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Specialized Functions of Lamina Propria Antigen-Presenting Cells in the Maintenance of Intestinal Immune-Homeostasis

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3 Abbreviations

AF Alexa Fluor

Ag Antigen

AID Activation-Induced cytidine Deaminase

AMP Anti-Microbial Peptides

APC Antigen-Presenting Cell

BM Bone marrow

cDC conventional dendritic cell

CDP Common DC precursor

CFA Complete Freund's Adjuvant

CSR Class-Switch Recombination

Cx Connexin

DC Dendritic Cell

DTH Delayed type hypersensitivity

EC Epithelial Cell

FBS Fetal Bovine Serum

Flt3 fms-like tyrosine kinase 3

Foxp3 Forkead Box P3

GALT Gut-Associated Lymphoid Tissue

GFP Green Fluorescent Protein

GI Gastrointestinal

GJ Gap Junction

GM-CSF Granulocyte Macrophage Colony - Stimulating Factor

i.g. Intra gastrically

i.f. Intra-footpad

IBD Inflammatory Bowel Disease

IEC Intestinal Epithelial Cell

IL Interleukin

IRAK1 IL-1 Receptor Associated Kinase 1

LP Lamina Propria

M-CSF Macrophage Colony Stimulating Factor

MDP Macrophage - DC precursor

MLN Mesenteric Lymph Node

MMP Matrix Metalloproteinase

Mϕ Macrophages

NLR Nucleotide - binding domain and Leucine - rich repeat containing Receptor

OVA Ovalbumin

PAMP Pathogen-Associated Molecular Pattern

PP Peyer's Patch

PRR Pattern Recognition Receptor

RA Retinoic Acid

RLR Retinoic Acid - Inducible gene-I - like Receptor

s.c. Subcutaneous

SED Sub - Epithelial Dome

SHM V(D)J somatic hypermutation

SI Small Intestine

sIgA Secretory IgA

spp species

TG-β Transforming Growth Factor

TLR Toll-like receptor

T_{reg} T Regulatory cell

4 Abstract

The intestinal immune system is daily threatened by the exposure to potentially harmful agents, such as food antigens and the gut flora. They represent, indeed, a potent immunogenic stimulus that the immune system has to tolerate.

In the Gut-Associated Lymphoid Tissue, the cooperation and mutual interaction of specific environmental factors and peculiar populations of antigen-presenting cells renders the gut a suitable site for the maintenance of a tolerogenic milieu, where immunosuppressive mechanisms keep at bay unwanted deleterious immune responses.

The establishment of tolerance to food antigens mostly relies on the ability of specific subsets of mononuclear cells to take up antigens in the SI (Small Intestine) and to subsequently shape the immune response, which is initiated in the draining mesenteric lymph nodes.

Here, we describe a subset of resident intestinal macrophages, expressing CX_3CR_1, which are committed to antigen uptake. Furthermore, we show that in the absence of functional CX_3CR_1 receptor, which impairs the ability of these cells to sample the intestinal lumen, decreased antigen uptake and failure in the establishment of oral tolerance is observed in CX_3CR_1-deficient mice.

As $CX_3CR_1^+$ cells are sessile and poorly able to prime T cells, we hypothesized the possibility of gap junction-mediated antigenic material transfer to $CD103^+$ dendritic cells, which have been accounted for migratory tolerogenic cells. We show, indeed, that mononuclear cells from the SI can exchange peptides and gap-junction diffusible dyes. Furthermore, $CX_3CR_1^+$ macrophages and $CD103^+$ dendritic cells express a peculiar panel of connexins, among which connexin 43 is the most expressed.

We then generated mice lacking connexin 43 in $CD11c^+$ cells. These mice displayed no change in the frequency and activation state of the different populations of lamina propria cells but decreased the levels of peripheral T regulatory cells at steady-state. In addition, we observed impaired establishment of oral tolerance to ovalbumin. This indicates that DC-expression of Cx43 is required for the establishment of oral tolerance. Whether this is

due to a lessening in antigen transfer which impinges on the ability of $CD103^+$ dendritic cells to prime T cells towards a tolerogenic phenotype or to other Cx43-dependent functions of SI mononuclear cells are still open questions, which need to be addressed.

5 Introduction

The gastrointestinal system consists of a hollow muscular tube going from the mouth to the anus, primarily implicated in the digestive process. As a result of its main function, the mucosal surface of the GI tract is daily exposed to food antigens introduced with the diet. Such a high antigen load represents a potent activation stimulus the immune system has to tolerate and constantly cope with. Indeed, the inappropriate activation to such harmless antigens (Ag_s) would result in tissue damage and reduction of functional efficiency of the organ.

An additional layer of complexity at the intestinal level is conveyed by the commensal bacteria that colonize the gut and mutualistically share nutrients and ecosystem with their host. If only considering Pathogen - Associated Molecular Patterns expressed by these microorganisms, they are indistinguishable for the immune system from the pathogens that might be accidentally introduced *via* the oral route.

Thus, a highly specialized immune system that could properly respond to this variety of stimuli is needed, in order to mount an effective immune response against infectious microorganism, as well as to induce tolerance to commensal bacteria and food antigens. Hence, at steady state a sort of *immune privilege* [1] characterizes the intestinal environment where regulatory mechanisms control and, eventually, suppress the establishment of a response against innocuous antigens encountered at the periphery.

Immune hyporesponsiveness to bacterial and food Ag_s in the gut relies on diverse but interconnected mechanisms [2] and is established with a different range of action. Indeed, tolerance to fed Ag_s - usually referred as oral tolerance - is systemically spread, being the immune unresponsiveness maintained even when the same antigens are parenterally administered under immunogenic conditions [3, 4]. On the other hand, tolerance to commensal bacteria is restrained locally, as systemic immune “ignorance” towards bacterial Ag_s is pursued [5].

The maintenance of a balanced immune response in the gut is clinically relevant since its pathological alteration may lead to food hypersensitivities and allergies or inflammatory

bowel diseases (IBD), such as Crohn's disease and ulcerative colitis. Together with the contribution of environmental factors, IBD susceptibility depends indeed on genetic alteration that mostly concern the ability of the immune system to correctly deal with gut bacteria [6].

5.1 Tolerance to bacteria

The intestine is colonized by a heterogeneous microflora, a complex ecosystem containing over 1000 identified species, sparse in the stomach and the upper intestine and widely present in the large intestine [7]. For instance, concentrations of 10^9-10^{11} bacteria for gram of content are found in the human colon, most belonging to anaerobic genera, such as *Bacteroides, Bifidobacterium, Eubacterium, Peptostreptococcus,* and *Clostridium.*

The relationship between the host and its microflora outwits the simple symbiosis (non-harmful coexistence), but it is referred as mutualism [8]. Indeed, gut flora has been implicated in different functions that benefit the host, such as the absorption of complex macromolecules, the synthesis of vitamin K and some components of the vitamin B complex [9], and the prevention of the colonization by pathogenic bacteria. In fact, it hampers the overgrowth of potentially pathogenic members, as it physically occupies most available niches, limits resources and space for invading microorganisms and produces antimicrobial substances to inhibit their growth.

The acquisition of a fully diversified microbiota is instrumental to the postnatal development of the gut immune system during the first years of life. Particularly, recent studies conducted in mice have highlighted the role of some bacteria, such as Segmented Filamentous Bacteria (SFB), in their superior ability to induce the maturation of the intestinal immune system [10, 11]. Also T regulatory cells induction has been associated to particular bacterial species, such as *Clostridium* spp., or bacterial components, such as polysaccharide A derived from *B. fragilis*. [12, 13].

In the gut, distinctive anatomical and functional features well suit the need to tolerate and maintain a controlled homeostasis toward intestinal flora [5, 14], namely i) the presence of the epithelium that physically avoid the direct contact between the intestinal immune system and the microorganisms confined in the lumen; ii) secreted factors, such as the mucus, antimicrobial peptides and IgA_s, that limit the contact between the epithelium and the microbes; iii) system for the rapid detection and killing of bacteria that might penetrate the epithelial barrier, as in the case of tissue macrophages; iv) maintenance of systemic "ignorance" to bacteria (Figure 5-1). A general overview of these issues will be given in the next paragraphs.

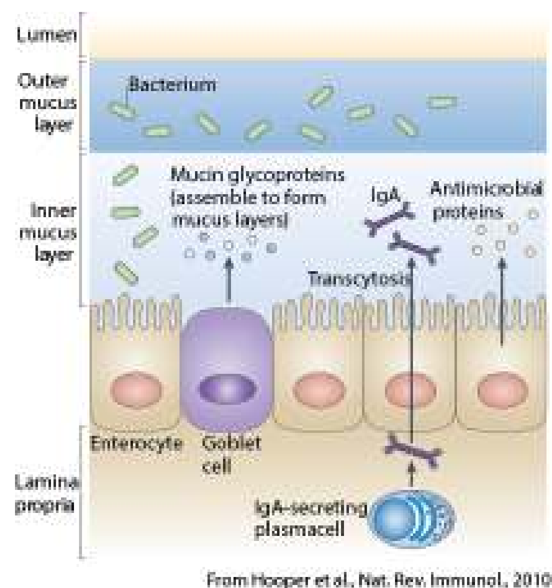


Figure 5-1 Immune mechanisms that contribute to the maintenance of intestinal homeostasis. Distinctive anatomical and fuctional features are important to tolerate intestinal microbes: i) the epithelium; ii) secreted factors, such as the mucus, anti-microbial peptides and $sIgA_s$; III) presence of highly phagocytic cells in the tissue; iv) maintenance of systemic ignorance to bacteria.

5.1.1 Epitehelium

The intestinal mucosa is lined by an extensive layer of columnar epithelium that provides both a barrier to pathogens and commensal bacteria, a selective transit for fluid and electrolyte absorption and secretion. The epithelium is a polarized surface, in contact at

the apical side with the luminal content and at the basolateral side with the immune compartment, consisting mostly of organized lymphoid tissue and populations of T and B lymphocytes and innate immune cells scattered throughout the lamina propria.

For a long time, the epithelium has been considered as just a mechanical barrier impeding the uncontrolled translocation of invading pathogens, such as bacteria, virus and parasites. The continuity of the layer is conferred by tight and adherens junctions located near the apical surface of the columnar epithelium. Cell - cell adhesion at these junctions is maintained by complexes of proteins, such as occludins, zonula occludens (ZO) - 1 and -2 and members of the claudin family.

Recently, this limited view has been broadened by the observation that the epithelium is not only a physical barrier but it takes an active part in immunological processes [15].

Indeed, epithelial cells - through the expression of different Pattern Recognition Receptors (PRR_s) - may recognize philogenetically conserved PAMPs, which are shared by pathogenic bacteria and non - pathogenic ones, and send signals to the mucosa beneath.

PRR_s are classified in three main groups, namely Toll - Like Receptors (TLR_s), Nucleotide-binding domain and Leucine - rich repeat containing Receptors (NLR_s) and Retinoic Acid - Inducible gene- I - like Receptors (RLR_s) [16]. They differ for their localization and ligand specificity; their expression is not limited to epithelial cells (EC_s), but they are found in different cell types of the immune system, such as monocytes, dendritic cells and macrophages [17]. TLR_s are membrane - bound, NLR_s and RLR_s are cytosolic.

How epithelial cells can discriminate between gut flora and pathogens is still a topic under active debate. Certainly, only bacteria that are able to infect EC_s and break the integrity of the epithelial monolayer can interact with receptors that that expressed intracellularly - such as NOD1 and NOD2 - or in the basolateral side of the epithelium - such as TLR5 [18]. More precisely, as a result of tissue damage, bacteria can permeate into the tissue, reach EC basolater side and start a pro - inflammatory cascade, as it has been described for TLR5 [19].

On the other hand, at steady - state microbes are confined into the lumen and basal PRR_s activation from the apical side is beneficial to the maintenance of EC integrity and

intestinal homeostesis to bacteria. For example, a polarity - dependent response has been shown *in vitro* for TLR9 [20]. When TLR9 is stimulated from the apical side, a defective activation of the NF-κB pathway and a peculiar gene expression profile of apically - vs. basolaterally - stimulated cells have been reported.

In vivo, this is paralleled by the observation of spontaneous development of colon inflammation or increased susceptibility to experimental colitis in several mouse models of PRR_s - deficiency, such as TLR-2, -5, -4, -9 and MyD88 knock - out mice [20-24]. Although the use of straight knockout mice prevents to fully discern EC_s' from immune cells' contribution to the phenotype, general defects in the absence of TLR signalling affect EC functions, including decreased proliferation and secretion of cytoprotective factors and increased apoptosis. Nonetheless, specific inhibition of NF-κB pathway in EC_s by IKKγ or IKKα / IKKβ deletion [25] recapitulates the observed phenotype in the previously mentioned knock - out mice.

Moreover, commensal and non - pathogenic bacteria dampen the induction of an inflammatory response mainly modulating NF-κB activation at different levels [26], for example post - transcriptionally inhibiting the pathway activator IL-1 Receptor Associated Kinase 1 (IRAK1) soon after birth in mice [27, 28], or stabilizing Iκ-Bα in the cytosol in human intestinal epithelial cell lines [29-31], or shuttling nuclear NF-κB subunit RelA out the nucleus [32]. Of note, prolonged stimulation with TLR agonists renders intestinal epithelial cells hyporesponsive to subsequent stimulation, due to reduced surface expression of the receptors and upregulation of negative regulators of the NF-κB pathway, such as Tollip [33].

5.1.2 Epithelial Cell - derived conditioning factors

As the epithelium strictly interacts with Antigen - Presenting Cells (APC_s) in the lamina propria (LP), conditioning by EC-derived products may provide an important mechanism for the maintenance of intestinal hyporesponsiveness by APC_s. For instance, such conditioning renders DCs able to induce a tolerogenic T cell response in absence of

infection, limiting the production of pro-inflammatory cytokines. Nonetheless, in the presence of inflammatory signals, inflammatory cytokines and chemokines released by IEC_s would recruit a new pool of “non-conditioned” dendritic cells, competent for sustaining an active immune response [15].

In vitro systems based on the co-culture of polarized epithelial cells with dendritic cells have led to the identification of factors that contributes to APC conditioning. For example, human colonic EC_s release thymic stromal lymphopoietin (TSLP), which drives a non-inflammatory Th2 response and inhibits the capacity of monocyte-derived DCs to produce IL-12, a Th1-polarizing cytokine [34, 35] even when infected with *S. Thyphimurium*.

Additionally, *Iliev et al.* showed that EC - conditioned dendritic cells promote T regulatory cell (Treg) cell differentiation, through a mechanism dependent on TSLP, Transforming Growth Factor (TGF)-β and Retinoic Acid (RA) in humans [35]. Decreased expression of Tslp, Tgfb1 and Aldh1a1 (which catalyzes the reaction from Vitamin A to Retinoic Acid) genes is observed in EC_s isolated from Ulcerative colitis and Crohn’s disease patients, suggesting an *in vivo* role of these mediators in intestinal homeostasis.

Besides, intestinal epithelial cells are able to shape the local humoral response secreting B-cell activating factor (BAFF, also known as TNFSF13B) and a proliferation-inducing ligand (APRIL, also known as TNFS13) [36] that promote class-switching of B cells toward the production of IgA_s. Furthermore, through the release of TSLP, EC_s induce dendritic cells themselves to release BAFF and APRIL [36].

Once secreted, IgA_s are bound to the polymeric immunoglobulin receptor (pIgR), translocated through IEC_s to the lumen and released by proteolitic cleavage of the pIgR.

5.1.3 The mucus layer

Secreted factors, such as mucins, anti - microbial peptides (AMP_s) and secretory IgA_s, are then released to further prevent luminal bacteria to contact EC surface.

Mucous and goblet cells along the GI tract release mucins, high molecular weight - proteins characterized by extensive glycosylation [37]. Varying along the intestinal length,

the mucus layer is characterized by different properties, including mucin composition, viscosity and thickness. In the colon, where high bacterial burden is observed, the mucus layer is thicker, mainly made up of MUC2, and easily distinguishable in two main sheets. The inner one is a densely packed matrix of membrane - anchored mucins, devoid of bacteria [38] and rendered even more inhospitable as it concentrates EC - derived anti - microbial peptides and $sIgA_s$. The external layer is looser and represents the habitat for intestinal flora.

The role of mucins in the protection against invading bacteria is highlighted by increased susceptibility to pathogen infection reported for MUC1 knockout mice [39, 40] or to spontaneous colitis for MUC2 knockout mice [41, 42]. Mucins may even bind pathogens, as in the case of MUC1, that can be released by human gastric epithelial cells and adhere to *Helicobacter pylori* to limit its invasiveness [43].

5.1.4 Antimicrobial peptides

Anti-microbial peptides, such as defensins, cathelicidins and C-type lectins, are constitutively produced by IEC_s or inducibly released after PRR engagement by Paneth cells - specialized EC_s localized at the base of the intestinal crypts [44, 45]. Various mechanisms account for AMP bactericidal activity, but often they interfere with membrane integrity and cell wall synthesis. Indeed, AMP polar structure, consisting of charged heads and a central hydrophobic region, allows their insertion in the bacterial membrane to form “pore - like” or “carpet - like” structures [46], making holes or covering bacteria that are finally killed. Besides, chemo-attractant and immunomodulatory properties have been reported for some defensins [47].

As highly redundant in their function, single knockout mice for AMP genes are ineffective to prove AMP importance in the maintenance of intestinal homeostasis. Only the use of approaches that directly target Paneth cells or the production / maturation of AMP have offered valuable tools to understand AMP role in controlling the intestinal microflora and prevent pathogens outgrowth. For instance, Paneth cells - deficient mice, that express a

diphtheria toxin fragment under the control of the Paneth cell - specific cryptidin-2 promoter, or Myd88$^{-/-}$ mice, that show lower expression of AMP_s in the small intestine, display increased translocation of commensal bacteria to the mesenteric lymphnodes (MLN_s) and increased systemic spreading of *S. typhimurium* [45]. In Myd88$^{-/-}$ mice, the observed phenotype is reverted by Paneth cell - specific re - expression of Myd88.

A similar phenotype is observed in mice lacking the gene for MMP-7 (Matrix Metalloproteinase-7), which is required for the processing of pro-α-defensins into their active form [48].

Interestigly, the increased susceptibility of Nod2 - deficient mice to *H. Hepaticus* infection is reverted by the expression of human α - defensin 5 (HD5) [49], suggesting a connection to the human pathology, as Crohn's disease patients with NOD2 susceptibility alleles display a reduced expression on α - defensins. In humans, also the genetic variants in the transcription factor TCF4, implicated in goblet cell maturation and function, has been associated with ileal Crohn's disease [50].

5.1.5 Secretory IgA

IgA_s represent a further line of defense to uncontrolled bacterial translocation through the epithelium and are abundantly found in the mucosal secretion. Their release into the gut lumen requires the formation of dimers of IgA_s *via* the interaction with the joining (J) chain and the recognition by the polymeric immunoglobulin receptor (pIgR) expressed by EC_s [51].

B cell development begins in the bone marrow, where the exons coding for immunoglobulin heavy-chain (IgH) and light-chain (IgL) variable regions are re - arranged from variable (V), diversity (D) and joining (J) segments, through a process known as V(D)J recombination. Properly arranged IgH and IgL are then expressed as IgM heavy and light chains on newly generated B cells.

After leaving the bone marrow, IgM - expressing naïve B cells further diversify their repertoire through V(D)J somatic hypermutation (SHM) and heavy chain class - switch

recombination (CSR), both requiring activation-induced cytidine deaminase (AID) enzymatic activity [52].

The process of SHM introduces point mutations at high rate in the variable regions of IgH and IgL genes and ends up with the selection of B cells producing high affinity antibodies; CSR consists in the substitution of the IgH constant (C)-region (Cμ) exons with downstream IgH C-region exons, such as Cγ, Cα and Cε. Hence, antigen specificity is preserved, yet the antibody isotype is changed to IgG, IgA or IgE, respectively.

In the GALT, induction of IgA^+ B cells takes place in organized lymphoid tissues, such Peyer's Patches and isolated Lymphoid Follicles (ILF_s) and unorganized regions, namely B cells sparse in the lamina propria [52].

Different signals are required for IgA class-switch recombination and induction of AID expression by B cells (Figure 5-2), including soluble factors, cytokines and / or ligation of CD40 expressed by B cells. Engagement of CD40 by its ligand CD40L present on T cells is dispensable for IgA CSR, as demonstrated by almost normal secretion of IgA_s in the gut of CD40 knockout mice [53] and of patients affected by congenital type-3 hyper-IgM syndrome characterized by CD40 deficiency [36].

Indeed, induction of AID expression by released factors and cytokines, such as Transforming Growth Factor (TGF)-β, APRIL, Retinoic Acid, interleukin (IL)-6 or IL-10 [52, 54], renders IgA CSR potentially independent by T cell. This is the case for IgA_s to commensal bacteria, whose release may be T cell-independent and necessitate direct interaction of dendritic cell (DC_s) with B cells [55, 56].

Macpherson et al. showed indeed that DC_s in the dome of PP_s are able to sample the commensal flora and convey live bacteria to the draining mesenteric lymphnodes, where they induce T cell - independent IgA production by B cells [55]. This process allows the containment of the bacteria to the MLN_s which act as a sort of "firewall" and prevent bacterial dissemination through the lymphatic and blood systems. Neverthelss, induced IgA^+ B cells can recirculate through the mucosal tissues and home back to the gut as the result of their expression of gut homing-receptors [57].

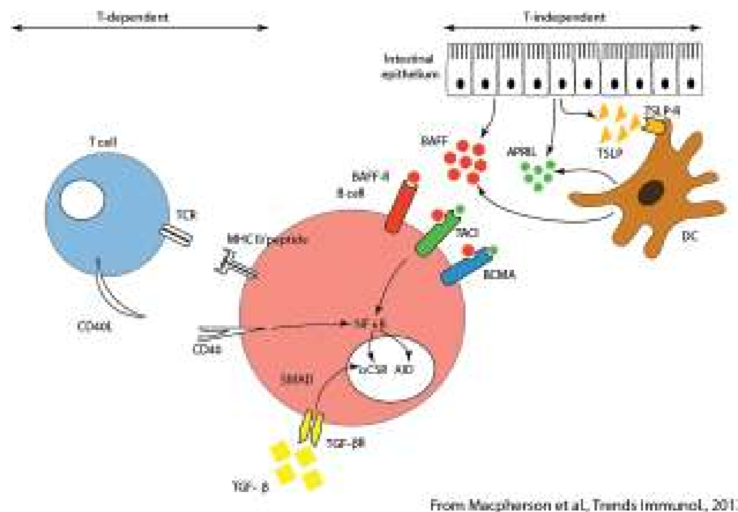


Figure 5-2 T cell-dependent and -independent mechanisms of Class-Switch Recombination in B cells. IgA class-switch recombination can be initiated through T cell-dependent or -independent pathways. The former is due to CD40-CD40L interaction between T and B cells and lead to subsequent NF-κB pathway activation. NF-κB pathway can be alternatively engaged by factors secreted by ECs, such as BAFF and APRIL, independently of T cells. Besides, TSLP secreted by IECs acts on dendritic cells which start producing BAFF themselves.

The role of IgA_s in the control of intestinal microflora has been investigated through a number of different approaches; in general, the lack of IgA_s usually results in the alteration of the commensal relationship between the intestinal microflora and its host.

To evaluate the effect of single components on this process, *Peterson et al.* took advantage of germ-free $RAG1^{-/-}$ mice monocolonized with *B. thetaiotamicron* implanted with a hybridoma releasing monoclonal IgA_s specific for a bacterium's capsular polysaccharide [58]. In the absence of IgA_s (i.e. mice without hybridoma), a potent innate immune response - exemplified by iNOS upregulation - is induced at the intestinal level and *vice versa* the bacterium needs to express enzymes required for the metabolism of host oxidative products. On the other hand, IgA production brings back the balance between host immune system and gut flora to the equilibrium, normalizing cytokine secretion.

Moreover, mice which are knockout for Aicda and lack IgA_s display an expansion of anaerobic bacteria, SFB among the others, which leads to the systemic hyperactivation of germinal center B cells [59, 60]. To dissect single contribution of CSR and SHM to this process, mice with point mutation in AID (AID^{G23S} mutant) were generated [61]. These

mice, which are characterized by normal CSR but defective SHM, show expansion of both aerobic and anaerobic bacteria in the feces and increased systemic dissemination of *Y. Enterocolitica*, though the effects being milder than the $AID^{-/-}$ mice. This suggests that diversification of the IgA repertoire through SHM, which has to shown to be dependent on the gut flora, T cells and Rorγt transcription factor [62], is necessary for the preservation of the host - microbes mutualism and for mucosal protection.

5.2 Oral tolerance

The general term “tolerance” refers to a series of mechanisms which take place in order to prevent the activation of the immune system to an antigen. It exists in three forms: central, peripheral and acquired.

The first line of selection occurs in the primary lymphoid organs, i.e. the thymus and the bone marrow, where newly developing T and B cells, respectively, are deleted before the completion of their maturation in case they recognize self antigens [63]. T cells recognizing self antigen with high affinity can alternatively be induced to anergy or start to express the transcription factor Forkead Box P3 (Foxp3) and become natural $Treg_s$.

When mature lymphocytes reach the periphery, additional mechanisms contribute to the maintenance of tolerance as a lifeline toward T and B cell clones which recognize self antigens with low affinity or tissue-specific Ag_s that they have not encountered in primary lymphoid organs.

Peripheral tolerance is mainly achieved by regulatory T cell - mediated suppression of autoreactive cells or by induction of hyporesponsiveness of lymphocytes which encounter antigens in the absence of co-stimulatory signals. In addition to this, autoreactive T cells may be negatively selected in the spleen or lymph nodes, or their recirculation may be impeded by anatomical barriers (as it is in the testes, brain and eyes) [64].

As previously described, the gut represents the primary route by which the organisms are exposed to antigens in the periphery, including food Ag_s and microflora. The process by which tolerance to fed antigens is induced is generally known as oral tolerance.

Depending on the dose and frequency of administration of the antigen, peripheral tolerance is induced by anergy [65] or deletion of antigen-specific T cells [66, 67] for high dose Ag_s and induction of regulatory T cells after low and repeated Ag exposure [68], but these mechanisms are not exclusive.

Tolerance in the gut is a complex process that requires the interaction of many factors and cell types. Specific subsets of APC_s, particularly dendritic cells, are conditioned by the local environment and shape the nature of the tolerogenic response in the GALT. The main sites where oral tolerance is induced are the intestinal lamina propria, where DC_s are loaded with the antigens, and the draining mesenteric lymph nodes, where migratory DC_s induce the differentiation of Foxp3$^+$ T regulatory cells (defined as induced $Treg_s$) and the expression of gut-homing receptors on T cells. Specific features of LP dendritic cells and macrophages that contribute to the establishment of tolerance will be described in Paragraphs 3.2.3 and 3.2.4.

