Chemokine expression profile of an innate granuloma.

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Keywords: granuloma, *Chromobacterium violaceum*, chemokines, CCR2

Impact statement

A successful innate granuloma requires CCR2 to organize the macrophage ring, and without CCR2, mice succumb to *Chromobacterium violaceum* infection.

Abstract

Granulomas are defined by the presence of organized layers of immune cells that include macrophages. Granulomas are often characterized as a way for the immune system to contain an infection and prevent its dissemination. We recently established a mouse infection model where *Chromobacterium violaceum* induces the innate immune system to form granulomas in the liver. This response successfully eradicates the bacteria and returns the liver to homeostasis. Here, we sought to characterize the chemokines involved in directing immune cells to form the distinct layers of a granuloma. We use spatial transcriptomics to investigate the spatial and temporal expression of all CC and CXC chemokines and their receptors within this granuloma response. The expression profiles change dynamically over space and time as the granuloma matures and then resolves. To investigate the importance of monocyte-derived macrophages in this immune response, we studied the role of CCR2 during *C. violaceum* infection. *Ccr2*$^{-/-}$ mice had negligible numbers of macrophages, but large numbers of neutrophils, in the *C. violaceum*-infected lesions. In addition, lesions had abnormal architecture resulting in loss of bacterial containment. Without CCR2, bacteria disseminated and the mice succumbed to the infection. This indicates that macrophages are critical to form a successful innate granuloma in response to *C. violaceum*.

Introduction

Granulomas are organized aggregates of immune cells defined by the presence of macrophages, with a variety of other features (i.e. necrosis and fibrosis) being more variable (Warren, 1976). The evolved function of the granuloma response is thought to be a protective mechanism by which immune cells sequester a foreign body or pathogen, walling-off the threat (Pagán & Ramakrishnan, 2018). Some pathogens are not successfully eliminated, however, leading to chronic granulomas that persist for months or sometimes even years. New in vivo models are needed to study the complicated mechanisms that coordinate the formation of protective granulomas, in order to understand the events that lead to the successful clearance of pathogens that initiate this response.

We seek to identify environmental pathogens that have immense virulence capacity but are defeated by the innate immune system. *Chromobacterium violaceum* is one such pathogen that invades host cells and replicates in the intracellular niche, but only causes morbidity and mortality in immunocompromised animals or individuals (Macher, 1982). We discovered that during infection, wildtype (WT) C57BL/6 mice develop necrotic liver granulomas in response to this ubiquitous soil microbe (Harvest et al., 2023; Maltez et al., 2015). As soon as 1 day post-infection (1 DPI), liver microabscesses can be macroscopically visualized. These lesions are composed primarily of neutrophils until approximately 3 - 5 DPI, when, importantly, monocytes traffic into the area and form a mature granuloma starting at 5 DPI. Once the resulting macrophage zone surrounds the infected lesion, bacterial burdens begin to decrease, suggesting that granuloma macrophages are an important cell type for the clearance of *C. violaceum*. By 21 DPI, virtually all mice clear the infection and resolve the granuloma pathology, leaving small collagen scars in place of lesions (Harvest et al., 2023). Though we identified neutrophils and macrophages as the key immune players in this model, much remains to be learned about the cellular mechanisms that initiate formation of the granuloma in response to *C. violaceum*, and what signals instruct immune cells to organize within the granuloma architecture. Indeed, by studying the granuloma response that successfully clears *C. violaceum*, we hope to identify critical cellular mechanisms that underlie the basic biology of the granuloma response.

Within the granuloma response to *C. violaceum*, neutrophils and then macrophages migrate and assemble in an organized manner. Cellular movement, or chemotaxis, must be carefully regulated during tissue development, homeostasis, and inflammatory responses (Hughes & Nibbs, 2018). Chemotaxis is controlled by small, secreted proteins called chemokines that signal through transmembrane chemokine receptors. Since their discovery in the 1980's, approximately 50 chemokines are now appreciated for their role in cellular chemotaxis (Zlotnik & Yoshie, 2012). The temporal and spatial expression of chemokines and chemokine receptors dictate cellular trafficking, and dysregulation of these systems is linked to many diseases (Turner et al., 2014).

As more chemokines have been identified, there have been multiple revisions to their nomenclature, and now a systematic naming of chemokines and their receptors is in wide use. Chemokines have conserved cysteine residues, and the current naming system categorizes four subfamilies based on the arrangement of these N-terminal cysteines: CXC, CC, XC, and CX_3C (Zlotnik & Yoshie, 2000). Though there are exceptions, most chemokines fit into one of these four subfamilies. Similarly, chemokine receptors fall into four subfamilies based on their chemokine ligand. The naming scheme has become complicated due to promiscuous ligand-receptor interactions, reassigning of mouse and human homologs after syntenic analysis, and divergent evolution of ligands in mice and humans (Nomiyama et al., 2013). Nonetheless, the detailed description of many chemokines and their receptors has been accomplished in both species. Herein, we focus on the mouse chemokines and their role in the *C. violaceum*-induced murine granuloma.

Inflammatory chemokines are those that are rapidly upregulated in the presence of infection or other inflammatory stimuli (David & Kubes, 2019). Several cell types can upregulate chemokines, creating a gradient of ligand that diffuses away from the point of infection. Still other cell types can respond to this gradient if/when they express the appropriate receptor. Furthermore, activated cells that migrate to the area can also upregulate expression of chemokines, creating a feed-forward loop to enhance cell recruitment. In addition to mediating chemotaxis, chemokines can induce a variety of other cellular responses including proliferation, oxidative burst, and even degranulation (Hughes & Nibbs, 2018). Lastly, it is now appreciated that chemokines also contribute to wound healing and resolution of inflammation, with coordinated efforts between neutrophils and macrophages to clean up debris and halt immune cell infiltration (Soehnlein & Lindbom, 2010).

Here, we use spatial transcriptomics to identify key genes that are upregulated in response to *C. violaceum*, and assess the importance of CCR2-dependent monocyte trafficking to the site of infection in the liver.

Results

Spatial transcriptomics of an innate granuloma

In our initial characterization of the granuloma response to *C. violaceum*, we used spatial transcriptomics (10x Genomics, Visium Platform) to identify genes that are upregulated at critical timepoints during infection, including 0.5, 1, 3, 5, 7, 10, 14, and 21 DPI (note: we excluded the 7 DPI timepoint from analysis because the granuloma in this capture area was not representative of typical 7 DPI granulomas histologically). A major advantage of this technology is the ability to conserve the spatial location of expression data by overlapping cDNA output with hematoxylin and eosin (H&E)-stained tissue sections (Figure 1A). Each capture area can collect nearly 5,000 barcoded spots, each spot being 55 μm in diameter. Though this is not single-cell resolution, the dataset successfully identified 16 unique clusters with differentially expressed genes (Figure 1B), representing cell types (e.g. hepatocytes and endothelial cells), and also representing spatial elements (e.g. necrotic core center, etc.). These unique clusters appear at various timepoints, with 10 DPI being the most enriched timepoint with 14 out of the 16 clusters present (Figure 1C). We further characterized the clusters by assigning appropriate cell types based on each cluster's gene expression profile and its location within the granuloma (original characterization performed in (Harvest et al., 2023), annotation shown in Figure 1C, D).

The spatial transcriptomics dataset was rich with candidate genes that could be critical for the successful granuloma response. Specifically, we were interested in the expression of chemokines and chemokine receptors that could be involved in the recruitment of key cell types, namely neutrophils and monocytes, to the site of infection within the liver. Indeed, immune cell trafficking is required for granuloma formation in various infectious and non-infectious models, and chemokines are the obvious candidates for facilitating this chemotaxis (Chensue, 2013).

To investigate various chemokines (Table 1) and chemokine receptors (Table 2), we used the Seurat package in RStudio to analyze gene expression over time and space. We used the SpatialFeaturePlot to assess relative gene expression within the granuloma at each timepoint. For example, *Pf4* (the murine homolog of *CXCL4*) is highly expressed at 10 DPI, corresponding with clusters 0, 6, 9, 11, and 15 (Figure 1E). Though chemokines and chemokine receptors are key facilitators of chemotaxis, other pro-inflammatory molecules such as damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) also direct cells to sites of inflammation. In fact, neutrophils respond to chemotactic molecules in a hierarchical manner, integrating a variety of signals and prioritizing end-target molecules (David & Kubes, 2019; Kolaczkowska & Kubes, 2013). Further demonstrating the complexity of chemotaxis, various adhesion molecules are also required for transmigration of cells out of the blood and into tissues. Indeed, we saw significant upregulation of a number of these genes in this model (Table 3). Though these chemoattractive and adhesion molecules are likely involved and could be explored in future studies, in this paper we focus on the chemokines and their receptors.

Expression of neutrophil-attractive chemokines

We observed high expression levels of chemokines involved in neutrophil trafficking (e.g., *Cxcl1* and *Cxcl2*) as early as 12 hours post-infection (0.5 DPI) (Figure 2A-B), which correlates with our previous data that neutrophils are the first immune cells to arrive in response to *C. violaceum* (Harvest et al., 2023). Two other ligands that also bind to CXCR2 are CXCL3 and CXCL5. In contrast to *Cxcl1* and *Cxcl2*, *Cxcl3* and *Cxcl5* show delayed expression peaking around 10 DPI (Figure 2C-D). In addition to temporal differences, the spatial location of chemokine expression varies within the lesion. For example, at 5 DPI *Cxcl1* is expressed more towards the periphery of the lesion, while *Cxcl2* is expressed more towards the center (Figure 2A-B). For all of these ligands, expression is absent by 21 DPI, which correlates with the time at which the majority of mice

clear the infection. Therefore, although these four chemokines all bind to CXCR2, they clearly demonstrate the complexity of different temporal and spatial expression profiles over the course of infection.

Expression of monocyte-attractive chemokines

We also investigated chemokines and receptors involved in monocyte trafficking (e.g., *Ccl2*, *Ccl7*, and *Ccl12*). Though all three of these ligands bind to CCR2, they had vastly different expression levels through the course of infection (Figure 3). *Ccl2* was the most highly upregulated, while *Ccl12* was expressed only at low levels, and *Ccl7* expression was somewhere in between (Figure 3A-C). Similar to the chemokines involved in neutrophil trafficking, these ligands are not expressed by 21 DPI.

Compilation of chemokine and receptor expression data

In order to summarize our findings in a way that facilitates comparisons, we used the SpatialFeaturePlot to visually rank the expression intensity of each chemokine and receptor as absent, low, medium, or high over the course of infection. Each rank was based on both the intensity of expression and the relative number of spots that expressed the gene. For example, *Cxcl1* expression was ranked as medium at 0.5 DPI, and ranked as high at 1 and 3 DPI based on the large presence of orange and red spots (Figure 2A). In contrast, *Cxcl3* was ranked as absent at 0.5 DPI, low at 1 DPI, and medium at 3 DPI based on the fewer spots that were orange or red (Figure 2C). We depicted these ranks as qualitative heatmaps (Figure 4A-D). The relative expression of various chemokines (Figure S1 – Figure S5) was much greater than the relative expression of their receptors (Figure S6 – Figure S7), which is expected because large quantities of chemokines are needed to create gradients in tissues, but comparatively low expression of chemokine receptors is sufficient to enable trafficking of cells that express the receptors. Therefore, we changed the scale to best visualize receptor expression.

One aspect of chemokine biology that makes understanding their function complicated is the promiscuity of certain ligands for multiple receptors, and vice versa. For example, CCL3, which is highly upregulated during infection with *C. violaceum*, can bind to CCR1 (along with several other chemokines), and CCL3 can also bind to CCR5 (again, along with several other chemokines). This promiscuity often makes it challenging to determine what unique or redundant roles each chemokine and chemokine receptor are playing. In order to simplify and graphically depict ligand and receptor interactions that seem relevant to the *C. violaceum*-induced granuloma, we listed the ligands that bind to the receptors that were expressed (Figure 4C-D). We colored each respective ligand based on its maximum expression ranking, regardless of the timepoint. This visualization allows for easier generation of hypotheses from this complex dataset.

Weakly expressed chemokines suggest that certain immune cells are dispensable

The chemokines that are not present or are only weakly expressed can also be informative (Figure 4A-B). Two chemokines that are important for migration to the lung, *Cxcl15* and *Cxcl17*, are both absent (as expected). Still other chemokines that are important for migration to the skin, lymph nodes, and mucosal tissues are also absent, namely *Ccl17*, *Ccl27b*, *Ccl21b-c*, and *Ccl28*, respectively (also as expected). Such negative data provides stronger confidence in the positive expression data for other chemokines.

In our previous studies, we showed that the adaptive immune response is dispensable to successfully form granulomas around, and then to eradicate, *C. violaceum* (Harvest et al., 2023). In agreement with those findings, several chemokines involved in T cell trafficking are absent or only expressed at low levels (i.e. *Cxcl11*, *Ccl1*, *Ccl22*, and *Ccl25*) (Figure 4A-B). On the other hand, other chemokines involved in T cell trafficking such as *Cxcl9* and *Cxcl10* are highly expressed during the first few days of infection, as is their receptor *Cxcr3* (Figure 4A, C). During primary infection, T cell recruitment is not essential for clearance and we found that T cells are not recruited in large numbers (Harvest et al., 2023). However, *Cxcl9* and *Cxcl10* could play a more important role during a secondary infection that involves the adaptive immune response. It is a curious observation that T cells are dispensable during primary infection because in *Mycobacterium tuberculosis*-induced granulomas, CD4+ T helper type 1 (Th1) cells are required to stimulate the antibacterial activity of macrophages (Pagán & Ramakrishnan, 2018). A key difference between granuloma formation in

response to *C. violaceum* compared to *M. tuberculosis* could be that *M. tuberculosis* is able to intracellularly infect macrophages, whereas *C. violaceum* is unable to circumvent pyroptosis of macrophages.

We did not observe basophils or eosinophils histologically during infection with *C. violaceum*, and this was again supported by the absence or low expression of chemokines involved in trafficking of these cell types (i.e. *Ccl11*, *Ccl24*, and *Ccl26*) (Figure 4B). CCR3, which is expressed mainly by eosinophils, plays a major role in the granuloma response to parasitic *Schistosoma mansoni* eggs (Chensue, 2013). During infection with *C. violaceum*, however, *Ccr3* is not expressed at any timepoint (Figure 4D), further supporting that eosinophils are not involved in the granuloma response to *C. violaceum*. Furthermore, granulomas that form in response to *M. tuberculosis* often contain follicular dendritic cells which secrete CXCL13 to recruit B cells via CXCR5 (Domingo-Gonzalez et al., 2016). However, *Cxcl13* is expressed at low levels, and *Cxcr5* is absent in the *C. violaceum* model (Figure 4A, C). These examples reveal chemokines that are likely dispensable in the context of *C. violaceum*.

Comparison of neutrophil- and monocyte-recruiting chemokines

To compare chemokines involved in neutrophil recruitment or monocyte recruitment, we further characterized *Cxcl1* and *Ccl2*, respectively (Figure 5). When comparing their SpatialFeaturePlots, *Cxcl1* and *Ccl2* had unique expression profiles corresponding to different cluster identities (Figure 2A, Figure 3A). To more easily visualize these differences in expression, we generated UMAP plots and violin plots (Figure 5A-D). Though there is some overlap, suggesting that some clusters express both *Cxcl1* and *Ccl2*, there are also some clusters that appear to express only one or the other (Figure 5A-B). For example, cluster 14 (a cluster enriched for hepatocytes) expressed high levels of *Cxcl1* but only low levels of *Ccl2* (Figure 5C-D). Furthermore, there are interesting differences in temporal expression; *Cxcl1* is highly expressed at 1 DPI while *Ccl2* expression peaks at 3 DPI (Figure 5E-F). Though gene expression does not necessarily correlate with the timing and intensity of protein expression, we expect CXCL1 and CCL2 protein levels to accumulate over time, which would allow proper chemokine gradients to form. Altogether, these data corroborate our previous findings that neutrophils traffic to the liver within 1 DPI, and monocytes traffic and form granulomas beginning at 3 DPI.

