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CCL2 recruitment of IL-6-producing CD11b$^+$ monocytes to the draining lymph nodes during the initiation of Th17-dependent B cell-mediated autoimmunity

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The development and function of Th17 cells are influenced in part by the cytokines TGF-β, IL-23 and IL-6, but the mechanisms that govern recruitment and activity of Th17 cells during initiation of autoimmunity remain poorly defined. We show here that the development of autoreactive Th17 cells in secondary lymphoid organs in experimental autoimmune myasthenia gravis – an animal model of human myasthenia gravis – is modulated by IL-6-producing CD11b$^+$ cells *via* the CC chemokine ligand 2 (CCL2). Notably, acetylcholine receptor (AChR)-reactive Th17 cells provide help for the B cells to produce anti-AChR antibodies, which are responsible for the impairment of the neuromuscular transmission that contributes to the clinical manifestations of autoimmunity, as indicated by a lack of disease induction in IL-17-deficient mice. Thus, Th17 cells can promote humoral autoimmunity *via* a novel mechanism that involves CCL2.

Key words: Autoantibodies · CCL2 · Myasthenia gravis · Th17

Supporting Information available online

Introduction

Helper CD4$^+$ T cells that are characterized by the production of IL-17 are commonly called Th17 cells [1–6]. Th17 cells have features that are distinct from those of both Th1 and Th2 lineages. For example, TGF-β and IL-6 are required for the generation of Th17 cells, and IL-23 supports the survival and expansion of these cells [1–8]. Recent data also suggest that IL-6 and TGF-β drive initial lineage commitment of Th17 cells, whereas IL-23 mediates the full acquisition of pathogenic function of Th17 cells [9]. Studies in several experimental models have indicated that IL-17 is a major mediator of tissue inflammation and autoimmune disease

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[4–6], but the cellular source of the cytokines and chemokines required for Th17 development and recruitment during initiation of autoimmunity have not been investigated as yet.

The CC chemokine ligand 2 (CCL2), initially known as monocyte chemoattractant protein 1 (MCP-1), is expressed by inflammatory cells at sites of tissue injury [10–13]. CCL2 directs the migration of monocytes, dendritic cells, NK cells and T cells, and coordinates inflammatory responses during infection [13]. Here we tested the possibility that CCL2-mediated homing of monocytes may affect Th17 cells in a mouse model of human myasthenia gravis (MG), the experimental autoimmune MG (EAMG) model. In MG and EAMG, the cardinal sign of weakness is mediated by circulating IgG autoantibodies to the acetylcholine receptor (AChR) or to other muscular antigens at the neuromuscular junction [14, 15].

We show here that CCL2 plays a critical role in coordinating cognate interactions among IL-6-producing $CD11b^+$ cells, autoreactive Th17 cells, and B cells. These interactions appear critical in the genesis of autoantibodies and in subsequent development of EAMG.

Results

Expression of CCL2 in EAMG

In C57BL/6 (B6) mice, EAMG presents as a protracted, progressive form of muscular weakness [16]. This presentation differs from the bi-phasic form of this disease characterized by early muscular inflammatory infiltration in Lewis rats [17]. Since the expression of CCL2 can be important for the pathogenesis of EAMG in the Lewis rat model [18], we measured CCL2 levels in EAMG B6 mice at the sites of injections as well as in secondary lymphoid organs (lymph nodes and spleen). By ELISA, CCL2 was hardly detectable in naïve mice (0.03 ± 0.02 pg/mg tissue). However, CCL2 expression was elevated in AChR/CFA-injected mice at the sites of immunization, in lymph nodes, and in spleens (Fig. 1).

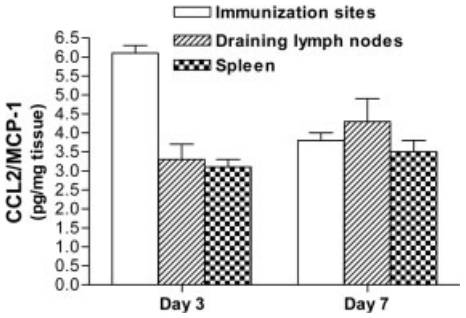

Figure 1. CCL2 expression in EAMG. C57BL/6 (B6) mice were injected with AChR in CFA and sacrificed 3 or 7 days later. Tissues from the injection sites as well as from spleens and lymph nodes were prepared as homogenates. The content of CCL2 in the supernatant was quantified by ELISA (n=4 mice/group. $^*p<0.05$).

CCL2-deficient mice developed mild muscular weakness upon induction of EAMG

To investigate directly whether CCL2 contributes to the development of EAMG, we immunized $CCL2^{-/-}$ mice and control B6 mice with AChR in CFA. Muscular weakness characteristic of MG was apparent in control mice around day 14 post immunization (p.i.), and became severe after booster immunizations on days 30 and 60, when the clinical score maximized at 2.5 ± 0.3. By contrast, no signs of muscular weakness were observed until day 25 p.i. in the $CCL2^{-/-}$ mice, and their disease remained mild (maximal clinical score of 0.6 ± 0.2, $p<0.01$) even after booster immunizations (Fig. 2A). Also, the $CCL2^{-/-}$ mice had less muscle AChR loss compared to control mice (Fig. 2B). Thus, $CCL2^{-/-}$ mice are developed milder behavioral and histological disease after the induction of EAMG.

B cell and autoantibody responses in $CCL2^{-/-}$ mice

In MG and EAMG, major immune effectors of the disease process at the neuromuscular junctions are circulating IgG autoantibodies reactive to AChR, whereas the cellular inflammatory infiltrates in the muscles are sparse and not considered essential for the impaired neuromuscular transmission, both in humans and mice [16, 19, 20]. Therefore, we measured anti-murine AChR IgG (and related isotypes) in $CCL2^{-/-}$ mice and control mice immunized

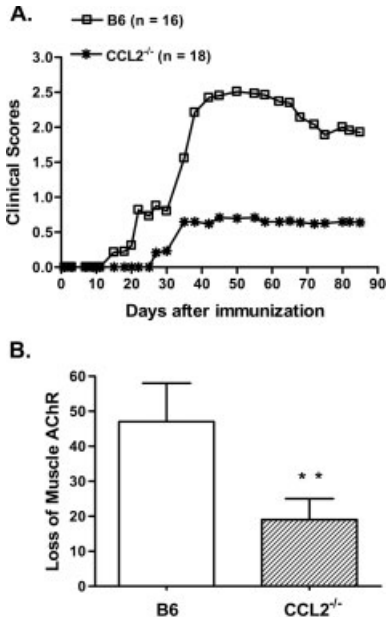

Figure 2. $CCL2^{-/-}$ mice are less susceptible to the induction of EAMG. B6 mice and $CCL2^{-/-}$ mice were immunized with AChR/CFA on days 0, 30 and 60 to induce EAMG. (A) Animals were then evaluated every 2–3 days for characteristic muscle weakness as described in *Materials and methods*. (B) Loss of muscle AChR content. The muscle tissues were removed from the mice in (A) at the termination of experiments and the percentage loss of muscle AChR was quantified. All results are expressed as mean values + SD. $^{**}p<0.01$.