Whether other secondary lymphoid organs are involved in the establishment of tolerance is still a controversial topic. As regard the role of PP_s, *Fujashi et al.* showed that oral tolerance to proteins is not induced in PP-null mice, generated by the treatment of pregnant mothers with LT-β fusion protein, but still tolerance to haptens is maintained [69]. On the other hand, surgical resection of Peyer's Patches in rats do not alter oral tolerance establishment [70]. The same ambiguous role is assigned to B cells that are the main immune components in the PP_s, as either normal [71, 72] or defective [73] establishment of oral tolerance has been reported for B-cell deficient μMT or B-cell depleted mice.

In addition to DC-mediated transport of antigens to MLN through the lymphatics, Ag_s may be drained in the bloodstream and reach the liver *via* the portal vein. Surgical alteration in the blood delivery to the liver has been shown, indeed, to impair oral tolerance in rats [74]. There, plasmacytoid DC_s have a primary role in oral tolerance to haptens, based on a mechanism that depends on anergy / depletion of Ag-specific CD8$^+$ T cells [75].

5.2.1 Dendritic cells: General overview

Immature dendritic cells are found in almost all peripheral tissues where they capture and process foreign and self-antigens. Despite the lack of antigen-specific receptors that characterize the adaptive compartment of the immune system, DC_s express non-clonally distributed receptors that allow their activation upon the encounter of foreign antigens or microbial products. This activation occurs through the recognition of phylogenetically conserved $PAMP_s$ expressed by microorganisms, through PRR_s, such as C-type lectins, mannose receptors and Toll-like receptors [76]. TLR engagement favors the phagocytosis by DC_s and induces the production of chemokines and cytokines that contribute to the initiation of the immune response and affect the nature of the subsequent adaptive response.

As a result of their ability to sense infections and present antigens to T cells, DC_s are often regarded to the link between the innate and adaptive immune system. However, they are not only involved in the initiation of primary immune responses, but also actively shape T cell-mediated responses and take part to the establishment of immunological tolerance [77].

At steady state, DC migratory properties are essential for the distinction between non-lymphoid tissue migratory DC_s and lymphoid-tissue resident DC_s.

Migratory DC_s carry out a sentinel function, as they patrol peripheral tissues, e.g. the skin and the mucosal surfaces, they sample Ag_s and *via* the lymphatics they migrate to the draining lymph nodes where they present the acquired antigens. Langerhans cells, dermal DC_s and intestinal DC_s are typical examples of migratory cells.

Spleen- and thymus- resident conventional DCs are of non-migratory type and develop in situ from precursors arriving from the blood [78]. Indeed, it is now generally accepted that lymphoid tissue cDCs derive from pre-DC_s *via* the common DC precursor (CDP) and Macrophage-DC precursor (MDP) [79, 80] (Figure 5-3).

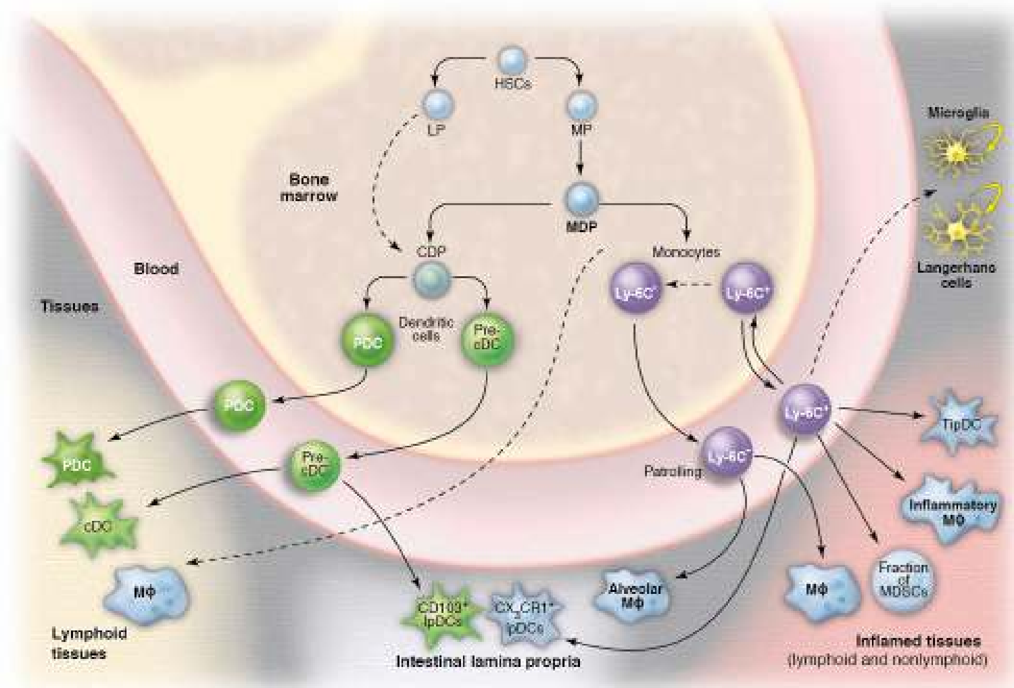


Figure 5-3 Differentiation of Dendritic cells and Macrophages in mice. In the bone marrow, hematopoietic stem cells (HSC_s) give rise to lymphoid (LP_s) and myeloid progenitor (MP_s) cells. MP_s further differentiate into Macrophage-Dendritic Cell Precursors (MDP_s) which may potentially give rise Monocytes and Common Dendritic cell Precursors (CDP_s). From CDP_s, pre-DC_s (precursors of cDC_s in the tissues) and PDC_s (Plasmacytoid Dendritic Cells) are originated. $Ly6C^+$ monocytes may generate different cellular type, depending whether they seed steady-state or inflamed tissues.

MDP, defined as Lin^- $cKit^{high}$ $CD115^+$ $CX_3CR_1^+$ $Flt3^+$, originates from bone-marrow progenitors with myeloid-restricted differentiation potential. Technical advances regarding the possibility to sort and re-transplant progenitor cells into recipient mice have allowed the definition of their developmental relationship, being MP (Myeloid Progenitors) > MDP > CDP > pre-DC_s > cDC_s. CDP_s lose the ability to give rise to monocytes, which instead originate tissue macrophages and $CX_3CR_1^+$ cells in the intestine [80].

DC_s present in the tissues and lymphoid organs at steady state are often classified according to cell surface receptor expression. Conventional DC_s in the spleen an lymph nodes express high levels of CD11c and three main subsets can be recognized according to their expression of CD4, CD8α and CD11b, namely $CD4^+$ $CD8\alpha^-$ $CD11b^+$, $CD8\alpha^+$ $CD4^-$ $CD11b^-$, and $CD4^-$ $CD8\alpha^-$ $CD11b^+$. $CD8\alpha^+$ DCs are specialized in MHC class I presentation, whereas $CD4^+$ DC subset is specialized in MHC class II presentation. Unlike

$CD4^+$ DC_s, $CD8\alpha^+$ DCs have also been shown to cross-present cell-associated antigens [81].

5.2.2 GALT Dendritic Cells

The intestinal immune system, generally referred as GALT (Gut-Associated Lymphoid Tissue), is usually distinguished in effector sites, consisting of lymphocytes residing in the intestinal epithelium and in the lamina propria, and organized tissues, that are responsible for the induction of the immune response, namely the Peyer's patches, the isolated lymphoid follicles and the mesenteric lymph nodes (MLNs) [82] (Figure 5-4).

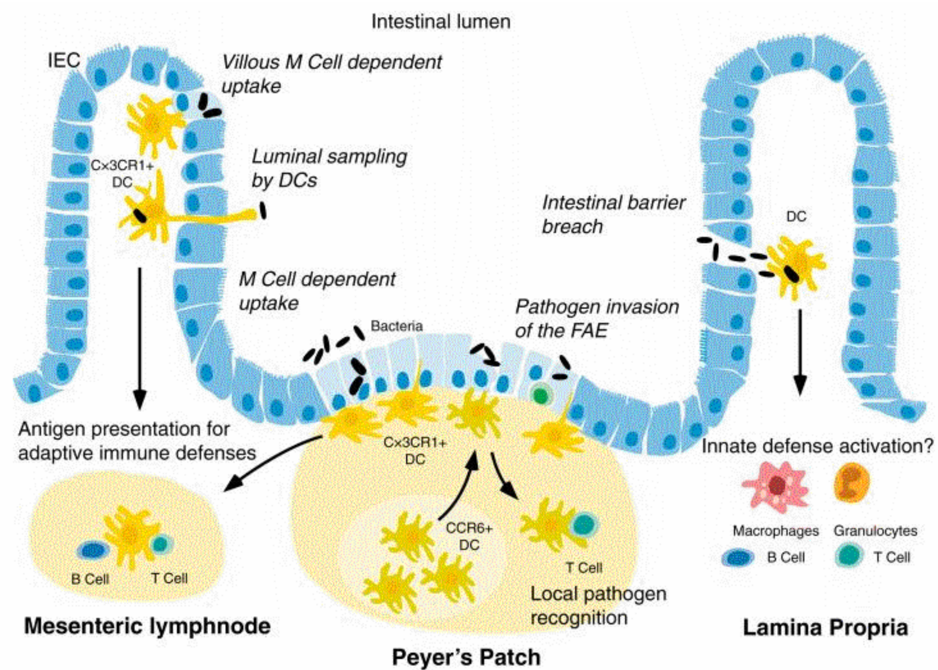


Figure 5-4 Anatomy of the immune system in the intestine. Effector sites (sparse lymphocytes in the epithelium and lamina propria) and inductive site (Peyer's Patches, Isolated Lymphoid Follicles and Mesenteric Lymph Nodes) are recognized.

Peyer's Patches (PP_s) consist of B cell areas forming extensively follicular structures interspersed by inter-follicular regions, where T cells reside [83]. The overlying epithelium, known as follicle-associated epithelium (FAE), includes specialized microfold (M) cells which are unable to present antigens directly – since they do not express the major

histocompatibility complex (MHC) class II – but rather they transport Ag_s through the epithelial layer to APCs beneath. M cells have been also observed in the absorptive epithelium [84], but their low number can hardly account for this required mechanism.

One of the most important features of immune regulation in the intestine is the presence of distinctive subsets of DCs in most part of the GALT. Despite this diversity, it has not been clarified yet whether certain subtypes may be specialized to drive different types of responses [85] or whether dendritic cells in general are able to integrate differential signals coming from the environment and modulate the subsequent response [86].

In murine PPs, at least four subtypes of $CD11c^+$ cells have been described, according to their expression of CD11b and CD8α surface markers: $CD11b^+$ $CD8\alpha^-$, $CD11b^-$ $CD8\alpha^+$, $CD11b^-$ $CD8\alpha^-$ subtypes. An additional population of $CD11c^{int}$ $CD8\alpha^+$ $B220^+$ plasmacytoid dendritic cells is also present at these sites [87].

These cell types have specialized functions: $CD11b^+$ $CD8\alpha^-$ $CCR6^+$ DC_s are located in the subepithelial dome (SED) and activate primarily IL-10 - producing Th2 cells; $CD11b^-$ $CD8\alpha^+$ $CCR6^-$ DC_s are localized in the T cell interfollicular regions (IFR_s) and activate Th1-type of responses; $CD11b^-$ $CD8\alpha^-$ $CCR6^+$ cells are located both in the SED and in the IFR_s, can activate Th1-type responses [88] and migrate to the FAE following *Salmonella* infection [89].

In the small intestine, different subtypes of DCs have been described, resembling the ones isolated from the Peyer's Patches. Recently, a coherent phenotypical characterization of lamina propria cells has been obtained on the basis of their expression of CX_3CR_1 and CD103 markers [90, 91], defining two not-overlapping subsets of dendritic cells and macrophages that differ for their origin, function and turnover rate. These subsets of LP cells will be described in Paragraph 3.2.3 and 3.2.4.

LP dendritic cells stand out for their *immunomodulatory* phenotype, since they express constitutively higher levels of interleukin (IL)-10 and interferon (IFN)-β than DC_s from PP_s or the spleen and show low expression of co-stimulatory markers on their surface, such as CD40, CD80 and CD86 [92]. Besides, a low amount of the pro-inflammatory cytokine IL-12 is always observed, even upon LPS treatment *in vitro*. Similarly, dendritic cells isolated

from the colon are found in an immature state and produce constitutively IL-10, whose expression is upregulated after Toll-like receptors engagement [93, 94].

Once dendritic cells in the lamina propria or in the PP_s have phagocytised antigens, they transport them to the mesenteric lymph nodes where they prime naïve T cell. Upon first-time encounter of an antigen, T cell response is characterized by the activation and clonal expansion of antigen-specific T cells. If the activation occurs in inflammatory conditions, T cells will continue to proliferate and establish a protective immune response.

On the other hand, at steady state, DCs that have sampled dietary, self or commensal bacteria antigens migrate to the MLN_s in the absence of any infection or inflammatory stimulus. This mechanism seems to accomplish for the induction of tolerance to ingested or innocuous antigens, since T cells expanded under tolerogenic circumstances fail to gain classical effector functions [95]. Expression of the chemokine receptor CCR7 by DC_s appears to be critical for their migration to MLN_s [95, 96].

5.2.3 Lamina propria CD103$^+$ Dendritic cells

CD103 (also called integrin α_E) has been associated with a subset of *bona fide* LP-DC_s which derives from pre-DC_s *via* the CDP and MDP and relies on Flt-3 ligand and GM-CSF for its development [79, 90, 91, 97].

The best characterized ligand for CD103, E-cadherin, is mainly expressed by epithelial cells in the lamina propria [98], thus suggesting a possible mechanism by which CD103-expressing cells (including DC_s and CD8$^+$ T cells) might be kept in the tissue.

GALT CD103$^+$ DC_s are endowed with tolerogenic functions contributing to the maintenance of intestinal tolerance [3, 99]. Indeed, they are able to induce the expression of gut homing molecules, such as CCR9 and $\alpha_4\beta_7$, on T lymphocytes, both in humans and mice, [100-102] and to drive the peripheral generation of Foxp3$^+$ T regulatory cells [103, 104], through a mechanism that depends on TGF-β and Retinoic Acid.

These soluble mediators are abundantly present in the intestinal tissues. For instance, epithelial cells [35, 105], stromal cells [54] and T regulatory cells in the GALT are major

producers of TGF-β. Usually, this cytokine is secreted in a latent inactive form, which needs the activation by several molecules, including MMP-9 and MMP-13, integrins ($\alpha_v\beta_8$), or thrombospondin-1.

Besides, CD103$^+$ dendritic cells are able themselves to produce TGF-β as they express *tgfb2* gene and other enzymes needed for the correct localization and release of latent TGF-β [104]. CD103$^+$ DC$_s$ express integrin $\alpha_v\beta_8$, as well [106, 107], and efficiently convert inactive TGF-β into its active form.

Retinoic Acid derives from dietary vitamin A, which is sequentially metabolized by two enzymes, namely ALDH and RALDH. The former catalyzes the oxidation of vitamin A to retinaldehyde, the latter oxidizes retinaldehyde to retinoic acid. These enzymes are expressed by GALT CD103$^+$ DC$_s$, particularly the RALDH2 isoform [97], and by stromal cells in the mesenteric lymph nodes, which mainly express the RALDH-1 and -3 isoforms [108, 109].

Vitamin A, introduced through the diet, is mostly stored in the liver and conveyed by the bile directly to the small intestine. There, it conditions CD103$^+$ DC tolerogenic properties, including *aldh1a2* expression and the induction of gut-homing receptors on T cells [110].

The ability of CD103$^+$ DC$_s$ to induce T regulatory cells is additionally dependent on their expression of IDO [111], an enzyme involved in tryptophan catabolism. IDO inhibition *in vivo* affects the establishment of tolerance to fed ovalbumin and exacerbates the outcome in two models of colitis (T cell-mediated and dextran sodium sulphate models - induced), thus providing further evidence for the tolerogenic role of these cells.

CD103$^+$ DC$_s$ in the lamina propria represent a heterogeneous population, further subdivided by the expression of CD8α and CD11b, in addition to CD11c and MHC-II as markers of conventional DC$_s$. Thus, CD103$^+$ DC$_s$ have been divided into two main subsets, one CD8α^{high} CD11b^{neg} and one CD8α^{neg} CD11b^{high} [112].

CD8α^{high} CD11b^{neg} cells mainly accumulate in the Peyer's Patches and in the Isolated Lymphoid Follicles [91]. Despite this first report, CD8α^+ DC$_s$ have been found in the intestinal lymph collected from mesenteric lymphoadenectomized mice by thoracic duct

cannulation [113]. in fact, *Cerovic at al.* showed that this DC subset is still present in Rorγt-deficient mice which lack PP_s and ILF_s.

CD8α^{high} CD11b^{neg} cells depend on Id2 and IRF8 transcription factors for their development [91, 114]. Moreover, in the lamina propria these cells are almost uniformly marked by the expression of the chemokine receptor XCR1 and are absent in Batf3-deficient mice [115, 116]. As CD8α$^+$ cDC_s in the lymphoid organs, they are endowed with superior ability of cross-presentation of cell-associated antigens [117].

The CD8α^{neg} CD11b^{high} subset expresses *aldhh1a2* and Toll-like Receptors -5 and -9. In response to flagellin, this subset induces the T cell-independent differentiation of naïve B cells to IgA$^+$ B cells and the polarization of T cells to Th1 and Th17 phenotype [112, 118].

CD103$^+$ DC_s are usually considered a migratory population, as they express CCR7 [91] and are found in the MLN-afferent lymph [97]. Although the migration occurs at steady state, it can be further increased by TLR stimulation. Accordingly, after oral infection with S. *Thyphimurium*, CD11b$^+$ CD103$^+$ DC_s are the only DC_s in the draining MLN_s bearing GFP-labeled Salmonella [91].

In the absence of CCR7, mice have reduced CD11b$^+$ CD103$^+$ DC_s in the MLN [91] and do not develop tolerance to fed antigens [95]. Hence, not only these cells are gifted with tolerogenic properties, but antigen transport to the MLN seems to be essential for the establishment of oral tolerance [95]. However, CD103$^+$ DC_s are not highly phagocytic by themselves [97], hence they might acquire antigens from other cells, possibly the CD103$^-$ counterpart or epithelial cells. As recently described by *McDole et al.*, goblet cells may possibly bridge dextran and low-molecular weight soluble antigens to CD103$^+$ DC_s in the lamina propria [119].

The tolerogenic features of CD103$^+$ DC_s are not preserved under inflammatory conditions, but these cells are turned into a pro-inflammatory state. For instance, in a model of T-cell transfer colitis, CD103$^+$ DC_s sorted from the mesenteric lymphnodes show lower expression of *aldh1a2* and *tgfb2* genes and reduced ability to induce Foxp3$^+$ Treg cells. Conversely, they polarize T cells to Th1 phenotype, similarly to the CD103$^-$ counterpart [120].

Furthermore, $CD11b^+$ $CD103^+$ DCs provide a first line innate defense to bacterial infection. In response to flagellin stimulation, $CD11b^+$ $CD103^+$ DCs isolated from the SI secretes pro-inflammatory cytokines, such as IL-12p40 and IL-23p19 [121], and mediates AMP secretion by epithelial cell *via* a mechanism that depend on IL-22.

5.2.4 CX_3CR_1 lamina propria cells

CX_3C Chemokine Receptor 1 (CX_3CR_1), also known as Fractalkine receptor, is mainly involved in leukocyte adhesion and migration. Its main ligand, Fractalkine or CX_3CL_1, is mostly expressed by epithelial and endothelial cells in the intestine in a membrane-anchored form, which can be cleaved by metalloproteases to generate a soluble chemokine with chemoattractive properties [122, 123].

The expression of the fractalkine receptor CX_3CR_1 has been for a long time associated with a subset of lamina propria dendritic cells capable of forming trans-epithelial projections, of capturing luminal bacteria, such as invasion-defective *Salmonella enterica* [124-127] and even of transmigrating to the gut lumen in a MyD88-mediated fashion [128]. Lumen sampling by $CX_3CR_1^+$ cells depends on the expression of the Fractalkine receptor and requires the formation tight junction (TJ)-like structures within intestinal epithelial cells through the expression of TJ proteins, such as occludins and claudins. Experiments performed with bone marrow chimeras highlighted the role of the epithelium in mediating DC extensions [126]. The mechanism is MyD88-dependent and is probably due to the release by EC_s of chemokines and chemical mediators, such as CCL20 (MIP-3α), which recruit cells *via* CCR6 receptor.

Phenotypical and functional characterization of $CX_3CR_1^+$ cells have challenged the initial definition of these cells as DC_s, as they share common features with macrophages [129]. Indeed, the lack of specific markers which univocally classify DCs and macrophages in the non-lymphoid organs has raised a heated debate about the definition of the identity of these cells. It is now generally accepted that in non-lymphoid organs CX_3CR_1 expression marks a subpopulation of LP-resident Mϕ, which derives from $Ly6C^{high}$ blood monocytes

and depends on Macrophage Colony Stimulating Factor (M-CSF) for its development [90, 91]. At steady state, they can be phenotipically distinguished as F4/80$^+$ MHC-II$^+$ $CX_3CR_1^+$ CD103$^-$ CD11b$^+$ cells, and some share with cDC$_s$ the expression of integrin α_x, known as CD11c [117, 129, 130].

However, not all $CX_3CR_1^+$ cells can be called macrophages, as recently *Cerovic et al.* found a population of $CX_3CR_1^{int}$ cells in the lymph collected from the thoracic duct in mice undergone mesenteric lymphadenectomy. These cells resemble CD103$^+$ DC$_s$, as they are migratory, prime efficiently T cells and respond to Flt3 [113], but differently from CD103$^+$ LP cells they polarize T cells to Th1 and Th17 phenotype.

$CX_3CR_1^+$ Mϕ are highly phagocytic and accumulate in response to microbiota-derived signals [131], as they are reduced in the small and large intestine of germ-free mice. They poorly prime naïve T cells, but preferentially support Th1 / Th17 T cell differentiation [131]. Even commensal-derived products, such as ATP, can favor Th17 response mediated by CD70^{high} $CX_3CR_1^+$ cells [132].

F4/80$^+$ $CX_3CR_1^{high}$ macrophages produce high levels of IL-10 and acquire an anti-inflammatory phenotype when stimulated with LPS [117, 133]. IL-10 has been linked to secondary Treg cell expansion in the intestinal mucosa after their generation in the MLNs, pointing out a role for $CX_3CR_1^+$ cells in oral tolerance establishment. Mice lacking the expression of functional CX_3CR_1 receptor, where production of IL-10 by these cells is abolished, fail indeed to develop tolerance to fed ovalbumin and to accumulate Treg$_s$ in the intestinal lamina propria [134].

Besides, also F4/80 knockout mice are not tolerized after Ag feeding, but in this case the underlying mechanism seems to be the defective generation of CD8$^+$ T regulatory cells [135].

In inflammatory conditions, Ly6C^{high} monocytes give rise to E-cadherin$^+$ F4/80^{int} $CX_3CR_1^{int}$ CD11b$^+$ macrophages that secrete pro-inflammatory cytokines, such as IL-12, IL-23 and TNF-α [117, 136]. A similar population of inflammatory macrophages, expressing TLR-2 and -4 and low to intermediate levels of CX_3CR_1 has been described to accumulate in the inflamed colon in a model of DSS colitis [137].

$Ly6C^{high}$ monocyte recruitment to the inflamed tissue depends on their expression of diverse receptor, such as CCR2 and CX_3CR_1. Accordingly, CCR2 - and CX_3CR_1 - deficient mice are less susceptible to experimental colitis after DSS treatment [137, 138] or in a T-cell mediated model [131], respectively, consistently with impaired recruitment of inflammatory precursors.

5.2.5 T Regulatory cells

It is now generally accepted that mucosal antigen administration induces the development of T regulatory cells that are fundamental for the establishment of oral tolerance. Treg cells comprise several subsets of both $CD4^+$ and $CD8^+$ T cells, which control and suppress effector immune responses. Based on their origin, two main subsets of Treg cells have been described: naturally occurring $CD4^+CD25^+$ regulatory T cells ($nTreg_s$), which develop in the thymus and suppress self-reactive T cells in the periphery, and inducible regulatory T cells ($iTreg_s$), which arise in the periphery from naïve T cells, after being in contact with regulatory cytokines and / or APC_s.

Indeed, generation of $Treg_s$ outside the thymus is often based on T cell activation in the absence of inflammation, a situation generally referred as sub-immunogenic, which may be achieved by antigen presentation in the absence of co-stimulatory signals or in specific “tolerogenic-prone” environments, such as the gut mucosa.

Despite the different origin, it is a hard task to discriminate between $nTreg_s$ and $iTreg_s$, as they share common markers, and to understand how they singularly participate to the maintenance of peripheral tolerance. Transcriptional profiling of *in vitro* induced $iTreg_s$ and *in vivo* isolated $nTreg_s$ has led to the identification of differential expressed genes, which contribute to the definition of the genetic signature of the two populations [139]. Among them, *Ikzf2* (Helios) and *Nrp1* (Neuropilin-1) are preferentially expressed by $nTreg_{s.}$. The transcription factor Helios was indeed proposed as a marker of thymic T regulatory cells [140], but more recently it has been shown to be expressed also by $iTreg_s$, depending on the conditions of activation [141]. Recent reports have, instead, confirmed higher

expression of *Nrp1* in $nTreg_s$ compared to $Foxp3^+$ $iTreg_s$ isolated from MLNs or the spleen [142, 143].

In order to functionally dissect single contribution of $nTreg_s$ and $iTreg_s$ to peripheral tolerance, mice lacking $nTreg_s$ where generated crossing TCR-transgenic mice to RAG - deficient mice. These mice have normal naïve B and T cells, are devoid of $nTreg_s$ and develop an allergic disease after immunization with Hemagglutinin (HA) Ag as they bear HA-specific monoclonal B cells [144]. Interestingly, oral tolerance is efficiently established in the absence of thymus-derived T cells; besides, $iTreg_s$ can be peripherally generated after antigen feeding and display suppressive activity *in vivo* and *in vitro*.

Inducible regulatory T cells have been classified into IL-10 producing T regulatory 1 (Tr1) cells, TGF-β secreting T helper 3 (Th3) cells and converted $Foxp3^+$ Regulatory T cells.

