Gut Microbes 1:6, 396-400; November/December 2010; © 2010 Landes Bioscience

What are CX_3CR1^+ mononuclear cells in the intestinal mucosa?

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Key words: dendritic cells, macrophages, gut immunology, CX_3CR1, commensal microbiota

Abbreviations: DC, dendritic cells; APC, antigen presenting cell; SILT, solitary intestinal lymph follicle; MLN, mesenteric lymph node; PP, peyer's patch; LP, lamina propria; cLP, colonic lamina propria; siL, small intestinal lamina propria; DSS, dextran sodium sulphate; cDC, conventional dendritic cells; pDC, plasmacytoid dendritic cells; MDP, macrophage dendritic cell precursors; CDP, common dendritic cell precursors

Submitted: 05/16/10

Revised: 09/22/10

Accepted: 10/13/10

Previously published online: www.landesbioscience.com/journals/gutmicrobes/article/13939

DOI: 10.4161/gmic.1.6.13939

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Addendum to: Niess JH, Adler G. Enteric flora expands gut lamina propria CX3CR1+ dendritic cells supporting inflammatory immune responses under normal and inflammatory conditions. J Immunol 2010; 184:2026–37; PMID: 20089703; DOI: 10.4049/jimmunol.0901936.

Intestinal dendritic cells (DC) and macrophages play a key role for the maintenance of intestinal integrity by initiating innate and adaptive immune responses. Although DC and macrophages have been viewed as distinct lineages, the reliability of surface markers for the definition of DC and macrophages has recently been questioned. Here, I will discuss the ontogeny and function of CX_3CR1^+ mononuclear cells in the small and large intestine.

The mucosal immune system faces the challenge of a continuous stimulation by microbial products from the commensal microbiota, fungi, viruses and occasionally pathogens.[1] There has been a necessity to define antigen-presenting cell (APC) subsets specialized for directly or indirectly mediating host defence by activating innate/adaptive immune responses.[2] The definition of APC subsets and immune responses has helped to explain bowel pathologies. Crohn disease is considered a TH1 disease and ulcerative colitis is thought to be a TH2 disease.[3] Increasingly complex descriptions of APC subsets have evolved based on (1) the marker profile (i.e., $CD103^+$, CX_3CR1^+, E-cadherin$^+$, $CD70^+$, SIRPalpha$^+$, $CD103^-$), (2) the functional phenotype (i.e., inducing TH1/TH17 T cell subsets or Foxp3 expressing regulatory T cell subsets) (3) the in vivo migration and (4) the ontogeny.[4-13] The reliability of these definitions has been recently questioned when the plasticity of immune cells has been appreciated. Plasticity of cytokine production of T helper subsets has been acknowledged, DC markers are expressed by NK cells and the plasticity of the mononuclear phagocyte system (MPS) has been discussed.[14-16] Hence, controversies have arisen on criteria by which mononuclear cells can be classified as either DC or macrophages.[17] This issue became a major challenge for dissecting mononuclear cell populations from the small and large intestinal lamina propria during normal steady state or inflammation. In particular these controversies can be highlighted on discussions concerning the role of CX_3CR1^+ mononuclear cells in the gut.

Are CX_3CR1^+ Mononuclear Cells a Distinct, Surface Marker-Defined Subset?

DC were identified in the 70's as phagocytic cells adapted for antigen presentation and distinct from macrophages. Surface markers, such as the integrin CD11c and MHC class II have been used as markers for the definition of DC. DC were traditionally further divided into $CD4^+$, $CD8^+$ and $CD4^-$ $CD8^-$ conventional DC (cDC) and $CD11c^+$ $B220^+$ plasmacytoid DC (pDC). Because CD11c is expressed by DC, macrophages and activated NK cells, the sole use of CD11c as a marker for the definition of DC has been challenged. The integrin CD11b is expressed by monocytes, macrophages but also by subsets of DC, granulocytes, NK cells and B1 cells.[16] Multi-color flow cytometry using multiple combinations of surface markers and advances in imaging technologies led to the identification of increasing numbers of mononuclear subsets. CX_3CR1^+ mononuclear cells have been identified by us as a major cell in the small intestinal lamina propria (siLP) and colonic lamina propria (cLP).[6] CX_3CR1^+ cells in the lamina propria (LP) have been described as DC because CX_3CR1^+ cells express CD11c,

