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## **CCR2 deficiency promotes exacerbated chronic erosive neutrophil-dominated chikungunya virus arthritis**

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26

**ABSTRACT**

27 Chikungunya virus (CHIKV) is a member of a globally distributed group of arthritogenic  
28 alphaviruses, that cause weeks to months of debilitating polyarthritis/arthralgia, which is  
29 often poorly managed with current treatments. The arthritic disease is usually characterised  
30 by high levels of the chemokine CCL2 and a prodigious monocyte/macrophage infiltrate.  
31 Several inhibitors of CCL2 and its receptor CCR2 are in development and may find  
32 application for treating certain inflammatory conditions, including autoimmune and viral  
33 arthritides. Herein we used CCR2<sup>-/-</sup> mice to determine the effect of CCR2 deficiency on  
34 CHIKV infection and arthritis. Although there were no significant changes in viral load or  
35 RNA persistence, and only marginal changes in anti-viral immunity, arthritic disease was  
36 substantially increased and prolonged in CCR2<sup>-/-</sup> mice when compared with wild-type mice.  
37 The monocyte/macrophage infiltrate was replaced in CCR2<sup>-/-</sup> mice by a severe neutrophil  
38 (followed by an eosinophil) infiltrate, and was associated with changes in expression of  
39 multiple inflammatory mediators (including CXCL1, CXCL2, G-CSF, IL-1 $\beta$ , IL-10). Loss  
40 of anti-inflammatory macrophages and their activities (eg efferocytosis) was also implicated  
41 in the exacerbated inflammation. Clear evidence of cartilage damage was also seen in  
42 CHIKV infected CCR2<sup>-/-</sup> mice, a feature not normally associated with alphaviral arthritides.  
43 Although recruitment of CCR2<sup>+</sup> monocyte/macrophages can contribute to inflammation, they  
44 also appear to be critical for preventing excessive pathology and resolving inflammation  
45 following alphavirus infection. Caution might thus be warranted when considering  
46 therapeutic targeting of CCR2/CCL2 for the treatment of alphaviral arthritides.  
47

48 **Importance statement**

49 Herein we describe the first analysis of a viral arthritis in mice deficient for chemokine  
50 receptor CCR2. CCR2 is thought to be central to the monocyte/macrophage dominated  
51 inflammatory arthritic infiltrates seen after infection with arthritogenic alphaviruses, such as  
52 chikungunya virus. Surprisingly, the viral arthritis caused by chikungunya virus in CCR2  
53 deficient mice was more severe, prolonged and erosive, and neutrophil dominated, with viral  
54 replication and persistence not significantly affected. Monocytes/macrophages recruited by  
55 CCL2 thus also appear to be important for both preventing even worse pathology mediated  
56 by neutrophils, and for promoting resolution of inflammation. Caution might thus be  
57 warranted when considering use of therapeutic agents that target CCR2/CCL2 or  
58 inflammatory monocyte/macrophages for treating alphaviral (and perhaps other viral)  
59 arthritides. Individuals with diminished CCR2 responses (due to drug treatment or other  
60 reasons) may also be at risk of exacerbated arthritic disease following an alphaviral infection.

61

62

63           Although many viruses can cause arthritis (56), few do so with the reliability of the  
64 arthritogenic alphaviruses, where symptomatic infection of adults is nearly always associated  
65 with rheumatic disease. This group of globally distributed, mosquito borne, positive-strand  
66 RNA viruses include the Australasian Ross River virus and Barmah Forest virus, the African  
67 o'nyong-nyong virus, the American Mayaro virus, the Sindbis virus family (which includes  
68 the Scandinavia Ockelbo and Pogosta viruses), and chikungunya virus (CHIKV) (54, 55).  
69 CHIKV has caused sporadic outbreaks every 2-50 years, which have generally been restricted  
70 to Africa and Asia. However, in 2004-2012, CHIKV caused the largest outbreak ever  
71 recorded for this virus, with an estimated 1.4-6.5 million patients and imported cases reported  
72 in nearly 40 countries, including USA, Japan and several European countries (7, 54, 59).  
73 CHIKV disease, and alphaviral rheumatic disease generally, is usually self-limiting and  
74 characterised by acute and chronic symmetrical peripheral polyarthralgia/polyarthritis, with  
75 acute disease often also associated with fever, myalgia and/or rash. Arthropathy can be  
76 debilitating, usually lasts weeks to months, is generally not erosive, but can be protracted (54,  
77 55).

78           Chemokine (C-C motif) receptor 2 (CCR2) is the receptor for a number of C-C motif  
79 chemokines, including CCL2, which is also known as monocyte chemoattractant protein-1  
80 (MCP-1). CCL2 recruits monocytes, basophils and T-cells to sites of inflammation and has  
81 been implicated as an important mediator in a range of inflammatory diseases including *inter*  
82 *alia* rheumatoid arthritis (RA), multiple sclerosis, atherosclerosis, asthma, neuropathic pain  
83 (53), obesity, diabetes (37) and cancer (66). A range of therapeutic agents that target CCR2  
84 or CCL2 are being developed (37, 53, 66), although initial trial results for CCR2 blockade in  
85 RA patients have been disappointing (23). Nevertheless, CCR2/CCL2 inhibition may  
86 ultimately find utility in the treatment of a range of conditions (37, 53, 66). Elevated CCL2  
87 and monocyte/macrophage infiltrates are also prominent features in several viral arthritides,

88 and are well described for alphaviral arthropathies (56), including CHIKV disease in mice,  
89 monkeys and humans (13, 22, 54). Treatments with anti-CCL2 antibody or bindarit, a new  
90 drug purported to target CCL2 production, were shown to be effective at ameliorating  
91 rheumatic disease in CHIKV and Ross River virus mouse models (43, 44). As CCL2  
92 generally appears to have no direct anti-viral activity (15, 26, 44, 56), targeting this  
93 chemokine and/or its receptor would appear attractive for treatment of viral arthritides.

94 The inflammation biology of CCR2/CCL2 has emerged to be complicated. Collagen-  
95 induced arthritis in CCR2<sup>-/-</sup> mice is more severe with increased macrophage and neutrophil  
96 infiltrates (39, 40), whereas in other arthritis models, inhibition of CCL2 ameliorated disease  
97 (14, 50). CCR2 deficiency was associated with reduced monocyte/macrophage infiltration in  
98 some settings (3, 38, 47), but not others (12, 39). More neutrophil infiltrates were seen in  
99 CCR2<sup>-/-</sup> mice in certain settings (12, 33, 39), unchanged in some (10), less in others (51). For  
100 dengue and influenza infections in CCR2<sup>-/-</sup> mice the viral load was unchanged, and the  
101 pathology was attenuated (15, 26). In contrast, cytomegalovirus and West Nile virus  
102 infections in these mice resulted in increased viral loads and pathology (17, 25).

103 Given the recent outbreak of CHIKV and the interest in using CCR2/CCL2 blockers for  
104 alphaviral arthritides and other diseases, we sought to determine the effect of CCR2  
105 deficiency in CHIKV arthritis using a recently established adult mouse model, which mimics  
106 many aspects of human disease (13).

107

## 108 **MATERIALS AND METHODS**

109 **Ethics statement.** Animals were handled in accordance with National Health and Medical  
110 Research Council (Australia) guidelines. Animals were kept in the modern Optimice caging  
111 system (Centennial, CO, USA). All animal experimentation was approved by the QIMR  
112 Berghofer animal ethics committee.

113       **Virus isolates and preparation.** The Reunion Island (LR2006-OPY1) CHIKV isolate  
114 was prepared as described (13). The virus preparations had undetectable endotoxin and  
115 mycoplasma contamination (19, 21). As CHIKV is a biosafety level 3 pathogen, all work  
116 with infectious virus was conducted in the BSL3 facility at QIMR Berghofer.