Tr1 may be generated *in vitro* and *in vivo* after priming in the presence of IL-10 [145]. Tr1 cells are characterized by the production of IL-10 and lack of Foxp3 expression [146]; in a model of *H. hepaticus*-induced colitis in $RAG2^{-/-}$ mice reconstituted with $CD4^+$ $CD45RB^{high}$ T cells, Tr1 cells actively suppress the intestinal pathology through an IL-10 - and TGF-β - dependent mechanism [147]. Besides, also expression of CTLA-4 by Tr1 cells contributes to their suppressive function and ability to control intestinal inflammation [148].

Th3 cells are characterized by the ability to produce TGF-β and have been initially described during oral tolerance induction [68]. Their suppressive properties and ability to secrete TGF-β are common features that Th3 cells share with $Foxp3^+$ Regulatory T cells; unfortunately, most of the characterization of Th3 cells has been performed before the introduction of Foxp3 as a marker of T Regulatory cells, hence they might represent the same population.

Among $iTreg_s$, $Foxp3^+$ $Treg_s$ have been in the last years the most attractive as they have been shown to be directly involved in oral tolerance establishment [134]. Phenotypically, they are defined as $CD4^+$ $CD25^+$ $Foxp3^+$; they develop *in vitro* from naïve $CD4^+$ $Foxp3^-$ T cells upon the interaction with certain subsets of DC_s, or *in vivo* after oral antigen administration [103, 104] or after Ag-targeting to $DEC205^+$ cells [149]. Particularly, in experiments performed with $RAG\text{-}1^{-/-}$ mice adoptively transferred with TCR-specific

congenic naïve T cells, after Ag feeding $Foxp3^+$ Treg cell conversion takes place preferentially in the GALT, mainly MLN_s and LP [103]. Hence, various environmental factors seem to influence the development of $Foxp3^+$ $Treg_s$ *in vivo;* minimal requirements for this process are represented by TGF-β and IL-2, both *in vivo* and *in vitro* [150].

Once generated, $iTreg_s$ home back to the intestinal lamina propria, where they are re-stimulated and expanded by IL-10 - producing $CX_3CR_1^+$ Mϕ [134]. The correct homing of $Foxp3^+$ $Treg_s$ depends on their expression of gut-homing receptors, such as $\alpha_4\beta_7$ or CCR9. Accordingly, β_7 - [134] and CCR9 [151] - deficient mice do not develop oral tolerance; this phenotype is definitely corrected after WT T cell transplantation.

5.3 Gap Junctions

5.3.1 Structure and Function

In a physiological environment, tissue integrity and homeostasis are preserved by the balanced response of cells to signals coming from the extracellular matrix, soluble mediators and other cells. In particular, communication among adjacent cells is mediated by adhesion complexes, such as adherens, tight and gap junctions (GJ_s) [152]. Adherens and tight junctions mainly control paracellular permeability and polarity, while GJ_s allow direct communication between cells. Indeed, small peptides, secondary messengers and metabolites may be transferred through GJ_s, contributing to electrical and biochemical coupling of cells.

GJ_s consist of hemichannels (known as connexons) that, when juxtaposed, form channels between two adjacent cells. Each hemichannel represents a complex of six connexin proteins, which share a common structure. In fact, connexins (Cx_s) are made of nine domains: the N-terminus, two extracellular loops and four transmembrane domains, which are highly conserved; a cytoplasmic loop and the C-terminus, which vary in length and sequence and specifically mediate the biological functions of Cx_s.

The cytoplasmic tail and loop can be post-translationally modified, especially by phosphorylation, which is relevant for the GJ assembly and the modulation of its physiological functions [153]. Besides, the C-terminus interacts with cytoskeletal elements and intracellular proteins, such as kinases, phosphatases, membrane receptors and cell signaling proteins, which highlight the role for gap junctions also in intracellular signaling, in addition to cell-cell communication [152].

Furthermore, hemichannels can form non-junctional channels in unopposed areas of the cell membrane, causing the release of a variety of factors such as ATP, glutamate, and NAD^+ into the extracellular space, especially under mechanical or ischemic stress [154].

Twenty-one Cx genes have been identified in humans, twenty in mice; their expression is tightly regulated during development and in a tissue-specific manner. Among them, only Cx43 is ubiquitously expressed; GJ formed by connexin 43 electrically couple heart muscle cells and control synchronous heart muscle contractions. Connexin 43-deficient mice show a plethora of defects and die postnatally as the result of ventricular arrhythmia [155].

5.3.2 Gap Junctions in the Immune System

As already described, GJ_s mediate the passage of small molecules, such as ions, metabolites, second messengers and peptides, so that electric, metabolic and immunological pieces of information are diffused.

The first two have been extensively characterized in nervous cells of the brain and in cardiac cells; however, the role of GJ_s in the immune system remain elusive and only few studies have been reported until now.

Immune cells express a defined panel of connexins [156], such as Cx43 and more rarely Cx40, Cx37 and Cx32, as shown in Table 1, and through GJ_s they are coupled to other immune cells or endothelial and stromal cells [157, 158].

Table 1 Connexin expression in cells of the immune system (from Neijssen et al., 2007)

Connexin isotype	Cell types	Function	Coupled cell types	References
Cx43	Bone marrow derived DC	Cross-presentation	Bone Marrow DCs - Tissue cells	Neijssen et al. (2005)
		DC activation	Bone Marrow DC - Bone Marrow DC	Matsue et al. (2006)
	Bone marrow stromal cells	Haematopoiesis	Bone marrow stromal cells - HSCs	Cancelas et al. (2000)
	Haematopoietic stem cells			
	Follicular DC	Germinal centre development	Follicular DCs - B cells	Krenacs et al. (1997)
			Follicular DCs - Follicular DCs	
	Monocytes	Cross-presentation	Monocytes - Tissue cells	Eugenin et al. (2003) and Neijssen et al. (2005)
	Tonsil DC		DCs - Tissue cells	
	Appendix DC		Langerhans cells - Tissue cells	
	Langerhans cells			
	Macrophages	Inflammation	Macrophages - Intestinal endothelium	Martin et al. (1998)
	Thymus epithelial cells	T cell development	TEC - T cell progenitor cells	Alves et al. (1995) and Fonseca et al. (2004)
	T cell progenitors			
	T cells	Maturation (?)	T cells - T cells	Oviedo-Orta et al. (2000)
	B cells	Antibody secretion	T cells - B cells	Oviedo-Orta et al. (2001)
	NK cells	Activation (?)	NK cells - NK cells	Oviedo-Orta et al. (2000)
	Mast cells	Unkown	Mast cells - Mast cells	Vliagoftis et al. (1999)
			Mast cells - Fibroblasts	
	Polymorphonucle ar neutrophils (PMN)	Extravasation	PMN - PMN	Jara et al. (1995)
			PMN - Endothelium	Zahler et al. (2003)
Cx40	T cells	Maturation (?)	T cells - T cells	Oviedo-Orta et al. (2002)
	B cells	Immunoglobulin secretion	T cells - B cells	
			B cells - B cells	
Cx37	Macrophages	Atherogenesis	Macrophages - Smooth muscle cells	Kwak et al. (2002)
	Monocytes	Prevention of atherosclerosis	Hemi-channel	Wong et al. (2006)
Cx32	Mast cells	Unknown	Mast cells - Mast cells	Vliagoftis et al. (1999)
			Mast cells - Surrounding cells	
Cx30.3	Thymocytes	Unknown	-	Fonseca et al. (2004)

Immune cells in the bone marrow communicate with stromal cells mainly *via* Cx43. Cx43 deficient fetuses at embryonic day 14.5 show reduced progenitor cells in the liver, suggesting a role for Cx43 in stem cell development [159]. In addition to this, $Cx43^{-/-}$ mice show defects in T and B lymphopoiesis [160].

Few findings about the expression of connexins by dendritic cells have been reported. For example, follicular DC_s upregulate Cx43 expression and form GJ_s among each other or with B cells in the germinal center upon antigen challenge [161].

Also bone marrow-derived dendritic cells form GJ_s *in vitro*, especially after stimulation of cells with LPS or IFN-γ. Inhibition of GJ formation dampens the activation of BM-DC_s, measured as CD40 expression [162]. In addition to this, *Neijssen et al.* have demonstrated that activated human monocytes are able to take up antigenic peptides through GJ_s from a peptide-expressing cell line and cross-present them to Ag-specific cytotoxic T cells [163]. Peptides need to fulfill some requirement in order to be transferred through GJ_s and subsequently presented on MHC-I, that is to be around 9 amino acids in length (about 1 kDa). Thus, GJ_s couple the antigen processing machinery of adjacent cells, sustaining cross-presentation.

6 Aim of the project

The role of GALT mononuclear cells in the establishment of oral tolerance has been extensively investigated in the past years and linked to peculiar functional determinants of these cells. In particular, while one subset of mononuclear cells is apt at antigen capture, another one is mostly involved in T cell priming towards a tolerogenic phenotype. In this thesis, our aim was to understand fine mechanisms underlying the phase of antigen uptake in the small intestine, as well as mechanisms of cooperation between the different mononuclear phagocytes, as the initial steps in the induction of oral tolerance to intragastrically-delivered antigens.

Hence, we are proceeding through these steps:

- Characterization of the SI cell subsets involved in antigen uptake, mostly focusing on LP subsets of $CX_3CR_1^+$ macrophages and $CD103^+$ dendritic cells;
- Evaluation of the effects of CX_3CR_1 deficiency on Ag uptake and oral tolerance establishment;
- Assessment of the possible transfer of antigens among lamina propria cells, through gap junctions, and its effect on the induction of tolerance to fed antigens.

7 Materials and Methods

7.1 Mice

6-7 weeks old female C57BL/6J mice were purchased from Charles River. $CX_3CR_1^{GFP/GFP}$ (B6.129P-Cx3cr1tm1Litt/J), $Cx43^{fl/fl}$ mice (B6.129S7-Gja1tm1Dlg/J) and CD11c-CRE mice (B6.Cg-Tg(Itgax-cre)1-1Reiz/J) were purchased by Jackson Laboratory. Animals were bred and maintained in our SPF (Specific Pathogen Free) animal facility. $Cx43^{fl/fl}$ mice and CD11c-CRE were genotyped after tail biopsies, following the protocol recommended by Jackon Laboratory. Tails were lysed and genomic DNA extracted.

Standard PCR was performed using the following primers:

Cx43 Forward CTTTGACTCTGATTACAGAGCTTAA

Cx43 Reverse GTCTCACTGTTACTTAACAGCTTGA

CD11c-CRE Forward ACTTGGCAGCTGTCTCCAAG

CD11c-CRE Reverse GCGAACATCTTCAGGTTCTG

All experiments were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC).

7.2 Tolerance Induction by intragastric Antigen Delivery

Mice were fed with 1 or 20 mg of ovalbumin in 200 µl DPBS by gavage on days 13, 10 and 7 before OVA/CFA immunization. Mice were immunized subcutaneously with 50 ug OVA in 100 ul PBS/CFA emulsion. After 7 days, mice were challenged by s.c. injection of 250 µg heat-aggregated OVA in 20 µl of PBS in the left hind footpad. Right footpad received PBS as a control. OVA-specific DTH was determined by footpad swelling 24 and 48 hours after challenge.

Total splenocytes were subsequently isolated and re-stimulated *in vitro* with 1 mg/ml ovalbumin. In particular, spleens were collected and smashed onto a 70 µm cell strainer.

Red blood cells were lysed using a hypotonic red cell lysis buffer. 50000 cells per each well were seeded in a 96-well plate.

After restimulation, supernatants were collected after 4 days and IFN-γ measured by ELISA using CBA FACSArray.

7.3 Intestinal loop and ovalbumin administration

$CX_3CR_1^{GFP/+}$ mice were anesthetized with 2.5% Avertin for the duration of the experiment. Segments of the small intestine were exposed and ligated at both extremities with surgical thread. 40 μg of ovalbumin-Alexa Fluor (AF) 647 (Molecular Probes) were injected into the loop, the intestines removed after two hours and processed for immunofluorescence staining.

When indicated, 250 μg of ovalbumin-Alexa Fluor 647 were administered intra-gastrically in 200 μl DPBS using a feeding needle.

7.4 Immunofluorescence staining

Tissues were fixed in 0.1 M L-Lysine pH 7.4 and 1% paraformaldehyde in PBS for 12 hours, dehydrated in 20% sucrose for 12 hours and frozen in OCT (Sakura Finetek). 10 μm-thick sections were cut and posed on Poly-L-Lysine coated microscope slides. Sections were blocked in 1% Bovine Serum Albumin and 1% Triton X-100 and were subsequently stained using the following antibodies: α-CD11c (Clone HL3, BD Pharmingen), α-CD11b (Clone M1/70, conjugated with Pacific Blue, eBioscience) and α-K^bOVA (25-D1.16, eBioscience).

α-K^bOVA was conjugated with AF568-labeled Fab fragments using the Zenon Mouse IgG Labeling Kit (Molecular probes) according to manufacturer's instruction. Sections were then extensively washed and incubated with secondary antibody against hamster anti-CD11c, which is Cy3 Goat anti-Armenian Hamster IgG (Jackson ImmunoResearch). Phalloidin and DAPI were added when indicated.

Confocal images were collected with a Leica TCS-SP2 (Leica Microsystem) with a 40x or 63x objectives and processed using Imaris 6.1.0. (Bitplane).

7.5 Isolation of small intestine lamina propria cells

Small Intestines were cut into small pieces and washed in separation medium (PBS, 1% fetal bovine serum, 1 mM DTT, 1 mM EDTA) for 15 min at 37°C. Tissues were subsequently incubated in digestion medium consisting of MEMα medium, 5% fetal bovine serum (FBS), 0.5 mg/ml collagenase type VIII (Sigma-Aldrich), 5 U/ml DNase I (Roche Diagnostics), 100 IU/ml penicillin and 100 μg/ml streptomycin for 30 min at 37°C by gentle shaking. Cells were passed through a mesh and centrifuged. Red blood cells were lysed with a hypotonic lysis buffer.

For FACS staining or cell sorting, cells were incubated with anti-FcR antibody (clone 24G2; BD Biosciences) and stained with the following primary antibodies: anti-CD45.2 (clone 104, eBioscience), CD11c (clone HL3, BD Pharmingen), MHC-II (clone M5/114.15.2, eBioscience), CD103 (clone M290, BD Pharmingen), CD11b (clone M1/70, eBioscience), K^bOVA (clone 25-D1.16, eBioscience), CD86 (clone GL1, BD Pharmingen), CD40 (clone HM40-3, BD Pharmingen) and CD83 (clone Michel-19, BD Pharmingen). DAPI was added to exclude dead cells.

Samples were acquired using FACSCanto (BD Biosciences) and analyzed by FlowJo (Treestar).

When indicated, MHC-II^+ $CD11c^+$ cells were sorted by FACSAria (BD Biosciences) into $CD11b^-$ $CD103^+$, $CD11b^+$ $CD103^+$, $CD11b^+$ $CX_3CR_1^{int}$ and $CD11b^+$ $CX_3CR_1^{high}$ populations.

Collected cells were sorted in complete medium (RPMI, 10% FBS, 1%P/S, 1% Glutamine, 50 μM β-Mercaptoethanol) and subsequently centrifuges at 2000 rpm for 10’. When proceeding with RNA extraction, cells were lysed in RTL Plus Buffer (Quiagen).

For $CD11c^+$ cell-enrichment, cells were incubated with anti-FcR antibody, then with anti-CD11c MACS beads (Miltenyi Biotec) and purified according to manufacturer’s instruction. Briefly, cells were incubated with CD11c MicroBeads in MACS Buffer for 15 minutes at

4°C, washed and passed onto a MS Column (Miltenyi Biotec). To get a better purity, cells were passed twice onto the colums.

7.6 Isolation and enrichment of $CD11c^+$ from MLN_s, spleen and PP_s

Murine MLNs, spleens and Peyer's Patches were digested in RPMI-1640 containing 5% FBS, 1 mg/ml collagenase D and 5 U/ml DNase I (Roche) for 20 min at 37°C.

Tissues were subsequently smashed and cell suspension centrifuged. Red blood cells were lysed with a hypotonic lysis buffer.

Cells were incubated with anti-FcR antibody (clone 24G2; BD Biosciences), followed by anti-CD11c MACS beads enrichment (Miltenyi Biotec). In order to obtain a higher purity, cells were passed twice onto the column.

7.7 Dye and Antigen Transfer Assays

$CD11c^+$ cells were enriched from small intestines of C57BL/6 mice (as previously described) and were divided into two pools: one was labeled with 15 µM DDAO (Molecular Probes) for 15 minutes at room temperature; the second was labeled with 0.5 µM Calcein-AM (Molecular Probes) in serum-free medium for 30 minutes at 37°C. After labeling, cells were extensively washed in complete medium.

The two pools were co-cultured for 30 minutes and calcein transfer was evaluated by FACS. When indicated, cells were pre-treated with 1-Heptanol (Sigma Aldrich), a gap junction inhibitor, at different concentration (0.5, 5 and 50 mM).

For the experiment of Ag transfer, $CD103^+$ DC_s and $CX_3CR_1^+$ LPC_s were sorted from $CX_3CR_1^{GFP/+}$ mice. They were respectively loaded with 100 µg ovalbumin-Alexa Fluor 647 for 1 hours and then washed extensively. Sorted subpopulations were subsequently co-cultured with the unlabelled counterpart for 1 hour and transfer of antigenic material was evaluated by FACS.

7.8 Staining for T regulatory cells

Cells suspensions were prepared from the small intestine and mesenteric lymph nodes, as previously described. Cells were subsequently permeabilized using the eBioscence Fix/Perm buffer for 30’. They were subsequently stained in Wash/perm Buffer (eBioscience) with anti-CD45.2 (clone 104, eBioscience), CD3 (clone 17A2, BD Pharmingen), CD4 (clone H129.19, BD Pharmingen), CD8 (clone 53-6.7, BD Pharmingen), CD25 (clone 3C7, BD Pharmingen) and Foxp3 (clone FJK-16s, eBioscience). Samples were acquired with FACSCanto (BD Biosciences) and analyzed using FlowJo (Tristar).

7.9 Quantitative RT- PCR analysis

RNA was extracted using the RNeasy Micro Kit (Qiagen). RNA was reverse transcribed with random hexamers and SuperScript III reverse transcriptase (Invitrogen). Quantitative real-time RT-PCRs were performed with the SYBR Green PCR kit on the Applied Biosystems 7500 RT-PCR System (Applied Biosystems). Results were quantified using the $2^{-\Delta\Delta CT}$ method [164]. The expression levels of the genes of interest were normalized to the expression levels of the reference Rlp32 gene. PCR experiments were performed in duplicate.

Primers that were used are indicated in the following table:

Gene	Forward (5'-3')	Reverse (5-3')
Cx23	ATCAGTTCCGGCCAATAACTC	ATCACAGTGTACACGGGCTTC
Cx26	GCTTCAGACCTGCTCCTTACC	GGTGGAGTGTTTGTTGACACC
Cx29	AAGGACACAACCGATGAATTG	TTGACTGCAAGCACCTTTACC
Cx30	AAGCCCTGGAGAACAAGACTC	CAAAGTCCTCCTGCTCATCAC
Cx30.2	AGGACGAGCAGGAGGAGTTC	AGGATGTGGAAGAGCCAGAAG

Cx30.3	CTTCCCTGTTAGTGGTCATGC	CAGCAGCCTTGAAGATGAGAC
Cx31	GACAACTTCTTCCCCATCTCC	ACCAATTCGATGATGAGCTTG
Cx31.1	TACGTCTTCAGCCTCTCGTTC	CAGCTCCACAAGGTTAAGCAG
Cx32	ACAGCCATTGGCCGAGTATG	TGTTGGTGAGCTACGTGCATT
Cx33	AGGAAGAACTGGAAGCTGCTC	GCCATCTCGAAGACAGACTTG
Cx36	TACTGCCCAGTCTTTGTCTGC	TACACCGTCTCCCCTACAATG
Cx39	TTGTTGGGATTCCTCATCATC	ACTGGGGAAAAGAGGTCGTAG
Cx40	AGAGCCTGAAGAAGCCAACTC	CGGAAAATGAACAGGACAGTG
Cx43	ACTTCAGCCTCCAAGGAGTTC	GGAGTAGGCTTGGACCTTGTC
Cx45	TTGGGAAAGCAACAAACAAAG	ATCTCCTCTAGCAGGCGAGTC
Cx46	GGCTGTGAGAACGTCTGCTAC	CCGCTCTTTCTTCTTCTCCTC
Cx47	AGCTCTGCCTTGTGCATCTC	GGTGGAATGATTGTGGATCTC
Cx50	GCAGCAAGAGAGAAAGACAGC	ACAGTGGAGTGCTCATTCACC
Cx57	AATTTACTGGGTGGCATCCTAGA	GGGAAAGCATCATCGTAACAGAT
Rlp32	AAGCGAAACTGGCGGAAAC	TAACCGATGTTGGGCATCAG

7.10 Evaluation of homologous recombination event at Cx43 locus in $Cx43^{fl/fl}$ CD11c-CRE mice

$CD11c^{+}$ cells were enriched from the small intestine of $Cx43^{fl/fl}$ $CD11c\text{-}CRE^{+}$ mice and control mice ($Cx43^{+/+}$ $CD11c\text{-}CRE^{+}$, $Cx43^{fl/fl}$ $CD11c\text{-}CRE^{-}$). They were subsequently lysed and genomic DNA was extracted using phenol-chloroform extraction. In order to evaluate deletion of exon 2 in Cx43 gene, standard PCR was performed using the following primers:

YL49 (5’-3’) GCTACTTCTTGCTTTGACTCTGATTA

YL50 (5’-3’) GCTCACTTGATAGTCCACTCTAAGC

The PCR reaction amplifies a fragment of 686 bp when exon 2 is deleted.

7.11 Statistics

Results were represented as Mean ± SEM or Mean ± SD (when indicated). Statistical significance was determined by the Student's *t* test using GraphPad Prism software. $^*p<0.05$, $^{**}p<0.01$, n.s. not significant.

8 Results

8.1 Characterization of antigen uptake in $CX_3CR_1^{GFP/+}$ and $CX_3CR_1^{GFP/GFP}$ mice

8.1.1 $CX_3CR_1^+$ cells are responsible for antigen uptake in the small intestine

Efficient induction of tolerance to food antigens relies on different processes, among which one of the main critical steps is represented by the ability of antigen presenting cells to uptake antigens in the lamina propria of the small intestine and to migrate through the lymphatics to the draining lymph nodes [95].

In order to assess whether a defined subset of lamina propria cells was responsible for the uptake of soluble antigens, we took advantage of $CX_3CR_1^{GFP/+}$ knock-in mice [165], which bear in heterozygosis the gene coding for Green Fluorescent Protein (GFP) under the control of the CX_3CR_1 promoter. The use of this mouse model allows the efficient *in vivo* tracking of cells expressing CX_3CR_1, which marks in the small intestine a population of sessile cells, generally considered resident macrophages.

Thus, $CX_3CR_1^{GFP/+}$ mice were subjected to intestinal ligation and a fluorescently-labeled protein, ovalbumin (OVA) conjugated with Alexa Fluor 647, was injected into the intestinal loop. After two hours, the loop was resected and tissues fixed and processed for imaging analysis.

10 µm-thick sections were initially stained with phalloidin to evaluate general integrity of the tissue. As shown in Figure 8-1, intestinal surgery did not alter epithelial layer continuity as suggested by the uninterrupted actin staining of the IEC_s.

As ovalbumin is fluorescently-labeled, it can be then visualized on the tissue sections; additional staining with anti-CD11c to localize DC_s was performed.

As depicted in Figure 8-2, Ag localization was restricted to $CX_3CR_1^+$ LP cells, preferentially to those positioned in the apical part of the villus. Indeed, a gradient of cells, which have

phagocytosed OVA, was highlighted going from the apical to the basal part of the villi, as may be expected considering antigen availability and accessibility in the intestinal lumen.

Three-dimensional reconstruction of the villus (Figure 8-3) clearly showed that OVA-AF647 was preferentially located in the cytosol of $CX_3CR_1^+$ cells, but not in other $CD11c^+$ cells present in the lamina propria.

This initial observation was confirmed in a time course experiment, where OVA-AF647 was administered in a more physiological setting *via* the oral route. Small intestines were collected at different time points after feeding and were processed for FACS analysis, which allowed a quantification of the antigen uptake process that was observed by imaging techniques. Generally, CX_3CR_1 and CD103 markers are used to discriminate the two main subsets of antigen presenting cells in the small intestine, namely CX_3CR_1-expressing Mϕ and $CD103^+$ DC_s.

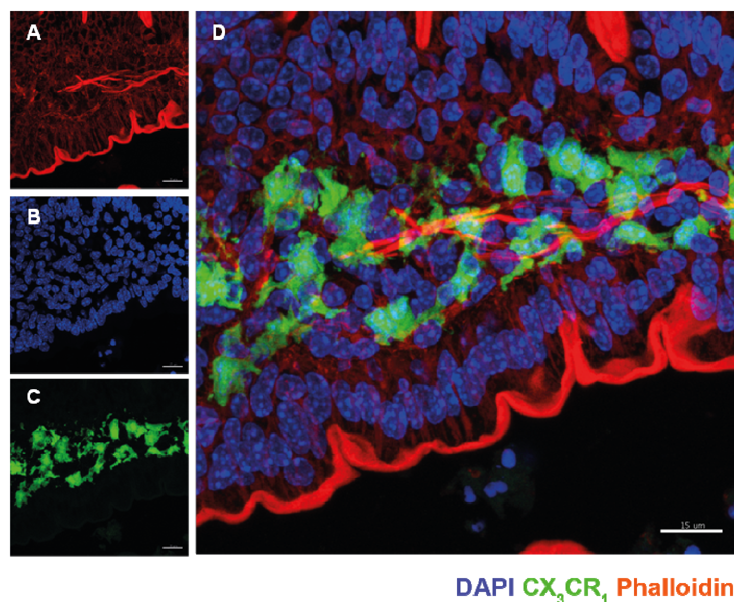


Figure 8-1 Evaluation of tissue integrity and epithelial layer continuity. The small intestine of $CX_3CR_1^{GFP/+}$ mice was exposed and ligated to form a loop. The loop was injected with 40 μg of ovalbumin conjugated with Alexa Fluor 647. After 2 hours, the loop was resected and fixed. Sections were stained with Phalloidin (**A**) and DAPI (**B**). Single channels are shown in panels A - C. Red, Actin (**A**); Blue, DAPI (**B**); Green, CX_3CR_1 (**C**). Merged images are shown in (**D**); Scale bar, 15 μm.

