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CC Chemokine Ligand 2 and Its Receptor Regulate Mucosal Production of IL-12 and TGF-β in High Dose Oral Tolerance

R. William DePaolo, Barrett J. Rollins, William Kuziel, and William J. Karpus

Oral tolerance is the result of a complex immunoregulatory strategy used by the gut and its associated lymphoid tissues to render the peripheral immune system unresponsive to nonpathogenic proteins, such as food or commensal bacteria. The mechanism of oral tolerance induction and maintenance is not well understood. We have previously shown that the chemokine, CC chemokine ligand 2 (CCL2), is important for the induction and maintenance of oral tolerance. To address the role CCL2 plays in oral tolerance, we used both CCL2−/− and CCR2−/− mice. Cells from the spleen, mesenteric lymph nodes, and peripheral lymph nodes of CCL2−/− and CCR2−/− mice fed high doses of OVA showed robust proliferative responses compared with cells from Ag-fed wild-type mice. CCL2−/− and CCR2−/− mice also produced high amounts of Th1 cytokines such as IL-2 and IFN-γ and very low amounts of IL-4 and IL-10. The ability of APCs from the gut of CCL2−/− and CCR2−/− OVA-fed mice to stimulate an indicator T cell line was evaluated. APCs from the Peyer’s patch of OVA-fed knockout animals could induce a Th1 cell response measured by an increase in proliferation and generation of IL-12 and IFN-γ with a concomitant reduction of TGF-β compared with wild-type controls that did not induce a Th1 response. These data indicate that CCL2 and signaling through its receptor CCR2 is critical for the induction of oral tolerance by regulating Ag presentation leading to a disruption in the balance of inflammatory and regulatory cytokines. The Journal of Immunology, 2003, 171: 3560–3567.

A critical feature of the immune system is the discrimination of self from nonself (1). This discrimination is mediated by immune tolerance, the mechanism that prevents the immune system from pathogenic reactivity against self-Ags. Central tolerance refers to the deletion of autoreactive T cells in the thymus by clonal deletion, however not all autoreactive cells are deleted in the thymus and they must be tolerated in the periphery. The mechanisms of peripheral tolerance include anergy (2) and suppression (3).

Oral tolerance is the immunological phenomenon that the mucosal immune system uses to maintain a hyporesponsive state against a number of nonpathogenic proteins that come in contact with the mucosal environment through ingestion of food proteins or colonization by commensal bacteria (4–6). This method of tolerance has been used to suppress the immune response in many models of autoimmunity by feeding self Ags or proteins (7). The mechanisms of immune suppression mediated by oral tolerance include anergy, which may occur when the TCR engages the MHC/Ag complex in the absence of proper costimulation (8–10) and/or through active cellular suppression, mediated through the secretion of soluble factors such as TGF-β (11), Th2 cytokines (12–15), and chemokines (16, 17).

Chemokines are small m.w. cytokines that induce chemotaxis of leukocytes (reviewed in Ref. 18). Members of the chemokine superfamily are subdivided into four families based on the position of the first two cysteines at the N terminus: CXC, CC, C, CX3C (19, 20). Chemokines are ligands for seven transmembrane-spanning receptors that induce signaling through a Gq-protein linked pathway (21). CC chemokine ligand 2 (CCL2) (monocyte chemotactic protein-1), a CC chemokine, is expressed by a wide variety of tissues and cells. Although it only binds one receptor, CCR2 (22), it is a chemotaxant for many cells of the immune system including most lymphocytes and macrophages (23, 24). CCL2, like many chemokines, has pleiotropic effects beyond cellular trafficking. CCL2 has been implicated in Th1/Th2 development (25) and costimulation (26, 27). In fact, CCL2 has been demonstrated to play a role in mucosal immunity because intestinal epithelial cells cultured with both pathogenic and nonpathogenic bacteria express CCL2 (28, 29). Our laboratory identified CCL2 as a molecule involved in mucosal tolerance by demonstrating expression in the gut-associated lymphoreticular tissue (GALT) after peptide feeding, as well as abrogating oral tolerance by neutralizing CCL2 (16). The mechanism by which CCL2 may contribute to oral tolerance is currently not well-understood. The present report demonstrates that CCL2 and its receptor are critical factors in the induction of oral tolerance and suggests that they play a role in suppressing APC-derived IL-12 and enhancing TGF-β expression, which determine the program of T cell effector function.