117       **Mouse infection, arthritis and antibody monitoring.** Female 6-10 week old mice were  
118 inoculated s.c. into each hind foot with CHIKV (LR2006-OPY1) (viral dose of  $10^4$  CCID<sub>50</sub> in  
119 40  $\mu$ l medium) and tissue viral titres and viraemia determined as described (13). Arthritis  
120 was monitored by measuring the height and width of the metatarsal area of the hind feet using  
121 digital calipers. The data is presented as a group average of the percentage increase in foot  
122 height x width for each foot compared with the same foot on day 0 (42). C57BL/6 mice were  
123 supplied by Animal Resources Centre (Perth, Australia). CCR2<sup>-/-</sup> mice on a C57BL/6  
124 background (3) were bred in-house at QIMR Berghofer. Antibody responses were  
125 determined using isotope-specific ELISA using inactivated whole CHIKV as antigen as  
126 described (62).

127       **Quantitative real-time reverse transcription PCR (qRT-PCR) analyses.** RNA  
128 isolation from feet, cDNA preparation and quantitative real-time PCR analyses were  
129 performed as described (13). The qRT-PCR analyses used 1  $\mu$ l ( $\approx$ 50 ng) of cDNA, 10  $\mu$ l of  
130 SYBR green Super mix-UDG (Invitrogen), 1  $\mu$ l of the kit's BSA solution, 6  $\mu$ l H<sub>2</sub>O, and 1  $\mu$ l  
131 of 10  $\mu$ M of forward and reverse primers. Each sample was analyzed in duplicate and  
132 normalized to RPL13A mRNA levels (13). Primer sequences are shown in Table S1 in the  
133 supplemental material.

134       **Histology and immunohistochemistry.** H&E, F4/80 and ApoTag staining (Millipore,  
135 Temecula, US) was undertaken as described previously (13, 34). Anti-Ly6G staining was  
136 undertaken using rat anti-mouse Ly6G (Abcam, Cambridge, MA, USA; #NMP-R14) and was  
137 detected in the same way as for F4/80. Leder (Naphthol AS-D Chloroacetate, Sigma, USA),

138 Chromotrope 2R (ProSciTech, Thuringowa, Australia) and Safranin O staining (BDH  
139 Laboratory Supplies, Poole, UK) used standard protocols. Stained whole foot sections were  
140 digitally scanned using Scan Scope XT digital slide scanner (Aperio, Vista, CA). Image  
141 analyses of whole foot sections in duplicate were undertaken using Aperio ImageScope  
142 Software (v10) and the Positive Pixel Count v9 algorithm using default “Strong” settings.

143 **MK0812 treatment.** MK0812 (Amandis Chemical, China) was dissolved in chloroform  
144 (5% in PBS final) and administered i.p. 30 mg/kg, daily days 1-6 post infection. Control  
145 mice received 5% chloroform in PBS.

146 **Microarray analyses.** Microarray analysis, identification of differentially expressed  
147 genes (DEGs) and Ingenuity Pathway Analyses (IPA) were performed as described  
148 previously (34). DEGs were obtained for day 7 post-infection relative to day 0, separately for  
149 WT and  $CCR2^{-/-}$  mice, using pooled RNA samples from feet of 4-6 mice for each time point  
150 and mouse strain. Genes with low expression levels in the microarray analysis ( $\log_2$   
151 expression  $< 7$ ) for both days 0 and 7 were removed. Gene set enrichment analysis (GSEA)  
152 was undertaken as described (34) using publicly available microarray raw data files from  
153 studies of synovial biopsies from healthy patients and patients with RA (GSE1919). Human  
154 genes were converted into mouse homologs utilizing the NCBI Homolo-Gene annotation  
155 (available at <http://www.ncbi.nlm.nih.gov/homologene>).

156 **Statistics.** Analysis was performed using IBM SPSS Statistics (version 19). The t test  
157 was used if the difference in the variances was  $< 4$ , skewness was  $> -2$ , and kurtosis was  $< 2$ .  
158 Where the data was non-parametric and difference in variances was  $< 4$  the Mann Whitney U  
159 test was used, if  $> 4$  the Kolmogorov-Smirnov test was used.

160

## 161 **RESULTS**

162 **Foot swelling in CHIKV infected wild-type and  $CCR2^{-/-}$  mice.** After CHIKV infection of



163 adult wild-type (WT) mice, a clearly discernable foot swelling is observed, which peaks on  
164 day 6/7 and largely resolves by day 10-14 (Fig. 1A, black squares) as reported previously  
165 (13). The same CHIKV infection of CCR2<sup>-/-</sup> mice resulted in a nearly 2 fold higher mean  
166 peak foot swelling (Fig. 1A, day 6), with differences clearly observable by eye (Fig. 1B).  
167 The swelling in CCR2<sup>-/-</sup> mice also resolved much more slowly, returning to normal after day  
168 40 (Fig. 1A). Foot swelling in CCR2<sup>-/-</sup> mice was significantly higher than WT mice for all  
169 time points from day 3 to 31.5 (Fig. 1A). Area under the curve calculations show that CCR2<sup>-</sup>  
170 <sup>-/-</sup> mice experience >4 fold more foot swelling than WT mice (not shown).

171 **Virus and viral RNA in WT and CCR2<sup>-/-</sup> mice.** No consistent and/or significant  
172 differences were seen between WT and CCR2<sup>-/-</sup> mice following CHIKV infection in (i)  
173 viraemia (Fig. 1C), (ii) virus titres in feet, muscle, spleen, and lymph node (Fig. 1D), or (iii)  
174 viral RNA levels in feet (Fig. 1E). CCR2 deficiency thus does not appear to have a  
175 significant effect on CHIKV replication or persistence of CHIKV RNA (54). The increased  
176 and more protracted disease seen in CCR2<sup>-/-</sup> mice (Fig. 1A), was thus not due to an increase  
177 in viral replication or increased persistence of viral RNA in these mice.

178 **Histology and immunohistochemistry of the cellular infiltrates in the arthritic feet**  
179 **of WT and CCR2<sup>-/-</sup> mice.** Histological examination of arthritic feet in WT mice shows a  
180 predominance of mononuclear cell infiltrates on day 6 (the day of peak arthritis) as described  
181 previously (13). This could be clearly seen by H&E staining of muscle (compare Fig. 2A  
182 with 2B and 2C). Staining of foot sections with F4/80 antibody (a monoclonal antibody  
183 recognising mouse monocyte/macrophages), also showed a large number of infiltrating  
184 F4/80<sup>+</sup> cells (Fig. 2D) as reported previously (13). Leder staining (Fig. 2E) showed that  
185 neutrophils were largely absent from infiltrates in day 6 feet of WT mice (Fig. 2E). ApoTag  
186 staining revealed some apoptosis primarily in cells with monocytic and fibroblastic  
187 morphology (Fig. 2F). Chromotrope2R staining (Fig. 2F) showed that eosinophils were

188 largely absent (Fig. 2G).

189 The cellular infiltrates in the feet of  $CCR2^{-/-}$  mice were quite distinct from those seen in  
190 WT mice. H & E staining showed a predominance of neutrophils (compare Fig. 2H with 2I  
191 and 2J), (many with hyper-segmented nuclei), with few  $F4/80^{+}$  cells detected in the infiltrates  
192 (Fig. 2K). Prodigious neutrophil infiltrates were also clearly seen by Leder staining (Fig.  
193 2L). ApoTag staining was more widespread, with frequent staining of cytoplasmic debris  
194 (Fig. 2M, left), suggestive of secondary necrosis. ApoTag staining was often also present in  
195 cells with polymorphonuclear morphology (Fig. 2M, right). The latter suggests inflammatory  
196 neutrophils undergoing apoptosis, a well described phenomena in inflammation biology (2).  
197 Chromotrope2R staining also revealed that a large number of eosinophils were present in the  
198 arthritic infiltrates in  $CCR2^{-/-}$  feet on day 21 post-infection (Fig. 2L).