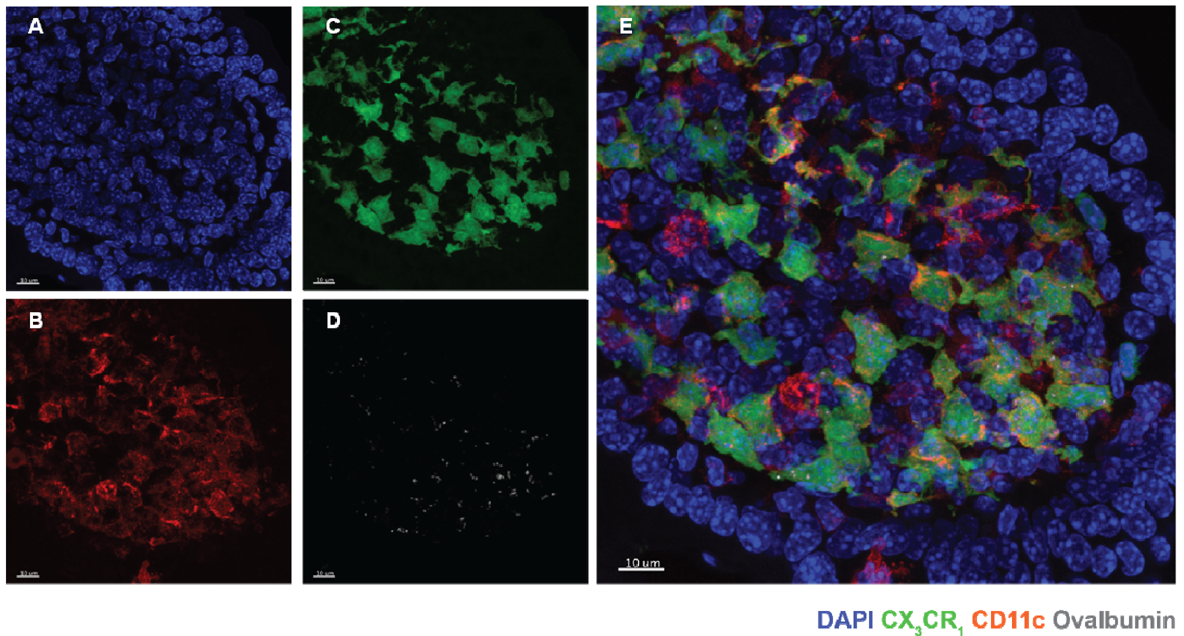
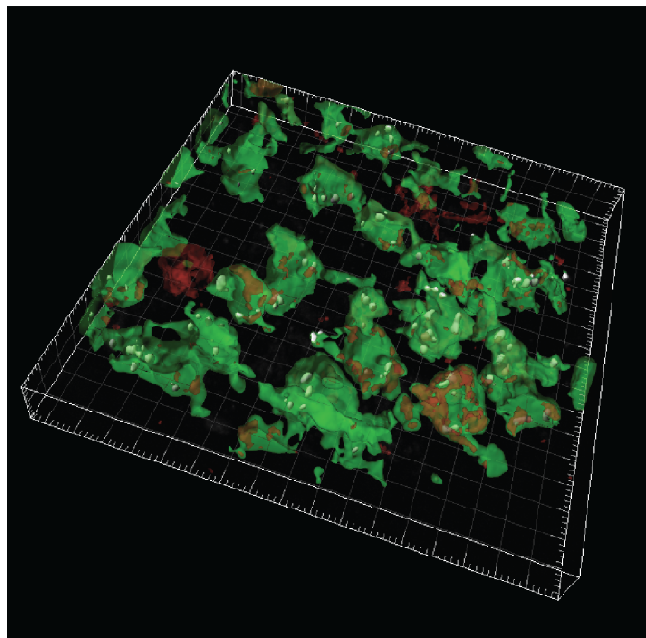


Figure 8-2 $CX_3CR_1^+$ cells uptake ovalbumin in the small intestine. $CX_3CR_1^{GFP/+}$ mice were anesthetized and their intestines were ligated. The intestinal loop was injected with 40 μg of ovalbumin conjugated with Alexa Fluor 647 and resected 2 hours later. Sections were stained with DAPI (**A**) and CD11c (**B**). Single channels are shown in panels A-D. Blue, DAPI (**A**); Red, CD11c (**B**); Green, CX_3CR_1 (**C**); Grey, Ovalbumin-AF647 (**D**). Merged images are shown in (**E**); Scale bar, 10 μm. Images are representative of two independent experiments, n = 2.



CX_3CR_1 CD11c Ovalbumin

Figure 8-3 OVA-AF647 is preferentially captured by $CX_3CR_1^+$ cells. Three-dimensional reconstruction of the SI villus in Figure 8-2 using Imaris 6.1.0.. The white grid marks the 3D volume, green marks $CX_3CR_1^+$ cells, red $CD11c^+$ cells. White dots stand for ovalbumin.

Thus, cells were isolated from mice fed with DPBS or ovalbumin-AF647 and stained with anti-CD103, CD11c and MHC-II antibodies. In the FACS analysis, MHC-II$^+$ CD11c$^+$ cells were initially gated; then, three subsets of CD103$^+$, $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ cells were highlighted.

Higher percentage of cells positive for OVA-AF647 was observed in the $CX_3CR_1^{high}$ and $CX_3CR_1^{int}$ subsets, while CD103$^+$ cells were almost negative. The kinetic of antigen uptake was rapid, with a peak between one and three hours after Ag administration (Figure 8-4, A-B).

When separating the three tracts which form the SI, namely duodenum, jejunum and ileum, and staining additionally for CD45 to differentiate epithelial cells, it was evident that antigen uptake was unevenly distributed along the small bowel. Indeed, among the total of OVA-AF647$^+$ cells, most were CD45$^-$, ranging from about 50% in the duodenum to almost 95-99% in the jejunum and ileum, outnumbering $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ cells.

In the duodenum, which is the first SI tract reached by the antigen, about 50% of OVA-AF647$^+$ cells were $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$, balancing EC uptake of the antigen (Figure 8-4, C).

Hence, we have described a subset of intestinal mononuclear cells, namely $CX_3CR_1^+$ LPC_s, which are mainly involved in Ag uptake in the small intestine.

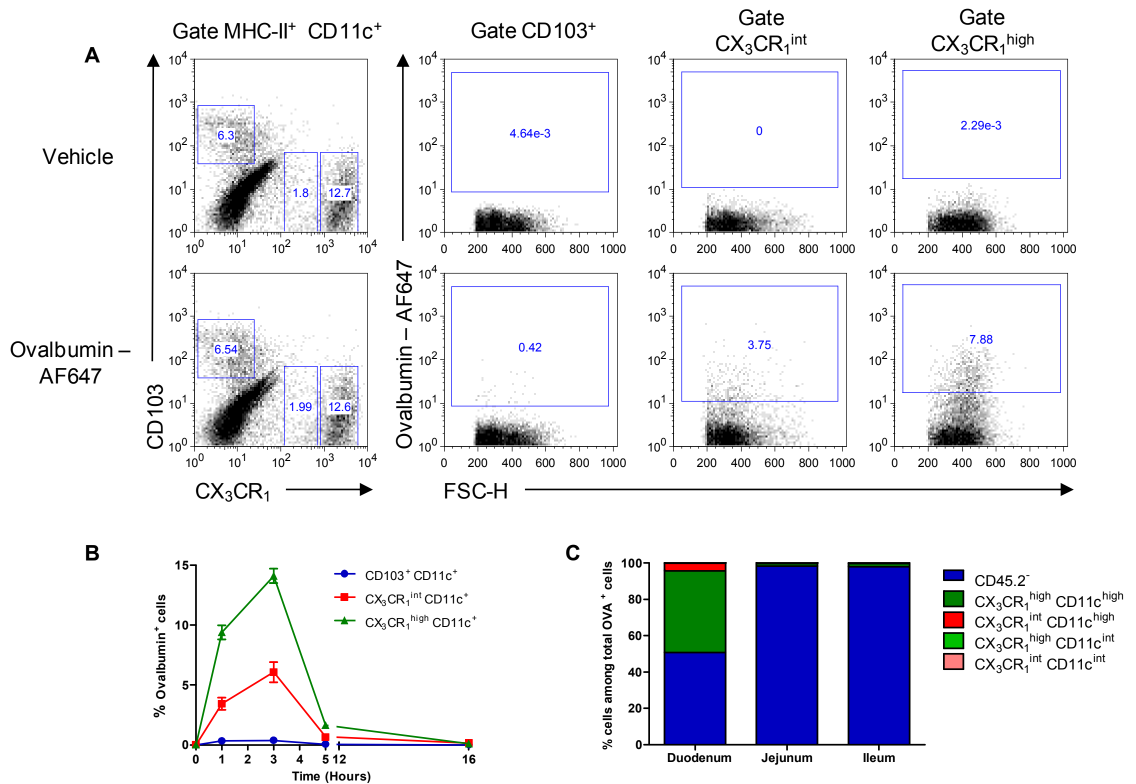


Figure 8-4 $CX_3CR_1^{high}$ and $CX_3CR_1^{int}$ cells take up OVA-AF647 in the intestinal lamina propria. **A-B**) 250 µg of ovalbumin-AF647 or DPBS (Vehicle) were i.g. administered to $CX_3CR_1^{GFP/+}$ mice and intestines were collected at the indicated time points. Cells were isolated from the small intestines, stained with anti-CD103, MHC-II and CD11c antibodies and analyzed by FACS. Percentage of OVA^+ cells in $CD103^+$, $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ subsets is shown in (**B**). Results are representative of two independent experiments, performed with 3 mice / group. Data are shown as Mean ± SEM. **C**) Duodenum, Jejunum and ileum were separately collected and stained with CD45 and CD11c antibodies. $Ovalbumin^+$ cells were gated and percentages of each subset among total OVA^+ cells are represented.

8.1.2 Ovalbumin presentation on MHC-I by lamina propria cells depends on antigen dose

We further characterized functional OVA peptide presentation on MHC-I taking advantage of the Anti-MHC Class I ($H\text{-}2K^b$)-SIINFEKL Antibody, known as anti-K^bOVA, 25-D1.16 [166]. As shown in Figure 8-5, along with $CX_3CR_1^+$ LPC_s (highlighted by yellow arrows), another subset of $CD11b^+$ LP cells was capable of OVA peptide presentation on MHC-I, as highlighted by white arrows. Certainly, CD11b marker alone is not sufficient to univocally define a subset of antigen presenting cells in the lamina propria. Nevertheless,

observing their localization, $CD11b^+$ cells are located where $CD11c^+$ and $CD103^+$ DC_s are expected in the small intestine.

Again, we proceeded to FACS analysis in order to more precisely characterize subsets of APC_s in the lamina propria presenting OVA peptide on MHC-I. Thus, $CX_3CR_1^{GFP/+}$ mice were fed with ovalbumin, either at low (1 mg) or high dose (20 mg), and intestines were dissected and processed after 8 hours.

Cells were then stained with MHC-II, CD11c, CD103 and CD11b antibodies to distinguish between $CX_3CR_1^+$ Mϕ and $CD103^+$ DC_s. After exclusion of dead cells, $MHC\text{-}II^+$ $CD11c^+$ cells were gated and four main subsets are recognized, $CD11b^-$ $CD103^+$, $CD11b^+$ $CD103^+$, $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ cells. $CX_3CR_1^+$ cells uniformly expressed CD11b (as shown in Figure 8-6).

K^bOVA antibody is not perfectly suited for FACS staining; nonetheless, a small shift in K^bOVA histogram was observed when cells display H-2K^b-SIINFEKL marker on their surface in a dose-dependent manner. Results were then reported as the difference in the Geometric Mean of K^bOVA fluorescence between OVA and Vehicle samples for each subset.

When mice were fed with high dose of ovalbumin, at different degrees all the four subsets presented OVA peptide on MHC-I. On the other hand, at low Ag dose, which is compatible with the dose used for the previous experiments, only $CD11b^+$ $CD103^+$ and $CX_3CR_1^{high}$ cells presented OVA peptide on MHC-I (Figure 8-7, A-B).

Hence, the ability of presenting ovalbumin peptides on MHC-I depends on Ag dose. While at high dose, this ability is shared among the different LP subsets, at low dose mostly $CX_3CR_1^{high}$ Mϕ and $CD11b^+$ $CD103^+$ DC_s present OVA peptides on MHC-I. We have previously shown that $CD11b^+$ $CD103^+$ DC_s do not take up antigens by themselves, suggesting alternative routes of Ag acquisition.

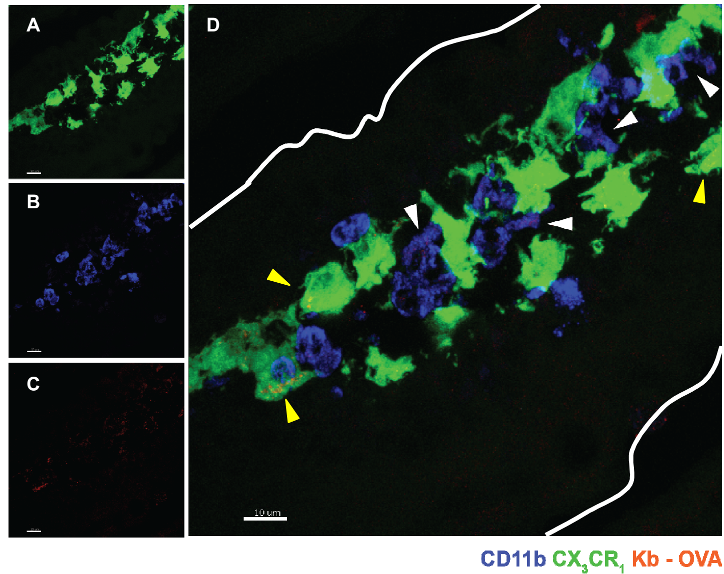


Figure 8-5 CD11b$^+$ cells in the lamina propria present OVA on MHC-I complex. Sections of the intestinal loop were stained with Anti-CD11b and Anti-MHC Class I (H-2K^b)-SIINFEKL (K^{b}OVA) Antibodies. Single channels are shown in the right panels: Green, CX_3CR_1 (**A**), Blue, CD11b (**B**), K^{b}OVA (**C**). Merge Picture is shown in (**D**). White lines represent the villus contour; cells where the K^{b}OVA signal colocalizes with CD11b signal are indicated by white arrows; cells where K^{b}OVA signal colocalizes with CX_3CR_1 are highlighted by yellow arrows. Scale bar, 10 μm.

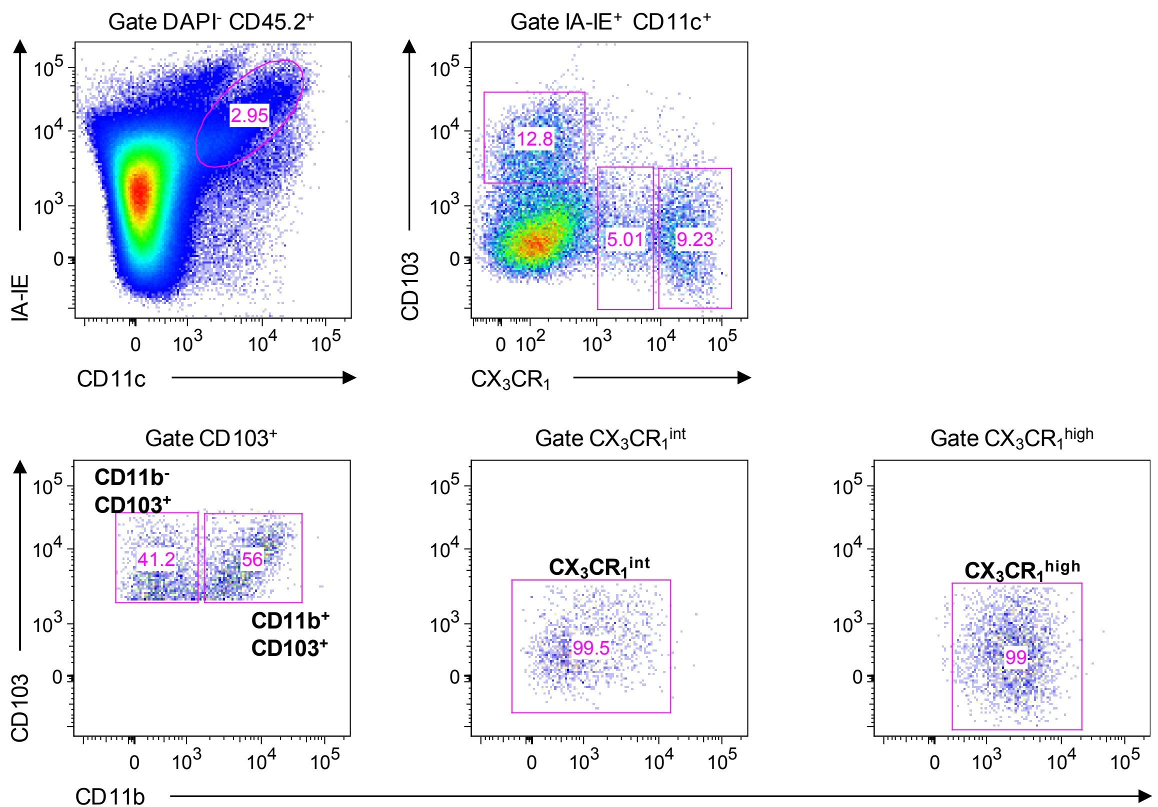


Figure 8-6 Gating strategy used for the definition of mononuclear cell subsets in the small intestine. Cell suspension, obtained by digestion of the tissue, is blocked with anti-CD16-CD32 antibody and stained with anti-CD45.2, CD11c, MHC-II, CD103 and CD11b antibodies. After exclusion of epithelial and dead cells (DAPI$^-$ CD45.2$^+$), MHC-II$^+$ CD11c$^+$ are gated. Three main

subsets are subsequently recognized: $CD103^+$, $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$. The three subsets are then divided based on their expression of CD11b; in particular, $CD103^+$ DC_s are divided as $CD11b^-$ $CD103^+$ and $CD11b^+$ $CD103^+$, while $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ Mϕ express uniformly CD11b.

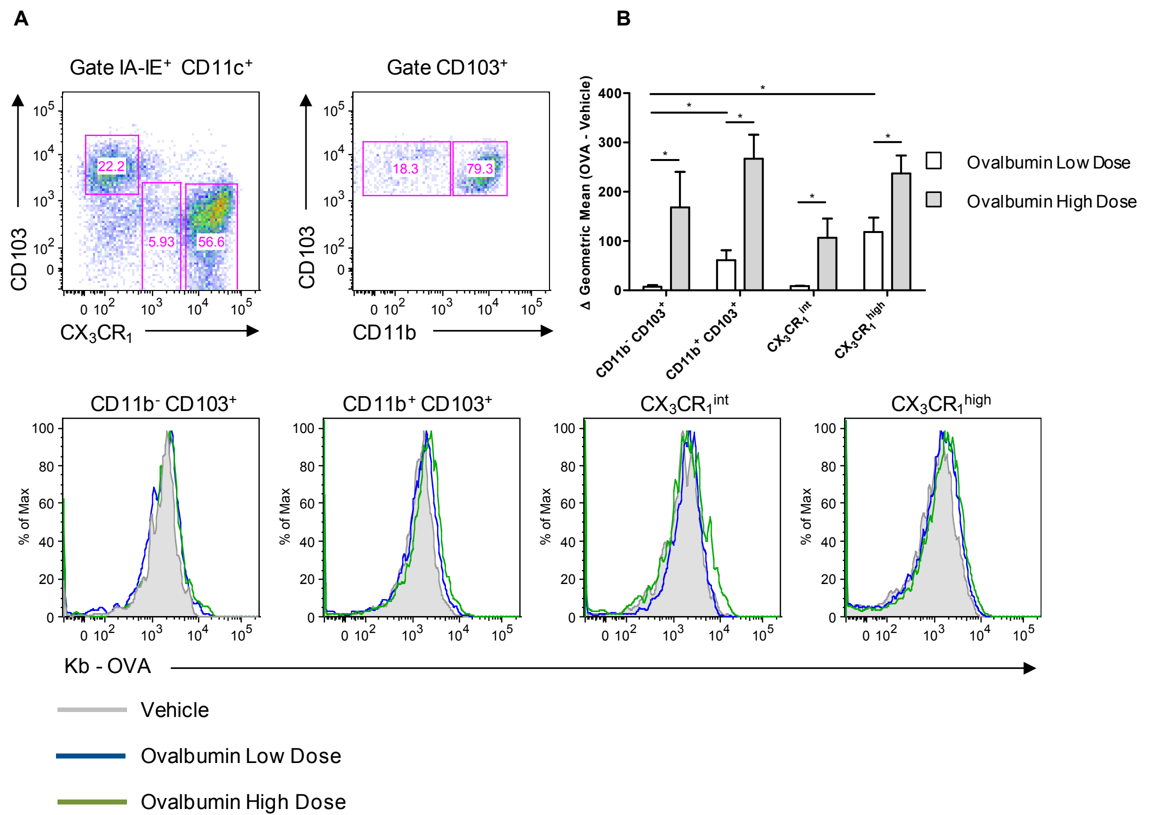


Figure 8-7 Ovalbumin presentation on MHC-I by LP cells depends on antigen dose. **A**) After 8 hours from feeding $CX_3CR_1^{GFP/+}$ with DPBS (Vehicle), low (1 mg) or high (20 mg) ovalbumin dose, small intestines were dissected and processed. Cells were stained with MHC-II, CD11c, CD103 and CD11b antibodies and analyzed by FACS. Gating strategy is depicted in (**A**): after excluding dead cells by DAPI staining, $MHC\text{-}II^+$ $CD11c^+$ were gated. They were subsequently distinguished in $CD11b^-$ $CD103^+$, $CD11b^+$ $CD103^+$, $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ subsets. Lower panels represent the histograms of K^bOVA fluorescence for each subset. Grey Line, Vehicle; Blue Line, Low Dose Ovalbumin; Green Line, High Dose Ovalbumin. **B**) Results are displayed as Mean ± SEM of the difference in the Geometric Mean of K^bOVA fluorescence between OVA and Vehicle samples for each subset. Results are representative of two independent experiments, 4 mice / group. * $p < 0.01$.

8.1.3 Uptake of ovalbumin by $CX_3CR_1^+$ cells is decreased in the absence of CX_3CR_1 receptor

CX_3CR_1-expressing cells have been for a long time considered a subset of intestinal DC_s that sample the luminal content and extend dendrites into the intestinal lumen in a CX_3CR_1-dependent manner. In the absence of CX_3CR_1 receptor, indeed, dendrites are not formed [124, 125]. Despite their recent redefinition as intestinal Mϕ, $CX_3CR_1^+$ LPC_s still maintain their functional properties.

We have previously shown that antigen uptake in the small intestine is preferentially carried out by $CX_3CR_1^+$ cells; thus, we next asked whether this process is dependent on luminal sampling and expression of functional CX_3CR_1 receptor by these cells.

We took advantage of $CX_3CR_1^{GFP/GFP}$ mice, which have both copies of the CX_3CR_1 gene substituted by the gene coding for GFP. $CX_3CR_1^{GFP/+}$ and $CX_3CR_1^{GFP/GFP}$ mice were fed with 250 µg of ovalbumin-AF647 and their intestines were removed after 1 h. Organs were processed to obtain a cell suspension, which was stained with MHC-II, CD11c, CD103 and CD11b antibodies. Cells were gated as shown in Figure 8-6.

For each subset, namely $CD11b^-$ $CD103^+$, $CD11b^+$ $CD103^+$, $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ cells, percentage of OVA^+ cells is reported (Figure 8-8). As regards $CD11b^-$ $CD103^+$ and $CD11b^+$ $CD103^+$ DC_s, their poor ability to take up antigens is confirmed and no difference is observed between CX_3CR_1-sufficient and -deficient mice. On the other hand, both $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ cells show decreased uptake of OVA-AF647 in $CX_3CR_1^{GFP/GFP}$ compared to $CX_3CR_1^{GFP/+}$ mice, suggesting the involvement of CX_3CR_1 receptor and hence cell protrusions in the sampling and uptake of soluble antigens from the gut lumen.

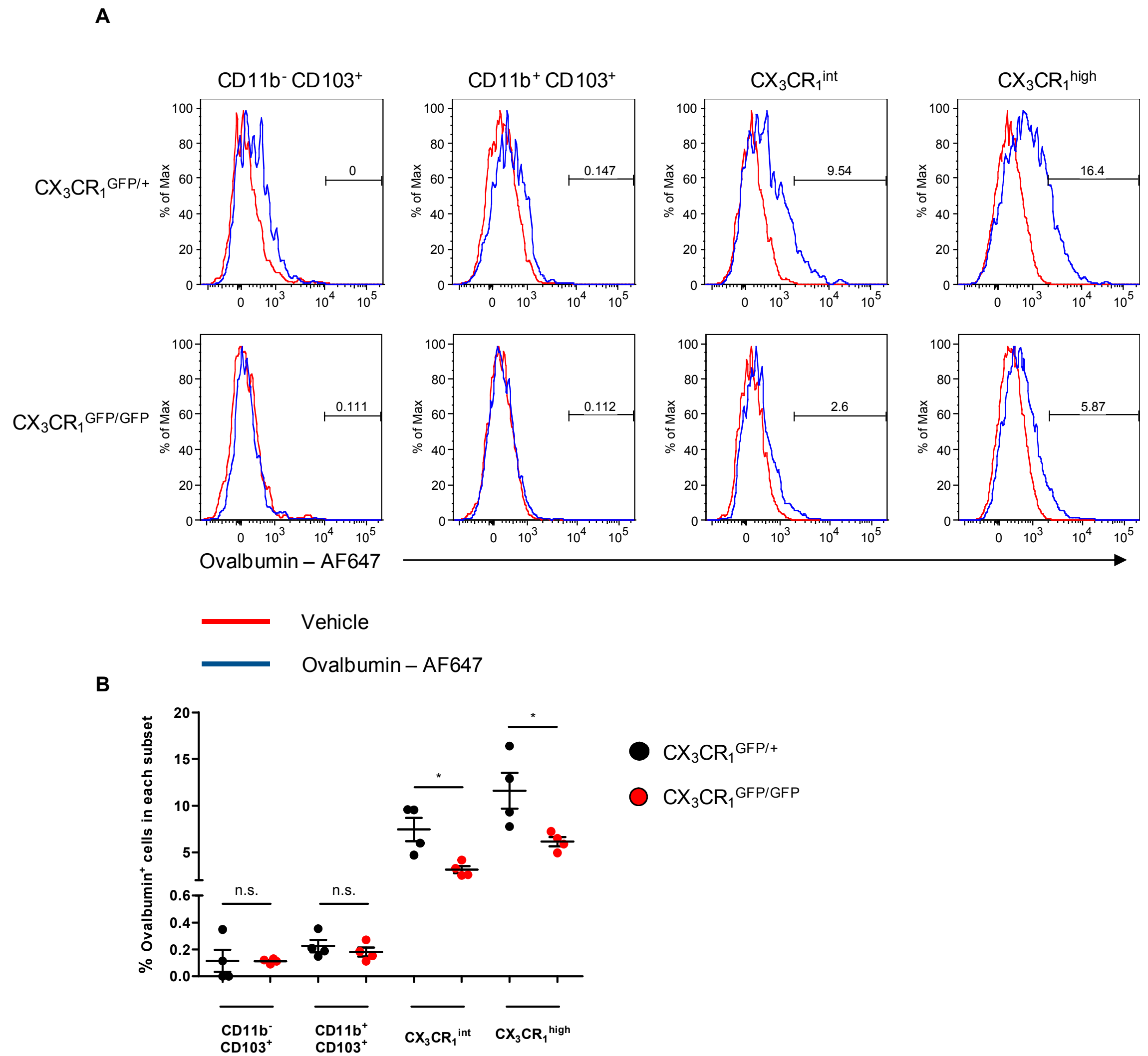


Figure 8-8 Uptake of Ovalbumin-AF647 by $CX_3CR_1^+$ cells in decreased in $CX_3CR_1^{GFP/GFP}$ mice. **A**) $CX_3CR_1^{GFP/+}$ and $CX_3CR_1^{GFP/GFP}$ mice were i.g. administered with 250 µg of AF647-conjugated ovalbumin. After 1 hour, intestines were processed for FACS analysis. After excluding dead cells and gating on MHC-II$^+$ CD11c$^+$ cells, cells were divided in fours subsets, based on their expression of CD103, CX_3CR_1 and CD11b. Histograms show OVA-AF647 fluorescence for each subset. **B**) Percentage of OVA$^+$ cells in each subset is shown. Data are presented as Mean ± SEM and are representative of two independent experiments, 4 mice / group.* $p < 0.01$; n.s. not significant.