Materials and Methods

Animals

Female C57BL/6 (H-2b) mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). CCL2 (H-2b) (30) and CCR2 (H-2b) (31) homozygous knockout mice as well as OTII TCR transgenic (H-2d) mice were previously described (32). All mice were maintained in the Center for Comparative Medicine (Northwestern University, Chicago, IL). CCL2 knockout mice and OTII transgenic mice have been backcrossed eight times onto the C57BL/6 background, whereas the CCR2 knockout mice have a C57BL/6 × 129 mixed genetic background. Mice were 6–7 wk-old at the initiation of the experiment and were maintained on standard laboratory chow and water ad libitum.

Abbreviations used in this paper: CCL2, CC chemokine ligand 2; GALT, gut-associated lymphoreticular tissue; i.g., intragastrically; BCC, bovine cytochrome c.
Animal care was provided in accordance with Northwestern University and National Institutes of Health guidelines.

Proteins

Chicken egg albumin grade III and bovine heart cytochrome c were purchased from Sigma-Aldrich (St. Louis, MO).

Antibodies

The following Abs were used, all obtained from BD PharMingen (San Diego, CA): CD16/32 (2.4G2, anti-mouse FeRγIIb/III), CD19 (1D3 FITC anti-mouse), CD11b (M1/70 allophycocyanin anti-mouse), CD40 (3/23 PE anti-mouse), CD80 (16-10A1 PE and purified anti-mouse), CD86 (GL-1 PE and purified anti-mouse), inducible costimulator (7E.17G9 BIO and purified anti-mouse), rat IgG2a (R35-95), and Armenian hamster IgG group 2 k (BSI-3).

Tolerance and immunization

Animals were given 25 mg of OVA in 0.5 ml of sterile PBS intragastrically (i.g.) with an 18-gauge round-tipped gavage needle, while control animals received 25 mg of bovine heart cytochrome c or saline. In some of the experiments, 7 days after receiving oral Ag, mice were immunized with 100 μg of OVA and 20 μg of Mycobacterium tuberculosis H37Ra inIFA (Sigma-Aldrich) at three sites on the dorsal flank.

RT-PCR

Total RNA was isolated from adherent cell populations from the mesenteric lymph node and Peyer’s patches using TRIzol reagent (Life Technologies, Grand Island, NY) and converted to cDNA (Clontech Laboratories, Palo Alto, CA). Reverse transcriptase was performed as previously described (33). 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 30 s at 72°C, with a final extension at 72°C for 3 min. CCL2 primer sequences include, sense 5'-TCTCTCTTCCTCCACCATGACG-3', and antisense 5'GGAAAATGATGTCACACCTTGC-3' (Life Technologies). PCR products were resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide.

Cell culture

Single cell suspensions from pooled lymph nodes, Peyer’s patches or spleens were isolated from mice by mechanical disruption through mesh stainless steel screens. RBC in the spleen samples were lysed by hypotonic shock in Tris-NCI (pH 7.3) and cells were resuspended in HBSS (Life Technologies). The cells were then cultured in 96-well microtiter plates (Corning-Costar, Acton, MA) at a density of 5 × 10⁴ viable cells/ml in DMEM (Life Technologies) containing 5% FCS (HyClone Laboratories, Logan UT), 2 mM l-glutamine (Life Technologies), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 0.1 M nonessential amino acids (Life Technologies), and 5 × 10⁻⁵ M 2-ME in a humidified atmosphere containing 7.5% CO₂. For measurement of proliferation, the cells were pulsed with 1 μCi [³H]Tdr (ICN Radiochemicals, Irvine, CA) after 72 h, harvested after 96 h and [³H]Tdr uptake was detected using a Packard Topcount microplate scintillation counter (Packard Instruments, Meriden, CT). Results are presented as the mean ± SEM of triplicate wells.

