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CCR5 Regulates High Dose Oral Tolerance by Modulating CC Chemokine Ligand 2 Levels in the GALT

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Oral tolerance is a complex immunoregulatory mechanism used by the gut and its associated lymphoid tissue to remain in a hyporesponsive state (1–4). It is thought that oral tolerance helps prevent reactivity against nonpathogenic food proteins and commensal bacteria. The mechanism of oral tolerance is not clearly defined. However, many studies suggest regulation by suppressive cytokines such as IL-4, IL-10, and TGF-β (5–11), while others suggest that clonal deletion and/or anergy occur (12–14).

Experimental autoimmune encephalomyelitis (EAE) is a CD4+ Th cell-mediated demyelinating disease of the CNS that serves as a model for multiple sclerosis (15, 16). Oral tolerance has been used experimentally to protect from EAE (17), as well as other autoimmune diseases (18, 19), by feeding immunodominant peptides or whole self proteins before disease induction. However, it has been proven difficult to suppress ongoing disease. Additionally, human trials using oral tolerance strategies have failed to provide protection from multiple sclerosis (20). A more complete understanding of the mechanism of oral tolerance would be useful for further development of clinical studies that use this approach.

We have previously demonstrated a role for CCL2 (MCP-1) in oral tolerance, using both neutralizing anti-CCL2 Ab (21, 22) and CCL2 knockout mice (23). The results suggested that CCL2 suppressed IL-12 and augmented TGF-β expression in GALT-derived APCs after Ag feeding. Regulation of CCL2 by CCL5 (RANTES) has been demonstrated in astrocyte cultures (24). In the present study, we found that CCR5, the receptor for CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5, is essential to the induction of oral tolerance, and that the lack of oral tolerance seen in CCR5−/− mice is related to CCL5 regulation of CCL2 expression.

Materials and Methods

Animals

Female C57BL/6 (H-2b) mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). CCR5−/− (H-2b) homozygous knockout mice were previously described (25). CCR5−/− mice have been backcrossed eight times onto the C57BL/6 background and were maintained in the Center for Comparative Medicine at Northwestern University. All mice were 6–7 wk old at the initiation of the experiment and were maintained on standard laboratory chow and water ad libitum. Animal care and use were performed in accordance with Northwestern University and National Institutes of Health guidelines.

RT-PCR

Total RNA was isolated from adherent cell populations from the mesenteric lymph node (MLN) and Peyer’s patches (PP) using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and reverse transcribed to cDNA (BD Clontech, Palo Alto, CA). CCL2 primer sequence is as follows: sense, 5'-TCTCTCCTCCCCACCCAGACG-3'; antisense, 5'-GGAAAAATG GATTCACACCTTGC-3'. PCR conditions were: 94°C for 3 min, followed by 40 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, with a final extension at 72°C for 3 min. PCR products were visualized by ethidium bromide (Molecular Probes, Eugene, OR) and 2% agarose gel (Invitrogen Life Technologies) electrophoresis.

Cell culture

Peritoneal macrophages were obtained by lavage using HBSS (BioWhittaker, Walkersville, MD), supplemented with 1% FCS (HyClone, Logan, UT), and cultured in 48-well microwell plates (Corning-Costar, Acton, MA) at a density of 5 × 10^5 cells/ml in DMEM (Invitrogen Life Technologies) containing 5% FCS (HyClone, Logan, UT), 2 mM L-glutamine (Invitrogen Life Technologies), 100 U/ml penicillin (Invitrogen Life Technologies), 0.1 M nonessential amino acids (Invitrogen Life Technologies), and 5 × 10^-5 M 2-ME; Methionine (met)-RANTES (A. Proudfoot, Serono Pharmaceutical Research Institute, Geneva, Switzerland) was prepared and added to the triplicate cultures at 0.1, 0.01, and 0.001 µg/ml. Cells were incubated at 37°C in a humidified atmosphere containing 7.5% CO₂.

