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Souphalone Luangsay
Dartmouth College

Lloyd H. Kasper
Dartmouth College

Nicolas Rachinel
Dartmouth College

Laurie A. Minns
Dartmouth College

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CCR5 Mediates Specific Migration of *Toxoplasma gondii*–Primed CD8$^+$ Lymphocytes to Inflammatory Intestinal Epithelial Cells

SOUPHALONE LUANGSAY,* LLOYD H. KASPER,* NICOLAS RACHINEL,* LAURIE A. MINNS,* FRANCK J. D. MENNECHET,* ALAIN VANDEWALLE,† and DOMINIQUE BUZONI–GATEL§

*Departments of Microbiology and Immunology and Medicine, Dartmouth Medical School, Lebanon, New Hampshire; †INSERM Unité 478, Faculté de Médecine Xavier Bichat, Paris, France; and §Département de Parasitologie, Institut Pasteur, and Département de Santé Animale, Institut National de la Recherche Agronomique, Paris, France

Background & Aims: ***Toxoplasma gondii*, an obligate intracellular parasite, can invade intestinal epithelial cells and elicit a robust Th1 immune response. In this model of intestinal inflammation, CD8$^+$ intraepithelial lymphocytes (IELs) secrete transforming growth factor (TGF)-β, which appears necessary for the maintenance of homeostasis in the intestine. However, the mechanism responsible for the IEL migration to the inflamed intestine is still unclear. *Methods:* An in vitro coculture cell system was used to quantify the IEL attraction by an infected intestinal epithelial cell line (m-IC$_{cl2}$). We used CCR5-deficient mice to determine which chemokine receptor–chemokine interaction could be responsible for the recruitment of antigen-specific CD8$^+$ IELs to the small intestine for the promotion of parasite clearance and host recovery. *Results:* We observed increased expression of several chemokine receptors (CCR1, CCR2, CCR5, CXCR3) in the infected ileum. In particular, CCR5 expression was markedly increased in antigen-primed CD8$^+$ IELs. Experiments using recombinant chemokines as well as blocking antibodies showed that macrophage inflammatory protein (MIP)-1α and MIP-1β were critical for their homing. CD8$^+$ IELs isolated from CCR5-deficient mice (CCR5−/−), despite their high production of TGF-β and overexpression of activation markers, were impaired in their ability to migrate in vitro to the m-IC$_{cl2}$ monolayer or in vivo to the inflamed intestine after adoptive transfer. *Conclusions:* Our data emphasize the biologic role of CCR5 as an important component in the migration of intraepithelial CD8$^+$ T cells and the regulation of the inflammatory response following parasite infection.**

A principal biologic function of the intestinal mucosal surface is to maintain immunologic homeostasis in response to foreign antigens. The enterocytes lining the villus wall of the intestine form a tight barrier against potentially invasive pathogens. Chemokines secreted at the surface of intestinal epithelial cells (IECs) play a critical role in the initiation and modulation of the immune response to various pathogens.[1] In humans, IECs from inflamed intestines secrete inflammatory cytokines; in mice, intestinal lesions due to invasive pathogens are more frequently characterized by acute neutrophil infiltrations associated with increased levels of macrophage inflammatory protein (MIP)-2 produced by infected IECs.[2,3] Other chemokines may act as chemoattractants for macrophages/monocytes/neutrophils and/or lymphocytes.[4]

Intestinal intraepithelial lymphocytes (IELs), mainly composed of CD8α$^+$ subsets of lymphocytes (CD8αα$^+$ and CD8αβ$^+$ IELs), are present in the gastrointestinal tract and express chemokine receptors, including CCR2, CXCR3, CCR5, and CCR9. Some of these receptors are up-regulated during intestinal inflammation and are perhaps critical in lymphocyte localization within intestinal mucosa. Recently, a specialized interaction has been described between both CD8$^+$ IELs and CD4$^+$ T cells from the lamina propria expressing CCR9 and IECs through the interaction with its ligand, the thymus-expressed chemokine (CCL25), which appears to be highly expressed in crypt intestinal cells.[5,6] Such specific chemokine-lymphocyte receptor interaction in the normal gastrointestinal tract suggests that the expression of chemokines by differentiated epithelium represents an

Abbreviations used in this paper: CFSE, 5(6) carboxyfluorescein diacetate, succinimidyl ester; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; IL, interleukin; IP-10, interferon γ–inducible 10-kilodalton protein; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; RANTES, regulated upon activation, normal T cell expressed and secreted protein; TGF, transforming growth factor; WT, wild-type.

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important mechanism for specialized immune responses. This interaction may allow for homeostatic balance in the normal gut.[6,7]

Toxoplasma gondii, an obligate intracellular parasite acquired by oral infection and responsible for acute and lethal ileitis in susceptible C57BL/6 mice, can invade IECs, eliciting a robust immune response that is associated with increased production of chemokines.[8,9] In *T. gondii*–infected and –inflamed intestines, the composition of infiltrating cells is predominantly represented by $CD8^+$ IELs and $CD4^+$ lymphocytes, which confer the main components of protective immunity to *T. gondii*.[9–11] The $CD8^+$ IELs have been shown to promote host recovery and parasite clearance. When adoptively transferred, infiltrating CD8αβ TCRαβ IELs provide long-term immunity and protect mice against a lethal parasite challenge in a transforming growth factor (TGF)-β–dependent manner.[8,10] The preferential recruitment of these cells to infected intestinal sites appears to be critical for the control of the inflammatory cellular response.[12] Although the α4β7 and αEβ7 integrins expressed at the cell surface of lymphocytes may partially explain IEL trafficking,[12] other molecules including IEC-expressed chemokines may act as chemoattractants.[8,9]

In this study, we show that the secretion of beta chemokines MIP-1α (CCL3) and MIP-1β (CCL4) by enterocytes can orchestrate the recruitment of $CD8^+$ IELs via CCR5 to enhance the immune response against *T. gondii* infection. These results show the immunologic potential of IECs expressing specific chemokines for the trafficking and control of intestinal IELs in this pathogen-driven model of intestinal inflammatory disease.