Coculture of APCs and T cells

Single cell suspensions of Peyer’s patches were prepared as described above. The cell suspension was then depleted of CD4 and CD8 cells by magnetic sorting. Briefly, cells were incubated at 10⁶cells/ml with anti-CD4 and anti-CD8 magnetic-conjugated beads (Miltenyi Biotec, Auburn, CA). After 20 min of rotation at 4°C, the samples were placed on a magnet for 2 min and the supernatants were collected, washed, and counted. The APC were then irradiated at 3000 R and resuspended at 4 × 10⁶ cells/ml. OVA-specific T cells were derived from TCR transgenic OTII mice. Briefly, spleens were harvested and prepared as described above. Cell suspensions were incubated at 37°C for 1 h in a tissue culture flask to separate macrophages and dendritic cells from lymphocytes by adherence. The cells were incubated with anti-CD8 and anti-B220 magnetic-conjugated beads to deplete CD8 T cells and B cells. After 20 min of rotation at 4°C, the sample was placed on a magnetic column and the supernatant was collected, washed, and counted. This method gave a yield of a 96% pure population of CD4⁺T cells as measured by flow cytometry. The CD4⁺T cells were seeded at 2 × 10⁵ cells/well in a 96-well plate with different dilutions of APC. The cells were pulsed with 1 μCi [³H]Tdr (ICN Radiochemicals, Irvine, CA) after 72 h, harvested after 96 h and [³H]Tdr uptake was determined using a Packard Topcount microplate scintillation counter (Packard Instruments). Results are presented as the mean ± SEM of triplicate wells.

Chemokine and cytokine ELISA

Assessment of CCL2 was quantified from tissue samples using previously described ELISA (34, 35). Briefly, mucosal 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 and washed 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 CCL2 were generated using a series of dilutions of purified recombinant CCL2 (R&D Systems, Minneapolis, MN). The detection limit of these assays was 31.25 pg/ml and this ELISA was specific and did not cross-react with any other cytokine or chemokines. Chemokine levels in mucosa and spleen were quantified by comparison to the standard curve and expressed as picograms per milliliter of tissue. Assessment of cytokine production was determined from lymph node and spleen supernatants harvested 48 h after stimulation with 0 and 100 μM OVA. The supernatants were tested for the presence of IL-2, IFN-γ, 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, IFN-γ, 48.8, and IL-4, 31.3 pg/ml.

Flow cytometry

Cells (0.5–1 × 10⁶) harvested from mesenteric lymph nodes, peripheral lymph nodes (axial, brachial, inguinal), spleen, and Peyer’s patches were washed and incubated with anti-mouse FeRγIIb/III (CD16/32 clone 2.4G2; BD PharMingen) for 15 min at 4°C to prevent nonspecific Fc binding. Cells were washed in PBS with 10% Na₂ and BSA (Sigma-Aldrich) and labeled directly with mAbs specific for lymphocyte surface markers (CD4, CD8, CD19, and CD11b) at 4°C for 15 min. The cells were washed and resuspended in 0.5 ml of isotonic buffered saline; expression was determined on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences).

Statistics

Statistical analysis was performed using a one-way ANOVA with Tukey tests to determine statistical significance for multiple comparisons. A p value of <0.05 was considered significant.

Results

CCL2 mRNA is up-regulated after feeding oral Ag

CCL2 has been shown to play an important role in oral tolerance induced in SJL mice immunized with self Ag (16). To assess the presence of CCL2 in OVA-fed C57BL/6 mice, animals were fed 25 mg of OVA, 25 mg of bovine cytochrome c (BCC) or saline by i.g. gavage. These mice were killed at either 6 h or 2 days after feeding and assessed for tissue-specific CCL2 mRNA production by RT-PCR. As shown in Fig. 1, CCL2 mRNA was seen in the Peyer’s patches of mice regardless of fed Ag (either OVA (O) or BCC (B)) as early as 6 h after feeding and this expression was maintained at 2 days post-Ag feeding. There was no CCL2 mRNA present in peripheral immune sites such as spleen (Fig. 1). Mice fed saline (P) showed no increase in CCL2 mRNA at either time point or in any organ examined (Fig. 1). These data demonstrate that CCL2 mRNA is specifically up-regulated in the mucosal tissue of C57BL/6 mice following Ag feeding.

