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Critical role for the microbiota in CX_3CR1^+ intestinal mononuclear phagocyte regulation of intestinal T cell responses

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Summary

The intestinal barrier is vulnerable to damage by microbiota-induced inflammation that is normally restrained through mechanisms promoting homeostasis. Such disruptions contribute to autoimmune and inflammatory diseases including inflammatory bowel disease. We identified a regulatory loop whereby, in the presence of the normal microbiota, intestinal antigen-presenting cells (APCs) expressing the chemokine receptor CX_3CR1 reduced expansion of intestinal

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Contributions

G.E.D., M.H.K, and D.R.L designed experiments and wrote the manuscript with input from all co-authors. G.E.D., M.H.K, C.G., A.A.H., designed, performed and analyzed the experiments. W.J.W., H.F.P, H.W.S. performed experiments. R.S.L, M.L.B. designed and analyzed the experiments. D.S. performed blinded histology scoring on colitis sections. K.W.S. provided bacterial strains and analyzed experiments.

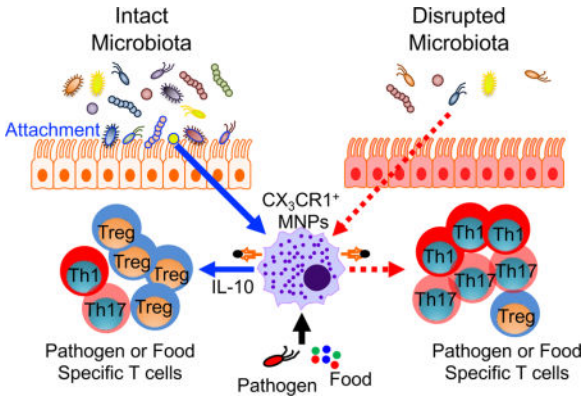
Declaration of Interests

The authors declare no competing interests.

microbe-specific T helper-1 (Th1) cells and promoted generation of regulatory T cells responsive to food antigens and the microbiota itself. We identified that disruption of the microbiota resulted in CX_3CR1^+ APC-dependent inflammatory Th1 cell responses with increased pathology after pathogen infection. Colonization with microbes that can adhere to the epithelium was able to compensate for intestinal microbiota loss indicating that, while microbial interactions with the epithelium can be pathogenic, they can also activate homeostatic regulatory mechanisms. Our results identify a cellular mechanism by which the microbiota limits intestinal inflammation and promotes tissue homeostasis.

eToc

Host microbiota interactions regulate many aspects of immunity. Kim et al. demonstrate that microbial adhesion to intestinal epithelium helps regulate the balance between pro-and anti-inflammatory T cell responses through induction of IL-10 by a subset of intestinal antigen presenting cells.



Introduction

The gastrointestinal tract is continuously exposed to a variety of potential immune stimuli including antigens from diet, commensal bacteria, and pathogens. This high antigenic load requires balance between inflammatory responses to harmful pathogens and tolerance to commensal microorganisms or food in order to maintain immune homeostasis and promote a healthy gut (Round and Mazmanian, 2009). Failure to regulate inflammatory T cells against environmental antigens is thought to drive the development of intestinal disorders including inflammatory bowel disease (IBD) (Sun et al., 2015; Wing and Sakaguchi, 2010). Immune cells with the capacity to suppress excess inflammation are crucial to limit inflammatory diseases (Wing and Sakaguchi, 2010).

T helper 1 (Th1) cell responses, while crucial for clearance of intracellular pathogens, are associated with autoimmune and inflammatory disorders (Cope et al., 2011). Interferon-γ(IFN-γ), the prototypic Th1 cell effector cytokine, can induce tissue pathology associated with infectious disease (Dolowschiak et al., 2016). IFN-γ directly increases epithelial permeability both in vivo and in vitro (Beaurepaire et al., 2009) resulting in increased food antigen, bacteria and bacterial products entering the mucosa alongside elevated local

immune responses (Sartor, 2007). Therefore, IFN-γ must be tightly regulated to mediate pathogen clearance while limiting unintended tissue damage.