Neutrophil chemotaxis

We next wanted to investigate whether the upregulated neutrophil-recruiting chemokines are important during infection. However, there are many challenges when studying chemokines. As previously mentioned, ligands and receptors often show promiscuity in that one receptor may bind multiple ligands, which makes it difficult to completely abrogate chemotaxis through inhibiting a single ligand. Further, although chemokine-specific antibodies exist (Fox et al., 2009; Mollica Poeta et al., 2019; Vales et al., 2023), neutralizing such large quantities of ligand can be challenging. Therefore, instead of attempting to block chemokine ligands, we chose to target chemokine receptors. In fact, the promiscuity of ligands and receptors means that targeting one chemokine receptor has the potential to impact more than one ligand of interest (Figure 4C-D). Nevertheless, targeting receptors is also challenging due to poor solubility of many receptor antagonists (Li et al., 2019).

During infection with *C. violaceum*, neutrophils appear in the liver within 1 DPI. However, it is still unclear what signals initiate their migration into the liver. Though a large number of neutrophils are already present in the blood during homeostasis, additional neutrophils expressing CXCR2 exit the bone marrow in response to endothelial cell (EC)-derived CXCL1 and CXCL2 (David & Kubes, 2019). Furthermore, tissue-resident macrophages can also express CXCL1, CXCL2, and various leukotrienes in response to infection (Soehnlein & Lindbom, 2010). Though *Cxcr2* knockout mice exist, they have abnormalities (Cacalano et al., 1994). Therefore, to assess the role of CXCL1, CXCL2, CXCL3, and CXCL5 in neutrophil trafficking during infection with *C. violaceum*, we used a CXCR2 inhibitor. Reparixin is an allosteric inhibitor of CXCR1 and CXCR2 that has been shown to inhibit neutrophil trafficking during ischemia-reperfusion injury and acid-induced acute lung injury (Bertini et al., 2004; Zarbock et al., 2008; Hosoki & Sur, 2018). We pre-treated mice with reparixin or saline (PBS) 1 day before infection, then infected mice with *C. violaceum* followed by daily treatment with reparixin or PBS (Figure S8A). We then harvested livers and spleens at 3 DPI to assess

bacterial burdens. Though there was no difference in CFU for the liver, a few CFU were recovered from the spleens of two reparixin-treated mice (Figure S8B), which, though this was not statistically significant, is unusual for WT mice. Based on these results, we hypothesized that reparixin would have a stronger effect at 1 DPI (Figure S8C), before the infection causes excessive damage to the liver. At 1 DPI, we again saw no difference in bacterial burdens in the liver of reparixin-treated mice (Figure S8D). To verify that reparixin affected neutrophil numbers in the liver and spleen, we used flow cytometry to quantify Ly6G$^+$ neutrophils (Figure S8E). We observed differences in the number of neutrophils between PBS-treated female and male mice, so data were analyzed disaggregated for sex. Though reparixin might have caused a subtle decrease in neutrophil numbers in the liver and spleen at 1 DPI, the results were variable between mice (Figure S8F-G). In our hands, reparixin was poorly soluble in PBS, which could account for some of the variability. Because monocytes also express CXCR2, albeit to a much lesser extent than neutrophils, we also stained for CD68. There was no marked difference in macrophage numbers in the liver or spleen between PBS- and reparixin-treated mice (Figure S8H-I).

Altogether, it is clear that reparixin was not a successful inhibitor of neutrophil recruitment during infection with *C. violaceum*. The role of CXCR1/2 and their ligands could be further studied using knockout mice. Regardless, other chemoattractants likely contribute to neutrophil recruitment as well. Indeed, neutrophils migrate in response to a variety of pro-inflammatory DAMPs and PAMPs (Kolaczkowska & Kubes, 2013). Importantly, formyl peptide receptors such as FPR2 promote neutrophil migration in response to bacterial infection in the liver (Lee et al., 2023). In support of this, formyl peptide receptors (FPRs) are upregulated in this model (Table 3).

CCR2 is essential for monocyte trafficking and defense against *C. violaceum*

Previously, we noticed that the appearance of organized macrophages at approximately 5 DPI correlates with a subsequent decrease in bacterial burdens (Harvest et al., 2023). We also observed that *Nos2*$^{-/-}$ mice, which lack the ability to express inducible nitric oxide synthase (iNOS), succumb to infection beginning at 7 DPI, a timepoint when the granuloma matures with a thicker macrophage ring (Harvest et al., 2023). Though neutrophils can also express iNOS (Saini & Singh, 2018), these data suggested that macrophages are playing a critical protective role. We therefore hypothesized that monocyte trafficking to the site of infection is a key event in clearing the infection. There are several candidate chemokines that could attract monocytes to the site of infection, and these chemokines bind to several different receptors (Table 1, Figure 4D). We chose to focus on the chemokine receptor CCR2 because of its known role in monocyte migration out of the bone marrow (Serbina & Pamer, 2006). Importantly, *Ccr2*$^{-/-}$ mice have intact tissue-resident macrophage populations but are unable to recruit additional monocytes in the event of infection (Kurihara et al., 1997).

To assess the role of monocyte trafficking to lesions in the liver, we infected *Ccr2*$^{-/-}$ mice with *C. violaceum*. Strikingly, *Ccr2*$^{-/-}$ mice were highly susceptible and succumbed to infection beginning at 5 DPI, with all mice dying by 9 DPI (Figure 6A), which is more severe than phenotype in *Nos2*$^{-/-}$ mice (Harvest et al., 2023). This is in contrast to *Yersinia pseudotuberculosis* models in which deletion of *Ccr2* has the opposite phenotype, and loss of monocytes is actually protective (Zhang et al., 2018). This also contrasts with *M. tuberculosis* models where loss of *Ccr2* has no effect on survival in some contexts (Domingo-Gonzalez et al., 2016; Scott & Flynn, 2002). At 5 DPI, *Ccr2*$^{-/-}$ mice had increased liver burdens (Figure 6B), and bacterial dissemination into the spleen (Figure 6C). We also observed that *Ccr2*$^{-/-}$ mice had abnormal lesions which were more numerous and larger than the lesions of WT mice (Figure 6D).

We used flow cytometry to assess macrophage (CD68$^+$) and neutrophil (Ly6G$^+$) numbers in the liver, spleen, and blood of mice at 5 DPI (Figure 6E). Uninfected WT and uninfected *Ccr2*$^{-/-}$ mice had similar macrophage numbers in the liver (Figure 6F), likely representing the tissue-resident Kupffer cell population, as well as similar numbers of splenic macrophages (Figure 6H). However, upon infection, the livers of *Ccr2*$^{-/-}$ mice had markedly less macrophages and drastically more neutrophils compared to the livers of WT mice (Figure 6F-G). This trend was also observed in the spleen (Figure 6H-I) and blood (Figure 6J-K), showing that failure to recruit monocytes leads to enhanced neutrophil recruitment. Interestingly, infected *Ccr2*$^{-/-}$ mice did have slightly more macrophages in the liver, spleen, and blood compared to uninfected *Ccr2*$^{-/-}$ mice (Figure 6F,

H, J), suggesting that loss of CCR2 does not completely abrogate monocyte recruitment. Alternatively, this expansion could represent emergency hematopoiesis and proliferation of pre-existing cell populations in these tissues (Boettcher & Manz, 2017).

C. violaceum in the liver cannot be contained without macrophages

In our previous characterization of granulomas in WT mice, we identified three distinct zones using immunohistochemistry (IHC): necrotic core (NC), coagulative necrosis (CN), and macrophage zone (M) (Harvest et al., 2023). By 5 DPI, all three layers are distinctly visible through hematoxylin and eosin (H&E) staining (Figure 7A, Figure S9A). Furthermore, we consistently see containment of *C. violaceum* within the necrotic core (Figure 7B, Figure S9B), which overlaps with pronounced Ly6G staining (Figure 7C, Figure S9C). Importantly, by 5 DPI the macrophage zone is clearly visible in WT mice, showing that macrophages surround the granuloma and form a protective barrier between the coagulative necrosis zone and healthy hepatocytes outside the infected lesion (Figure 7D, Figure S9D). Compared to WT mice, lesions in *Ccr2*$^{-/-}$ mice lack these distinct zones. Though *Ccr2*$^{-/-}$ mice had larger areas of necrotic debris, the coagulative necrosis zone was largely absent from most lesions (Figure 7E, Figure S9E). In previous studies, we also observed sporadic clotting in WT mice (Harvest et al., 2023), and this clotting was even more abundant in *Ccr2*$^{-/-}$ mice (Figure S10C). Excessive clotting, in addition to elevated bacterial burdens and sepsis, could also cause mortality in these mice by pulmonary embolism. Strikingly, lesions in *Ccr2*$^{-/-}$ mice had abnormal budding morphology, which stained very strongly for *C. violaceum* (Figure 7F, Figure S9F) and Ly6G neutrophils (Figure 7G, Figure S9G). In fact, many puncta that appear to be individual bacteria were visualized (Figure S10A).

Though we were able to visualize the Kupffer cell population scattered throughout the liver, an organized macrophage zone was absent from the majority of lesions in *Ccr2*$^{-/-}$ mice (Figure 7H, Figure S9H). These Kupffer cells are likely the CD68^{+} cells identified by flow cytometry (Figure 6F). A rare *Ccr2*$^{-/-}$ mouse that survived to 7 DPI also had few macrophages (Figure S10B, D), in contrast to WT mice that display mature granulomas with thick macrophage zones at this timepoint (Harvest et al., 2023). Importantly, without distinct coagulative necrosis or macrophage zones, *C. violaceum* staining extends well outside the center of each lesion. In fact, numerous bacteria were identified in immune cells immediately adjacent to the healthy hepatocyte layer (Figure 7F, Figure S9F, Figure S10A). This staining, along with the elevated CFU burdens, suggests that without CCR2, monocyte recruitment fails, and the lack of a macrophage zone leads to loss of bacterial containment. Despite the excessive number of neutrophils in the liver, spleen, and blood of *Ccr2*$^{-/-}$ mice (Figure 6G, I, K), these mice are unable to clear the infection and ultimately succumb.

Previously, we observed abnormal lesion architecture in *Casp1-11*^{DKO} and *Gsdmd*$^{-/-}$ mice with budding morphology and loss of bacterial containment (Harvest et al., 2023) that is remarkably similar to the architecture observed in *Ccr2*$^{-/-}$ mice (Figure 7F, Figure S9F, Figure S10D). However, the *Ccr2*$^{-/-}$ mice survive a few days longer and thus develop even larger lesions over time. Together, these data suggest that macrophage recruitment and pyroptosis are both essential in defense against, and containment of, *C. violaceum*. In addition, because the *Ccr2*$^{-/-}$ mice succumb in a timeframe similar to that seen with *Nos2*$^{-/-}$ mice, this supports our hypothesis that it is nitric oxide derived from granuloma macrophages that is specifically required for bacterial clearance. Altogether, these data indicate that without monocytes trafficking to the site of infection, *C. violaceum* is able to replicate and spread into adjacent hepatocytes, resulting in ever-expanding lesions. These in vivo data support the transcriptomics dataset and provide proof-of-concept that upregulated genes, specifically chemokines, are critical to the formation of the granuloma.

Discussion

Here, we demonstrate that macrophages are essential for clearance of *C. violaceum* from the infected liver, and for protection against dissemination into the spleen. Loss of CCR2-dependent monocyte trafficking results in a loss of bacterial containment, ultimately leading to uncontrolled bacterial replication in the liver, evidenced by elevated CFU burdens and increased lesion size.

There are many questions that still remain about the individual and coordinated efforts of neutrophils and macrophages during infection with *C. violaceum*. It is likely that the tissue resident Kupffer cells and infected hepatocytes are the first cells to sound the alarm, calling for neutrophils. The initial recruitment of neutrophils likely involves chemokines (i.e. CXCL1 and CXCL2) redundantly with other chemoattractants such as formylated peptides and leukotrienes. However, these neutrophils are unable to clear the infection despite being recruited in large numbers.

Based on our data, CCR2 is an essential chemokine receptor for monocyte trafficking in response to *C. violaceum*, but we have not yet determined which ligand(s) mediate this response. CCL2 and CCL7 can both bind to CCR2 to induce monocyte trafficking. Importantly, pro-inflammatory cytokines and PAMPs can induce CCL2 expression by most cell types (Shi & Pamer, 2011). In agreement, we see upregulation of *Ccl2* in several clusters, further suggesting that CCL2 may be a critical chemokine that promotes monocyte recruitment in response to *C. violaceum*. In contrast, *Ccl7* is expressed by fewer clusters, and to a lesser degree, and its expression is slightly delayed compared to *Ccl2*. Deletion of either ligand partially diminished monocyte trafficking in response to *Listeria monocytogenes* infection, but the individual role of each ligand was unclear (Jia et al., 2008). Future studies using *C. violaceum* could further elucidate the unique or redundant roles of CCL2 and CCL7. Lastly, adoptive transfer experiments in the context of *Listeria* infection showed that *Ccr2*$^{-/-}$ monocytes are still able to traffic to the site of infection in the spleen (Serbina & Pamer, 2006) and liver (Shi et al., 2010). During *C. violaceum* infection, we have not yet determined whether CCR2 is required for migration once monocytes have left the bone marrow, as CCR2 is required for this initial egress. We saw a subtle increase in the number of macrophages in the liver of infected *Ccr2*$^{-/-}$ mice. Though macrophage numbers in *Ccr2*$^{-/-}$ tissues remain considerably lower than seen in WT mice, there are two explanations for the subtle increase: 1) loss of CCR2 may not completely abrogate monocyte recruitment, as monocytes could be migrating via other chemokine receptors, or 2) tissue-resident macrophages, or even tissue-resident hematopoietic stem cells, could undergo emergency hematopoiesis and proliferate in response to infection (Boettcher & Manz, 2017). More studies are needed to assess the origin of this small population of macrophages in *Ccr2*$^{-/-}$ mice. Regardless, this small population of macrophages is not sufficient to protect against infection with *C. violaceum*.

In other granuloma models, the role of CCR2 is less clear. Loss of CCR2-dependent monocyte trafficking enhances clearance of *Yersinia pseudotuberculosis* (Zhang et al., 2018), which is a surprising result as typically macrophages would be expected to be important to clear infections. The role of CCR2 during *M. tuberculosis* infection is strain-dependent, and also varies depending on the dose and route of infection (Dunlap et al., 2018; Scott & Flynn, 2002). Though there are similarities between these infection models and *C. violaceum*, there are numerous differences. For example, expression of specific chemokines in response to *M. tuberculosis* differs from those we observe in response to *C. violaceum*, especially chemokines that attract T cells (Kang et al., 2011). A key concept in the *M. tuberculosis* field is that a delicate balance exists between cellular recruitment to control infection, and excess inflammation that causes disease symptoms (Monin & Khader, 2014). Furthermore, excess recruitment of monocytes to *M. tuberculosis*-induced granulomas leads to increased bacterial replication due to the ability of *M. tuberculosis* to inhibit degradation within phagosomes in which it resides (Domingo-Gonzalez et al., 2016; Slight & Khader, 2013). In contrast, *C. violaceum* appears to lack sufficient virulence factors to enable it to replicate within macrophages (Batista & da Silva Neto, 2017). Importantly, while *M. tuberculosis* bacterial burdens plateau at 21 DPI, almost all mice clear *C. violaceum* by this timepoint. Though decades of research have been dedicated to investigating *M. tuberculosis*, fewer studies involving other granuloma-inducing pathogens have been performed. As we continue to study the cellular mechanisms that allow for successful granuloma formation and clearance of *C. violaceum*, it will be interesting to compare the two pathogens, as future studies could shed light on key differences that result in successful pathogen clearance.

In WT mice, neutrophil recruitment wanes as the granuloma matures, which coincides with clearance of *C. violaceum*. However, in the *Ccr2*$^{-/-}$ mice, we see elevated neutrophil numbers at 5 DPI, suggesting that neutrophils are continuously recruited in the absence of macrophages. Under normal circumstances, endocytosis of chemokines by endothelial cells helps to diminish chemokine gradients, limiting prolonged neutrophil recruitment (Kolaczkowska & Kubes, 2013). Future studies could investigate the various signals that diminish neutrophil recruitment in WT mice during clearance, and why this fails in *Ccr2*$^{-/-}$ mice. Another

interesting component of the granuloma response is the spatial arrangement of neutrophils and macrophages within the granuloma. In vitro studies found that CCR1 and CCR5 differentially affected monocyte localization within a transwell system, implying that a system exists for fine-tuning the exact location of macrophages within inflamed tissues (Shi & Pamer, 2011). These receptors are highly upregulated in the *C. violaceum*-induced granuloma and are thus good candidates for balancing the localization of macrophages between the coagulative necrosis zone and healthy tissue outside the granuloma.