Materials and Methods

Mice and *T. gondii* Infection

Six- to 8-week-old female C57BL/6 mice and B6129F2/J-Cmkbr5tmlKuz (CCR5−/−) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). CCR1-deficient mice (CCR1−/−)[13] were backcrossed to the C57BL/6 background for 6 generations and housed under approved conditions of the Animal Research Facility at Dartmouth Medical School (Lebanon, NH). Age- and sex-matched B6129PF2/J mice (Jackson Laboratory) were used as control wild type (WT) for CCR5−/− mice. Mice were infected orally with 35 cysts from the 76K strain of *T. gondii*.

Lymphocyte Isolation

Unprimed or primed IELs were isolated from 4–8 naive or day-7 postinfected C57BL/6, WT, CCR5−/−, or CCR1−/− mice as previously described.[11] $CD8\alpha^+$ and $CD8\beta^+$ IELs were purified by incubation with either anti-CD8α microbeads (Miltenyi Biotec, Auburn, CA) or anti-CD8β antibody (BD Pharmingen, San Diego, CA) followed by anti-rat immunoglobulin G microbeads (Miltenyi Biotec) and separated with a magnetically activated cell sorting column (Miltenyi Biotec). $CD8\alpha^+$ and $CD8\beta^+$ staining (BD Pharmingen) confirmed 98% pure populations. Surface expression was assessed with CCR5, αE (CD103), CD25, Ly-6C, and Ox40 (CD134) (BD Pharmingen).

Culture of m-IC_{cl2} Cells and Infection by *T. gondii*

IECs from the m-IC_{cl2} cell line (C57BL/6, H-2^b)[14] were seeded upside down on collagen I–coated Transwell filters (5×10^5 cells/filter; ID, 6.5 mm; pore size, 3 μm; Costar Transwell, Fisher Scientific, Pittsburgh, PA) and cultured for 10 days in a modified defined medium as previously described.[8,9] The integrity of the infected monolayer was confirmed microscopically before each experiment and infected with 2:1 parasites cells. For the in vitro infection, tachyzoits from the RH strain of *T. gondii* were used. All experiments were performed on 3 separate filters for each condition tested and then repeated in at least 3 different experiments.

In Vitro Migration Assay

Unprimed and primed $CD8\alpha^+$ IELs incubated with a cell dye 5-chloromethylflurorescein diacetate (10 μmol/L) (Molecular Probes, Eugene, OR) were added to the upper chamber of confluent m-IC_{cl2} cells grown on Transwell as previously described (5×10^5 IELs/filter; Costar Transwell) without or with blocking antibodies to MIP-1α and MIP-1β (1 and 10 μg/mL) or with their irrelevant control anti-goat immunoglobulin G (R&D Systems, Minneapolis, MN). m-IC_{cl2} cells and IELs were incubated for 4 hours at 37°C. To visualize the IELs in the monolayer, filters were stained with propidium iodide and rhodamine-phalloidin (Molecular Probes) and observed by confocal laser scanning microscopy (MRC 1024; Bio-Rad, Hercules, CA). Ten fields (481 × 481 μm) were examined for each condition. The average number of 5-chloromethylflurorescein diacetate–tagged IELs was quantified, and all experiments were performed at least 3 times for each condition tested.

In Vivo Migration Assay

Primed and unprimed $CD8\alpha^+$ IELs were incubated with 5(6)-carboxyflurorescein diacetate, succinimidyl ester (CFSE) 15 μmol/L (Molecular Probes). Labeled IELs (5×10^6) were injected intravenously into the tail of WT recipient mice (3 mice per group) orally challenged 24 hours later by *T. gondii*. IELs from recipient mice were purified 48 hours after challenge, and the percentage of CFSE-labeled IELs was analyzed by flow cytometry.

Chemotaxis Assay

IELs (5×10^5) were added in the upper chamber of Transwell filters (ID, 6.5 mm; pore size, 3 μm; Costar Transwell) without the m-IC_{cl2} monolayer. The lower chamber was filled with conditioned medium from infected m-IC_{cl2} cells or with medium supplemented with various concentrations of RANTES, MIP-1α, and MIP-1β (1, 10, or 100 ng/mL) (R&D Systems). IELs recovered in the lower chamber after 2 hours were counted by flow cytometry. A known number (2×10^5) of beads (Nile red, 10–14 μm size; Spherotech, Libertyville, IL) was added to each sample before fluorescence-activated cell sorter counting to determine the absolute number of migrating cells. The percentage of migrating cells per condition was calculated as follows: % Migrating IELs = Absolute Number of Migrating Cells/Total Cells Added to the Upper Chamber; Migration Index of IELs = % Migrating Cells With Chemokine/% Migrating Cells With Chemokine-free Medium.

Chemokine and Cytokine Expression

TGF-β secretion from 24-hour cultured IEL (10^6) supernatants was assessed by enzyme-linked immunosorbent assay (R&D Systems) following the manufacturer's instructions. Total RNA was isolated using TRIzol reagent according to the manufacturer's protocol (Life Technology, Grand Island, NY). Specific messenger RNA (mRNA) expression from whole small intestines and isolated IELs was evaluated using a RiboQuant Multi-Probe Ribonuclease Protection Assay System Kit (BD Pharmingen) as described by the manufacturer. A total of 10 μg of RNA from intestine or IELs was analyzed. Bands were quantified by densitometric analysis with NIH Image (National Institutes of Health, Bethesda, MD) normalized to L32 and glyceraldehyde-3-phosphate dehydrogenase gene expression.