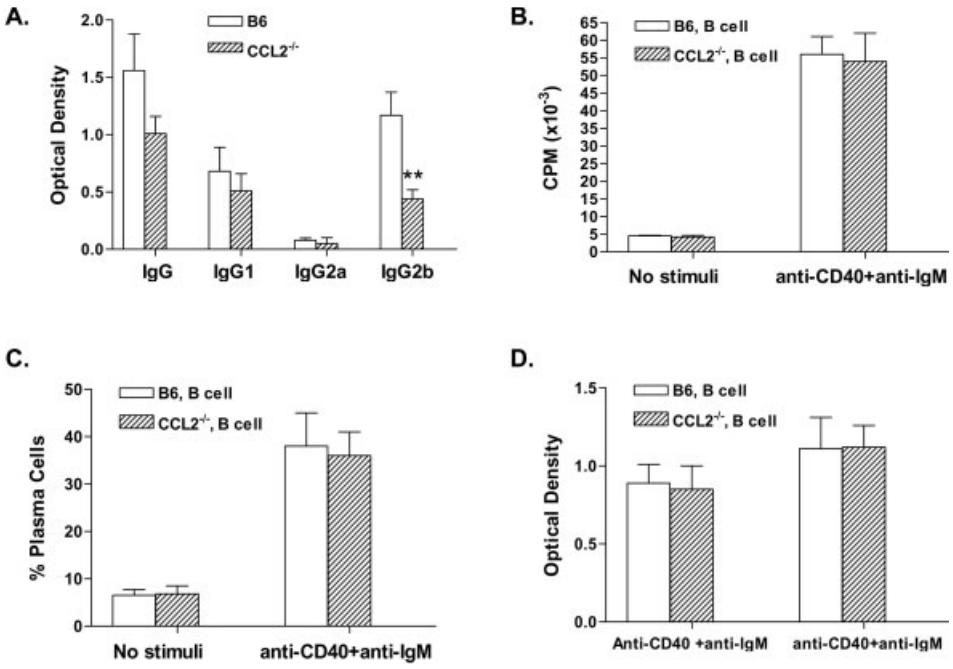

Figure 3. B cells are functionally intact in the CCL2$^{-/-}$ mice. CCL2$^{-/-}$ mice and control mice were immunized with AChR/CFA as described in *Materials and methods*. (A) Serum samples were collected from mice as decribed in Fig. 2 at the termination of the experiments. Anti-AChR IgG antibodies and IgG isotypes were determined by ELISA (n=16–18) on AChR-coated plates. Mice were sacrificed on day 14 p.i., and lymph nodes cells were cultured with or without B-cell stimuli. (B) Proliferation of B cells measured as [^{3}H] thymidine incorporation. (C) Plasma cell differentiation after polyclonal B cell stimulation measured by FACS. (D) Antibody production in the culture supernatants of plasma cells from spleen (left bars) and lymph nodes (right bars) (n=4/group). All results are expressed as mean values ± SD. $^{**}p<0.01$.

with AChR. Compared with controls, CCL2$^{-/-}$ mice had only a marginal reduction of total anti-AChR IgG levels ($p<0.06$) (Fig. 3A). Although the levels of anti-AChR IgG1 and IgG2a were similar in the two groups, the amounts of anti-AChR IgG2b were markedly lower in the CCL2$^{-/-}$ mice ($p<0.01$) (Fig. 3A). The same results were obtained for anti-torpedo AChR IgG in AChR-immunized control and CCL2$^{-/-}$ mice (control *vs*. CCL2$^{-/-}$ mice: IgG, 1.6 ± 0.5 *vs*. 0.7 ± 0.2, p=0.42; IgG2b, 1.2 ± 0.2 *vs*. 0.3 ± 0.1, p=0.008).

CCL2 exerts its function through its receptor CCR2 *in vivo* [11, 13]. CCR2 expression has been recently described on B cells and influences B cell functions [21, 22]. To determine whether the altered autoantibody response in CCL2$^{-/-}$ mice could be linked to CCR2 expression on B cells, we purified B cells from control mice and CCL2$^{-/-}$ mice for studies of proliferation and production of antibodies. As shown in Fig. 3B, B cells from control mice and from CCL2$^{-/-}$ mice proliferated at a similar extent after BCR and CD40 cross-linking. The number of plasma cells generated from control and CCL2$^{-/-}$ B cells after BCR stimuli were similar (Fig. 3C), and so were the levels of total IgG and IgG2b released from plasma cells (Fig. 3D). Thus, CCL2 deficiency does not impair the capacity of B cells to proliferate, differentiate into plasma cells, or release IgG antibodies. Although the intrinsic features of antibody responses are preserved in CCL2$^{-/-}$ mice, it is possible that CCL2$^{-/-}$ B cells may have an altered capacity in migration during the immune response, since B cells express CCR2. To examine this possibility, we quantified CD19$^+$ B cells in several peripheral lymphoid organs (spleen and lymph nodes) during the course of EAMG. There was no significant alteration in B cell percentage and numbers between control mice and CCL2$^{-/-}$ mice (Supporting Information Fig. 1). It was thus unlikely that the observed phenotype of EAMG in CCL2$^{-/-}$ mice was due to the defects in B cell migration.

Impaired Th17 responses in CCL2$^{-/-}$ mice

The altered levels of IgG autoantibodies in the AChR-primed CCL2$^{-/-}$ mice, associated with an intact B cell proliferation and differentiation, led us to investigate the phenotype of T cells in this model.

In the absence of CCL2, T cell proliferation was not significantly altered, as reflected by CFSE dilution experiments (Supporting Information Fig. 2). Nonetheless, the preferential reduction of the levels of anti-AChR IgG2b suggested altered Th cell function. CCL2 plays a role in the development of both Th2 and Th1 cells [11, 23]. In our model, IFN-γ production by CD4$^+$ T and CD8$^+$ T cells in response to AChR stimulation was marginally impaired in CCL2$^{-/-}$ mice (Fig. 4A and B; Supporting Information Fig. 3), and both CCL2$^{-/-}$ mice and controls produced similar amounts of IL-4 (Fig. 4D and E).