CCL2 and CCR2 knockout mice are not tolerated by orally administered Ag

Previous work in our laboratory has shown that neutralization of CCL2 at the time of feeding PLP139–151, an encephalitogenic
peptide, abrogates oral tolerance and results in loss of protection from experimental autoimmune encephalomyelitis in a susceptible strain of mice (16). This evidence, taken together with the expression of CCL2 after Ag feeding suggested a role for CCL2 in the induction and/or maintenance of oral tolerance. To further explore the role of CCL2 and CCR2 in the development of oral tolerance, CCL2−/−, CCR2−/−, and wild-type C57BL/6 mice were fed 25 mg of OVA and control animals were fed 25 mg of BCC. Seven days after feeding, all mice were primed with 100 mg of OVA in CFA. Seven days after priming (14 days after tolerance induction), mice were killed and OVA-specific recall proliferative responses were measured from mesenteric lymph node, peripheral lymph node, and spleen cells to assess tolerance. Our definition of oral tolerance for this particular study is the absence of a recall Ag-specific proliferative response from mice fed the specific Ag compared with mice fed a control Ag. As shown in Fig. 2, wild-type control mice fed OVA show a significant reduction in the recall proliferative response to OVA compared with control Ag-fed mice that demonstrated robust Ag-specific recall proliferation. In contrast, CCL2−/− and CCR2−/− mice that were fed OVA and primed with OVA in CFA showed a robust OVA-specific proliferative response that was equal to control Ag-fed mice primed with OVA in CFA. These data suggest that deletion of the CCL2 or CCR2 genes impairs the induction of oral tolerance as measured by Ag-specific recall T cell proliferative response and instead appears to prime T cell reactivity.

Following Ag feeding, CCL2−/− and CCR2−/− mice do not show immune deviation

One feature of high dose oral tolerance is immune deviation of cytokine secretion (36), whereby mice primed with Ag and CFA will normally produce Th1 cytokines, but a tolerized animal will produce Th2 cytokines after priming. We wanted to evaluate whether CCL2−/− and CCR2−/− mice had the typical immune deviation seen in orally tolerized wild-type mice. To evaluate the expression patterns of Th1 and Th2 cytokines, CCL2−/−, CCR2−/−, and wild-type mice were fed OVA and challenged with OVA and CFA in vivo 7 days later. Lymphocytes were recovered, restimulated with OVA in vitro, and evaluated for cytokine expression by ELISA. As shown in Fig. 3, T cells derived from mesenteric lymph nodes of C57BL/6 mice fed 25 mg of OVA produced very low levels of both IL-2 and IFN-γ but high levels of IL-4 and IL-10. Splenic T cells from tolerized wild-type control mice showed the same cytokine secretion pattern (Fig. 3). T cells from the mesenteric lymph node and spleen of both OVA-fed CCL2−/− and CCR2−/− mice produced significantly higher amounts of IL-2 and IFN-γ when compared with Ag-fed wild-type controls (Fig. 3). However, T cells from the mesenteric lymph node and spleen of both OVA-fed CCL2−/− and CCR2−/− mice produced minimal amounts of IL-4 and IL-10, when compared with OVA-fed wild-type control mice (Fig. 3). These data suggest that in the absence of CCL2 and its receptor, Ag feeding does not induce immune deviation as measured by the up-regulation of Th2-associated cytokines.

**FIGURE 1.** CCL2 mRNA expression is up-regulated following Ag feeding. C57BL/6 mice were fed 25 mg of OVA (O), BCC (B), or PBS (P) by i.g. gavage. Peyer’s patches and spleens were harvested at the indicated time points and assayed for CCL2 mRNA expression using RT-PCR. Amplified cDNA was electrophoresed through 2% agarose gel and visualized by ethidium bromide. Results represent three animals pooled per group and are representative of two identical experiments.

**FIGURE 2.** CCL2−/− and CCR2−/− mice are not tolerized to orally administered OVA. Mice were fed 25 mg of OVA or 25 mg of BCC (Sham) and 7 days later primed with 100 μg of OVA in CFA. Seven days after priming, mesenteric lymph nodes (A and D), peripheral lymph nodes (B and E), and spleens (C and F) were harvested and cultured with various concentrations of OVA to determine the Ag-specific proliferative recall response. These data are representative of three identical experiments. * A value of p < 0.05 denotes statistical significance in comparison to OVA-fed wild-type mice to OVA-fed CCL2−/− and CCR2−/− animals.
Ag feeding alone primes an inflammatory response in both the GALT and periphery of CCL2−/− and CCR2−/− mice

