Inhibition of Generation of Cytotoxic T Lymphocyte Activity by a CCL19/Macrophage Inflammatory Protein (MIP)-3β Antagonist

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Chemokines constitute a group of over 40 secreted peptides that are important for the control of leukocyte migration both during homeostasis and inflammation. Recent studies have implicated the ligands CCL19 and CCL21 and their receptor, CCR7, in the specific migration of naive lymphocytes and mature dendritic cells to secondary lymphoid organs during immune homeostasis. However, the role that these molecules play during immune priming is not well understood. In this study, using CCL19(8–83), a novel N-terminal truncation mutant, we have investigated the role of CCL19 in a primary allogeneic immune response, a response of particular relevance to transplant rejection. This antagonist specifically inhibited wild type CCL19-induced chemotaxis and intracellular calcium mobilization without affecting that of CCL21. The treatment of mice with CCL19(8–83) did not globally inhibit the recruitment of cells into lymph nodes; however, it inhibited the generation of cytotoxic T lymphocytes toward allogeneic dendritic cells. This is the first evidence that CCL19 plays a role in immune priming.

The rapid initiation of a primary antigen-specific T cell immune response is dependent on the coordinated and efficient interaction between antigen presenting DCs and naive T cells. This process is promoted by colocalization of these cells in specific microenvironments within secondary (2°) lymphoid tissues. Following antigen-specific activation, effector lymphocytes must also be guided to the site of infection. Members of the chemokine superfamily play a critical role in these processes (1, 2). Chemokines can be classified into two groups based on expression and function: 1) inflammatory/inducible and 2) homeostatic/constitutive (3–6). In general, inflammatory chemokine expression is induced at peripheral sites in response to inflammatory stimuli where they regulate recruitment of effector leukocytes into the tissue. In contrast, homeostatic chemokines are constitutively expressed within lymphoid organs where they regulate lymphocyte and DC trafficking. In particular, they are involved in maintaining homeostatic trafficking and positioning of cells both within 2° lymphoid organs and in the peripheral tissues for antigen sampling and immune surveillance (5, 7).

Two chemokines of particular importance in lymphocyte and DC trafficking are the homeostatic chemokines CCL19 macrophage inflammatory protein (MIP)-3β and CCL21 (secondary lymphoid organ chemokine), which share a common receptor, CCR7 (2, 8, 9). Both CCL19 and CCL21 are constitutively expressed in lymphoid organs, particularly by cells throughout the T cell zones (10–13). In addition to this observation, CCL21 is also expressed in high endothelial venules in the lymph nodes and in lymphatic endothelial cells lining the afferent lymphatics in peripheral tissues. Studies on CCR7−/− and plt (paucity of lymph node T cells) mice have demonstrated the importance of these ligands and CCR7 in the correct development of microarchitecture within 2° lymphoid organs as well as in naive T cell and DC homing to lymph nodes (1, 14, 15). However, using these models, it has proven impossible to distinguish between the roles of CCL21 and CCL19 in the immune system. The plt mouse is deficient in both CCL21 and CCL19, and the CCR7−/− mouse does not allow discrimination between the two ligands. The results of recent studies using neutralizing anti-CCL21 antibodies indicate the importance of CCL21 for mature DC trafficking from the skin to draining lymph nodes and in the development of delayed-type hypersensitivity responses to hapten (16, 17), and transgenic studies have demonstrated that ectopic expression of either CCL19 or CCL21 under the control of the rat insulin promoter in the pancreas results in lymphocyte and dendritic cell recruitment and lymphoid neogenesis (15, 18, 19). However, the biological function of CCL19, particularly the question of whether or not it is redundant to CCL21, remains unresolved.