Since the cytokine IL-17 is an important mediator of tissue inflammation and Th17 cells are important in the pathogenesis of several autoimmune conditions [1–6], we tested the role of these

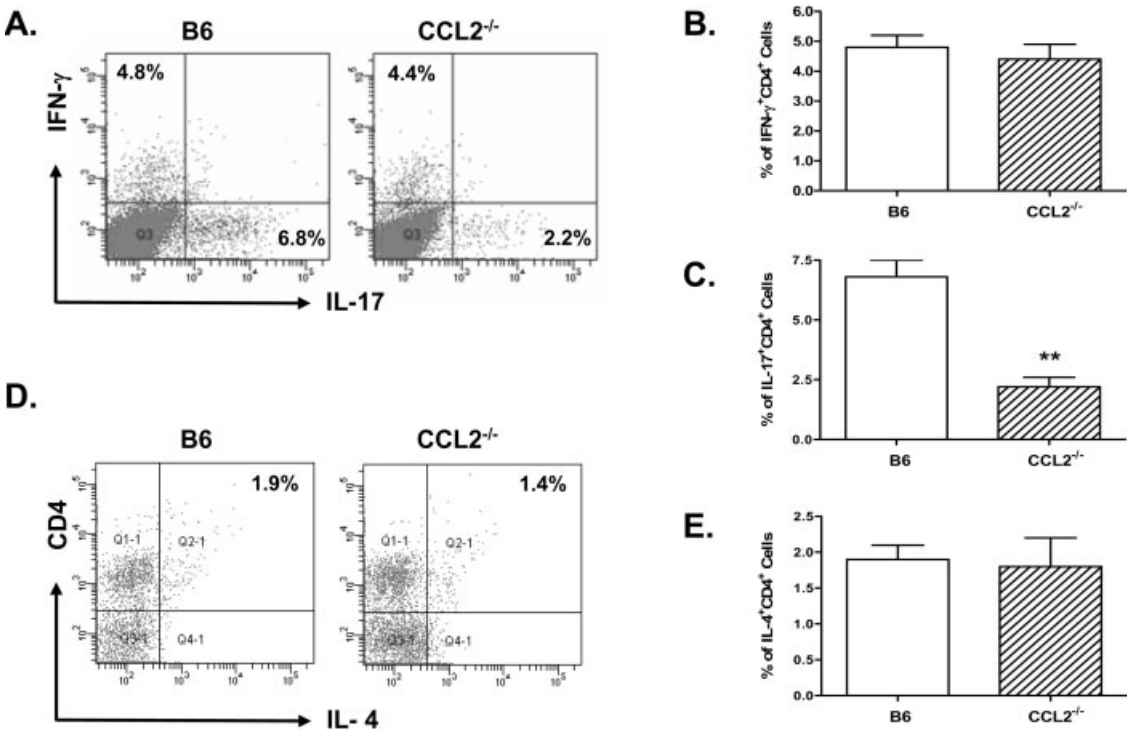

Figure 4. Th17 responses are impaired in the CCL2$^{-/-}$ mice. Splenocytes from B6 and CCL2$^{-/-}$ mice immunized with AChR/CFA were harvested at day 7–14 p.i. and stimulated with10 μg/mL AChR in culture medium for 3–5 days. For intracellular cytokine staining, cells were restimulated with PMA/ionomycin/brefeldin A for further 5 h, then IFN-γ-, IL-4-, and IL-17-producing cells were detected by flow cytometry as described in *Materials and methods*. Results were shown in representative plots from three separate experiments (n=4–6 mice/group) and bar graphs for summary data. (A–C) AChR-induced IFN-γ and IL-17 production by CD4$^+$ cells. (D, E) AChR-induced IL-4 production by CD4$^+$ cells. $^{**}p<0.01$.

cells in EAMG, because it is not known how and/or whether Th17 cells contribute to this disease.

We first compared Th17 cell responses to AChR between wild-type (B6) and CCL2$^{-/-}$ T cells. Interestingly, CCL2$^{-/-}$ T cells failed to mount an effective Th17 response to AChR (Fig. 4A and C). To determine whether CCL2$^{-/-}$ T cells have intrinsic defects in Th17 differentiation, we sorted CD4$^+$CD44$^+$ T cells from control mice and CCL2$^{-/-}$ mice and cultured them in the presence or absence of IL-6 and TGF-β for 48 h. Levels of IL-17 were similar for control T cells and CCL2$^{-/-}$ T cells (8834 ± 322 *vs*. 8923 ± 202 ng/mL), showing that CCL2$^{-/-}$ T cells do not have an intrinsic defect in Th17 differentiation. The failure in mounting Th17 cells in CCL2$^{-/-}$ mice must thus derive from other factors.

CCL2 modulates the homing of CD11b$^+$ cells to lymphoid organs

Phenotypic commitment of Th17 cells is dependent on IL-6 [1–6]. To study the mechanism underlying the impaired generation of Th17 cells in AChR-immunized CCL2$^{-/-}$ mice, we quantified IL-6 in several cell types of EAMG mice. As shown in Fig. 5A, CD11b$^+$ cells and, to a lesser degree, CD11c$^+$ cells were the predominant sources of IL-6. The production of IL-6 in the supernatants from cultured spleen and lymph node cells of CCL-2$^{-/-}$ mice was significantly lower than that of controls (Fig. 5B).

Since CCL2$^{-/-}$ mice had low levels of IL-6, a cytokine mainly produced by CD11b$^+$ cells, we sought to address whether CCL2 deficiency altered the homing of CD11b$^+$ cells to lymph nodes or spleens, *i.e*., causing a secondary reduction of IL-6 production. We found no significant difference in the numbers and percentages of CD4$^+$, CD8$^+$, NK1.1$^+$CD3$^-$ (NK cells) or NK1.1$^+$CD3$^+$ (NKT) cells in lymph nodes of control mice and CCL2$^{-/-}$ mice (Supporting Information Fig. 4). However, the numbers of CD11b$^+$ and CD11b$^+$CD11c$^+$ cell populations were reduced in spleens (Fig. 5C) and the lymph nodes of CCL2$^{-/-}$ mice (Supporting Information Fig. 5). These data suggested that CCL2 deficiency may interfere with homing of CD11b$^+$ cells and CD11b$^+$CD11c$^+$ cells to peripheral lymphoid organs, which in turn reduces IL-6 availability for development of Th17 cells.

