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Characterization of Conventional and Atypical Receptors for the Chemokine CCL2 on Mouse Leukocytes

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Chemokine-directed leukocyte migration is crucial for effective immune and inflammatory responses. Conventional chemokine receptors (cCKRs) directly control cell movement; atypical chemokine receptors (ACKRs) regulate coexpressed cCKRs; and both cCKRs and ACKRs internalize chemokines to limit their abundance in vivo, a process referred to as scavenging. A leukocyte’s migratory and chemokine-scavenging potential is determined by which cCKRs and ACKRs it expresses, and by the ligand specificity, signaling properties, and chemokine internalization capacity of these receptors. Most chemokines can bind at least one cCKR and one ACKR. CCL2 can bind to CCR2 (a cCKR) and two ACKRs (ACKR1 and ACKR2). In this study, by using fluorescent CCL2 uptake to label cells bearing functional CCL2 receptors, we have defined the expression profile, scavenging activity, and ligand specificity of CCL2 receptors on mouse leukocytes. We show that qualitative and quantitative differences in the expression of CCR2 and ACKR2 endow individual leukocyte subsets with distinctive CCL2 receptor profiles and CCL2-scavenging capacities. We reveal that some cells, including plasmacytoid dendritic cells, can express both CCR2 and ACKR2; that Ly6Chi monocytes have particularly strong CCL2-scavenging potential in vitro and in vivo; and that CCR2 is a much more effective CCL2 scavenger than ACKR2. We confirm the unique, overlapping, ligand specificities of CCR2 and ACKR2 and, unexpectedly, find that cell context influences the interaction of CCL7 and CCL12 with CCR2. Fluorescent chemokine uptake assays were instrumental in providing these novel insights into CCL2 receptor biology, and the sensitivity, specificity, and versatility of these assays are discussed. The Journal of Immunology, 2014, 193: 400–411.

Precise temporospatial leukocyte positioning is crucial for physiological and pathological immune and inflammatory responses. The chemokine family of secreted chemotactants plays a central role in orchestrating this process by controlling leukocyte navigation into, within, and between tissues (1, 2). There are >40 chemokines in mammals, which, based on the organization of cysteine residues in the mature protein, are subdivided into four subfamilies (CC, CXC, C, and CX3C). They signal through heptahelical G protein–coupled receptors that decorate leukocyte surfaces, and 18 receptors have been identified that can induce cell migration after binding their cognate chemokine ligand. These conventional chemokine receptors (cCKRs) are usually specific for a single chemokine subfamily, and there are 10 CC chemokine receptors, 6 CXC chemokine receptors, and 1 receptor each for C and CX3C chemokines. Four atypical chemokine receptors (ACKRs) also exist that were previously called DARC, D6, CXCR7, and CCRL1 and that have recently been renamed ACKR1, ACKR2, ACKR3, and ACKR4, respectively (2, 3). ACKRs structurally resemble cCKRs but cannot directly initiate migratory responses. Instead they scavenge, sequester, or transport chemokines to control cCKR-driven responses, and they can also, in some contexts, regulate coexpressed cCKRs (3). Chemokine scavenging is not restricted to ACKRs. cCKR activation is accompanied by internalization of chemokine/cCKR complexes, and, interestingly, migrating cells can use cCKRs to actively scavenge the chemokines that are driving their migration (4). Moreover, like ACKRs, cCKRs have been shown to modulate chemokine abundance in vivo through ligand uptake (5–7).

Interactions between chemokines and their receptors are complex. Many chemokines bind multiple receptors, and some cCKRs and ACKRs show remarkable ligand promiscuity (2). This is prominent among chemokines and receptors that regulate leukocyte trafficking during inflammation. A leukocyte’s response to a specific inflammatory chemokine will depend on which cCKRs and ACKRs it carries; the level of expression and specificity of these receptors; and their ability to translate chemokine binding into biological responses. In addition, the extent of chemokine scavenging mediated by its cCKRs and ACKRs will determine how effectively it modifies chemokine abundance. Subsets of leukocytes are likely to show qualitative and quantitative differences in these parameters that will dictate how they respond to, and regulate, chemokines. In this study, we have examined these issues by exploring how leukocytes interact with the chemokine CCL2. CCL2 is a key proinflammatory chemokine that can direct the migration of a variety of leukocytes, including subsets of monocytes, dendritic cells, NK cells, and T cells (2, 8–14). Responses to...
CCL2 are mediated by the cCKR CCR2, but CCL2 can also bind to ACKR1 and ACKR2. CCR2 is activated by other chemokines (e.g., CCL7 and CCL12 in mice), and ACKR1 and ACKR2 show broad specificity for inflammatory chemokines (3). ACKR1 is not expressed by leukocytes; it is found on RBCs, where it acts as a chemokine buffer (3, 15), and blood vessel endothelial cells, where it participates in chemokine transcytosis (3, 16, 17). Lympathic endothelial cells are a prominent source of ACKR2 (18, 19), but they are also expressed by mouse innate-like B cells (marginal zone [MZ] and B1 B cells) and can suppress the migration of these cells (20). It is unclear whether other mouse leukocytes express ACKR2, but this could contribute to the many indispensable in vivo functions that have been defined for ACKR2 (3). CCR2 plays a particularly prominent role in the biology of inflammatory Ly6C<sup>high</sup> monocytes. It mediates their recruitment into inflamed tissues, but it is also important for their mobilization from the bone marrow (BM) under steady state conditions (10, 13, 14). Interestingly, ACKR2 has also been implicated in regulating homoeostatic monocyte release from mouse BM and circulating monocyte count in humans (21, 22).

