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Non-redundant role of the chemokine receptor CX3CR1 in the anti-inflammatory function of gut macrophages☆

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ABSTRACT

Mucosal immunity at the intestinal level is constantly challenged by the presence of external food and microbial antigens and must be kept under strict control to avoid the rise of aberrant inflammation. Among cells of the innate immunity, macrophages expressing the chemokine receptor CX3CR1 are strategically located near the gut epithelial barrier. These cells contribute to the maintenance of homeostasis by producing the anti-inflammatory cytokine IL-10; however, their role in the control of full blown inflammation and tissue injury is controversial. In this study we investigated mice proficient or deficient for the expression of the CX3CR1 receptor in a model of dextran sulphate sodium (DSS) induced acute colitis. We found that KO mice ($CX3CR1^{GFP/GFP}$) had a more severe disease compared to WT mice ($CX3CR1^{GFP/+}$), both in terms of histological examination of colonic tissues and leukocyte infiltration, with an expansion of macrophages and CD4-Th17 lymphocytes. The expression of several inflammatory mediators (IL-1β, IL-6, IFNγ, iNOS) was also significantly upregulated in KO mice, despite higher IL-10 production. Overall, our study demonstrates that macrophages expressing a functional CX3CR1 receptor have an important and non-redundant role in controlling the abnormal intestinal inflammation that may lead to tissue damage.

1. Introduction

The intestine is an exclusive tissue able to maintain the delicate balance between immune system activation and tolerance against the very huge amount of harmless antigens that are in contact with the intestinal lumen, including food antigens and microflora (Kuhl et al., 2015).

It is fundamental for our health that the immune system is able to face potential dangerous antigens with a robust and appropriate response, but the same reaction against harmless antigens would be very dangerous and give rise to allergies or chronic inflammation eventually leading to permanent tissue damage. Many immune cells participate to the maintenance of gut homeostasis, having the role of recognizing if an antigen is a potential danger for the organism, and being silent in response to non-dangerous antigens (Tlaskalova-Hogenova et al., 2011).

In this landscape, mononuclear phagocytes play a central part in this discrimination process. Mononuclear phagocytes are detected in the entire gastrointestinal tract, in particular in the *lamina propria* in close contact with the enterocytes that compose the crypts (Bain and Mowat, 2014; Rua and McGavern, 2015). Overall they represent the most abundant leukocyte population in the intestine and the largest population of macrophages in the entire body (Hume, 2006). In particular, they are able to keep the immune system silent and allow a peaceful coexistence with the microflora but, in case of need, they are able to trigger and to sustain the immune response (Kuhl et al., 2015) (Sheikh and Plevy, 2010). Two main subsets are present: dendritic cells (DC) identified with the marker CD103, and macrophages (Mϕ) identified by F4/80.

A specific population of macrophages in the gut expresses the chemokine receptor CX3CR1 and has the ability to protrude transepithelial dendrites into the lumen and sample luminal antigens (Niess et al., 2005; Chieppa et al., 2006; Mazzini et al., 2014).

Abbreviation: CNS, central nervous system; DC, dendritic cells; D.A.I., disease activity index; DSS, dextran sulphate sodium; GFP, green fluorescent protein; IL-10R, IL-10 receptor; KO, knock out; MLN, mesenteric lymph nodes; Mϕ, macrophages; OVA, ovalbumin.

☆ CX3CR1 macrophages have an important and non-redundant role in homeostasis and in controlling the abnormal intestinal inflammation.

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The nature of these cells has been a matter of debate; the current view is that CX3CR1^{+} cells are macrophages and not DC, as they also express F4/80 and are negative for CD103, though a small population of CX3CR1^{+} F4/80^{-} cells has been described (Mowat and Bain, 2011; Persson et al., 2010). On the other side, not all DCs are CD103^{+} (Schulz et al., 2009; Hume, 2008). Thus, these two markers need to be associated with others to properly identify specific subsets. It is very important to define which cell population is involved in homeostasis maintenance or in the regulation of immune responses. For instance, only DCs are able to migrate to regional lymph nodes where they can induce tolerance to antigens or, in case of need, trigger an immune response (Rescigno et al., 2001; Niess et al., 2005; Rimoldi et al., 2005; Rescigno and Chieppa, 2005; Diehl et al., 2013; Mazzini et al., 2014).

Macrophages are plastic cells able to generate distinct functional programs in response to various stimuli (Marelli et al., 2015; Gordon et al., 2014). A remarkable feature of intestinal CX3CR1+ macrophages is to maintain gut homeostasis by the production of the anti-inflammatory cytokine IL-10. Recently, Zigmond et al., raised the issue of the importance for gut Mϕs to sense IL-10 with the IL-10 receptor (IL-10R). They proposed that an IL-10-based autocrine loop acts directly on intestinal macrophages. Consequently, if gut Mϕs are not able to detect IL-10, the autocrine mechanism of regulation is lost and inflammation is triggered (Zigmond et al., 2014).