To examine whether the absence of CCL2 allows for the enhanced induction of inflammatory cytokine expression in the gut and periphery, control and knockout mice were fed 25 mg of OVA or BCC. Six hours or 2 days following Ag feeding, cells from the Peyer’s patches and spleens were restimulated in vitro with 1 mg/ml OVA and culture supernatants were examined for cytokine expression by ELISA. These mice did not receive an in vivo challenge of Ag and CFA. The results shown in Fig. 4 demonstrate that Peyer’s patch cells from C57BL/6 wild-type control mice fed OVA did not produce IL-12 or IFN-γ, yet splenic-derived cells produced both inflammatory cytokines. In contrast, Peyer’s patch-derived cells from CCL2−/− and CCR2−/− mice produced significantly higher amounts of both IL-12 and IFN-γ at 6 h and 2 days after OVA feeding compared with wild-type mice. Production of IL-12 and IFN-γ is Ag-specific as cells from BCC-fed mice restimulated with 1 mg/ml OVA did not produce either cytokine.

Because the regulatory cytokine TGF-β and Th2 cytokines such as IL-4 and IL-10 have been shown to play an important role in oral tolerance (37–40), we decided to examine production of this factor following OVA feeding in both CCL2−/− and CCR2−/− mice. Control and knockout mice were fed 25 mg of OVA or BCC. Six hours or 2 days following Ag feeding, cells from the Peyer’s patches were restimulated in vitro with 1 mg/ml OVA and culture supernatants were examined for TGF-β expression by ELISA. The results shown in Fig. 3 demonstrate that Peyer’s patch-derived cells from wild-type mice fed OVA produced TGF-β in contrast to those cells derived from CCL2−/− and CCR2−/− mice that produced significantly lower amounts of the cytokine. IL-4 and IL-10 were not detected in any of the samples. These data indicate that in the absence of CCL2 and CCR2, a proinflammatory response is

![Graph showing cytokine production](image-url)
favored in the gut following Ag feeding as determined by enhanced IL-12 and IFN-γ and decreased TGF-β expression.

**Gut APCs from CCL2−/− and CCR2−/− animals can induce a T cell response and produce IL-12**

The production of IL-12 and IFN-γ in cultures of Peyer’s patch cells following OVA feeding and peripheral Ag challenge suggested that there was an induction of an inflammatory response in the GALT of CCL2−/− and CCR2−/− animals rather than the tolerance seen in wild-type mice. We wanted to examine whether CCL2 affected gut-derived APC-mediated costimulation and activation of Ag-specific T cells. A role for CCL2 in costimulation and Ag presentation has previously been suggested (26, 27). To evaluate a role for CCL2 in the regulation of Ag presentation in our model of oral tolerance, mice were fed 25 mg of OVA or BCC. Six hours following feeding, the Peyer’s patches were removed and cocultured with Ag-specific T cells from OTII mice, which express a transgenic TCR specific for OVA323–339. These cells were used as an indicator cell line for OVA-specific proliferation. Fed animals were not challenged with Ag and no exogenous OVA was added to the in vitro cultures, except that Ag-pulsed APC from wild-type mice were used as a positive control. This design directly tests the function of APC derived from Ag-fed CCL2−/−, CCR2−/− and wild-type mice for their ability to induce OVA-specific T cell proliferation and cytokine production. Previous data from our laboratory indicated that when CCL2 is neutralized at the time of Ag feeding there is a decrease in IL-4 production concomitant with an increase in IL-12 (16). Additional evidence that CCL2 has the potential to regulate IL-12 production derives from adenovirus-driven overexpression of CCL2 at the site of immunization during granulomatous responses. Increasing amounts of CCL2 reduced the Th1 granulomatous response, affecting both IFN-γ and IL-12 (41). These data suggested to us that CCL2 may be altering the cytokine milieu of the gut and thereby regulating APC and/or T cell function. To specifically address this area, Peyer’s patch APC from OVA-fed wild-type, CCL2−/− or CCR2−/− were cultured with OVA-specific CD4+ T cells (OTII) and the supernatants were assessed for production of IL-12 and IFN-γ. The results shown in Fig. 5A and B, demonstrate there was very little IL-12 and IFN-γ, respectively, produced and/or induced by OVA-fed APC. In contrast, the cultures containing APCs from OVA-fed CCL2−/− and CCR2−/− mice induced robust IL-12 and IFN-γ expression. It is well-known that Th2 cytokines and TGF-β play an important role as suppressive cytokines in the induction of oral tolerance. To specifically address the role of CCL2 in the regulation of TGF-β and Th2 cytokine expression, Peyer’s patch APC from OVA-fed wild-type, CCL2−/− or CCR2−/− were cultured with OVA-specific CD4+ T cells (OTII) and the supernatants were assessed for production of TGF-β by ELISA. The results shown in Fig. 5C demonstrate that there was detectable TGF-β expression induced by OVA-fed wild-type APCs. In contrast, APCs from OVA-fed CCL2−/− and CCR2−/− mice had significantly depressed TGF-β expression when compared with wild-type cultures. There was no detectable protein expression of IL-4 or IL-10 in any of the cultures (data not shown).