Theoretically, immunostaining could be used to profile expression of CCR2 and ACKR2 on mouse leukocytes. However, effective anti-mouse ACKR2 Abs are not available, and Abs provide no insight into receptor specificity or activity, that is, whether the detected receptors can bind chemokine, transduce signals, and mediate scavenging. Moreover, alternative splicing, posttranslational modification, or heterodimerization could mask Ab epitopes on receptors that are competent for chemokine binding. The use of fluorescently labeled chemokines overcomes these restrictions and limitations. We used AlexaFluor-647–tagged CCL2 (CCL2<sub>AF647</sub>) to reveal ACKR2 expression by innate-like B cells (20). Binding of CCL2<sub>AF647</sub> at 4 °C was insufficiently sensitive to detect ACKR2, and cells had to be allowed to internalize CCL2<sub>AF647</sub> by incubation at 37°C. Significantly, this showed that ACKR2 was functional with respect to the binding and internalization of CCL2 (20). This is critical for chemokine scavenging and driven by constitutive ACKR2 trafficking to and from the cell surface (23). Some CCR2-dependent CCL2<sub>AF647</sub> uptake was also observed in our previous work (20). The labeled cells carry CCR2 molecules that bind and internalize CCL2<sub>AF647</sub>, so, because internalization of CCR2 requires chemokine-induced signaling (24), these CCR2 molecules must presumably be capable of initiating intracellular signals upon CCL2 binding. Therefore, unlike Ab staining, CCL2<sub>AF647</sub> uptake assays specifically identify cells carrying functionally competent cCKRs and ACKRs for CCL2. Moreover, the extent of uptake reflects a cell’s chemokine-scavenging potential, and the inclusion of unlabeled competitor chemokines allows receptor specificity to be defined.

In this work, we have systematically determined which mouse leukocytes express functionally competent CCL2 receptors. We have compared the ex vivo and in vivo CCL2-scavenging potential of different leukocyte subsets and revealed the contribution of CCR2 and ACKR2 to CCL2 receptor activity. We have also examined whether the ligand specificity of CCR2 and its sensitivity to chemokine exposure are influenced by the cellular context in which the receptor is expressed. These studies have provided novel insights into the expression, regulation, ligand specificity, and scavenging potential of CCL2 receptors.

Materials and Methods
Animals and in vivo procedures

Wild-type (WT) and Ccr2<sup>−/−</sup> C57BL/6 mice were bred and maintained under specific pathogen-free conditions at the Central Research Facility, University of Glasgow. Ccr2<sup>−/−</sup> mice were originally from The Jackson Laboratory (stock number 004999) (25). In all experiments, 8- to 12-wk-old male mice were used. For in vivo expansion of plasmacytoid dendritic cells (pDCs), ~2 × 10<sup>5</sup> Flt3L-producing B16FL cells (26) (provided by O. Pabst, Hannover Medical School, Hannover, Germany) were injected s.c. into WT mice, and tumor growth was monitored for 10–14 d until sacrifice. For in vivo fluorescent chemokine uptake, WT mice were injected via the tail vein with 1 μg CCL2<sub>AF647</sub> in 100 μl PBS, or with 100 μl PBS alone, and sacrificed 2 h later. All procedures had received approval from Glasgow University’s ethical review boards and were performed under license in accordance with the U.K. Home Office regulations.

Cell isolation

Single-cell suspensions of mouse spleen, lymph node, and BM were prepared, as previously described (20). BM was lysed in spleen and BM samples by incubating cells in BM lysis buffer (Sigma-Aldrich) for 1 min at room temperature. Mouse peripheral blood was harvested by terminal cardiac puncture using a 1-ml syringe with a 25-gauge needle that had previously been flushed with 0.5 M EDTA (pH 7.5). BM was lysed by adding 9 vol ammonium chloride solution (StemCell Technologies) to the blood and incubating on ice for 10 min. Cells were then washed twice by centrifugation at 400 × g for 30 min at 4°C. Cells were resuspended in complex RPMI, and viable cells were counted on a hemocytometer using trypan blue exclusion.

Chemoattractants

CCL2<sub>AF647</sub> (Almac Sciences) is a chemically synthesized form of human CCL2 that carries AlexaFluor-647 on its extreme C terminus. It has equivalent bioactivity to unlabeled human CCL2 in in vitro chemotaxis assays (20). Unlabeled chemokines were from PeproTech or R&D Systems.

Fluorescent chemokine uptake

A total of 1–2 × 10<sup>6</sup> cells was incubated in the dark for 1 h at 37°C or 4°C in 50 μl binding buffer (complete RPMI with 20 mM HEPES [pH 7.2]) containing 25 nM CCL2<sub>AF647</sub> (Almac Sciences), with or without unlabeled chemokine competitor (20). Cells were then washed in binding buffer and stained with fluorescently labeled Abs, as described below. In some experiments, cells were preincubated with unlabeled chemokine at 37°C for 30 min, washed three times at 4°C in binding buffer, and then incubated with fluorescent chemokine, as above.

Abs and flow cytometry

Cells were incubated in ice-cold FACS buffer (PBS containing 1% FCS, 0.02% NaN<sub>3</sub>, and 5 mM EDTA) with Fc block (BD Biosciences) at 4°C for 15 min. Cells were then stained with Abs for 15 min at 4°C and washed twice with FACS buffer. Where necessary, cells were incubated in secondary detection reagents for 15 min at 4°C, before being washed twice with FACS buffer. To allow dead cell exclusion, the cell viability dye Vioprobe (BD Biosciences) was added after Ab labeling or cells were labeled with fixable viability dyes (eBiosciences) before Ab staining, each according to the manufacturer’s instructions. The following Abs, labeled with various fluorophores, were used (clone names are in parentheses): anti-mouse CCR2 (7G6,6, and D11b (M1/70) were from BD Biosciences; CD19 (1D3), CD4 (GK1.5), and CD3 (17A2), and SiglecH (440c) were from eBioscience. Unstained cells, cells stained with one fluorescent Ab, and “fluorescent minus one” controls were used in all experiments to allow appropriate acquisition parameters to be established, and to aid gating during data analysis. “Fluorescent minus one” controls contained all Abs except one, which was replaced with an equivalent quantity of an isotype-matched Ab control. Data were acquired on a Miltenyi Biotec MACSQuant or BD Biosciences LSRII and analyzed using FlowJo software (Tree Star). Dead cells and cell doublets/clusters were excluded from all analyses.