Peptides

Myelin oligodendrocyte glycoprotein (MOG) 35–55 (MEGVYRSPFS RVHLYRNGK) and OVA 323–339 (LSGAVHAAHAGLAGAGR) were
purchased from Peptides International (Louisville, KY); amino acid composition was verified by mass spectrometry, and purity was >98%.

**Tolerance and immunization**

Induction of tolerance was performed by administering 2 mg of MOG35–55 or 2 mg of OVA323–339 dissolved in 0.5 ml of PBS by intragastric gavage. To induce EAE, mice were immunized with 200 μg of MOG35–55 emulsified in CFA containing 4 mg/ml Mycobacterium tuberculosis (Difco, Kansas City, MO) s.c. and given 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) i.v. on the day of and 2 days after immunization. Individual animals were graded according to clinical severity, as follows: grade 0, no abnormality; grade 1, limp tail; grade 2, limp tail and hind limb weakness; grade 3, partial hind limb paralysis; grade 4, complete hind limb paralysis. In some experiments, mice were treated with 100 μg of met-RANTES 1 h before Ag feeding and immunized with MOG35–55 to induce EAE 7 days later.

**Chemokine and cytokine ELISA**

CCL2, CCL3, CCL4, and CCL5 were quantified from tissue samples by specific ELISA (26, 27). Briefly, PP and MLN samples were harvested and homogenized in 1 ml of PBS and clarified by centrifugation (400 × g) for 10 min. Flat-bottom microtiter plates were coated with capture Ab diluted in sodium phosphate coating buffer and incubated at 4°C overnight. Non-specific binding sites were blocked by incubation with 10% FCS in PBS for 1 h at 37°C, and samples were subsequently added in triplicate and incubated for 2 h at 37°C. HRP-conjugated anti-CCL2 Ab was added and incubated for an additional 1 h at 37°C. Plates were developed using o-phenylenediamine substrate, and absorbance was read at 490 nm. Standard curves for the chemokines were generated using a series of dilution of purified recombinant chemokine (R&D Systems, Minneapolis, MN). The detection limit of these assays is 31.25 pg/ml, and this ELISA is specific and does not cross-react with any other cytokine or chemokines. Chemokine levels in mucosa and spleen were quantified by comparison with the standard curve and expressed as pg/ml tissue. Culture supernatants were tested for the presence of IL-2, IFN-γ, IL-12, TNF-α, and IL-4 by commercial ELISA kits (Endogen, Cambridge, MA). The detection limit of the cytokine ELISA kits was as follows: IL-2, 15.6 pg/ml; IFN-γ, 48.8 pg/ml; IL-12, 156 pg/ml; TNF-α, 31.3 pg/ml; and IL-4, 31.3 pg/ml.

**Statistical analysis**

Comparisons of disease and disease incidence were analyzed by the χ² analysis, using Fisher’s exact probability test. Statistical significance of cytokine levels, proliferations, and disease onset was analyzed using Student’s t test for comparisons of two means. Values of p ≤ 0.05 were considered significant.

**Results**

**Ligands for CCR5 are up-regulated in the GALT after Ag feeding**

To assess the role of chemokines in oral tolerance, levels of CCL3, CCL4, and CCL5 were measured from wild-type mice after feeding 2 mg of MOG35–55 or saline. Six hours and 2 days after Ag feeding, PP and MLN were harvested and homogenized in 1 ml of sterile PBS, and the clarified supernatants were analyzed by ELISA. Mice that received oral PBS did not produce any CCL3, CCL4, or CCL5 at either 6 h or 2 days postpeptide gavage (Fig. 1). Feeding MOG35–55 induced a significant amount of CCL5 in the GALT at both 6 h and 2 days (Fig. 1A). CCL4 was significantly increased 2 days after Ag feeding (Fig. 1B); however, there was no increase in CCL3 in the wild-type mice at either time point (Fig. 1C). These data indicate that both CCL4 and CCL5, but not CCL3, are up-regulated in the GALT following high dose Ag feeding. CCR5<sup>−/−</sup> mice are not protected from EAE when fed MOG35–55