IL-6$^{-/-}$ mice fail to mount AChR-induced Th17 responses

In a previous study, IL-6$^{-/-}$ mice were found resistant to the induction of EAMG [24]. Our current data taken in the content of recent findings [9] suggest that the resistance to EAMG could derive from faulty development of Th17 cells. Since this could be secondary to IL-6 deficiency, we measured autoantigen-induced Th17 responses in IL-6$^{-/-}$ mice immunized with AChR/CFA. Consistent with previous reports [24], IL-6$^{-/-}$ mice were resistant

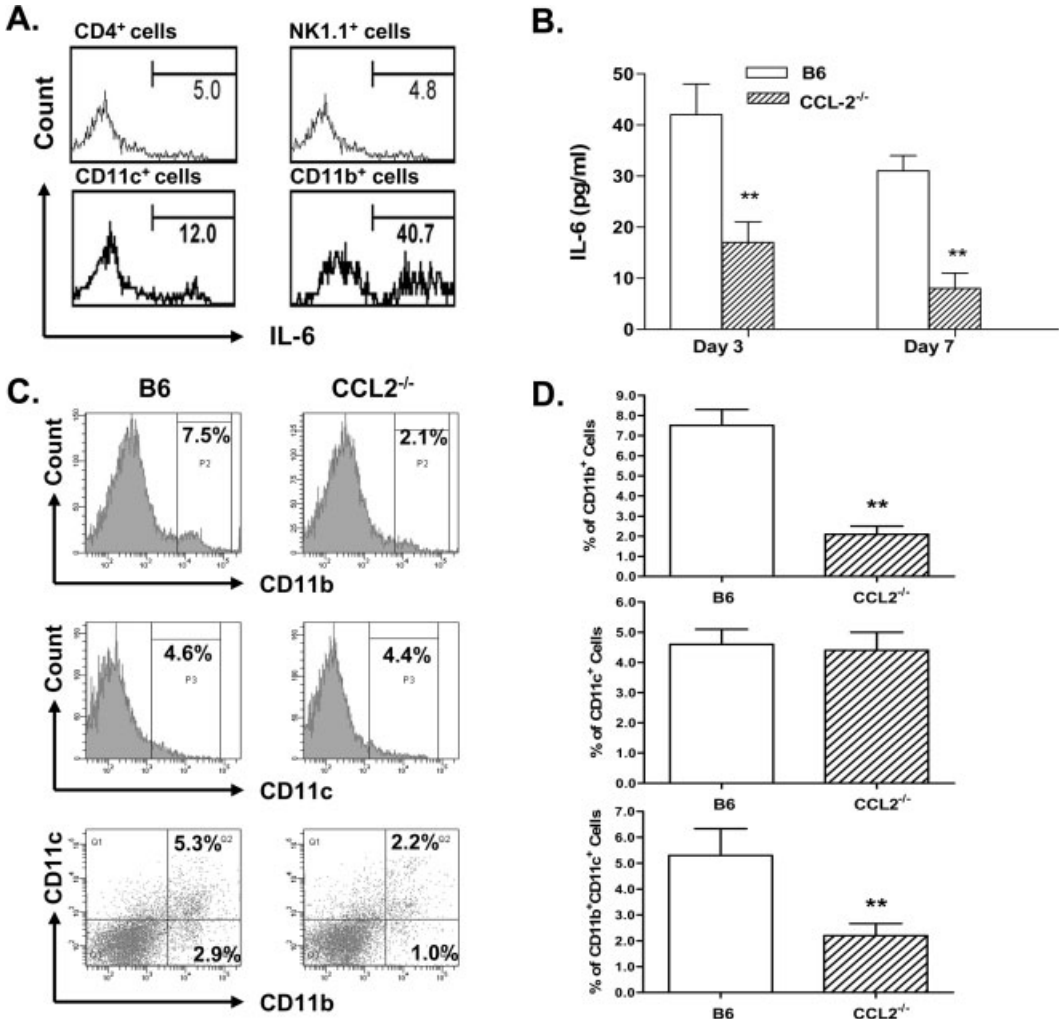

Figure 5. CCL2 controls homing of IL-6-producing CD11b$^+$ cells. B6 and CCL2$^{-/-}$ mice were immunized with AChR/CFA to induce EAMG and sacrificed on day 7–14 p.i. (A) Production of IL-6 by CD4$^+$ cells, NK1.1$^+$ cells, CD11c$^+$ cells and CD11b$^+$ cells from B6 mice was quantified by intracellular IL-6 staining. (B) IL-6 released from draining lymph nodes was measured in B6 mice as well as CCL-2$^{-/-}$ mice by ELISA (n=4–7 each/group). (C, D) Single-cell suspensions of spleen were stained with PE-Cy7-labeled anti-mouse CD11b and allophycocyanin-labeled anti-mouse CD11c before analysis by FACSAria using Diva software. Results represent three independent experiments with reproducible outcomes. $^{**}p<0.01$.

to the induction of EAMG. Importantly, IL-6$^{-/-}$ mice had impaired capacity to mount AChR-induced production of IL-17 in CD4$^+$ cells, while IFN-γ production was not altered significantly (Fig. 6). These results indicate a role of IL-6 in the development of Th17 cells and not Th1 responses in EAMG.

IL-17 facilitates autoantibody responses and clinical EAMG independently of IFN-γ

Next, we injected recombinant IL-17 into AChR-primed mice during the T cell priming period, and then compared the outcome to that of mice given only PBS injections. Significantly, recipients of IL-17 developed severe EAMG and elevated titers of AChR-specific IgG and IgG2b, as compared to controls (Fig. 7A and B).

We and others [25–27] have previously found that the development of EAMG is not dramatically altered by IFN-γ deficiency. To investigate whether the effects of IL-17 on EAMG were related to or depended on IFN-γ, we treated IFN-γ$^{-/-}$ mice with IL-17 during EAMG induction, using the same regimen used for the B6 mice. We found that IFN-γ$^{-/-}$ mice and controls responded with elevated anti-AChR antibody production and clinical EAMG at similar magnitudes (Fig. 7C and D). These data indicate that IL-17 rather than IFN-γ influences humoral autoimmune responses in EAMG.