199 **Quantitation of histology and immunohistochemistry of the cellular infiltrates in the**  
200 **arthritic feet of WT and  $CCR2^{-/-}$  mice.** To quantitate the features described above, the  
201 indicated number of feet were processed and stained in parallel, and slides were digitally  
202 scanned (Aperio) and analysed using the Positive Pixel Count algorithm (Fig. 3).  $F4/80$   
203 staining increased substantially in WT feet on day 6 post-infection (as reported previously  
204 (13)). This did not occur in  $CCR2^{-/-}$  feet, with  $F4/80$  staining significantly higher in WT feet  
205 compared with  $CCR2^{-/-}$  feet at this time (Fig. 3A). These data support the widely expressed  
206 view that the prodigious monocyte/macrophage infiltrate seen in WT mice after infection  
207 with arthritogenic alphaviruses is dependent on  $CCL2/CCR2$  expression (13, 22, 24, 43, 44,  
208 54, 56). We cannot formally exclude the possibility that the reduced monocyte/macrophage  
209 recruitment in  $CCR2^{-/-}$  mice is, at least in part, due to the reduced level of circulating  
210 monocytes in these animals (25). However, pixel count of  $F4/80$  staining of feet day 6 post  
211 infection after adoptive transfer (prior to infection) of WT  $CCR2^{+/+}$  splenocytes ( $3 \times 10^7$ ) into  
212  $CCR2^{-/-}$  mice gave a mean value of  $0.172 \pm SE 0.02$  Brown pixels/ $\mu m^2$  (settings as in Fig.

213 3A), whereas after adoptive transfer of CCR2<sup>-/-</sup> splenocytes into CCR2<sup>-/-</sup> mice this value was  
214  $0.119 \pm \text{SE } 0.006$  Brown pixels/ $\mu\text{m}^2$  (i.e. very similar to CCR2<sup>-/-</sup> mice on day 6, see Fig. 3A)  
215 (data not shown). The difference between the two adoptive transfer groups was significant  
216 ( $p=0.015$ ,  $n=8$  feet from 4 mice per group). Pixel counts of Ly6C staining of the same feet  
217 also indicated a  $\approx 25\%$  reduction in neutrophil infiltration in CCR2<sup>-/-</sup> mice given WT cells, but  
218 this only approached significance ( $p=0.057$ ) (data not shown).

219 F4/80 staining increased significantly in CCR2<sup>-/-</sup> feet on days 21 and 29 post infection  
220 (Fig. 3A). Rather than being associated with monocyte/macrophages, these increases were  
221 likely associated with infiltrating eosinophils (see below), which also stain with F4/80  
222 antibody (data not shown) as reported previously (30).

223 Leder staining showed a dramatic increase in CCR2<sup>-/-</sup> feet on day 6 post-infection and  
224 was still significantly elevated on day 14, consistent with the increase in neutrophils seen by  
225 H&E staining (Fig. 3B). In WT mice Leder staining was slightly elevated day 6, but was  
226 substantially lower than in CCR2<sup>-/-</sup> feet on days 6 and 14 (Fig. 3B). Immunohistochemistry  
227 using a neutrophil-specific anti-Ly6G antibody and image analysis confirmed the stark and  
228 significant contrast in neutrophil infiltrate between WT and CCR2<sup>-/-</sup> feet on day 6 (data not  
229 shown). To investigate how important neutrophils were for the increased foot swelling seen  
230 in these mice (Fig. 1A), neutrophils were depleted using an anti-Ly6G antibody (administered  
231 day 4, 5, and 6 post infection; 0.3 mg per day), with a  $\approx 85\%$  reduction in circulating  
232 neutrophil numbers achieved on day 7. However, this led to slightly increased foot swelling  
233 (days 5-7), with widespread haemorrhage and edema evident (data not shown). Neutrophil  
234 depletion in CCR2<sup>-/-</sup> mice post CHIKV infection thus appears to promote a new pathology  
235 (42), complicating any analysis of the arthritogenic role of neutrophils using this approach.

236 ApoTag staining of apoptotic cells was significantly higher in CCR2<sup>-/-</sup> feet on day 6, and  
237 was also significantly elevated on day 14, whereas in WT mice there was no significant

238 difference between any of the time points shown (34) (Fig. 3C). Chromotrope2R staining  
239 (eosinophils) was slightly elevated on days 6 and 14 in WT mice, but was substantially  
240 elevated in CCR2<sup>-/-</sup> mice on day 21 and 29 (Fig. 3D).

241 In summary, the increased and prolonged arthritic disease seen in CCR2<sup>-/-</sup> mice was  
242 associated with loss of infiltrating monocytes/macrophages, a severe neutrophil infiltrate  
243 followed by an eosinophil infiltrate, with more apoptotic cells also seen during peak disease.

244 **Treatment with a CCR2 antagonist.** To determine whether treatment with a CCR2  
245 antagonist might have a similar effect on neutrophil infiltration as that seen in CCR2<sup>-/-</sup> mice,  
246 WT mice were treated with MK0812, a CCR2 antagonist tested (without clinical benefit) in  
247 rheumatoid arthritis patients (65). The drug has been shown to reduce the peripheral blood  
248 monocyte frequency and monocyte recruitment to sites of inflammation in animal models  
249 (31, 63). On day 6 post-infection a significant increase in Ly6G staining was seen in feet of  
250 WT mice treated with MK0812, when compared with control treatment (Fig. 3E). This  
251 observation supports the view that reduction in CCR2 signalling can lead to increased  
252 neutrophil infiltration following CHIKV infection.

253 **Cartilage damage in CHIKV infected CCR2<sup>-/-</sup> mice.** In contrast with most  
254 autoimmune arthritides, alphaviral arthritides (and most viral arthritides (56)) are generally  
255 not associated with joint damage (54). Joint damage was also not seen in CHIKV-infected  
256 WT mice in the current study (6/6 feet examined) (Fig. 4A), or in previous studies (13, 42) or  
257 in CHIKV-infected monkeys (22). However, H&E staining of joints from CHIKV-infected  
258 CCR2<sup>-/-</sup> mice day 45 post-infection often showed (in 6/6 feet examined) loss of chondrocytes  
259 with empty lacunae in one or more foot joints (Fig. 4, arrows). Clear signs of cartilage  
260 damage at the articular surfaces (never seen in WT mice) was also occasionally observed in  
261 CCR2<sup>-/-</sup> mice (twice in 6 feet) (Fig. 4B, arrowhead). Loss of cartilage collagen (as revealed  
262 by Safranin O staining) was not observed in WT mice (Fig. 4C), but was clearly seen in at

263 least 1, occasionally 2, joints per foot in CCR2<sup>-/-</sup> mice (6 feet examined) (Fig. 4D, blue areas  
264 indicated by arrows). CCR2 deficiency thus increased the severity of CHIKV arthritis, with  
265 cartilage damage clearly apparent.

266 **Anti-viral Th1/Th2/Th17 responses in CHIKV-infected WT and CCR2<sup>-/-</sup> mice.**

267 CHIKV infections induce a strongly Th1-biased immune response (13, 22), with CCR2<sup>-/-</sup>  
268 mice reported to have impaired Th1 responses (3, 18, 46). However, analysis of the anti-  
269 CHIKV IgG2c (Th1) and IgG1 (Th2) titres (48, 62), illustrated that the dominant IgG2c  
270 responses were not significantly different in CCR2<sup>-/-</sup> mice (Fig. 5A). Anti-CHIKV-specific  
271 IgG1 levels were, however, significantly elevated in CCR2<sup>-/-</sup> mice (Fig. 5A), perhaps  
272 consistent with the increased eosinophilia seen in these mice (Fig. 3D). Serum IFN $\gamma$  levels  
273 (measured as described (13)) and neutralizing antibody levels (measured as described (62))  
274 were not significantly different between WT and CCR2<sup>-/-</sup> mice (data not shown).

