arthritis in both collagen-induced arthritis and autoantibody-induced arthritis [35,46]. The lack of consistent efficacy of CCR2 blockade in different models [47] suggest that perhaps the depletion of CCR2⁺ monocytes might provide more efficacious treatments. Indeed, the depletion of CCR2⁺ monocytes with MC-21 markedly improved established arthritis [36], consistent with our results in the aSTA model.

Previously, Angyal et al. used 2P imaging to lymphocyte recruitment to the ankle during proteoglycan-induced arthritis [48]. Our work provides the first real-time description of neutrophil trafficking dynamics *in vivo* during arthritis induction and implicates a role for CCR2⁺ monocytes in regulating neutrophil extravasation during aseptic inflammation. Identifying the molecular mechanism(s) through which CCR2⁺ monocytes regulate neutrophil recruitment is the focus of ongoing studies. Several possibilities exist for both direct and indirect effects. For example, the binding of monocyte β2 integrins to endothelial ICAM-1 has been shown to induce endothelial cell activation and reactive oxygen species production leading to altered endothelial shape [49], which may facilitate neutrophil extravasation. Alternatively, CCR2⁺ monocytes may act indirectly by secreting cytokines and chemokines to potentiate neutrophil transmigration [50]. In fact, multiple cellular and molecular mechanisms are likely to exist and may vary in importance depending on the inflammatory stimulus and the tissue context. Indeed, our results demonstrated that CCR2⁺ monocytes were required for efficient neutrophil extravasation and trafficking during aseptic

arthritis, but were dispensable for recruitment in response to acute bacterial infection. This difference may reflect the relative magnitude of the inflammatory response to bacterial challenge or may imply the existence of distinct leukocyte recruitment mechanisms. Our findings raise the possibility that targeting monocytes could provide safer and more selective anti-inflammatory therapies for autoimmune diseases such as inflammatory arthritis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Peggy Gieselman for animal breeding and care and Andy Gelman (Wash U) and Mark Thiede (Pfizer) for helpful advice. This work was supported by the Washington University in St Louis/Pfizer, Inc. Biomedical Agreement and the NIH R01 AI0776003 (MJM).

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Fig. 1.

The accelerated serum transfer arthritis mouse model recapitulates important features of the *i.v.* K/B $\times$ N serum transfer model. (A-C) The recruitment of neutrophils (green) at the indicated time points in the paw of LysM-GFP mice. (A) 45 min after serum transfer, neutrophils (arrowhead) arrest within vessels (red). (B) 20 h, extravascular neutrophils (arrowheads) migrate through the collagen rich interstitium (blue). (C) 72 h, neutrophils form dynamic clusters near the joint (arrowheads). The dotted lines indicate the joint space based on the second harmonic generation signal. $n = 7$, (Scale bar = 40 μ m). (D) Arthritis development in *i.v.* versus *s.c.* serum transfer models. Arthritis was evaluated by measuring an increase in ankle thickness. Data are expressed as the mean change in ankle thickness $\pm$ SD; $n = 8$. (E) Representative hematoxylin-eosin staining of a tarsal joint. Leukocyte influx within joint synovium is indicated (asterisk). In addition, a focal area of bone resorption is visible (open arrows), $n = 3$. (F) The loss of chondrocytes and cartilage matrix is apparent (closed arrows), as well as multifocal areas of cartilage ulceration (asterisks) with fibrovascular (pannus) invasion of the bone marrow space (open arrows), $n = 3$. (G-I) Neutrophil (green) recruitment in the paw of LysM-GFP mice after *s.c.* serum transfer. (G) 40 min after serum transfer, neutrophils (arrowhead) arrest within vessels (red). (H) 60 min, neutrophils migrate away from extravasation sites through interstitial tissue. (I) 6 h, neutrophils cluster near the joint (arrowheads), $n = 8$. (Scale bar = 40 μ m).

Fig. 2.

Neutrophil trafficking behavior in the paw of LysM-GFP mice after *s.c.* K/BxN serum transfer. Time-lapse images show, (A) the recruitment and migration dynamics of neutrophils. Representative cell tracks with indicated cell positions (arrowheads), $n = 8$. (Scale bar = $40 \mu\text{m}$). Time stamps show relative time in min:sec. (B) Representative extravascular neutrophil tracks imaged 45–75 min after serum injection. The plot is an overlay of 49 individual neutrophil tracks normalized to their starting positions. Tracks from three independent experiments are shown in different colors. (C) Meandering index and (D) median track velocity are plotted for data in (B). Flow cytometric analysis of freshly disaggregated joint myeloid cells (E) gated on CD11b⁺ cells and assessed for Ly6G⁺ neutrophils at 2 h ($T = 2$) after *s.c.* serum injection and (F) gated on CD11b⁺ and CD115⁺ to assess the infiltration of Ly6C^{high} vs. Ly6C^{low} monocytes.

Fig. 3.