MHC II, the costimulatory molecules CD80 and CD86, and have morphological features of DC after in vitro cultures.[6] Recent Giemsa stains revealed CX_3CR1^+ cells with macrophage characteristics such as a vacuolar cytoplasm.[12] We used in our original description of the CX_3CR1^+ cells density centrifugation adapted to the density of DC. The isolation of mononuclear cells from the LP is a difficult procedure which needs to be considered for the interpretation of reported results. In our recent publication we have changed the isolation procedures and identified a $CD11c^-$ CX_3CR1^+, a $CD11c^+$ CX_3CR1^+ $CD68^+$ $F4/80^+$ and a small fraction of $CD11c^+$ CX_3CR1^+ $CD68^-F4/80^-$ cells in the cLP.[18] CX_3CR1^+ cells in the cLP are hence a heterogeneous cell population with a major fraction of cells that express the myeloid markers CD68 and F4/80. As CX_3CR1 is expressed (at different levels) by different LP cell subsets, CX_3CR1 expression per se can in my view not be used as a marker for the definition of a mononuclear subset in this tissue.

Can $CD103^+$ and CX_3CR1^+ Cells be Defined as Distinct Subsets?

Before I will discuss if CX_3CR1 expression indicates a distinct myeloid subset, it needs to be considered that multiple cell types express CX_3CR1. CX_3CR1 is expressed by macrophage dendritic cells precursors (MDP), pDC, $CD4^-$ $CD8^-$, $CD4^+$, $CD8^+$ cDC, monocytes/macrophages, NK cells and T cells.[18-22] Agace and colleagues have identified a $CD11c^{high}$ MHC class II^{high} expressing cell in the siLP and mesenteric lymph nodes (MLN) that was defined by the expression of CD103 (the integrin αE).[5] Intraepithelial lymphocytes and LP T cells are also characterized by CD103 expression. Hence, CD103 is not a surface molecule that solely defines DC. $CD103^+$ $CD11c^+$ cells in LP cell preparations can be divided into $CD11b^+$ and $CD11b^-$ cells.[12] Mice lacking solitary isolated lymphoid tissues (SILT) and Peyer's Pacthes (PP) ($Id2^{-/-}$ animals) have reduced numbers of $CD103^+$ $CD11b^-$ myeloid cells in LP preparations indicating that $CD103^+$ $CD11b^-$ are located in SILT and PP.[12] Despite a small fraction of $CD103^+$ CX_3CR1^+ mononuclear cells in $RAG^{-/-}$ animals CD103 and CX_3CR1 are expressed by distinct subsets in the siLP and cLP.[12,13,18] More CX_3CR1^+ cells than $CD103^+$ DC can be found in the siLP and cLP in the steady state but approximately equal numbers of CX_3CR1^+ $CD11c^+$ and $CD103^+$ $CD11c^+$ cells are found.[11,18] In the siLP, CX_3CR1^+ mononuclear cells are associated with the intestinal epithelium, located in the villous core and present in the SILT. $CD103^+$ $CD11c^+$ cells are located more central within the LP of the villous.[11] In the colon CX_3CR1^+ mononuclear cells form a network beneath the epithelium around intestinal crypts.[18] CX_3CR1^+ mononuclear cells are also present in colonic lymphoid aggregates. The exact location of $CD103^+$ cells in the cLP has not been determined. The turnover of $CD103^+$ cells in the siLP is rapid indicating that this population is replenished by blood borne precursors.[23] Turnover rates of CX_3CR1^+ mononuclear cells appear to be slower as shown in transplant experiments.[11] Together, this evidence indicates that CD103 and CX_3CR1 are expressed by distinct LP cells under steady state conditions.

Are CX_3CR1^+ Cells Associated with Specific Functions?