Lastly, this dataset inspires a number of new hypotheses related to granuloma resolution and tissue repair after bacterial clearance. Chemokines undergo a variety of post-translational modifications, such as glycosylation, nitration, citrullination, and proteolytic cleavage, which can either enhance or abrogate their activity (Vanheule et al., 2018). For example, nitration of CCL2 and CCL3 by peroxynitrite was shown to reduce monocyte and neutrophil chemotaxis, respectively (Sato et al., 1999, 2000). Furthermore, binding to atypical receptors can also affect chemokine availability, representing another mechanism to resolve inflammation (Hansell et al., 2006; Ulvmar et al., 2011). Of particular interest is the implication of matrix metalloproteinases (MMPs) in regulating chemokine functions. MMPs can not only directly cleave chemokines, they can also cleave various chemokine-binding proteins that help establish the chemokine gradient (Parks et al., 2004). Several studies have found that MMPs can cleave chemokines to alter their function, either increasing or decreasing their receptor binding activity. For example, MMP-2 cleaves both CXCL12 and CCL7, abolishing their ability to induce chemotaxis (McQuibban et al., 2000, 2001); importantly, all three of these genes are upregulated during *C. violaceum* infection (Table 1, Table 3). Furthermore, MMP-2 and MMP-9 have been extensively studied in the context of lung inflammation, both of which are important to limit tissue damage (Greenlee et al., 2006). MMP-9 has also been shown to promote or inhibit liver fibrosis and wound repair, depending on the context (Feng et al., 2018). An unsolved mystery during infection with *C. violaceum* is how the chemotaxis of neutrophils and monocytes is abrogated when the infection is cleared, and how wound repair and resolution is initiated. Future studies could characterize the role of MMPs during resolution, especially MMP-9 and its various targets in relation to wound repair.

Analysis of a spatial transcriptomics dataset revealed the upregulation of many chemokines and their receptors during murine infection with *C. violaceum*. Here, we show that CCR2 is an essential chemokine receptor for monocyte trafficking, which enables the formation of mature granulomas with organized macrophage zones. Importantly, loss of organized macrophages leads to loss of bacterial containment. This work has given new insight into the function of chemokines during granuloma formation, and this model of *C. violaceum*-induced granuloma formation will be useful in exploring the unique and redundant roles of chemokines during infection.

Materials and Methods

Key Resources Table				
Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
strain, strain background (*Mus musculus*)	Wildtype C57BL/6 mice (WT)	Jackson Laboratory	Ref# 000664	
strain, strain background (*Mus musculus*)	*Ccr2*^{RFP} (*Ccr2*$^{-/-}$)	Jackson Laboratory	Ref# 017586	
strain, strain background (Bacteria)	*Chromobacterium violaceum (C. violaceum)*	ATCC	Ref# 12472	
antibody	Rat anti-mouse Ly6G in BV421™	BD Biosciences	Ref# 562737	1:300 (Flow)
antibody	Rat anti-mouse CD68 in FITC	BioLegend®	Ref# 137005	1:300 (Flow)
antibody	Rabbit anti-*C. violaceum*	Cocalico Biologicals	Custom polyclonal antibody	1:2,000 (IHC)
antibody	Rat anti-Ly6G	BioLegend®	Ref# 127601	1:300 (IHC)
antibody	Rabbit anti-CD68	Abcam	Ref# ab125212	1:200 (IHC)
commercial assay or kit	Avidin/Biotin Blocking Kit	Vector Laboratories	Ref# SP-2001	
commercial assay or kit	SignalStain® Boost IHC Detection Reagent (HRP, Anti-Rabbit)	Cell Signaling	Ref# 8114	
commercial assay or kit	ImmPRESS® HRP Goat Anti-Rat Detection Kit	Vector Laboratories	Ref# MP-7404	

commercial assay or kit	DAB Substrate Kit, HRP	Vector Laboratories	Ref# SK-4100	
commercial assay or kit	H&E Stain Kit (Modified Mayer's Hematoxylin and Bluing Reagent)	Abcam	Ref# ab245880	
chemical compound, drug	Reparixin	MedChemExpress	Ref# HY-15251	
software, algorithm	RStudio	Posit PBC		
software, algorithm	FlowJo™	BD Biosciences		
software, algorithm	Prism 9	GraphPad		
software, algorithm	Fiji	ImageJ		
other	Collagenase Type IV	Gibco™	Ref# 17104019	
other	1X DMEM, +4.5 g/L D-Glucose, +L-Glutamine, +110 mg/L Sodium Pyruvate	Gibco™	Ref# 11995-065	
other	1X RPMI Medium 1640, +L-Glutamine	Gibco™	Ref# 11875-093	
other	PenStrep +10,000 Units/mL Penicillin, +10,000 µg/mL Streptomycin	Gibco™	Ref# 15140-122	
other	HyClone™ Characterized Fetal Bovine Serum	Cytiva	Ref# SH30396.03	
other	1X DPBS, -Calcium Chloride, -Magnesium Chloride	Gibco™	Ref# 14190-144	
other	70 µm Cell Strainers	Genesee Scientific	Ref# 25-376	
other	40 µm Cell Strainers	Genesee Scientific	Ref# 25-375	
other	Percoll®	GE Healthcare	Ref# 17-0891-01	

other	1X RBC Lysis Buffer	eBioscience™	Ref# 00-4333-57	
other	Falcon™ Round-Bottom Polystyrene Test Tubes	Thermo Scientific™	Ref# 14-959-1A	
other	Mouse BD Fc Block™	BD Biosciences	Ref# 553142	1 µg
other	Intracellular Fixation & Permeabilization Buffer	eBioscience™	Ref# 88-8824-00	
other	10% Neutral Buffered Formalin	VWR®	Ref# 16004-128	
other	Epredia™ Xylene	Fisher Chemical™	Ref# 99-905-01	
other	ImmEdge® Pen	Vector Laboratories	Ref# H-4000	
other	Normal Goat Serum Blocking Solution, 2.5%	Vector Laboratories	Ref# S-1012	
other	SignalStain® Antibody Diluent	Cell Signaling	Ref# 8112	
other	Permount®	Fisher Chemical™	Ref# SP15-100	

Analysis of spatial transcriptomics dataset

To further analyze the spatial transcriptomics dataset generated in (Harvest et al., 2023), we used the Seurat package in RStudio to analyze gene expression over time and space. UMAP plots, SpatialDimPlots, SpatialFeaturePlots, and Violin plots were all used to visualize gene expression data.

Ethics statement and mouse studies

All mice were housed in groups of two – five according to IACUC guidelines at UNC-Chapel Hill or at Duke University, with approved protocols in place. Wildtype C57BL/6 mice (referred to as WT; from Jackson Laboratories) or *Ccr2*^{RFP} mice (referred to as *Ccr2*$^{-/-}$; originally generated in (Saederup et al., 2010)) were used as indicated. Mice were moved to a BSL2 facility a minimum of three days prior to treatment. For experiments involving infection, mice were monitored every twenty-four hours for signs of illness. After the appearance of symptoms, mice were monitored every twelve hours. Mice showing sever signs of illness were euthanized according to previously established euthanasia criteria.

Treatment of mice with reparixin

Stock solutions of reparixin were prepared in PBS with gentle warming for a final concentration of 20 mg/kg in 200 µL PBS. Mice were injected subcutaneously with 200 µL of appropriate reparixin stock or with 200 µL PBS (control).

Preparation of inoculum

Bacteria were grown overnight on brain heart infusion (BHI) agar plates (*C. violaceum* ATCC strain 12472) or at 37°C and stored at room temperature for no more than two weeks. To prepare infectious inocula, bacteria were cultured in 3 mL BHI broth with aeration overnight at 37° before being diluted in PBS to indicated infectious inoculum.

468 **In vivo infections**
469 For in vivo infections, 8-10-week-old, age- and sex-matched mice were infected as previously described
470 (Harvest et al., 2023). Mice were infected intraperitoneally (IP) with indicated number of bacteria in 200 µL
471 PBS. Whole livers and spleens were harvested at indicated timepoints.

472 **Plating for CFUs**
473 At the indicated days post-infection (DPI), mice were euthanized and the spleen and liver were harvested for
474 quantification of bacterial burdens as previously described (Harvest et al., 2023). Briefly, spleens were placed
475 in a 2 mL homogenizer tube with 1 large metal bead and 1 mL sterile PBS, and whole livers were placed in a 7
476 mL homogenizer tube with 1 large metal bead and 3 mL sterile PBS. Tube weights were recorded before and
477 after tissue harvest to normalize CFUs/volume/tissue. After homogenizing, 1:5 serial dilutions were performed
478 in sterile PBS, and dilutions were plated on BHI in triplicate or quadruplicate. The following day, bacterial
479 colonies were counted and CFU burdens calculated.

480 **Flow cytometry**
481 At the indicated days post-infection (DPI), mice were euthanized and the spleen, liver, and whole blood were
482 harvested for flow cytometry as previously described (Harvest et al., 2023). For experiments involving whole
483 blood, cardiac puncture was used to collect 100 µL whole blood prior to perfusion with PBS through the vena
484 cava as described in (Mendoza et al., 2022). Briefly, whole livers were minced on ice using scissors and
485 incubated in digestion buffer (100 U/mL Collagenase Type IV in DMEM supplemented with 1 mM $CaCl_2$ and 1
486 mM $MgCl_2$) for 40 minutes in a 37° water bath with intermittent vortexing. Digested livers were homogenized
487 through a 40 µm cell strainer, followed by washing with RPMI (supplemented with 1X Pen/Strep and 1% FBS)
488 and centrifugation at 300xg for 8 minutes. Leukocytes from the liver were further isolated using a Percoll®
489 gradient where samples were resuspended in 45% Percoll® with an 80% Percoll® underlay, and spun at
490 800xg for 20 minutes with no brake. For spleens, tissues were mechanically homogenized through a 70 µm
491 strainer, followed by washing and centrifugation at 300xg for 5 minutes. Red blood cells were lysed with 1X
492 RBC Lysis Buffer according to product manual (note: whole blood was stained with Ly6G at room temperature
493 prior to RBC lysis. Blood samples were treated identically to liver and spleen samples thereafter). Liver and
494 spleen samples were counted using trypan blue, and $1x10^6$ cells per tissue per mouse were stained for various
495 cell markers: Mouse BD Fc Block™ (1 µg), rat anti-mouse Ly6G in BV421™ (1:300), rat anti-mouse CD68 in
496 FITC (1:300) for 30 minutes. For CD68, staining was performed using Intracellular Fixation & Permeabilization
497 Buffer according to product manual. For each sample, 10,000 events were acquired on a BD LSRFortessa X-
498 20 Cell Analyzer at the Duke Flow Cytometry Core Facility. Samples were analyzed using FlowJo™ (for
499 Windows, version 10.7.1).

500 **Histology and immunohistochemistry**
501 To prepare paraffin-embedded tissues, whole livers were harvested at the indicated day post-infection (DPI)
502 and submerged in 20 mL of 10% neutral buffered formalin. Samples were gently inverted every day for a
503 minimum of three days before being transferred to tissue cassettes and given to the Histology Research Core
504 at the University of North Carolina at Chapel Hill. The research core performed tissue embedding, serial
505 sectioning, slide mounting, and staining of hematoxylin and eosin (H&E) samples. For immunohistochemistry
506 (IHC), serial sections were then processed and stained as described in (Harvest et al., 2023). Washes were
507 performed in 1X TBS-T. Primary antibodies were diluted in SignalStain® antibody diluent, and included: rabbit
508 anti-*C.violaceum* (1:2000), rat anti-Ly6G (1:300), and rat anti-CD68 (1:200). Slides were incubated in primary
509 antibody overnight at 4° in a humidity chamber. Prior to staining with secondary antibody, endogenous
510 peroxidase activity was blocked using 3% H_2O_2. Slides were incubated in secondary antibody (SignalStain®
511 Boost HRP anti-rabbit or ImmPRESS® HRP anti-rat) at room temperature for 30 minutes. Incubation with DAB
512 Substrate Kit was performed for 30 seconds to 2 minutes, depending on the intensity of signal. Slides were
513 counter-stained with hematoxylin for 5 seconds to 1 minute, depending on the intensity of the DAB, and then
514 dipped in bluing reagent for 1 minute. After dehydration, slides were covered with Permount® mounting
515 medium and a coverslip. Importantly, WT slides and *Ccr2*$^{-/-}$ slides were stained side-by-side.

516
517

518 **Microscopy**
519 Histology samples were analyzed on a KEYENCE All-in-One Microscope BZ-X800. Images were further
520 analyzed in Fiji by ImageJ.

521 **Statistics**
522 Statistical analysis was performed using GraphPad Prism 9.5.1. For survival analysis, the Mantel-Cox test was
523 used to compare WT and *Ccr2*$^{-/-}$ mice. For bacterial burdens, data were first assessed for normality using the
524 Shapiro-Wilk test. For two groups, a two-tailed t-test (or Mann-Whitney for abnormally distributed data) was
525 used.

526 **Data availability statement:** All study data are included in the article and deposited on LabArchives, available
527 upon request. Relevant source code will be shared upon request.

528 **Author contributions:** Conceptualization, M.E.A. and E.A.M.; Data curation, M.E.A., C.K.H., C.J.B.; Formal
529 analysis, M.E.A. and C.J.B.; Funding acquisition, E.A.M. and C.K.H.; Investigation, M.E.A.; Methodology,
530 M.E.A., C.K.H.; Software, M.E.A. and C.J.B.; Supervision, E.A.M.; Validation, M.E.A.; Visualization, M.E.A.;
531 Writing – original draft, M.E.A.; Writing – review & editing, M.E.A. and E.A.M.

532 **Funding:** This research was funded by NIH grants AI139304, AI136920, AI175078 (E.A.M.), and AI133236-
533 04S1 (C.K.H.). The APC was funded by startup funds from Duke University School of Medicine.

534 **Acknowledgements:** We thank Michael Dee Gunn for key discussions regarding chemokines and Ashley
535 Moseman for sharing mice. We thank Heather N. Larson for mouse colony maintenance. Original graphics
536 created with BioRender.com.

537

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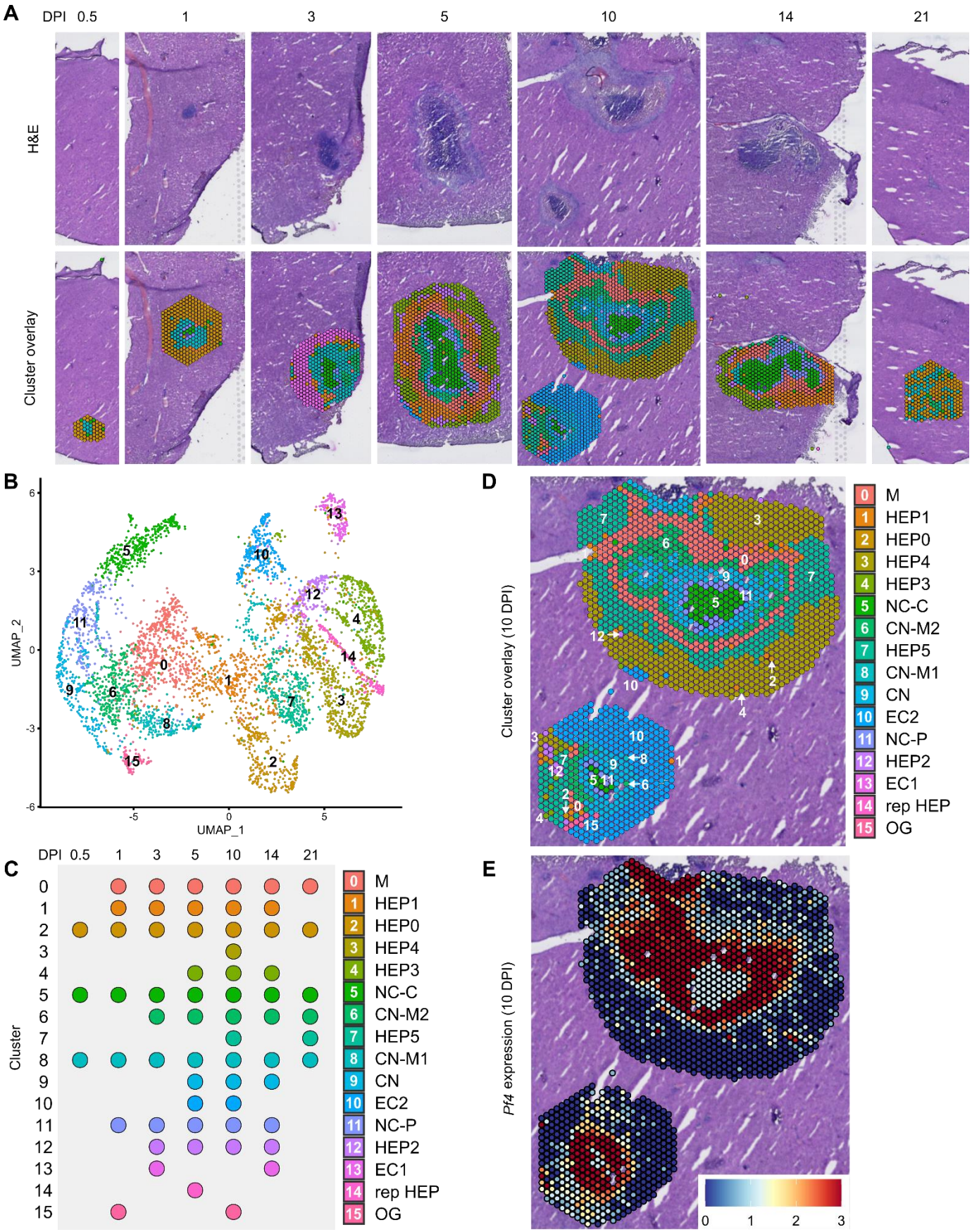

Figure 1. Spatial transcriptomics dataset reveals 16 unique clusters during infection with *C. violaceum*. (**A**) SpatialDimPlots showing hematoxylin and eosin (H&E) and cluster overlay of spatial transcriptomics data corresponding to various days post-infection (DPI). Each circle is an individual barcoded spot that is 55 µm in diameter. (**B**) UMAP plot of 16 unique clusters identified based on differentially expressed genes during the course of infection. (**C**) Temporal

684 expression of each cluster, and characterization of predominant cell types present and/or location of each cluster (initial
685 characterization performed in Harvest et al., 2023); spot size is arbitrary. Macrophage zone (M), hepatocyte (HEP),
686 representative HEP (rep HEP), necrotic core center (NC-C), NC-periphery (NC-P), coagulative necrosis (CN), CN-
687 macrophage (CN-M), endothelial cell (EC), outside granuloma (OG). (**D**) SpatialDimPlot at 10 DPI as in (A), showing
688 cluster overlay and annotated with cluster identity. (**E**) SpatialFeaturePlot at 10 DPI, showing expression of *Pf4* (murine
689 homolog of *CXCL4*).