The use of synthetic chemokine antagonists has proven useful in elucidating the role of specific chemokines in several different aspects of the immune response. The N-terminal deletion converts CCL2 monocyte chemotactic protein (MCP)-1 and CCL5 (RANTES (regulated on activation normal T cell expressed and secreted)) into the antagonists of native CCL2, CCL5, CCL8 (MCP-3), and CCL3 (MIP-1α), respectively (20, 21), whereas retention of the initiating methionine converts CCL5 into a receptor antagonist (22). These antagonists have been shown to prevent the entry of human immunodeficiency virus into target cells (23, 24) and to inhibit the development of arthritis in animal models (25, 26). In addition, we have shown that truncated CXCL1 (gro) and CXCL4 (platelet factor 4) analogs antagonizing mUCXCR2 inhibit neutrophil recruitment to subcutaneous tissue and the peritoneal cavity in response to pro-inflammatory agents (27). We now report the development of a specific CCL19 antagonist that has enabled us to examine the role of CCL19 in vivo, independently of CCL21. Using this antagonist, we demonstrate a role for CCL19 in CD8+ T cell activation in response to allogeneic DCs.
The results of these studies indicate for the first time an important non-redundant role for CCL19 in the allogeneic immune response.

MATERIALS AND METHODS

Reagents—All of the synthetic murine peptides used were made as described previously (29). The CCL24 peptide control peptide was also a synthetic peptide, the sequence of which was identical to human CCL24, with the exception that the four conserved cysteines of the native peptide were substituted with alanines (29). Human CCL19-C4 was a generous gift from Dr. Timothy Springer (Center for Blood Research, Harvard Medical School, Boston, MA).

Cell Lines—Female BALB/c nude mice (H-2d), aged 8–12 weeks, were obtained from the Central Animal House at the University of Adelaide, South Australia. The animals were housed in conventional rodent rooms at the University of Adelaide where they were provided with food and water ad libitum. A conditionally immortalized dendritic cell line transgenic for the thermolabile mutant of the SV40 large T antigen, raised from a CBA background (H-2k), was used to induce an allogeneic immune response in BALB/c mice (28).

In Vitro Chemotaxis Assay—Transwell chemotaxis assays using primary splenocytes were conducted as previously described (29). Single cell suspensions were placed in RPMI 1640 medium supplemented with 10% fetal calf serum. Following 24 h of culture, cells were labeled by incubating with Calcein-AM (2 × 10−5 M of final concentration; Molecular Probes) for 30 min at 37 °C. Cells were resuspended to 5 × 106 viable cells/ml in chemotaxis buffer and subjected to Transwell chemotaxis assays (6.5-mm-diameter filter, 5 μm pore size, Corning, NY). 600 μl of chemotaxis buffer containing the indicated concentration of CCL19, CCL21, and/or chemokine mutant were added to the lower chambers of a Transwell plate. After adding 100 μl of labeled cells to the upper chambers, the assay was conducted for 3 h at 37 °C and cells were collected from the lower chamber. The cells in the lower chamber were quantified by transferring to a 96-well microtiter tray and measuring fluorescence. The percentage migration of cells was calculated by comparison with a standard curve obtained from cells added at known decreasing concentrations directly to the lower chamber of the Transwell plate.

Induction and Manipulation of the Allogeneic Immune Response—Confluent flasks of the tsDC cell line were differentiated at 39 °C for 24 h and then detached from the surface of the flask, washed, and resuspended to 2 × 106 viable cells/ml in endotoxin-free PBS. BALB/c mice were injected with 25 μl of cells subcutaneously per footpad. Control footpads received 25 μl of endotoxin-free PBS. On the day following injection with PBS or tsDCs (day 1), brdomyoeyrinidium (Sigma) was dissolved in water to 0.8 mg/ml and fed to the mice ad libitum for 2 days before replacement with fresh solution on day 3. One day prior to injection with either PBS or tsDCs, half of the mice were injected intraperitoneally with 100 μg of CCL19, and the other half were treated with 100 μg of CCL21. This treatment was repeated every second day until termination of the experiment. At days 2 and 5 post-injection, the mice were sacrificed and the draining lymph nodes (popliteal) were removed for analysis.