Histologic Assessment of Intestinal Inflammation

Intestines (3 per group) were immediately fixed overnight at 4°C in 10% formalin. Samples were embedded in paraffin, cut into longitudinal and cross sections, and stained with H&E for histologic examination. All morphologic observations were from 4 different experiments with at least 100 villi evaluated per sample. Only specimens exhibiting longitudinally oriented sections through the crypts were measured. Histologic inflammatory score ranging from 0 to 4 as previously described[15] was applied in a blinded fashion to estimate intestinal inflammation: 0, no inflammation; 1, slight infiltrating cells in lamina propria with focal acute infiltration; 2, mild infiltrating cells in the lamina propria with increased blood flow and edema; 3, diffuse and massive infiltrating cells leading to disturbed mucosal architecture; 4, crypt abscess and superficial necrosis of the intestinal villi.

Parasite Load

DNA was extracted from small intestine using the DNeasy Tissue Kit (Qiagen, Valencia, CA). A total of 50 μg of total DNA from tissues was analyzed using primers specific for *Toxoplasma* B1 gene[16,17] (SYBR Green PCR Core Reagents; PE Biosystems, Warrington, England). A standard curve using the known parasite number was used to determine actual parasite burden.

Statistical Analysis

Results are expressed as the mean ± SD. Statistical differences between groups were analyzed using the Student t test. A value of $P < 0.01$ was considered significant.

Results

Chemokine Receptor and Chemokine Expression in Response to *Toxoplasma gondii* Infection

Tachyzoite-infected m-IC_{cl2} cells secrete a large array of chemokines.[8,9] To investigate the functional relevance of this response in the small intestine, the expression of chemokines and chemokine receptors were analyzed in naive and day-7 *T. gondii*–infected mice. Small intestines from naive C57BL/6 mice (without *T. gondii*) displayed no detectable MIP-1β, MIP-2, and monocyte chemoattractant protein (MCP)-1 mRNA expression and low levels of RANTES, MIP-1α, and interferon gamma–inducible 10-kilodalton protein (IP-10) mRNA expression (Figure 1*A*). In contrast, small intestines from infected mice (with *T. gondii*) displayed a significant increase in MCP-1 and IP-10 mRNA expression and, to a lesser extent, MIP-1α, MIP-1β, MIP-2, and RANTES mRNA (Figure 1*A*). Consistent with the overproduction of their ligands, parasite infection in the whole intestine (with *T. gondii*) resulted in a significant increase in CCR5 expression, the receptor for RANTES, MIP-1α and MIP-1β, CCR2, the ligand for MCP-1, and, to a lesser extent, CCR1, the ligand for RANTES, and MIP-1α (Figure 1*B*). CXCR3 mRNA was weakly expressed in naive and infected mice despite the overexpression of its ligand IP-10 (Figure 1*A* and *B*). Additionally, CCR9 and CXCR2 expression were equally expressed in naive and infected mice (Figure 1*B*). Neither CCR9 expression (Figure 1*B*) nor its ligand TECK expression in both small intestine and m-IC_{cl2} IEC line (data not shown) were modified after infection.

Chemokine receptor expression in isolated IELs was also analyzed and compared with the chemokine profile (Figure 1*C*). The levels of CCR5 expression and, to a lesser extent, CCR2 expression were greater in primed

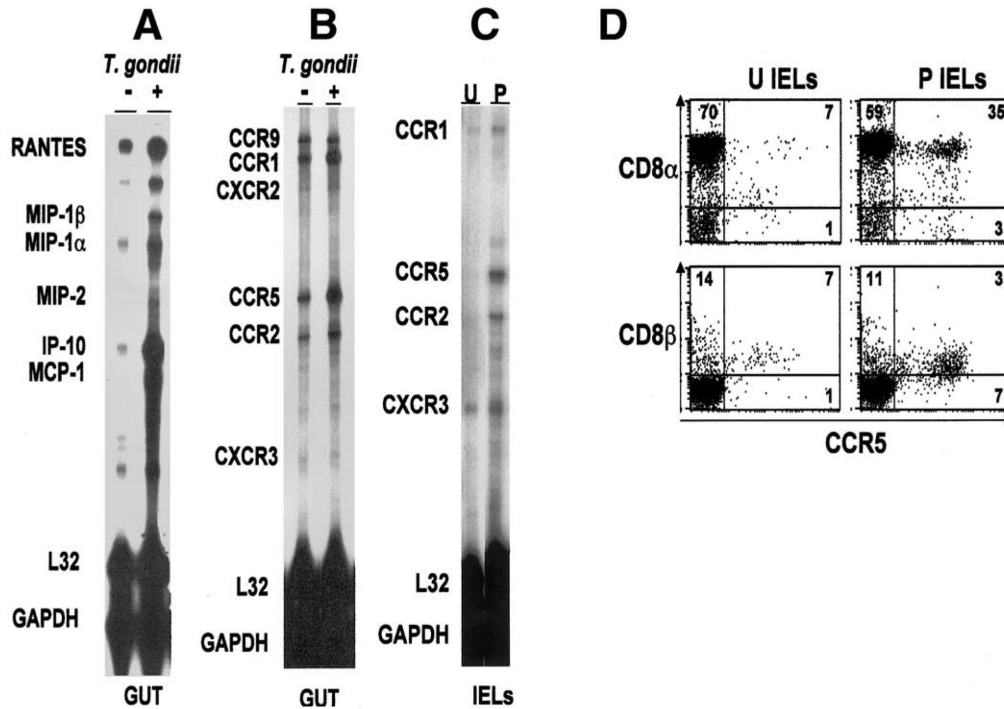

Figure 1. Chemokine and chemokine receptor expression in response to *T. gondii* infection. (*A*) Chemokine and (*B*) chemokine receptor expression was assessed by ribonuclease protection assay in the small intestine of naive (without *T. gondii*) and day-7 infected (with *T. gondii*) C57BL/6 mice or (*C*) in unprimed (U) and primed (P) IELs. (*D*) CCR5 expression was analyzed on the surface of unprimed or primed $CD8\alpha^+$ and $CD8\beta^+$ IELs. The images are representative illustrations from 3 to 5 separate experiments.

