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CCR9$^+$ T cells contribute to the resolution of the inflammatory response in a mouse model of intestinal amoebiasis

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ABSTRACT

Analysis of the mechanisms underlying the inflammatory response in amoebiasis is important to understand the immunopathology of the disease. Mucosal associated effector and regulatory T cells may play a role in regulating the inflammatory immune response associated to *Entamoeba histolytica* infection in the colon. A subpopulation of regulatory T cells has recently been identified and is characterized by the expression of the chemokine receptor CCR9. In this report, we used CCR9 deficient (CCR9$^{-/-}$) mice to investigate the role of the CCR9$^+$ T cells in a murine model of *E. histolytica* intestinal infection. Intracecal infection of CCR9$^{+/+}$, CCR9$^{+/-}$ and CCR9$^{-/-}$ mice with *E. histolytica* trophozoites, revealed striking differences in the development and nature of the intestinal inflammatory response observed between these strains. While CCR9$^{+/+}$ and CCR9$^{+/-}$ mice were resistant to the infection and resolved the pathogen-induced inflammatory response, CCR9$^{-/-}$ mice developed a chronic inflammatory response, which was associated with over-expression of the cytokines IFN-γ, TNF-α, IL-4, IL-6 and IL-17, while IL-10 was not present. In addition, increased levels of CCL11, CCL20 and CCL28 chemokines were detected by qRT-PCR in CCR9$^{-/-}$ mice. *E. histolytica* trophozoites were identified in the lumen of the cecum of CCR9$^{-/-}$ mice at seven days post infection (pi), whereas in CCR9$^{+/+}$ mice trophozoites disappeared by day 1 pi. Interestingly, the inflammation observed in CCR9$^{-/-}$ mice, was associated with a delayed recruitment of CD4$^+$CD25$^+$FoxP3$^+$ T cells to the cecal epithelium and lamina propria, suggesting that this population may play a role in the early regulation of the inflammatory response against *E. histolytica*, likely through IL-10 production. In support of these data, CCR9$^+$ T cells were also identified in colon tissue sections obtained from patients with amoebic colitis. Our data suggest that a population of CCR9$^+$CD4$^+$CD25$^+$FoxP3$^+$ T cells may participate in the control and resolution of the inflammatory immune response to *E. histolytica* infection.

Introduction

Amoebiasis, caused by the protozoan parasite *Entamoeba histolytica*, is an endemic disease of tropical developing countries with a prevalence of, in some cases, up to 50% in the general population. It is estimated to cause more than 100,000 deaths per year worldwide (WHO 2010). *E. histolytica* infects mainly the large intestine, however extra-intestinal infections, including the skin, brain and mostly the liver, can also occur (Garcia-Zepeda et al. 2007; Mortimer and Chadee 2010). In Mexico, recent epidemiological reports indicate that intestinal amoebiasis is one of the 20 main causes of disease with an incidence of 498 cases per 100,000 individuals (Sanchez Garay and Mujica Vargas 2009).

While the majority of individuals infected with *E. histolytica* remain asymptomatic, approximately 10% of patients will develop acute intestinal inflammation associated to diarrhoea and dysentery. The inflammatory immune response driven by innate immune cells appears to be sufficient to eliminate the parasite in most patients. However, a small percentage of these individuals will develop chronic amoebic colitis (CAC). CAC is characterized by the

Abbreviations: CAC, chronic amoebic colitis; FBS, fetal bovine serum; IEL, intra epithelial cells; KO, knock out; LP, lamina propria; MLN, mesentheric lymph nodes; qRT-PCR, quantitative real time-polymerase chain reaction; Treg, regulatory T cells; WT, wild type.

