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An Independent Subset of TLR Expressing CCR2-Dependent Macrophages Promotes Colonic Inflammation

Andrew M. Platt, Calum C. Bain, Yvonne Bordon, David P. Sester, and Allan Mcl. Mowat

Macrophages (Mφs) in the large intestine are crucial effectors of inflammatory bowel disease, but are also essential for homeostasis. It is unclear if these reflect separate populations of Mφs or if resident Mφs change during inflammation. In this study, we identify two subsets of colonic Mφs in mice, whose proportions differ in healthy and inflamed intestine. Under resting conditions, most F4/80+ Mφs are TLR2+ CCR2+ CX3CR1hi and do not produce TNF-α in response to stimulation. The lack of TLR expression is stable, affects all TLRs, and is determined both transcriptionally and posttranscriptionally. During experimental colitis, TLR2+ CCR2+ CX3CR1int Ly6Chi Gr-1+, TNF-α-producing Mφs come to dominate, and some of these are also present in the normal colon. The TLR2+ and TLR2− subsets are phenotypically distinct and have different turnover kinetics in vivo, and these properties are not influenced by the presence of inflammation. There is preferential CCR2-dependent recruitment of the proinflammatory population during colitis, suggesting they are derived from independent myeloid precursors. CCR2 knockout mice show reduced susceptibility to colitis and lack the recruitment of TLR2+ CCR2+ Gr-1+, TNF-α-producing Mφs. The balance between proinflammatory and resident Mφs in the colon is controlled by CCR2-dependent recruitment mechanisms, which could prove useful as targets for therapy in inflammatory bowel disease. The Journal of Immunology, 2010, 184: 6843–6854.

The two main forms of inflammatory bowel disease (IBD), Crohn’s disease and ulcerative colitis, are chronic, relapsing inflammatory disorders that remain difficult to manage despite recent therapeutic advances such as anti–TNF-α (1). Therefore, it is important to understand the mechanisms of tissue damage, the cells involved, and the processes of cellular infiltration and inflammation. Macrophage (Mφ) infiltration and activation are central features of IBD, participating in a chronic inflammatory response driven by recognition of commensal microbiota by proinflammatory CD4+ T cells (2). The subsequent production of TNF-α and other mediators by the activated Mφ plays a crucial role in the tissue damage (3, 4), and these are important targets for therapy. If it could be shown that a distinct population of Mφs is responsible for these inflammatory effects, targeting these cells could prove an even more direct and effective means of disease modification.

Mφs are also abundant in the normal intestine, especially the colon, where they sit in close proximity to the enormous population of commensal microbiota. Despite the resulting potential for Mφ activation, inflammation does not develop in the healthy colon, and, indeed, resident colonic Mφs have crucial physiological functions, both by restricting invasion by commensals and by maintaining epithelial integrity (5–7). This raises intriguing questions about how colonic Mφs can play such important but apparently distinct roles in healthy and inflamed intestine. Studies in humans have suggested that this may reflect the presence of two different populations of Mφs, one of which is inherently non-inflammatory (resident) and the other that has potent proinflammatory properties (8, 9). However, this has never been studied under defined experimental conditions, and the processes by which Mφ behavior can alter so dramatically between the resting and inflamed intestine remain unknown. One possibility is that the resident cells themselves change during inflammation; alternatively, the proinflammatory Mφ may reflect a distinct population of newly arrived cells that replace the inert, resident cells. If the latter is the case, it would be important to define these subsets at the cellular level, understand how their accumulation is controlled, and explore whether neutralizing the recruitment of these new cells might allow specific therapy against IBD.

That colonic inflammation could be driven by a new subset of Mφs has never been proven formally, but would be consistent with current ideas of Mφ biology, which suggest that there may be two independent subsets of tissue Mφ derived from different monocyte precursors. In mice, CX3CR1hi Ly6Clo CCR2lo monocytes are proposed to give rise to long-lived resident tissue Mφs, whereas CX3CR1lo Ly6Chi CCR2hi monocytes give rise to inflammatory Mφs in inflamed tissues such as the peritoneum, as well as dendritic cells in the gut (10, 11). A similar dichotomy has been described in humans (12–14). Although this pattern is consistent with the different properties of Mφs in the inflamed and resting...
Materials and Methods

Mice

C57BL/6 (B6) (CD45.2+), congenic C57BL/6.SJL (CD45.1+), CX3CR1gfp/+ (both obtained originally from Dr. W. Agace, University of Lund, Lund, Sweden), and CCR2 KO mice backcrossed nine times onto the B6 background (CD45.2+) (The Jackson Laboratory, Bar Harbor, ME) were maintained under specific pathogen-free conditions in the Central Research Facility at the University of Glasgow, Glasgow, Scotland. Unless stated otherwise, mice were first used at 6 to 8 wk of age. All experiments were performed according to United Kingdom Home Office and Local Ethical Committee regulations.

Generation of bone marrow-derived and peritoneal Mφs

Bone marrow Mφs (BMMs) were obtained from B6 mice by culture of bone marrow (BM) cells in 20% M-CSF (supernatant from the L929 cell line) at 37°C in 5% CO2 for 6 d. The cells obtained were typically >90% F4/80+ positive. Resting peritoneal Mφs were obtained by flushing out the peritoneal cavity of normal mice with 10 ml 1 ml i ice-cold EDTA/PBS.

Isolation of colonic lamina propria cells

The large intestines of mice were removed and washed in PBS (Life Technologies/Invitrogen, Paisley, U.K.), and the fat was removed. The intestines were opened longitudinally, washed in 2% FCS (Life Technologies) in HBBS (Life Technologies), and cut into 0.5-cm sections. Tissue was then placed in 10 ml HBBS/2% FCS and shaken vigorously, and the supernatant was discarded. A total of 10 ml fresh calcium/magnesium-free HBSS (Life Technologies) containing 2 mM EDTA (Sigma-Aldrich), DsRed (BD Biosciences) was added to the cells before acquisition to enable exclusion of dead cells. For intracellular staining, cells were first activated in vitro by TLR ligands in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich) for 4.5 h and stained for cell-surface markers. They were then fixed in 1% formaldehyde (BDH Laboratory Supplies, Letchworth, U.K.) in PBS for 10 min at 4°C and permeabilized with Perm stain (BD Pharmingen, San Diego, CA) before blocking with 10% FCS and staining with fluorochrome-conjugated anti-cytokine Abs at a 1:200 dilution. Dead cells were excluded by adding 0.1 μg/ml ethidium mononucleoside (Molecular Probes, Eugene, OR) prior to permeabilization. All cells were analyzed on an FACS calibur Flow Cytometer (BD Biosciences) using FlowJo software (Tree Star, Ashland, OR).