8.1.4 Induction of oral tolerance to ovalbumin is impaired in $CX_3CR_1^{GFP/GFP}$ mice

As the first step in the establishment of oral tolerance - antigen uptake in the SI lamina propria - is impaired in $CX_3CR_1^{GFP/GFP}$ mice, we wondered whether the lack of functional fractalkine receptor would affect the establishment of tolerance to fed antigens at steady-

state condition. Hence, we evaluated the Delayed type hypersensitivity (DTH) response to OVA in C57BL/6, $CX_3CR_1^{GFP/+}$ and $CX_3CR_1^{GFP/GFP}$ mice after administration of the same antigen through the oral route, as previously described by *Goubier et al.* [75]. Mice were given OVA i.g. at day -13, -10 and -7, immunized subcutaneously with OVA and Complete Freund's Adjuvant (CFA) at day 0 and challenged in the footpad with heat-aggregated OVA at day 7. Footpad swelling was measured after 24 and 48 hours. A scheme of DTH schedule is shown in Figure 8-9.

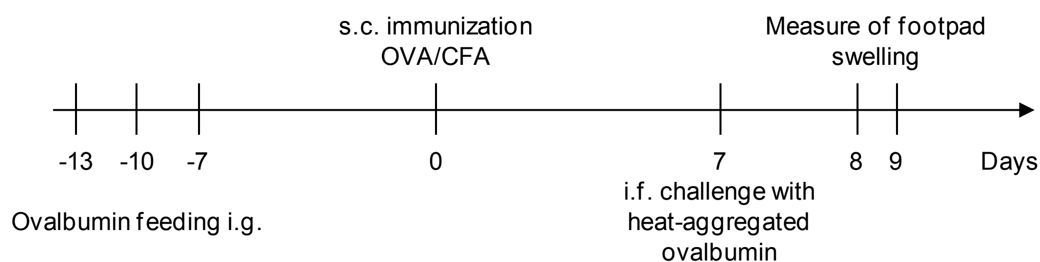


Figure 8-9 Schedule of Ovalbumin feeding, s.c. immunization and i.f. challenge. Control C57BL/6, $CX_3CR_1^{GFP/+}$ and $CX_3CR_1^{GFP/GFP}$ mice were fed with 20 mg ovalbumin at day -13, -10 and -7. At day 0, they were immunized subcutaneously with 50 µg OVA in Complete Freund's adjuvant and 7 days later were challenged with 250 µg of heat-aggregated OVA in their footpad. DTH response was measure as foodpad swelling after 24 and 48 hours from the challenge.

As shown in Figure 8-10, establishment of tolerance to fed OVA was observed in C57BL/6 and $CX_3CR_1^{GFP/+}$ mice. Indeed, i.g. administration of ovalbumin resulted in a decreased DTH response, measured as lessening in the footpad swelling. On the other hand, $CX_3CR_1^{GFP/GFP}$ mice displayed impaired induction of oral tolerance, as no significant difference was observed between DPBS- and OVA-fed mice.

Similar results were observed in the re-stimulation *in vitro* of splenocytes derived from immunized C57BL/6, $CX_3CR_1^{GFP/+}$ and $CX_3CR_1^{GFP/GFP}$ mice, fed or not with ovalbumin. In OVA-fed C57BL/6 and $CX_3CR_1^{GFP/+}$ mice, lowering of IFN-γ was observed compared to DPBS-fed mice, while no difference was reported for $CX_3CR_1^{GFP/GFP}$ mice (Figure 8-11).

Therefore, we have shown that CX_3CR_1-deficient mice display decreased Ag uptake by $CX_3CR_1^+$ cells in the SI and confirmed previous data that they fail to develop tolerance to fed antigens [134].

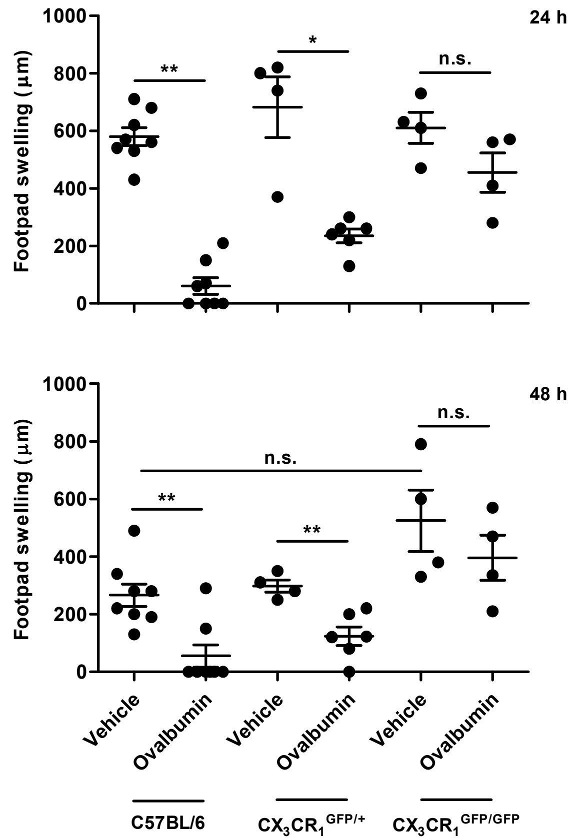


Figure 8-10 Induction of oral tolerance to ovalbumin is impaired in $CX_3CR_1^{GFP/GFP}$ mice. For C57BL/6, $CX_3CR_1^{GFP/+}$ and $CX_3CR_1^{GFP/GFP}$ mice, DTH response was evaluated. After 24 and 48 hours from i.f. challenge with heat-aggregated ovalbumin, footpad swelling was measured as difference in the thickness of OVA-challenged and DPBS-injected control footpad for each mouse. Results are displayed as Mean ± SEM and are representative of two independent experiments, 3 - 8 mice / group. * p < 0.05; ** p < 0.01; n.s. not significant.

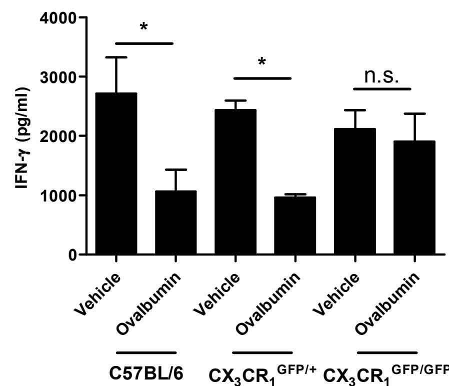


Figure 8-11 In vitro re-stimulation of splenocytes reveals failure of oral tolerance establishment in $CX_3CR_1^{GFP/GFP}$ mice. Splenocytes isolated from DPBS- or OVA-fed C57BL/6, $CX_3CR_1^{GFP/+}$ and $CX_3CR_1^{GFP/GFP}$ mice were re-stimulated *in vitro* with 1 mg/ml OVA. Supernatant was collected after 4 days and IFN-γ measured by ELISA.

8.2 Characterization of antigen transfer among lamina propria cells

8.2.1 Lamina propria cells exchange Ag peptides and a gap-junction diffusible dye

Although no or little antigen was detected in $CD103^+$ DC_s by FACS staining, we have now shown that in the small intestine $CD11b^+$ $CD103^+$ cells, together with $CX_3CR_1^{high}$ Mϕ, present antigens on MHC-I when Ag is administered at low dose.

Furthermore, previous studies have shown that $CD103^+$ DC_s have proven to be the only population of migratory cells endowed with tolerogenic properties once they have reached the MLN [103, 104].

Hence, due the uneven ability of lamina propria cells to phagocytose soluble antigens and functionally present them on MHC complexes, we hypothesized that the $CX_3CR_1^+$ cells may be able to exchange antigenic material with $CD103^+$ cells.

To test our hypothesis, we sorted SI $CD103^+$ (pooling $CD11b^-$ and $CD11b^+$ DC_s) and $CX_3CR_1^+$ cells (pooling $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ subsets) from $CX_3CR_1^{GFP/+}$ mice and, after loading *in vitro* with OVA-Alexa Fluor 647, cells were co-cultured for one additional hour. The transfer of fluorescent antigens was evaluated by FACS.

As shown in Figure 8-12, when $CX_3CR_1^+$ cells were loaded with ovalbumin and then co-cultured with $CD103^+$ DC_s (third column), around 1% of $CD103^+$ cells became positive for OVA, suggesting a transfer of antigenic material from $CX_3CR_1^+$ to $CD103^+$ cells.

Additionally, the experiment was performed also in the opposite direction, labeling first $CD103^+$ DC_s, and subsequently co-culturing them with $CX_3CR_1^+$ cells (forth column). Of note, *ex vivo* $CD103^+$ DC_s efficiently phagocytosed ovalbumin, differently from what observed *in vivo*, and transfered antigens to $CX_3CR_1^+$ cells, which become around 4% positive for OVA-AF647.

Therefore, *ex vivo* both subsets of $CX_3CR_1^+$ cells and $CD103^+$ DC_s show the capability to take up fluorescently-labeled ovalbumin and transfer it to their counterpart.

We then asked what possible mechanism might account for Ag_s exchange between $CX_3CR_1^+$ cells and $CD103^+$ DC_s. Often immune cells release exosomes or cytosol fragments in order to communicate to neighboring cells. Alternatively, since these two subsets of cells strictly interact in the tissue, one might think that direct connection of cytoplasm of adjacent cells can occur. Based on previous know-how acquired in our laboratory, we hypothesized that gap junction-mediated transfer may be responsible for Ag passage among $CX_3CR_1^+$ to $CD103^+$ cells in the small intestine. It has been previously shown, indeed, that GJ_s mediate peptide transfer of small peptides between bone- marrow derived dendritic cells [162] and sustain cross-presentation [163, 167].

In order to test our idea, we asked whether $CD11c^+$ cells purified from the small intestine could exchange a GJ-diffusible dye, namely calcein. The experiment was performed as depicted in Figure 8-13: the donor fraction of $CD11c^+$ cells (enriched from the small intestines of C57BL/6 mice) was loaded with calcein. The acceptor fraction was labeled with DDAO, a dye which cannot diffuse through GJ.

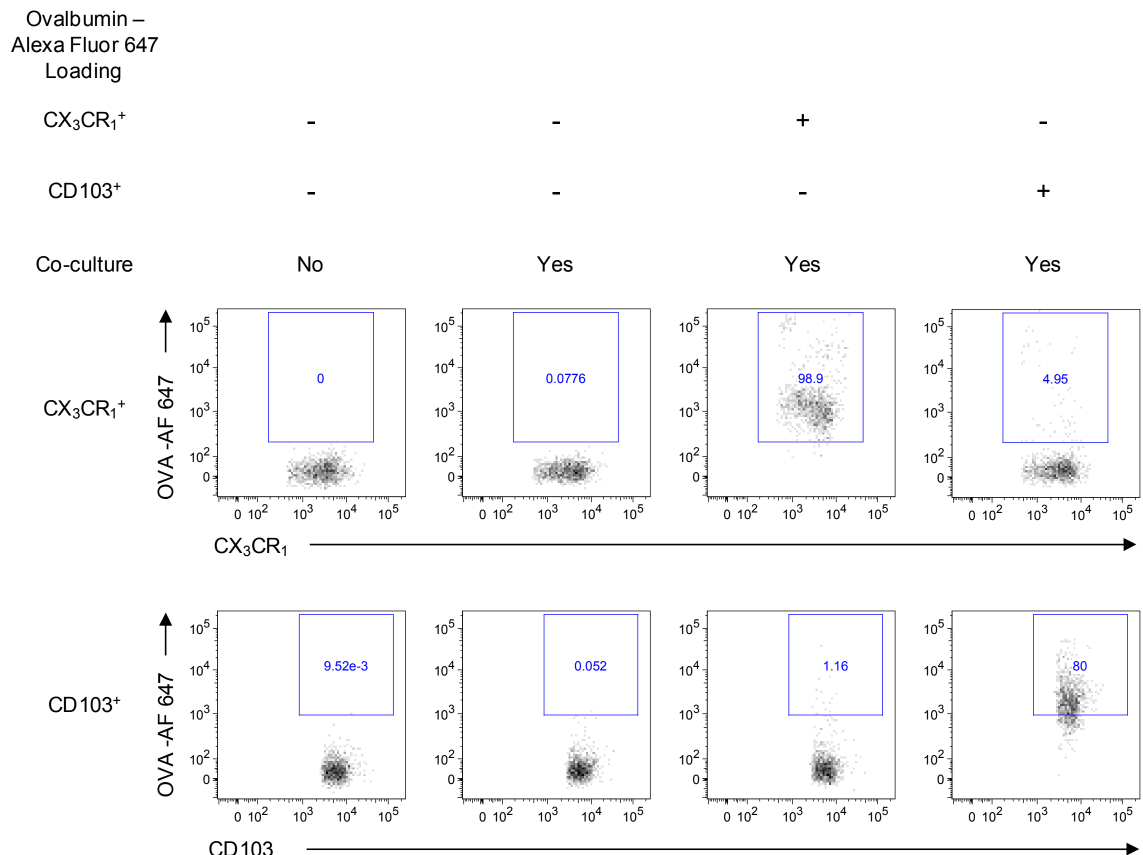


Figure 8-12 Ovalbumin peptides can be transferred from $CX_3CR_1^+$ LP cells to $CD103^+$ $CD11c^+$ DCs ex vivo, and viceversa. Sorted populations ($CX_3CR_1^+$ LPC_s and $CD103^+$ DCs) were cultured in the presence (+) or absence (-) of 100 μg/ml Alexa Fluor 647-conjugated ovalbumin for 1 hour

and then extensively washed. When indicated, sorted populations were mixed and co-cultured for 1 h (Yes=co-culture, No=not co-cultured). Transfer of OVA peptides is evaluated by FACS.

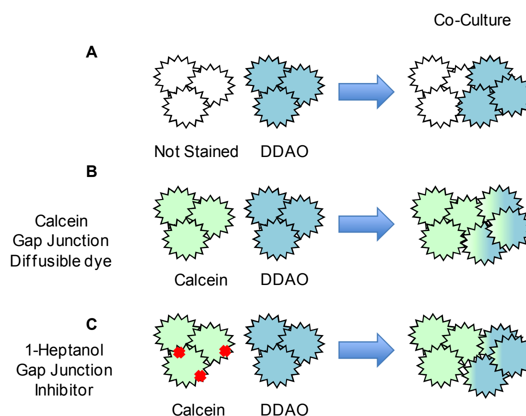


Figure 8-13 Experimental scheme to evaluate calcein exchange between pools of CD11c$^+$ cells isolated from the small intestine. CD11c-enriched from the SI of C57BL/6 mice were loaded with calcein (donor cells) and then co-cultured with DDAO-loaded CD11c$^+$ cells (acceptor cells), as in (**B**). As a control, DDAO$^+$ cells were co-cultured with not labeled CD11c$^+$ cells (**A**). Transfer of Calcein to DDAO$^+$ cells was evaluated by FACS. In (**C**), calcein-loaded cells were pre - treated with 1-Heptanol (at a concentration of 0.5, 5 or 50 mM), a GJ inhibitor, before the co-culture with DDAO$^+$ cells.

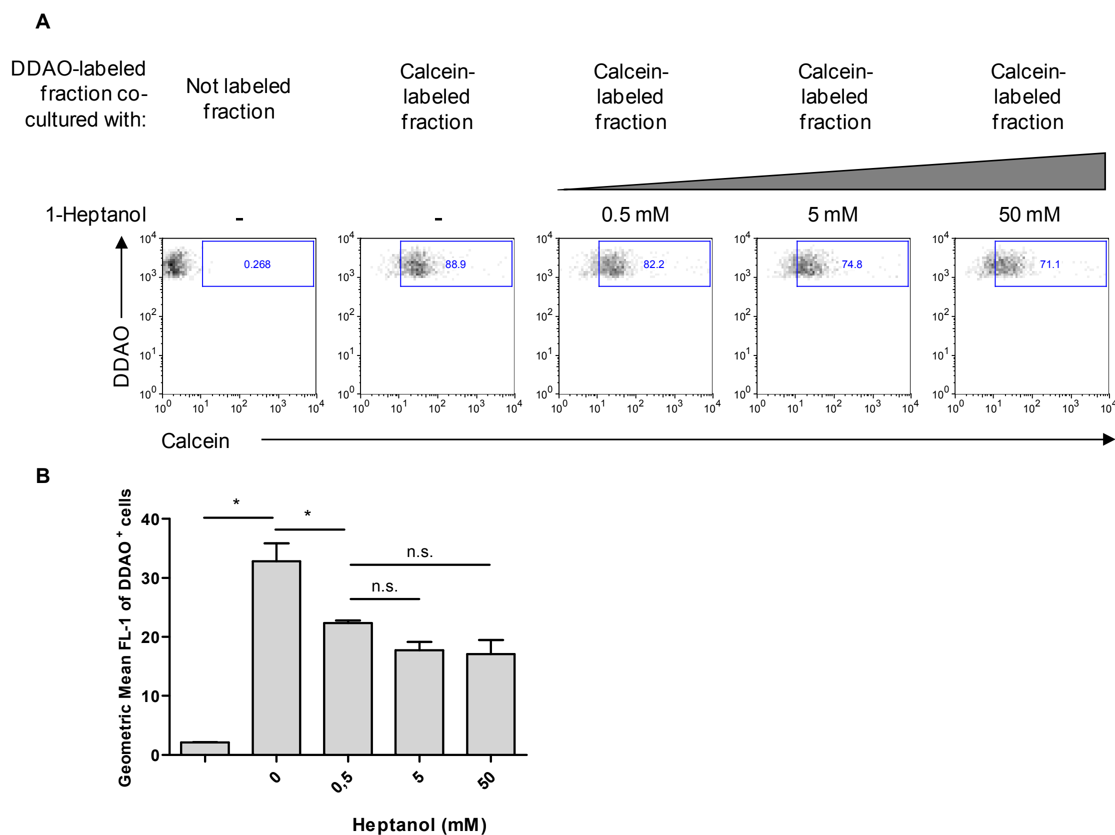


Figure 8-14 Calcein, a gap junction-diffusible dye, is exchanged ex vivo by pools of CD11c$^+$ cells in the small intestine. **A**) DDAO$^+$ cells were co-cultured with not labeled control cells or calcein-loaded cells for 30 minutes. Transfer of the dye to DDAO$^+$ cells was subsequently

evaluated by FACS. When indicated, increasing concentrations of 1-Heptanol were added. **B**) Mean ± SD of the Geometric Mean of FL-1 (Calcein) fluorescence for $DDAO^+$ $CD11c^+$ cells is indicated. Pooled results from three independent experiments are shown, each of them performed with $CD11c^+$ cells obtained from 4 C57BL/6 mice.

Donor and acceptor cells were mixed and calcein transfer to $DDAO^+$ cells was evaluated by FACS. As shown in Figure 8-14, calcein dye is transferred from $Calcein^+$ $CD11c^+$ cells to $DDAO^+$ $CD11c^+$ cells through gap junctions and the process is partly inhibited by the pre-treatment of cells with 1-Heptanol, a GJ-blocking agent. Inhibition is obtained already at a concentration of 0.5 mM of 1-Heptanol and it is not dose-dependent.

Taken together, this data suggest that $CX_3CR_1^+$ LP cells and $CD103^+$ DC_s are able to mutually exchange Ag_s and this process might be mediated by gap junctions.

8.2.2 Small intestine mononuclear cells express a peculiar panel of connexins

We next assessed which are the connexins expressed by dendritic cells in the small intestine compared to those from mesenteric LN_s, spleen and Peyer's Patches. Hence, $CD11c^+$ cells were enriched from these organs and processed for RNA extraction. In Figure 8-15, expression of connexins relative to Rlp32 housekeeping gene is shown. For each connexin, the expression pattern is highly variable among the different tissues. To simplify the results, a threshold was drawn in order to consider only those genes characterized by relative expression ≥ 0.001.

We then isolated $CD11c^+$ cells from different GI tracts. As depicted in Figure 8-16, among 20 different Connexins encoded in *M. musculus* genome, GALT DC_s express a peculiar panel of six connexins, being Cx32 and Cx43 mostly expressed in all the segments of the SI.

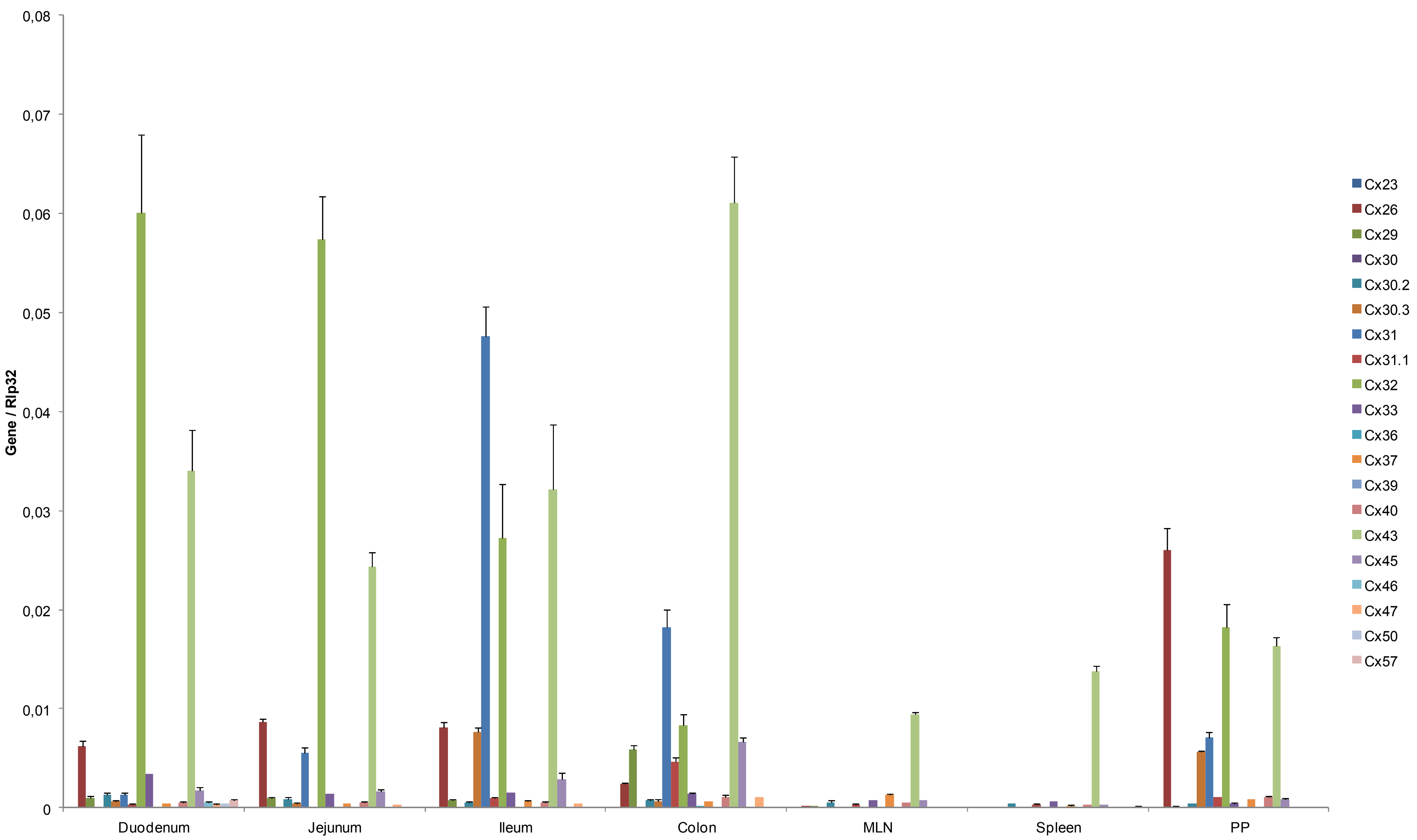

Gene / Rlp32
0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
Duodenum
Jejunum
Ileum
Colon
MLN
Spleen
PP
Cx23
Cx26
Cx29
Cx30
Cx30.2
Cx30.3
Cx31
Cx31.1
Cx32
Cx33
Cx36
Cx37
Cx39
Cx40
Cx43
Cx45
Cx46
Cx47
Cx50
Cx57

Figure 8-15 Connexin expression by CD11c+ cells in the GALT and spleen is tissue-specific. CD11c+ cells were enriched from the different tracts of the small intestine (duodenum, jejunum and ileum), colon, mesenteric lymph nodes, Peyer's Patches and spleen. RNA was extracted and retrotranscribed. Expression of Cx genes was evaluated by qPCR and normalized to the Rlp32 housekeeping gene using the $2^{-\Delta Ct}$ Method. Results are shown as Mean ± SD of relative expression values obtained for three different mice.