IL-17 restores EAMG severity of CCL2$^{-/-}$ mice to that of wild-type mice

If the failure to mount autoreactive Th17 responses contributes to milder EAMG in the CCL2$^{-/-}$ mice, provision of exogenous IL-17 to CCL2$^{-/-}$ mice should reverse this phenotype. To test this hypothesis, we treated CCL2$^{-/-}$ mice with IL-17 or PBS at the

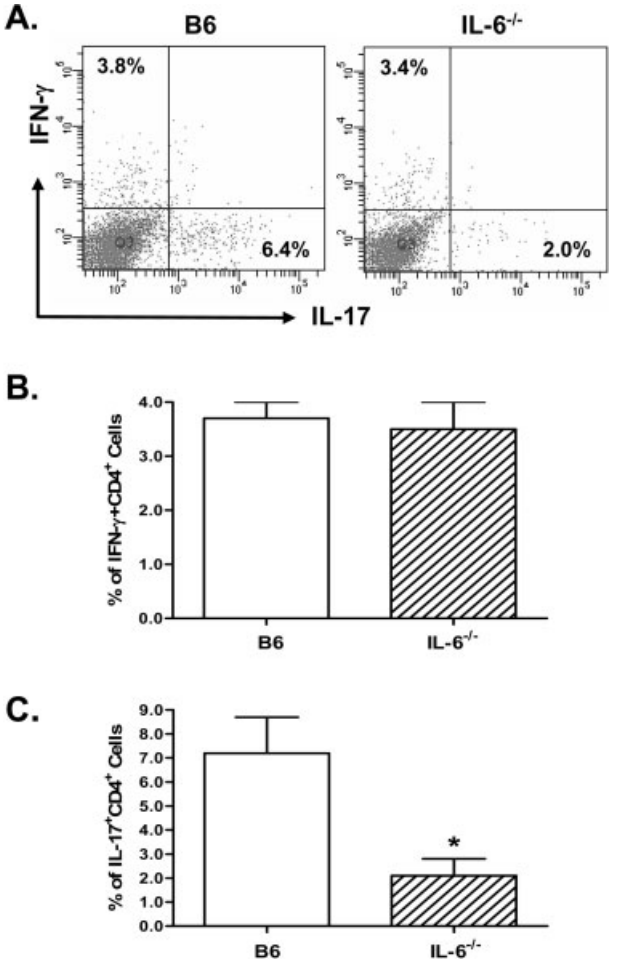

Figure 6. AChR-primed IL-6$^{-/-}$ mice fail to mount Th17 responses. B6 and IL-6$^{-/-}$ were immunized with AChR/CFA as described in Fig. 1. Mice were sacrificed on day 7–14 p.i. Production of IL-17 and IFN-γ by cultured spleen cells in response to AChR stimulation (10 μg/mL) was measured by intracellular cytokine staining. (A) Expression of CD4 *vs.* intracellular IL-17 (upper panel) and IFN-γ (lower panel) was compared within this gate. Representative plots are from one of three experiments using 2–3 mice/group. (B, C). CD4$^+$ cells expressing IFN-γ or IL-17 in control mice and IL-6$^{-/-}$ mice (n=4–6/group). *$p<0.05$.

time of induction of EAMG. Compared with PBS-treated CCL2$^{-/-}$ mice that exhibited milder EAMG, AChR-primed CCL2$^{-/-}$ mice treated with IL-17 exhibited early onset of muscular weakness (median day of onset: 13 ± 2 *vs.* 27 ± 4) that progressed to severe weakness over time (maximum severity 2.6 ± 0.3 *vs.* 1.5 ± 0.2, Fig. 7E). Treatment with IL-17 elicited higher anti-AChR IgG and prominent IgG2b responses (Fig. 7F). These data indicate that defective Th17 responses contribute to the reduced severity of muscular weakness in the CCL2$^{-/-}$ mice.

Autoreactive Th17 cells drive IgG2b antibody response *in vivo*

Transfer of myasthenogenic lymphocytes from humans with MG or from EAMG mice results in clinical EAMG [28, 29]. We isolated CD4$^+$ cells from control mice and CCL2$^{-/-}$ for transfer into RAG1$^{-/-}$ deficient mice (which are devoid of intrinsic T and B cells) [30]. The transferred CD4$^+$ cells preferentially produced Th17 in control mice but not in CCL2$^{-/-}$ mice. Naive B220$^+$ B cells were co-transferred into the RAG1$^{-/-}$ recipient mice. At 2 weeks following immunization with AChR/CFA, production of anti-AChR IgG and IgG isotypes were evaluated. Mice receiving T cells from CCL2$^{-/-}$ mice had marginally reduced levels of total anti-AChR IgG and significantly reduced levels of IgG2b (Table 1), compared to mice receiving T cells from control EAMG mice. Mice receiving T cells from CCL2$^{-/-}$ mice scarcely developed behavioral signs of EAMG (Table 1). In control experiments, transferred control CD4$^+$ cells maintained the Th17 phenotype in the recipient mice (percentages of IL-17$^+$CD4$^+$ cells: 7.2 ± 1.6% (control) *vs.* 1.3 ± 0.2% (CCL2$^{-/-}$), $p<0.01$). Collectively, these data suggest that autoreactive Th17 cells help B cells to produce pathogenic antibodies, particularly the IgG2b subtype.

IL-17-deficient mice develop mild EAMG

To further elucidate the role of IL-17/Th17 in EAMG, we immunized control mice and IL-17$^{-/-}$ mice with AChR and CFA and monitored the development of EAMG. Compared to control mice, IL-17$^{-/-}$ mice exhibited significantly milder EAMG, as reflected by evaluation of muscular weakness (Table 2). Production of IgG2b was impaired in the IL-17$^{-/-}$ mice (Table 2).

Table 1. CCL2$^{-/-}$ CD4$^+$ T cells are poor helpers for B cells[a)]

Source of T cells	No. of T cells	No. of mice/ group	Disease incidence (%)	Loss of muscle AChR	Mean maximal severity of EAMG (± SD)	Anti-AChR IgG[b)] (OD_{405})	Anti-AChR IgG2b[b)] (OD_{405})
B6	5×10^6	6	(6/6) 100	53.3 ± 12.7%	1.87 ± 0.22	1.84 ± 0.23	1.21 ± 0.20
CCL2$^{-/-}$	5×10^6	7	(1/7) 14.3	18.4 ± 9.6%	0.13 ± 0.07*	1.03 ± 0.30	0.31 ± 0.06*

[a)] CD4$^+$ cells were enriched and purified from AChR and CFA primed control mice and CCL2$^{-/-}$ mice and were co-transferred with 5×10^6 B220$^+$ B cells into RAG1$^{-/-}$ recipient mice.

[b)] Muscle weakness was monitored for 55 days following cell transfer, sera were collected at the termination of experiments for anti-AChR antibody ELISA. Loss of muscle AChR was quantified by RIA.

*$p<0.05$.