pDC purification and chemotaxis

pDCs were purified using a MACS Aria (BD Biosciences) as live singlet B220<sup>−</sup>CD11c<sup>−</sup>Ly6C<sup>−</sup>CD11b<sup>−</sup>SiglecH<sup>+</sup> cells to >95% purity. pDCs were resuspended to 10<sup>5</sup>/ml in chemotaxis buffer (RPMI 1640 plus 0.1% BSA and 25 mM HEPES [pH 7.2]), and 100 μl was added to inserts of a 24-well Transwell chemotaxis plate (5-μm pores) sitting above 600 μl chemotaxis buffer containing 0–50 mM CCL2. Plates were incubated for 3 h at 37°C. Migrated cells were retrieved from the lower chamber, washed, resuspended...
in 200 μl FACS buffer, and stained with Abs, as above. Cells were counted on a Miltenyi Biotech MACSQuant set to analyze a defined sample volume. Data were analyzed using FlowJo software.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software. The statistical tests used are indicated in the figure legends. The p values <0.05 were considered to demonstrate statistically significant differences between groups.

Results

CLL2<sup>AF647</sup> uptake identifies mouse leukocytes expressing functionally competent CCL2 receptors

First, we sought to identify leukocytes bearing functionally competent CCL2 receptors in the spleen, BM, and blood of WT mice at steady state. Single-cell suspensions were prepared and incubated with CLL2<sup>AF647</sup>. To determine whether CCR2 was responsible for mediating CLL2<sup>AF647</sup> uptake, cells from Ccr2<sup>−/−</sup> mice were also used. Ccr2<sup>−/−</sup> samples were also included that contained excess unlabeled mouse CCL2 in addition to CLL2<sup>AF647</sup>. ACKR2, unlike CCR2, binds CCL2 and CLL2<sup>AF647</sup>, so any ACKR2-mediated CLL2<sup>AF647</sup> uptake by Ccr2<sup>−/−</sup> cells will be blocked by CCL2<sup>20</sup>. After CLL2<sup>AF647</sup> uptake, cells were stained with anti-Ly6C Abs because we anticipated that Ly6C<sup>high</sup> monocytes would demonstrate CCR2-dependent CLL2<sup>AF647</sup> uptake. Indeed, as shown in Fig. 1, most Ly6C<sup>high</sup> cells in spleen, BM, and blood internalized substantial quantities of CLL2<sup>AF647</sup> in a CCR2-dependent fashion and clearly had higher CLL2-scavenging potential than any other cells in the samples. Lower levels of CLL2<sup>AF647</sup> uptake were observed among CCR2<sup>−/−</sup> or ACKR2-dependent CLL2<sup>AF647</sup> uptake and compared the extent of uptake between different leukocyte populations. When specific leukocyte populations in the spleen were examined (identified as shown in Supplemental Fig. 2), five distinct patterns of CLL2<sup>AF647</sup> labeling were seen (Fig. 2). First, virtually all Ly6C<sup>high</sup> monocytes showed very strong CCR2-dependent CLL2<sup>AF647</sup> uptake. Second, most cells expressing the NK cell marker DX5, along with subsets of γδ T cells, CD11b<sup>+</sup> conventional dendritic cells (cDCs), and CD8<sup>+</sup> eDCs, were capable of CCR2-dependent CLL2<sup>AF647</sup> uptake (Fig. 2A, 2B), although the amount of CLL2<sup>AF647</sup> internalized per cell was substantially less than that achieved by Ly6C<sup>high</sup> monocytes (Fig. 2A). A few WT CD4<sup>+</sup> and CD8<sup>+</sup> T cells also internalized CLL2<sup>AF647</sup>, and this was reduced in Ccr2<sup>−/−</sup> cells (Fig. 2A, 2B). Third, some cell types, including neutrophils, showed no CCR2-dependent CLL2<sup>AF647</sup> uptake (Fig. 2A, 2B, data not shown). A fourth pattern of CLL2<sup>AF647</sup> uptake was seen among B cells. None of the splenic cell types discussed above showed any evidence of ACKR2 activity in Ccr2<sup>−/−</sup> or WT mice, as there was no significant reduction of CLL2<sup>AF647</sup> uptake in the presence of CCL2<sup>20</sup> (data not shown), but, consistent with our previous work (20), nearly all CLL2<sup>AF647</sup> uptake by CD2<sup>20</sup> MZ B cells was ACKR2 dependent (Fig. 2C, 2D). In addition, a small population of CD21<sup>−/low</sup> B cells showed strong CCR2-dependent CLL2<sup>AF647</sup> uptake that resembled that achieved by Ly6C<sup>high</sup> monocytes (Fig. 2C, 2D). Ccr2 transcripts are found in immature T1 B cell precursors (27, 28), but, to our knowledge, expression of CCR2 protein by mouse B cells has not been previously reported. Finally, analysis of pDCs and macrophages provided evidence of a fifth CLL2<sup>AF647</sup> uptake profile. CLL2<sup>AF647</sup> internalization by these cells was reduced by Ccr2 deletion but could be lowered further by inclusion of CCL2<sup>20</sup> (Fig. 2E, 2F). Thus, pDCs and macrophages can express CCR2 and ACKR2.