275 qRT-PCR analysis of T-bet, a key transcription factor for Th1 cells (27), showed a clear  
276 increase in WT mice on day 6 (peak arthritis) (Fig. 5B, T-bet), consistent with the  
277 arthritogenic role of CD4 T cells and IFN $\gamma$  reported previously (34). However, T-bet mRNA  
278 induction and levels were not significantly different (from WT mice) in CCR2<sup>-/-</sup> mice (Fig.  
279 5B, T-bet), supporting the view that anti-CHIKV Th1 responses were not significantly  
280 impaired in CCR2<sup>-/-</sup> mice.

281 Expansion of Th17 cells were reported to be responsible for the increase in neutrophil  
282 infiltration and exacerbated arthritic disease seen in collagen-induced arthritis in CCR2<sup>-/-</sup>  
283 mice (40). However, no significant increases in ROR $\gamma$ T mRNA were observed in either  
284 mouse strain on day 6 post-infection (Fig. 5B, ROR $\gamma$ T). ROR $\gamma$ T is a key transcription factor  
285 for Th17 cells (41).

286 **Changes in inflammatory mediators in the arthritic feet of CCR2<sup>-/-</sup> mice.** Increased  
287 neutrophil infiltrates in CCR2<sup>-/-</sup> mice have been associated with elevated levels of (i)

288 neutrophil-attracting chemokines CXCL1 (KC) and/or CXCL2 (macrophage inflammatory  
289 protein 2-alpha) (12, 33, 39), and/or (ii) G-CSF (47), a key growth factor for neutrophil  
290 development and recruitment involved in inflammatory arthritis (11). IL-1 $\beta$  can also  
291 promote neutrophil recruitment (45), and IL-10 can inhibit neutrophil recruitment (1). qRT-  
292 PCR analyses showed that significantly more CXCL1, CXCL2, G-CSF and IL-1 $\beta$ , and less  
293 IL-10, was present in the arthritic feet of CCR2<sup>-/-</sup> mice (Fig. 5C), suggesting multiple  
294 mediators were involved in the switch from the predominantly monocyte/macrophage to  
295 neutrophil infiltrates. (Serum IL-10 was also elevated earlier in the course of infection in WT  
296 mice – data not shown). qRT-PCR analysis also showed that Arg-1 expression (a marker of  
297 M2 macrophages), although up-regulated in WT feet as expected (52), was not up-regulated  
298 in CCR2<sup>-/-</sup> feet (Fig. 5C, Arg-1). M2 macrophages are associated with resolution of  
299 inflammation (2).

300 **Global gene expression changes induced by CHIKV infection.** Microarray analysis  
301 was undertaken to determine what genes were up- and down-regulated in the arthritic feet of  
302 CHIKV-infected WT and CCR2<sup>-/-</sup> mice. The genes up- or down-regulated on day 7 post-  
303 infection (one day after peak arthritis to capture genes associated with resolution initiation)  
304 relative to day 0 were determined for both mouse strains. A total of 2406 genes were up- or  
305 down-regulated following CHIKV infection in WT and/or CCR2<sup>-/-</sup> mice. When the fold up-  
306 or down-regulation of these differentially expressed genes (DEGs) was compared between  
307 WT and/or CCR2<sup>-/-</sup> mice, 81% showed less than 2 fold difference, illustrating that most genes  
308 were similarly regulated in feet day 7 post-infection in the two mouse strains. However,  
309 consistent with the histological analyses, monocyte/macrophage-associated genes and  
310 pathways were more up-regulated/significant in WT feet, and neutrophil-associated genes  
311 and pathways were more up-regulated/significant in CCR2<sup>-/-</sup> feet (see Fig. S1 in the  
312 supplemental material). CHIKV infection-associated induction of multiple chemokines and

313 chemokine receptors was also different in the two mouse strains, with these differences again  
314 consistent with the contrasting cellular infiltrates in WT and CCR2<sup>-/-</sup> mice (see Table S2 in  
315 the supplemental material).

316 To gain a better understanding of the differences in infection-associated gene regulation  
317 between CCR2<sup>-/-</sup> and WT mice, the differences in fold changes in gene expression between  
318 the two strains for all 2406 DEGs was expressed as a ratio. For 181 genes (7.5% of all  
319 DEGs) the “the fold change in CCR2<sup>-/-</sup> feet” divided by “the fold change in WT feet” was  
320 >1.5 (Fig. 6, top left). For 920 genes (38% of all DEGs) “the fold change in WT feet”  
321 divided by “the fold change in CCR2<sup>-/-</sup> feet” was >1.5. The former 181 genes thus represent  
322 genes that were more induced in CCR2<sup>-/-</sup> mice than in WT mice, and the latter 920 genes  
323 represent genes that were less induced in CCR2<sup>-/-</sup> mice than in WT mice. IPA analysis of the  
324 181 genes more induced in CCR2<sup>-/-</sup> mice, revealed canonical pathways associated with  
325 granulocyte, IL-17A in autoimmunity, RA, and inflammation (Fig. 6, IPA pathways, top; see  
326 Table S3 in the supplemental material). Although autoimmune pathways were identified  
327 (consistent with the worse pathology), CCR2<sup>-/-</sup> mice did not develop anti-nuclear antibodies  
328 (data not shown). IPA analysis of the 920 genes less induced in CCR2<sup>-/-</sup> feet revealed  
329 canonical pathways associated with monocyte/macrophage, pattern recognition,  
330 autoimmunity, B and T cell (mostly Th cell), NK cell, muscle and inflammation pathways  
331 (Fig. 6, IPA pathways, bottom; see Table S4 in the supplemental material). Many of these  
332 pathways are consistent with the aforementioned changes in infiltrates and inflammation.  
333 The identification of muscle pathways likely reflects increased muscle damage (34) in CCR2<sup>-/-</sup>  
334 feet. The identification of NK, T and B cell pathways in this gene set also suggests reduced  
335 recruitment of these cells (13) into the infected feet in CCR2<sup>-/-</sup> mice; T cells can also express  
336 CCR2 (5).

337 The same 181 and 920 gene sets were subjected to Ingenuity upstream regulator analysis.

338 STAT1 and 3 were identified as upstream regulators in both gene sets (see Tables S6 and S7  
339 in the supplemental material), consistent with STAT1 and 3 regulating a distinct set of genes  
340 in monocyte/macrophages and neutrophils (16). Several muscle-associated upstream  
341 regulators were identified in the 181 gene set (with negative z-scores) and in the 920 gene set  
342 (with positive z-scores), again indicating increased muscle damage in  $CCR2^{-/-}$  feet. NF- $\kappa$ B  
343 pathways were prominent upstream regulators identified in the 181 gene set (see Table S5 in  
344 the supplemental material), consistent with the NF- $\kappa$ B-dependent expression of the multiple  
345 chemokines and proinflammatory mediators seen in this gene set. Perhaps surprising (given  
346 no change in viral loads - Fig. 1C,D,E) was the identification of STAT1, IRF1, IRF3 and  
347 IRF7 as upstream regulators in the 920 genes less induced in  $CCR2^{-/-}$  mice (see Table S6 in  
348 the supplemental material). Reduced STAT1 signalling is perhaps consistent with the  
349 slightly lower IFN $\alpha/\beta$  receptor and IFN $\gamma$  expression in  $CCR2^{-/-}$  feet. Reduced interferon  
350 signalling is also supported by a smaller overall fold up-regulation of interferon-associated  
351 genes in  $CCR2^{-/-}$  feet (see Fig. S2 in the supplemental material).