CX_3CR1^+ mononuclear cells are close associated with the intestinal epithelium. CX_3CR1^+ mononuclear cells are more efficient than $CD103^+$ DC in sampling luminal antigens in the LP. In the PP, lysozyme-expressing CX_3CR1^+ cells are located in the subepithelial dome regions associated with highest expression of MHC class II and expression of co-stimulatory molecules; this population is most efficient in sampling luminal antigens.[24] After sampling of Salmonella CX_3CR1^+ $CD11b^-$ mononuclear cells migrate into the intestinal lumen to expel pathogens from the host.[25] In our hands CX_3CR1^+ $CD11c^+$ cells are characterized by CD11b expression. TH17 cells are most frequent in the LP of the terminal ileum.[26,27] CX_3CR1^+ $CD70^+$ LP cells induce the differentiation IL-17 secreting CD4 T cells in presence of ATP in in vitro assays.[4] Macrophage-derived IL-10 inhibits the production of pro-inflammatory cytokines by CX_3CR1^+ $CD11b^+$ $CD11c^+$ mononuclear cells.[28] IL-10 producing $CD11c^+$ $CD11b^+$ $F4/40^+$ (that are likely CX_3CR1^+ mononuclear cells) are required for maintaining Foxp3 Treg cells indicating CX_3CR1^+ mononuclear cells are able to promote TH17 differentiation and to maintain Foxp3 regulatory cells. In Crohn disease patients $CD14^+$ CX_3CR1^+ $CCR9^+$ mononuclear cells [that express DC markers such as DEC-205 (CD205) and DC-SIGN (CD209) and monocyte/macrophage markers such as CD14] produce IL-23 and TNFα and induce the differentiation of IFNγ secreting CD4 T cells.[29,30] $CD11c^+$ MHC class II^+ cells are able to present antigens directly to effector/memory T cells within the intestinal mucosa.[31] Migration of mononuclear cells to the MLN seems not required for the activation of effector/memory CD4 T cells but their role in regulating adaptive immunity needs further examinations. CX_3CR1^+ mononuclear cells seem to adapt their cytokine profile to challenges provided by the local environment. Plasticity of CX_3CR1^+ mononuclear cells may be a key feature of the mucosal immune system to maintain intestinal homeostasis and combat pathogen challenges.

Does CX_3CR1 Surface Expression Correlate with the Function of Mononuclear Cells?

Fractalkine (CX_3CL1) is expressed by intestinal endothelial and epithelial cells and plays a role in lymphocyte migration and adhesion.[32] Binding of CX_3CL1 to CX_3CR1 activates the NFκb pathway and induces the release of pro-inflammatory mediators, such as IL-8, by epithelial cells.[22] The stimulation of mononuclear cells with CX_3CL1 induces the release of TNF, IFNγ, IL-6 and iNOS in a dose-dependent manner.[33,34] $CX_3CR1^{-/-}$ animals have an increased susceptibility to pathogens, such as Salmonella and Listeria.[6,35] Dextran sodium sulphate (DSS) colitis is attenuated in $CX_3CR1^{-/-}$ animals. The attenuated DSS colitis is associated with reduced TNF, IL-6 and iNOS expression by macrophages and DC.[36] In accordance transfer colitis is attenuated in $CX_3CR1^{-/-}$ animals associated with reduced numbers of IFNγ and IL-17A secreting CD4 T cells in the cLP.[18] Of note, polymorphism of *CX_3CR1* is associated with Crohn

disease.[37] Animals that harbor CX_3CR1^+ cells and lack $CD103^+$ DC are characterized by accelerated DSS colitis.[13] Surface expression of CX_3CR1 is not only a phenotypic marker of mononuclear cells in the LP. Distinct functions of APC subsets in the LP correlates with phenotypic markers; i.e., the release of proinflammatory cytokines by CX_3CR1^+ mononuclear cells depends in part on the binding of fractalkine/CX_3CL1 to CX_3CR1. CX_3CR1 expression by mononuclear cells is in my view associated with the function of CX_3CR1^+ mononuclear cells.

Are CX_3CR1^+ Mononuclear Cells Specialized for Antigen Sampling?

Intestinal mononuclear cells are close associated with the intestinal epithelium. In the ileum intestinal mononuclear cells are extending dendrites into the intestinal lumen.[38] Rescigno and colleagues have visualized these processes in small intestinal loops infected with Salmonella.[39] Constitutive p40 promoter and IL-23 activation has been observed in $CD11c^+$ cells extending processes into the lumen located in the ileum of transgenic mice.[40] We confirmed the presence of transepithelial dendrites by ex vivo confocal imaging in CX_3CR1-GFP reporter animals.[6] The number of transepithelial processes increased from the upper to the lower small intestinal and were preferentially observed in the terminal ileum of CX_3CR1-GFP reporter animals. In part, intestinal epithelial cell derived CX_3CL1 is involved in the formation of transepithelial processed because the number of transepithelial processes is reduced in CX_3CR1-deficient animals. Transepitethelial dendrites can be also observed in MHC II—EGFP reporter animals.[6] Chieppa and colleagues have challenged the view that the formation of transepithelial processes solely depends on CX_3CR1.[41] Challenging mice with pathogens, such as Salmonella or Aspergillus, or with TLR ligands increases the numbers of transepithelial dendrites.[6,41-43] Myd88-deficient animals have reduced numbers of transepithelial dendrites. Treatment of animals with antibiotics led to the reduction of transepithelial processes.[41] In accordance the number of transepithelial processes is significantly reduced in germ-free animals.[18] Microbial products are the major driving force for the formation of transepithelial processes. In the colon the formation of transepithelial processes has only been observed after the infection of animals with *Trichuris muris*.[44] Colonic mononuclear cells are located beneath the epithelium and have processes directed into but not through the epithelium in the steady state. CX_3CR1^+ mononuclear cells play a role in sampling luminal antigens in the ileum; their role in luminal antigen acquisition in the colon is currently unknown. LP CX_3CR1^+ mononuclear cells are phagocytic. If LP CX_3CR1^+ mononuclear cells only scavenge and degrade ingested material, or also present in an immunogenic form epitopes processed from this phagocytosed material remains to be established.