Similar analyses were undertaken on cells from BM, blood, and skin-draining lymph nodes (Fig. 3, data not shown). CLL2<sup>AF647</sup> uptake profiles of leukocyte subsets in lymph nodes were broadly similar to those seen in the spleen (data not shown). In BM, CLL2<sup>AF647</sup> uptake was restricted to CD11b<sup>+</sup> and CD11c<sup>+</sup> cells (Fig. 3A, 3C, 3D). The restricted profile of CD11b<sup>+</sup> cells was consistent with the presence of CCR2<sup>AF647</sup> expression on a subset of CD11b<sup>+</sup> cells in the spleen and BM. However, CD11c<sup>+</sup> cells in the BM showed broad expression of CCR2<sup>AF647</sup> uptake, with readily identifiable subsets with differing CCL2 receptor activity. Interestingly,
FIGURE 2. CCL2<sub>AF647</sub> uptake identifies splenic leukocyte subsets expressing CCR2 and/or ACKR2. WT and Ccr2<sup>−/−</sup> (CCR2 KO) splenocytes were incubated with CCL2<sub>AF647</sub> (±10-fold molar excess of unlabeled CCL22), stained with fluorescently labeled Abs, and examined by flow cytometry. Dead cells and cell doublets have been excluded from all data. (A and E) Overlaid CCL2<sub>AF647</sub> uptake profiles of WT and CCR2 KO splenic leukocyte subsets identified by the surface immunophenotype indicated. (B) Mean percentage (+SD) of CCL2<sub>AF647</sub>-positive cells in splenic leukocyte subsets (n = 3). CCL2<sub>AF647</sub>-positive WT cells were defined based on arbitrary gates set using equivalent populations of CCR2 KO splenocytes. The percentage of CCL2<sub>AF647</sub>-positive CCR2 KO cells remaining in this gate is shown in the white columns. *p < 0.05, **p < 0.01, ***p < 0.001 using Student t test. (C) Dot plots of live splenic B cells (CD19<sup>+</sup>) showing CCL2<sub>AF647</sub> uptake against CD21 expression. R1 and R2 identify cells with specific CCL2<sub>AF647</sub> uptake properties that are discussed in Results. (D) Mean percentage (+SD) of CCL2<sub>AF647</sub>-positive cells in R1 and R2 (n = 3). (F) Mean percentage (+SD) of CCL2<sub>AF647</sub>-positive WT and CCR2 KO splenic macrophages and pDCs (n = 3). CCL2<sub>AF647</sub>-positive WT and CCR2 KO cells were defined based on arbitrary gates set using equivalent populations of CCR2 KO splenocytes that had been incubated with CCL2<sub>AF647</sub> and an excess (Figure legend continues).
all BM cells expressing active CCR2 in WT mice, particularly Ly6C\textsuperscript{hi} monocytes and pDCs, showed evidence of ACKR2 activity, as revealed by the ability of CCL2 to inhibit CCL2\textsuperscript{AF647} uptake by Ccr2\textsuperscript{−/−} cells (Fig. 3A, 3C). Analysis of Ccr2\textsuperscript{−/−} cells provided a reliable reflection of ACKR2 expression by WT cells because unlabeled CCL2 reduced CCL2\textsuperscript{AF647} uptake by a similar amount when Ccr2\textsuperscript{−/−} and WT BM cells were compared (data not shown), although, as described below, Ccr2 deficiency was associated with a reduction in the ACKR2 activity of pDCs.

Circulating peripheral blood cells had a CCL2\textsuperscript{AF647} uptake profile that was similar to BM (Fig. 3B, 3D), although there were several notable differences. First, CCR2 activity was lower on most Ly6C\textsuperscript{low} monocytes. Second, in contrast to CD11b\textsuperscript{−/−}Gr1\textsuperscript{low} CD115\textsuperscript{+} Ly6C\textsuperscript{+} cells in the BM, cells with this surface phenotype in the blood had a CCR2-dependent CCL2\textsuperscript{AF647} uptake profile comparable to Ly6C\textsuperscript{hi} monocytes. Third, active ACKR2 was only reproducibly detectable on pDCs.

Collectively, these data reveal marked qualitative differences in CCL2 receptor usage between leukocyte subsets. In addition, by using the extent of CCL2\textsuperscript{AF647} uptake to gauge scavenging potential, it is clear that CCR2 is capable of mediating much more CCL2 scavenging than ACKR2, and that Ly6C\textsuperscript{hi} monocytes have a greater capacity for CCL2 scavenging than any other leukocyte subset examined.

**CCL2 receptors in mouse pDCs**

We were interested in the biphasic CCL2\textsuperscript{AF647} uptake profiles of pDC and the evidence that these cells can express CCR2 and ACKR2. Previous work using the anti-mouse CCR2 Ab MC-21 (29) has reported that CCR2 can only be detected on 15–25% of mouse BM pDCs (30, 31), whereas a slightly higher proportion of pDCs in the spleen and lymph node carries surface CCR2 (31). However, our data indicate that virtually all WT pDCs carry functionally competent CCL2 receptors and that pDCs can be subdivided into two subsets based on CCL2\textsuperscript{AF647} uptake (CCL2\textsuperscript{hi} and CCL2\textsuperscript{lo}). We were satisfied with our definition of pDCs as CD11c\textsuperscript{+}B220\textsuperscript{+}Ly6C\textsuperscript{+}CD115\textsuperscript{+} cells, because they expressed SiglecH and PDCA1 (CD317/BST2), two surface proteins highly restricted to pDCs (Supplemental Fig. 4A). These pDC markers were absent from most CD11c\textsuperscript{+}B220\textsuperscript{−} cells lacking Ly6C or expressing CD11b (Supplemental Fig. 4A). To further examine CCL2 receptors on pDCs, we undertook CCL2\textsuperscript{AF647} uptake experiments in the presence or absence of excess unlabeled CCL2, using cells from WT and Ccr2\textsuperscript{−/−} BM, spleen, blood, inguinal lymph node, and mesenteric lymph node (Fig. 4). Biphasic CCL2\textsuperscript{AF647} uptake profiles were seen for WT pDC populations from all anatomical locations. In WT lymph nodes and blood, CCL2 only significantly reduced CCL2\textsuperscript{AF647} uptake by CCL2\textsuperscript{lo} pDCs. This was seen with WT splenic and BM pDCs as well, although the size of the CCL2\textsuperscript{hi} pDC population was also reduced when CCL2 was present. These data indicated that CCL2\textsuperscript{AF647} uptake by WT CCL2\textsuperscript{lo} pDCs was mediated primarily by ACKR2 and that, consistent with the observations of others (30, 31), only a subset of WT pDC expresses CCR2. However, it was notable that the CCL2\textsuperscript{lo} pDC population was also affected by Ccr2 deletion. In all tissues examined, Ccr2\textsuperscript{−/−} pDCs displayed levels of CCL2\textsuperscript{AF647} uptake that were markedly lower than the majority of WT pDCs, including most of the CCL2\textsuperscript{lo} pDCs. Thus, WT pDCs can express functionally competent CCR2 and/or ACKR2, and genetic deletion of CCR2 reduces ACKR2-mediated CCL2\textsuperscript{AF647} internalization by these cells.