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presence of multiple discrete lesions of varying stages ranging from superficial erosion of colonic epithelia to deep flask-shaped ulcers involving the mucosa and part of the muscularis mucosa and the serosa. Amoebic colitis progression has been related to Th2 type immune responses, while the resolution of the infection in this phase of the disease, appears to be mediated by a Th1 response (Espinosa-Cantellano and Martinez-Palomo 2000; Garcia-Zepeda et al. 2007; Mortimer and Chadee 2010).

The mechanisms that determine progression *versus* resolution of the infection are poorly understood. In this regard, it has been demonstrated that regulatory cytokines such as IL-10, may be a key determinant of resistance as it was observed in a murine model of *E. histolytica* infection (Hamano et al. 2006). Analysis of IL-10$^{-/-}$ mice infected with *E. histolytica* showed an increase in the parasite load and the degree of mucosal inflammation compared to WT animals. Although these results may indicate that IL-10 has a role in maintaining host resistance and promoting resolution of the inflammatory immune response in the intestinal mucosa, it is not clear whether a population of CD4$^+$CD25$^+$FoxP3$^+$ Treg cells, IL-10 producers, may also be involved in this process. It has been reported that Treg cells migrate into the intestinal mucosa through the use of CCR2, CCR4, CCR5, CCR6 and CCR7 chemokine receptors (Izcue et al. 2009; Kitamura et al. 2010). Specifically, CCR4 and CCR6 have proved to be important in the recruitment of Treg cells to the colon mucosa under inflammatory conditions, since adoptive transfer of CCR4$^{-/-}$ and CCR6$^{-/-}$ Treg cells are unable to resolve colitis *in vivo* (Yuan et al. 2007; Kitamura et al. 2010). Recently, CCR9 has also been identified in both Treg cells and T cells of regulatory phenotype (such as Th3) in human PBMCs and after being recruited into the small intestine in mouse models of intestinal inflammation (Papadakis et al. 2003; Alford et al. 2008; Kang et al. 2009).

However, it is not known whether CCR9$^+$ T cells may play a role in regulating inflammation during protozoa infection in the colon. To investigate the specific role of CCR9$^+$ T cell populations during infections in the colon, we generated an *E. histolytica* infection model in CCR9$^{-/-}$ (KO) mice. We found that, in the absence of CCR9, *E. histolytica* infected mice developed a chronic inflammation associated with parasite presence in the lumen and progressive destruction of the cecal mucosa. This inflammatory response was associated with reduced expression of IL-10 and a significant decrease in the number of intraepithelial (IE) CD4$^+$CD25$^+$FoxP3$^+$ Tregs in CCR9$^{-/-}$ mice.

Our results suggest that a population of CCR9$^+$CD4$^+$CD25$^+$FoxP3$^+$ IEL Tregs may play an important role in the resolution of infection and in the control of inflammation against *E. histolytica*.

Materials and methods

Mice

C57BL/6 CCR9$^{-/-}$ mice were generated as described previously (Wurbel et al. 2001). Eight to ten week old CCR9$^{-/-}$ mice and their heterozygous littermates were used in all experiments. C57BL/6 age-matched mice (WT) were used as controls (The Jackson Laboratories, Bar Harbour, ME). Animals were kept in pathogen-free conditions in the animal research facility of the Instituto de Investigaciones Biomédicas, UNAM. All animals were handled in strict accordance with good animal practice, as defined by the Animal Experimental Bio-Ethics Guidelines of the Instituto de Investigaciones Biomédicas, UNAM. This study was reviewed and approved by the Animal Experimental Bio-Ethics Committee of the Instituto de Investigaciones Biomédicas, UNAM.

Parasite culture and intracecal inoculation

Axenic cultures of *E. histolytica* strain HM1-IMSS were maintained in TYI-S-33 medium, according to standard protocols (Diamond et al. 1978). For all intracecal inoculations, axenic trophozoites were grown to the log phase, counted under a light microscope with a haemocytometer and adjusted to 25×10^6 trophozoites/ml in TYI-S-33 medium. 5×10^6 trophozoites were intracecally injected after laparotomy, as described (Rivero-Nava et al. 2002).