Are DC Defined by their Ability to Prime Naïve T Cells?

$CD103^+$ DC are more efficient than CX_3CR1^+ cells in priming CD4 and CD8 T cell responses.[11] In vitro co-culture experiments have shown that $CD103^+$ DC isolated from the MLN and LP play an important role in the induction of CCR9 and the integrin $\alpha4\beta7$ on CD4 and CD8 T cells. $CCR9^+$ and $\alpha4\beta7^+$ CD4 and CD8 T cells are programmed to home to gut tissues.[5] $CD103^+$ DC also induce the differentiation of $Foxp3^+$ regulatory T cells.[10,45] The numbers of $CCR9^+$ CD4 and CD8 T cells and $Foxp3^+$ Treg cells are not reduced in the LP of Batf3-deficient animals (that lack $CD103^+CD11b^-$ cells) challenging the view that $CD103^+$ $CD11b^-$ cells play a major role in generating gut-homing T cells and $Foxp3^+$ Treg cells.[46] Under inflammatory conditions $CD103^+$ DC induce the differentiation of TH17 cells and IFNγ producing TH1 cells.[47] Intestinal inflammation may hence abrogate the tolerogenic properties of intestinal $CD103^+$ DC. Not only DC but also macrophages are able to stimulate naive CD4 and CD8 T cells in vivo to proliferate, develop effector function and differentiate into memory cells.[48,49] IFNγ stimulated $CD14^+$, $F4/80^+$ and $CD11b^+$ macrophage cell lines generate TH1 responses after pulsing with antigen.[50] Together, published work indicated that macrophages and DC are able to prime naïve T cells. In my view the ability of a cell to activate naïve T cells is not a sole criterion for the definition of DC or macrophages.

Are DC Defined by Migration to the MLN?

DC pick antigens in peripheral sites transport the antigens to draining lymph nodes, where T cells are primed. Migration of DC from the intestinal LP to the MLN depends on the chemokine receptor CCR7. Schulz and colleagues demonstrated by confocal imaging and flow cytometry analyses of cells collected from the draining lymph that $CD103^+$ but not mononuclear cells with high CX_3CR1 expression migrate to the MLN in the steady state. A small fraction of cells with low CX_3CR1 expression was found in lymph vessels. In MLN pDC, $CD4^+$, $CD8\alpha^+$ and $CD4^-$ $CD8\alpha^-$ cDC express CX_3CR1.[18] After oral infection of CX_3CR1-GFP reporter animals with invasive and non-invasive Salmonella mutants red fluorescent Salmonella can be identified within CX_3CR1^+ cells of the MLN.[6] *E. coli* F-18 is localized within CX_3CR1^+ cells of the MLN after colonization of CX_3CR1-GFP animals with the apathogenic commensal *E. coli* F-18. When CX_3CR1^+ cells from lymphoid tissues are compared to CX_3CR1^+ cells from the lamina propria the CX_3CR1 expression levels vary between lymphoid and non-lymphoid cells. In my opinion the question arises if cells with distinct CX_3CR1 expression are distinct subsets or if alternatively the tissue microenvironment influences the degree of CX_3CR1 expression by a given cell. After performing acute inflammatory and scavenging roles in the peritoneal cavity inflammatory macrophages emigrate to draining lymph nodes. The migration of macrophages from the inflamed mesothelium to the lymphatics depends in part on the integrin MAC-1 (CD11b).[51] The migration behaviour of CX_3CR1^+ mononuclear cells has been only investigated in the steady state but not during inflammation. Further studies are required examining the migration pattern of intestinal mononuclear cells from the inflamed cLP and siLP to draining lymph nodes and to the liver via the portal vein.

Are CX_3CR1^+ Mononuclear Cells Generated by Specific Pathways?