**Ly6C\textsuperscript{hi} monocytes efficiently scavenge CCL2\textsuperscript{AF647} in live mice**

We next considered whether the scavenging potential of leukocytes revealed by ex vivo CCL2\textsuperscript{AF647} uptake assays accurately reflected their scavenging potential in vivo. To do this, CCL2\textsuperscript{AF647} was injected i.v. into live WT mice, and leukocytes in the spleen, BM, and blood were assessed by flow cytometry. Control mice received carrier (PBS) alone. As in the ex vivo CCL2\textsuperscript{AF647} uptake assays, more CCL2\textsuperscript{AF647} was internalized by Ly6C\textsuperscript{hi} cells than by Ly6C\textsuperscript{−/−} cells (dot plots; Fig. 5), and the overall profile of CCL2\textsuperscript{AF647} labeled cells was similar to that seen after ex vivo labeling (compared Fig. 5 with Fig. 2). The Ly6C\textsuperscript{hi} and CCL2\textsuperscript{AF647}-positive cells in spleen, blood, and BM were Ly6C\textsuperscript{hi} monocytes, as expected (histogram plots; Fig. 5). All Ly6C\textsuperscript{hi} monocytes in BM and blood were CCL2\textsuperscript{AF647} positive. However, some splenic Ly6C\textsuperscript{hi} monocytes were poorly labeled perhaps because the injected CCL2\textsuperscript{AF647} did not access the subcapsular red pulp where these cells are known to reside (32).

Thus, Ly6C\textsuperscript{hi} monocytes have the greatest capacity for CCL2 scavenging in vivo, whereas CCR2- or ACKR2-mediated CCL2 uptake by Ly6C\textsuperscript{−/−} cells only makes a minor contribution to the total CCL2 scavenging achieved by leukocytes.

**Cell-specific interactions between CCR2 and its ligands**

Next, we explored the nature of the interaction of CCL7 and CCL12 with CCR2 and ACKR2 on mouse leukocytes. Fluorescently labeled versions of these chemokines were not available, so we examined this indirectly by assessing the ability of unlabeled versions of these chemokines to interfere with CCL2\textsuperscript{AF647} uptake. We initially assessed CCL2\textsuperscript{AF647} uptake by WT splenocytes coincubated with or without 25 nM unlabeled CCL2, CCL7, or CCL12 (Fig. 6A). Ccr2\textsuperscript{−/−} splenocytes incubated with CCL2\textsuperscript{AF647} alone were included as a control. Interestingly, each competitor chemokine left a unique profile of residual CCL2\textsuperscript{AF647} uptake. All three chemokines substantially reduced uptake by Ly6C\textsuperscript{hi} cells, although CCL7 and CCL12 were somewhat less effective than CCL2. As expected, inclusion of unlabeled CCL2 as a competitor had more impact than CCR2 deletion on CCL2\textsuperscript{AF647} uptake by Ly6C\textsuperscript{−/−} cells because of its ability to interfere with ACKR2-mediated uptake (20). However, CCL12 and CCL7 were much less effective than CCL2 at blocking CCL2\textsuperscript{AF647} uptake by Ly6C\textsuperscript{−/−} cells. The ligand specificity of ACKR2 contributes to these differences: 25 nM CCL2 and CCL12 completely inhibited CCL2\textsuperscript{AF647} uptake by MZ B cells, whereas CCL7 had no effect (data not shown). However, the data indicated that CCL7 and CCL12 differ from CCL2 in their ability to interact with CCR2.

To explore this in more detail, we compared the impact of a range of concentrations of unlabeled chemokine on the high CCR2-dependent CCL2\textsuperscript{AF647} uptake of splenic Ly6C\textsuperscript{hi} monocytes and, in the same samples, on the lower levels of CCL2\textsuperscript{AF647} uptake achieved by CD11b\textsuperscript{+}Ly6C\textsuperscript{−} cells (Fig. 6B, 6C). There are no ACKR2-expressing splenocytes in the CD11b\textsuperscript{+}Ly6C\textsuperscript{−} population, and their CCL2\textsuperscript{AF647} uptake is mediated by CCR2 expressed primarily by NK cells (33) and cDCs (Supplemental Fig. 2). When Ly6C\textsuperscript{hi} monocytes were examined, even the lowest concentration...
FIGURE 3. Cells expressing CCL2 receptors in mouse BM and blood. Cells from WT or Ccr2−/− (CCR2 KO) BM (A and C) or blood (B and D) cells were incubated with CCL2AF647 (± 10-fold molar excess of unlabeled CCL22), stained with fluorescently labeled Abs, and examined by flow cytometry. Dead cells and cell doublets have been excluded from all data. Leukocyte subsets were identified using the surface immunophenotype indicated to the right of each histogram overlay and using the gating strategy shown in Supplemental Fig. 3. (A and B) Representative overlaid histograms of CCL2AF647 uptake profiles for each of the populations indicated. (A) BM; (B) blood. (C and D) Mean percentage (+SD) of CCL2AF647-positive cells in each leukocyte subset (n = 3). CCL2AF647-positive WT cells were defined based on arbitrary gates set using equivalent populations of CCR2 KO BM cells that had been incubated with CCL2AF647 and CCL22: the percentage of cells in this gate is shown (gray columns). Three or more repeat experiments generated similar datasets. Data were analyzed using one-way ANOVA with Tukey posttest, *p < 0.05, ***p < 0.001.
(≈1.5 nM) of all chemokines inhibited some CCL2AF647 uptake, and 25 nM reduced uptake to levels only slightly higher than Ccr2−/− cells (Fig. 6B). CCL2 was marginally more effective than CCL7 or CCL12 at higher concentrations. With CD11b+Ly6C− cells, CCL2 showed a dose-response curve for inhibition of CCL2AF647 uptake that was similar to that seen with Ly6Chigh monocytes. However, CCL7 and CCL12 were much less effective competitors (Fig. 6C). Even when they were present at 25 nM, these two chemokines only partially inhibited CCL2AF647 uptake by these cells. Thus, the way that CCL7 and CCL12 interact with CCR2 depends on which cell type is expressing the receptor.