IELs compared with the unprimed IELs (Figure 1*C*). Expression of CCR1 and CXCR3 were almost unchanged in response to parasite infection (Figure 1*C*).

Flow cytometry was then used to determine which IEL subsets expressed CCR5 receptor. Isolated $CD8\alpha^+$ IELs were gated for CCR5 and $CD8\beta^+$ expression. In accor-

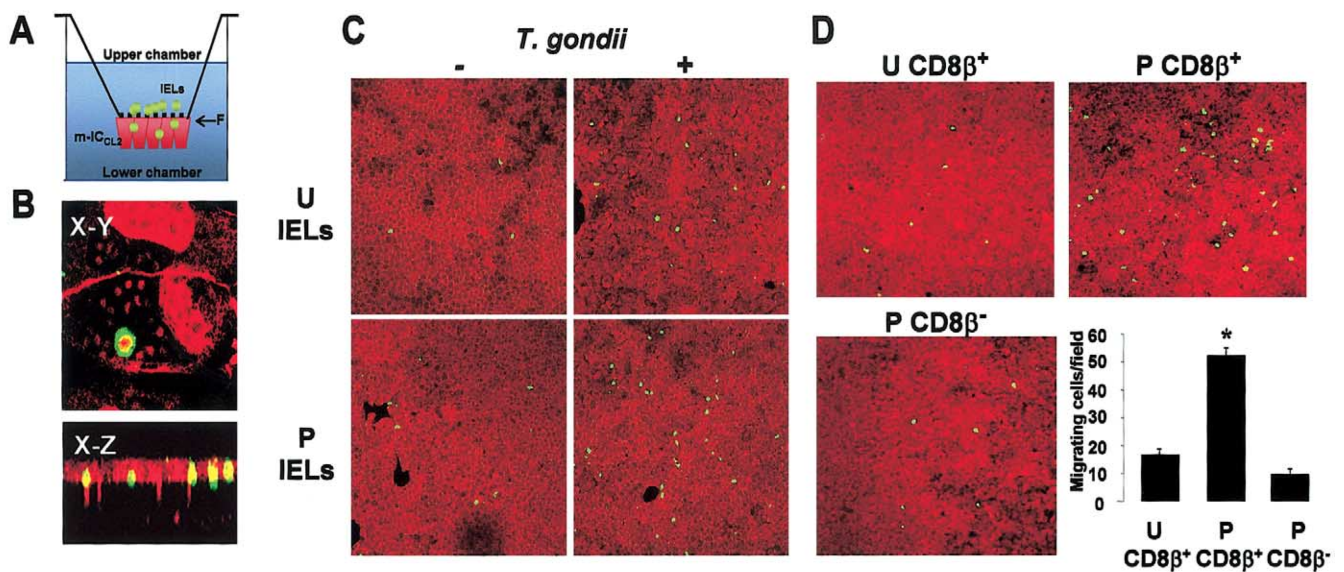

Figure 2. IEL migration into intestinal epithelial m-IC_{cl2} cells. (*A* and *B*) The epithelial monolayer m-IC_{cl2} (in *red*) and the migrating IELs (*green-yellow*) were visualized by confocal microscopy and scanned longitudinally (x-z) or laterally (x-y). (*C*) m-IC_{cl2} epithelial cells were infected (with *T. gondii*) or not (without *T. gondii*) with the parasite before adding the primed (P) or unprimed (U) IELs. (*D*) $CD8\alpha\beta^+$ IEL migration into infected intestinal epithelial m-IC_{cl2} cells. *Bars* show the mean ± SD of migrating cells. $*P < 0.01$ comparison vs. unprimed $CD8\beta^+$ and primed $CD8\beta^-$.

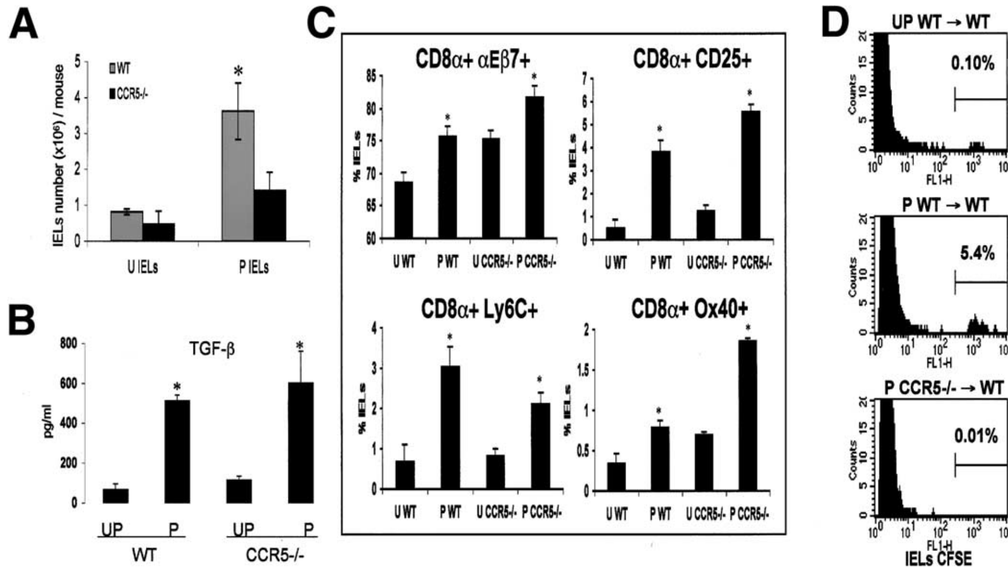

Figure 3. IELs isolated from CCR5−/− mice are activated by *T. gondii* infection but are unable to migrate in vivo. (*A*) Number of IELs isolated from naive (unprimed IELs [UIELs]) or day-7 infected (primed IELs [PIELs]) 129B6F2/J (WT) and CCR5−/− mice. $*P < 0.01$. (*B*) TGF-β secretion of unprimed and primed IELs (10^6) isolated from WT and CCR5−/− mice (CCR5−/−). (*C*) Surface expression analyzed by flow cytometry of αEβ7, CD25, Ly6C, and Ox40 in unprimed (U) and primed (P) IELs isolated from WT and CCR5−/− mice. (*D*) Unprimed or primed CFSE-stained $CD8\alpha^+$ IELs from WT or CCR5−/− mice were injected intravenously into WT recipient mice respectively (unprimed WT → WT, primed WT → WT, primed CCR5−/− → WT). The percentage of donor CFSE-tagged IELs within the IEL population of the recipient mice that have migrated to the intestine was measured by flow cytometry. The results are representative of 2 different experiments.