CX3CR1^{+} macrophages in the intestine derive from circulating classical blood monocytes (Ly6C^{high}) that can differentiate in both inflammatory and resident macrophages. Bain et al. demonstrated that blood Ly6C^{Hi} monocytes give rise to all different types of macrophages in healthy and inflamed colon, and that the marker CX3CR1 is upregulated by local signals (Bain et al., 2013). The CX3CR1 receptor can be expressed at different levels: resident CX3CR1^{int} macrophages constitute a pool able to generate both inflammatory and resident CX3CR1^{Hi} phagocytes, depending on signals in the milieu, and that a population of CX3CR1^{int} macrophages is able to expand during colitis (Bain et al., 2013).

However, the exact role of these macrophages in the control of aberrant inflammation, and if and how they participate to mount an immune response is still unclear and contrasting results are available. Kayama et al., demonstrated that CX3CR1^{+} myeloid cells are able to prevent intestinal inflammation in a model of T-cell dependent colitis (Kayama et al., 2012). Similarly, Medina-Contreras et al. showed that CX3CR1^{+} Mϕs play a central role in preventing the translocation of commensal bacteria to mesenteric lymph nodes, limiting the colitogenic Th17 responses (Medina-Contreras et al., 2011). Conversely, other studies demonstrated that CX3CR1^{+} cells are important to activate CD4T cell during antigen-driven colitis (Rossini et al., 2014; Mazzini et al., 2014).

Niess et al., showed an increased susceptibility to pathogenic bacteria, such as Listeria and Salmonella, in CX3CR1 KO mice (Niess et al., 2005); they further demonstrated that absence of the CX3CR1 receptor drives to higher vulnerability to Citrobacter rodentium caused by low production of IL-22 and consequent reduction of innate lymphoid cells (ILCs) having a protective role on the epithelium (Manta et al., 2013). Finally, Kostadinova et al., demonstrated that CX3CR1^{+} Mϕs contribute to elicit a pro-inflammatory response by producing iNOS (Kostadinova et al., 2010). Thus, the studies investigating the role of this resident CX3CR1^{+} phagocyte population in the development of inflammation, gave contrasting results, highlighting the complexity of the gut immune landscape. To further clarify this issue, in this study we used mice proficient or deficient for the CX3CR1 receptor, in a model of acute colitis, and characterized the leukocyte infiltrate and the inflammatory cytokines within the injured colons.

2. M&M

2.1. Mice

Procedures involving animals and their care conformed to institutional guidelines in compliance with national (4D.L.N.116, G.U., Suppl. 40, 18-2-1992) and international law and policies (EEC Council Directive 2010/63/EU, OJ L 276/33, 22.09.2010; NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 2011). All efforts were made to minimize the number of animals used and their suffering.

All mice used were of the C57BL/6 background. Six to eight-week old male were purchased from The Jackson Laboratory (Italy). We compared CX3CR1$^{+/GFP}$ (WT) to CX3CR1$^{GFP/GFP}$ (KO). Mice were maintained in a specific-pathogen free facility.

2.2. DSS-induced colitis

C57BL/6 CX3CR1 WT and KO mice were used to generate a model of colitis. Six- to eight-week-old male mice with an initial weight of 18–20 g received 3% of dextran sulphate sodium (DSS-MP biomedical, United Kingdom) dissolved in drinking water for 7 days. Mice were sacrificed at day 7. During the experiment, mouse weight loss, stools blood and consistency were monitored twice a week to provide an assessment of colitis severity during the treatment. Hemoccult SENSA test from Beckman Coulter (Italy) was used to detect occult blood and to evaluate stools consistency (Supplementary Table S1).

2.3. Histological analysis

After mouse sacrifice, colons were isolated and excised for histological analysis and immunofluorescence. Colons were harvested, flushed of luminal contents, and opened with a longitudinal cut; next, they were rolled up transversely *(swiss roll)*, with the distal region inside the *swiss roll*. Tissues were then fixed in 4% paraformaldehyde (PFA) overnight at 4 °C and dehydrated first in 30% sucrose in PBS$^{-/-}$, for 6 h and then in 40% sucrose in PBS$^{-/-}$, overnight. Next, the colonic tissues were embedded in OCT (optimum cutting temperature compound, Diapath, Italy) and stored at −80 °C.

To evaluate the histological architecture of the inflamed colon 8 μm thick frozen sections were cut with cryostat, placed on a glass slide and stained with hematoxylin and eosin (H&E stain).

Sections were fixed with 4% formalin and then stained with hematoxylin and eosin. Slides were observed with microscope (4x) and colitis score was assessed (Supplementary Fig. S1).

2.4. Immunofluorescence

For immunofluorescence staining, frozen tissue sections (8 μm) were firstly rehydrated and then were permeabilized and blocked with 0.1% Triton X-100 (Sigma Aldrich, Italy), 2% Bovine Serum Albumin (BSA), and 5% Normal Goat Serum in PBS$^{+/+}$ for 30 min in a dark incubation chamber. Sections were washed for 5 min in PBS$^{+/+}$ Tween-20 0,05% (washing buffer) and subsequently incubated with the primary antibody(ies) in washing buffer for 1 h at room temperature. After three washes in washing buffer, sections were incubated with fluorophore-coniugated secondary antibody(ies) at room temperature for 1 h in the dark (1:2000). Finally, nuclei counter-stained with DAPI, washed out the excess of DAPI with water and mounted with a fluorescence preserving medium (Fluorsave, Calbiochem, United Kingdom). Images were captured with the Olympus FluoView^{TM} FV1000 confocal microscope. The acquired