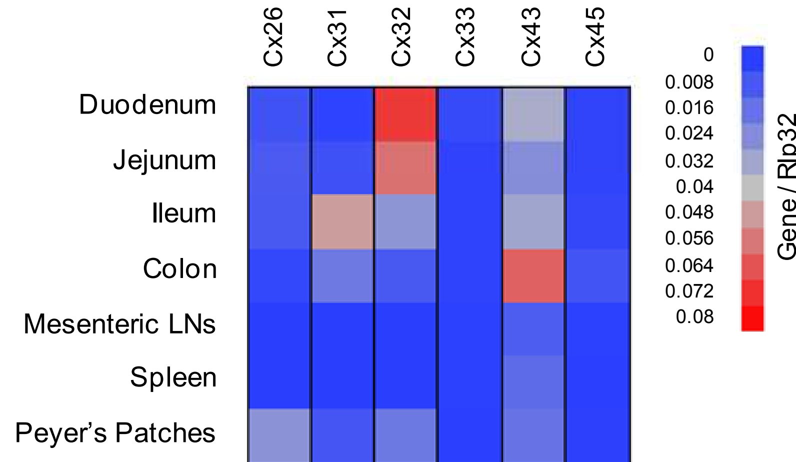


Figure 8-16 CD11c+ cells isolated from the GALT and spleen express a defined panel of six connexins. Connexin genes were filtered based on their relative expression to the housekeeping gene (≥ 0.001) and represented as color-coded plot following the scale displayed on the right (from blue to red). Results represent the mean of expression values obtained for three different mice.

We next sorted SI MHC-II+ CD11c+ cells from $CX_3CR_1^{GFP/+}$ mice, following the scheme indicated in Figure 8-6, and evaluated the expression of the six connexins in CD11b- CD103+, CD11b+ CD103+, $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ subpopulations.

While Cx26, -31 and -33 were broadly expressed in all the subsets, some displayed a subset-specific expression. For instance, Cx32 and -45 were primarily expressed by $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ subpopulations; differently, Cx43 was mainly expressed by $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ Mϕ and CD11b+ CD103+ DC_s (Figure 8-17).

Therefore, we have shown that expression of connexins is highly tissue- and subset-specific. Moreover, six connexins (Cx26, -31, -32, -33, -43 and -45), and in particular Cx32 and -43, are the most expressed by CD11c+ cells enriched from the GALT and are expressed at different degrees by SI mononuclear cell subsets.

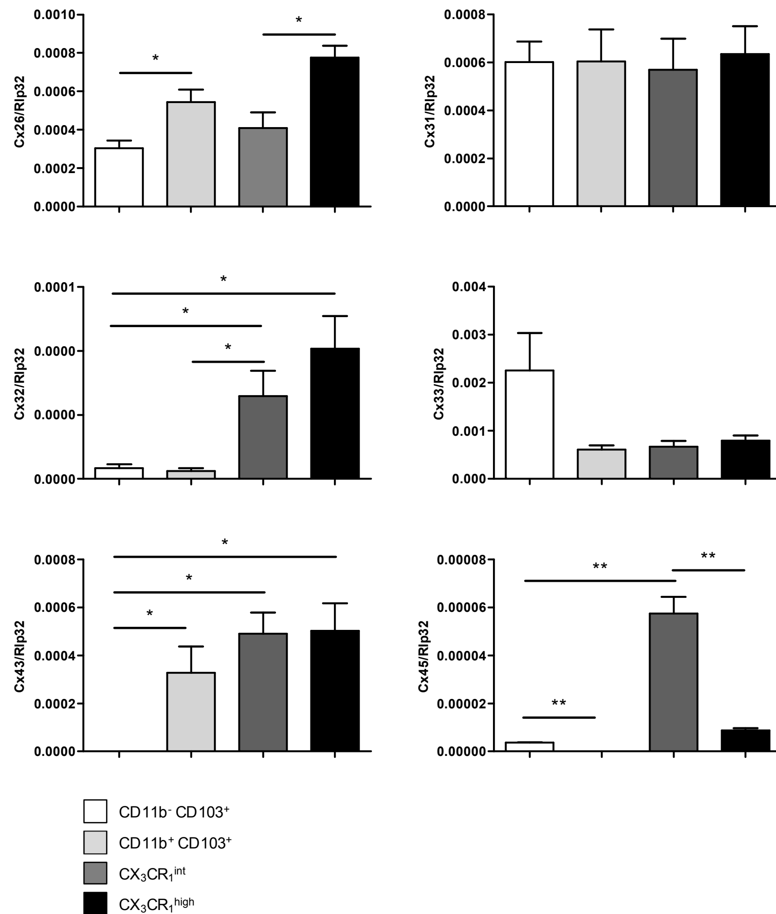


Figure 8-17 SI mononuclear cells differentially express Cx genes. Sorted subsets of DC_s and Mϕ from the small intestine of $CX_3CR_1^{GFP/+}$ mice ($CD11b^- CD103^+$, $CD11b^+ CD103^+$, $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$) were lysed and processed for RNA extraction. Expression of the panel of 6 connexins was evaluated and reported relative to the expression of the housekeeping Rlp32 gene. Data are shown as Mean ± SEM of three samples derived from independent sortings. * $p < 0.05$, ** $p < 0.01$

8.3 Characterization of mice lacking Cx43 in CD11c - expressing cells

8.3.1 Generation of mice lacking Cx43 in CD11c - expressing cells

We have hypothesized that GJ-mediated transfer of antigenic material may occur from $CX_3CR_1^+$ cells, which are endowed with phagocytic ability and uptake soluble antigens from the intestinal lumen, to $CD103^+$ DC_s, particularly to the CD11b-expressing subset, which do not uptake soluble antigens but still present Ag peptides in complex with MHC-I at low antigen dose. Besides, we have now observed that a panel of six connexins is

differentially expressed among lamina propria cells, and the only one that is concomitantly expressed by $CX_3CR_1^+$ (both $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$) and $CD11b^+$ $CD103^+$ DC_s is connexin 43.

In order to test whether antigen transfer mediated by GJ_s, and in particular by connexin 43, might be relevant to the first steps of antigen uptake in the small intestine, and more generally to the establishment of oral tolerance, $Cx43^{fl/fl}$ mice (B6.129S7-$Gja1^{tm1Dlg}$/J from Jackson Laboratory) were bred to transgenic CD11c-CRE mice (B6.Cg-Tg(Itgax-cre)1-1Reiz/J) to get $Cx43^{fl/fl}$ CD11c-CRE mice, characterized by deletion of exon 2 in the connexin 43 gene only in CD11c-expressing cells, as a result of loxP site-specific recombination by CRE recombinase (as shown in Figure 8-18).

CD11c-driven deletion should target dendritic cells and all the CD11c-expressing immune cells.

Efficient deletion of the exon 2 of connexin 43 was evaluated either by PCR on genomic DNA (Figure 8-19) and by qPCR (Figure 8-20) on RNA extracted from $CD11c^+$ cells enriched from the small intestine of $Cx43^{fl/fl}$ CD11c-CRE mice.

As shown in Figure 8-19, an amplicon of 686 bp is amplified by YL49/YL50 primers on genomic DNA, as a result of exon 2 deletion. Correctly, that band is observed only in the sample derived from $Cx43^{fl/fl}$ CD11c-CRE mice.

Accordingly, Cx43 expression is reduced in $CD11c^+$ cells enriched from the small intestine of $Cx43^{fl/fl}$ CD11c-CRE mice, compared to control mice (Figure 8-20).

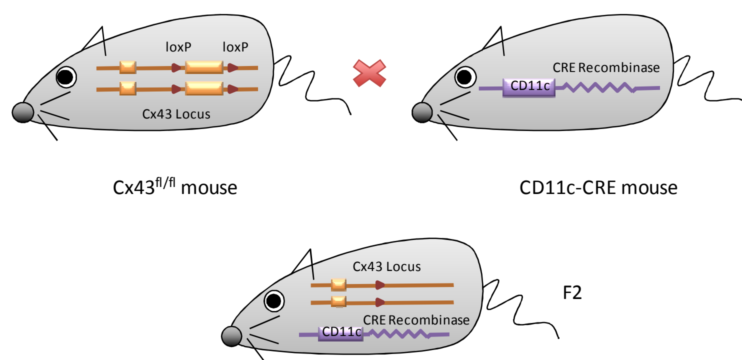


Figure 8-18 Breeding strategy to obtain Cx43-CD11c mice. $Cx43^{fl/fl}$ and CD11c-CRE mice were bred to obtain a generation F1 of mice, characterized by heterozygosity of the Cx43 locus. Mice from generation F1 were then bred to obtain a generation F2, where positive mice (bearing Cx43

locus in homozygosis and CRE transgene under the control of CD11c promoter) were screened by genotyping.

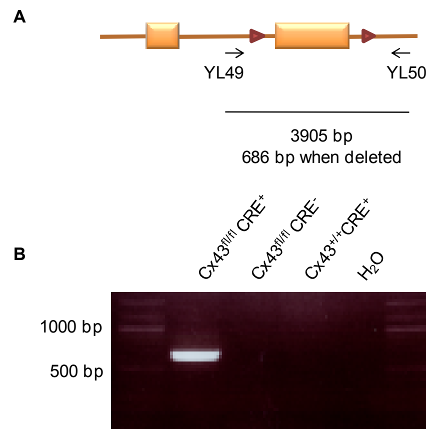


Figure 8-19 Evaluation of efficient deletion of exon 2 of Cx43 gene on genomic DNA extracted from SI CD11c$^+$ cells. A) Efficient deletion of exon 2 of Cx43 gene can be evaluated by PCR on genomic DNA. When exon 2 is deleted, the two loxP sites are brought together and YL49/YL50 primers amplify a region of around 700 bp on the genome. **B**) CD11c$^+$ cells were enriched from the small intestine of Cx43$^{fl/fl}$ CRE$^+$ mice and genomic DNA was extracted. CD11c$^+$ cells from Cx43$^{fl/fl}$ CRE$^-$ and Cx43$^{+/+}$ CRE$^+$ were used as negative controls. When exon 2 of Cx43 gene is deleted, the 686 bp band is visualized.

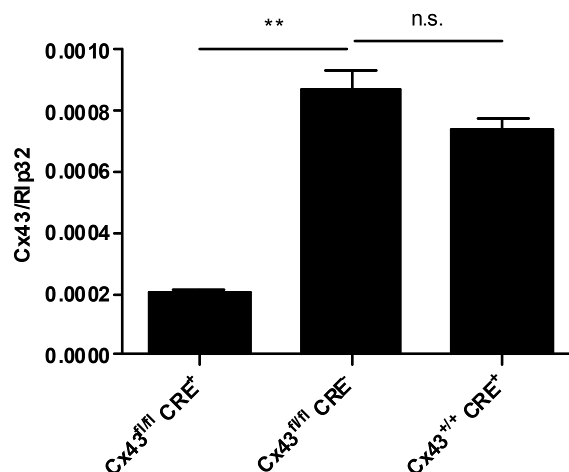


Figure 8-20 Evaluation of Cx43 expression in CD11c$^+$ cells enriched from the SI of Cx43$^{fl/fl}$ CD11c-CRE mice. CD11c$^+$ cells were enriched from the SI of Cx43$^{fl/fl}$ CD11c-CRE mice and RNA was extracted. Cx43 expression is normalized to Rlp32 housekeeping gene. CD11c$^+$ cells from Cx43$^{fl/fl}$ CRE$^-$ and Cx43$^{+/+}$ CRE$^+$ were used as positive controls. Data are shown as Mean ± SEM, from two independent experiments. ** $p < 0.01$; n.s. not significant.

8.3.2 $Cx43^{fl/fl}$ CD11c-CRE mice do not show major differences in LP and MLN DC subsets

Lack of connexin 43 has been associated with evident defects of the hematopoietic stem cell compartment during embryonic development [160]. In our model Cx43 deletion occurs later during DC development, particularly when CD11c begins to be expressed that is at the stage of pre-DC_s. Nonetheless, we asked whether deletion of Cx43 gene in dendritic cells may result in alterations of DC peripheral subsets.

We firstly analyzed small intestines of $Cx43^{+/+}$ CD11c-CRE and $Cx43^{fl/fl}$ CD11c-CRE mice. Cells were harvested from the organs and stained to evaluate their expression of CD45.2, CD11c, MHC-II, CD103 and CD11b markers. Three main subsets of $MHC\text{-}II^+$ $CD11c^+$ cells were distinguished based on their expression of CD103 and CD11b, namely $CD11b^-$ $CD103^+$, $CD11b^+$ $CD103^+$ and $CD11b^+$ $CD103^-$ (Figure 8-21, A).

As shown in Figure 8-21, no significant difference was observed either in the percentage of $MHC\text{-}II^+$ $CD11c^+$ cells (Figure 8-21, B) and in the relative abundance of single subsets (Figure 8-21, C).

The same analysis was repeated for mesenteric lymph nodes; hence, cells obtained from digestion of the organs were stained and differentiated on the basis of the previous mentioned markers. Four subsets of $MHC\text{-}II^+$ $CD11c^+$ dendritic cells were observed in the MLN: $CD11b^-$ $CD103^+$, $CD11b^+$ $CD103^+$, $CD11b^+$ $CD103^-$ and $CD11b^-$ $CD103^-$ (Figure 8-22, A). Again, no significant difference was observed both in the percentage of $MHC\text{-}II^+$ $CD11c^+$ DC_s (Figure 8-22, B) and of single DC subsets (Figure 8-22, C), except for the $CD11b^+$ $CD103^-$ subset.

Besides, we evaluated the expression of co-stimulatory molecules, such as CD86, CD40 and CD83, in SI subsets as it has been reported that chemical inhibition of Cx43 in BM-DC_s causes a down-regulation of co-stimulatory molecules, even in the presence of LPS or IFN-γ stimulation [162]. As observed in Figure 8-23, we did not observe significant difference in the expression of co-stimulatory molecules, except for a slight reduction in CD86 expression in $CD11b^+$ $CD103^-$ cells.

Hence, Cx43 deletion generally affect neither dendritic cell balance in the peripheral organs we have considered, namely small intestine and mesenteric lymph nodes, nor the expression of co-stimulatory molecules by LP mononuclear cell subsets.

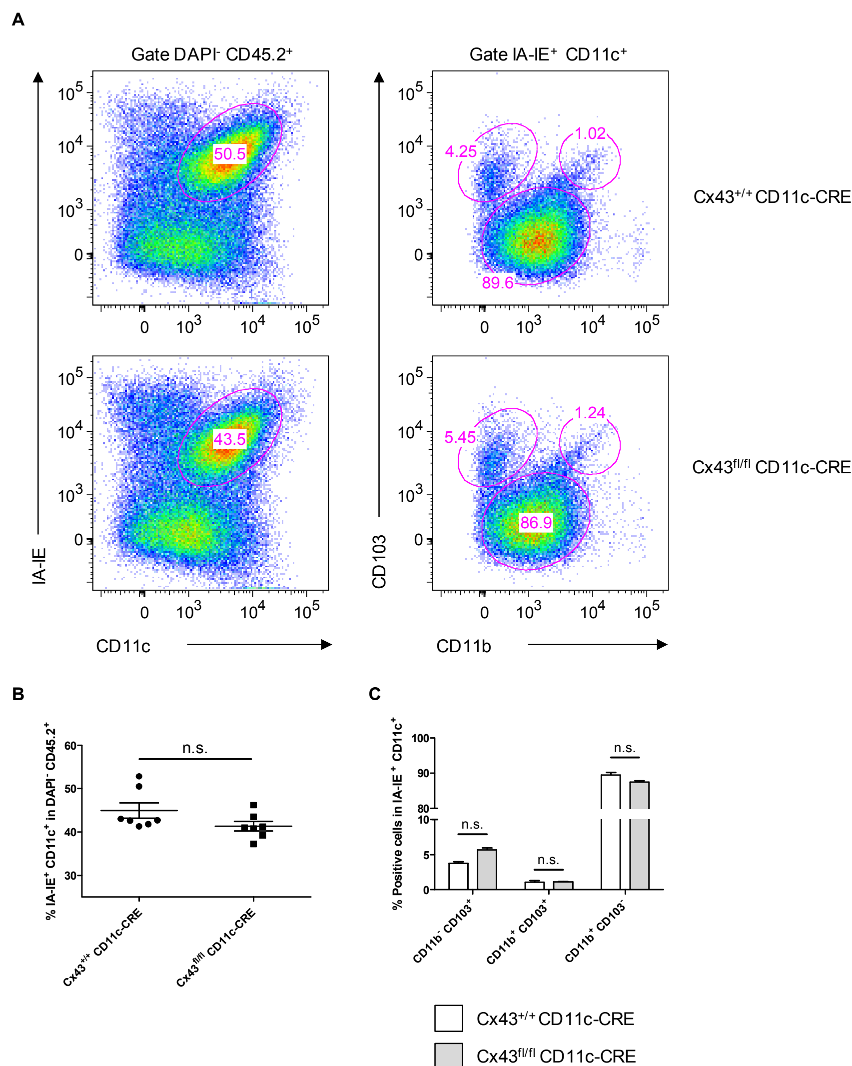


Figure 8-21 Cx43$^{fl/fl}$ CD11c-CRE mice do not display differences in intestinal DC subsets. A) Cells were obtained from the small intestine of Cx43$^{fl/fl}$ CD11c-CRE mice and control Cx43$^{+/+}$ CD11c-CRE and subsequently stained with anti-CD45.2, CD11c, MHC-II, CD103 and CD11b. In the analysis, after excluding dead and epithelial cell (DAPI$^-$ CD45.2$^+$), MHC-II$^+$ CD11c$^+$ cells were gated. MHC-II$^+$ CD11c$^+$ cells are then divided based on their expression of CD11b and CD103. Three main subsets of CD11b$^-$ CD103$^+$, CD11b$^+$ CD103$^+$ and CD11b$^+$ CD103$^-$ are recognized. **B**) The graph represents the Mean ± SEM of the percentage of MHC-II$^+$ CD11c$^+$ in the gate DAPI$^-$ CD45.2$^+$. **C**) Mean in the percentage of each subset relative to MHC-II$^+$ CD11c$^+$ gate is shown for Cx43$^{fl/fl}$ CD11c-CRE and Cx43$^{+/+}$ CD11c-CRE mice. Results are shown as Mean ± SEM and are representative of two independent experiments, 7 mice / group. * $p < 0.05$; n.s. not significant.

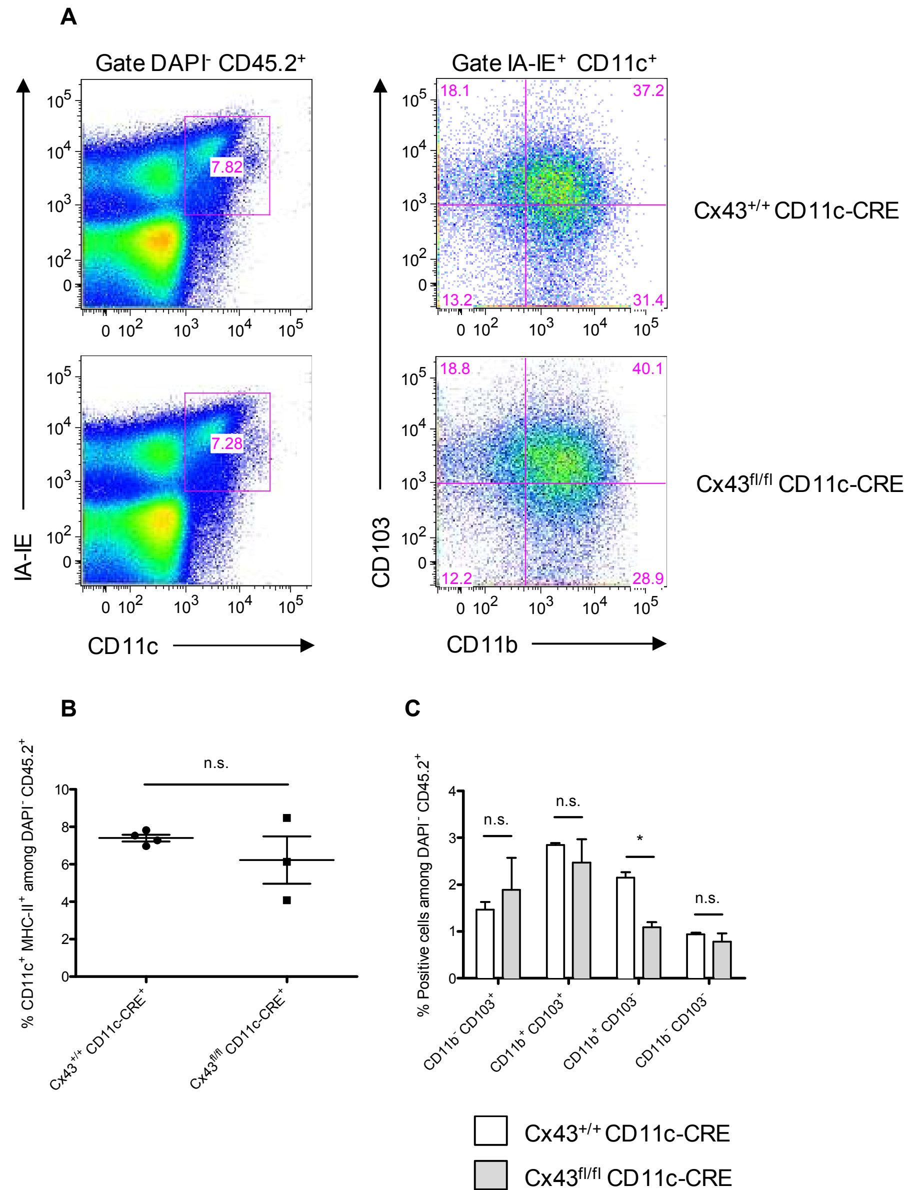


Figure 8-22 Cx43$^{fl/fl}$ CD11c-CRE mice do not display differences in MLN DC subsets. **A**) Cells obtained from the digestion of mesenteric lymph nodes were stained in order to evaluate their expression of CD45.2, CD11c, MHC-II, CD103 and CD11b. Dead and stromal cells were excluded, gating on DAPI$^-$ CD45.2$^+$ cells. Among MHC-II$^+$ CD11c$^+$ cells, four subsets are distinguished based on their expression of CD103 and CD11b: CD11b$^-$ CD103$^+$, CD11b$^+$ CD103$^+$, CD11b$^+$ CD103$^-$ and CD11b$^-$ CD103$^-$. **B**) Percentage of MHC-II$^+$ CD11c$^+$ among live CD45.2$^+$ cells is shown. **C**) Percentage of CD11b$^-$ CD103$^+$, CD11b$^+$ CD103$^+$, CD11b$^+$ CD103$^-$ and CD11b$^-$ CD103$^-$ cells is shown as Mean ± SEM for each subsets. Data are representative of two independent experiments, 3-4 mice/group. * $p < 0.05$, n.s. not significant.

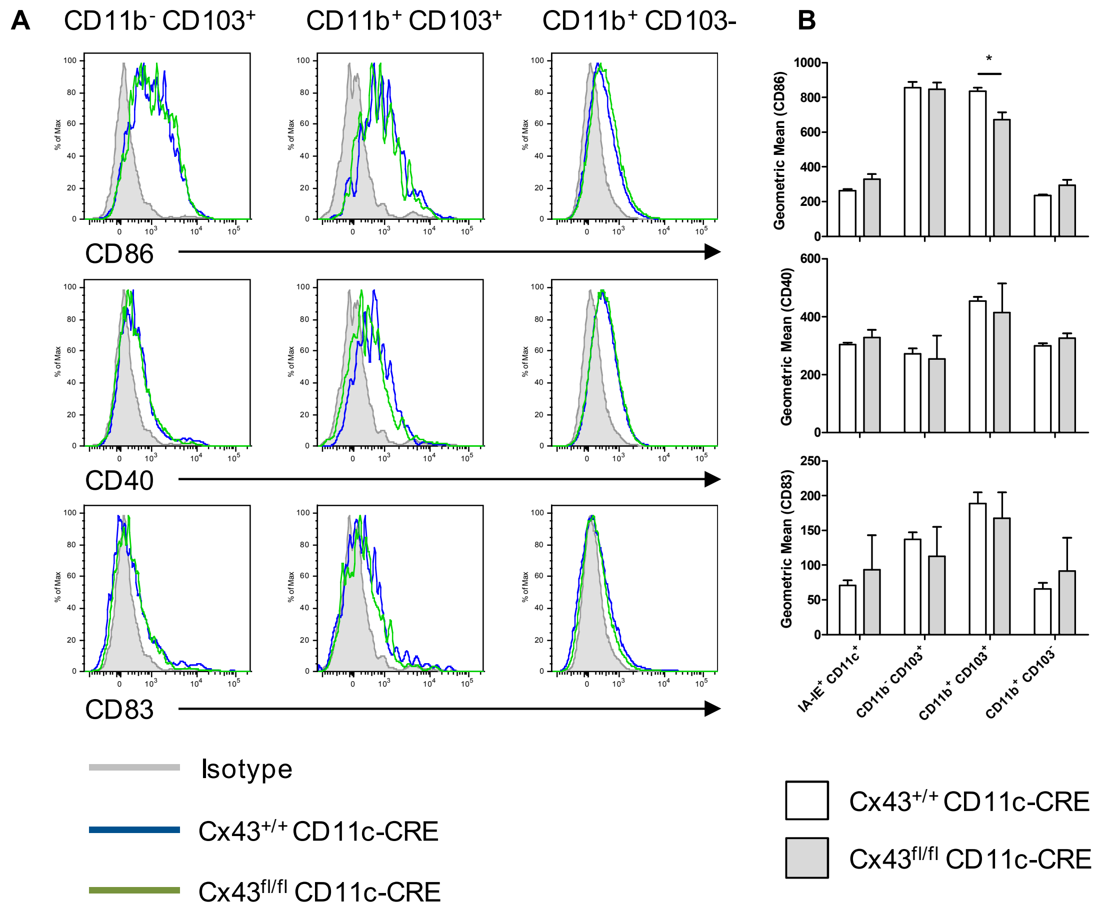


Figure 8-23 Expression of co-stimulatory molecules by LP subsets is not changed in Cx43$^{fl/fl}$ CD11c-CRE mice. **A**) Small intestine from Cx43$^{+/+}$ CD11-CRE and Cx43$^{fl/fl}$ CD11c-CRE were processed and stained with anti-CD45.2, CD11c, MHC-II, CD103 and CD11b. Besides, expression of CD86, CD40 and CD83 co-stimulatory markers were evaluated. Histograms of CD86, CD40 and CD83 expression for each subset are shown. The grey line represents the isotype control, the blue line stands for Cx43$^{+/+}$ CD11c-CRE, the green for Cx43$^{fl/fl}$ CD11c-CRE. **B**) Expression of CD86, CD40 and CD83 is reported as difference in the Geometric Mean between stained sample and isotype control for each subset. Data are representative of two independent experiments, 3-4 mice / group. * $p < 0.05$.