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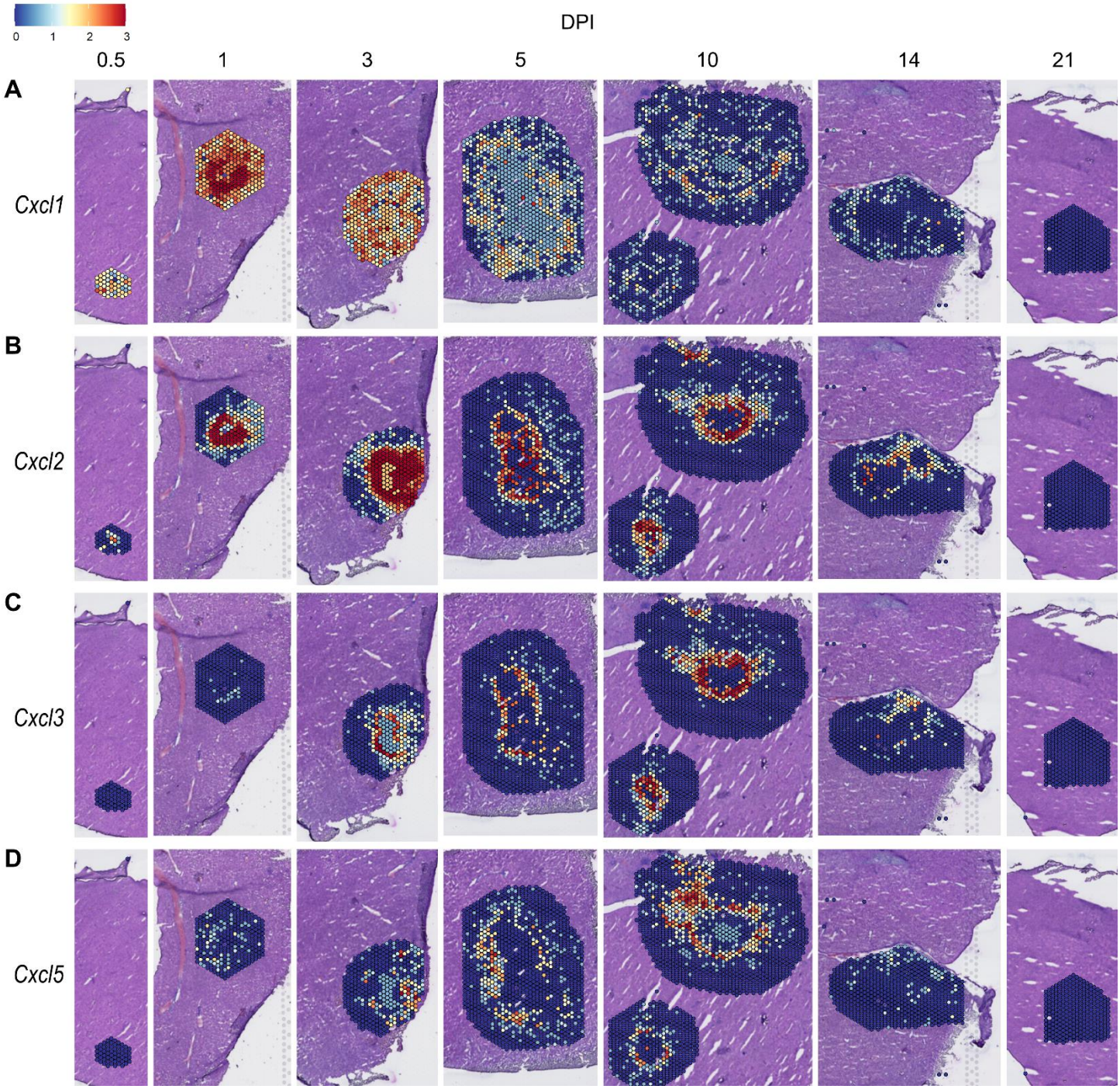


Figure 2. Chemokines involved in neutrophil recruitment are upregulated during infection. SpatialFeaturePlots displaying gene expression data of CXCR2 ligands (i.e. *Cxcl1*, *Cxcl2*, *Cxcl3*, and *Cxcl5*) at various days post-infection (DPI). Scale set at 0 – 3.0 expression.

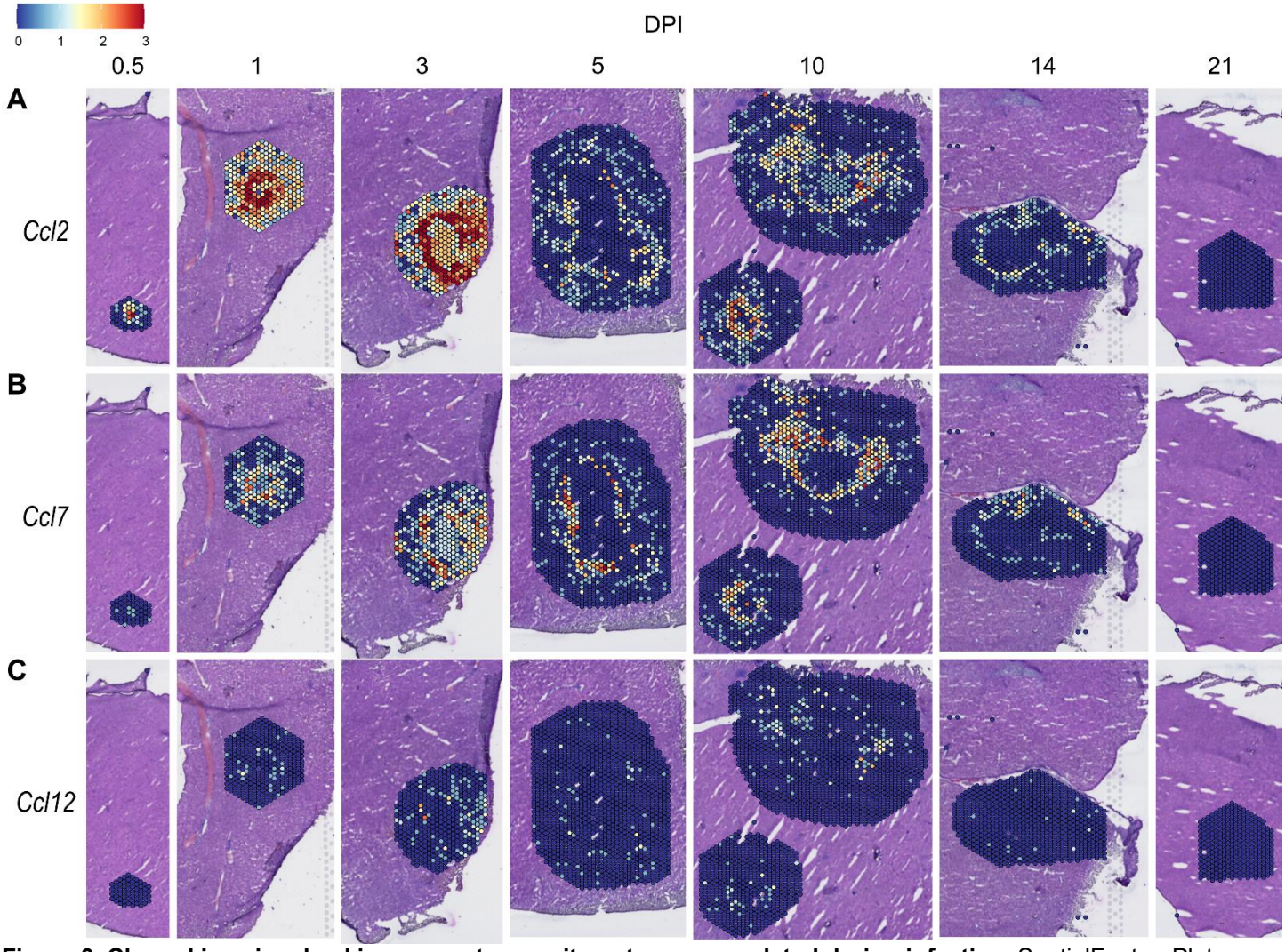


Figure 3. Chemokines involved in monocyte recruitment are upregulated during infection. SpatialFeaturePlots displaying gene expression data of CCR2 ligands (i.e. *Ccl2*, *Ccl7*, and *Ccl12*) at various days post-infection (DPI). Scale set at 0 – 3.0 expression.

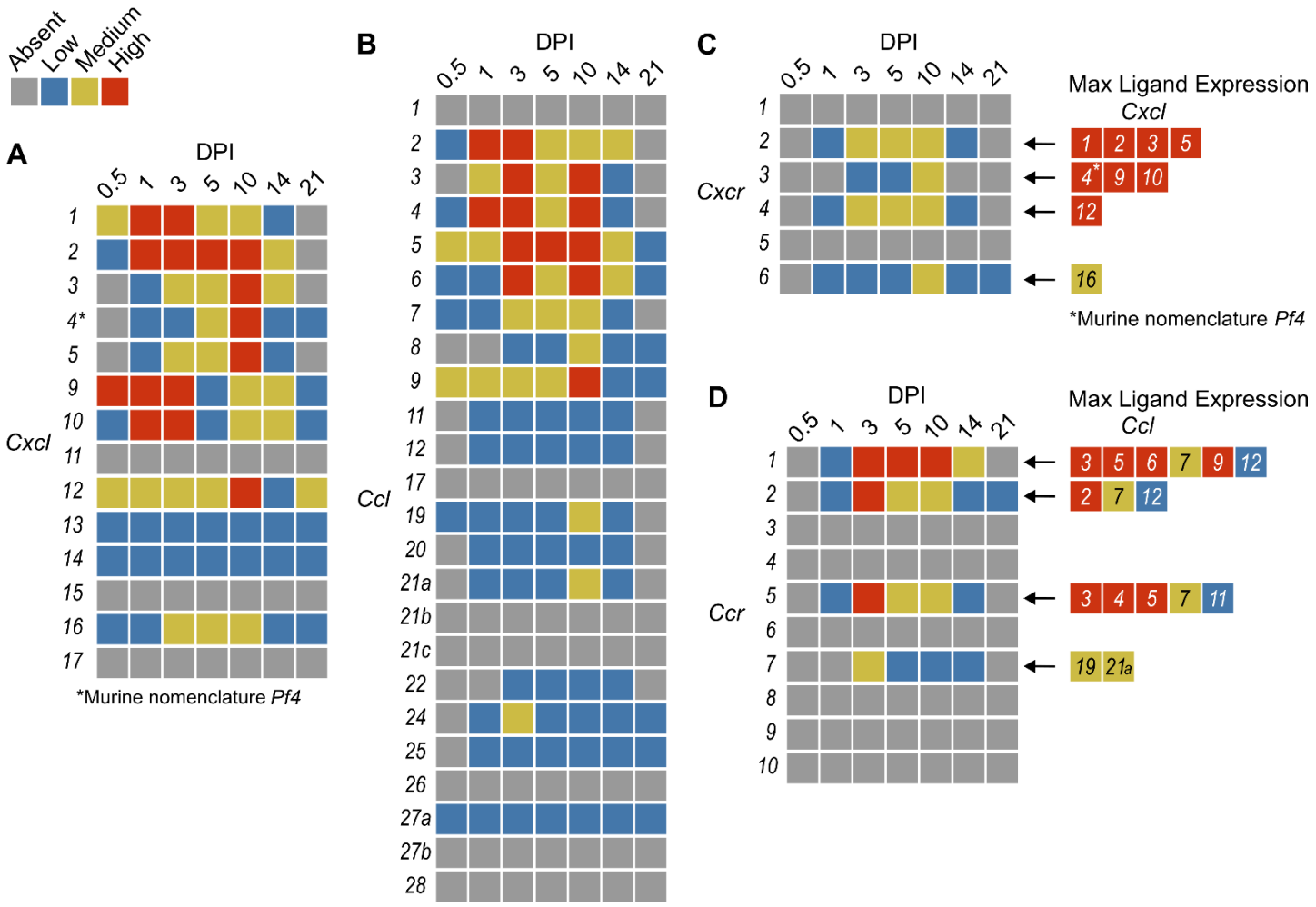


Figure 4. Qualitative heatmaps of chemokine and receptor expression during infection. Expression in SpatialFeaturePlots was visually ranked as absent (grey), low (blue), medium (yellow), or high (red) for (**A**) CXCL family chemokines, (**B**) CCL family chemokines, (**C**) CXC chemokine receptors, and (**D**) CC chemokine receptors. Visual rankings were based on both the intensity of expression and the relative number of spots that expressed the gene. (A-B) Scale set at 0 – 3.0 expression; (C-D) Scale set at 0 – 2.0 expression. Arrows indicate Ligand – Receptor interactions. Ligands are color-coded based on the maximum expression level reached at any time during the course of infection.