Cytotoxicity Assay—The target cells, tsDCs, cultured at 33 °C were resuspended in PBS and labeled with the fluorescent dye, Calcein-AM (2 μm of final concentration), in a 37 °C water bath for 40 min. Following two washes in PBS, subconfluent monolayers of labeled target cells were generated in 96-well trays by aliquoting 5 × 105 viable tsDCs into each well in a 100-μl volume. The cells were allowed to adhere for at least 5 h, after which the medium was aspirated and 200 μl of effector cells (lymphocytes from extracted nodes) in RPMI 1640 medium, 5% fetal calf serum were added at a concentration of 5 × 104 cells/well. The assay was incubated for 48 h at 37 °C, and then the culture medium was aspirated and lymphocytes and target cell debris were washed away by immersing trays in four consecutive baths of PBS. 100 μl of PBS was added to each well, and degree of target cell monolayer remaining was quantified by measuring fluorescence emission at 520 nm on a Molecular Imager FX (Bio-Rad), allowing the percent cytotoxicity to be determined.

Calcium Mobilization—Cells were resuspended at 2 × 106 viable cells/ml in Hanks’ balanced salt solution and incubated with Fura-2/AM (2 μm of final concentration; Molecular Probes) for 30 min at 37 °C. Calcium mobilization was assayed after one or sequential stimulation with chemokines or chemokine analogs by recording intracellular calcium-related fluorescence changes on an Aminco-Bowman Series 2 luminescence spectrometer. Displacement Assay—Cells were resuspended to 4 × 106 viable cells/ml in PBS containing 0.04% sodium azide and 1% bovine serum albumin (mouse staining buffer). Fc receptors were blocked by incubation for 30 min at room temperature with 50 μg/ml of murine γ-globulin, and then 50 μl of cells were aliquoted into round-bottomed polystyrene tubes. Cells were then incubated with CCL19-Fc for 10 min on ice followed by an additional 10-min incubation on ice with increasing concentrations of the ligands CCL19, CCL21, CCL19(Asn→Glu) or CCL19(Asp→Glu). The cells were then washed with mouse-staining buffer before being incubated with fluorescein isothiocyanate-conjugated anti-human IgG and incubated for 30 min on ice. Cells were then washed twice in mouse-staining buffer followed by a rinse in protein-free staining buffer and immediately analyzed by flow cytometry. Labeled cells were analyzed on a BD Biosciences FACS Canto, and the data were analyzed using FlowJo WEASEL software (Treestar).

RESULTS AND DISCUSSION

To develop the assay for characterization of an antagonist, chemotactic dose response curves to both muCCL19 and muCCL21 were generated in primary mouse splenocytes. The expected bell-shaped curves were obtained in response to both CCL19 and CCL21 (Fig. 1A). CCL19 induced detectable migration at 0.01 μM and optimal chemotaxis at 0.3 μM, affecting the migration of ~50% of the total input cells. In comparison, CCL21-induced migration was detectable between 0.1 and 0.3 μM and optimal chemotaxis required 3 μM CCL21. However, over a series of eight individual experiments, this resulted in the migration of 20% more cells than that observed in response to CCL19 (i.e. 70% of total input cells). In a parallel set of experiments, flow cytometric analysis was performed on cells harvested from Transwell chemotaxis assays to test whether CCL19 or CCL21 induced selective migration of CD4+ or CD8+ T lymphocytes. No selective migration of either cell type was observed when comparing the percentage of input cells with the percentage of CD4 and CD8 cells obtained from the bottom chamber of the Transwell plate (data not shown). Concentrations of 0.2 and 0.5 μM were chosen for CCL19 and CCL21, respectively, for in vitro chemotaxis inhibition assays to identify and characterize a CCL19 antagonist. A series of truncation mutants of muCCL19 were synthetized and analyzed for their ability to antagonize CCL19- and/or CCL21-induced chemotaxis. These mutants were truncated at the N terminus, generating a series of seven mutants (Fig. 1B). The shortest mutant, CCL19(8–83), commenced at the first conserved cysteine and this cysteine was acetylated to increase stability of the truncated protein. The four longest mutants (CCL19(8–83)–CCL19(8–83)) were each able to directly induce chemotaxis in a manner similar to that of wild type CCL19. In contrast, neither CCL19(8–83), CCL19(7–73), nor CCL19(6–83) induced chemotaxis of naive splenocytes (Fig. 1C). Therefore, these three latter mutants were tested as potential antagonists of CCL19-mediated chemotaxis (Fig. 1D). Each of these mutants inhibited CCL19-mediated chemotaxis. However, the optimal concentration varied among the mutants with the general trend being the shorter the mutant the lower the concentration required to inhibit chemotaxis. CCL19(8–83), was the least agonistic mutant and was a highly effective antagonist of CCL19-mediated chemotaxis when compared with the other two mutants and therefore was selected for a further more detailed analysis. Over a large number of experiments, CCL19(8–83) consistently and significantly inhibited wild type CCL19-mediated chemotaxis at a concentration as low as 1.5
Fig. 1. Development and characterization of mCCL19 truncation mutants. A, splenocytes were purified from BALB/c mice and incubated in RPMI 1640 medium. Following 24 h of culture, cells were stained with 2 μM Calcein-AM and subjected to Transwell chemotaxis assays using mCCL19, mCCL21, or chemotaxis buffer alone. The percent migration was determined fluorometrically as described under "Materials and Methods." Values are the mean ± S.E. (CCL19, n = 12; CCL21, n = 8). B, the CCL19 mutants used in this study. C, the ability of CCL19 mutants to stimulate chemotaxis of primary mouse splenocytes (n = 2 for CCL19_6–83 and CCL19_7–83; n = 4 for CCL19_8–83). D, the ability of CCL19(6–83), CCL19(7–83), and CCL19(8–83) to antagonize wild type CCL19-induced chemotaxis (n = 2 for CCL19_6–83, and CCL19_7–83; n = 14 for CCL19_8–83). E, lack of antagonism of CCL19_8–83 toward wild type CCL21 (n = 4).