A dynamic interaction between the host and microbiota shapes the development and responsiveness of both the mucosal and systemic immune systems (Round and Mazmanian, 2009; Sommer and Bäckhed, 2013). Studies in germ-free mice demonstrate reduced immune cellularity as well as a lack of organized structures such as B cell germinal centers, indicating an essential role for the microbiota (Round and Mazmanian, 2009; Sommer and Bäckhed, 2013). The microbiota also allows for proper induction of protective immunity against pathogens while also limiting aberrant responses against the microbiota and self-antigens. For example, production of interleukin-10 (IL-10), a critical immunoregulatory molecule, which down regulates IFN-γ responses, requires the microbiota (Grainger et al., 2013). Additionally, the microbiota is critical for induction of intestinal regulatory T cells (Tregs) as they are absent in germ-free mice (Atarashi et al., 2011). Disrupted signaling downstream from the microbiota, such as through loss of toll-like receptor pathways, is thought to drive disease pathology including in IBD (Zhou et al., 2016). We and others have found that the microbiota is required for proper intestinal barrier repair through innate lymphoid cell production of IL-22 (Longman et al., 2014; Ouyang et al., 2011). We also found that the microbiota serves to limit trafficking of antigen presenting cells (APCs) from the intestinal lamina propria to the mesenteric lymph node (MLN), a site of T cell priming (Diehl et al., 2013). As altered migration has the potential to regulate inflammatory T cell responses against intestinal antigens, we sought to understand the cellular signaling network between the microbiota and induction of T cell responses against intestinal antigens. Using antibiotics to disrupt the microbiota, we established that the presence of the intact microbiota led to reduction in Th1 cell responses against intestinal pathogens. This reduced response depended on chemokine receptor CX_3CR1-expressing intestinal mononuclear phagocytes (MNPs). In the presence of the intact microbiota, CX_3CR1^+ MNPs limited T effector expansion and promoted the differentiation of Treg cells against soluble antigens and the microbiota itself. In contrast, after microbiota disruption, CX_3CR1^+ MNPs promoted pathogen-specific Th1 cell expansion and contributed to tissue pathology. Antigen presentation and IL-10 production by CX_3CR1^+ MNPs were required to mediate these effects. We further defined a requirement for microbial adhesion to intestinal epithelial cells for induction of this regulatory loop. These data demonstrate a conditioning of CX_3CR1^+ MNPs by the intact microbiota that restrains pro-inflammatory Th1 cell responses and promotes anti-inflammatory Treg cell responses to sustain intestinal homeostasis.

Results

The microbiota limits Th1 cell responses against intestinal pathogens

To understand how the microbiota impacts induction of intestinal immunity, we utilized infection with the intestinal pathogen *Salmonella* Typhimurium with or without prior antibiotic treatment to disrupt intestinal microbes. Antibiotics reduced fecal microbiota to the level of detection by qPCR (Figure S1A). We utilized antibiotic treatment because of immune defects, including loss of intestinal APCs, in germ free animals (Bain et al., 2014; Round and Mazmanian, 2009). In our previous work, we have described an increased