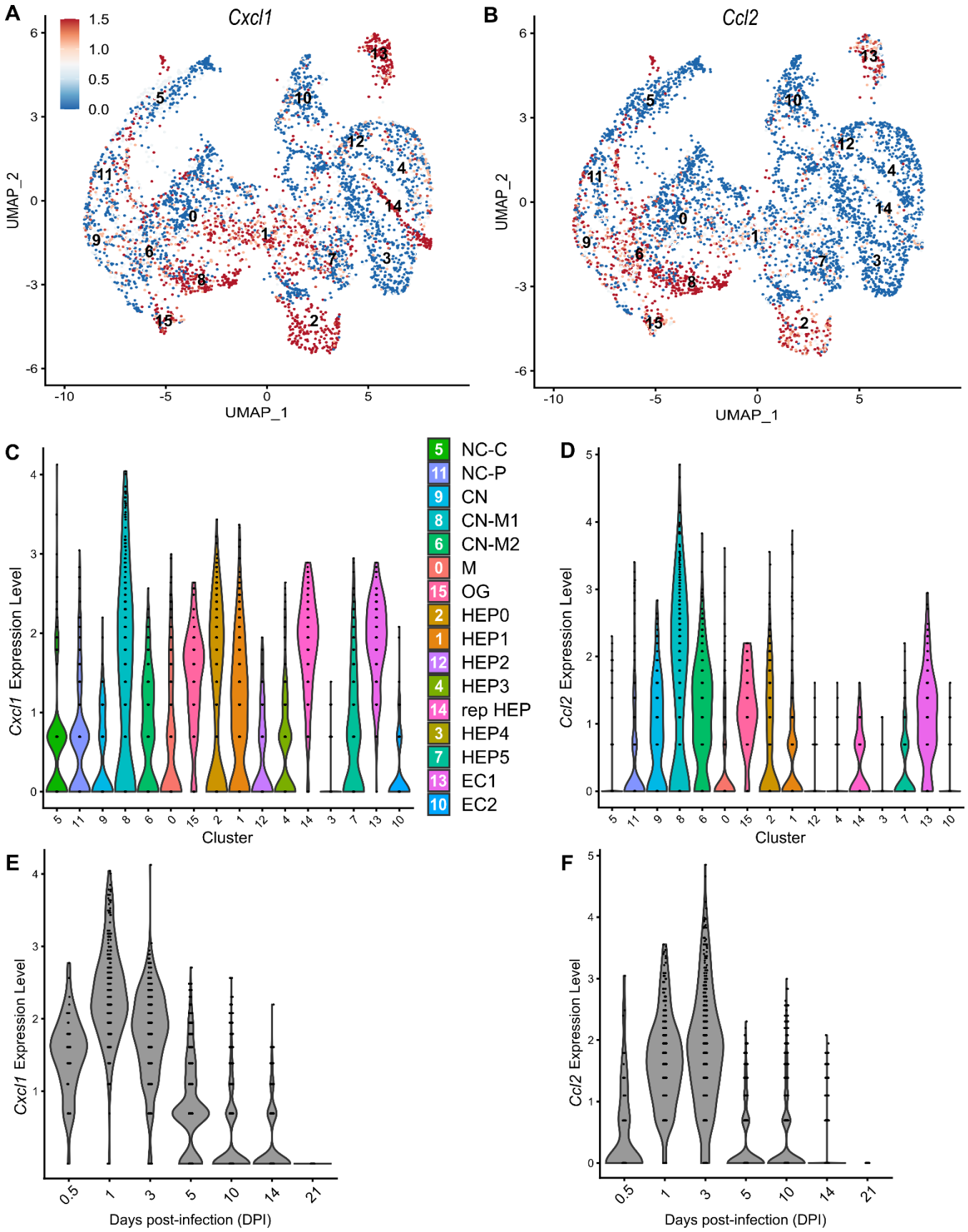


Figure 5. Chemokines involved in monocyte recruitment peak after chemokines involved in neutrophil recruitment. Comparative analysis of *Cxcl1* (A, C, and E) and *Ccl2* (B, D, and F). (**A-B**) UMAP plots of 16 unique clusters showing expression level of each gene. Maximum expression level set to 1.5; annotated with cluster identity. (**C-D**) Violin plots of 16 unique clusters showing expression level of each gene. (**E-F**) Violin plots of various days post-infection (DPI) showing expression level of each gene.

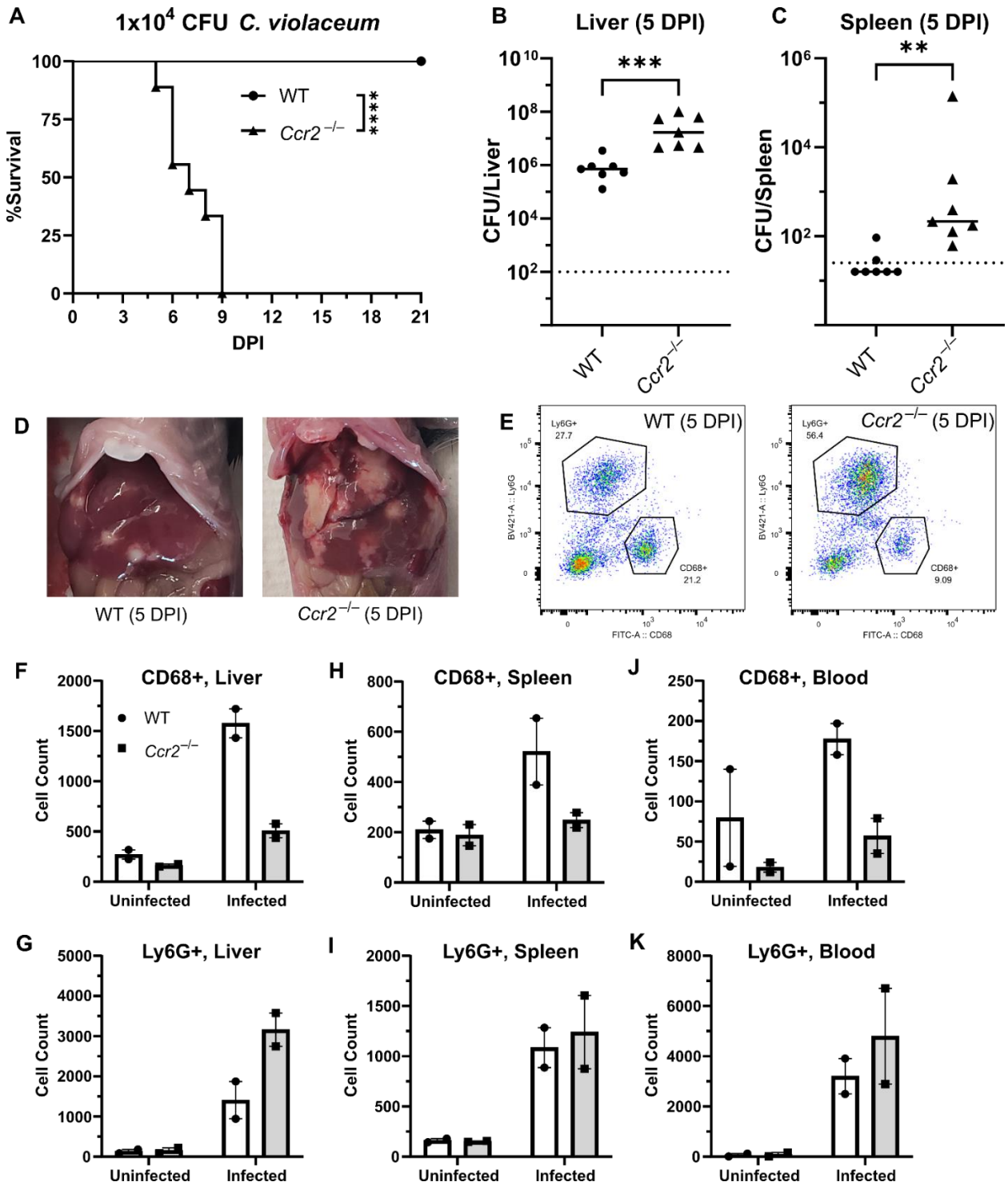
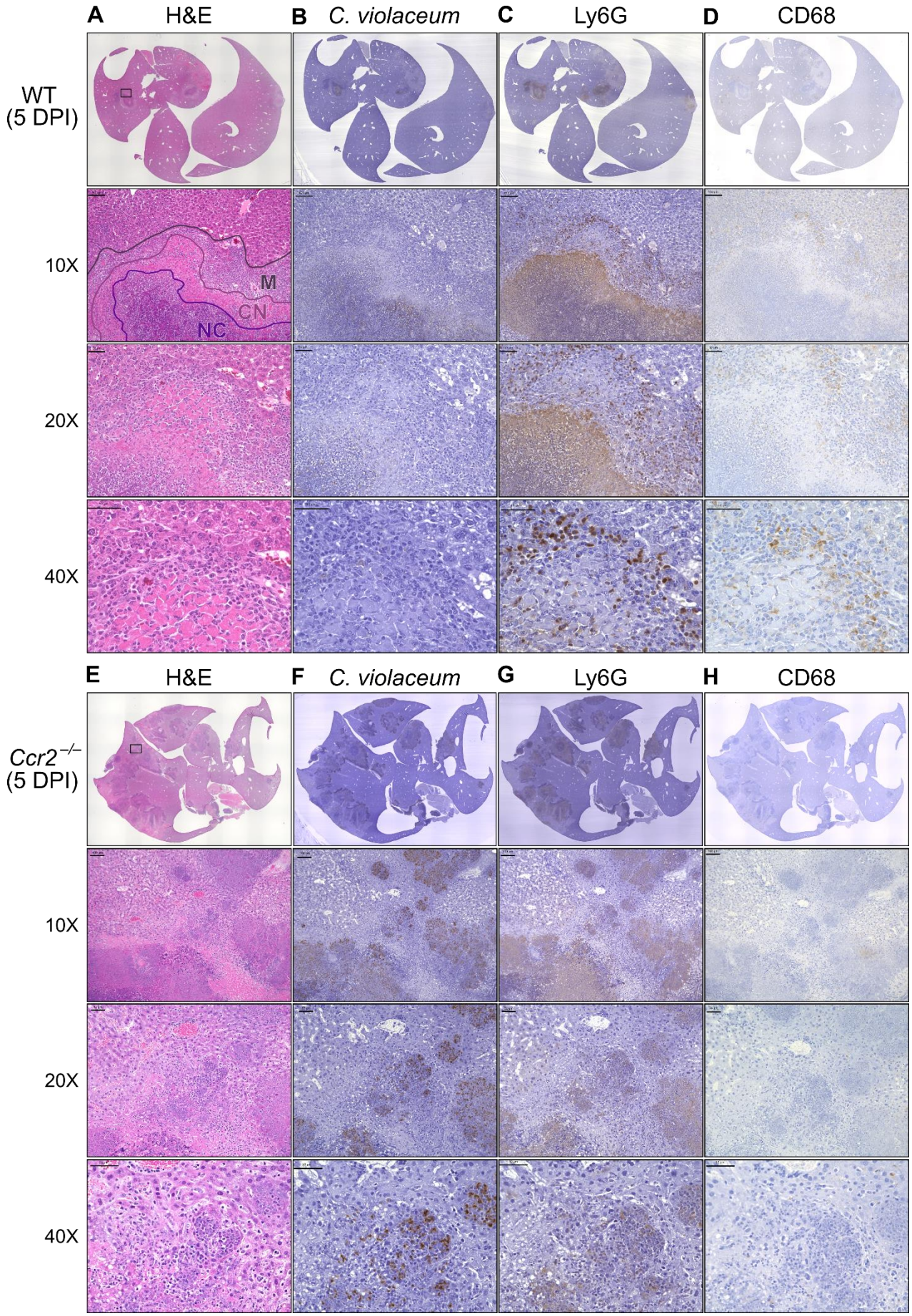


Figure 6. CCR2 and monocyte recruitment is essential for a successful granuloma response to *C. violaceum*. Wildtype (WT) and *Ccr2*$^{-/-}$ mice were infected intraperitoneally (IP) with 1x10^4 CFU *C. violaceum*. (**A**) Survival analysis of WT (N = 10) and *Ccr2*$^{-/-}$ (N = 9) mice. Two experiments combined. Mantel-Cox test, ****p<0.0001. (B-K) Livers and spleens were harvested 5 days post-infection (DPI). Bacterial burdens in the (**B**) liver and (**C**) spleen of WT and *Ccr2*$^{-/-}$ mice. Two experiments combined. Each dot represents one mouse. (B) Two-tailed t test (normally distributed data); ***p=0.0002. (C) Mann-Whitney (abnormally distributed data); **p=0.0012. Dotted line, limit of detection. Solid line, median. (**D**) Gross images of WT and *Ccr2*$^{-/-}$ livers 5 DPI. (**E**) Gating strategy for analysis of neutrophil (Ly6G$^+$) and macrophage (CD68$^+$) numbers via flow cytometry. Liver samples from infected mice shown. Macrophage numbers in (**F**) liver, (**H**) spleen, and (**J**) blood. Neutrophil numbers in (**G**) liver, (**I**) spleen, and (**K**) blood. (F-K) Two experiments combined using only female mice. Each dot represents one mouse, with 10,000 events collected per sample. Line represents mean ± standard deviation.



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726 **Figure 7. Loss of CCR2-dependent monocyte trafficking results in abnormal granuloma architecture and failure of**
727 **bacterial containment.** WT and *Ccr2*$^{-/-}$ mice were infected intraperitoneally (IP) with 1x10^{4} CFU *C. violaceum* and livers

728 were harvested 5 days post-infection (DPI). Serial sections of livers stained by hematoxylin and eosin (H&E) or various
729 IHC markers for (**A-D**) WT male and (**E-H**) *Ccr2*$^{-/-}$ male. Necrotic core (NC), coagulative necrosis zone (NC), macrophage
730 zone (M). For 10X, scale bar is 100 µm. For 20X and 40X, scale bar is 50 µm. Representative of two experiments with 2 –
731 4 mice per group, and multiple granulomas per section.