Induction of DSS colitis

C57BL/6, CX3CR1gfp/+ and CCR2 KO mice received 2% DSS salt reagent grade (molecular mass 36,000–50,000 kDa) (MP Biomedical, Solon, OH) ad libitum in sterile drinking water for 7 d. Controls received water alone. Animals were monitored daily and scored for clinical disease based on the following parameters: 1) weight loss (0–3); 2) diarrhea (0–3); and 3) rectal bleeding (0–3).

Assessment of cell turnover in vivo

After administration of BrdU (BD Pharmingen) (1 mg/ml i.p. or 0.8 mg/ml in drinking water), the incorporation of BrdU was measured using the BrdU Flow Kit (BD Pharmingen) as per the manufacturer’s instructions. Cells in cell division were detected by permeabilization and staining with PE-conjugated anti–Ki-67 Ag (BD Pharmingen).

Assessment of BM cell trafficking in vivo

A 1:1 mixture of BM cells from wild-type (WT) CD45.1+ B6.SJL and CCR2 KO (CD45.2+) mice was labeled with 5 μM CellTrace Far Red DDAO-SE (Invitrogen) in HBBS at room temperature for 5 min, then washed twice with 5% FCS/PBS. A total of 12 × 10^6 labeled cells in 0.2 ml PBS was then injected i.v. into WT B6 (CD45.2+) mice that had received 2% DSS in their drinking water for the previous 5 d or into control mice that had received normal water. Cells were harvested from the spleens and colons of recipient mice 24 h later and stained with anti-F4/80 (PE), and anti-CD45.1 (FITC). The percentages of Far Red-labeled, CD45.1+ and F4/80+ cells were determined by flow cytometry, and dead cells were excluded from analyses by staining with propidium iodide.

Measurement of cytokines and chemokines in colon cultures

Colon samples were collected under sterile conditions and washed in PBS (Life Technologies) supplemented with 1% penicillin/streptomycin (Life Technologies). Three segments from the distal colon of 1 cm in length were placed in 24 flat-bottom well culture plates (Costar, Cambridge, MA) containing fresh RPMI 1640 (Life Technologies) supplemented with 1% penicillin/streptomycin and incubated at 37°C for 24 h. Culture supernatants were then harvested, centrifuged at 13,000 × g, and stored at −20°C. Subsequently, TNF-α was measured using ELISA, and CCL2 levels were quantified using Luminex Multiplex bead assay (BioSource International) according to the manufacturer’s instructions.
Measurement of cytokine and chemokine production by ELISA and Luminex

 Supernatants from cell cultures were harvested and stored at −20°C until cytokine production was quantified using sandwich ELISA as previously described (15). Cytokine concentrations in test supernatants were determined with a standard curve constructed using serial dilutions of the standard cytokines. For Luminex, CCL2, CCL3, CCL4, CCL5, CCL19, CXCL10, fibroblast growth factor, GM-CSF, IFN-γ, IL-1α, the β form of pro-IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p40 and p70), IL-13, IL-17, KC, monokine induced by IFN-γ, and TNF-α production was quantified simultaneously using the Multiplex Bead Assay (BioSource International) and analyzed using a Luminex XMAP system (BioSource International) according to the manufacturer’s instructions.

Analysis of mRNA expression by PCR

 Total RNA was extracted from purified F4/80+ Mψs using the RNeasy Micro or Mini Kit (Qiagen, West Sussex, U.K.) as per the manufacturer’s instructions. Contaminating genomic DNA was removed on-column with the RNase-free DNase Set (Qiagen). cDNA was reverse transcribed from DNase-treated RNA using Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. End-product PCR amplifications were performed using the FTGENE95D thermocycler (Techne Cambridge, Duxford, Cambridge, U.K.). Using the primers listed in Supplemental Table II, PCR samples were run on a 2% agarose gel (2% Tris-acetate-EDTA, 0.1× TBE) with 16.8 × 10−6 M ethidium bromide (Sigma-Aldrich) in 0.5× TBE buffer (0.275% boric acid [Fisher Scientific, Letchestershire, U.K.] 2 mM EDTA, 0.5% Tris base [Fisher Scientific]), and analyzed using the Gel Logic 200 imaging system (Eastman Kodak, Hemel Hempstead, U.K.).

Quantitative/real-time PCR

 Relative levels of mRNA were examined using TaqMan chemistry, using the primers and fluorogenic probes described in Supplemental Table II, PCR reactions were performed in a 96-well fast plate in the ABI Prism 7900 Sequence Detector (Applied Biosystems, Foster City, CA) on fast mode. Each PCR reaction was performed in triplicate using the following cycle conditions: 2 min at 50°C and 10 min at 94°C, followed by a total of 40 cycles of 15 s at 94°C and 1 min at 60°C. Cycle threshold values were calculated and data analyzed using the RQ Manager software (Applied Biosystems), and samples were normalized by their reference reporter hypoxanthine-guanine phosphoribosyltransferase.

Statistical analysis

 Results are shown as means ± SD unless stated otherwise, and groups were compared using a Student two-tailed paired t test or one-way ANOVA followed by a Bonferroni multiple comparison test. The rate of Mψ turnover was calculated using linear regression analysis, in which best-fit values were used to calculate the difference between slopes.