CCL5, CCL4, and CCL3 all have the ability to bind and signal through the chemokine receptor CCR5 (28). Because two of the three ligands for CCR5 were up-regulated in the GALT after Ag feeding, we wanted to assess the role of CCR5 in oral tolerance induction. CCR5<sup>−/−</sup> mice develop EAE (29), so we decided to use lack of disease development as a readout for the induction of oral tolerance. CCR5<sup>−/−</sup> and wild-type mice were fed 2 mg of MOG35–55 or OVA320–339, as a control peptide, 7 days before immunization with 100 μg of MOG35–55 in CFA plus pertussis toxin to induce EAE. The results shown in Fig. 2A demonstrate that control peptide-fed wild-type and CCR5<sup>−/−</sup> mice immunized with MOG35–55 develop severe EAE with an incidence of 100% (Fig. 2B). Feeding MOG35–55 protected wild-type mice from developing severe EAE (Fig. 2A). The incidence of disease development in this group was 14% (Fig. 2B). However, deletion of CCR5 reverses the protective effect of feeding MOG35–55 peptide, and those mice developed EAE identical with control peptide-fed mice (Fig. 2A) with an incidence of 100% (Fig. 2B). These
results demonstrate that CCR5 expression is necessary for the induction of high dose oral tolerance.

**CCR5−/− mice have altered immune deviation**

We further evaluated the effect of CCR5 deletion on immune responses following Ag feeding. One feature of high dose oral tolerance is immune deviation, whereby mice primed with Ag and CFA normally produce Th1-associated cytokines, but an orally tolerized animal will produce Th2 cytokines after priming (30). When a subset of control peptide-fed wild-type mice from the experiment shown in Fig. 2 displayed peak clinical EAE, MLN cells and splenocytes were harvested from all four groups of mice and were stimulated in vitro with MOG35–55 to determine their ability to produce cytokines. The results shown in Fig. 3 indicate that wild-type mice fed MOG35–55 produced significantly less IL-2 and IFN-γ and significantly more IL-4 than control Ag-fed wild-type mice. However, MOG35–55-fed CCR5−/− mice showed no change in IL-2, IFN-γ, or IL-4 compared with either control peptide-fed CCR5−/− mice or control peptide-fed wild-type mice. Therefore, they did not demonstrate an Ag-specific T cell cytokine phenotype seen in orally tolerized mice. These data suggest that in the absence of CCR5, Ag feeding does not induce immune deviation as measured by the up-regulation of Th2 cytokines.

**CCR5−/− mice produce less mucosal CCL2 after Ag feeding**

Chemokines have been described principally as chemoattractant cytokines (28). We considered the possibility that the absence of CCR5 was altering leukocyte trafficking into or out of the gut following Ag feeding, and thus accounting for the lack of tolerance induction and cytokine deviation. However, when PP, MLN, and spleen were assessed by flow cytometry for leukocyte subpopulations, there were no differences in numbers of CD4, CD8, CD11b, γδ T cells, and CD19 populations between any of the groups at 2 and 5 days after Ag feeding (data not shown). Because there were no changes in lymphocyte trafficking in the GALT following Ag feeding in the CCR5−/− mice, we hypothesized that there may be defects in inflammatory and/or regulatory cytokine. We have previously demonstrated a role for CCL2 in the induction of high dose oral tolerance through a mechanism of mucosal IL-12 regulation (22, 23). CCL2 was not found to be a critical molecule for the induction of low dose oral tolerance; however, deletion of CCL2 or its receptor did impair the production of IL-4 after repeated low dose feedings (31). Both models suggest that CCL2 functions to control cytokine production. Therefore, we explored a potential role of CCR5 stimulation in mucosal cytokine regulation by hypothesizing that CCR5−/− mice have a deficiency in the ability to produce CCL2, which could potentially affect inflammatory cytokine expression. In the first set of experiments to address this possibility, wild-type and CCR5−/− mice were fed 2 mg of MOG35–55 or saline as a control; 2 days later, intestinal mucosa was harvested and homogenized, and the clarified supernatants were analyzed for IL-12 and CCL2 by ELISA. Consistent with our previous findings (23), wild-type mice fed saline showed neither IL-12 nor CCL2 production (Fig. 4). Wild-type mice fed 2 mg of MOG35–55 produced low amounts of IL-12 (Fig. 4A) and high amounts of CCL2 (Fig. 4B) in the GALT. However, CCR5−/− mice fed MOG35–55 produced high levels of IL-12 (Fig. 4A) and low levels of CCL2 (Fig. 4B). These data suggest that the absence