8.3.3 Lessening of T regulatory cells in the mesenteric lymph nodes is caused by lack of Cx43 in CD11c$^+$ cells

In order to gain a general view regarding T cell responses in Cx43$^{fl/fl}$ CD11c-CRE, we proceeded with the characterization of different T cell subsets at steady-state. We first analyzed T regulatory cells populations in the small intestine and mesenteric lymph nodes. Hence, cells were stained with anti-CD3, CD4 and CD8 to define CD4$^+$ T helper

and CD8$^+$ T cytotoxic T cells, respectively. CD25 and Foxp3 were used as markers for T regulatory cells.

As shown in Figure 8-24, in the small intestine of Cx43$^{fl/fl}$ CD11c-CRE, there were no differences in T regulatory cell frequency, both CD4$^+$ and CD8$^+$, compared to control mice. On the other hand, a significant decrease in CD25$^+$ Foxp3$^+$ T regulatory cells, both CD4$^+$ and CD8$^+$, was observed in the mesenteric lymph nodes (Figure 8-25).

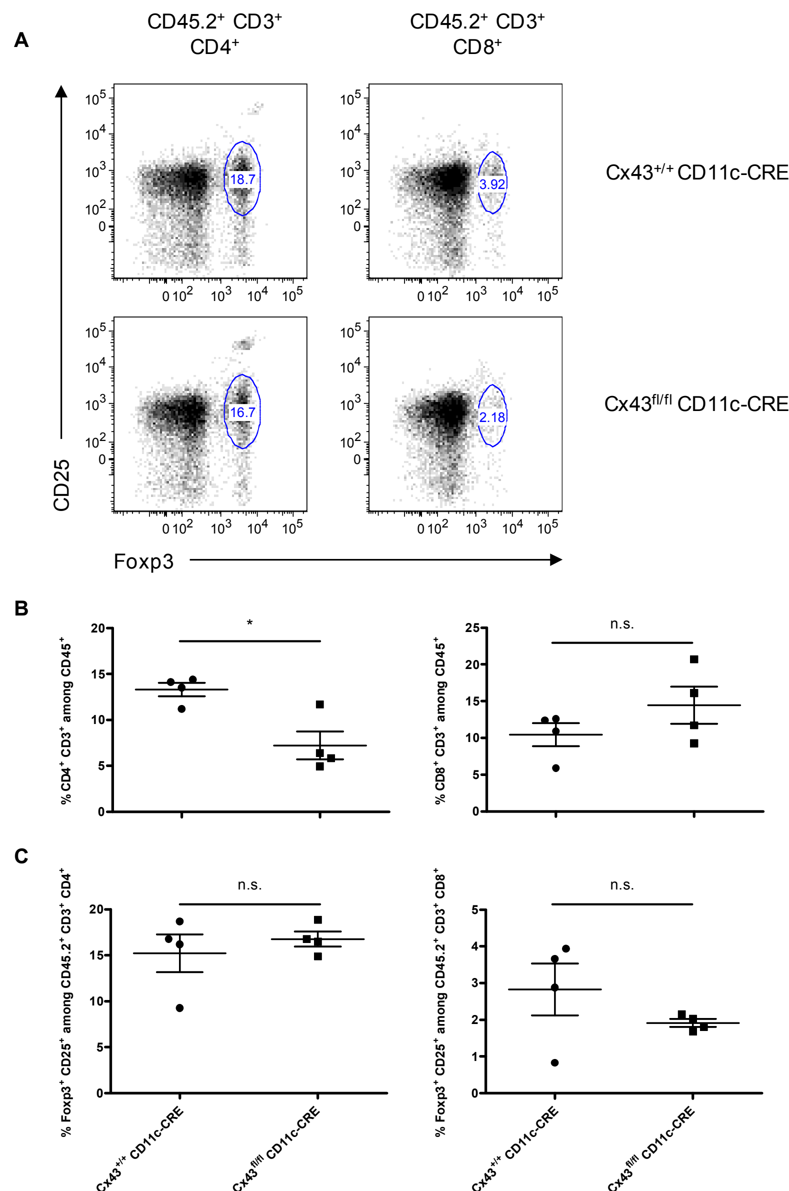


Figure 8-24 Lack of Cx43 in CD11c$^+$ cells does not alter peripheral T regulatory cell population in the small intestine. A) Mononuclear cells were isolated from the small intestine of Cx43$^{+/+}$ CD11c-CRE and Cx43$^{fl/fl}$ CD11c-CRE and stained with anti - CD45.2, CD3, CD4, CD8, Foxp3 and CD25 antibodies. CD45.2$^+$ CD3$^+$ T cells were gated and subsequently divided as CD4$^+$ and CD8$^+$ T cells. For each population, frequencies of Foxp3$^+$ CD25$^+$ Tregs are shown. **B**) Graphs

show the frequencies of CD4$^+$ (on the left) and CD8$^+$ T (on the right) cells among CD45.2$^+$ cells. C) Graphs represent the percentage of Foxp3$^+$ CD25$^+$ among CD4$^+$ (on the left) and CD8$^+$ (on the right) T cells. Data are representative of two independent experiments, 4 mice / group. * $p < 0.05$, n.s. not significant.

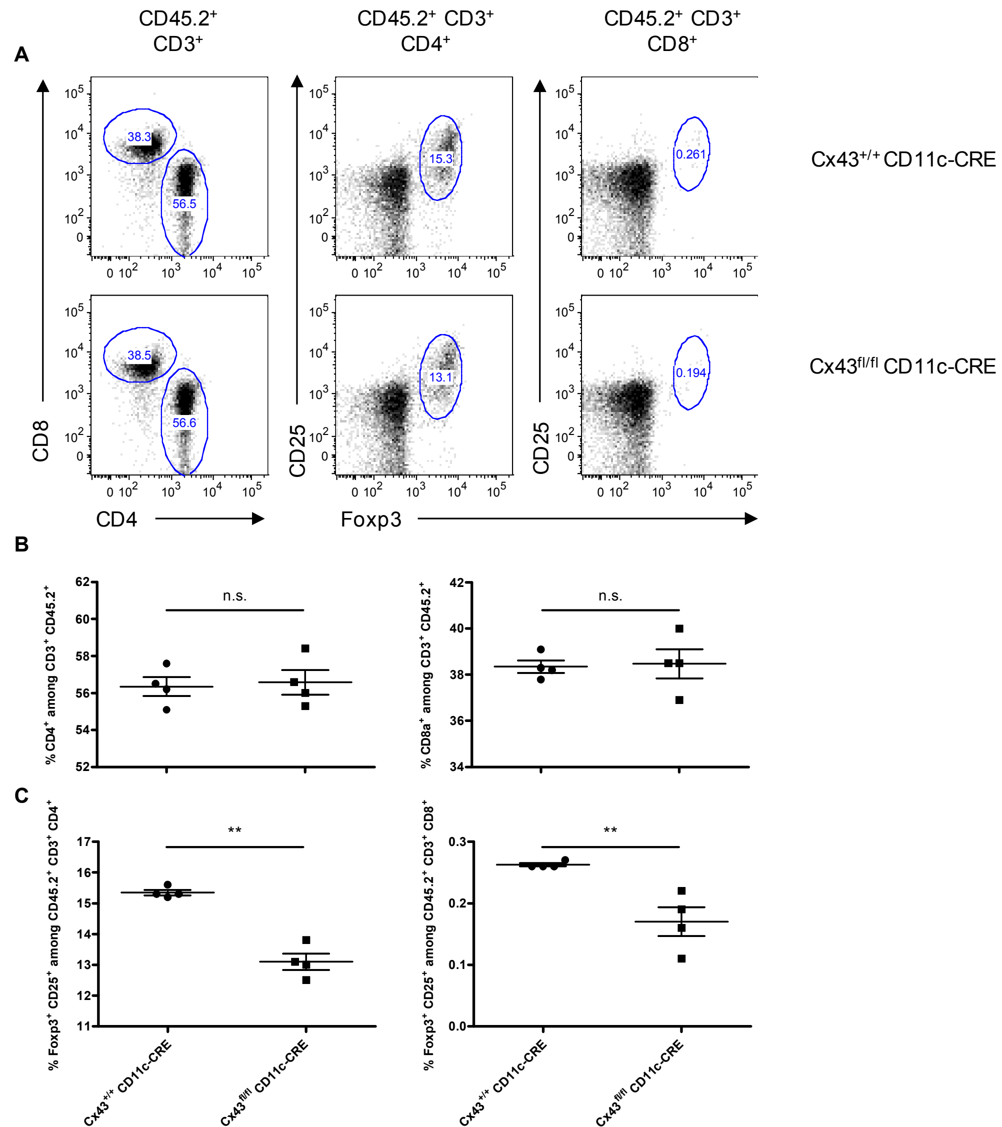


Figure 8-25 Lack of Cx43 in CD11c$^+$ cells is associated with decreased frequency in the Treg cell population in the mesenteric lymph nodes. **A**) Cells obtained from the digestion of MLN_s were stained with anti-CD45.2, CD3, CD4, CD8, Foxp3 and CD25 antibodies. CD3$^+$ cells were divided in CD4$^+$ and CD8$^+$ T cells and frequency of Foxp3$^+$ CD25$^+$ Tregs was evaluated for each population. **B**) The graph shows the percentage of CD4$^+$ (on the left) and CD8$^+$ T (on the right) cells among CD45.2$^+$ CD3$^+$ T cells. **C**) Frequencies of Foxp3$^+$ CD25$^+$ Tregs relative to CD4$^+$ CD3$^+$ (on the left) or CD8$^+$ CD3$^+$ T (on the right) cells are shown. Data are representative of two individual experiments, performed with 4 mice / group. ** $p < 0.01$; n.s. not significant.

Although really far to be conclusive, data suggest that a peripheral defect in T regulatory cells might be a consequence of Cx43 deletion in CD11c^{+} cells. What is the mechanism by which Cx43 deletion in dendritic cells affects Treg$_{s}$ balance, whether it regards the peripheral generation of iTregs and how it is related to antigen-specificity of T regulatory cell induction are general issues that still need to be addressed.

8.3.4 Tolerance to fed antigens is not established in Cx43$^{fl/fl}$ CD11c-CRE mice

Given the reduced level of CD4^{+} Treg cells in the MLN, we hypothesized that also tolerance induction may be impaired in Cx43$^{fl/fl}$ CD11c-CRE mice.

As a general read out for the establishment of tolerance to i.g. administered Ag$_{s}$, we performed a DTH protocol, in order to summarily evaluate the possible contribution of Cx43 deletion in CD11c^{+} cells in oral tolerance. Indeed, if antigens are transferred in the SI lamina propria to CD103^{+} DC$_{s}$, which are the key players of the tolerogenic response generated in the MLN, and Cx43 is involved in that exchange, the net result would be defective oral tolerance in Cx43$^{fl/fl}$ CD11c-CRE mice.

Thus, mice were administered three times with 1 mg ovalbumin every second day and immunized s.c. with ovalbumin mixed to Complete Freund's Adjuvant after 7 days, according to the schedule depicted in Fifure 8-9. Mice were then challenged in the footpad with heat-aggregated ovalbumin or DPBS as a control and footpad swelling was measured as a read out for DTH after 24 and 48 hours.

As expected, decrease in the footpad swelling of control mice (Cx43$^{+/+}$ CD11c-CRE) was reported after ovalbumin feeding, compared to DPBS-fed mice. On the other hand, Cx43$^{fl/fl}$ CD11c-CRE mice showed impairment in oral tolerance establishment, as no significant difference was observed between ovalbumin- and vehicle-fed mice.

These results indicate that, when Cx43 is deleted from CD11c+ cells, oral tolerance is not correctly generated. We still do not know at which level Cx43 deletion acts, which are the DC cell subsets involved, whether it is the result of defective Ag exchange in the SI or

whether it implies general defects in T cell priming and peripheral T regulatory cell induction.

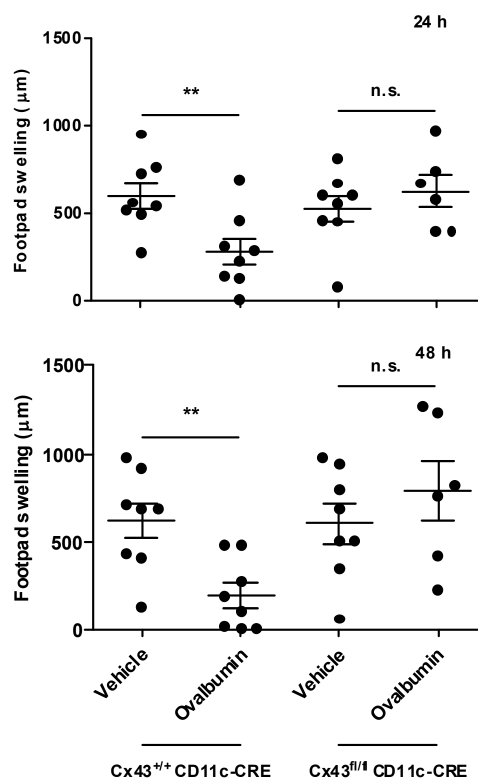


Figure 8-26 Induction of oral tolerance to ovalbumin is impaired in Cx43$^{fl/fl}$ CD11c-CRE mice. DTH response was evaluated in Cx43$^{+/+}$ and Cx43$^{fl/fl}$ CD11-CRE mice. Mice were fed with 1 mg ovalbumin for three times every second day and immunized s.c. with CFA+OVA after 7 days. Seven days later, mice were challenged with heat-aggregated ovalbumin in the footpad and footpad swelling was measured after 24 and 48 hours. Difference in the thickness of OVA-challenged and DPBS-injected control footpad for each mouse is reported. Results are displayed as Mean ± SEM and are representative of one experiment, 6 - 8 mice / group. ** $p < 0.01$; n.s. not significant.

9 Discussion

9.1 Role of $CX_3CR_1^+$ cells in antigen uptake and oral tolerance establishment

The generation of oral tolerance to fed antigens relies on antigen presentation to T cells by APC_s in the gut-draining lymph nodes. This implies the need for antigens to be taken up at the site of administration (the small intestine) and delivered to the mesenteric LN_s [95].

Using ovalbumin as a model antigen, we demonstrated that its uptake is limited to $CX_3CR_1^+$ macrophages in the intestinal lamina propria. Thus, as a result of their close proximity to the intestinal lumen and due to their ability to extend their dendrites outside the epithelium [97, 124, 126], $CX_3CR_1^+$ cells represent the first line by which the immune system deals with the luminal content. This seems to be the case also during infections, as $CD11c^+$ $CX_3CR_1^+$ cells have been shown to hold back *S. Typhimurium* burden especially in its early phase [127, 168] and, eventually, to migrate intra-luminally and capture the bacteria [128].

Differently from what previously shown [97], in our hands antigen uptake by $CX_3CR_1^+$ cells (both $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ Mϕ) seems partially dependent on their expression of functional fractalkine receptor and cell protrusion formation [125]. In fact, a decrease in the percentage of OVA^+ $CX_3CR_1^+$ cells is observed in CX_3CR_1-deficient mice.

In our experiments, we used a sub-optimal dose of fluorescently-labeled ovalbumin administered *via* the oral route, which may favor Ag uptake by these cells *via* dendrite formation rather than EC paracellular or transcellular leakage.

As a result of defective antigen uptake, we showed that CX_3CR_1-deficient mice do not develop oral tolerance to fed antigens, in agreement to what has been previously published by *Hadis et al.*. Indeed, it was demonstrated that in CX_3CR_1-deficient mice, T regulatory cells generated in the mesenteric lymph nodes are able to home to the intestinal lamina propria but fail to undergo secondary expansion, as a consequence of

diminished IL-10 production by $CX_3CR_1^+$ cells. We suggest that, in addition to this mechanism, at low antigen administration, there is an impairment of T regulatory cell development as a consequence of reduced uptake of ovalbumin in the absence of CX_3CR_1.

In our system, we still lack formal proof that decreased antigen uptake by $CX_3CR_1^+$ macrophages is linked to impaired peripheral T regulatory cell induction. It would be interesting to define whether primary generation of T regulatory cells in the GALT or $Treg_s$ Ag-specific secondary expansion in the small intestine is affected by decreased antigen uptake by $CX_3CR_1^+$ cells. This issue will be addressed using an Ag-specific T cell transfer model in CX_3CR_1-sufficient and -deficient mice fed with low ovalbumin dose.

9.2 Role of epithelial cells in antigen uptake

Our data do not exclude a role for epithelial cells in antigen handling. The intestinal epithelium has been shown, indeed, to mediate antigen transport by paracellular or transcellular pathways [169]. Additionally, intestinal epithelial cells can release exosome-like vescicles, called “tolerosomes”, which have been isolated on the supernatant of Ag-treated IEC lines or from the serum of Ag-fed animals [170, 171].

Besides, it has been recently demonstrated that goblet cells are able to deliver antigens directly to $CD103^+$ DC_s in the intestinal lamina propria through the formation of GAP_s (Goblet cell-associated Antigen Passages), but the mechanism seems to be restricted to dextran and low-molecular weight Ag_s [119].

In our experiments, OVA^+ EC_s were detected by FACS, but not by immunofluorescence, suggesting alternative ways in which the antigens are intracellularly processed by EC_s and $CX_3CR_1^+$ macrophages. The latter, indeed, display cytosolic aggregates that may favor their visualization by immunofluorescence. On the other hand, EC_s might rapidly degrade and crumble the antigens, allowing their detection by FACS but not by immunofluorescence.

Moreover, EC_s which have taken up fluorescently-labeled ovalbumin outnumber OVA^+ $CX_3CR_1^+$ cells in the jejunum and ileum, but not in the duodenum. This is in line with previous observations made by *Chieppa et al*. [126], who pointed out that most of the protrusions by DC_s at steady-state are localized in the first tract of the small intestine. Hence, in this region antigen uptake by $CX_3CR_1^+$ cells may be preferentially dependent on cell protrusion formation. This would explain why only in the duodenum antigen uptake by $CX_3CR_1^+$ cells counterbalances that of epithelial cells.

9.3 Antigen presentation and transfer by mononuclear cells in the lamina propria

While antigen uptake was a characteristic associated primarily to $CX_3CR_1^+$ cells, we unexpectedly found that when ovalbumin was administered at high dose (20 mg), all the subsets of mononuclear cells in the small intestine, namely $CD11b^-$ $CD103^+$ DC_s, $CD11b^+$ $CD103^+$ DC_s, $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ macrophages presented OVA peptide on MHC-I at different degrees, suggesting a spread diffusion of antigenic peptides in the APC subsets.

On the other hand, also at low antigen dose, $CX_3CR_1^{high}$ macrophages and $CD11b^+$ $CD103^+$ DC_s were preferentially positive for K^bOVA. Thus, based on functional peptide presentation on MHC-I, the antigen dose influences ovalbumin presentation by lamina propria mononuclear cells but still indicates that CD103+ cells are capable of retrieving the antigen in the form of peptides.

This observation made us consider possible ways on how $CD11b^+$ $CD103^+$ DC_s acquire antigens, as they were not taking up ovalbumin by themselves.

Given this functional difference between $CX_3CR_1^+$ $M\phi$ and $CD103^+$ DC_s in antigen handling in the GALT, our hypothesis is that the former, which efficiently take up and process antigens, are then capable of transferring antigens in the form of peptides to $CD103^+$ DC_s that - due to their inner localization in the villi - might not be able to directly phagocytose ovalbumin.

Ag presentation by $CD103^+$ DC_s appear to be fundamental for $Treg_s$ induction in the GALT, as they were initially described to be the only DC subset able to migrate to the MLN_s [97] in a CCR7- [96, 100] and $S1P_1$-$S1P_3$-dependent manner [172] and efficiently prime naïve $CD4^+$ and $CD8^+$ T cells [100, 101]. One might now argue that because also a subset of $CD11b^+$ $CD103^-$ $CX_3CR_1^{int}$ cells has been reported to display classical DC features, as it is migratory and efficiently prime naïve T cells, may be involved in Treg induction. However, differently from $CD103^+$ DC_s that drive primarily Tregs, $CD11b^+$ $CD103^-$ $CX_3CR_1^{int}$ cells seem to polarize T cells toward Th1/Th17 rather than to a T regulatory phenotype [113].

How the transfer of peptides between LP mononuclear cells occurs is not known; however, it is really frequent for immune cells to exchange cytosol or membrane components in order to communicate between each other. For instance, human dendritic cells can form filopodia-like structures, called tunnelling microtubules (TNT_s) [173], which allow to ions and surface proteins to be transferred among cells.

Besides, mouse dendritic cells have been shown to release exosomes, which are originated from the late endosomal compartments fusing with the plasma membrane [174]. Released exosomes can reach distant sites and are relevant for their immunomodulatory properties [175].

Furthermore, adjacent cells can transfer ions, metabolites and small peptides through gap junctions, which are specialized intercellular connections constituted by hemi-channels of connexins directly linking the cytoplasm of neighboring cells. GJ_s have been shown to favor intercellular peptide coupling and transfer to professional APC for cross-presentation [163].

Because we found that $CD103^+$ cells present the OVA peptide but do not seem to have the whole protein, it is likely that antigenic transfer occurs in the form of peptides thus favoring the possibility that antigen exchange may occur through gap junctions established between $CX_3CR_1^+$ LPC_s and $CD103^+$ DC_s.

Constrains regarding the size of exchanged peptides affects GJ-mediated transfer (9 amino acids in length and 1 KDa as molecular weight), allowing them to be presented in complex with MHC-I [163]. Nevertheless, variation in the conformational structure of peptides from globular to linear may allow the transfer of longer peptides [176], but whether they can be presented in complex with MHC-II has not been addressed yet.

We observed that SI mononuclear cells can exchange calcein (a GJ diffusible dye) as well as ovalbumin peptides and express a peculiar panel of Cx_s. Among them, Cx43 is mostly expressed both by $CX_3CR_1^+$ Mϕ (both $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$) and $CD11b^+$ $CD103^+$ DC_s.

In order to understand the specific contribution of Cx43 in GJ formation between the mononuclear subsets in the LP and its possible involvement in antigen handling in the small intestine, we have generated conditional Cx43 knock-out mice in CD11c-expressing cells, crossing CD11c-CRE (B6.Cg-Tg(Itgax-cre)1-1Reiz/J) with $Cx43^{fl/fl}$ mice (B6.129S7-$Gja1^{tm1Dlg}$/J).

We are aware that in the gut $CX_3CR_1^+$ Mϕ are expressing variable levels of CD11c, which may hamper the efficiency of Cx43 deletion in this subset. Particularly in the colon, subsets of $CX_3CR_1^{high}$ $CD11c^-$ macrophages have been described [117]. However, from our cell preparations from the small intestine, we observe that both $CX_3CR_1^{int}$ and $CX_3CR_1^{high}$ cells express intermediate to high levels of CD11c.

The initial characterization of $Cx43^{fl/fl}$ CD11c-CRE mice has shown no defects both in the frequency and activation state of mononuclear cell subsets in the SI and MLN compared to control mice. However, we found lessened T regulatory cells, both $CD4^+$ and $CD8^+$. The decrease in T regulatory cells is accompanied by hampered ability of these mice to develop oral tolerance.

Several scenarios may explain this observation: 1. Cx43 may be involved in the transfer of antigenic peptides from $CX_3CR_1^+$ to $CD103^+$ cells and hence in the absence of antigen transfer DC_s are unable to prime Treg cells; 2. Cx43 may be involved in T cell priming and hence when DC_s lack Cx43 also priming is affected; 3. Gap junctions have been involved in cyclic AMP release from DC_s to T cells for Treg induction [177] and hence in the

absence of Cx43 T regs cannot be induced. We also need to determine whether Cx43 deletion in $CD11c^+$ cells is affecting only MHC-I presentation by $CD103^+$ DC_s or also MHC-II presentation.

In conclusion, we have characterized two different aspects of Ag uptake in the SI lamina propria, namely ovalbumin uptake by $CX_3CR_1^+$ macrophages and antigenic material transfer to $CD103^+$ DC_s Our results suggest that the preliminary phases of antigen handling in the lamina propria are fundamental for the correct establishment of tolerance to food antigens. Further experiments are required to fully understand the role of Cx43 in either the transfer of antigenic peptides or the activation of T regulatory cells *in vivo*.

10 Bibliography

1. Mellor, A.L. and D.H. Munn, *Creating immune privilege: active local suppression that benefits friends, but protects foes.* Nat Rev Immunol, 2008. **8**(1): p. 74-80.
2. Tanaka, K. and H. Ishikawa, *Role of intestinal bacterial flora in oral tolerance induction.* Histol Histopathol, 2004. **19**(3): p. 907-14.
3. Rescigno, M., *Dendritic cells in oral tolerance in the gut.* Cell Microbiol, 2011. **13**(9): p. 1312-8.
4. Pabst, O. and A.M. Mowat, *Oral tolerance to food protein.* Mucosal Immunol, 2012. **5**(3): p. 232-9.
5. Hooper, L.V. and A.J. Macpherson, *Immune adaptations that maintain homeostasis with the intestinal microbiota.* Nat Rev Immunol, 2010. **10**(3): p. 159-69.
6. Kaser, A., S. Zeissig, and R.S. Blumberg, *Inflammatory bowel disease.* Annu Rev Immunol, 2010. **28**: p. 573-621.
7. Baron, S., et al., *Medical Microbiology*1996, Galveston: The University of Texas Medical Branch at Galveston.
8. Hooper, L.V. and J.I. Gordon, *Commensal host-bacterial relationships in the gut.* Science, 2001. **292**(5519): p. 1115-8.
9. Burkholder, P.R. and I. McVeigh, *Synthesis of Vitamins by Intestinal Bacteria.* Proc Natl Acad Sci U S A, 1942. **28**(7): p. 285-9.
10. Ivanov, II, et al., *Induction of intestinal Th17 cells by segmented filamentous bacteria.* Cell, 2009. **139**(3): p. 485-98.
11. Gaboriau-Routhiau, V., et al., *The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses.* Immunity, 2009. **31**(4): p. 677-89.
12. Atarashi, K., et al., *Induction of colonic regulatory T cells by indigenous Clostridium species.* Science, 2011. **331**(6015): p. 337-41.