352 **Comparison of gene expression profiles of feet from CHIKV infected  $CCR2^{-/-}$  and**  
353 **WT mice and synovial biopsies from human RA patients.** The DEGs identified for  $CCR2^{-/-}$   
354 mice and the DEGs identified for WT mice (both comparing day 7 with day 0) were each  
355 separately compared with a publicly available gene set from a microarray study of RA  
356 patients (34). Gene set enrichment analysis (GSEA) software was used to determine whether  
357 the rank-ordered list of genes in the RA study (i.e. genes ranked by their fold change in  
358 expression in RA patients relative to healthy individuals) were enriched in the DEGs  
359 identified in  $CCR2^{-/-}$  and WT mice. Nominal p values and false discovery rates (FDR) are  
360 provided for comparisons of both up- and down-regulate genes (Fig. 6B). In all cases there  
361 was significant enrichment, suggesting the high degree of similarity between the gene  
362 signatures of RA and CHIKV arthritis in WT mice (as reported previously (34)) was largely



363 retained for CCR2<sup>-/-</sup> mice. (Previous analyses (34) used a consensus signature from 2  
364 CHIKV isolates, whereas the data in Fig. 6B represents an analysis for the Reunion Island  
365 isolate alone to allow comparison with data from CCR2<sup>-/-</sup> mice).

366

## 367 **DISCUSSION**

368 This paper describes the first analysis of a viral arthritis in CCR2<sup>-/-</sup> mice, and illustrates that  
369 deficiency in this gene results in a more severe, prolonged and erosive arthritis. The  
370 prodigious monocyte/macrophage infiltrate seen in WT mice was almost entirely replaced by  
371 a neutrophil infiltrate in CCR2<sup>-/-</sup> mice, with this switch associated with dysregulation of a  
372 range of inflammatory and anti-inflammatory pathways. Recruitment of CCR2<sup>+</sup> monocytes  
373 during CHIKV arthritis, although likely contributing to acute inflammatory disease (54, 56),  
374 thus also protects against even worse neutrophil-mediated pathology.

375 CXCL1, CXCL2, G-CSF, and IL-1 $\beta$  were increased in the arthritic feet of CCR2<sup>-/-</sup> mice,  
376 whereas IL-10 was decreased, suggesting multiple mediators were involved in promoting  
377 neutrophil recruitment (1, 11, 12, 33, 39, 45, 47). The reduction in IL-10 and lack of Arg-1  
378 induction, also suggests a paucity of (CCR2<sup>+</sup> derived) M2 and resolving macrophages and  
379 their anti-inflammatory activities in CCR2<sup>-/-</sup> mice. This contention is supported by array data  
380 that shows less up-regulation of a number of M2 and resolving macrophage markers in  
381 CCR2<sup>-/-</sup> feet (eg TGF $\beta$ , Ym1, Msr1) (see Fig. S3 in the supplemental material). M2  
382 macrophages have high efferocytosis activity, with efferocytosis a key driver of inflammation  
383 resolution (2). The increase in ApoTag staining in CCR2<sup>-/-</sup> mice also suggests that  
384 efferocytosis is compromised in these mice, with CCL2 having previously been shown to  
385 promote efferocytosis (57). Evidence for secondary necrosis is also present in CCR2<sup>-/-</sup> feet  
386 (Fig. 2M), and usually occurs when efferocytosis is compromised. Secondary necrosis itself  
387 promotes inflammation by releasing a number of soluble inflammatory mediators (32).

388 The eosinophil infiltration that follows the neutrophil infiltration in CCR2<sup>-/-</sup> mice may be  
389 promoted by the tissue damage induced by the neutrophils and/or secondary necrosis, and  
390 may help down-regulate the inflammatory response in these animals (29). Eosinophilic  
391 arthritis has been reported previously, and is usually associated with a benign course of  
392 disease (60).

393 Exactly how CCR2<sup>+</sup> monocytes recruited during CHIKV infection, and/or the  
394 macrophages that develop from them (and/or other CCR2<sup>+</sup> cells), might prevent excessive  
395 neutrophil infiltration and pathology in WT mice remains unclear. IL-10 may be involved  
396 (1), but is unlikely to be the only player, with foot swelling and neutrophil infiltration only  
397 slightly elevated (the latter not significantly) in IL-10<sup>-/-</sup> mice (data not shown). Treatment of  
398 CCR2<sup>-/-</sup> mice with Anakinra (an IL-1 receptor antagonist) had no significant affect on peak  
399 foot swelling (data not shown). Anti-inflammatory macrophages might suppress neutrophil  
400 recruitment by *inter alia* secreting (i) a series of proresolving lipid mediators (49) and/or (ii)  
401 proteases that cleave neutrophil-attracting chemokines (36). The reduction in interferon  
402 signalling seen CCR2<sup>-/-</sup> mice may also be involved as loss of IFN $\alpha/\beta$  receptor signalling has  
403 been shown to promote production of neutrophil-recruiting cytokines/chemokines in a  
404 number of infections (6, 64). Treg cells may be involved as (i) depletion of CCR2<sup>+</sup> Tregs has  
405 been shown to exacerbate collagen-induced arthritis (5), (ii) anti-inflammatory macrophages  
406 can promote Treg expansion via TGF $\beta$  and IL-10 (28, 36), (iii) T cells are important in  
407 driving CHIKV arthritis (34), and (iv) Th cell pathways were decreased in CCR2<sup>-/-</sup> mice (Fig.  
408 6), with the microarray analysis unable to differentiate between Th and Treg cells. The  
409 increased IgG1 responses in CCR2<sup>-/-</sup> mice also supports the view that T cell activities were  
410 altered in CHIKV-infected CCR2<sup>-/-</sup> mice. Further work is needed before the mechanisms and  
411 cells involved and their relative importance are fully elucidated, with other cell types (eg  
412 myeloid-derived suppressor cells (36)) potentially also involved. What does emerge from

413 these studies is that in CHIKV arthritis (and perhaps other viral arthritides (56)),  
414 inflammation-recruited CCR2<sup>+</sup> monocyte/macrophages appear to be important for limiting  
415 neutrophil recruitment into the infected and inflamed joint tissues. This contrasts with  
416 observations in other arthritis models where these cells promote neutrophil recruitment (61).

417 Treatment of WT mice with clodronate (one day prior to CHIKV infection) to deplete  
418 monocyte/macrophages was previously shown to reduce foot swelling day 6/7 after CHIK  
419 infection (13); data that would seem at odds with the observations presented herein.  
420 However, the recent recognition that CD4 T cells and IFN $\gamma$  play important roles in driving  
421 arthritis in this CHIKV mouse model (34), and the ability of clodronate treatment to suppress  
422 Th1 responses (4), might suggest the observation above (13) has more to do with reduced  
423 Th1 responses than reduced monocyte/macrophage infiltration (9). (Th1 responses were not  
424 significantly affected in CHIKV infected CCR2<sup>-/-</sup> mice). Clodronate treatment of WT mice  
425 also extended the viraemia (13), whereas viraemia was not significantly different in CCR2<sup>-/-</sup>  
426 and WT mice. Clodronate treatment was recently shown to affect germinal centres (35), with  
427 compromised antibody responses perhaps explaining the extended viraemia (13).