Results

Intestinal Mψs differ between resting and inflammatory conditions

 To begin to understand how local Mψ populations might vary under normal and pathological conditions, we compared Mψs from the resting colon with those found after the induction of colitis by feeding 2% DSS. Large numbers of F4/80+ Mψs were present in the LP of the normal mouse colon, especially in the area immediately below the epithelium (Fig. 1A); these were readily detectable in preparations isolated from the colon, accounting for ~27% of all CD45+ cells (0.61 × 106 ± 0.3 F4/80+ cells/mouse; Fig. 1B). Mice fed DSS began to lose weight after 5 d and developed diarrhea, rectal bleeding, shortening of the colon, and loss of normal intestinal architecture (data not shown). This was accompanied by a large infiltrate of additional F4/80+ Mψs into the inflamed colon, which was already significant by day 4 of colitis and increased further by day 7 (Fig. 1A, 1B).

 The increased infiltration by Mψs in colitis was accompanied by the production of TNF-α by the inflamed mucosa (Fig. 1C). This was found only in the distal colon, where the DSS-induced inflammation was focused, and TNF-α production was negligible in either the distal or proximal colon of control mice. Intracellular staining showed that ~12% of colon cells were producing TNF-α spontaneously by day 7 of colitis, the majority of which were F4/80+ CD11b+ and had the forward light scatter/side scatter characteristics of monocytes/Mψs on FACS analysis (Fig. 1D and data not shown). By days 5 and 7 of disease, ~50% of F4/80+ cells produced TNF-α spontaneously, and further in vitro stimulation with LPS did not alter the frequency of these cells (Fig. 1D, 1E). In contrast, TNF-α-producing cells were absent from control colon, even after stimulation with LPS in vitro. The appearance of inflammation and TNF-α-producing cells was associated with infiltration by recently divided Mψs, as shown by BrdU pulse-chase experiments. A total of 4.1 ± 0.6% of the total F4/80+ Mψs population was recruited to the resting colon LP was labeled by a single 24-h pulse with BrdU, and this proportion increased dramatically during colitis, with 16.8 ± 5.1% and 18.9 ± 4.7% F4/80+ Mψs being BrdU+ after a 24-h pulse of BrdU given on days 3 and 5 of disease, respectively (Fig. 1F, 1G). Together, these results show that the presence of inflammation produces intense infiltration of the colonic mucosa by Mψs that differ markedly from their resident counterparts in terms of cytokine production and turnover.

Unique unresponsiveness of intestinal Mψs to stimulation

 The findings above showed that a major difference between Mψs from inflamed and normal colon was in their ability to produce TNF-α. To explore this in more detail, we examined whether the unresponsiveness of normal murine colonic Mψs extended to other cytokines and stimuli. As expected, in vitro-generated BMMs and resting peritoneal exudate cell (PEC) Mψs produced high levels of TNF-α, IL-6, and IL-12p70 after stimulation with LPS and/or IFN-γ, as well as the proinflammatory chemokines CCL5, CCL19, CXCL10, fibroblast growth factor, GM-CSF, IFN-γ, and TNF-α, as well as intracellular TLR3 and TLR9, none of these mediators under any of the conditions. This was confirmed at the cellular level and using BLP and MDP, the ligands for TLR2 and intracellular nucleotide-binding oligomerization domain 2, respectively, as stimuli. Thus, virtually no F4/80+ Mψs produced high levels of TNF-α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p40 and p70), IL-13, IL-17, KC, monokine induced by IFN-γ, and TNF-α immediately below the epithelium (Fig. 1A, 1B). More colonic Mψs from normal mice produced little or none of these mediators under any of the conditions. This was confirmed at the cellular level and using BLP and MDP, the ligands for TLR2 and intracellular nucleotide-binding oligomerization domain 2, respectively, as stimuli. Thus, virtually no F4/80+ Mψs produced high levels of TNF-α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p40 and p70), IL-13, IL-17, KC, monokine induced by IFN-γ, and TNF-α after a 24-h pulse of BrdU given on days 3 and 5 of disease, respectively (Fig. 1F, 1G). Together, these results show that the presence of inflammation produces intense infiltration of the colonic mucosa by Mψs that differ markedly from their resident counterparts in terms of cytokine production and turnover.
expressed TLR4 (Supplemental Fig. 1A), but overall, very few of these cells were TLR4+ (mean 3.4 ± 0.9%). The lack of TLR2 and 4 expression by colonic Mφs did not require constant exposure to the local microenvironment, as FACS-sorted colonic Mφs failed to express these proteins after overnight culture in medium (Supplemental Fig. 1A). In addition, it was not due to the enzymatic digestion protocol used to obtain colonic Mφs, as identical treatment of PEC Mφs had no effect on their expression of TLR (Supplemental Fig. 1B).

To examine whether the lack of TLR expression occurred at the transcriptional or posttranscriptional level, we assessed mRNA expression by purified colonic, BM, and PEC Mφs (90–95% positive for F4/80). End-product PCR analysis indicated the presence of TLR1–7 and TLR9 mRNA in all populations, although TLR5 appeared less intense than the others, and TLR7 mRNA was virtually absent from colonic Mφs (Supplemental Fig. 2A). Quantitative PCR analysis of TLR2, -4, and -9 mRNA as representative surface and intracellular TLRs showed that BMM expressed high levels of mRNA for all these TLRs, as did PEC Mφs, but at lower levels (Fig. 3E). In contrast, colonic Mφs expressed virtually no TLR2 or TLR4 mRNA, but expressed similar levels of TLR9 mRNA to those found in BMM, despite identical defects in protein expression for the different TLRs. Thus, the hyporesponsiveness to stimulation is associated with the global absence of TLR protein expression by most resident Mφs. This is a stable phenotype that appears to be regulated at the transcriptional and/or posttranscriptional level depending on the individual TLR.