Tissue processing and histochemical analysis

Infected and control mice were sacrificed at days 1, 7, and 20. The cecum was dissected and processed for histochemistry. The tissue was frozen at −70 °C in OCT compound (Tissue Teck, Sakura Finetek) and 6–8 μm cryostat sections were obtained. Tissue sections were stained with haematoxylin/eosin (H/E) to assess the cellular infiltrate. Mucus production was revealed by periodic acid Schiff (PAS) staining. The degree of mucosal inflammation observed was scored as follows: 0, normal; 1, mucosal hyperplasia; 2, increase of inflammatory cells in the mucosa and submucosa; 3, marked increase of inflammatory cells in the mucosa and submucosa; 4, destruction of the cecal mucosa by inflammation (Hamano et al. 2006). Human tissue specimens were obtained from patients with fulminant amoebic colitis undergoing surgical resections of the ileum and colon (Ventura-Juarez et al. 2007). Tissues were obtained from fully informed patients undergoing surgical resections of the ileum and colon for amoebic lesions. The data obtained were analyzed anonymously. This study was reviewed and approved by the ethical committee of the hospitals involved as well as by the IRB of the Universidad Autónoma de Aguascalientes, Aguascalientes, México. Tissue sections were obtained and processed as described elsewhere (Sierra-Puente et al. 2009). Analysis of CCR9 protein expression was performed using a mouse anti-human CCR9 antibody (R&D) and a goat anti-mouse-HRP secondary antibody.

Real time PCR analysis

Cecal tissue obtained from infected mice at different time points was homogenized and total RNA was obtained using Trizol (Invitrogen), according to manufacturer's protocol. cDNA was synthesized from RQ-DNAse (Promega) treated total RNA using M-MLV reverse transcriptase (Promega) and Oligo-dT (Invitrogen). RT-PCR was performed using SYBR Green PCR master mix (Applied Biosystems) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems), as indicated by manufacturer. Relative expression of chemokines, chemokine receptors and cytokines was determined using the ΔΔCT method described (Livak and Schmittgen 2001; Schmittgen and Livak 2008). β-Actin was used as housekeeping gene. Primer sequences for selected genes: β-actin, F: 5′-GG GTCAGAAGGATTCCTATG-3′, R: 5′-GGTCTCAAACATGATCTGGG-3′; *CCR9*: F: 5′-TCCGAAGGGATCTGGTGAAG-3′, R: 5′-GAATGAAACC-CACTGGGCC-3; *CCR10*: F: 5′-TCAATCCGGTGCTTTATGCC-3′, R: 5′-AGCAGCCTCCGCAGGT C-3′; CCR3: F: 5′-TTCTC-ACCA-GGAAGAAACGGA-3′, R: 5′-GGAGGTGACTGAGGTGATTGC-3′; *CCR6:* F: 5′-GATGCTGCTCCTGGCCTG-3′, R: 5′-AAGAT TTGGTTGC-CTGGA-CG-3′; *CCL25:* F:5′-GCCTGGTTGCCTGTTTTGTT-3′; R: 5′-CAGCA-GTCTTCAAAGGCACT-3′; *CCL28*: F: 5′-CAGGGCTCACACTCATGGCT-3′; R: 5′-GCCATGGGAAGTATGGCC TTC-3′; *CCL11:* F: 5′-TCCAC-AGCGCTTCTATTCCTG-3′; R: 5′-GGAGCCTGGG TGAGCCA-3′; CCL20: F: 5′-TTTTGGGATGGAATTGGACAC-3′; R: 5′-TGCAGGTGAAGCCTT CAACC-3′; *IFN*-γ: F: 5′-AACGCTACACACTGCATCTTGG-3′, R: 5′-GCCGTGGAGTA ACAGCC-3′; *TNF*-α; F: 5′-GGCAGGTCTACTTTGGAGT CATTGG-3′,