428 The reduction in interferon signalling in CCR2<sup>-/-</sup> mice was surprising as IFN $\alpha/\beta$  receptor,  
429 STAT1, IRF3 and IRF7-associated signalling (but not IFN $\gamma$  (34)) are important for anti-viral  
430 activity against CHIKV (42), and viral titres and RNA levels were unchanged in CCR2<sup>-/-</sup>  
431 mice (Fig. 1C,D,E). Macrophages are, whereas neutrophils are generally not, infected with  
432 CHIKV, so viral RNA-mediated IRF3 and IRF7 stimulation via the cytoplasmic IPS-1  
433 pathway (42) would not occur in neutrophils. In contrast to monocytes/macrophages,  
434 neutrophil TLR3 expression is also low, both in humans (58) and mice  
435 (<http://www.immgen.org/databrowser/index.html>), with TLR3 signalling (upstream of IRF3  
436 and 7) known to be active during CHIKV infection (42). Conceivably, the reduced interferon  
437 signalling thus simply reflects fewer CCR2<sup>+</sup> monocytes/macrophages being infected and

438 responding to infection in CCR2<sup>-/-</sup> feet, with infection of these cells making a relatively minor  
439 contribution to overall viral load (Fig. 1C,D,E). One might speculate that resident tissue  
440 macrophages (whose recruitment is CCR2-independent) may be a more important target of  
441 CHIKV infection and/or persistence (22, 52).

442 The increase in IL-17 pathway activation in CHIKV infected CCR2<sup>-/-</sup> feet did not appear  
443 to be due to increased levels of Th17 cells (as was reported for in collagen-induced arthritis  
444 (40)). Recently, immune complexes present during acute arthritis were shown to induce IL-  
445 17 production by neutrophils (20). Similar levels of neutralising antibody responses were  
446 raised against CHIKV in WT and CCR2<sup>-/-</sup> mice (data not shown), so immune complexes are  
447 likely to be present in CCR2<sup>-/-</sup> mice and may thus be responsible for triggering IL-17  
448 production by the infiltrating neutrophils.

449 The widespread dysregulation of inflammatory processes observed in CHIKV infected  
450 CCR2<sup>-/-</sup> mice, suggests care might be warranted when considering therapeutic agents that  
451 target CCR2/CCL2 for treating alphaviral arthritides (44) (and perhaps other arthritides (12)).  
452 The association of (i) neutrophil-recruiting chemokines, IL-8 (homologue of murine CXCL1)  
453 and CCL3, both elevated in CCR2<sup>-/-</sup> mice (see Table S2 in the supplemental material), and  
454 (ii) IL-6, which was higher in the serum of CCR2<sup>-/-</sup> than WT mice post infection (data not  
455 shown), with chronic human CHIKV arthropathy (8), perhaps adds to these concerns.  
456 Complications might also arise when patients who are being treated with such agents (for  
457 other conditions) become infected with an arthritogenic alphavirus.

458

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469 **REFERENCES**

470

471

472 **FIGURE LEGENDS**

473

474 **FIG 1.** Disease, virus replication and RNA persistence. (A) The mean percentage increase  
475 in foot swelling over time for wild-type (WT) (n=16 feet, 8 mice) and  $CCR2^{-/-}$  mice (n=14  
476 feet, 7 mice). Asterisks (\*) indicates significant differences ( $p<0.01$ ) by t test or  
477 Kolmogorov-Smirnov test; the choice of test at each time point was dependent on the  
478 skewness, kurtosis and variance of the data being compared (see Statistics in Materials and  
479 Methods). (A single experiment, representative of 3 repeat experiments is shown). (B)  
480 Images of WT and  $CCR2^{-/-}$  feet on day 0 and day 6 (peak arthritis). (C) Peripheral blood  
481 viremia (n=7 for  $CCR2^{-/-}$  mice, n=8-14 for WT mice). (D) Virus titres in the indicated tissues  
482 at the indicated times post infection (n=3-6 mice per time point) (NT - not tested). (E) qRT-  
483 PCR analyses of CHIKV RNA in feet. Feet were taken at the indicated times (n=3-6) and the  
484 level of CHIKV positive-strand RNA determined using qRT-PCR analyses and primers  
485 specific for E1 (a major CHIKV structural protein); data was normalised to RPL13A mRNA  
486 levels.

487

488 **FIG 2.** Histology and immunohistochemistry of rheumatic lesions in CHIKV infected WT  
489 and  $CCR2^{-/-}$  mice. (A-G) WT mice. (H-N)  $CCR2^{-/-}$  mice. (A, H) H&E staining of muscle  
490 from uninfected mice. (B and I) H&E staining of muscle day 6 post infection. (C, J) high  
491 magnification of H&E staining of muscle day 6 post-infection. (D, K) F4/80 antibody  
492 (monocyte/macrophage) staining of connective tissue day 6 post-infection. (E, L) Leder  
493 staining (neutrophils) of connective tissue day 6 post-infection. (F, M) ApoTag staining  
494 (apoptotic cells) day 6 post-infection (F, two images, M, three images). (G, N)  
495 Chromotrope2R staining (eosinophils) of connective tissue day 21 post-infection.

496

497 **FIG 3.** Histology quantitation. (A) Image analysis (strong brown) of F4/80 staining of  
 498 whole foot sections from WT and CCR2<sup>-/-</sup> mice at the indicated times post-infection. Dotted  
 499 line indicates upper limit of non-specific staining in control slides. (n=3-5 feet from different  
 500 mice per time point and mouse strain, 2 sections per foot analysed). Statistics by  
 501 Kolmogorov-Smirnov test. (B) Image analysis (strong red) of Leder staining of whole foot  
 502 sections of WT and CCR2<sup>-/-</sup> mice at the indicated times post-infection. (n as above).  
 503 Statistics by Kolmogorov-Smirnov, Mann Whitney U or t tests. (C) Image analysis (strong  
 504 brown) of ApoTag staining of whole foot sections of WT and CCR2<sup>-/-</sup> mice at the indicated  
 505 times post-infection. (n as above). Statistics by Kolmogorov-Smirnov tests. (D) Image  
 506 analysis (strong red) of Chromotrope2R staining of whole foot sections of WT and CCR2<sup>-/-</sup>  
 507 mice at the indicated times post-infection. (n as above). Statistics by Kolmogorov-Smirnov  
 508 or Mann Whitney U tests. (E) WT mice were treated with the CCR2 antagonist MK0815 or  
 509 diluent (Control) after CHIKV infection and feet stained with anti-Ly6G (a neutrophil  
 510 specific marker), followed by image analysis. Statistics by Kolmogorov-Smirnov test (n=4/6  
 511 feet per group).

512

513 **FIG 4.** Cartilage damage in CCR2<sup>-/-</sup> feet. (A) H & E staining of joint from WT mouse day  
 514 45 post-infection (SS – synovial space). (B) H & E staining of joint from CCR2<sup>-/-</sup> mouse day  
 515 45 post-infection. Arrows indicate empty chondrocytic lacunae within the hyaline cartilage.  
 516 Arrowheads show cartilage damage at the articular surface. (C) Safranin O staining  
 517 (cartilage) of joint from WT mouse day 45 post-infection. (D) Safranin O staining (cartilage)  
 518 of joint from CCR2<sup>-/-</sup> mouse day 45 post-infection. Arrows show loss of red collagen  
 519 staining (replaced by blue staining) at the articular surfaces.

520

521 **FIG 5.** Immune and inflammatory mediator responses. (A) CHIKV-specific IgG2c and



522 IgG1 titres 7 weeks post-infection as determined by ELISA. Statistics by Mann Whitney U  
523 test (n=5 mice per group assayed in duplicate). (B) qRT-PCR analysis of T-bet (Th1 T cell  
524 transcription factor) and ROR $\gamma$ T (Th17 transcription factor) at the indicated times post-  
525 infection. Figure legend as in A. Statistics by Kolmogorov-Smirnov tests (n=4/5 mice and  
526 feet per group and time point assayed in duplicate). (C) qRT-PCR analysis of the indicated  
527 species at the indicated times post-infection. Figure legend as in A. Statistics by  
528 Kolmogorov-Smirnov or t tests (n=4/5 mice and feet per group and time point assayed in  
529 duplicate).