The functional differences between colonic Mφs in healthy and inflamed intestine were accompanied by alterations in TLR expression. In contrast to normal colon Mφs, the majority of F4/80+ cells from mice with DSS colitis expressed TLR2 on day 4 of colitis, and this was still the case at the peak of disease on day 7 (69.1 ± 10.6% and 75.9 ± 15.1%, respectively; Fig. 3F, 3G). A significant proportion of F4/80+ cells consistently expressed surface TLR4 during colitis, but no intracellular TLR3 and -9 expression could be detected in F4/80+ cells from colitic

FIGURE 1. Mφ properties and turnover in the resting and inflamed colon. A, F4/80+ Mφs (red) in the distal colon of B6 mice given water or 2% DSS for 7 d to induce colitis (original magnification ×20). Arrow indicates the epithelium, with nuclei stained blue. B, Absolute numbers of F4/80+ cells isolated from colonic LP of control mice and mice fed DSS for 4 or 7 d. The data shown are the means ± 1 SD for four mice/group. C, Production of TNF-α by explants of proximal or distal colon from mice fed water or DSS for 7 d. Results are shown as the mean cytokine concentration (pg/ml) ± 1 SD for three mice/group. D, Intracellular TNF-α production by F4/80+ cells from the colon of control mice and from mice on day 7 of DSS colitis assessed after culture in medium or 1 µg/ml LPS for 4.5 h. TNF-α+ cells in colitis were all CD11b+. E, Frequency of spontaneous and LPS-induced TNF-α+ cells among F4/80+ Mφs isolated from control or inflamed colon on day 7 of DSS colitis. The data shown are the mean percentage of F4/80+ cells that also express TNF-α ± 1 SD for four mice/group. F and G, In vivo incorporation of BrdU by Mφs from normal and inflamed colon. Representative dot plots of colonic F4/80+ and BrdU+ cells from control mice and mice fed DSS for 5 d, injected with 1 mg BrdU i.p, and culled 1 d later (F). Mean percentages ± 1 SD of colonic F4/80+ cells that have incorporated BrdU in four mice/group after injection with BrdU on day 3 or 5 of colitis. The data shown are representative of four independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 as determined by one-way ANOVA, followed by Bonferroni’s multiple comparison test.
mucosa (Supplemental Fig. 3A–C). These data indicate that TLR expression by colonic Mϕs is highly dependent on the presence of inflammation, with a dramatic increase in TLR2-expressing Mϕs during colitis. This could reflect the presence of independent subsets of TLR+ and TLR− Mϕs, or alternatively, TLR− resident Mϕs could change phenotype during inflammation.

**TLR2 expression defines unrelated Mϕ subsets with distinct turnover dynamics**

To address how these Mϕ populations might be related, we examined for evidence that they might give rise to each other by exploring their turnover kinetics in vivo. After a single pulse-chase with BrdU, there was a striking dichotomy between the uptake shown by TLR2+ and TLR2− subsets in the resting colon, with significantly more TLR2+ F4/80+ cells having proliferated than the majority TLR2− subset (6.0 ± 0.8% BrdU+ versus 1.3 ± 0.5%, respectively; Fig. 4A, 4B). This equated to >70% of the F4/80+BrdU+ cells expressing TLR2. The proportion of TLR2+ F4/80+ cells incorporating BrdU after a single pulse increased significantly during colitis (21.0 ± 5.7% and 21.9 ± 2.4% on days 3 and 5 respectively; Fig. 4A, 4B), and on day 5–6, >96% of the F4/80+BrdU+ cells expressed TLR2. In contrast, there was no significant increase in BrdU uptake by the TLR2− fraction of colonic Mϕs during colitis (4.7 ± 1.4% and 5.9 ± 5.0% on days 3 and 5, respectively). Local proliferation seemed unlikely to account for the large increase in BrdU+ cells in the inflamed colon, as very few Ki-67+ F4/80+ cells could be seen in the resting colon, and there was only a minor change in colitis (0.5 ± 0.1% and 1.5 ± 0.3% respectively; Fig. 4C).

To gain a better idea of the turnover of Mϕ subsets in the normal and inflamed colon, we used a long-term BrdU administration protocol in which control or DSS-fed mice were fed BrdU continuously throughout the course of colitis. In normal colon, the percentage of total F4/80+ Mϕs that were BrdU+ rose steadily from 4.0 ± 0.2% on day 1 to 18.4 ± 2.9% and 24.1 ± 2.7% after 4 and 6 d, respectively (Supplemental Fig. 3F). A similar increase in BrdU uptake by total F4/80+ cells also occurred in colitic mice, rising to levels significantly above those in controls on days 4 and 6 of colitis (26.7 ± 3.3% and 39.8 ± 3.8%, respectively). This corresponded to overall turnover rates of the total F4/80+ population of 4.1 ± 0.4% Mϕs per day under resting conditions and 7.1 ± 0.5% Mϕs per day during colitis (data not shown). Dramatic differences between the Mϕ subsets defined by TLR2 expression were seen when long-term BrdU incorporation was compared. As before, the TLR2+ F4/80+ subset showed greater initial BrdU uptake than the equivalent TLR2− subset in control mice (7.8 ± 0.8% on day 1), and this rose significantly to 37.3 ± 2.6% and 44.0 ± 5.9% after 4 and 6 d of administering BrdU, respectively (Fig. 4D). These values were not altered by the induction of colitis, with identical BrdU uptake at all times by TLR2+F4/80+ cells from DSS-treated and control colons. BrdU incorporation by the TLR2− subset of F4/80+ cells also rose significantly in control colon over time, from 1.9 ± 0.3% on day 1 to 8.8 ± 3.3% and 16.6 ± 3.2% after 4 and 6 d, respectively (Fig. 4D). These levels were significantly lower than the equivalent values among the TLR2+ cells at all times (p = 0.0003). As with the TLR2+ population, BrdU incorporation by TLR2− cells was not altered during colitis and was significantly lower than that of the TLR2+ subset at all times (p ≤ 0.0001). Linear regression analysis showed that TLR2+ Mϕs had significantly higher overall turnover rates than TLR2− Mϕs and that this was not dependent on the presence of inflammation (7.4 ± 0.9% and 8.4 ± 0.9% TLR2+ Mϕs per day versus 2.9 ± 0.4% and 2.5 ± 0.5% TLR2− Mϕs per day in resting and DSS colon). Thus, the subsets of colonic Mϕ defined by TLR2 expression have quite distinct population kinetics in vivo, and these are independent of the presence of inflammation. Together, these findings indicate that the subsets may be stable and distinct populations of myeloid cells.