The results shown in Fig. 5D demonstrate APCs from the GALT of OVA-fed wild-type mice could not induce an OVA-specific proliferative response in the indicator T cell line specific for OVA323–339. In contrast, APCs from OVA-fed mice lacking CCL2 or its receptor induced a vigorous T cell response. These data suggest that CCL2 may regulate functions of APCs necessary to induce T cell proliferation and effecter function.

To induce an optimal T cell proliferative response, a second signal must be present in addition to TCR recognition of Ag and BCC. The ligation of CD28 on a T cell by B7.1 and/or B7.2 on the APC is known as the major costimulatory signal. The role of co-stimulatory molecules has been explored in oral tolerance with...
B7.2 required for induction of low dose oral tolerance and CTLA-4 required for high dose oral tolerance (42, 43). To help understand the potential role of CCL2 in the regulation of B7.1 and B7.2 molecules in oral tolerance induction, animals were fed 25 mg of OVA or PBS and either 6 h or 2 days after Ag feeding; cell surface B7 expression was assessed by flow cytometry. B7.1 and B7.2 expression on mucosal CD19+ or CD11b+ cells was not different between wild-type, CCL2−/− or CCR2−/− mice (data not shown). These data suggest that CCL2 regulates gut APC function through mechanisms other than B7 costimulatory molecule expression.

CD11b+CD11c+ cells from the Peyer’s patch of CCL2−/− and CCR2−/− animals produce IL-12 and induce a T cell response

The Peyer’s patch contains many different types of APCs, some with distinct capabilities. To further understand which APC population is mediating the induction of a T cell response in the CCL2−/− and CCR2−/− cocultures rather than the suppression seen in wild-type mice, the Peyer’s patches were phenotyped by flow cytometry after depletion of T cells. CD19-positive cells comprised 67% of the cultures, macrophages were identified by CD11b staining and comprised ~8%, dendritic cells were identified by CD11b and CD11c staining and made up 10% of the population (data not shown). To understand which population may be mediating the IL-12 production and induction of IFN-γ in the CCL2−/− and CCR2−/− mice, the cocultures were depleted of either B cells (CD19+ cells) or macrophages and DCs (both CD11b+CD11c−-depleted) by using magnetic beads. In cultures depleted of CD11b+ and CD11c− cells, there was no detectable cytokine secretion (Fig. 6A–C) nor was there any Ag-specific proliferation (Fig. 6D) indicating that the Ag presentation seen in the CCL2−/− and CCR2−/− cultures was not mediated by B cells. However, when Peyer’s patch cells from CCL2−/− and CCR2−/− were depleted of B cells and contained macrophages and DCs there was an induction of a T cell response as well as IL-12 expression. TGF-β was only produced in cocultures with macrophages and DC derived from wild-type OVA-fed mice with little to no production of TGF-β from cultures containing CCL2−/− and CCR2−/− macrophages and DCs (Fig. 6C). Additionally, the ability to induce a T cell response in CCL2−/− and CCR2−/− mice fed OVA is mediated by macrophages and DC but not B cells (Fig. 6D). IL-4 and IL-10 do not appear to play a role in this process as their expression could not be detected in any of the samples. Taken together these data suggest that CCL2 may control oral tolerance through up-regulation of TGF-β together with down-regulation of IL-12 resulting in suppression of an inflammatory immune response.