dance with mRNA analysis, primed IELs ($CD8\alpha^+$) expressed more CCR5 on their surface than unprimed IELs (35% and 7%, respectively) and showed that this 5-fold increase in CCR5 was mostly due to the increased expression of CCR5 in the primed $CD8\alpha\beta^+$ subset (Figure 1*D*).

Migration of IELs Into IECs

An in vitro coculture system was used to study the specific chemokine/ligand interactions in the attraction of IELs within the IEC layer (Figure 2*A* and *B*). We used the mouse m-IC_{cl2} cell line derived from intestinal crypt cells.[14] m-IC_{cl2} cells overexpress a variety of chemokines (MIP-2, MCP-3, MIP-1β, MCP-1, IP-10, and MIP-1α) in response to tachyzoite infection[8] and are a reasonable alternative to study the migration of IELs. Confluent m-IC_{cl2} cells were infected through their apical surface with *T. gondii* tachyzoites, and primed or unprimed green-tagged 5-chloromethylfluorescein diacetate IELs were added in the upper chamber of the Transwell. As a result, the infiltrating IELs present in the IEC layer exhibited a yellow-green staining (Figure 2*A* and *B*). As shown in Figure 2*C*, confocal analyses showed that the number of "migrating" primed or unprimed IELs was greater ($P < 0.01$) in confluent *T. gondii*–infected m-IC_{cl2} cells (unprimed IELs, 7.5 ± 0.7 cells/field; primed IELs, 30 ± 2.8 cells/field) than in noninfected m-IC_{cl2} cells (unprimed IELs, 4 ± 1.7 cells/field [$P < 0.01$]; primed IELs, 5 ± 1.4 cells/field [$P < 0.01$]). Furthermore, the number of "migrating" IELs detected in the infected m-IC_{cl2} layers was greater ($P < 0.01$) using primed than unprimed IELs. Similar experiments using purified subsets of $CD8^+$-enriched lymphocytes showed that significantly more primed $CD8\beta^+$ IELs than unprimed $CD8\beta^+$ or $CD8\beta^-$ IELs were able to migrate in infected m-IC_{cl2} monolayers ($P < 0.01$) (Figure 2*D*).

Role of CCR5 and Its Specific Ligands in the Chemoattraction of IELs by Infected IECs

To evaluate the specific role of CCR5 in IEL trafficking, we analyzed the migrating capacities of IELs isolated from the small intestines of WT and CCR5−/− mice. The role of CCR5 in IEL trafficking was first investigated in vivo. The number of primed IELs recovered from infected CCR5−/− mice (unprimed, $0.47 \times 10^6 \pm 0.36$; primed, $1.41 \times 10^6 \pm 0.5$) was significantly reduced ($P < 0.01$) compared with the WT mice (unprimed, $0.815 \times 10^6 \pm 0.08$; primed, $3.617 \times 10^6 \pm 0.79$) (Figure 3*A*). Although reduced in number, the IEL population isolated from the CCR5−/− mice could still be activated by the infection. TGF-β production by the IEL population is the hallmark of their

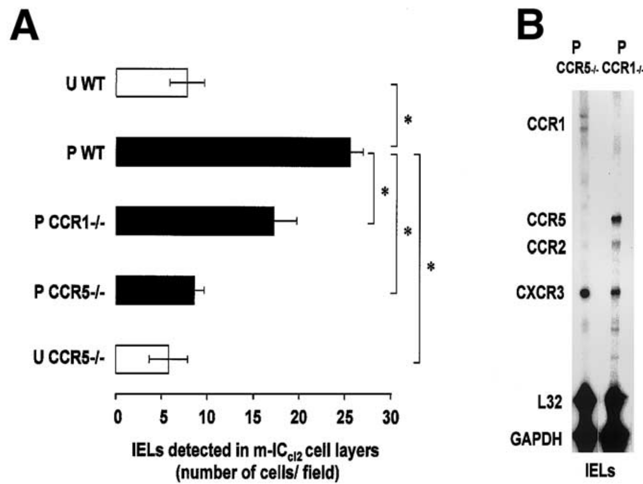

Figure 4. CCR5−/− IEL migration into infected IECs. (*A*) Quantification of the migration in *T. gondii*–infected m-IC_{cl2} monolayer of unprimed (U) or primed (P) CD8α^+ IELs isolated from 129B6F2/J (WT), CCR5−/−, or CCR1−/− mice. $*P < 0.01$. (*B*) Chemokine receptor expression in primed IELs isolated from day-7 infected CCR5−/− and CCR1−/− mice (n = 5). The results are representative of 3 different experiments.

activation after *T. gondii* infection.[8] As expected, primed CCR5−/− IELs produced significantly more TGF-β than unprimed CCR5−/− IELs and secrete the same amount of TGF-β as primed WT controls (Figure 3*B*). Other activation markers were also examined (Figure 3*C*) and showed overexpression of αE integrin, CD25, Ly-6C, and Ox40 on the surface of the primed CCR5−/− IEL population, indicating that they are activated following infection.