Further evidence of differences in CCR2 between cell types emerged when the ability of preincubation with CCL7 and CCL12 to prevent CCR2-mediated CCL2AF647 uptake was examined (Fig. 7). Splenocytes were exposed to unlabeled chemokine for 30 min at 37˚C and washed thoroughly at 4˚C, and then their CCL2AF647 uptake properties were assessed. Compared with coincubation (Fig. 6), much higher concentrations of chemokine were required to inhibit CCL2AF647 uptake using this preincubation approach. For example, pre-exposure to 12.5 nM CCL2 had barely any impact on subsequent CCL2AF647 uptake (Fig. 7B, 7C), but nearly completely blocked CCL2AF647 internalization when it was included during the uptake period (Fig. 6B, 6C). Moreover, only preincubation with 250 nM CCL2 inhibited uptake by WT cells to a level approaching that seen by Ccr2−/− cells (Fig. 7). However, as in the coincubation experiments, preincubation with each chemokine left a distinct CCL2AF647 uptake profile (Fig. 7A). CCL2 was more effective than CCL7 or CCL12 at reducing uptake by Ly6Chigh monocytes (Fig. 7B), and this difference between chemokines was even more striking when CD11b+Ly6C− cells were examined. With these cells, exposure to 100 nM CCL7 or CCL12 was unable to prevent any subsequent uptake of CCL2AF647, and only minimal inhibition was achieved at 250 nM, whereas equivalent concentrations of CCL2 resulted in strong suppression of CCL2AF647 uptake (Fig. 7C).

We also wished to explore the impact of CCL2 and CCL7 on the surface anti-CCR2 immunoreactivity of Ly6Chigh monocytes and CD11b+Ly6C− cells from WT spleens. Only one of four commercially available anti-mouse CCR2 Abs tested showed specificity for CCR2 (determined by comparing the flow cytometry profiles of untreated WT and Ccr2−/− cells [data not shown]), and whereas this Ab (monoclonal 475301) provided robust detection of CCR2 on Ly6Chigh monocytes, it was limited in its ability to detect CCR2 on untreated CD11b+Ly6C− cells. Nonetheless, it allowed us to explore whether exposure to chemokine resulted in changes in anti-CCR2 Ab binding to WT Ly6Chigh monocytes, and whether CCL2 and CCL7 differed in their ability to modulate this binding (Fig. 7D, 7E). When used at 12.5 nM, CCL2 and CCL7 both reduced subsequent anti-CCR2 Ab binding to Ly6Chigh monocytes by 40–50% (Fig. 7D). However, at higher concentrations (50 or 250 nM), CCL2 was able to further reduce anti-CCR2 Ab binding to these cells, but CCL7 was far less effective (Fig. 7D).
were obtained if Ly6<sup>high</sup> monocytes pre-exposed to CCL2 or CCL7 were allowed to internalize CCL2<sub>AF647</sub> before binding of the anti-CCR2 Ab was examined (Fig. 7E). Thus, CCL2 and CCL7 clearly differ in their ability to modify CCR2 on the surface of Ly6<sup>high</sup> monocytes, and this could contribute to the differences in CCL2<sub>AF647</sub> uptake seen after pretreatment with these chemokines.

Collectively, these data reveal that CCL2, CCL7, and CCL12 have unique properties that are determined, at least in part, by the ligand-binding capacity of ACKR2 and the cell type--specific ligand recognition properties and ligand-specific responsiveness of CCR2.