μg/ml (7.5-fold excess of antagonist w/w or 7.5:1 ratio). When the antagonist was used at a 30:1 ratio, chemotaxis was inhibited by ~65%. At 50 μg/ml (250:1 ratio), CCL19_8–83 almost completely inhibited CCL19-mediated chemotaxis (approximately 83%). To examine the specificity of antagonism of CCL19-mediated chemotaxis, a control peptide, CCL2_4Ala, in which the conserved cysteines were mutated to alanine was used in these experiments. CCL2_4Ala did not inhibit CCL19-induced cellular migration (Fig. 1E). Because CCL19 and CCL21 both interact with CCR7, the effect of CCL19_8–83 on CCL21-induced chemotaxis was investigated. CCL19_8–83 did not antagonize CCL21-mediated chemotaxis (Fig. 1E). Furthermore, CCL19_8–83 had no effect on the ability of the closely related CC chemokine, CCL20 (MIP-3α), to induce chemotaxis (data not shown). Finally, it was found that CCL19_8–83 inhibited CCL19-induced chemotaxis of both CD4+ and CD8+ T cells (data not shown).

A CCL19-Fc fusion protein was generated, which was able to bind CCR7 in the same manner as its native ligands, with the fused Fc portion, allowing detection by flow cytometry. A dose-dependent binding curve for CCL19-Fc to splenocytes was generated (data not shown). The dose resulting in maximum binding was chosen for further studies in which the ability of CCL19, CCL21, or CCL19_8–83 to displace the fusion protein was examined. Cells were preincubated with CCL19-Fc followed by an incubation with increasing concentrations of the native ligands (CCL19 and CCL21) or the CCL19 analog (CCL19_8–83). The cells then were analyzed by flow cytometry to determine the level of binding of CCL19-Fc. In the absence of competitive ligand, ~40% of cells from a spleen were found to be positive for CCL19-Fc (Fig. 2A and B). CCL19, CCL21, and CCL19_8–83 were all able to displace the CCL19-Fc in a dose-dependent manner with the order of potency being CCL19 > CCL19_8–83 > CCL21 (Fig. 2C). However, the control peptide, CCL2_4Ala, did not displace the fusion protein even at the highest concentration tested. Overall, these data indicate that CCL19_8–83 displaces wild type CCL19 to approximately the same extent as does CCL21.