**Distinct phenotypic properties of TLR2+ and TLR2− Mϕs in the healthy and inflamed colon**

To extend our understanding of these Mϕ subsets in more detail, we compared their phenotype in healthy and inflamed colon. More than 90% of TLR2+F4/80+ cells were class II MHC+ in resting colon

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**FIGURE 2.** Unresponsiveness of resident intestinal Mϕs to stimulation. A. Production of TNF-α, IL-6, and IL-12p70 by colonic Mϕs. PEC Mϕs, and BMMs following stimulation in vitro. BMMs and MACS-purified F4/80+ Mϕs from PEC or colon were cultured for 20 h in medium, 1 μg/ml LPS, 100 U/ml IFN-γ, or LPS + IFN-γ, and cytokine production was measured by ELISA. Results are shown as the mean cytokine concentrations ± 1 SD for three replicates/group. B. BMM and colonic LP cells were cultured in medium, 1 μg/ml LPS, 1 μg/ml BLP, or 10 μg/ml MDP for 4.5 h in the presence of brefeldin A, and intracellular TNF-α was assessed by flow cytometry. Histograms are gated on live F4/80+ cells with the numbers showing the percentages of F4/80+ cells that expressed TNF-α; the shaded histograms represent staining with isotype control Ab. The data shown are representative of three independent experiments. **ppp < 0.001; *pp < 0.01; **p < 0.001 as determined by one-way ANOVA, followed by Bonferroni’s multiple comparison test.”
compared with <40% of the TLR2− cells (Supplemental Fig. 3D). Resting TLR2+ F4/80+ Mφs also expressed the costimulatory molecules CD40, CD80, and CD86, which were virtually absent from the TLR2− cells (Supplemental Fig. 3D). This phenotypic dichotomy was maintained during colitis, in which the majority of TLR2+ F4/80+ cells was strongly class II MHC+ and expressed detectable amounts of CD40, CD80, and CD86, and only low levels of CD80 even in colitic mucosa (Supplemental Fig. 3D). Most TLR2− cells were also CD14hi both under resting conditions and in colitis, with much higher proportions of these cells expressing CD14 and CD11c by PEC Mφs than by BMMs (Supplemental Fig. 1B and data not shown).

A further striking and stable difference between the TLR2+ and TLR2− subsets was in their expression of CCR2, with around 75% of the F4/80+ TLR2+ cells being CCR2+ in both normal and inflamed colon (Fig. 5A). In contrast, only 22.6 ± 4.5% of the TLR2− population expressed CCR2 in the healthy colon, and this did not alter significantly during DSS colitis (27.5 ± 3.0% and 16.6 ± 4.3% on days 3 and 7, respectively). Significantly higher proportions of the TLR2+ subset also expressed Gr-1 both in healthy and inflamed colon compared with the TLR2− subset (Fig. 5B). The relative abundance of TLR2+ and TLR2− Mφs was reflected in distinctive patterns of expression of the monocyte subset markers CX3CR1 and Ly6C (10). Thus, whereas the majority of F4/80+ Mφs in the colon of resting CX3CR1gfp/+ mice expressed high levels of CX3CR1 and were Ly6Clo/Ly6C−, the expression of CX3CR1 by F4/80+ Mφs was much more heterogeneous during DSS colitis, in which novel populations of CX3CR1int and CX3CR1lo cells appeared (Fig. 5C). Of these, the CX3CR1int cells were the largest group, and uniquely, these were Ly6Chi, supporting the appearance of TLR2+ CCR2+ inflammatory Mφs in colitis. TLR2 expression on the subsets was stable in both resting and colitic intestine, as no changes in TLR2 levels were found when sorted TLR2+ and TLR2− Mφs were cultured overnight in either medium or the CCR2 ligand CCL2 (Supplemental Fig. 1C, D and data not shown). Thus, the Mφ populations in healthy and inflamed colon.
colon are phenotypically distinctive and can be characterized by their stable expression of TLR2 among other markers. TLR2 status predicts the functional status of Mφs

To assess the functions of these phenotypically defined subsets, we examined their production of TNF-α by intracellular staining. As noted earlier, the majority of cells producing TNF-α spontaneously in the inflamed colon were F4/80+, and further analysis showed that virtually all of these were in the TLR2+ subset (Fig. 6A,6B). Indeed, up to 60% of TLR2+ F4/80+ cells produced TNF-α spontaneously on days 5 and 7 of colitis, whereas very few TLR2- cells produced TNF-α even in colitis (4.1 ± 1.8% and 6.1 ± 6% on days 5 and 7, respectively).

FIGURE 4. TLR2 expression defines two Mφ populations in the colon with distinct kinetics. A and B, BrdU incorporation by TLR2+ and TLR2- subsets of colonic Mφs in control and inflamed colon. Mice were fed water or DSS, injected with 1 mg BrdU i.p. on day 3 or 5, and culled 1 d later. Representative TLR2 and BrdU staining by F4/80+ cells from the colon of mice fed DSS for 6 d (A). Mean percentages ± 1 SD of F4/80+TLR2+ and F4/80+TLR2- cells that have incorporated BrdU from four mice/group fed DSS for 4 or 6 d (B). C, Colonic LP cells from B6 mice fed water or DSS for 5 d were stained for F4/80 and permeabilized for the detection of Ki-67 by flow cytometry. Results are shown as the mean percentages ± 1 SD of live F4/80+ cells that are Ki-67+ from three mice/group. D, Population kinetics of TLR2+ and TLR2- subsets of Mφs in normal and inflamed colon. Control or DSS fed mice were given a single injection of 1 mg BrdU i.p. on day 0 and then drinking water containing 0.8 mg/ml BrdU thereafter. Colon LP cells were harvested 24, 96, or 144 h later and analyzed for the expression of F4/80, TLR2, and BrdU incorporation by flow cytometry. Results shown are the mean percentages ± 1 SD of live gated F4/80+TLR2+ and F4/80+TLR2- cells positive for BrdU from three mice/group. Data are representative of four (A, B) and two (C, D) independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 as determined by Student two-tailed unpaired t test (C), a one-way ANOVA, followed by Bonferroni’s multiple comparison test (B), and linear regression analysis (D).