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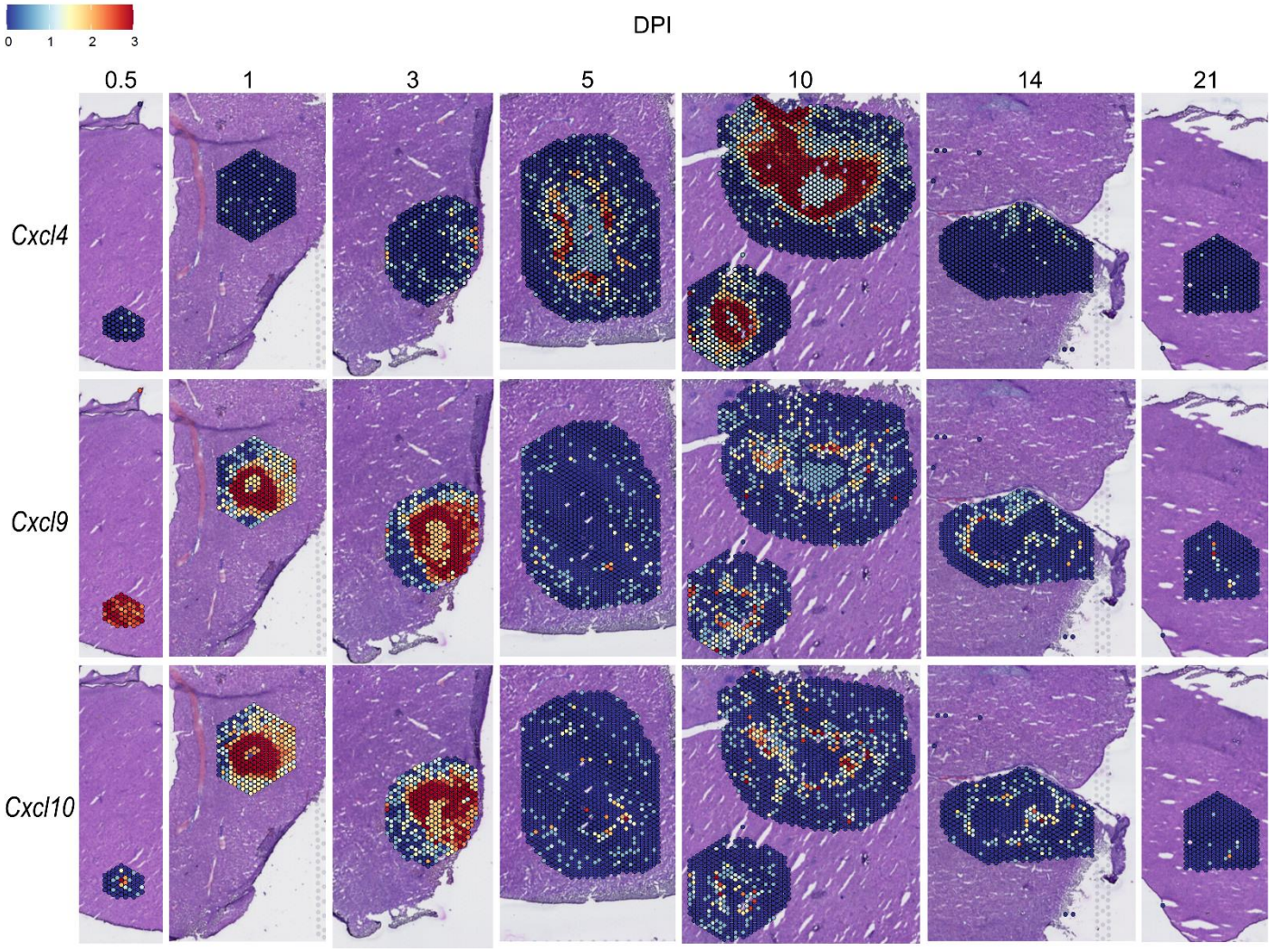
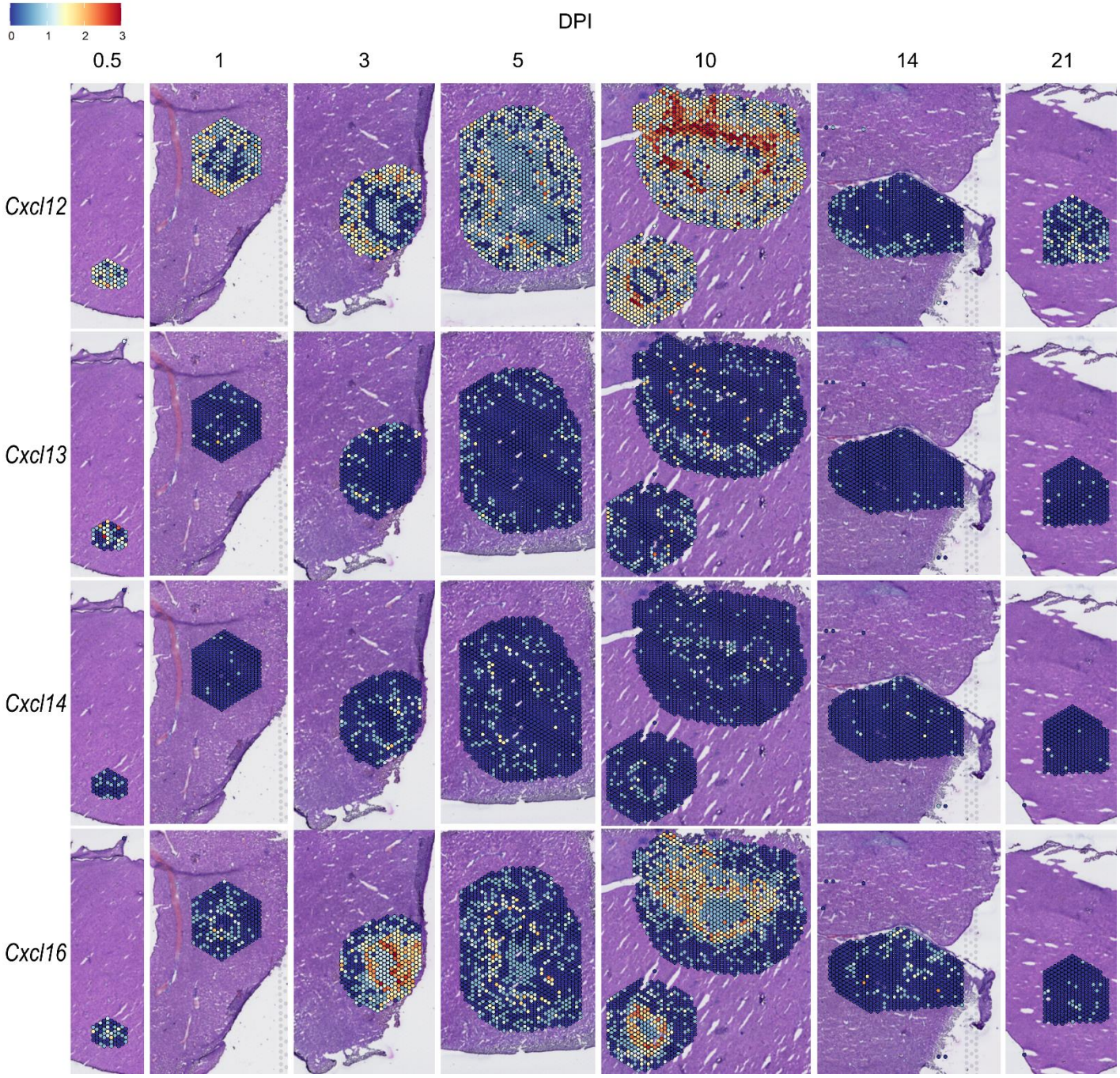
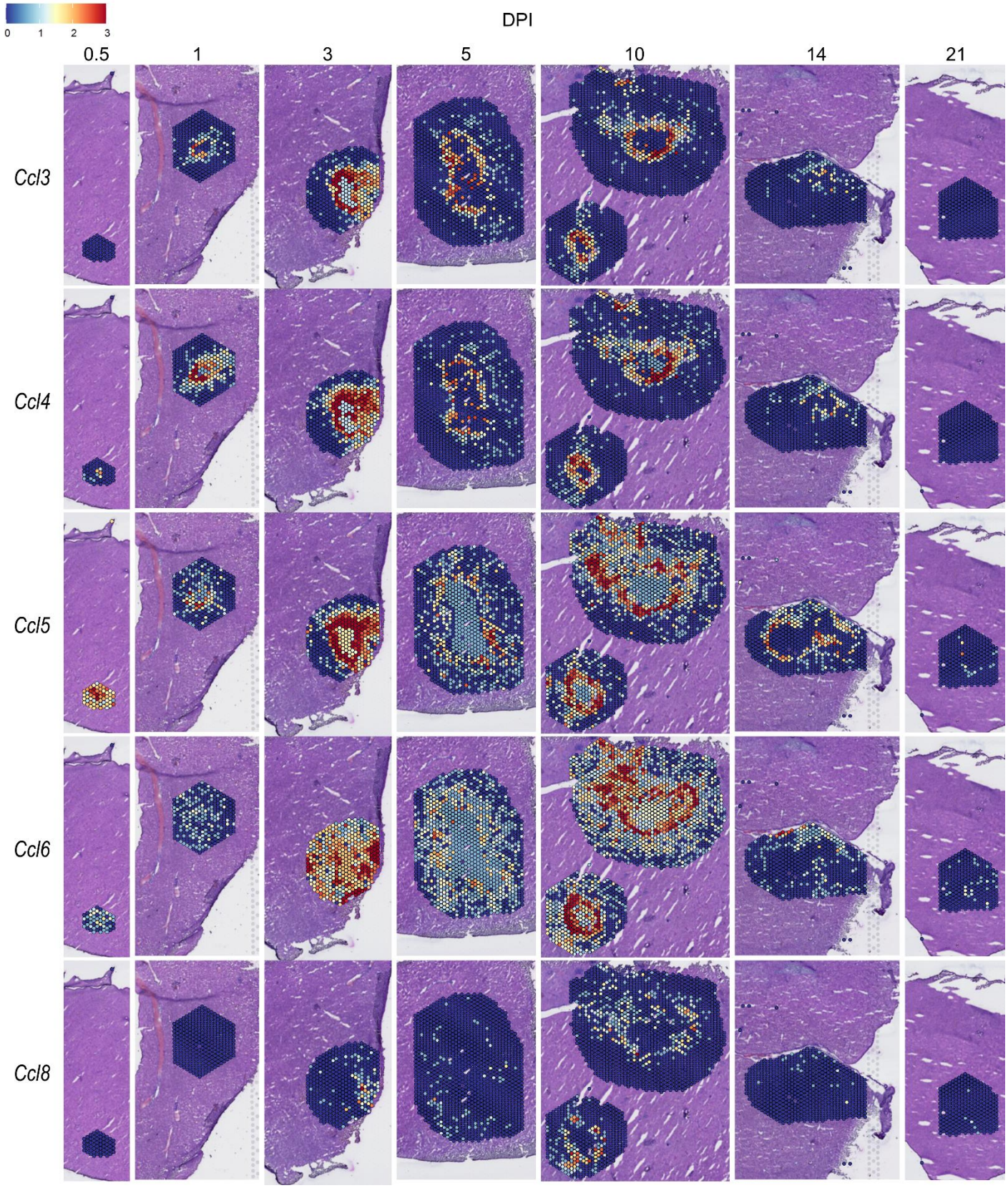


Supplemental Figure 1. Spatial expression of CXCR3 ligands. SpatialFeaturePlots displaying gene expression data of CXCR3 ligands (i.e. *Cxcl4*, *Cxcl9*, and *Cxcl10*) at various days post-infection (DPI). Scale set at 0 – 3.0 expression.



Supplemental Figure 2. Spatial expression of *Cxcl12*, *Cxcl13*, *Cxcl14,* and *Cxcl16*. SpatialFeaturePlots displaying gene expression data of selected *Cxcl* family members at various days post-infection (DPI). Scale set at 0 – 3.0 expression.



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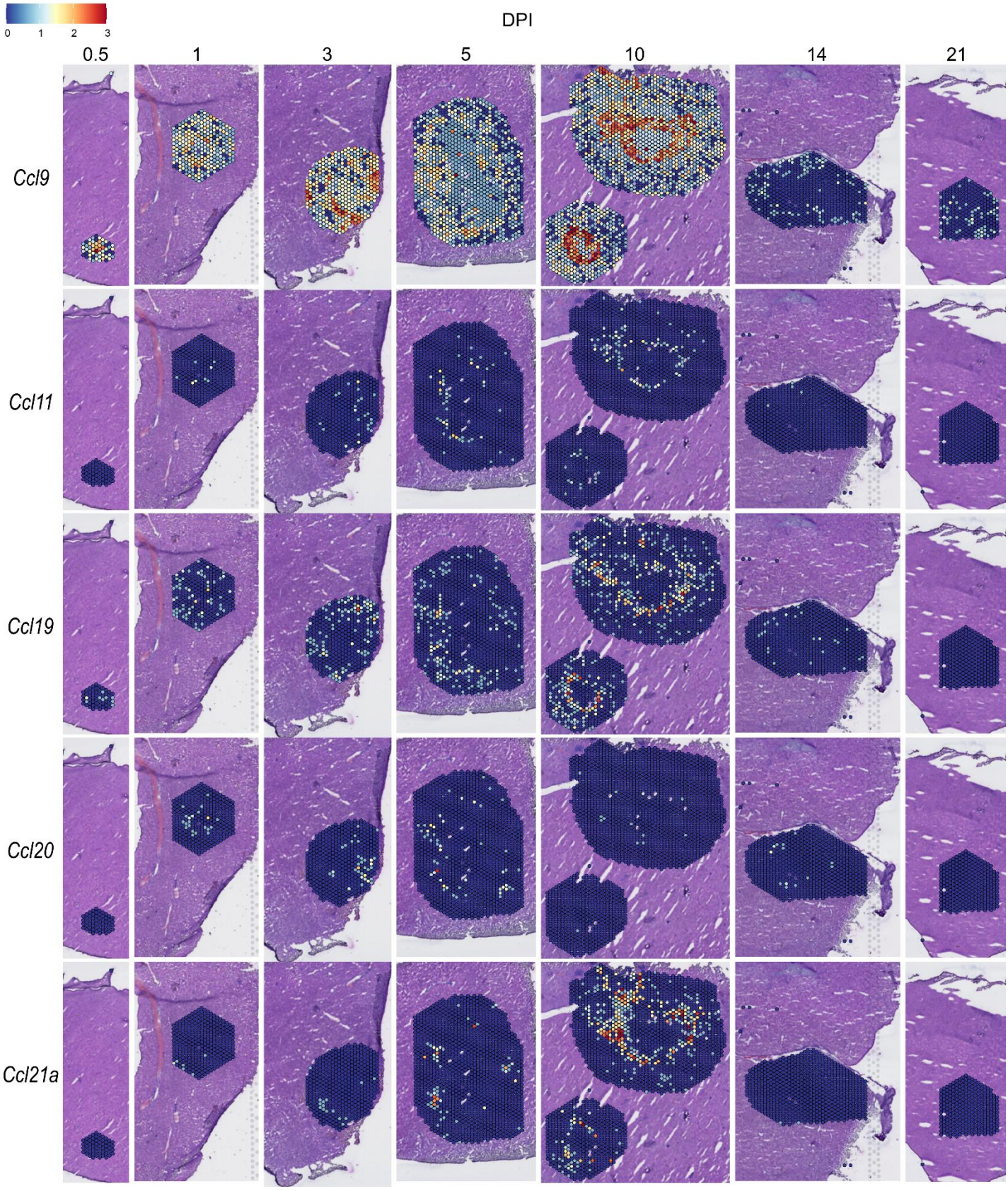
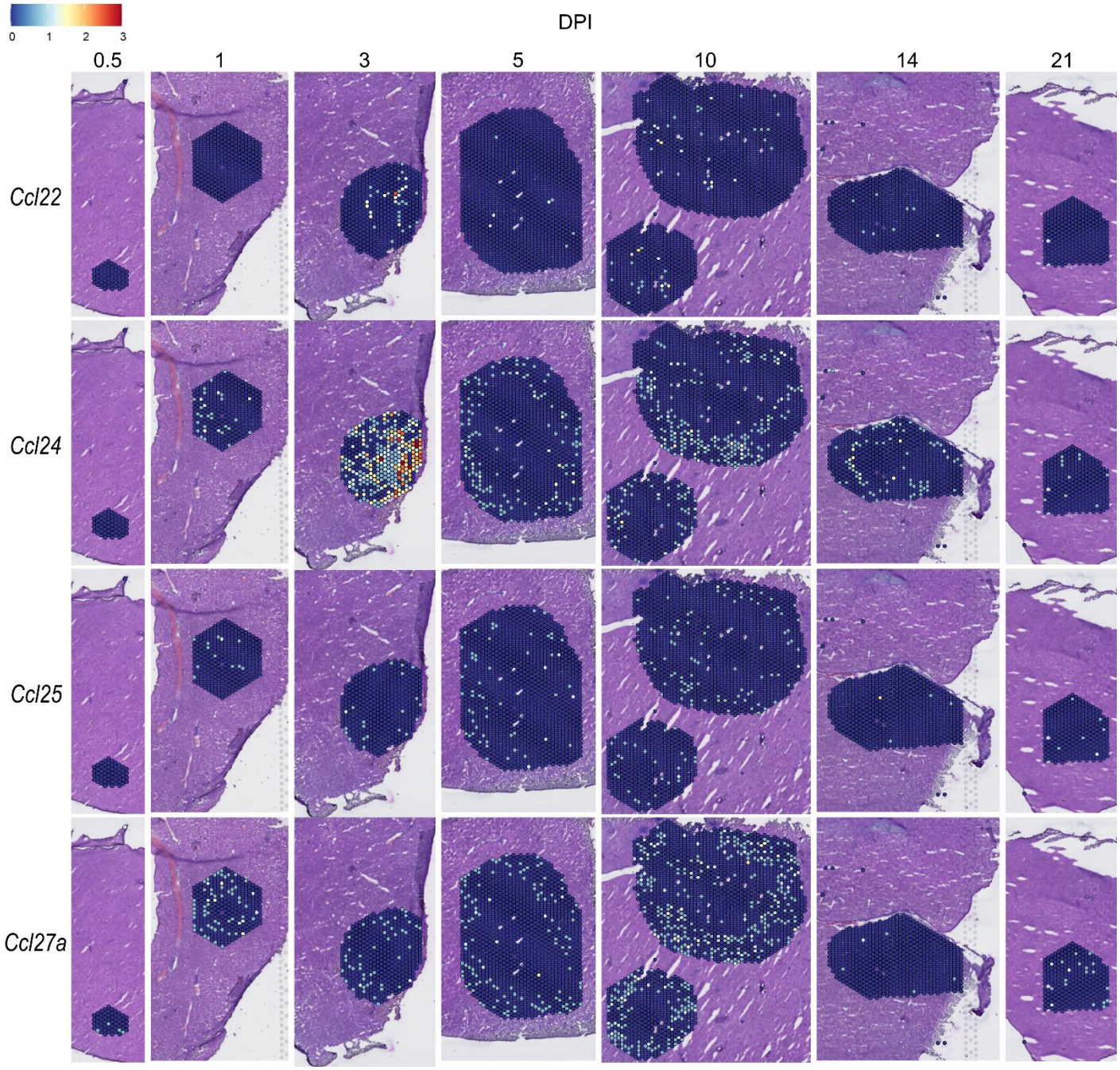
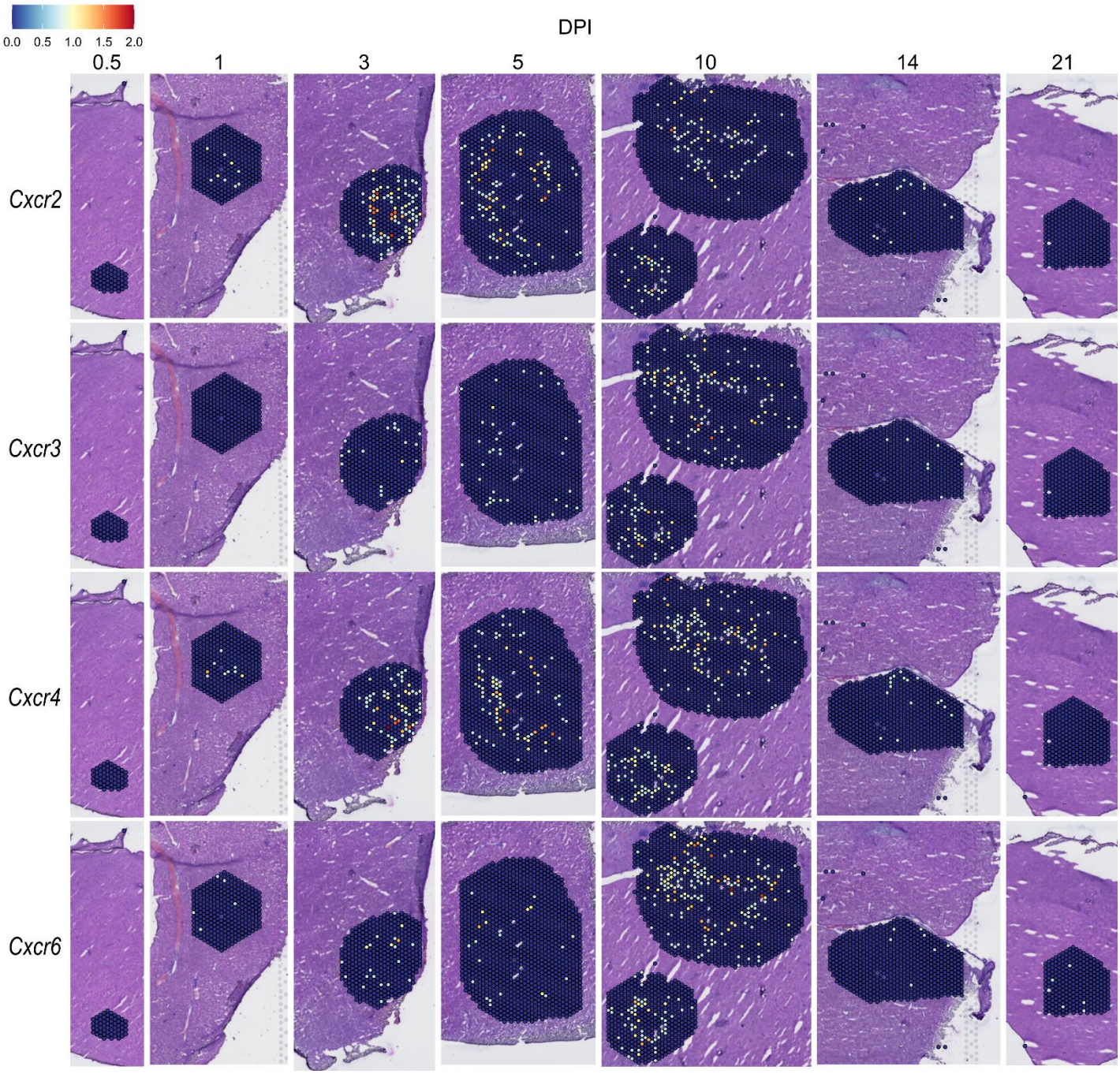
742 **Supplemental Figure 3. Spatial expression of *Ccl3, Ccl4, Ccl5, Ccl6,* and *Ccl8*.** SpatialFeaturePlots displaying gene
743 expression data of selected *Ccl* family members at various days post-infection (DPI). Scale set at 0 – 3.0 expression.