**FIGURE 2.** Oral tolerance is not induced in CCR5−/− mice fed MOG35–55. C57BL/6 or CCR5−/− mice received 2 mg of MOG 35–55 or OVA323–39 (sham) intragastrically 7 days before immunization with MOG35–55 in CFA. Mice were evaluated for clinical signs of EAE on a four-point scale. The data are expressed as the mean clinical disease score for all mice as a function of days after immunization (A) or as percentage of clinical disease incidence (B). Both mean clinical disease and disease incidence were significantly decreased in only the MOG35–55-fed wild-type mice compared with other groups (p < 0.05). The data shown are representative of three separate experiments.

**FIGURE 3.** CCR5−/− mice do not exhibit immune deviation when fed MOG35–55. Spleen (■) and MLN (□) were harvested from wild-type and CCR5−/− mice at the peak of acute disease. A total of 5 × 10⁶ cells was restimulated in vitro with 50 μg/ml MOG35–55 for 48 h, and supernatants were analyzed for IL-2, IFN-γ, and IL-4 by ELISA. *, Denotes p < 0.05. The data shown are representative of three independent experiments.
of CCR5 alters the cytokine milieu of the GALT through reduced CCL2 production and concomitant enhancement of IL-12 levels.

**CCR5−/− macrophages from the GALT produce less CCL2 after MOG35–55 feeding**

We wanted to directly investigate whether adherent cell populations from CCR5−/− mice have a defect in CCL2 production following oral Ag administration. Plastic-adherent cells, including macrophages and dendritic cells, were isolated from the PP and MLN 2 days after feeding either 2 mg of MOG35–55 or saline. Real-time PCR was performed for expression of CCL2 mRNA. The results in Fig. 5 demonstrate macrophages and dendritic cells from MOG35–55-fed wild-type mice up-regulated CCL2 mRNA expression in both the PP and MLN compared with cells from saline-fed controls. Additionally, macrophages and dendritic cells from both the PP and MLN of MOG35–55-fed CCR5−/− mice had a significantly lower amount of CCL2 mRNA expression. These data directly demonstrate that CCR5−/− macrophages and dendritic cells derived from the GALT produce less CCL2 after Ag feeding.

In **vivo treatment with a CCR5 antagonist before Ag feeding abrogates oral tolerance and inhibits mucosal CCL2 and IL-12**