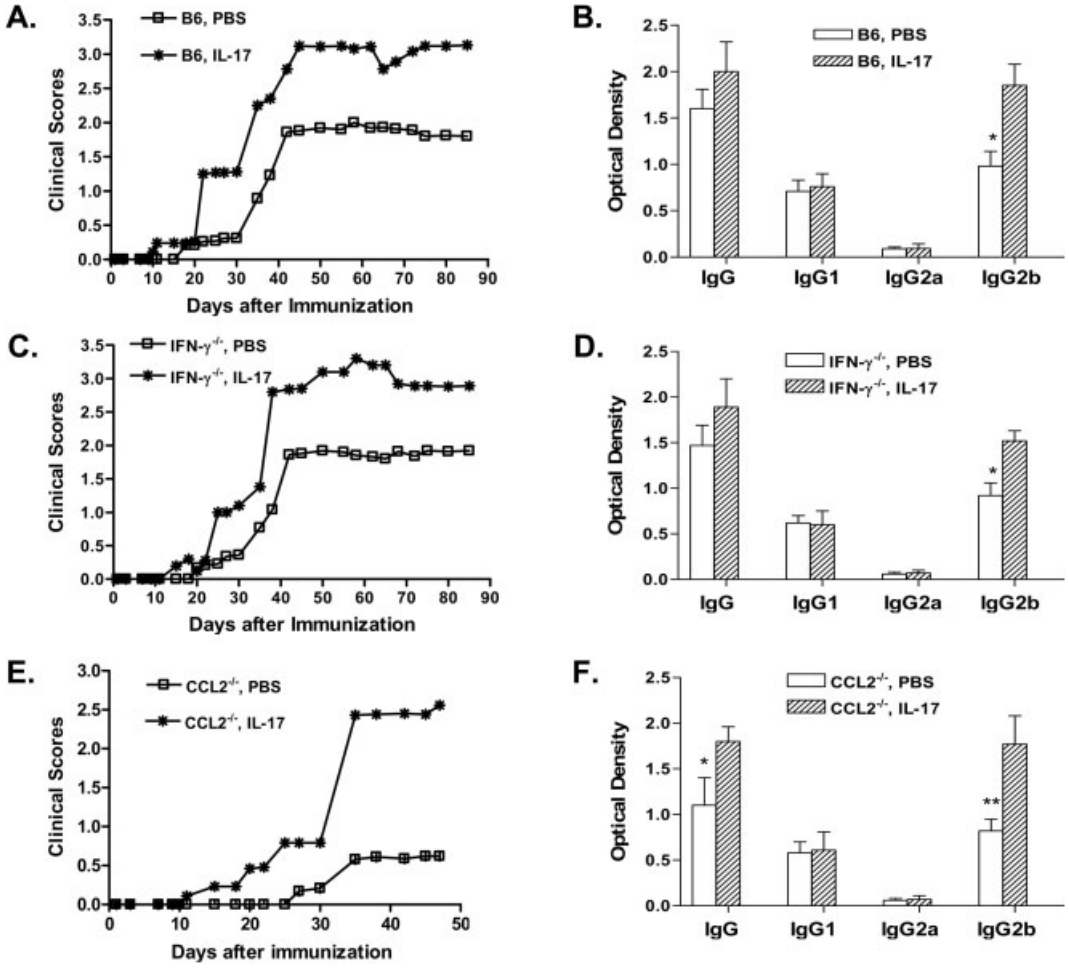

Figure 7. Role of IL-17 in EAMG. Groups of B6 (A, B), IFN-$\gamma^{-/-}$ (C, D) and CCL-$2^{-/-}$ (E, F) mice were immunized with AChR/CFA and received IL-17 or PBS starting at the date of immunization (see *Material and methods*). Determination of development of EAMG (A, C, E) and production of anti-AChR antibodies (B, D, F) (n=8/group). $^{*}p<0.05$, $^{**}p<0.01$.

Discussion

This study implicates Th17 responses in B cell-dependent (auto)immunity, and establishes a novel role for CCL2 in the development of Th17 responses. Increasing evidence supports the role of Th17 cells in several models of inflammation and autoimmunity. Here we show that the chemokine CCL2, which is elevated during inflammation, is required for the recruitment of IL-6-producing $CD11b^{+}$ cells involved in the generation of autoreactive Th17 cells in EAMG. Our model implies that in the lymph node $CD11b^{+}$ APC can produce IL-6 to polarize Th cells toward a Th17 phenotype, and then the Th17 cells can provide help to B cells for the production of pathogenic IgG2b antibodies mediating functional blockade at the neuromuscular junction.

It has long been known that autoimmune diseases such as EAMG are associated with elevated production of proinflamma-

Table 2. IL-$17^{-/-}$ mice exhibit mild EAMG with reduced incidence[a)]

Mice	No. of mice/ group	Disease incidence (%)	Loss of muscle AChR	Mean maximal severity of EAMG (± SD)	Anti-AChR IgG (OD_{405})	Anti-AChR IgG2b (OD_{405})
B6	8	(7/8) 88%	47.6 ± 9.2%	1.64 ± 0.33	1.91 ± 0.34	1.35 ± 0.41
IL-$17^{-/-}$	4	(0/4) 0	10.0 ± 2.3%	0.09 ± 0.05**	0.94 ± 0.20	0.22 ± 0.03**

[a)] Mice were immunized with AChR and CFA. Muscle weakness was monitored for 35 days following the immunization, and sera were collected at this time for anti-AChR antibody ELISA. Loss of muscle AChR was quantified by RIA.
$^{**}p<0.01$.

tory mediators by several cell types. However, the understanding of the intermediate events between immunogenic exposure to autoantigen and subsequent downstream activation of pathogenic immune response has been limited.

We found that CCL2-deficient mice developed milder behavioral and histological disease following the induction of EAMG. This result was surprising because monocyte infiltrates, the hallmark of CCL2 action, are not prominent in EAMG muscle [24]. This defect was not caused by impaired function of autoreactive B cells, which remained intrinsically intact in the absence of CCL2. However, their capacity to produce anti-AChR IgG antibody, in particular IgG2b, was drastically reduced following immunization. Incidentally, IgG2b is highly myasthenogenic [31].

The involvement of CCL2 in several experimental models of inflammation and autoimmunity is well established, and appears to partly depend on the ability of this chemokine to direct the inflammatory influx to target organs and to promote Th1 and/or Th2 cells [10–13]. The autoimmune model of EAMG differs from the other systems studied for at least two reasons. First, the muscular weakness characteristic of EAMG is primarily mediated by anti-AChR IgG antibodies produced by autoreactive B cells [19]. Second, because the inflammatory infiltrates are sparse and unrelated to the neuromuscular junctions in EAMG, their contribution to muscular weakness, if any, is likely minute [19]. As such, the B cell-mediated autoimmune process of EAMG, associated with limited inflammation, may represent a system with unique characteristics for studying the immune role of CCL2 independently of inflammation in tissue.