Discussion

Our current results demonstrate that CCL2 is a direct effector molecule in high dose oral tolerance as OVA-fed mice deficient for either the chemokine itself or its receptor were not rendered orally tolerant when peripherally challenged with specific Ag (Fig. 2). The genetic deficiency of either CCL2 or CCR2 resulted in enhanced IL-12 and IFN-γ but decreased TGF-β production by Peyer’s patch cells obtained from OVA-fed mice (Fig. 4). This regulation is confined to the gut mucosa as this observation was not seen in splenic cells (Fig. 4). This dysregulation of inflammatory and regulatory cytokine expression by mucosal-derived cells affected the immune deviation normally seen in OVA-fed wild-type mice (Fig. 3). CCL2 appears to regulate mucosal-derived APC as these cells from OVA-fed CCL2−/− or CCR2−/− mice induced significant Ag-specific proliferation of a TCR transgenic indicator T cell when compared with APC from wild-type mice (Fig. 5D). CCL2 appears to regulate mucosal-derived APC cytokine balance as these cells from OVA-fed CCL2−/− or CCR2−/− mice expressed enhanced IL-12 and an induction of IFN-γ, but these cells from OVA-fed wild-type culture depleted of either B cells or CD11b+CD11c+ cells. This repression was not mediated by CD11b+CD11c+ cells. However, when Peyer’s patch cells from CCL2−/− and CCR2−/− were depleted of B cells and contained macrophages and DCs there was an induction of a T cell response as well as IL-12 expression. TGF-β was only produced in cocultures with macrophages and DC derived from wild-type OVA-fed mice with little to no production of TGF-β from cultures containing CCL2−/− and CCR2−/− macrophages and DCs (Fig. 6C). Additionally, the ability to induce a T cell response in CCL2−/− and CCR2−/− mice fed OVA is mediated by macrophages and DC but not B cells (Fig. 6D). IL-4 and IL-10 do not appear to play a role in this process as their expression could not be detected in any of the samples. Taken together these data suggest that CCL2 may control oral tolerance through up-regulation of TGF-β together with down-regulation of IL-12 resulting in suppression of an inflammatory immune response.

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decreased TGF-β expression when cocultured with TCR transgenic indicator T cells compared with APC from OVA-fed wild-type mice (Fig. 5, A–C). Collectively these results indicate that CCL2 plays a role in the induction of high dose oral tolerance by regulating the mucosal IL-12 and TGF-β balance.

CCL2 could be functioning at a number of levels to control high dose oral tolerance, including the inductive and maintenance phases. The inductive events following feeding of an Ag take place in the Peyer’s patch (44). In fact, TGF-β, IL-4, and IL-10 are all increased in the GALT of mice within 6 h after feeding Ag (45). In low dose tolerance, CCL2 is not a critical factor, however IL-4 production is impaired when CCL2 or its receptor are deleted (46). The former observation is consistent with what has been previously demonstrated (30). We decided to investigate the role CCL2 may play in the inductive events of high dose oral tolerance immediately following Ag feeding without subsequent Ag priming in the presence of adjuvant. We previously demonstrated that neutralization of CCL2 at the time of Ag feeding abrogated oral tolerance, increased IL-12 production and decreased IL-4 in the GALT (16). However, in those experiments, mice were primed with Ag in adjuvant and the cellular mechanism of CCL2 regulation was less clear. Based on our current results, we believe that CCL2 exerts its function through the mucosal-derived APC as peripheral APC showed no CCL2-dependent coordinate regulation of inflammatory and regulatory cytokines. Our present study implicates CCL2 as a direct effector molecule in oral tolerance, because knocking out its receptor has an identical effect on the tolerogenic state of the mouse.

Previous studies using the CCR2−/− mice suggested that there was a defect in type 1 (Th1) cytokine production (47). Two possibilities were offered to explain this result. The first, was a defect in T cell differentiation to a Th1 profile. The second possibility, and the more likely explanation, was that there may be differences in migration of APCs to the draining lymph nodes, thus promoting a Th1 phenotype of the effector cells (48) suggesting that there is no inherent Th1 defect in the CCR2−/− leukocytes. In the present report, we considered that there may be leukocyte trafficking differences in OVA-fed CCL2−/− and CCR2−/− mice. However, differences in migration of macrophages, dendritic cells, and B cells was not observed after high dose Ag feeding in the gut or peripheral lymphoid organs after (data not shown), leading us to conclude that the role CCR2 may play in our model of high dose oral tolerance is regulation of APC-derived cytokines.