We have previously demonstrated that local gut production of CCL2 is crucial to high dose oral tolerance by down-regulating IL-12 production (22). met-RANTES is a selective CCR5 antagonist that has an extremely low binding affinity for CCR1 (32) and is unable to inhibit chemotaxis to the CCR3 ligand, eotaxin (32). However, met-RANTES was able to block RANTES-induced chemotaxis, demonstrating the CCR5 selectivity of this antagonist (32). To further explore the role of CCR5 in modulating oral tolerance, we treated wild-type C57BL/6 mice with 100 μg of met-RANTES 1 h before feeding 2 mg of MOG35–55 or OVA323–339, as a control. Seven days following Ag feeding, all of the mice were immunized with MOG35–55 in CFA to induce EAE. As shown in Fig. 6, animals fed MOG and treated with met-RANTES prior were not orally tolerized and demonstrated clinical disease (Fig. 6A), with an incidence of 80% (Fig. 6B) that was significantly higher than the tolerized control animals. At the peak of clinical disease, mice from each group were sacrificed and splenocytes were restimulated in vitro with MOG35–55. Splenocytes from animals fed MOG35–55 and treated with the CCR5 antagonist displayed significantly elevated IFN-γ production compared with the orally tolerized control animals (Fig. 6C). To understand whether antagonizing CCR5 had the same effect on mucosal production of CCL2 and IL-12 as seen in the CCR5−/− animals, mice were given 100 μg of met-RANTES 1 h before Ag feeding and then fed MOG35–55 or saline. Two days after Ag feeding, GALT was homogenized, and levels of CCL2 and IL-12 were examined by ELISA. The results shown in Fig. 7A demonstrate that met-RANTES treatment of MOG35–55-fed wild-type mice reduced expression of CCL2. Correspondingly, the level of IL-12 in the GALT was increased (Fig. 7B). Because treatment with met-RANTES before Ag feeding increased IL-12 levels, we wanted to determine whether this treatment abrogated oral tolerance. Remaining mice from each group were immunized with MOG35–55, and 7 days later Ag-specific proliferation and IFN-γ levels were measured. These data demonstrate that blocking CCR5 in vivo before feeding abrogates oral tolerance induction and alters the cytokine milieu of the gut by inhibiting CCL2 and promoting production of the inflammatory cytokine IL-12 as well as allowing differentiation of Th1 lymphocytes.
To gain further insight into CCR5 regulation of CCL2 expression, we assessed the ability of met-RANTES to antagonize CCR5 regulation of CCL2 production by activated macrophages in vitro. Normal peritoneal macrophages were isolated from wild-type mice and activated in vitro with LPS in the presence of met-RANTES. After 24 h in culture, the supernatants were harvested and assessed for chemokine expression by ELISA. The results shown in Fig. 8 demonstrated that met-RANTES was unable to inhibit CCL3, CCL4, or CCL5 expression at any dose tested. In contrast, met-RANTES inhibited CCL2 expression in a dose-dependent fashion. These data suggest that antagonizing the chemokine receptor, CCR5, leads to a decrease in CCL2 production, and support the earlier observations that CCR5 function is critical to oral tolerance induction through the regulation of CCL2 expression.

Discussion
In the present study, we demonstrate that CCR5 is a critical receptor for the induction of high dose oral tolerance. Not only are two of the three CCR5 ligands up-regulated in the GALT after Ag feeding (Fig. 1), but CCR5−/− mice fed MOG35–55 peptide before immunization did not develop oral tolerance and were not protected from developing EAE (Fig. 2). These results have led us to conclude that CCR5 activation regulates oral tolerance induction through the modulation of CCL2 expression. Support for this idea derives from the findings that MOG35–55-fed CCR5−/− mice produce lower amounts of CCL2 in the GALT compared with wild-type mice (Figs. 4 and 5), as well as the result in which the
CCR5 antagonist, met-RANTES, inhibited GALT CCL2 expression following MOG35–55 feeding in wild-type mice (Fig. 7).

Previous reports have suggested that CCL5 activation of its receptor CCR5 can modulate the up-regulation of other chemokines in dendritic cell subsets (33), as well as in cultures of astrocytes (24). The latter study demonstrated increases in CCL2 when astrocytes were stimulated with CCL5; however, when the astrocytes were derived from CCR5<sup>−/−</sup>, CCR3<sup>−/−</sup>, or CCR1<sup>−/−</sup> animals, there was still an increase in CCL2 transcripts, suggesting the response is not solely dependent on a single receptor. Specifically, within macrophage populations, Zhou et al. (34) observed that CCR5<sup>−/−</sup> macrophages had minor defects in clearing *Listeria* and defects in secretion of GM-CSF, IL-1β, and IL-6. Finally, they reported that CCR5<sup>−/−</sup> animals had an enhanced delayed-type hypersensitivity response, suggesting that signaling through CCR5 may have a down-modulatory role in T cell-dependent immune responses (34).