We report that the regulation of the immune response to AChR by CCL2 is sufficient to alter the magnitude of the symptoms of EAMG, yet CCL2 does not directly affect the function of autoreactive B cells in EAMG, as indicated by the fact that upon BCR-cross-linking, $CCL2^{-/-}$ B cells proliferated efficiently, differentiated into plasma cells, and released antibodies at a level comparable with controls. Rather, an altered function of Th17 cells was most likely responsible for the observed impaired autoantibody response.

CCL2 has been reported to affect both Th1 and Th2 cells [11, 23]. We add to this list Th17 cells. We think that since we used B6 mice, which are a Th1-prone strain (and immunization with CFA further drove Th1 responses), CCL2 might have had limited effects on Th2 cells in this system. On the other hand, the production of autoantibodies to AChR is clearly a T cell-driven process, which must rely in part on Th1 responses, given our finding of preferential IgG2b autoantibodies [32]. Although earlier work had indicated that ectopic expression of IFN-γ at the neuromuscular junction provoked MG-like disease [33], our current understanding of the role of IFN-γ in EAMG is far from conclusive. For example, mice deficient of IFN-γ [25] or IFN-γ receptor [26] are resistant to EAMG, yet some groups have shown that IFN-γ deficiency may not significantly alter the course of disease [27, 34]. Thus, while Th1 responses may possibly play a role in the disease depending on the experimental system used, the question arises on whether other inflammatory cytokine(s) with potent pro-pathogenic effects, such as IL-17, are required for the expression of EAMG. We found that AChR-primed $CCL2^{-/-}$ mice failed to mount an effective Th17 response, which correlated with an impaired anti-AChR IgG2b response associated with milder disease. The failure to mount productive Th17 responsiveness most likely came from impaired homing of the IL-6-producing $CD11^{+}$ cells to the secondary lymphoid organs, as $CCL2^{-/-}$ T cells maintained the capacity to produce IL-17 after stimulation. Also, AChR-primed $IL\text{-}6^{-/-}$ mice had significantly reduced autoantigen-reactive Th17 responses, and IL-17 injection enhanced both the humoral response to AChR and clinical EAMG in B6 mice as well as in the IFN-$\gamma^{-/-}$ mice. Finally, $CCL2^{-/-}$ $CD4^{+}$ T cells failed to help B cells to produce disease inducing IgG2b.

It has been reported that $IL\text{-}6^{-/-}$ mice had impaired anti-AChR IgG2b response upon immunization with AChR [24]. The question arises as whether the impaired IgG2b response is due to the lack of IL-6, or altered IL-17 response in $IL\text{-}6^{-/-}$ mice, or both. Injection of IL-17 can partially restore the capacity to mount an IgG2b response in $IL\text{-}6^{-/-}$ mice and reestablish susceptibility to the induction of EAMG in these mice. These results emphasized the importance of IL-17 in the generation of IgG2b response and the expression of clinical EAMG.

Th17 cells express CCR2 [35]. It is plausible that abnormal migration of Th17 cells in the $CCL2^{-/-}$ mice also contributes to the mild muscular weakness in this strain. This possibility is currently under investigation.

We have not examined the development of EAMG in $CCR2^{-/-}$ mice. Several published works have shown that $CCR2^{-/-}$ mice are resistant to experimental autoimmune diseases, including the B cell-dominant lupus model [36, 37]. These observations support our current findings and emphasize the importance of CCR2-CCL2 pathway in the development of autoimmunity.

The present study focused on the role of CCL2 in recruiting IL-6-producing cells capable of promoting generation of autoreactive Th17 responses in B cell-dependent (auto)immunity. We found, further, that Th17 plays a central role in humoral immunity in EAMG. We propose that inflammation associated with immunization recruits $CD11b^{+}$ mononuclear phagocytes to lymph nodes, a phenomenon that depends on CCL2. In the lymph nodes of wild-type mice, APC present the autoantigen to Th0 cells and make IL-6, in conjunction with other cytokines including TGF-β and IL-23, which polarizes Th cells toward a Th17 phenotype. The Th17 cells are then required as helpers for the production of high titers of pathogenic anti-AChR IgG2b antibodies from B cells. In the light of new finding by Hsu *et al.* [38], IL-17 may also promote the development of germinal centers. Antibodies to AChR are ultimately responsible for the block at the neuromuscular junction that leads to muscular weakness. It will be important to address next whether CCL2 may be a specific therapeutic target to modulate clinical MG.

Materials and methods

Mice

C57BL/6 (B6) mice deficient of MCP-1 (MCP-1$^{-/-}$) [11], IL-6 (IL-6$^{-/-}$), IFN (IFN-$\gamma^{-/-}$) and RAG (RAG-$\gamma^{-/-}$) [30] were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-17$^{-/-}$ mice were provided by Dr. Yoichiro Iwakura [5]. All mutant mice had been backcrossed to those of the B6 background for at least ten generations. Only female mice of 7–8 weeks of age (at the initiation of the experiments) were used. All mice were bred and maintained in pathogen-free conditions at the animal facilities of the Barrow Neurological Institute in accordance with the Institutional Animal Care and Use Committee regulations.

Antigens and peptides

AChR was purified from the electric organs of *Torpedo californica* (Pacific Biomarine, Venice, CA) by affinity chromatography on a α-cobratoxin-agarose resin (Sigma, St. Louis, MO) [39, 40]. The isolated product was pure as judged by SDS-PAGE. The AChR$\alpha_{146–162}$ (LGIWTYDGTKVSISPES) peptide was synthesized by Biosynthesis Inc. (Lewisville, TX).

Induction, clinical and laboratory evaluation of EAMG

Mice were injected subcutaneously (s.c.) between the shoulders and back with 20 μg AChR in CFA in a total volume of 100 μL. After 1 month, all mice were boosted once with 20 μg AChR in IFA s.c. at four sites on shoulders and thighs [38, 39]. The mice were observed every other day in a blinded fashion for signs of muscle weakness characteristic of EAMG. Clinical manifestations of EAMG were graded from 0 to 3 [39]: 0, no definite muscle weakness; 1+, normal strength at rest but weak with chin on the floor and inability to raise the head after exercise consisting of 20 consecutive paw grips; 2+, as grade 1+ and weakness at rest; and 3+, moribund, dehydrated, paralyzed. To verify the myasthenic nature of the weakness, we injected i.p. edrophonium chloride (Reversol; Organon, West Orange, NJ), a cholinesterase inhibitor that immediately increases the strength of mice that have clinical EAMG. To further verify the extent of EAMG, we quantified the muscle AChR content by radioimmunoassay using ^{125}I-labeled α-bungarotoxin, (Amersham Corp., Arlington Heights, IL) [27].