FIGURE 5. TLR2 expression defines two Mφ populations in the colon with distinct phenotypic properties in healthy and inflamed intestine. Expression of CCR2 (A) and Gr-1 (B) by TLR2+ and TLR2- F4/80+ cells from the colonic LP of mice fed water or DSS for 3 or 7 d. Results shown are the mean percentages ± 1 SD of F4/80+TLR2+ and F4/80+TLR2- cells that express CCR2 or Gr-1 for four mice/group. C, CX3CR1 expression by F4/80+ cells and Ly6C expression on CX3CR1-defined subsets of F4/80+ cells from the colon of control CX3CR1gfp/+ mice and from CX3CR1gfp/+ mice fed DSS for 4 d. The data are representative of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 by Student two-tailed unpaired t test and one-way ANOVA, followed by Bonferroni’s multiple comparison test.
As the subsets of colonic M\(\phi\)s do not change phenotypically, functionally, or kinetically during inflammation, we hypothesized that the accumulation of TLR2\(^+\) cells in colitis may reflect increased recruitment of these cells, rather than differentiation of the resident population. Specifically, we investigated whether the increased accumulation of TLR2\(^+\) M\(\phi\)s in colitis required the CCR2 chemokine receptor that was expressed selectively by these cells. To do this, we cotransferred Far Red-labeled CD45.1\(^+\) WT and CD45.2\(^+\) CCR2KO BM cells into control and day 5 colitic CD45.2\(^+\) mice and isolated cells 24 h later (Fig. 7A). Donor-derived F4/80\(^+\) cells were found in both resting and DSS-treated colon, and, not unexpectedly, this was more evident in the inflamed mucosa (data not shown). However, transferred CCR2KO cells were much less efficient at infiltrating the inflamed colon compared with transferred WT cells (Fig. 7B, 7C), with an 80% reduction in accumulation of CCR2 KO donor F4/80\(^+\) cells compared with that of WT donor cells (Fig. 7C). This CCR2-dependent infiltration of colitic mucosa was associated with an increase in the local levels of CCL2 (Supplemental Fig. 4).

The requirement for CCR2 in the recruitment of the precursors of TLR-expressing M\(\phi\)s to the inflamed colon was confirmed by examining DSS colitis in CCR2KO mice. Unlike WT mice, the CCR2KO animals showed no increase in the numbers of TLR2\(^+\) M\(\phi\)s during colitis (Fig. 7D, 7E), and there was no infiltration by F4/80\(^+\)TLR2\(^+\)BrdU\(^+\) or F4/80\(^+\)TLR2\(^-\) TNF-\(\alpha\)–producing cells (Fig. 7F, 7G). Importantly, CCR2KO mice had dramatically reduced colon shortening, weight loss, and clinical disease scores after induction of DSS colitis compared with WT mice (Fig. 7H, 7I, Supplemental Fig. 4A, 4B). In contrast, the numbers of TLR2\(^+\) M\(\phi\)s were identical during colitis in the two strains, and similar numbers of both M\(\phi\) subsets were also found in the healthy colon of WT and KO mice (Fig. 7D, 7E). Transferred CCR2KO and WT leukocytes also appeared to have...
equivalent abilities to enter the F4/80+ population of normal colon, although the low numbers of donor cells found under these circumstances made precise quantitation difficult (data not shown). Together, these results indicate that DSS colitis correlates directly with the CCR2-mediated accumulation of a recently divided, TLR-expressing, and TNF-α-producing subset of Mφs, which appears quite distinct from the resident population seen in the healthy colon.

**Discussion**

Different phenotypic populations of Mφs have been shown to exist in the human colon, and it has been suggested that the balance
between them may be altered during inflammation by an influx of new, inflammatory cells (8, 9, 16). However, this has never been proven formally, and the relationship between these inflammatory Mφs and the initial, resident Mφ population has not been addressed. In this study, we provide evidence that there are two independent subsets of colonic Mφs that are distinguished by their expression of TLR2 and for which relative numbers alter during inflammation.

As others have reported in humans and mice (17–19), we found that Mφs in normal colon were hyporesponsive to inflammatory stimuli, failing to produce proinflammatory cytokines and chemokines after stimulation with LPS, IFN-γ, or the nucleotide-binding oligomerization domain 2 ligand MDP. This was associated with little surface TLR4 expression and no intracellular TLR3 and -9 expression, confirming and extending findings in humans that showed no expression of TLR2, TLR4, or CD14 protein by normal colonic Mφs (20). However, TLR3 and -9 have not been examined in colonic Mφs previously, and our results indicate that the failure to express TLR is a much more global phenomenon than previously realized. In addition, TLR expression by colonic Mφs appeared to be controlled at both the transcriptional and posttranscriptional level. Previous studies on mRNA expression by human intestinal Mφs have produced conflicting results, as one report showed an absence of TLR1–5 mRNA in colonic Mφs (20). However, another report suggested that small intestinal Mφs expressed TLR2 and 4 mRNA (21), perhaps reflecting differences in TLR mRNA production depending on the region of the gastrointestinal tract. Similar studies have not been carried out in mice, and ours is also the first to use quantitative methods to analyze intestinal Mφs. Little is known about how TLR expression is controlled in intestinal Mφs, but the transcription of TLR4 by human Mφs is determined by the PU.1 transcription factor and the IFN consensus sequence-binding protein (22), whereas TLR2 transcription in murine Mφs can be regulated by NF-κB activation (23). Therefore, it would be important to explore the role of these transcriptional control elements in colonic Mφs and examine which posttranscriptional processes might also be involved. Preliminary investigations indicate that the noncoding regions of the relevant TLR mRNA do not contain patterns consistent with regulation by defined micro-RNAs or RNA binding proteins, suggesting that these RNA-editing mechanisms may not control TLR expression by colonic Mφs (A. McIn. Mowat and A.M. Platt, unpublished observations).