Salmonella-specific Th1 cell response in mice orally infected with a non-invasive mutant *Salmonella* after antibiotic disruption of the microbiota (Figure S1B and (Diehl et al., 2013)). Non-invasive *Salmonella* is defective in the type III secretion system required for entry across epithelial barriers and requires host trafficking to enter tissues and reach the MLN (Diehl et al., 2013; Galan, 2001; Vazquez-Torres et al., 1999). As we previously described, antibiotic depletion of the microbiota results in increased non-invasive *Salmonella* in the gut and MLN (Figure 1A and S1C and (Diehl et al., 2013)). Using non-invasive *Salmonella*, we therefore could not determine if the differential immune response we observed was due to changes in tissue bacterial load or differences in local immunity. For this we needed an intestinal pathogen that demonstrates equivalent penetration into host tissues in the presence of the intact and disrupted microbiota. Invasive *Salmonella* is able to penetrate to the MLN (Vazquez-Torres et al., 1999) and we asked if it reached equivalent bacterial numbers after antibiotic disruption of the microbiota. In contrast to infection with non-invasive *Salmonella*, infection with invasive *Salmonella* results in equivalent bacteria titers in the MLN in the presence of the intact or disrupted microbiota (Figure 1A). However, even in the presence of comparable antigen load, we found an increased *Salmonella* specific Th1 cell response in intestine and MLN of mice with disrupted microbiota (Figure 1B). This indicates increased bacterial penetration to the tissues was not driving the increased Th1 cell response. We previously demonstrated that infection with noninvasive *Salmonella* after antibiotic treatment allowed for increased MLN trafficking of CX_3CR1^+ MNPs (Figure S1D and (Diehl et al., 2013)). After infection with invasive *Salmonella*, we observed increased CX_3CR1^+ MNPs in the MLN in animals with both normal and disrupted microbiota (Figure 1C). Therefore, we conclude that infection with invasive *Salmonella* would allow us to identify how microbiota signals impacted CX_3CR1^+ MNP function and the induction of intestinal inflammatory T cell responses. Thus, we utilized invasive *Salmonella* for rest of our studies.

CX_3CR1^+ MNPs drive Th1 cell expansion in response to intestinal pathogens after microbiota disruption

To understand the role that CX_3CR1^+ MNPs play in the increased *Salmonella*-specific T cell response, we utilized mice in which CX_3CR1^+ MNPs or all CX_3CR1^+ cells could be selectively depleted after treatment with diphtheria toxin (DT) (Figure S2A and (Diehl et al., 2013; Longman et al., 2014). We administered DT to antibiotic-treated littermate *Cx3cr1*-STOP-DTR mice with (depletion of CX_3CR1^+ MNPs) or without *Itgax*-cre (no depletion) before infecting them orally with invasive or non-invasive *Salmonella*. After depletion of CX_3CR1^+ MNPs, we observed decreased non-invasive *Salmonella* titers in the MLN (Figure S2B and (Diehl et al., 2013) along with decreased *Salmonella* specific T cell responses (Figure S2C). In contrast, after infection with invasive *Salmonella* in antibiotic-treated mice, depletion of CX_3CR1^+ MNPs had no impact on MLN bacterial load (Figure 2A). However, depletion of CX_3CR1^+ MNPs led to reduction in *Salmonella*-specific T cell responses (Figure 2B) and intestinal inflammation (Figure 2C, D). These findings led us to hypothesize that, after microbiota depletion, increased MLN CX_3CR1^+ MNPs were driving enhanced Th1 cell responses against invasive *Salmonella*. We performed similar experiments with *Cx3cr1*-DTR mice in which treatment with DT results in depletion of all CX_3CR1^+ cells

including monocytes (Figure S2A). Similar to CX_3CR1^+ MNP depleted mice, loss of all CX_3CR1^+ cells led to decreased *Salmonella*-specific T cell responses (Figure S2D).

We next wanted to understand whether CX_3CR1^+ MNPs drive Th1 cell expansion or differentiation of *Salmonella*-specific T cells after microbiota depletion. We orally infected antibiotic treated mice with intact or depleted CX_3CR1^+ MNPs with *Salmonella* expressing a previously characterized 2W1S:I-A^b $CD4^+$ T cell epitope to allow for tracking of pathogen-specific T cells (Moon et al., 2007). No expansion of 2W1S specific T cells was observed in the absence of infection with 2W1S *Salmonella* or after CX_3CR1^+ MNP depletion (Figure S3A). We analyzed the expansion and differentiation of MLN and intestinal 2W1S-specific T cells after 2W1S *Salmonella* infection by flow cytometry. We observed expansion of 2W1S specific T cells in the MLN and intestine, the majority of which were T-bet^+ Th1 cells producing IFN-γ (Figure 2E, F). 2W1S Th1 cell expansion was blunted after depletion of CX_3CR1^+ MNPs, but expanded cells still produced IFN-γ albeit in reduced numbers (Figure 2E, F). We did not observe differential expansion or alterations in effector T cells that did not recognize the 2W1S antigen (Figure S3B). These data support a role for CX_3CR1^+ MNP driven expansion of *Salmonella*-specific Th1 cells after microbiota disruption.