The regulation of CCL2 by CCL5 is a novel and interesting mechanism through which oral tolerance induction may be regulated. We propose the following model whereby activation of CCR5 by either CCL4 and/or CCL5 serves to modulate CCL2 expression, which in turn regulates oral tolerance induction by influencing the balance between mucosal inflammatory cytokines such as IL-12 and regulatory cytokines such as TGF-β. After Ag is ingested and taken up by the M cells in the PP, there is local up-expression of CCL2, CCL3, CCL4, and CCL5. Ag ingestion also up-regulates these particular chemokines in the MLN. At this point, we do not know whether CCL3, CCL4, or CCL5 expression precedes that of CCL2. Nevertheless, CCL4 and CCL5 are potent agonists for CCR5 that is expressed by macrophages in the PP and MLN. We believe that CCL4 and CCL5 can activate CCR5 to modulate CCL2 expression by macrophages. It appears that CCR5 activation does not totally regulate CCL2 expression, as there is a low level of CCL2 production in the GALT of MOG35–55-fed CCR5<sup>−/−</sup> mice (Fig. 4). CCL2 serves to concomitantly down-regulate GALT IL-12 expression while allowing the up-regulation of TGF-β (23). It is this cytokine balance in the GALT that promotes the induction of high Ag dose oral tolerance. When CCR5 is either deleted (Fig. 4) or its activation antagonized (Fig. 7), there is less CCL2 expression, thereby shifting the GALT cytokine balance toward IL-12 and the promotion of immunity and the abrogation of high dose oral tolerance induction. We believe this to be specific to CCR5 and not other receptors for CCL5 such as CCR1 or CCR3 because met-RANTES has only a weak affinity for CCR1, is ineffective at inhibiting CCR3-mediated chemotaxis, but effectively inhibits CCL5-mediated chemotaxis (32, 35).

All three CCR5 ligands, CCL3, CCL4, and CCL5, have differential effects on mucosal and systemic immunity. When these chemokines were administered intranasally with OVA, it was found that CCL4 and CCL5, but not CCL3, could elicit both systemic IgA and mucosal Ab-forming cells that produce secretory IgA (36, 37). However, CCL4 skewed the response toward a Th2-like phenotype, producing high amounts of IL-4 from both mucosal and systemic lymph nodes, whereas CCL5 and CCL3 skewed the response toward a Th1-like phenotype, in which high amounts of IFN-γ and very little IL-4 or IL-10 were produced (36, 37). In the present study, we have demonstrated that CCL4 and CCL5 are increased after Ag feeding in GALT tissues, yet CCL3 is not. To further support the idea that CCL3 does not function to regulate the induction of high dose oral tolerance, CCL3<sup>−/−</sup> mice are tolerized by feeding high dose MOG35–55 (data not shown). Because both CCL4 and CCL5 can activate CCR5, we are currently testing whether both receptor ligands have similar ability to modulate GALT CCL2 expression and thereby regulate the IL-12/TGF-β response.

**FIGURE 8.** CCR5 antagonist inhibits CCL2 production from LPS-stimulated macrophages. Macrophages were harvested and prepared, as previously described (Materials and Methods). The cells were plated in triplicate and activated with LPS, and graded concentrations of met-RANTES were added to the wells. Twenty-four-hour culture supernatants were used to assess chemokine production (CCL3, A; CCL4, B; CCL5, C; and CCL2, D) by ELISA. The data shown are representative of three separate experiments. *, Denotes *p* < 0.05 compared with LPS stimulation in the absence of met-RANTES.
balance that we believe determines whether feeding high doses of Ag results in tolerance or immunity.

Our results indicate that signaling through CCR5 can act as a rheostat in regulating levels of CCL2 that may help to inhibit IL-12 production by local macrophages and dendritic cells in GALT tissue. This idea has implications in the design of both vaccines, in which the promotion of immunity is desired, as well as immunotherapy, in which the inhibition of immunity is the goal. The use of a CCR5 antagonist in conjunction with a mucosal vaccine could lead to high levels of IL-12 in the gut after vaccine administration, and provide a mechanism whereby a more robust immune response is generated. Similarly, the use of a CCR5 agonist in conjunction with high dose oral Ag might be able to tip the GALT cytokine balance during ongoing autoimmune disease toward anti-inflammatory and result in a diminution of autoreactivity. We are currently exploring these possibilities.

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