The ability of mucosal-derived APC from CCL2−/− and CCR2−/− mice to stimulate an Ag-specific T cell response suggests that CCL2 may be regulating high dose oral tolerance through Ag presentation. This regulation could occur on a number of levels. First, regulation may function through alterations in costimulatory molecule expression and/or function. B7.2 (42) and CTLA-4 (43) have been well-documented as necessary for the induction of low Ag dose and high Ag dose oral tolerance, respectively. Whether the exact mechanism involves initial T cell stimulation before tolerance or direct activation of a down-regulatory signal is not well-understood. A possible link between chemokines and costimulatory molecule expression has been previously demonstrated; Taub et al. (26, 27) have shown that β chemokines function to enhance the expression of costimulatory molecules that may ligate CTLA-4 and turn off an immune response. However, we do not think CCL2 is functioning to regulate B7 expression on APC in the present system. Following Ag feeding, there were no differences in B7.1 and B7.2 expression on APC among CCL2−/−, CCR2−/−, or wild-type mice (data not shown).

Rather than affecting B7-dependent costimulation, we believe CCL2 is directly regulating mucosal cytokine expression. Previous work has shown that naïve T cells cultured with Ag and CCL2 skew to a Th2 phenotype (25, 49). More specifically, within models of oral tolerance, neutralization of CCL2 at the time of feeding led to an increase in IL-12 production and a subsequent decrease in IL-4 (16). There is also evidence that suggests inflammatory cytokines, as well as CCL2, may exist within a regulatory axis that includes TGF-β (38, 40, 50). Based on our observations, we favor a model where Ag is taken up by mucosal APC resulting in CCL2 up-regulation. The expression of CCL2 subsequently down-regulates IL-12 production by the APC and augments TGF-β production, resulting in Ag-specific immune tolerance. The increase in TGF-β secretion could be a direct effect of CCL2 rather than a secondary effect of suppressing IL-12. In support of this idea was the observation that rat lung fibroblasts stimulated with CCL2 had significantly higher amounts of TGF-β mRNA and these cells secreted TGF-β in a dose-dependent manner when incubated with increasing concentrations of CCL2 (51). Additionally, mice over-expressing IL-13 in lung tissue produce high levels of TGF-β in bronchoalveolar lavage fluid. However, when IL-13 transgenic mice are also CCR2-deficient there was a significant decrease in TGF-β, suggesting IL-13 induces CCL2 expression which in turn regulates TGF-β (52).

The idea that CCL2 may play a role in up-regulating TGF-β secretion and down-regulating IL-12 has very important consequences for oral tolerance and potential therapies for autoimmunity. TGF-β has long been implicated as a major factor in controlling immune responses as well as oral tolerance. TGF-β can suppress IL-2-dependent proliferation (52, 53), decrease macrophage activation (54), and can resolve or suppress an inflammatory response (55, 56). A reciprocal relationship between TGF-β and IL-12 is known to exist (40) where systemic administration of anti-IL-12 after Ag feeding increases TGF-β independent of IL-4 expression (38). This relationship exists even in cultures of naïve CD4+ T cells with IL-12 and TGF-β where the T cells show decreased IFN-γ production (57), suggesting that TGF-β is inhibiting the effects of IL-12 on Th1 development. The results reported by Gonnella et al. (46) for low dose oral tolerance in CCL2−/− and CCR2−/− mice suggest that CCL2 is important for induction of IL-4, but not for low dose oral tolerance, whereas in our work CCL2 suppresses IL-12 production leading to increased Th2 differentiation and TGF-β secretion. Although many reports suggest that TGF-β is a major effector cytokine involved in the mechanism of tolerance induction using repeated low dose feedings, the present report demonstrates TGF-β expression following a high dose of oral Ag. TGF-β may be functioning to suppress IL-2-dependent proliferation leading to anergy (58) and recent studies have begun to report the presence of CD4+CD25+ T regulatory cells in high dose oral tolerance (59). The mechanistic role of TGF-β in our model of high dose oral tolerance is currently under further investigation.

In summary, our findings show that CCL2 is a necessary factor for the induction of high dose oral tolerance. Removal of CCL2 or its receptor abrogate tolerance as demonstrated by Ag-specific T cell responses and inhibits the traditional immune deviation seen in oral tolerance. The mechanism that CCL2 may be regulating oral tolerance appears to be through regulation of both IL-12 and TGF-β and seems to be at the level of CD11b+ and/or CD11b+CD11c+ APCs.

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References