To determine if antigen presentation by CX_3CR1^+ MNPs was essential for this function, we bred *Cx3cr1*-creERT2 mice (Parkhurst et al., 2013) to mice with a conditional allele for MHCII I-A^b (Hashimoto et al., 2002). Treatment with 4-hydroxy tamoxifen (4OHT) resulted in loss of MHCII expression specifically in CX_3CR1^+ cells (Figure S3C). *Cx3cr1*-creERT2 $MHCII^{flox/wt}$ (wildtype MHCII expression on CX_3CR1^+ cells) and *Cx3cr1*-creERT2 $MHCII^{flox/-}$ (loss of MHCII expression on CX_3CR1^+ cells) were treated with antibiotics and 4OHT before oral *Salmonella* infection. We found that MHCII loss did not impact intestinal or MLN CX_3CR1^+ MNP numbers or *Salmonella* titers in the MLN in antibiotic treated mice (Figure S3D, E). However, $CD4^+$ T cells from the MLN and intestine of antibiotic treated mice in which CX_3CR1^+ MNPs lack MHCII expression had decreased *Salmonella* specific IFN-γ production, as was observed for mice lacking CX_3CR1^+ MNPs (Figure S3F). This demonstrates that antigen presentation by CX_3CR1^+ MNPs directs *Salmonella*-specific Th1 cell responses after microbiota disruption.

CX_3CR1^+ MNPs limit intestinal inflammation in the presence of an intact microbiota

We next wanted to know if CX_3CR1^+ MNPs played a similar role in induction of intestinal Th1 cell responses against pathogens in the presence of an intact microbiota. To determine this, we infected mice with intact or depleted CX_3CR1^+ MNPs with *Salmonella* to assess induction of *Salmonella*-specific T cell responses. In contrast to our results with antibiotic treatment, mice depleted of CX_3CR1^+ MNPs with a normal microbiota had substantially increased T cell production of IFN-γ in both the MLN and small intestine as compared to mice with normal numbers of CX_3CR1^+ MNPs (Figure 3A). We observed similar increased Th1 cell responses from mice in which all CX_3CR1^+ cells were depleted (Figure S4A). To ask if CX_3CR1^+ MNPs limited immune responses to intestinal microbes other than *Salmonella*, we infected mice with intact or depleted CX_3CR1^+ MNPs with *Helicobacter hepaticus*, which is non-pathogenic in wild-type mice, and measured *Helicobacter*-specific

IFN-γ by ELISA. As with mice infected with *Salmonella*, mice depleted of CX_3CR1^+ MNPs had increased *Helicobacter*-specific T cell production of IFN-γ (Figure 3B).

To determine if this increased Th1 cell response in the absence of CX_3CR1^+ MNPs was the result of enhanced expansion or increased differentiation towards a Th1 cell phenotype of *Salmonella*-specific T cells, we orally infected mice with intact or depleted CX_3CR1^+ MNPs with 2W1S *Salmonella* and analyzed 2W1S-specific T cells by flow cytometry. In the absence of CX_3CR1^+ MNPs, we observed increased expansion of 2W1S-specific T cells in both the MLN and small intestine, the majority of which were T-bet$^+$ and produced IFN-γ, (Figure 3C, D) indicating that these cells limit expansion of *Salmonella*-specific T cells, thereby restraining the induction of Th1 cell responses against pathogenic intestinal microbes.