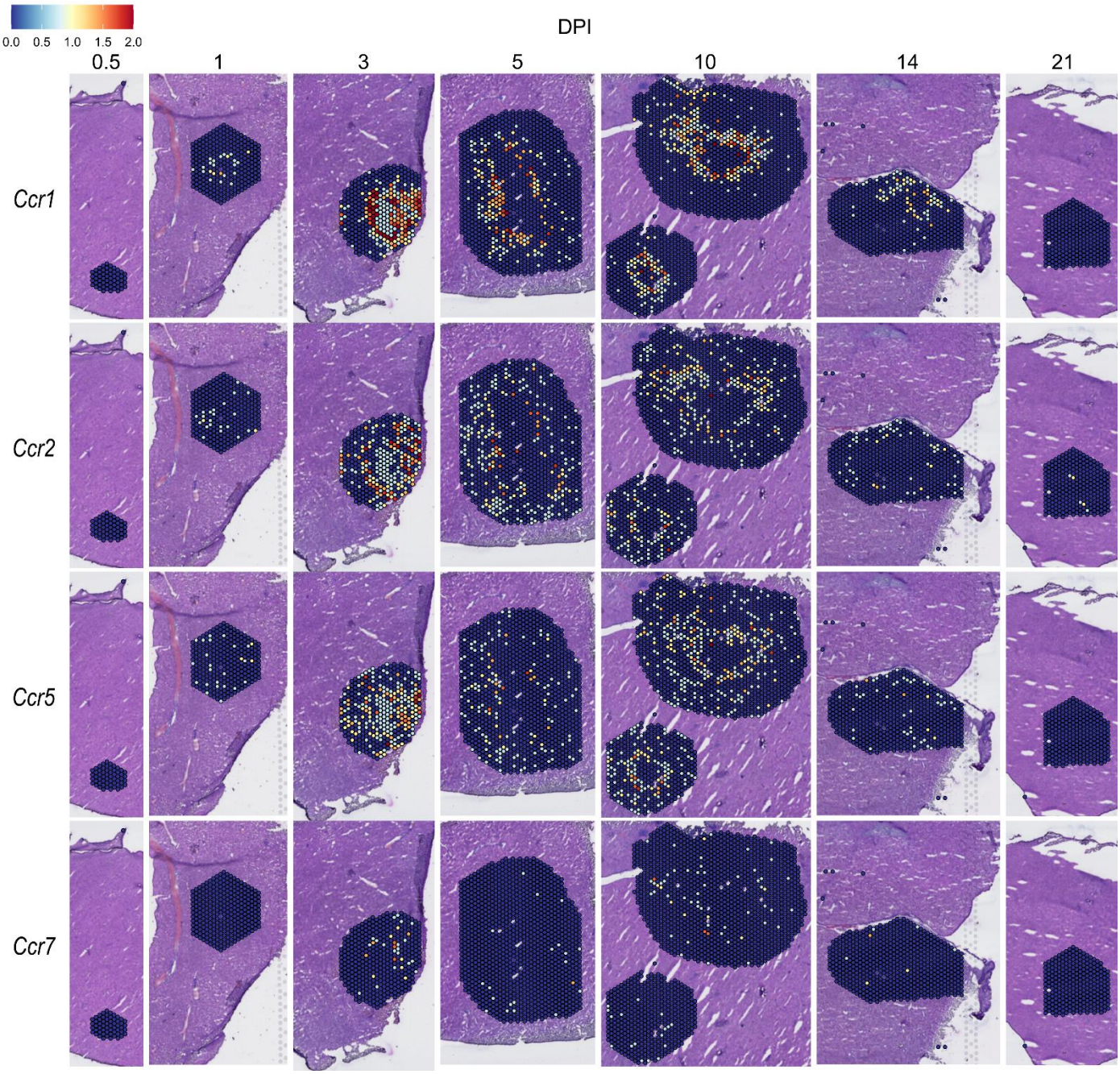
Supplemental Figure 4. Spatial expression of *Ccl9, Ccl11, Ccl19, Ccl20,* and *Ccl21a.* SpatialFeaturePlots displaying gene expression data of selected *Ccl* family members at various days post-infection (DPI). Scale set at 0 – 3.0 expression.



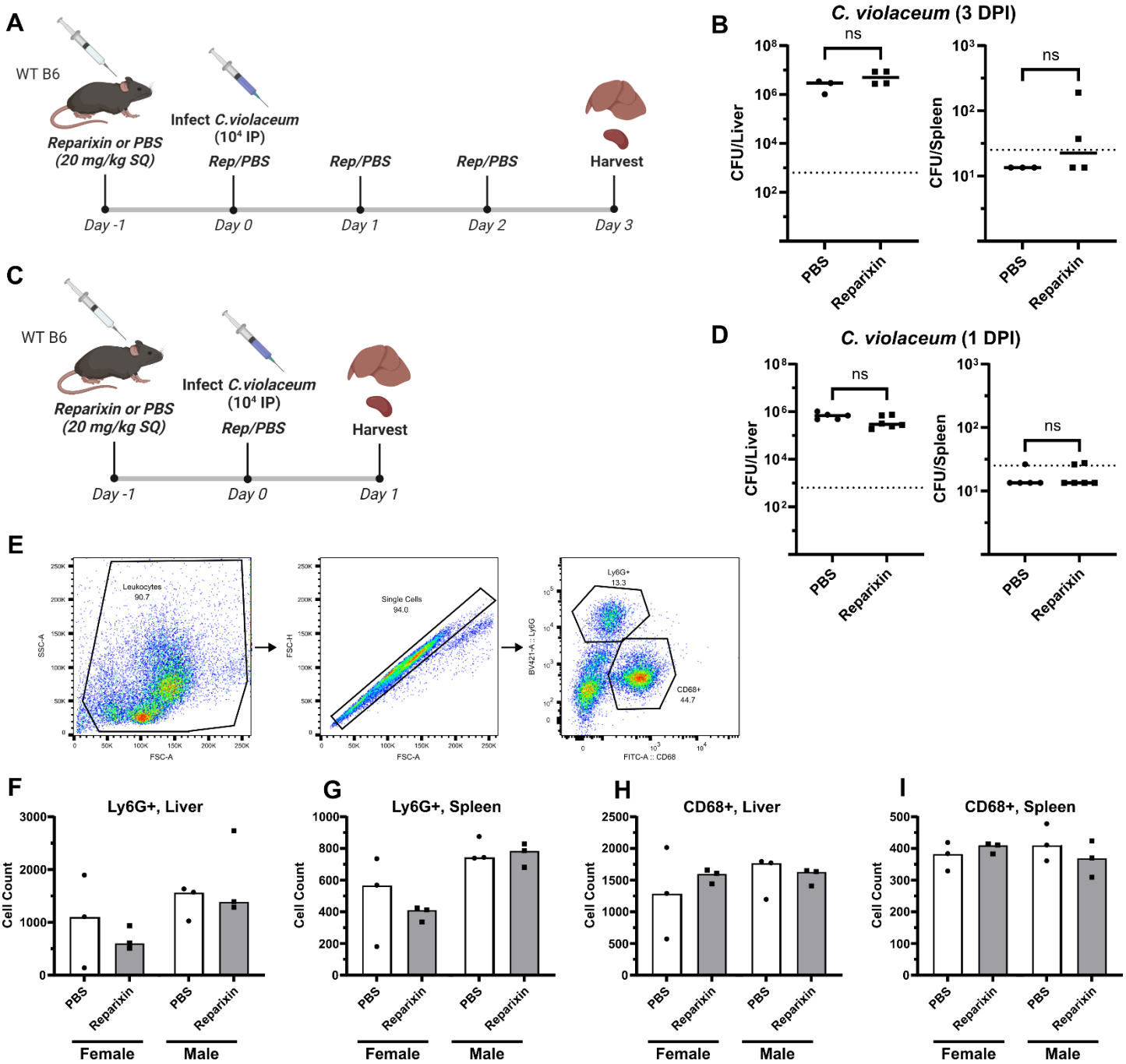
Supplemental Figure 5. Spatial expression of *Ccl22, Ccl24, Ccl25,* and *Ccl27a*. SpatialFeaturePlots displaying gene expression data of selected *Ccl* family members at various days post-infection (DPI). Scale set at 0 – 3.0 expression.



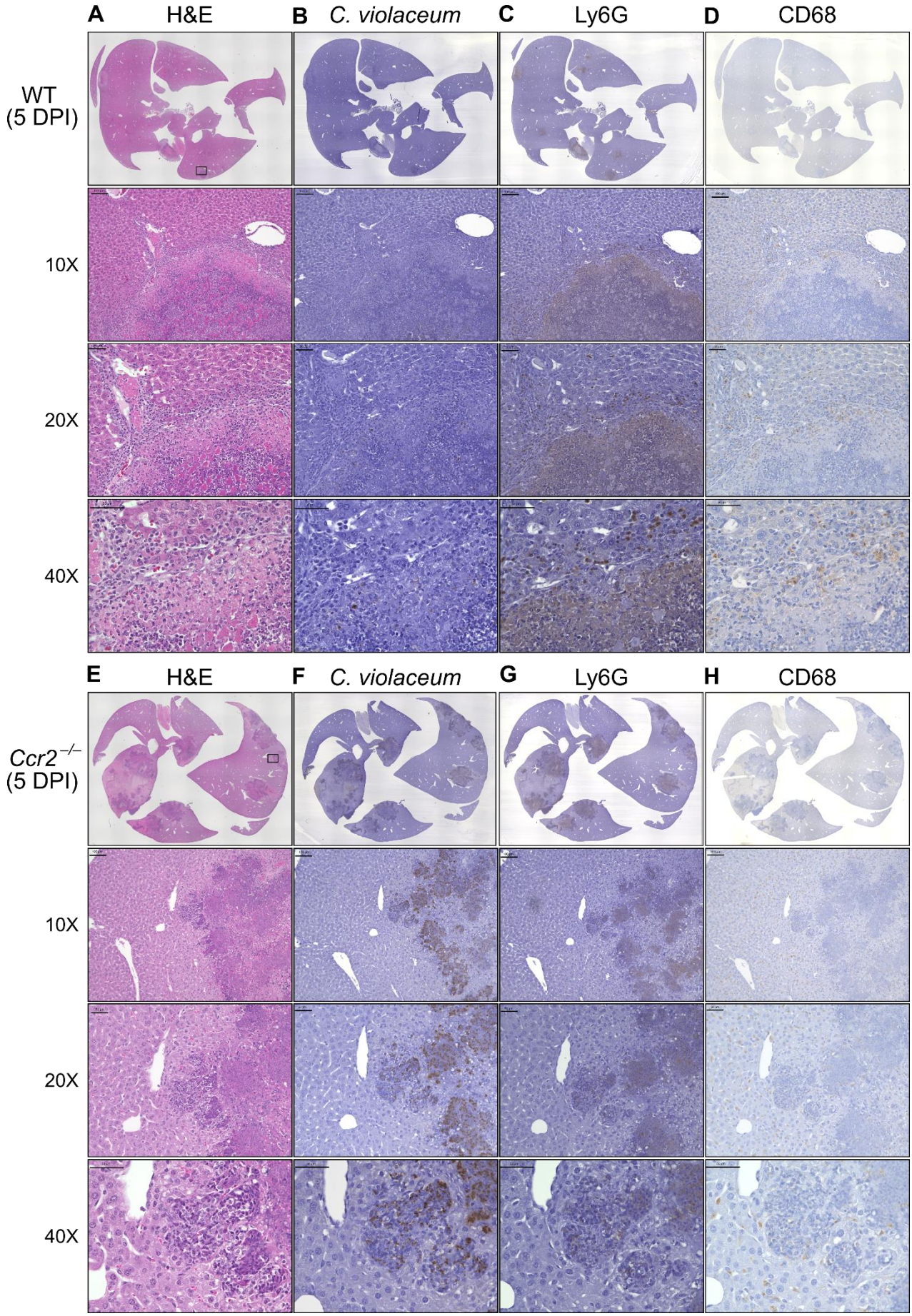
Supplemental Figure 6. Spatial expression of *Cxcr* family members. SpatialFeaturePlots displaying gene expression data of selected *Cxcr* family members at various days post-infection (DPI). Scale set at 0 – 2.0 expression.



Supplemental Figure 7. Spatial expression of *Ccr* family members. SpatialFeaturePlots displaying gene expression data of selected *Ccr* family members at various days post-infection (DPI). Scale set at 0 – 2.0 expression.