An exception to the general lack of TLR expression was that a small proportion of Mφs in normal colon were TLR2+. In parallel, there was some TNF-α production when resting Mφs were stimulated with the TLR2 ligand BLP. Our subsequent studies indicated that the populations of Mφs identified on the basis of TLR2 expression were stable and independent subsets with distinct phenotypes, functions, and ontology. The TLR2+ Mφs that dominated the normal mucosa lacked expression of CD11c or costimulatory molecules, and only a minority expressed class II MHC. They also had a slow turnover rate in vivo as shown by BrdU incorporation and did not express CCR2 or require it for their presence in the healthy or inflamed mucosa. In comparison, the minority subset of TLR2− Mφs was uniformly CD14+ and class II MHC+ and expressed higher levels of CD11c, CD40, CD80, and CD86, as well as having a high turnover rate in vivo. Significantly, they all also expressed high levels of CCR2. During colitis, the TLR2+ subset became the majority population of Mφs, particularly in the distal colon, where disease is most severe, and presumably reflecting the higher level of microbiota in this location. Under these conditions, the TLR2+ Mφs produced significant amounts of TNF-α and were BrdU+, with these cells accounting for most of the TNF-α–producing cells present in the colitic mucosa. Although some TNF-α–producing cells expressed low or negligible levels of F4/80, most were CD11b+ and had the morphological characteristics of Mφs. F4/80B+ cells of this kind were not present in the resting colon, and it is possible that they may represent recently recruited monocytes, which are known to express reduced levels of F4/80 (24). Significantly, TLR2+ Mφs were unable to produce TNF-α, even after the induction of DSS colitis and/or stimulation in vitro. There are no previous studies of TLR expression and function by colonic Mφs in mouse models of colitis, but the TLR2CD11c+class II MHC+ Mφs we found are similar to the inflammatory Mφ subset that has been described in Crohn’s disease and in inflamed mouse intestine (9, 16, 25–29). These cells have been shown to have a wide range of inflammatory properties, including the induction of Th1 and Th17 responses via the production of IL-1, IL-6, and IL-23 and the ability to present Ag to CD4+ T cells and induce granuloma formation in vivo. Because of these functions, this cell type could be an extremely important cell to neutralize in inflammation, and its origin and differentiation would be important to define accurately.

Our results indicate that the accumulation of these cells to the inflamed colon reflects selective recruitment of a distinct subset of myeloid precursor rather than local expansion of the TLR2+ cells already present in the normal intestine or altered differentiation of the normally unresponsive, TLR2− Mφs. Notably, the TLR2− and TLR2+ subsets had quite distinct phenotypic characteristics that were stable and not dependent on the presence of inflammation. Only the TLR2+ subset expressed the Gr-1 and CCR2 markers, which characterize the inflammatory subset of blood monocytes in mice that are believed to represent an independent lineage from the CX3CR1th Ly6CGr-1− CCR2th monocyte population that gives rise to tissue-resident Mφs (10). Although this paradigm has become widely accepted, it has not been studied previously in resting or inflamed intestine, and thus, it is significant that we found the resident population of CX3CR1th Mφs to be largely replaced by CX3CR1th Ly6Cth Mφs in colitis. Further evidence that the TLR2-positive and -negative colonic Mφs have independent origins comes from our BrdU studies and from our experiments using adoptively transferred CCR2KO cells. The higher turnover of TLR2+ Mφs in vivo was independent of the presence of inflammation, and the kinetics of BrdU accumulation by the subsets remained quite separate during long-term pulsing in vivo, suggesting that the subsets did not differentiate into the opposite population. Increased local proliferation of the resident subsets also did not appear to account for these differences, as although Ki-67 expression was slightly increased in colitic Mφs, this was unlikely to be extensive enough to account for the marked differences in Mφ numbers and BrdU uptake. Together with our findings that the TLR2−expressing subsets from either inflamed or resting colon could not interconvert in vitro, these results support the view that the increased numbers of TLR+ Mφs are due to selective accumulation of distinct precursors that had divided recently outside the mucosa before entering from the blood monocyte pool. In support of this idea, the small population of CD14+ Mφs in the normal human intestine is also believed to represent recently recruited monocytes (16, 30).

The BrdU−, TNF-α–producing, TLR2+ Mφs expressed CCR2, and their accumulation in inflamed intestine was completely abrogated in CCR2KO mice. This was not due to a need for CCR2 in the egress of myeloid precursors from the BM (31), as transferred CCR2KO leukocytes showed a marked defect in their ability to repopulate the inflammatory Mφ subset in mice with colitis, and the increased infiltration by TLR2CCR2+ Mφs was
associated with increased mucosal levels of the CCR2 ligand (CCL2), something also found in human IBD mucosa (32). Again, these results are consistent with the Ms in inflamed colon being derived from the distinctive lineage of CX3CR1−CCR2+ monocytes (10). Importantly, the lack of recruitment of TNF-α-producing TLR+ Ms during colitis was associated with resistance to DSS colitis in CCR2KO mice. Our study is consistent with previous findings that combined blockade of CCR2, CCR5, and CXCR3 in vivo reduces DSS-induced colitis (33) and that CCR2-deficient mice are less susceptible to DSS colitis (34). However, these earlier studies did not examine the role of CCR2 neutralization in isolation, nor were the mechanisms underlying the effects of the absence of CCR2 explored. As protective immunity against oral Toxoplasma gondii infection is also absent in CCR2KO mice and requires the CCR2-dependent recruitment of inflammatory Ms to the intestine (35), this receptor clearly plays a central role in the recruitment of Ms in both protective and pathological immune responses in the intestine.