Consistent with that observed in the chemotaxis assays, the mobilization of intracellular calcium induced by CCL19 was inhibited by CCL19_8–83, whereas CCL21-induced calcium mobilization was not inhibited (Fig. 3). Furthermore, CCL19_8–83 alone did not induce calcium mobilization, confirming its lack of agonistic activity. When cells were treated with wild type CCL19 or wild type CCL21, a consistent increase in the level of intracellular calcium was observed (Fig. 3, A and B). Prior exposure of the cells to the control peptide, CCL2_4Ala, had no effect on the ability of either of the native ligands to induce
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Calcium mobilization, demonstrating that the control peptide was unable to nonspecifically antagonize the CCR7 ligands (Fig. 3C, D). However, when cells were treated with CCL19 (8–83) prior to treatment with the native ligands, the increase in intracellular free calcium induced by wild type CCL19 was completely inhibited (Fig. 3E), whereas the increase in intracellular free calcium induced by wild type CCL21 was unaffected (Fig. 3F). A summary of three experiments is shown in Fig. 3G.

Previously, CCL19 and CCL21 have been indistinguishable in terms of their individual functions within the immune system; therefore, the ability of CCL19 (8–83) to antagonize CCL19 but not CCL21 enabled the examination of the role of CCL19 independently of CCL21. To investigate this finding, an allogeneic immune response was induced in BALB/c mice by injection of foreign dendritic cells (CBA-derived) into the footpads of mice that had been treated with either the control peptide, CCL2 (4Ala) or CCL19 (8–83). The draining lymph nodes were extracted, and various parameters were determined. In preliminary experiments, the appropriate dose of alloDCs and the time course of the allogeneic response were assessed and days 2 and 5 post-immunization were chosen for further analysis (data not shown).

Mice were either untreated (no chemokine treatment, no alloDCs) or treated with 100 μg (intraperitoneal injection) of either CCL2 (4Ala) (control) or CCL19 (8–83) the day prior to footpad injection of alloDCs. The chemokines were re-administered every 2 days for the duration of the experiment. This dosing regimen was chosen on the basis of our previous experience with other chemokine mutants (25, 27).

The weight of the lymph nodes and the number of viable cells present were assessed on day 2 post-immunization with alloDCs in mice treated as described above with either CCL2 (4Ala) or CCL19 (8–83) (Fig. 4A, data not shown for lymph node weight). These data were compared with the same parameters found in naïve mice (control). The number of cells was found to be the same in all of the conditions tested, although there was a trend (not statistically significant) for more cells to be present in the nodes from CCL19 (8–83)-treated mice. In contrast, the immunization of mice with alloDCs for 5 days resulted in a significant increase in the lymph node weight and the number of viable cells in the draining lymph nodes in animals treated with either CCL2 (4Ala) or CCL19 (8–83) (Fig. 4B; data not shown for lymph node weight). Moreover, treatment with CCL19 (8–83) exhibited the same trend toward increasing the number of viable cells present in the draining lymph nodes upon immunization.

Cytotoxic activity of draining lymph node cells toward alloDCs in vitro was increased by both days 2 and 5 post-immunization with alloDCs (Fig. 5, A and B). However, the level of cytotoxic activity was significantly greater on day 5 compared with day 2. In contrast to that observed with respect to cellular accumulation in the draining lymph nodes, cells isolated from mice treated with CCL19 (8–83) exhibited a significantly lower level of cytotoxicity toward the alloDCs when compared with CCL2 (4Ala)-treated mice.

The majority of information on the in vivo function of the CCL19/CCL21/CCR7 system has been derived from studies on either CCR7−/− mice or plt mice, neither of which distinguish between CCL19 and CCL21 (1, 14, 15). Studies in transgenic mice have revealed that overexpression of either CCL19 or CCL21 leads to lymphoid neogenesis (15, 18, 19). However, to date, information defining the specific roles for CCL19 in vivo...
during the generation of an immune response is lacking. The results of the present study using a specific CCL19 antagonist provide important and novel insights into the biology of CCL19. First, our ability to specifically target CCL19 using a novel CCL19 antagonist has revealed an important role for CCL19 in lymphocyte and lymph node biology that is independent of CCL21. Second, CCL19 appears to play an important role in CTL priming because the inhibition of CCL19 activity during a primary allogeneic response decreases the level of cytotoxicity toward alloDCs.