Supplemental Figure 8. Reparixin does not inhibit neutrophil chemotaxis into the liver of infected mice. (**A**) Schematic of the experimental procedure. Mice were injected subcutaneously (SQ) with 20 mg/kg of reparixin, or with PBS. The following day, mice were infected intraperitoneally (IP) with $1x10^4$ CFU of *C. violaceum* and treated again with reparixin or PBS. Mice were treated daily thereafter until harvesting on day 3 post-infection. (**B**) Bacterial burdens in the liver and spleen of PBS- or reparixin-treated mice at 3 days post-infection (DPI). (**C**) Schematic of the experimental procedure as in A, except mice were harvested on day 1 post-infection. (**D**) Bacterial burdens in the liver and spleen of PBS- or reparixin-treated mice at 1 DPI. (**E**) Gating analysis of neutrophil ($Ly6G^+$) and macrophage ($CD68^+$) numbers via flow cytometry. Neutrophil numbers in the (**F**) liver and (**G**) spleen. Macrophage numbers in the (**H**) liver and (**I**) spleen. Each dot represents one mouse, with 10,000 events collected per sample. Line at median. (B and D) Dotted line, limit of detection. Solid line, median. Mann-Whitney (abnormally distributed data) for all except liver CFU at 1 DPI, which was analyzed using a two-tailed t test (normally distributed data). Not significant (ns). (B) Liver, p=0.6286; spleen, p=0.4286. (D) Liver, p=0.0641; spleen, p=0.8485. (B) One experiment. (D) Two experiments combined. (F-I) Two experiments combined.



Supplemental Figure 9. Loss of CCR2-dependent monocyte trafficking results in abnormal granuloma architecture and failure of bacterial containment. WT and *Ccr2*$^{-/-}$ mice were infected intraperitoneally (IP) with 1x10^{4}

774 CFU *C. violaceum* and livers harvested 5 days post-infection (DPI). Serial sections of livers stained by hematoxylin and
775 eosin (H&E) or various IHC markers for (**A-D**) WT female and (**E-H**) *Ccr2*$^{-/-}$ female. For 10X, scale bar is 100 μm. For 20X
776 and 40X, scale bar is 50 μm.

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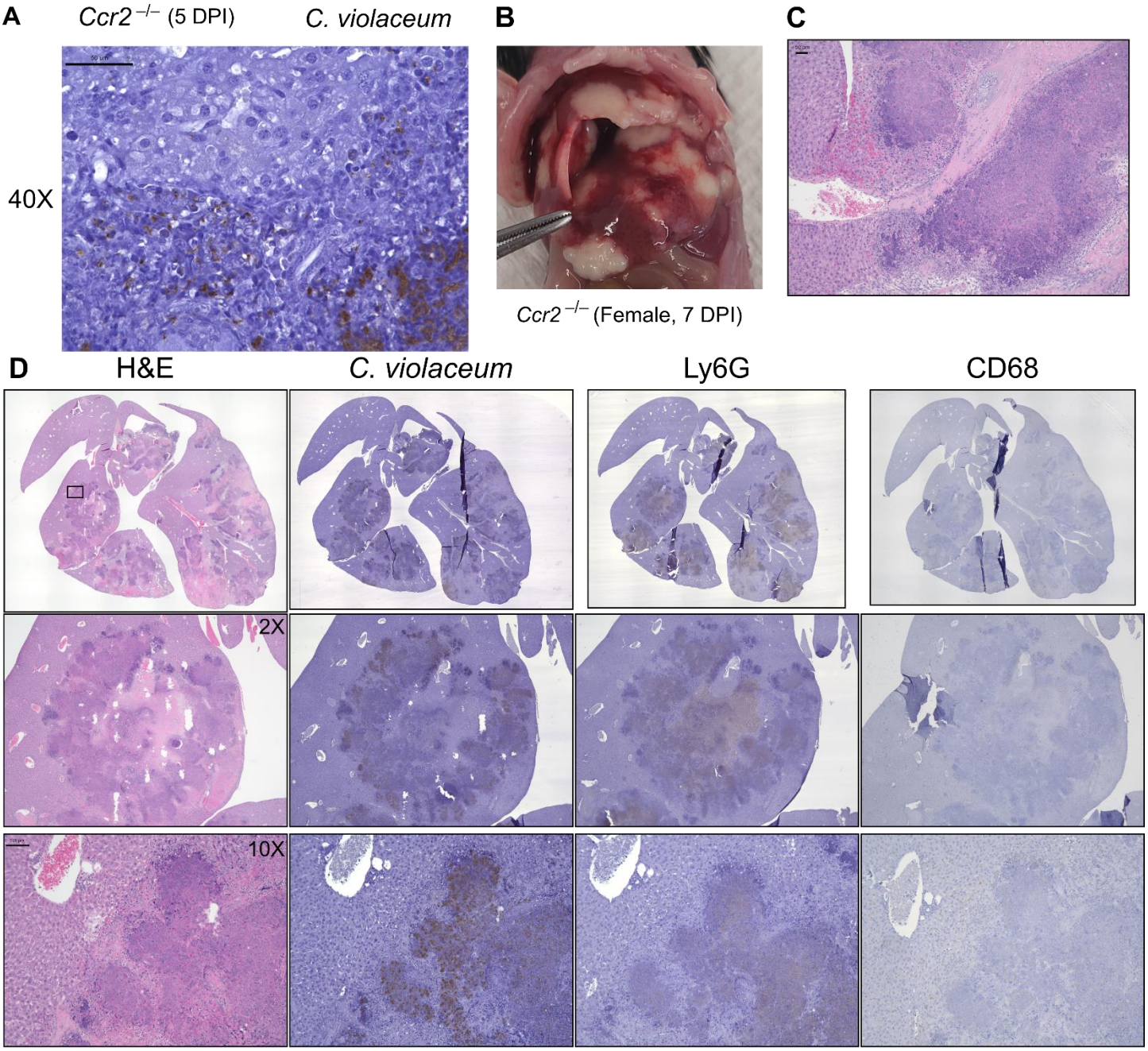

Supplemental Figure 10. *Ccr2*$^{-/-}$ mice have increased necrosis and clotting. *Ccr2*$^{-/-}$ mice were infected intraperitoneally (IP) with 1x10^4 CFU *C. violaceum*. (**A**) Liver section from *Ccr2*$^{-/-}$ male mouse 5 days post-infection (DPI), stained for *C. violaceum*; zoom showing individual puncta of *C. violaceum*. (**B-D**) A *Ccr2*$^{-/-}$ female mouse from survival curve in Figure 6A that was sacrificed at 7 DPI according to euthanasia criteria. (B) Gross pathology. (C) Liver section stained by hematoxylin and eosin (H&E) showing clotting. (D) Serial sections of liver stained with H&E or various IHC markers. For 10X, scale bar is 100 µm.

787 **Table 1. Expression level of chemokine ligands during infection with *C. violaceum*.** Expression was visually ranked
788 as absent, low, medium, or high based on SpatialFeaturePlots. Maximum expression rank recorded here. Table generated
789 from: (David & Kubes, 2019; Hughes & Nibbs, 2018; Sokol & Luster, 2015; Zlotnik & Yoshie, 2000, 2012). Lymph node
790 (LN); Natural killer cell (NK); NK T cell (NKT); innate lymphoid cell (ILC); dendritic cell (DC).

Ligand	Max Expression	Alias & Main Functions
Cxcl1	High	(NAP-3) Neutrophil migration
Cxcl2	High	(MIP-2) (MIP2-α) Neutrophil migration; 90% identical to *Cxcl1*; involved in wound healing
Cxcl3	High	(MIP2-β) Neutrophil migration; migration and adhesion of monocytes
Cxcl4	High	(*Pf4*) Neutrophil and monocyte migration; released by platelets; wound repair & coagulation; angiogenesis
Cxcl5	High	(LIX) Neutrophil migration; connective tissue remodeling
Cxcl9	High	Th1, CD8, NK, monocyte migration; closely related to CXCL10 and CXCL11
Cxcl10	High	Th1, CD8, NK, monocyte migration
Cxcl11	Absent	Th1, CD8, NK, monocyte migration
Cxcl12	High	(SDF-1) Lymphocyte migration; bone marrow homing
Cxcl13	Low	B cell migration within follicles of lymphoid tissues; highly expressed in liver, spleen, LN
Cxcl14	Low	Monocyte migration to skin; potent activator of DC
Cxcl15	Absent	Neutrophil migration during inflammation of lungs
Cxcl16	Med	NKT and ILC migration and survival; found in red pulp of the spleen
Cxcl17	Absent	Monocyte and DC migration in the lung
Ccl1	Absent	(TCA3) T cell trafficking
Ccl2	High	(MCP1) Monocyte trafficking
Ccl3	High	(MIP-1α) Macrophage and NK cell migration
Ccl4	High	(MIP-1β) Macrophage and NK cell migration
Ccl5	High	(RANTES) Macrophage and NK cell migration; also chemotactic for T cells, eosinophils, basophils
Ccl6	High	(C10) Myeloid cell differentiation; monocyte, T cell, and eosinophil chemotaxis
Ccl7	Med	(MCP3) (MARC) Monocyte mobilization
Ccl8	Med	(MCP2) Th2 response; skin homing
Ccl9	High	(MIP-1γ) (MRP-2) DC migration
Ccl11	Low	(Eotaxin) Eosinophil and basophil migration; selectively recruits eosinophils
Ccl12	Low	(MCP5) Inflammatory monocyte trafficking
Ccl17	Absent	(ABCD2) (TARC) T cell chemotaxis; lung and skin homing
Ccl19	Med	(MIP-3β) T cell and DC migration to LN
Ccl20	Low	(MIP-3α) Th17 responses; B cell and DC homing to gut-associated lymphoid tissue
Ccl21a	Med	(TCA4) T cell and DC migration to LN
Ccl21b	Absent	Very similar to *Ccl21a*
Ccl21c	Absent	Identical to *Ccl21b*
Ccl22	Low	(ABCD1) Th2 response and migration; monocyte, DC, NK migration; produced by monocytes and DC
Ccl24	Med	(MPIF-2) (Eotaxin-2) Eosinophil and basophil migration
Ccl25	Low	(TECK) T cell homing to gut; T cell development; thymocyte, macrophage, and DC migration
Ccl26	Absent	(Eotaxin-3) Eosinophil and basophil migration
Ccl27a	Low	T cell migration to skin
Ccl27b	Absent	T cell migration to skin
Ccl28	Absent	(MEC) T and B cell migration to mucosal tissues
Cx3cl1	Low	(Fractalkine) NK, monocyte, and T cell migration
Xcl1	Low	(Lymphotactin) Cross-presentation by CD8+ DCs

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792 **Table 2. Expression level of chemokine receptors during infection with *C. violaceum*.** Expression was visually
793 ranked as absent, low, medium, or high based on SpatialFeaturePlots. Maximum expression rank recorded here. Table
794 generated from: (David & Kubes, 2019; Hughes & Nibbs, 2018; Sokol & Luster, 2015; Zlotnik & Yoshie, 2000, 2012).
795 Natural killer cell (NK); innate lymphoid cell (ILC); dendritic cell (DC); plasmacytoid DC (pDC); lymph node (LN); red blood
796 cell (RBC).

Receptor	Max Expression	Alias, Cellular Expression & Main Functions
Cxcr1	Absent	(IL8R-α) Neutrophil, monocyte, NKs, mast cell, basophil, CD8 T cells; Neutrophil migration and activation
Cxcr2	Med	(IL8R-β) Neutrophil, monocyte, NKs, mast cell, basophil, CD8 T cells; B cell and neutrophil migration; neutrophil egress from BM
Cxcr3	Med	Various T cells, NKs, pDCs, B cells; effector T cell migration and activation
Cxcr4	Med	Most leukocytes; bone marrow homing and retention
Cxcr5	Absent	B cells, T cells; T and B cell migration within LN to B cell zones
Cxcr6	Med	Various T cells, ILCs, NKs, plasma cells; T cell and ILC function
Ccr1	High	Monocyte, macrophage, neutrophil, Th1, basophil, DC
Ccr2	High	Monocyte, macrophage, Th1, DC, basophil, NK; monocyte migration, Th1 immunity
Ccr3	Absent	Highly expressed on eosinophils and basophils; allergic airway; eosinophil trafficking
Ccr4	Absent	Various T cells, monocytes, B cells, DCs; T cell homing to skin and lung
Ccr5	High	Monocytes, macrophages, various T cells, NK, DC, neutrophils, eosinophils; adaptive immunity
Ccr6	Absent	Various T cells, DCs, NKs; DC and B cell maturation and migration; adaptive immunity
Ccr7	Med	Various T cells, DCs, B cells; migration of adaptive lymphocytes and DCs to lymphoid tissues
Ccr8	Absent	Various T cells, monocytes, macrophages; surveillance in skin; expressed in the thymus
Ccr9	Absent	T cells, thymocytes, B cells, DCs, pDCs; T cell migration to gut; key regulator of thymocyte migration and maturation
Ccr10	Absent	T cells, melanocytes, plasma cells; immunity at mucosal sites, especially skin
Xcr1	Low	DCs; antigen cross-presentation
Cx3cr1	Low	Monocytes, macrophages, microglia, DCs, T cells; migration and adhesion of leukocytes; marker of anti-inflammatory monocytes; thought to promote a patrolling phenotype and pro-survival signals
Atypical Receptors		
Ackr1	Low	(DARC) RBCs, endothelial cells, neurons; chemokine scavenging, neutrophil transmigration; chemokine transcytosis on lymphatic endothelium and RBCs
Ackr2	Low	Endothelial cells, DCs, B cells, macrophages; chemokine scavenging
Ackr3	Low	(Cxcr7) Stromal cells, B cells, T cells, neurons, mesenchymal cells; pro-survival, adhesion, shaping CXCR4 gradients; involved in CXCR4 gradients
Ackr4	Low	(Ccrl1) Epithelial cells, leukocytes, astrocytes, microglia; chemokine scavenging and transcytosis; chemokine scavenging in thymus
Ccrl2	High	Chemokine receptor-like protein; binds chemerin; related to CCR1; expressed on neutrophils and monocytes

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800 **Table 3. Expression level of selected chemoattractive molecules during infection with *C. violaceum*.** Expression
801 was visually ranked as absent, low, medium, or high based on SpatialFeaturePlots. Maximum expression rank recorded
802 here. Table generated from: (Bui et al., 2020; David & Kubes, 2019; Parks et al., 2004; Wang et al., 2018). Dendritic cell
803 (DC); plasmacytoid DC (pDC); Kupffer cell (KC); natural killer cell (NK); syndecan 1 (SDC1).

Other	Max Expression	Alias, Cellular Expression & Main Functions
Fpr1	High	(Formyl peptide receptor 1) Expressed on myeloid cells and lymphocytes; widely expressed by neutrophils, eosinophils, basophils, monocytes, and platelets (among others); involved in leukocyte chemotaxis and activation
Fpr2	Med	(Formyl peptide receptor 2) Expressed on neutrophils, eosinophils, monocytes, macrophages, T cells; involved in leukocyte chemotaxis and activation
C5ar1	Med	(Complement C5a receptor 1) Expressed on basophils, DCs, mast cells, non-immune cells; involved in leukocyte chemotaxis and activation
Ltb4r1	Low	(Leukotriene B4 receptor) Expressed on neutrophils, macrophages, T cells; involved in leukocyte chemotaxis and activation
Cmklr1	Low	(Chemerin chemokine-like receptor 1) Expressed mainly on myeloid cells; present in thymus, bone marrow, spleen, fetal liver, and lymphoid organs; involved in migration of macrophages, DCs, and pDCs
Mmp2	High	(Gelatinase A) Inactivates CXCL12, CCL7; degrades S100A9
Mmp8	Med	(Neutrophil collagenase) Stored in secondary granules; cleaves and enhances CXCL5; inactivates CXCL-9 and CXCL-10
Mmp9	High	(Gelatinase B) Mainly expressed by neutrophils; cleaves and enhances CXCL5; cleaves SDC1 to promote neutrophil infiltration; inactivates CXCL4 and CXCL1; inactivates CXCL-9 and CXCL-10; upregulated during respiratory epithelial healing; also expressed by KCs
Mmp12	High	(Macrophage elastase) Activates TNF release from macrophages
Mmp13	Med	(Collagenase 3) Inactivates CXCL-12; inactivates CCL2, CCL8, CCL13
Itgam	Med	(CR3A) (Cd11b) Regulates adhesion and migration of monocytes, granulocytes, macrophages, NKs; involved in complement system
Mif	High	(Macrophage migration inhibitory factor) Binds to CXCR2 and CXCR4 to promote chemotaxis of leukocytes
Icam1	High	(Intracellular adhesion molecule 1) Promotes leukocyte migration from circulation to sites of inflammation
S100a8	High	Heterodimerizes with S100a9; involved in leukocyte recruitment and inflammation
S100a9	High	Heterodimerizes with S100a8; involved in leukocyte recruitment and inflammation

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