IL-17 injection

Recombinant murine IL-17 was purchased from eBioscience (San Diego, CA). At EAMG induction, mice received daily 1 μg IL-17 (in PBS)/mouse i.p. for 7 consecutive days. Control mice received equal volumes of PBS.

Measurement of anti-AChR IgG antibodies

Anti-murine AChR IgG antibodies and anti-torpedo AChR IgG antibodies were detected by ELISA as described previously [39, 40]. Briefly, microtiter plates (Corning Glass Works, Corning, NY) were coated with 100 μL/well murine AChR (0.5 μg/mL) or torpedo AChR (2 μg/mL) at 4°C overnight. After blocking with 10% FBS, serum samples were added and incubated for 2 h at room temperature. Plates were then incubated for 2 h with biotinylated rabbit anti-mouse IgG, IgG1, IgG2a, and IgG2b (Invitrogen, Carlsbad, CA), followed by alkaline phosphatase-conjugated ABC reagent (Dakopatts; R&D systems, Minneapolis, MN), and color developed with *p*-nitrophenyl phosphate. Results were expressed as OD at 405 nm.

CCL2 ELISA

The s.c. injection sites and draining lymph nodes were removed from sacrificed control mice and CCL2$^{-/-}$ mice, weighed, homogenized in lysis buffer (PBS with 1 mM PMSF, 0.01 mg/mL aprotinin, and 0.01 mg/mL leupeptin), sonicated, and centrifuged. The supernatant was assayed for CCL2 immunoreactivity by ELISA (Quantikine; R&D systems, Minneapolis, MN) following the manufacturer's instructions [12].

B cell proliferation and differentiation

To determine the extent of B cell proliferation, 10^5 purified CD20$^+$CD27$^+$ B cells were cultured in 96-well round-bottom plates and stimulated with 24 μg/mL goat-anti-mouse IgM alone or in combination with 5 μg/mL anti-mouse CD40 [41]. After 3–5 days of culture, [^{3}H]thymidine was added to the culture for additional 18 h. [^{3}H]Thymidine uptake was measured using a liquid scintillation counter. In some experiments, stimulated and control cultures were stained after various intervals with anti-CD19 PerCP-Cy5.5 (1D3) and allophycocyanin-conjugated anti-CD138 (281–2) to visualize plasma cells, which were identified as CD19$^{low/-}$CD138$^+$ cells [42].

Plasma cell differentiation in AChR-primed control mice and CCL2$^{-/-}$ mice

Purified B cells from lymph nodes were either left unstimulated or stimulated with a combination of anti-CD40 and IgM. After 6 days of culture, B cells were identified as CD19$^+$ and plasma cells as CD19$^{low/-}$CD138$^+$.

FACS analysis

For cytokine staining [43], single-cell suspensions ($\sim 30 \times 10^6$ cells) were prepared and labeled with 0.5 μM CFSE at 37°C for 10 min. Cells with or without CFSE were incubated at 37°C for 4 days in round-bottom plates (2×10^6 cells/well) with or without antigens (10 μg/mL AChR, $AChR_{\alpha 146-162}$, or Con A 2.5 μg/mL), and stimulated with PMA (20 ng/mL)/ionomycin (1 μg/mL)/brefeldin A (5 μg/mL) for another 5 h at 37°C. After harvesting, cells were stained for surface markers with fluorochrome-conjugated mAb including anti-CD3-PE/Cy5 (17A2), anti-CD4-APC/Cy7 (GK1.5), anti-CD8α-PE/Cy7 (53–6.7), and anti-NK1.1-PE (PK136) (BD Bioscience). Isotype-matched negative mAb were used as controls. For intracellular cytokine staining, after fixation and permeabilization with the Cytofix/Cytoperm kit (BD Bioscience), anti-IFN-γ-Alexa 647 (XMG1.2), anti-IL-4-Alexa 647 (11B11), anti-IL-17-PE (TC11–18H10.1) mAb (BD Bioscience) were used. Flow cytometry data were collected on a FACSAria cytometer (BD Bioscience,) and analyzed with FACSDiva software. To determine the percentage of cells producing cytokine, the value obtained with isotype control was subtracted from that with specific antibody.

Th17 differentiation *in vitro*

Spleen cells from control B6 mice or $CCL2^{-/-}$ mice were cultured for 3–4 days in medium with plate-bound anti-CD3 (145–2C11, 4 μL/mL) plus anti-CD28 (PV-1, 2 μg/mL) plus irradiated syngeneic splenocytes (2000 rad) as APC and stimulated with recombinant cytokines IL-6 (30 ng/mL) and TGF-β1 (3 ng/mL) or IL-23 (20 ng/mL). IL-17 production in the supernatant was measured by commercially available ELISA kit (R&D Systems). Intracellular analysis of IL-17 produced by $CD4^+$ T cells was also evaluated by FACS analysis. In brief, polarized whole spleen cells were further stimulated for 5 h with PMA/ionomycin/BFA (20 ng/mL PMA, 1 μg/mL ionomycin, 5 μg/mL BFA) at 37°C. Cells were stained with surface markers anti-CD4-PeCy7 (RM4–5), and then with intracellular cytokine anti-IL-17-PE mAb (TC11–18H10.1) (BD Bioscience). Results were analyzed by flow cytometry [9, 44].

Adoptive transfer

Lymphocytes were pooled from spleen and lymph nodes of multiple control mice and $CCL2^{-/-}$ mice that had been immunized with AChR and CFA. $CD4^+$ cells were isolated with magnetic beads (Miltenyi Biotec, Auburn, CA). Similarly, $B220^+$ cells were isolated from naïve B6 cells. These cells were co-injected i.v. into $RAG1^{-/-}$ mice. The purity of transferred cells was verified by flow cytometry (96%).

Statistical analyses

Differences between groups were analyzed by the Student's *t*-test. Clinical scores were analyzed using the nonparametric Mann-Whitney *U* test. Differences between groups with respect to disease incidence were analyzed by Fisher's exact test. The level of significance was considered $p < 0.05$.

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Abbreviations: **AChR:** acetylcholine receptor · **B6:** C57BL/6 mice · **CCL2:** CC chemokine ligand 2 · **EAMG:** experimental autoimmune myasthenia gravis · **MG:** myasthenia gravis · **p.i.:** post immunization

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