CCL19<sub>8-83</sub> clearly antagonized wild type CCL19 activity toward naïve splenocytes, displacing the binding of CCL19-Fc and inhibiting wild type-induced chemotaxis and calcium mobilization. However, this antagonist did not inhibit the stimulatory effects of CCL21 on naïve splenocytes. In a recent study, a CCL21 antagonist that inhibited both chemotaxis and calcium mobilization induced by CCL21 was identified (30). In that study, it was also shown that the administration of the antagonist significantly reduced the development of graft-versus-host disease in mice (30) and the authors concluded that CCL21 played an important role in graft-versus-host disease. However, in preliminary studies, we have synthesized and characterized CCL21 mutants and have demonstrated that the N-terminal truncation mutants of CCL21 not only inhibit CCL21-mediated chemotaxis but also CCL19-mediated chemotaxis (data not shown). Therefore, because the antagonist used in the study by Sasaki et al. (30) does not discriminate between the functions of the two ligands, it is still possible that the
inhibition of graft-versus-host disease observed in that study may be due to antagonism of CCL19 and not CCL21.

The mechanism by which CCL19(8–83) interferes with CTL generation is not clear. The phenotype we observed using this antagonist was not what would be predicted based on the results of studies on the CCR7−/− and plt mice. Those mice displayed severely disrupted lymphoid organ architecture with a complete lack of defined T and B cell zones and significantly fewer lymphocytes in the secondary lymphoid organs. DC trafficking to the T cell zones was also severely affected. Despite this finding, a chronic absence of the CCR7 system does not inevitably lead to the inhibition of the immune response. Although antibody, contact sensitivity, and delayed-type hypersensitivity responses are diminished in CCR7−/− mice (1), CTL and antibody responses to LCMV, VV, and VSV are normal in the plt mouse (31). The increased CTL response against the latter viruses observed in the plt mouse suggests that either the role of CCL19 in viral immunity is different from that in allogeneic immunity (in which we observed a decrease in CTL activity) or a chronic absence of both CCL19 and CCL21 leads to a fundamentally different outcome compared with the pharmacological intervention used in the present study. The results of our studies indicate that the administration of CCL19(8–83) does not significantly alter the number of lymphocytes within the draining lymph node following immunization (Fig. 4). Moreover, extensive immunohistochemistry experiments revealed no discernable alteration in the distribution of either CD8+ or CD4+ T cells within the draining lymph nodes. Together, these observations suggest a more subtle effect on the immune response than that observed in either of the mutant mouse strains.

The patterns of expression of CCL19 and CCL21 may provide some insight into our observations. CCL21 is expressed in the draining lymphatics, on high endothelial venules, and by stromal cells in the T cell zones of the secondary lymphoid organs (15).
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CCL19 is also expressed by stromal cells in the T cell zones (13). However, interdigitating DCs appear to also produce CCL19 (14, 32). Therefore, it is possible that CCL19 production by interdigitating DCs is critical for the appropriate positioning of naive and recently activated T cells in the T cell zones for efficient clonal selection and expansion to occur. Such a function would explain that antagonism of CCL19 during the allogenic immune response does not prevent T cells from entering the 2nd lymphoid organs and the T cell zones (CCL21 is still active). However, if CCL19 is critical for the multiple T cell-DC interactions that are required for effective clonal selection and differentiation of T cells to occur, antagonism could inhibit CTL generation.

In summary, the results of this study using a novel antagonist provide important insights into the biological role of the homeostatic chemokine CCL19. Previously, it has been impossible to discriminate between the effects of CCL21 and CCL19; however, the development of this specific antagonist provides a useful tool not only for the examination of the CCL19/CCL21/CCR7 system but also as a proof-of-concept for therapeutic modulation of the immune system.

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