The role of CCR5 was further investigated in adoptive transfer experiments. IELs isolated from naive or infected WT and CCR5−/− mice were injected into WT recipient mice challenged 24 hours later. IELs from the donor mice were previously stained with CFSE to allow their detection among the recipient mice IEL population 48 hours after challenge. IELs isolated from the unprimed WT IEL recipient mice (unprimed WT → WT) showed very low levels of CFSE IELs (0.1%) compared with the primed WT IEL recipient mice (primed WT → WT) (5.4%) (Figure 3*D*). In contrast, primed CCR5−/− IELs (primed CCR5−/− → WT) seemed to be absent in the IEL population of the recipient mice (0.01%). This shows in vivo that CCR5 receptor expressed by primed IELs might be necessary for trafficking back to the infected intestine.

To confirm the low ability of primed CCR5−/− IELs to migrate into the infected enterocyte monolayer, we used our in vitro model. Unprimed IELs from both WT and CCR5−/− mice failed to invade the infected m-IC_{cl2} monolayer (Figure 4*A*). As well, the number of primed CCR5−/− IELs detected in the m-IC_{cl2} layer was significantly lower ($P < 0.01$) than that of primed WT IELs (Figure 4*A*), suggesting that CCR5 determined the subset of IELs that migrate into the intestinal cell layer. Because CCR1 ligands such as MIP-1α and RANTES partially overlap with those specific for CCR5, the ability of IELs isolated from infected CCR1−/− mice to migrate into m-IC_{cl2} layers was also assessed as controls. These primed IELs had a level of CCR5 expression similar to primed WT IELs (Figure 4*B*) and were activated like the CCR5−/− primed IELs (data not shown). The number of primed CCR1−/− IELs invading the infected m-IC_{cl2} monolayer was reduced ($P < 0.01$) compared with the primed WT IELs, although significantly greater ($P < 0.01$) than that of primed CCR5−/− IELs (Figure 4*A*). These results indicated that the chemokine receptor CCR1 has only a minor influence in the migration of IELs compared with CCR5.

The potential attraction of the 3 CCR5 ligands, MIP-1α, MIP-1β, and RANTES in this process was then assessed (Figure 5). Primed CD8α^+ IELs expressing CCR5 were isolated and added in the upper chamber of non–collagen-coated Transwell filters devoid of IECs. Increasing concentrations (1, 10, and 100 ng/mL) of recombinant MIP-1α, MIP-1β, or RANTES were added to the lower chamber of the Transwell. The migration index of the primed IELs was assessed by flow cytometry. A dose-dependent chemoattractive effect of MIP-1α and MIP-1β but not of RANTES was observed (Figure 5*A*). MIP-1α had a greater chemotactic effect on IELs ($P < 0.01$) than MIP-1β (Figure 5*A*). Although RANTES had no chemoattractive effect on IELs, ribonuclease protection assay and enzyme-linked immunosorbent assay analysis showed high level of RANTES mRNA expression among the primed IEL population (data not shown). As attested by real-time polymerase chain reaction, m-IC_{cl2} cells stimulated with RANTES (100 ng/mL) showed a significant increase ($P < 0.01$) in MIP-1α but not in MCP-1 mRNA expression (data not shown).

To better assess the chemoattractant role of chemokines secreted by IECs, conditioned media from the apical or the basal side of infected (6-hour) m-IC_{cl2} cells were collected and transferred to the lower chamber of a Transwell where primed CD8α^+ IELs were added to the upper chamber. In these experimental conditions, twice as many IELs were attracted by the conditioned basal medium than by the apical medium ($P < 0.01$) (Figure 5*B*). These results suggested that infected m-IC_{cl2} exhib-

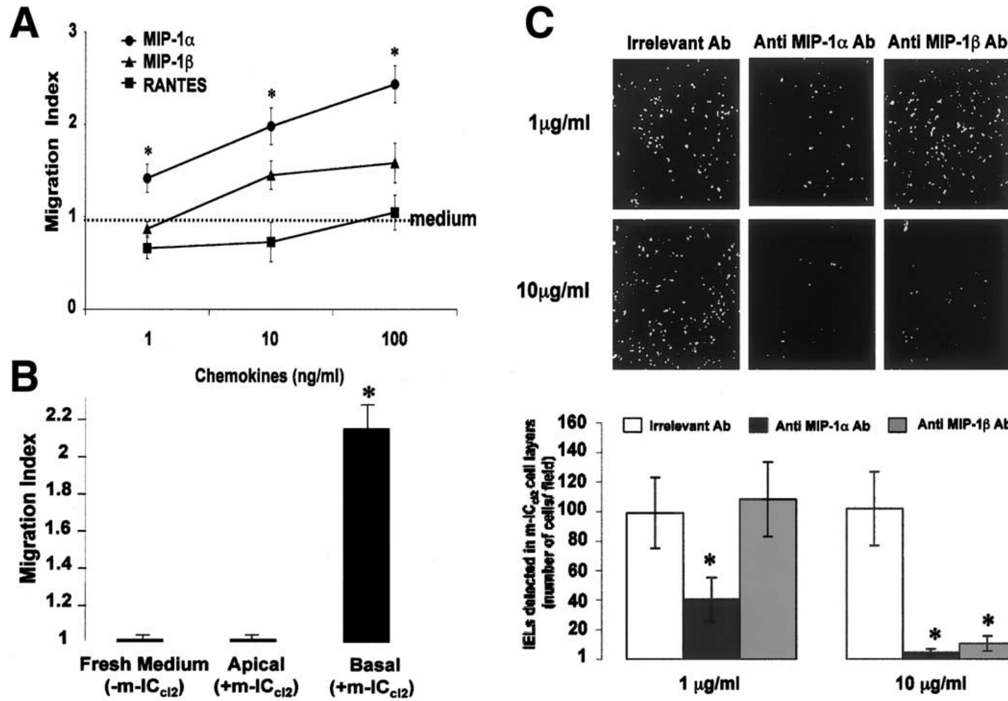

Figure 5. Migration of $CD8\alpha^+$ IELs to CCR5 ligands. (*A*) Primed IELs were added to the upper chamber of a Transwell, whereas the lower chamber was filled with medium supplemented or not with increasing concentrations of RANTES, MIP-1α, and MIP-1β (1, 10, or 100 ng/mL). (*B*) Apical or basal secretion from intestinal epithelial m-IC_{cl2} cells was added to the lower compartment. The *bars* represent the migration index to fresh medium (without m-IC_{cl2}) and conditioned apical or basal medium from *T. gondii*–infected m-IC_{cl2}cells (with m-IC_{cl2}). (*C*) Infected m-IC_{cl2} cells and IELs were incubated with or without blocking antibodies to MIP-1α or MIP-1β (1 and 10 μg/mL) or with an irrelevant antibody. $*P < 0.01$ represents statistical differences with the condition using the irrelevant antibody.