**Discussion**

Fluorescent chemokines complement existing tools, such as anti-chemokine receptor Abs or reporter gene knockin mice, that are typically used to identify cells expressing chemokine receptors (34–36). However, our study shows that fluorescent chemokine uptake assays have a number of added benefits. First, because they exploit the inherent specificity of chemokines for their...
FIGURE 7. Ligand- and cell type–specific modification of CCR2 behavior. (A) Representative flow cytometry profiles showing CCL2AF647 uptake by WT and Ccr2<sup>−/−</sup> (CCR2 KO) splenocytes that had been preincubated for 30 min with or without 250 nM unlabeled CCL2, CCL7, or CCL12, as indicated. Cells were separated according to Ly6C expression. Red boxes gate CCL2AF647-positive Ly6C<sup>+</sup> cells, and the percentage of cells in this gate, as a proportion of live cells, is shown. (B and C) Left panels, Representative overlaid histogram profiles showing CCL2AF647 uptake by (B) Ly6C<sup>high</sup> monocytes or (C) CD11b<sup>+</sup>Ly6C<sup>−</sup> cells from WT and Ccr2<sup>−/−</sup> (CCR2 KO) spleens preincubated for 30 min with or without a range of concentrations of unlabeled CCL2, CCL7, or CCL12, as indicated. The right panels show the average mean fluorescent intensity (MFI) (±SD) of CCL2AF647 uptake by Ly6C<sup>high</sup> monocytes or CD11b<sup>+</sup>Ly6C<sup>−</sup> cells (n = 3 WT mice). CCL2AF647 uptake by CCR2 KO cells is shown by the gray dotted line. Data were analyzed by two-way ANOVA with Bonferroni posttest, *p < 0.05, ***p < 0.001 (green, CCL2 versus CCL7; orange, CCL2 versus CCL12). (D) WT and Ccr2<sup>−/−</sup> splenocytes were incubated for 30 min with or without unlabeled CCL2 or CCL7 (12.5, 50, or 250 nM), and Ly6C<sup>high</sup> (Figure legend continues)
receptors, we find that they are easier to control than antibody-mediated detection methods. In our hands, CCL2<sup>AF647</sup> uptake is more sensitive, reliable, and reproducible than immunostaining with commercial anti-mouse CCR2 Abs as a way of detecting mouse cells expressing CCR2. Moreover, cells can be identified that express ACKR2, for which there is no Ab available for use in mice. Second, the only cells labeled are those carrying functionally competent chemokine receptors, that is, receptors able to bind and internalize the labeled chemokine. ACKR2 internalizes chemokine without chemokine-induced signaling, but activation of CCR2 is required for the internalization of CCL2/CCR2 complexes. Thus, cells showing CCR2-dependent CCL2<sup>AF647</sup> uptake carry CCR2 molecules capable of initiating intracellular signaling upon CCL2 binding. Third, all cells carrying functionally competent cCKRs and/or ACKRs for the labeled chemokine are labeled in these assays. As a result, they provide a comprehensive picture of how that chemokine is sensed and regulated by complex mixtures of cells. Fourth, they reveal the chemokine-scavenging potential of different leukocyte subsets. Ly6<sup>C</sup>high monocytes are the most effective CCL2 scavengers, and CCR2 mediates much more scavenging than ACKR2. Finally, fluorescent chemokines can be used to define the ligand specificity of cCKRs and ACKRs on primary cells and compare how chemokines interact with these receptors when expressed by different cell types in the same sample. Our data indicate that CCL2, CCL7, and CCL12 will have distinct properties in vivo because of the unique ways in which they interact with ACKR2 and CCR2. For all these reasons, we consider fluorescent chemokine uptake assays to be a particularly versatile method that has broad applicability in the detection and analysis of chemokine receptors on primary cells. Indeed, we have successfully used them to detect and characterize CCR1, CCR5, CCR7, CXCRR2, CXCR3, or ACKR4 on primary cells and found that single fluorescent chemokines work across a variety of mammalian species (D.L. Asquith, S.A. Bryce, M. Clarke, L.B. Ford, C.A.H. Hansell, and R.J.B. Nibbs, unpublished observations).

As expected, functionally competent CCR2 is expressed by all Ly6<sup>C</sup>high monocytes in BM, blood, and spleen. Ly6<sup>C</sup>low monocytes in mouse blood show heterogeneous CCR2 activity. These cells develop from circulating Ly6<sup>C</sup>high monocytes, during which cytes in mouse blood show heterogeneous CCR2 activity. These Ccr2<sup>−/−</sup> mice show CCR2 activity among Ly6<sup>C</sup>low monocytes is a reflection of their differentiation potential, and define their contribution to immune defects in Ccr2<sup>−/−</sup> mice.

Some populations of leukocytes, such as neutrophils, lack CCL2 receptors, but several others, such as γδ T cells, cDCs, and DX5<sup>+</sup> (NK) cells, contain subsets carrying functionally competent CCR2. These CCR2<sup>+</sup> cells have the potential to migrate in response to CCL2, and CCR2 on γδ T cells, cDCs, and NK cells is known to be indispensable in certain contexts (8, 9, 11). Innate-like B cells express ACKR2, but not CCR2, but both these CCL2 receptors are clearly expressed by some populations of cells, including pDCs and BM Ly6<sup>C</sup>lowCD115<sup>+</sup> monocytes.

pDCs can be separated into CCL2<sub>low</sub> and CCL2<sub>high</sub> subsets. In general, in WT mice, CCL2<sub>low</sub> pDCs express ACKR2, whereas CCL2<sub>high</sub> pDCs carry CCR2, but some splenic and BM pDCs appear to coexpress these receptors. pDCs migrate toward CCL2 in vitro (31) (Supplemental Fig. 4B, 4D), and CCL2 recruits pDCs into the skin after imiquimod treatment to contribute to tumor cell killing (12). We have not been able to determine whether CCL2<sub>low</sub> and CCL2<sub>high</sub> pDCs show differential chemotactic activity, because the exposure to CCL2<sup>AF647</sup> required for their fractionation prevents subsequent migratory responses. We could not separate these pDC subsets in other ways either, because differential CCL2<sup>AF647</sup> uptake activity does not cosegregate with other markers that can divide the pDC population into functionally distinct subsets (e.g., CD4, CD8, CD9, CCR9, Ly49Q) (data not shown), although it was notable that nearly all the rare CCR9<sup>+</sup> pDCs are in the CCL2<sub>high</sub> subset (Supplemental Fig. 4E). CCR9<sup>+</sup> pDCs can act as dendritic cell precursors and give rise to cDCs and pDCs (40, 41), whereas CCR9<sup>+</sup> pDCs are reportedly enriched for tolerogenic activity (40, 42, 43) and can home to the small intestine (30). CCR2 is responsible for CCL2-induced cell migration, but the role of ACKR2 on pDCs is unclear. It may act solely as a scavenger, but it was only weakly active in this regard in the CCL2<sup>AF647</sup> uptake assays. Other functions are perhaps more likely, and it is interesting that ACKRs, including ACKR2, have been shown to modulate coexpressed cCKRs (3). For example, CXCR4 and ACKR3 (the cCKR and ACKR, respectively, for CXCL12) are coexpressed by migrating interneurons during brain development, and deletion of Ackr3 disrupts CXCR4-mediated signaling (44). Conversely, when Ackr2 is deleted, B1 B cells become more responsive to CXCL13 and can, unlike WT B1 B cells, respond weakly to some proinflammatory chemokines (20). Thus, ACKR2 on pDCs may control coexpressed cCKRs, including CCR2 and the closely related receptor CCR5, which is expressed by all pDCs, shares ligands with ACKR2 (e.g., CCL3 and CCL4), and controls pDC release from the BM (31). Experiments are underway to explore these ideas and to examine whether the CCL2<sub>low</sub> and CCL2<sub>high</sub> subsets represent functionally distinct populations of pDCs.