530

531 **FIG 6.** Microarray analysis of arthritic feet from WT and CCR2<sup>-/-</sup> mice. (A) Genes  
532 differentially expressed on day 7 post infection (compared to day 0) for WT and CCR2<sup>-/-</sup> feet  
533 were determined by microarray analysis. 2406 differentially expressed genes were up or  
534 down-regulated in WT and/or CCR2<sup>-/-</sup> feet. For 181 of these genes “fold change in CCR2<sup>-/-</sup>  
535 mice”/“fold change in WT mice” was >1.5 fold (top left); (for 603 DEGs this ratio was >1).  
536 For 920 of these genes the “fold change in WT mice”/“fold change in CCR2<sup>-/-</sup> mice” was  
537 >1.5 fold (bottom right); (for 1794 genes this ratio was >1, for 9 the ratio was =1). IPA  
538 canonical pathway analyses of the 181 genes more induced in CCR2<sup>-/-</sup> feet (top right) and the  
539 920 genes less induced in CCR2<sup>-/-</sup> feet (bottom left) are shown, with pathways grouped into  
540 themes. Pathway details are shown in Tables S3 and S4 in the supplemental material.  
541 (B) Comparison of gene signatures of chikungunya virus (CHIKV) arthritis in WT and  
542 CCR2<sup>-/-</sup> mice with a gene signature from RA patients. Gene Set Enrichment Analysis  
543 showed statistically significant enrichment of up-regulated genes (UP CCR2<sup>-/-</sup> 163 genes and  
544 UP WT 341 genes in “core enrichment”; overlap 150) and down-regulated genes (DOWN  
545 CCR2<sup>-/-</sup> 82 genes and DOWN WT 76 genes in “core enrichment”) from CHIKV arthritis in  
546 the up- and down-regulated genes, respectively, identified in synovial biopsies from RA

547 patients. FDR, false discovery rate.

548

549

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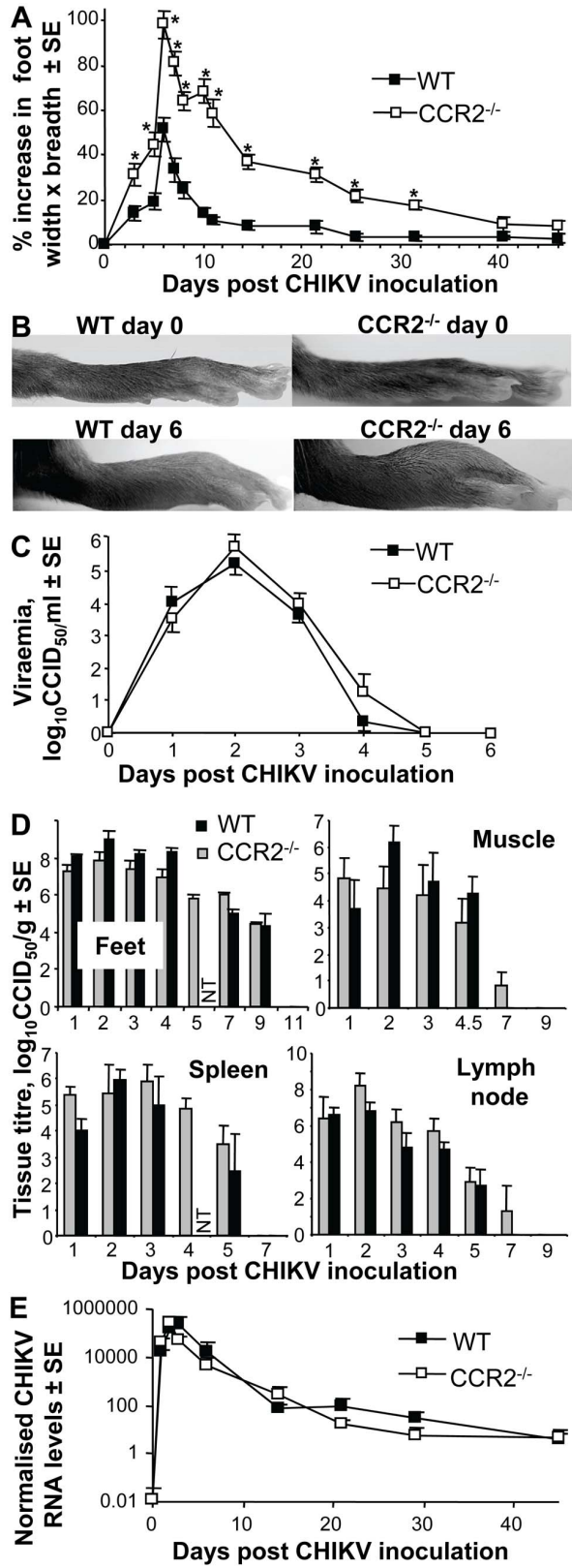
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Figure 1