ited a preferential polarized basal secretion of chemokines involved in IEL trafficking. MIP-1α and MIP-1β were also blocked, and the migration of primed subsets of $CD8\alpha^+$ IELs in infected m-IC_{cl2} cells was evaluated. The basal addition of increasing concentrations of anti–MIP-1β or anti–MIP-1α antibodies inhibited the migration of IELs when compared with the untreated control. The blockade of the chemokine secretion with anti–MIP-1β or anti–MIP-1α antibodies was concentration dependent (Figure 5*C*). Anti–MCP-1 antibody was also used in this system to verify the specificity of the blocking experiment because CCR2, the ligand of MCP-1, was also weakly expressed on CCR5−/− IELs compared with WT primed IELs. In this experiment, anti–MCP-1 antibody could not block the IEL migration (data not shown).

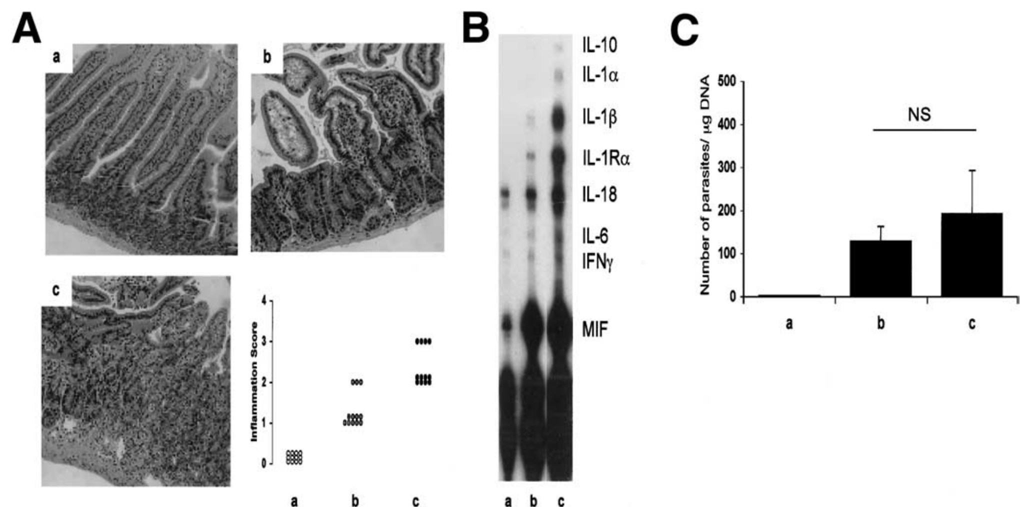

Figure 6. Histopathology, cytokine expression, and parasite load following *T. gondii* infection in WT or CCR5−/− mice. (*A*) Histology score, (*B*) expression of cytokines, and (*C*) parasite load assessed by quantitative real-time polymerase chain reaction were estimated from small intestines of (a) naive WT, (b) day-7 infected WT, and (c) CCR5−/− mice (n = 12 per group). The results are representative of 2 different experiments.

Consequences of the Lack of CCR5 Receptor Expression on *T. gondii* Pathogenesis

IELs via the secretion of TGF-β are critical in preventing inflammation of the small intestine as well as necrosis of the villi following parasite infection. We therefore investigated whether the absence of CCR5 expression on the IEL surfaces would affect the pathophysiology of the infection. Consistent with the lower number of primed IELs, CCR5−/− mice showed increased inflammation and tissue damage (Figure 6*A*). The intestinal lesions correlated with a higher production of inflammatory cytokines in the gut of CCR5−/− mice (interleukin [IL]-1, IL-6, IL-18, interferon gamma) (Figure 6*B*). We previously reported the role of the IELs in parasite clearance in CBA mice.[11] However, a very low number of IELs ($\sim10^4$) can account for the parasiticidal effect (personal observation, July, 2002). Indeed, despite the lower number of primed IELs, there was no significant difference between the parasite load of WT and CCR5−/− infected mice (Figure 6*C*).

These results indicate that CCR5 is crucial for the specific migration of IELs to the site of inflammation following *T. gondii* infection, exerting a protective effect in the control of inflammatory cell infiltration in the lamina propria.

Discussion

Oral infection with tissue cysts of *T. gondii* elicits a lethal inflammatory response in certain strains of mice.[11,18,19] *T. gondii*–primed IELs traffic to the infected intestine and participate in the protective mechanisms that clear the parasites and also reduce the deleterious consequences of an excess inflammatory response.[11] The migration of leukocytes into sites of inflammation involves a series of cellular and molecular events and interactions that are still not fully understood. This study provides in vivo and in vitro evidence of a pivotal role for CCR5 in IEL migration into *T. gondii*–infected IECs. We also show that MIP-1α and MIP-1β, secreted by the infected IECs, are required to elicit the most effective mobilization of the CD8β^+ IEL subset expressing the appropriate CCR5 receptor.