Conventional DC, pDC and monocytes originate from a common bone marrow progenitor, the macrophage dendritic cells precursor (MDP). MDP give rise to common DC precursors (CDP), from which cDC and pDC but not monocytes originate. CDP can develop in pre-DC that are committed to the development of cDC but not pDC. Recent work has examined the role of CDP, pre-DC and monocytes in CD11c depleted animals (diphteria toxin treated CD11c-DTR mice).[12,13] MDP gave rise to $CD103^+$ and CX_3CR1^+ myeloid cells. Adoptive transfer of CDP and pre-DC supported the appearance of $CD103^+CD11b^-$ and $CD103^+CD11b^+$ DC. The development of $CD103^+$ cells is under control of FMS-like tyrosine kinase 3 (Flt3) and granulocyte-macrophage-colony-stimulating factor (GM-CSF) because $CD103^+$ DC are reduced in *Flt3r*- and GM-CSF (*csf2r*)-deficient animals. $CD103^-CD11b^+MHC\ II^+$ LP cells (that are CX_3CR1^+ and CX_3CR1^- negative cells) are reduced in M-CSF receptor (*Csf1r*) deficient animals. Genetic deletion of the transcription factors *Id2*, *Irf8* and *Batf3* is associated with reduced numbers of $CD103^+$ $CD11b^-$ cells in the LP and extraintestinal tissues, such as the skin, lung and kidney.[12,46] This data suggested that peripheral $CD103^+$ $CD11b^-$ cells are related to $CD8\alpha^+$ lymphoid DC. Engrafted $Ly6C^{hi}$ monocytes give rise to CX_3CR1^+ mononuclear cells. Hence, the development of $CD103^+$ DC is under control of Flt3 and GM-CSF. In contrast M-CSF controls the development of CX_3CR1^+ LP cells. Hence, $CD103^+$ DC and CX_3CR1^+ mononuclear cells in non-lymphoid tissues are generated in different pathways.

Can CX_3CR1^+ Mononuclear Cells be Defined as Macrophages or DC?

The definition of CX_3CR1^+ mononuclear cells as macrophages or DC based on marker profile, functional phenotype, in vivo migration and ontogeny has led to confusion. In part the plasticity, adaptability and heterogeneity of macrophages and DC have contributed to these difficulties. The definition of CX_3CR1^+ mononuclear cells into macrophages or DC may seem on one hand arbitrary; on the other side it has implication for disease pathogenesis and therapeutic intervention when this task is carefully considered. If DC are the only APC that shape adaptive immunity targeting these cells for the development of vaccines and inhibiting autoimmunity is important. Targeting macrophages may interfere with their capability to produce pro-inflammatory cytokines without affecting adaptive immunity. CX_3CR1^+ mononuclear cells have macrophage- and DC-like characteristics. CX_3CR1^+ mononuclear cells are able to promote TH17 differentiation and maintain Foxp3 regulatory cells.[4,29] CX_3CR1^+ mononuclear cells do not express CCR7, are less efficient than $CD103^+$ DC in migration to MLN and are less efficient than $CD103^+$ DC in priming naïve T cells. The functional characterization of CX_3CR1^+ mononuclear cells is currently based on isolation from the LP and on carrying out in vitro co-culture assays. Cell isolation techniques from the intestinal LP do not carefully consider the location of CX_3CR1^+ mononuclear cells in the gut. Discrimination of the CX_3CR1^+ cells associated with the epithelium and extending processes into the intestinal lumen from CX_3CR1^+ cells located in the LP or within LP is currently a major challenge. Improved imaging and adoptive transfer technologies needs to be developed allowing the characterization of CX_3CR1^+ mononuclear cells in vivo. Improved techniques will increase our knowledge on CX_3CR1^+ mononuclear cells providing the opportunity to specifically target CX_3CR1^+ cells during intestinal inflammation for the potential benefit of patients with Crohn disease or ulcerative colitis. Of course, there might be also disadvantages in strategies targeting a specific cell type. Disastrous infections with pathogens may develop after specific targeting of mononuclear cells. CX_3CR1^+ mononuclear cells in the LP are in my view phagocytes specialized for the sampling of luminal antigens. If CX_3CR1^+ mononuclear cells only scavenge ingested materials or process and present epitopes to T cells are currently highly controversial issues. Current technologies need to be improved and new technologies developed to solve those issues. The controversies on the classification of CX_3CR1^+ mononuclear cells as DC or macrophages will help to increase the understanding of their role in mucosal immunology with possible implications for the development of vaccines, pathogen defence and target strategies for the treatment of patients with inflammatory bowel disease.

Acknowledgements

I would like to thank G. Adler for continuous support. J.H.N. is supported by DFG grants Ni575/6-2 and Ni575/7-1.

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