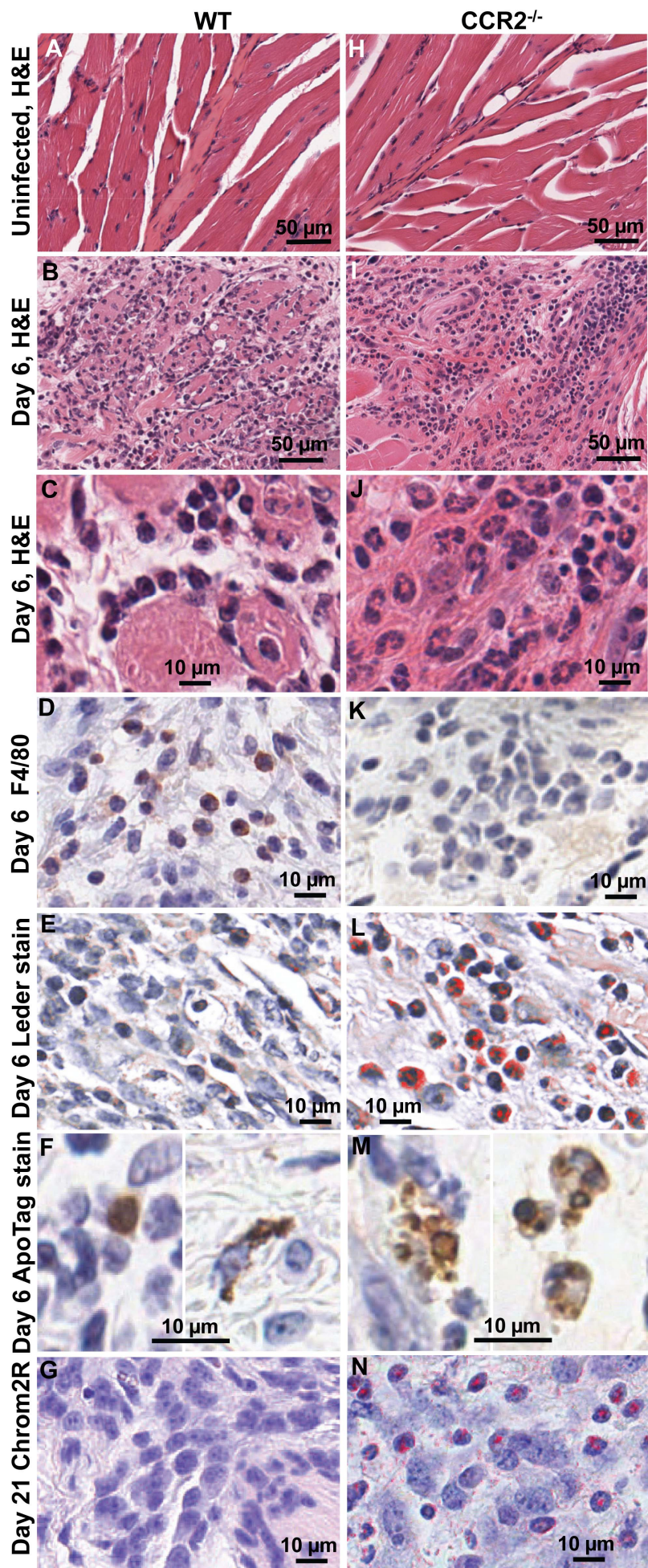


Figure 2



Figure 3

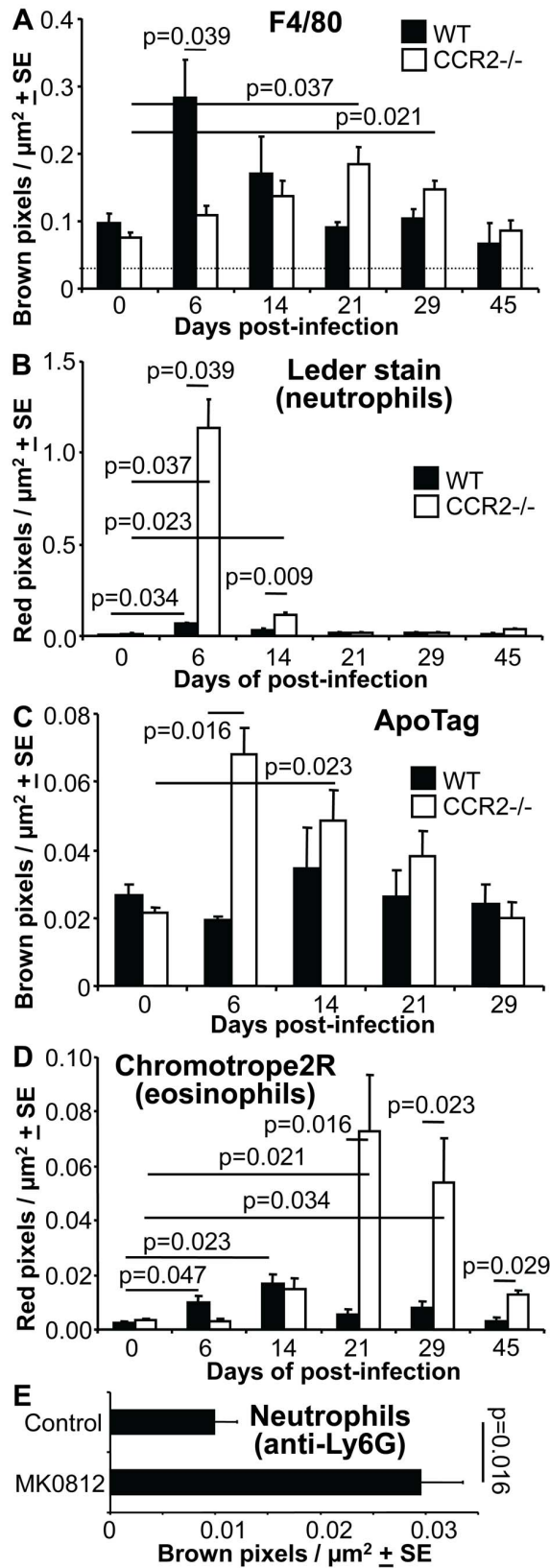


Figure 4

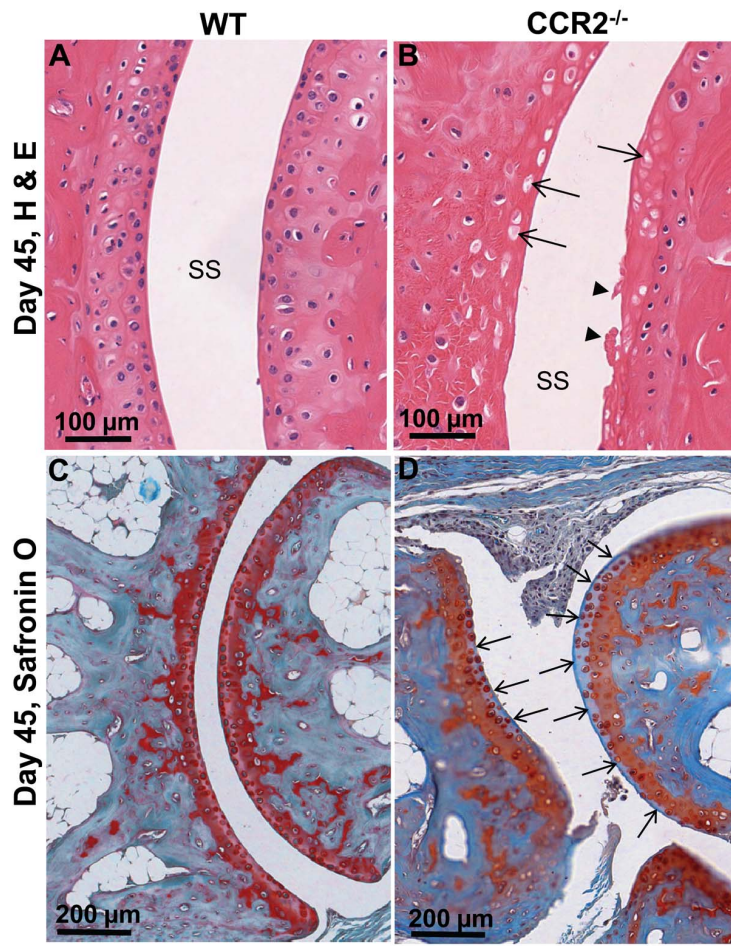


Figure 5

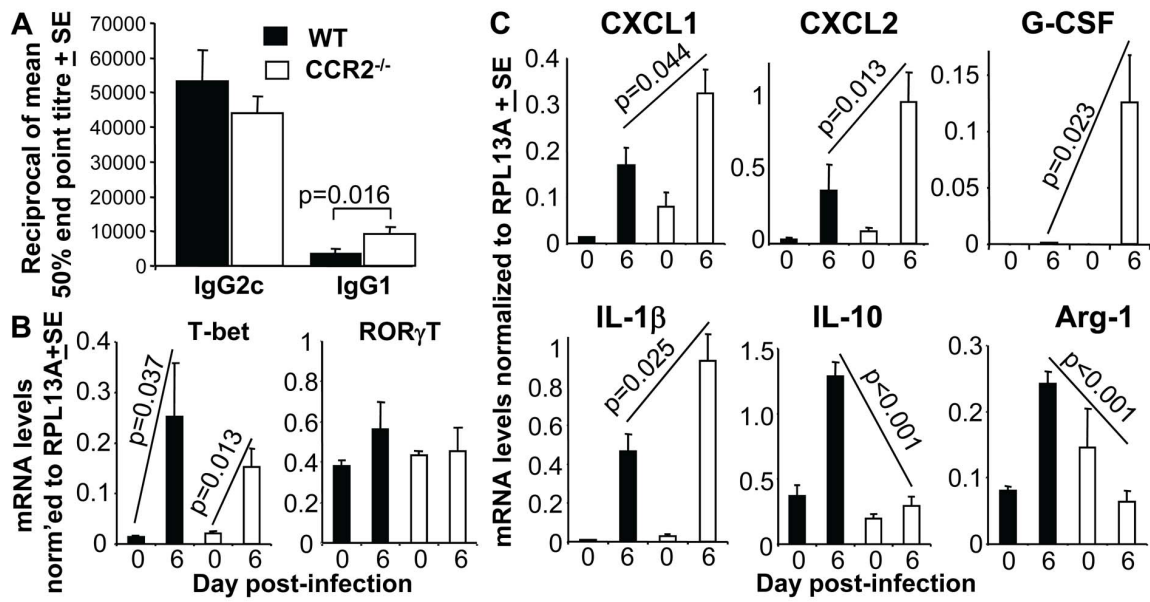


Figure 6