CL-mediated monocyte depletion prevents K/BxN serum induced arthritis. (A) Co-localization of Q-dot⁺ cells (red, indicated by white arrowhead) with neutrophils (green). The magenta arrowheads indicate sites of neutrophil extravasation. Scale bar = 20 μ m, $n = 5$. Time stamps show relative time in min:sec. (B) 45 min post injection of 655-nm nontargeted Q-dots, Q-dot⁺ cells in peripheral blood are predominantly CD115⁺ monocytes, $n = 5$. (C) Circulating monocyte numbers after daily *i.v.* CL-treatment (initial dose of 200 μ l followed by 100 μ l) assessed by flow cytometry. Peripheral blood cells were stained with CD115 (clone AFS98) and appropriate isotype control (BD PharMingen), $n = 5$. (D) The effect of CL-treatment in the aSTA model. Mice were pretreated with *i.v.* CL or PL and then challenged *s.c.* with K/BxN serum. CL-treatment fully blocked serum induced ankle swelling. Data are expressed as mean change in ankle thickness $\pm$ SD, $n = 7$. (E) 18 h after serum injection, foodpads were harvested, digested and single cell suspensions stained with antibodies to CD11b and Ly6G. CL-treatment reduced the recruitment of Ly6G high monocytes, but not Ly6G low monocytes.

Fig. 4.

CL-treatment inhibits neutrophil extravasation during arthritis. (A) Time-lapse 2P images were acquired 40 min after serum transfer. Neutrophils (green) arrested along the vessel (red), but displayed impaired extravasation and disrupted chemotaxis in parenchymal tissues. Representative cell tracks are shown and cell positions indicated (arrowheads), $n = 5$. (Scale bar = 40 μm). Time stamps show relative time in min:sec. (B) The percentage of intravascular neutrophils in CL and PL-treated mice after serum transfer. Time-lapse videos were captured beginning 40 min after *s.c.* serum transfer and the percentage of intravascular neutrophils that co-localized with blood vessels was analyzed using Velocity software, $n = 5$. (C) Representative tracks of extravascular neutrophils in CL-treated (left panel) and control PL-treated mice (right panel) imaged 45–75 min after serum injection. Plots show an overlay of 36 individual neutrophil tracks normalized to their starting positions. Tracks from three independent experiments are shown in different colors. (D) Meandering index and (E) median track velocity of neutrophils from CL-treated (open square) and PL-treated (open triangle) mice are plotted for tracks in (C). (F) Neutrophil trafficking behaviors in *i.v.* CL-pretreated mice 30–50 min after *s.c.* *Lm* challenge. Representative cell tracks are shown and cell positions indicated (arrowheads). Time stamps show relative time in min:sec, $n = 5$. (Scale bar = 40 μm). (G) The meandering index and (H) the median track velocity of neutrophils from CL-treated (open square) and PL-treated (open triangle) mice are plotted, $n = 5$.

Fig. 5. CX₃CR1-GFP cell trafficking behavior in the steady-state and after *s.c.* serum transfer. (A) Intravascular behavior of CX₃CR1-GFP cells (green) in the steady-state and (B) 40 min after serum transfer. Representative cell tracks are shown and cell positions indicated (arrowheads), $n = 8$. (Scale bar = 40 μ m). The median track velocity of CX₃CR1-GFP cells inside the vessel was $5.996 \pm 2.07 \mu$ m/min. (C) Day 5 after serum transfer, CX₃CR1-GFP cells localized near the joint (arrows). Dotted lines indicate the joint space based on the second harmonic generation signal, $n = 5$. (Scale bar = 40 μ m). (D) Arthritis development in $Cx3cr1^{gfp/gfp}$ and control mice and (E) after treatment with blocking antibody to CX₃CR1 or isotype matched control antibody. Mice were injected with 50 μ l of K/B $\times$ N serum *s.c.* and ankle thickness was measured daily. Data are expressed as the mean change in ankle thickness $\pm$ SD, $n = 6$. (F) Anti-CX₃CR1 blocking antibody did not inhibit neutrophil (green) intravascular arrest and (G) extravascular trafficking after *s.c.* serum transfer. Representative cell tracks are shown and cell positions indicated (arrowheads). Time stamps show relative time in min:sec, $n = 5$. (Scale bar = 40 μ m).

Fig. 6.

MC-21 monoclonal antibody treatment impairs neutrophil extravasation and subsequent chemotaxis. Mice were pretreated with either MC-21 monoclonal antibody or isotype control antibody (Iso). (A) Time-lapse images were recorded in LysM-GFP mice 40 min after serum injection. Neutrophils (green) displayed impaired extravasation (asterisks) from blood vessels (outlined by magenta dotted lines) and reduced directional migration away from vessels. Representative cell tracks are shown and cell positions indicated (white arrowheads). Cell clusters near sites of extravasation are indicated by yellow arrowheads, $n = 5$. (Scale bar = 40 μm). Time stamps show relative time in min:sec. (B) Representative tracks of extravascular neutrophils in the tissue of mice treated with MC-21 or control antibody imaged 45–75 min after serum injection. An overlay of 36 individual neutrophil tracks normalized to their starting positions. Tracks from three independent experiments are displayed in different colors. (C) Meandering index and (D) median track velocity of neutrophils from MC-21-treated (open square) and isotype control-treated (open triangle) mice are plotted for tracks in (B). (E and F) Neutrophil recruitment and trafficking to Lm challenge is not inhibited by MC-21-pretreatment. Mice were imaged 45 min after challenge. Representative cell tracks are shown and cell positions indicated (arrowheads). Scale bar = 40 μm , $n = 5$. (G) Meandering index and (H) median track velocity of neutrophils from MC-21-treated (open square) and isotype control-treated (open triangle) mice are plotted, $n = 5$.