Our data reveal the CCL2-scavenging potential of individual leukocyte subsets. On a cell-by-cell basis, and after labeling ex vivo or in vivo, Ly6<sup>C</sup>high monocytes internalize much more CCL2<sup>AF647</sup> than any other leukocyte population. These cells are
abundant in blood and lymphoid tissues and use CCR2 to navigate into inflamed tissues in large numbers. Because their CCL2-AM647 uptake can only be effectively blocked by relatively high concentrations of CCR2 ligand (Fig. 7), they are likely to be able to scavenge chemokines through CCR2 during, and after, their recruitment into tissues. Indeed, elegant experiments performed by Volpe et al. (4) demonstrated that migrating monocytes internalize substantial quantities of fluorescent CCL2, while retaining their ability to respond to gradients of this chemokine. Collectively, these data indicate that chemokine scavenging by CCR2 in vivo, which has been shown to regulate chemokine abundant at steady state and during inflammation induced by intratracheal LPS challenge or the implantation of allogeneic tissue (5–7), is most likely driven primarily by Ly6C<sup>high</sup> monocytes. In contrast, ACKR2, which is considered to be a professional chemokine scavenger (3), has only very low CCL2-scavenging potential on leukocytes. It seems unlikely that it will have a major impact on chemokine abundance in a tissue and may instead operate only at specific microanatomical niches, such as the splenic MZ. Indeed, anatomically restricted ACKR2-mediated scavenging serves a key role in the skin (3, 45). Lymphatic endothelial cells in this tissue express ACKR2 to prevent them from becoming coated in inflammatory chemokines. This is important because it stops leukocytes accumulating around these vessels and interfering with the flow of tissue fluid and mature dendritic cells from the skin (45).

CCR2, CCL7, and CCL12 clearly differ in the way they interact with leukocytes. CCL2 and CCL12, but not CCL7, are ligands for ACKR2 (20), and the interaction of CCL7 and CCL12 with CCR2 is influenced by cell background. The data suggest that CCR2 exists in two forms. DX5<sup>+</sup> (NK) cells and cDCs in the splenic CD11b<sup>+</sup> Ly6C<sup>+</sup> population express a version of CCR2 that is more readily activated by CCL2 than by CCL7 or CCL12. In contrast, because low concentrations of CCL2, CCL7, or CCL12 (up to 12.5 nM) coincubated with CCL2<sup>AM647</sup> show very similar abilities to block fluorescent chemokine uptake by Ly6C<sup>high</sup> monocytes, it appears that the dominant form of CCR2 on these cells does not readily discriminate between these three chemokines. However, at higher concentrations (50 nM or above), CCL2 is more effective than CCL7 at reducing anti-CCR2 Ab binding to these cells, suggesting that it drives more extensive CCR2 internalization under these conditions. The molecular bases and biological implications of these observations are under investigation. In humans, alternative splicing generates two isoforms of CCR2 (termed CCR2A and CCR2B) (46), but there is no evidence that equivalent diversity exists in mice. However, differences in posttranslational processing or heterodimerization could conceivably result in cell-specific differences in CCR2 behavior. Human CCR2 can heterodimerize and hetero-oligomerize with human CCR5 and CXCR4, and negative binding cooperativity exists between these receptors in transfected cell lines and primary human cells (47–50). According to microarray data available through Immgen (www.immgen.org), the CCR2<sup>+</sup> cell types examined in our study express CXCR4 but differ in their level of CCR5 expression. Thus, the precise nature of the dimers and higher order structures involving CCR2 could vary between Ly6C<sup>high</sup> monocytes and CD11b<sup>+</sup>Ly6C<sup>+</sup> cells to modulate the behavior of CCR2. Moreover, the ability of CCL7 and CCL12 to interact with a broader array of chemokine receptors than CCL2 may also be relevant.

We expect that the high expression and enhanced ligand-binding properties of CCR2 on Ly6C<sup>high</sup> monocytes will make these cells more responsive than other CCR2<sup>+</sup> cells to low concentrations of CCL7 and CCL12. Interestingly, under resting conditions, all cells whose steady state trafficking is affected by deficiency in Ccr2 (i.e., Ly6C<sup>high</sup> monocytes, CD115<sup>+</sup> cells in the BM, and the circulating CD115<sup>+</sup> Ly6C<sup>high</sup> monocyte-like cells discussed above) highly express a form of CCR2 in WT mice that interacts efficiently with CCL2, CCL7, and CCL12 (data not shown) (10, 13, 14). It is possible that this form of CCR2 is specifically required for monocyte navigation out of the BM, a process regulated by CCL2 and CCL7 (13, 14). It might also endow Ly6C<sup>high</sup> monocytes with greater sensitivity than other CCR2<sup>+</sup> cells when it comes to CCR2-dependent recruitment into inflamed tissues, and it will be of interest to see whether other leukocytes switch to a Ly6C<sup>high</sup> monocyte-like form of CCR2 to help facilitate their migration during inflammation or infection. Studies are also underway to examine whether the ligand recognition properties of other chemokine receptors are, like CCR2, subject to cell-specific modulation.

Chemokine receptors are attractive therapeutic targets in many diseases. Some chemokine receptor antagonists have reached the clinic, but many others have failed, and evidence of effective inhibition chemokine receptor function in vivo is often lacking (51). Future efforts in this area will benefit from work that builds a greater understanding of the expression and functional properties of cCKRs and ACKRs in humans and experimental animals. Fluorescent chemokines will be a valuable tool in this type of work. Moreover, because they detect functionally active chemokine receptors, fluorescent chemokines could be used to assess receptor activity in blood samples during preclinical and clinical trials. This would determine whether the drug being used has true potency against its target in vivo. We think that this could be particularly useful in trials that fail to ameliorate disease, because robust evidence of in vivo drug activity will strengthen the conclusion that the receptor under investigation is not a good target in that disease. It will also mean that the drug can be used in subsequent trials in other disease settings with the knowledge that it is genuinely blocking receptor activity in the patients.

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Disclosures
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References