13. Round, J.L. and S.K. Mazmanian, *Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota.* Proc Natl Acad Sci U S A, 2010. **107**(27): p. 12204-9.

14. Rescigno, M., *Gut commensal flora: tolerance and homeostasis.* F1000 Biol Rep, 2009. **1**: p. 9.

15. Iliev, I.D., G. Matteoli, and M. Rescigno, *The yin and yang of intestinal epithelial cells in controlling dendritic cell function.* J Exp Med, 2007. **204**(10): p. 2253-7.

16. Lavelle, E.C., et al., *The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis.* Mucosal Immunol, 2010. **3**(1): p. 17-28.

17. Medzhitov, R., *Toll-like receptors and innate immunity.* Nat Rev Immunol, 2001. **1**(2): p. 135-45.

18. Gewirtz, A.T., et al., *Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression.* J Immunol, 2001. **167**(4): p. 1882-5.

19. Rhee, S.H., et al., *Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation.* Proc Natl Acad Sci U S A, 2005. **102**(38): p. 13610-5.

20. Lee, J., et al., *Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells.* Nat Cell Biol, 2006. **8**(12): p. 1327-36.

21. Cario, E., G. Gerken, and D.K. Podolsky, *Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function.* Gastroenterology, 2007. **132**(4): p. 1359-74.

22. Fukata, M., et al., *Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis.* Am J Physiol Gastrointest Liver Physiol, 2005. **288**(5): p. G1055-65.

23. Rakoff-Nahoum, S., et al., *Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis.* Cell, 2004. **118**(2): p. 229-41.

24. Vijay-Kumar, M., et al., *Deletion of TLR5 results in spontaneous colitis in mice.* J Clin Invest, 2007. **117**(12): p. 3909-21.

25. Nenci, A., et al., *Epithelial NEMO links innate immunity to chronic intestinal inflammation.* Nature, 2007. **446**(7135): p. 557-61.
26. Ivanov, II and D.R. Littman, *Modulation of immune homeostasis by commensal bacteria.* Curr Opin Microbiol, 2011. **14**(1): p. 106-14.
27. Lotz, M., et al., *Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells.* J Exp Med, 2006. **203**(4): p. 973-84.
28. Chassin, C., et al., *miR-146a mediates protective innate immune tolerance in the neonate intestine.* Cell Host Microbe, 2010. **8**(4): p. 358-68.
29. Neish, A.S., et al., *Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination.* Science, 2000. **289**(5484): p. 1560-3.
30. Tien, M.T., et al., *Anti-inflammatory effect of Lactobacillus casei on Shigella-infected human intestinal epithelial cells.* J Immunol, 2006. **176**(2): p. 1228-37.
31. Kumar, A., et al., *Commensal bacteria modulate cullin-dependent signaling via generation of reactive oxygen species.* EMBO J, 2007. **26**(21): p. 4457-66.
32. Kelly, D., et al., *Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA.* Nat Immunol, 2004. **5**(1): p. 104-12.
33. Otte, J.M., E. Cario, and D.K. Podolsky, *Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells.* Gastroenterology, 2004. **126**(4): p. 1054-70.
34. Rimoldi, M., et al., *Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells.* Nat Immunol, 2005. **6**(5): p. 507-14.
35. Iliev, I.D., et al., *Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells.* Gut, 2009. **58**(11): p. 1481-9.
36. He, B., et al., *Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL.* Immunity, 2007. **26**(6): p. 812-26.
37. Linden, S.K., T.H. Florin, and M.A. McGuckin, *Mucin dynamics in intestinal bacterial infection.* PLoS One, 2008. **3**(12): p. e3952.

38. Johansson, M.E., et al., *The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria.* Proc Natl Acad Sci U S A, 2008. **105**(39): p. 15064-9.

39. McAuley, J.L., et al., *MUC1 cell surface mucin is a critical element of the mucosal barrier to infection.* J Clin Invest, 2007. **117**(8): p. 2313-24.

40. McGuckin, M.A., et al., *Muc1 mucin limits both Helicobacter pylori colonization of the murine gastric mucosa and associated gastritis.* Gastroenterology, 2007. **133**(4): p. 1210-8.

41. Van der Sluis, M., et al., *Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection.* Gastroenterology, 2006. **131**(1): p. 117-29.

42. Bergstrom, K.S., et al., *Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa.* PLoS Pathog, 2010. **6**(5): p. e1000902.

43. Linden, S.K., et al., *MUC1 limits Helicobacter pylori infection both by steric hindrance and by acting as a releasable decoy.* PLoS Pathog, 2009. **5**(10): p. e1000617.

44. Ayabe, T., et al., *Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria.* Nat Immunol, 2000. **1**(2): p. 113-8.

45. Vaishnava, S., et al., *Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface.* Proc Natl Acad Sci U S A, 2008. **105**(52): p. 20858-63.

46. Bevins, C.L. and N.H. Salzman, *Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis.* Nat Rev Microbiol, 2011. **9**(5): p. 356-68.

47. Yang, D., et al., *Mammalian defensins in immunity: more than just microbicidal.* Trends Immunol, 2002. **23**(6): p. 291-6.

48. Wilson, C.L., et al., *Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense.* Science, 1999. **286**(5437): p. 113-7.

49. Biswas, A., et al., *Induction and rescue of Nod2-dependent Th1-driven granulomatous inflammation of the ileum.* Proc Natl Acad Sci U S A, 2010. **107**(33): p. 14739-44.

50. Koslowski, M.J., et al., *Innate antimicrobial host defense in small intestinal Crohn's disease.* Int J Med Microbiol, 2010. **300**(1): p. 34-40.

51. Macpherson, A.J. and E. Slack, *The functional interactions of commensal bacteria with intestinal secretory IgA.* Curr Opin Gastroenterol, 2007. **23**(6): p. 673-8.

52. Macpherson, A.J., M.B. Geuking, and K.D. McCoy, *Homeland security: IgA immunity at the frontiers of the body.* Trends Immunol, 2012. **33**(4): p. 160-7.

53. Bergqvist, P., et al., *Gut IgA class switch recombination in the absence of CD40 does not occur in the lamina propria and is independent of germinal centers.* J Immunol, 2006. **177**(11): p. 7772-83.

54. Fagarasan, S., et al., *In situ class switching and differentiation to IgA-producing cells in the gut lamina propria.* Nature, 2001. **413**(6856): p. 639-43.

55. Macpherson, A.J. and T. Uhr, *Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria.* Science, 2004. **303**(5664): p. 1662-5.

56. Macpherson, A.J., et al., *A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria.* Science, 2000. **288**(5474): p. 2222-6.

57. Mora, J.R., et al., *Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells.* Science, 2006. **314**(5802): p. 1157-60.

58. Peterson, D.A., et al., *IgA response to symbiotic bacteria as a mediator of gut homeostasis.* Cell Host Microbe, 2007. **2**(5): p. 328-39.

59. Fagarasan, S., et al., *Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora.* Science, 2002. **298**(5597): p. 1424-7.

60. Suzuki, K., et al., *Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut.* Proc Natl Acad Sci U S A, 2004. **101**(7): p. 1981-6.

61. Wei, M., et al., *Mice carrying a knock-in mutation of Aicda resulting in a defect in somatic hypermutation have impaired gut homeostasis and compromised mucosal defense.* Nat Immunol, 2011. **12**(3): p. 264-70.
62. Lindner, C., et al., *Age, microbiota, and T cells shape diverse individual IgA repertoires in the intestine.* J Exp Med, 2012. **209**(2): p. 365-77.
63. Hogquist, K.A., T.A. Baldwin, and S.C. Jameson, *Central tolerance: learning self-control in the thymus.* Nat Rev Immunol, 2005. **5**(10): p. 772-82.
64. Mueller, D.L., *Mechanisms maintaining peripheral tolerance.* Nat Immunol, 2010. **11**(1): p. 21-7.
65. Melamed, D. and A. Friedman, *Direct evidence for anergy in T lymphocytes tolerized by oral administration of ovalbumin.* Eur J Immunol, 1993. **23**(4): p. 935-42.
66. Chen, Y., et al., *Peripheral deletion of antigen-reactive T cells in oral tolerance.* Nature, 1995. **376**(6536): p. 177-80.
67. Gutgemann, I., et al., *Induction of rapid T cell activation and tolerance by systemic presentation of an orally administered antigen.* Immunity, 1998. **8**(6): p. 667-73.
68. Chen, Y., et al., *Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis.* Science, 1994. **265**(5176): p. 1237-40.
69. Fujihashi, K., et al., *Peyer's patches are required for oral tolerance to proteins.* Proc Natl Acad Sci U S A, 2001. **98**(6): p. 3310-5.
70. Enders, G., T. Gottwald, and W. Brendel, *Induction of oral tolerance in rats without Peyer's patches.* Immunology, 1986. **58**(2): p. 311-4.
71. Hashimoto, A., et al., *Successful priming and tolerization of T cells to orally administered antigens in B-cell-deficient mice.* Cell Immunol, 2001. **207**(1): p. 36-40.
72. Peng, H.J., et al., *B-cell depletion fails to abrogate the induction of oral tolerance of specific Th1 immune responses in mice.* Scand J Immunol, 2000. **51**(5): p. 454-60.

73. Gonnella, P.A., H.P. Waldner, and H.L. Weiner, *B cell-deficient (mu MT) mice have alterations in the cytokine microenvironment of the gut-associated lymphoid tissue (GALT) and a defect in the low dose mechanism of oral tolerance.* J Immunol, 2001. **166**(7): p. 4456-64.

74. Yang, R., et al., *Intestinal venous drainage through the liver is a prerequisite for oral tolerance induction.* J Pediatr Surg, 1994. **29**(8): p. 1145-8.

75. Goubier, A., et al., *Plasmacytoid dendritic cells mediate oral tolerance.* Immunity, 2008. **29**(3): p. 464-75.

76. Janeway, C.A., et al., *Immunobiology*2001, New York and London: Garland Science.

77. Banchereau, J., et al., *Immunobiology of dendritic cells.* Annu Rev Immunol, 2000. **18**: p. 767-811.

78. Shortman, K. and S.H. Naik, *Steady-state and inflammatory dendritic-cell development.* Nat Rev Immunol, 2007. **7**(1): p. 19-30.

79. Geissmann, F., et al., *Development of monocytes, macrophages, and dendritic cells.* Science, 2010. **327**(5966): p. 656-61.

80. Liu, K., et al., *In vivo analysis of dendritic cell development and homeostasis.* Science, 2009. **324**(5925): p. 392-7.

81. den Haan, J.M., S.M. Lehar, and M.J. Bevan, *CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo.* J Exp Med, 2000. **192**(12): p. 1685-96.

82. Mowat, A.M., *Anatomical basis of tolerance and immunity to intestinal antigens.* Nat Rev Immunol, 2003. **3**(4): p. 331-41.

83. Iwasaki, A., *Mucosal dendritic cells.* Annu Rev Immunol, 2007. **25**: p. 381-418.

84. Kelsall, B.L. and M. Rescigno, *Mucosal dendritic cells in immunity and inflammation.* Nat Immunol, 2004. **5**(11): p. 1091-5.

85. Niess, J.H. and H.C. Reinecker, *Dendritic cells in the recognition of intestinal microbiota.* Cell Microbiol, 2006. **8**(4): p. 558-64.

86. Coombes, J.L. and K.J. Maloy, *Control of intestinal homeostasis by regulatory T cells and dendritic cells.* Semin Immunol, 2007. **19**(2): p. 116-26.

87. Bilsborough, J. and J.L. Viney, *Gastrointestinal dendritic cells play a role in immunity, tolerance, and disease.* Gastroenterology, 2004. **127**(1): p. 300-9.

88. Iwasaki, A. and B.L. Kelsall, *Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine.* J Exp Med, 2000. **191**(8): p. 1381-94.

89. Salazar-Gonzalez, R.M., et al., *CCR6-mediated dendritic cell activation of pathogen-specific T cells in Peyer's patches.* Immunity, 2006. **24**(5): p. 623-32.

90. Varol, C., et al., *Intestinal lamina propria dendritic cell subsets have different origin and functions.* Immunity, 2009. **31**(3): p. 502-12.

91. Bogunovic, M., et al., *Origin of the lamina propria dendritic cell network.* Immunity, 2009. **31**(3): p. 513-25.

92. Chirdo, F.G., et al., *Immunomodulatory dendritic cells in intestinal lamina propria.* Eur J Immunol, 2005. **35**(6): p. 1831-40.

93. Rigby, R.J., et al., *Production of interleukin (IL)-10 and IL-12 by murine colonic dendritic cells in response to microbial stimuli.* Clin Exp Immunol, 2005. **139**(2): p. 245-56.

94. Takenaka, S., et al., *Dendritic cells derived from murine colonic mucosa have unique functional and phenotypic characteristics.* J Immunol, 2007. **178**(12): p. 7984-93.

95. Worbs, T., et al., *Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells.* J Exp Med, 2006. **203**(3): p. 519-27.

96. Jang, M.H., et al., *CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes.* J Immunol, 2006. **176**(2): p. 803-10.

97. Schulz, O., et al., *Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions.* J Exp Med, 2009. **206**(13): p. 3101-14.

98. Dogan, A., Z.D. Wang, and J. Spencer, *E-cadherin expression in intestinal epithelium.* J Clin Pathol, 1995. **48**(2): p. 143-6.

99. Scott, C.L., A.M. Aumeunier, and A.M. Mowat, *Intestinal CD103+ dendritic cells: master regulators of tolerance?* Trends Immunol, 2011. **32**(9): p. 412-9.

100. Johansson-Lindbom, B., et al., *Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing.* J Exp Med, 2005. **202**(8): p. 1063-73.

101. Jaensson, E., et al., *Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans.* J Exp Med, 2008. **205**(9): p. 2139-49.

102. Annacker, O., et al., *Essential role for CD103 in the T cell-mediated regulation of experimental colitis.* J Exp Med, 2005. **202**(8): p. 1051-61.

103. Sun, C.M., et al., *Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid.* J Exp Med, 2007. **204**(8): p. 1775-85.

104. Coombes, J.L., et al., *A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism.* J Exp Med, 2007. **204**(8): p. 1757-64.

105. Iliev, I.D., et al., *Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning.* Mucosal Immunol, 2009. **2**(4): p. 340-50.

106. Paidassi, H., et al., *Preferential expression of integrin alphavbeta8 promotes generation of regulatory T cells by mouse CD103+ dendritic cells.* Gastroenterology, 2011. **141**(5): p. 1813-20.

107. Worthington, J.J., et al., *Intestinal dendritic cells specialize to activate transforming growth factor-beta and induce Foxp3+ regulatory T cells via integrin alphavbeta8.* Gastroenterology, 2011. **141**(5): p. 1802-12.

108. Hammerschmidt, S.I., et al., *Stromal mesenteric lymph node cells are essential for the generation of gut-homing T cells in vivo.* J Exp Med, 2008. **205**(11): p. 2483-90.

109. Molenaar, R., et al., *Lymph node stromal cells support dendritic cell-induced gut-homing of T cells.* J Immunol, 2009. **183**(10): p. 6395-402.

110. Jaensson-Gyllenback, E., et al., *Bile retinoids imprint intestinal CD103+ dendritic cells with the ability to generate gut-tropic T cells.* Mucosal Immunol, 2011. **4**(4): p. 438-47.

111. Matteoli, G., et al., *Gut CD103+ dendritic cells express indoleamine 2,3-dioxygenase which influences T regulatory/T effector cell balance and oral tolerance induction.* Gut, 2010. **59**(5): p. 595-604.

112. Fujimoto, K., et al., *A new subset of CD103+CD8alpha+ dendritic cells in the small intestine expresses TLR3, TLR7, and TLR9 and induces Th1 response and CTL activity.* J Immunol, 2011. **186**(11): p. 6287-95.

113. Cerovic, V., et al., *Intestinal CD103(-) dendritic cells migrate in lymph and prime effector T cells.* Mucosal Immunol, 2012.

114. Ginhoux, F., et al., *The origin and development of nonlymphoid tissue CD103+ DCs.* J Exp Med, 2009. **206**(13): p. 3115-30.

115. Crozat, K., et al., *Cutting edge: expression of XCR1 defines mouse lymphoid-tissue resident and migratory dendritic cells of the CD8alpha+ type.* J Immunol, 2011. **187**(9): p. 4411-5.

116. Bachem, A., et al., *Expression of XCR1 Characterizes the Batf3-Dependent Lineage of Dendritic Cells Capable of Antigen Cross-Presentation.* Front Immunol, 2012. **3**: p. 214.

117. Rivollier, A., et al., *Inflammation switches the differentiation program of Ly6Chi monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon.* J Exp Med, 2012. **209**(1): p. 139-55.

118. Uematsu, S., et al., *Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5.* Nat Immunol, 2008. **9**(7): p. 769-76.

119. McDole, J.R., et al., *Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine.* Nature, 2012. **483**(7389): p. 345-9.

120. Laffont, S., K.R. Siddiqui, and F. Powrie, *Intestinal inflammation abrogates the tolerogenic properties of MLN CD103+ dendritic cells.* Eur J Immunol, 2010. **40**(7): p. 1877-83.

121. Kinnebrew, M.A., et al., *Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense.* Immunity, 2012. **36**(2): p. 276-87.

122. Muehlhoefer, A., et al., *Fractalkine is an epithelial and endothelial cell-derived chemoattractant for intraepithelial lymphocytes in the small intestinal mucosa.* J Immunol, 2000. **164**(6): p. 3368-76.

123. Kim, K.W., et al., *In vivo structure/function and expression analysis of the CX3C chemokine fractalkine.* Blood, 2011. **118**(22): p. e156-67.

124. Rescigno, M., et al., *Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria.* Nat Immunol, 2001. **2**(4): p. 361-7.

125. Niess, J.H., et al., *CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance.* Science, 2005. **307**(5707): p. 254-8.

126. Chieppa, M., et al., *Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement.* J Exp Med, 2006. **203**(13): p. 2841-52.

127. Hapfelmeier, S., et al., *Microbe sampling by mucosal dendritic cells is a discrete, MyD88-independent step in DeltainvG S. Typhimurium colitis.* J Exp Med, 2008. **205**(2): p. 437-50.

128. Arques, J.L., et al., *Salmonella induces flagellin- and MyD88-dependent migration of bacteria-capturing dendritic cells into the gut lumen.* Gastroenterology, 2009. **137**(2): p. 579-87, 587 e1-2.

129. Mowat, A.M. and C.C. Bain, *Mucosal macrophages in intestinal homeostasis and inflammation.* J Innate Immun, 2011. **3**(6): p. 550-64.

130. Medina-Contreras, O., et al., *CX3CR1 regulates intestinal macrophage homeostasis, bacterial translocation, and colitogenic Th17 responses in mice.* J Clin Invest, 2011. **121**(12): p. 4787-95.

131. Niess, J.H. and G. Adler, *Enteric flora expands gut lamina propria CX3CR1+ dendritic cells supporting inflammatory immune responses under normal and inflammatory conditions.* J Immunol, 2010. **184**(4): p. 2026-37.

132. Atarashi, K., et al., *ATP drives lamina propria T(H)17 cell differentiation.* Nature, 2008. **455**(7214): p. 808-12.

133. Denning, T.L., et al., *Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses.* Nat Immunol, 2007. **8**(10): p. 1086-94.

134. Hadis, U., et al., *Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria.* Immunity. **34**(2): p. 237-46.

135. Lin, H.H., et al., *The macrophage F4/80 receptor is required for the induction of antigen-specific efferent regulatory T cells in peripheral tolerance.* J Exp Med, 2005. **201**(10): p. 1615-25.

136. Siddiqui, K.R., S. Laffont, and F. Powrie, *E-cadherin marks a subset of inflammatory dendritic cells that promote T cell-mediated colitis.* Immunity, 2010. **32**(4): p. 557-67.

137. Platt, A.M., et al., *An independent subset of TLR expressing CCR2-dependent macrophages promotes colonic inflammation.* J Immunol, 2010. **184**(12): p. 6843-54.

138. Kostadinova, F.I., et al., *Crucial involvement of the CX3CR1-CX3CL1 axis in dextran sulfate sodium-mediated acute colitis in mice.* J Leukoc Biol, 2010. **88**(1): p. 133-43.

139. Haribhai, D., et al., *A central role for induced regulatory T cells in tolerance induction in experimental colitis.* J Immunol, 2009. **182**(6): p. 3461-8.

140. Thornton, A.M., et al., *Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells.* J Immunol, 2010. **184**(7): p. 3433-41.

141. Gottschalk, R.A., E. Corse, and J.P. Allison, *Expression of Helios in peripherally induced Foxp3+ regulatory T cells.* J Immunol, 2012. **188**(3): p. 976-80.

142. Weiss, J.M., et al., *Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells.* J Exp Med, 2012.

143. Yadav, M., et al., *Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo.* J Exp Med, 2012.

144. Mucida, D., et al., *Oral tolerance in the absence of naturally occurring Tregs.* J Clin Invest, 2005. **115**(7): p. 1923-33.

145. Groux, H., et al., *A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis.* Nature, 1997. **389**(6652): p. 737-42.

146. Vieira, P.L., et al., *IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells.* J Immunol, 2004. **172**(10): p. 5986-93.

147. Maloy, K.J., et al., *CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms.* J Exp Med, 2003. **197**(1): p. 111-9.

148. Read, S., V. Malmstrom, and F. Powrie, *Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation.* J Exp Med, 2000. **192**(2): p. 295-302.

149. Kretschmer, K., et al., *Inducing and expanding regulatory T cell populations by foreign antigen.* Nat Immunol, 2005. **6**(12): p. 1219-27.

150. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.* J Exp Med, 2003. **198**(12): p. 1875-86.

151. Cassani, B., et al., *Gut-tropic T cells that express integrin alpha4beta7 and CCR9 are required for induction of oral immune tolerance in mice.* Gastroenterology, 2011. **141**(6): p. 2109-18.

152. Dbouk, H.A., et al., *Connexins: a myriad of functions extending beyond assembly of gap junction channels.* Cell Commun Signal, 2009. **7**: p. 4.

153. Mese, G., G. Richard, and T.W. White, *Gap junctions: basic structure and function.* J Invest Dermatol, 2007. **127**(11): p. 2516-24.

154. Gaietta, G., et al., *Multicolor and electron microscopic imaging of connexin trafficking.* Science, 2002. **296**(5567): p. 503-7.

155. Reaume, A.G., et al., *Cardiac malformation in neonatal mice lacking connexin43.* Science, 1995. **267**(5205): p. 1831-4.

156. Oviedo-Orta, E. and W. Howard Evans, *Gap junctions and connexin-mediated communication in the immune system.* Biochim Biophys Acta, 2004. **1662**(1-2): p. 102-12.

157. Jara, P.I., M.P. Boric, and J.C. Saez, *Leukocytes express connexin 43 after activation with lipopolysaccharide and appear to form gap junctions with endothelial cells after ischemia-reperfusion.* Proc Natl Acad Sci U S A, 1995. **92**(15): p. 7011-5.

158. Ploemacher, R.E., et al., *Hematopoiesis: Gap Junction Intercellular Communication is Likely to be Involved in Regulation of Stroma-dependent Proliferation of Hemopoietic Stem Cells.* Hematology, 2000. **5**(2): p. 133-147.

159. Cancelas, J.A., et al., *Connexin-43 gap junctions are involved in multiconnexin-expressing stromal support of hemopoietic progenitors and stem cells.* Blood, 2000. **96**(2): p. 498-505.

160. Montecino-Rodriguez, E., H. Leathers, and K. Dorshkind, *Expression of connexin 43 (Cx43) is critical for normal hematopoiesis.* Blood, 2000. **96**(3): p. 917-24.

161. Krenacs, T., et al., *Direct cell/cell communication in the lymphoid germinal center: connexin43 gap junctions functionally couple follicular dendritic cells to each other and to B lymphocytes.* Eur J Immunol, 1997. **27**(6): p. 1489-97.

162. Matsue, H., et al., *Gap junction-mediated intercellular communication between dendritic cells (DCs) is required for effective activation of DCs.* J Immunol, 2006. **176**(1): p. 181-90.

163. Neijssen, J., et al., *Cross-presentation by intercellular peptide transfer through gap junctions.* Nature, 2005. **434**(7029): p. 83-8.

164. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Res, 2001. **29**(9): p. e45.

165. Jung, S., et al., *Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion.* Mol Cell Biol, 2000. **20**(11): p. 4106-14.

166. Porgador, A., et al., *Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody.* Immunity, 1997. **6**(6): p. 715-26.

167. Saccheri, F., et al., *Bacteria-induced gap junctions in tumors favor antigen cross-presentation and antitumor immunity.* Sci Transl Med, 2010. **2**(44): p. 44ra57.

168. Niess, J.H. and H.C. Reinecker, *Lamina propria dendritic cells in the physiology and pathology of the gastrointestinal tract.* Curr Opin Gastroenterol, 2005. **21**(6): p. 687-91.

169. Menard, S., N. Cerf-Bensussan, and M. Heyman, *Multiple facets of intestinal permeability and epithelial handling of dietary antigens.* Mucosal Immunol, 2010. **3**(3): p. 247-59.

170. Karlsson, M., et al., *"Tolerosomes" are produced by intestinal epithelial cells.* Eur J Immunol, 2001. **31**(10): p. 2892-900.

171. Van Niel, G., et al., *Intestinal epithelial exosomes carry MHC class II/peptides able to inform the immune system in mice.* Gut, 2003. **52**(12): p. 1690-7.

172. Rathinasamy, A., et al., *The origin and maturity of dendritic cells determine the pattern of sphingosine 1-phosphate receptors expressed and required for efficient migration.* J Immunol, 2010. **185**(7): p. 4072-81.

173. Watkins, S.C. and R.D. Salter, *Functional connectivity between immune cells mediated by tunneling nanotubules.* Immunity, 2005. **23**(3): p. 309-18.

174. Thery, C., et al., *Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73.* J Cell Biol, 1999. **147**(3): p. 599-610.

175. Zitvogel, L., et al., *Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes.* Nat Med, 1998. **4**(5): p. 594-600.

176. Neijssen, J., B. Pang, and J. Neefjes, *Gap junction-mediated intercellular communication in the immune system.* Prog Biophys Mol Biol, 2007. **94**(1-2): p. 207-18.

177. Bopp, T., et al., *Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression.* J Exp Med, 2007. **204**(6): p. 1303-10.