Our results also raise questions about which subset of Ms is required for maintaining homeostasis in the normal intestine, as well as how the two subsets can coexist in the healthy mucosa. With their high phagocytic activity and inability to produce proinflammatory cytokines, it is believed that the major physiological role of resident colonic Ms is to clear away any commensal microbiota that have penetrated the epithelial barrier without producing inflammation (17). Further protection against inflammatory reactions to commensal organisms is provided by IL-10R signaling in Ms (3), whereas their expression of class II MHC in the absence of costimulatory molecules could allow resistant intestinal Ms to act as tolerogenic APCs. Indeed, small intestinal F4/80+CD11b+CD11c+Ms-like cells can induce the differentiation of FoxP3+ regulatory cells in vitro (36), and mice lacking F4/80 do not develop oral tolerance to protein Ags due to defective induction of CD8+ T regulatory cells (37). Intestinal Ms can also contribute to local homeostasis in a more active manner via enhancing the production of mediators such as PGE2, which promote epithelial cell renewal and barrier integrity (6). As a consequence, prior depletion of intestinal mononuclear phagocytes enhances susceptibility to DSS colitis, possibly secondary to increased chemokine-induced neutrophil infiltration (38). Somewhat paradoxically, these anti-inflammatory effects appear to require commensal bacteria-induced TLR signaling, as depletion of TLR2, TLR4, or MyD88 in BM-derived cells exacerbates experimental colitis (39, 40). Collectively, these results indicate a specific physiological role for the TLR-expressing subset in maintaining epithelial function and repair. However, as we found that TLR2+ Ms from normal colon could produce small amounts of TNF-α constitutively, it is difficult to understand why this does not lead to overt inflammation in the healthy gut. One possible explanation is that there are simply too few of these cells under normal conditions, and so their effects are diluted out or are even actively suppressed by the inert majority. The low numbers of TLR2+ Ms could be because CCR2 ligands are relatively low in the normal mucosa (32), thus restricting their entry. Additionally or alternatively, our BrdU studies showed that the TLR2 subset had a rapid turnover rate in the healthy mucosa, meaning that these cells are not likely to remain present for long periods, either because they die or leave. A final possibility is that these cells enter the mucosa in a fully responsive state, but are then conditioned by the environment to lose TLR expression.

Conditioning effects of this kind are known to play a crucial role in maintaining hyporesponsiveness in intestinal dendritic cells (41), and TGF-β from intestinal stromal cells has been shown to downregulate proinflammatory cytokine production and CD14 expression by monocytes (17). Understanding the cellular and molecular basis of these and the other processes that control colonic Ms behavior will be important steps in understanding intestinal physiology and pathology.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1: Pattern of TLR expression by colonic mϕ is stable and is not dependent on enzymatic isolation.

A) Total F4/80⁺ cells from colonic LP were FACS-sorted and examined for the expression of surface TLR2 and 4 by flow cytometry before or after culture in medium for 24hrs.

B) PEC mϕ were isolated and some were treated with the same enzymatic protocol used to obtain colonic LP cells, before being examined for the expression of F4/80, TLR2, TLR4 and class II MHC by flow cytometry.

Histograms show the levels of expression of the markers on live-gated F4/80⁺ cells and the shaded histograms represent staining with isotype control antibodies. Results are representative of 2 independent experiments.

C, D) TLR2⁺ F4/80⁻ cells from control (C) or day 7 colitis colon (D) were FACS-sorted and cultured overnight in medium or with 1μg/ml CCL2, before being assessed for TLR2 expression. Representative dot plots of cells from control colon (C) or colitis (D) pre-sort, post-sort and after culture in medium or CCL2.

Supplementary Figure 2: Expression of TLR mRNA expression by different macrophage populations.

A) mRNA was extracted from BMM or from MACS-purified F4/80⁺ PEC mϕ and colonic mϕ before being examined for the expression of TLR1-7 and 9 by end-product PCR.
Supplementary Figure 3: Macrophage phenotype and turnover in the inflamed colon.

Colonic LP cells from B6 mice fed DSS for 5 days were assessed for the expression of surface TLR4 (A), and intracellular TLR3 (B) and TLR9 (C) by flow cytometry. Histograms show marker expression by live-gated F4/80+ cells and shaded histograms represent staining with isotype control antibodies. D) Colonic LP cells from mice fed water or DSS for 5 days were analysed for the expression of CD40, CD80, CD86, class II MHC and CD14. Histograms show the proportions of live-gated F4/80+ cells positive for each marker among the TLR2+ (thick line) and TLR2- (thin line) cells; shaded histograms represent staining with isotype control antibodies. E) Expression of CD11c by live-gated F4/80+TLR2+ (thick line) and F4/80+TLR2- (thin line) cells from control colons and from mice fed DSS for 7 days. The numbers represent the proportion of each subset expressing CD11c. F) B6 mice were given a single injection of 1mg BrdU i.p. on day 0 and drinking water containing 0.8mg/ml BrdU was administered from day 0 onwards. Some mice then had DSS added to the water, while controls received BrdU in water alone. Colon LP cells were harvested 24hrs, 96hrs or 144hrs later and analysed for the expression of F4/80 and BrdU incorporation by flow cytometry. Results shown are the mean percentages ± 1 SD of F4/80− cells positive for BrdU from 3 mice/group. ns = not significant; *p<0.05; **p<0.01, as assessed by linear regression analysis. Data are representative of 2-3 independent experiments.
Supplementary Figure 4: Role of CCR2 in colonic macrophage infiltration and colitis.

A) DSS colitis in WT and CCR2KO mice. Mean disease scores (A) and histology (B) (calculated as in the Materials and Methods) ± 1 SD for 6 mice/group fed DSS for 6 days.

C) Release of CCL2 from colon explants from the inflamed mucosa. Mice received 2% DSS in the drinking water for 6 days. On days 3 and 6, explants of colon were cultured for 24 h and culture supernatants were harvested. The levels of CCL2 in these supernatants were determined by Luminex Multiplex bead assays. Mean CCL2 levels ± 1 SD for 3 mice/group fed DSS for 3 or 6 days.

Data are representative of at least 2 independent experiments.
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**Supplementary Table 1:** Antibodies used in flow cytometry
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**Supplementary Table 2:** Primers used for end-product (top) and real time (bottom) PCR