The immune system has evolved specialized cellular and molecular mechanisms for targeting and regulating immune responses at the epithelial surface. Selective expression of chemokines by differentiated epithelium represents an important mechanism for this immune process. IELs constitutively express CCR9, CXCR3, CCR2, and CCR5.[6,7,20,21] In our *T. gondii*–induced intestinal inflammation model, we observe a substantial increase in CCR5 expression by antigen-primed IELs and show the specific role of CCR5 receptor for the $CD8^+$ IEL trafficking in an infectious context. In the small intestine of C57BL/6 mice, *T. gondii* infection triggers expression of both CC and CXC chemokines, including RANTES (CCL5), MIP-1α (CCL3), MIP-1β (CCL4), MCP-1 (CCL2), IP-10 (CXCL10), and MIP-2. The expression of these chemokines by infected intestinal cells is also consistent with the observed increase of their receptor counterparts in day-7 *T. gondii*–infected small intestines, especially the more selective CCR5 overexpression in isolated IELs. A number of studies have identified the role of CCR5 and of its corresponding chemokines in the recruitment of T cells to inflammatory sites.[22–26] CCR5 as an important regulator of leukocyte trafficking is described in other studies showing that CCR5 deficiency may have deleterious consequences if associated with the cell trafficking involved in the regulation of the immune response.[24,26–29] Furthermore, CCR5 ligation has been shown to play a key role in the induction of IL-12 synthesis by dendritic cells and is also believed to be involved in the establishment of interferon-dependent resistance to *T. gondii*.[30,31]

CCR5 expression on the surface of *T. gondii*–primed IELs represents an important factor in the mediation of IEL trafficking and localization into infected enterocytes. Primed IELs isolated from CCR5-deficient mice have lost the ability in vitro to migrate into m-IC_{cl2} cell layers or in vivo to traffic back into the inflamed intestine after adoptive transfer and to display several potential protective effects. Indeed, after adoptive transfer of primed IELs, C57BL/6 mice are protected against the development of acute ileitis observed after *T. gondii* oral infection.[11,12] The protective mechanism involves TGF-β production.[8] *T. gondii*–primed CCR5−/− IELs produce TGF-β. However, their reduced number in the CCR5−/− infected intestine is likely due to a lower recruitment at the place of the infection and may account for the more severe inflammatory damages observed compared with WT mice. An increase number of $CD4^+$ T cells at day 7 in the lamina propria population and a reduced number of $CD8^+$ T cells was also observed in CCR5−/− mice after treatment with dextran sodium sulfate,[32] suggesting that CD8 T cells can influence the inflammatory response. Although decreased in number, IELs are still present and activated in infected CCR5−/− mice, indicating the involvement of additional trafficking mechanisms. IELs are also shown to

participate in parasite clearance through cytotoxic mechanisms in CBA mice.[11] This effect requires a very low number of IELs because the adoptive transfer of 10^4 primed IELs is sufficient to prevent the cerebral infection of CBA mice. Despite the reduced number of IELs in CCR5−/− mice, there is no difference of parasite load between CCR5−/− and WT mice. The present findings provide further evidence for a functional role of this receptor as part of the regulatory mechanism involved in the migration and trafficking of intraepithelial $CD8^+$ lymphocytes into the inflamed intestine following parasite infection.

The direct interaction between parasite-infected IECs and IELs was shown by transmigration assay using m-IC_{cl2} cells grown on permeable filters. This mouse intestinal cell line exhibits a number of important properties and functions specific of intestinal crypt cells.[14,33,34] The migration of antigen-primed CD8αβ TCRαβ IELs through the intact m-IC_{cl2} cell layer appears to be polarized in a basal-to-apical direction. A similar basolateral secretion of MIP-2 induced by lipopolysaccharide has already been reported in our model of m-IC_{cl2} cells,[34] and CD8αβ TCRαβ IELs have been found to be the main IEL subset detected in *T. gondii*–infected intestines.[10] The results of the present study also show that the migration of IELs requires specific CC receptors/chemokine interactions because anti–MIP-1α and anti–MIP-β antibodies inhibit the chemoattraction of IELs induced by infected IECs. Although RANTES has no chemoattractant effect on primed IELs, it increases the secretion of MIP-1α by the m-IC_{cl2} cells as previously shown.[35] We also found that the inhibition of RANTES by an anti-RANTES antibody totally inhibited the migration of primed IELs into the m-IC_{cl2} layer (data not shown), suggesting that RANTES behaves as an amplification factor for IEL-mediated MIP-1α and/or MIP-1β chemoattraction.

The sum of data collected from naive and infected WT and CCR5−/− mice together with the migration assays using cultured m-IC_{cl2} cells indicate that the migration of $CD8\beta^+$ IELs requires the specialized interaction of CCR5 with its ligands. Such interaction appears to be specific because the expression of other chemokine receptors, such as CCR9 and CXCR3, remains unchanged following *T. gondii* infection. A CXCR3/IP-10 interaction has been believed to be involved in the cellular trafficking for T cells.[36] However, CCR5−/− IELs have lost their migratory capacity despite the same level of CXCR3 expression as the primed WT IELs. CCR1 may interfere with IEL attraction, but its expression remains low compared with that of CCR5 in primed IELs. CCR2 is also potentially interesting in mediating IEL attraction in response to MCP-1, but its expression on primed IELs is significantly lower than that of CCR5 and anti–MCP-1 blocking antibody is inefficient in altering IEL trafficking into the infected IECs.

In conclusion, complementary in vitro and in vivo studies have shown both an essential and specific interaction between CCR5 receptor with its corresponding MIP-1α and MIP-1β ligands. This unique interplay of the epithelial cell chemokine and the CCR5 receptor results in the subsequent migration of CD8αβ TCRαβ IELs into the parasite-induced inflamed mucosal intestinal barrier.

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Address requests for reprints to: Lloyd H. Kasper, M.D., Departments of Microbiology/Immunology, Dartmouth Medical School, Lebanon, New Hampshire 03756. e-mail: Lloyd.kasper@dartmouth.edu; fax: (603) 650-6223.

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