To determine whether antigen presentation by CX_3CR1^+ MNPs is required to limit T cell responses in the presence of the intact microbiota, we infected mice with normal expression of MHCII or lacking MHCII expression on CX_3CR1^+ MNPs with *Salmonella* and assayed for *Salmonella*-specific T cell responses. As with depletion of CX_3CR1^+ MNPs, in the presence of an intact microbiota, loss of antigen presentation by CX_3CR1^+ MNPs to CD4$^+$ T cells led to enhanced Th1 cell response after *Salmonella* infection (Figure S4B). These data demonstrate a critical role for CX_3CR1^+ MNPs, in the presence of the intact microbiota, in limiting Th1 cell responses against intestinal microbes that are both pathogenic as well as potentially pathogenic such as pathosymbionts.

CX_3CR1^+ MNPs induce anti-inflammatory T cell responses against soluble oral antigens

One of the hallmark immune responses in the gastrointestinal tract is the induction of oral tolerance. Oral tolerance was described over a hundred years ago as the ability of an orally administered antigen to inhibit subsequent priming of immune responses against that antigen (Pabst and Mowat, 2012). As CX_3CR1^+ MNPs, in the presence of the intact microbiota, limited anti-microbial inflammatory immune responses by way of antigen presentation, we wanted to understand their role in inducing tolerogenic immune responses against soluble oral antigens. To assess induction of oral tolerance in animals lacking CX_3CR1^+ MNPs, we fed ovalbumin (OVA) to mice with intact or depleted CX_3CR1^+ MNPs. The animals were then challenged with OVA in complete Freund's adjuvant and rechallenged via footpad injection of OVA to induce a delayed type hypersensitivity (DTH) response. We observed a reduced DTH response in OVA fed mice with intact CX_3CR1^+ MNPs but not in mice depleted of CX_3CR1^+ MNPs, indicating that CX_3CR1^+ MNPs are required for induction of tolerance to fed proteins (Figure 4A). We did not observe defective oral tolerance in mice deficient in the chemokine receptor CX_3CR1 (Figure S4C).

To assess the direct impact of CX_3CR1^+ MNPs on the induction of antigen-specific Treg cells, we transferred OVA-specific OTII T cells into mice with intact or depleted CX_3CR1^+ MNPs fed OVA. The proportion of OTII cells that differentiated to FoxP3$^+$ Treg cells was significantly reduced in the absence of CX_3CR1^+ MNPs resulting in a marked reduction in the absolute number of Treg cells (Figure 4B, C). While there was no difference in the number of host-derived Treg cells in the lymph nodes or intestinal tissue after OVA feeding or depletion of CX_3CR1^+ MNPs (Figure S4D), we found a substantial increase in OTII cells

in the MLN and small intestines of animals depleted of CX_3CR1^+ MNPs (Figure 4B, C). Thus, in line with our findings in *Salmonella*-infected mice with an intact microbiota, CX_3CR1^+ MNPs restrict proliferation of oral antigen-specific T cells. They further promote the differentiation of antigen specific Treg cells.

To confirm a requirement for antigen presentation by CX_3CR1^+ MNPs in directing Treg cells differentiation in response to soluble oral antigens, we transferred OTII T cells into mice with normal or lacking MHCII expression on CX_3CR1^+ MNPs fed OVA. There was no difference in resident Treg cells in animals lacking MHCII expression on CX_3CR1^+ cells (Figure S4E). However, there was a marked decrease in the proportion of OTII Treg cells in mice lacking MHCII expression on CX_3CR1^+ cells as compared to mice with normal MHCII expression on CX_3CR1^+ cells (Figure S4F, G). In contrast to depletion of CX_3CR1^+ MNPs, loss of MHCII expression on CX_3CR1^+ cells did not result in increased expansion of OTII cells (Figure S4G), suggesting that while MHCII expression on CX_3CR1^+ cells is required for induction of Treg cells, other interactions between CX_3CR1^+ MNPs and T cells limit T cell proliferation.

To understand the impact of the microbiota on induction of tolerogenic immune responses against soluble oral antigens, we evaluated oral tolerance as above but in animals with antibiotic disrupted microbiota. In antibiotic treated animals, we found OVA feeding was insufficient to prevent the DTH response, indicating a reduced induction of tolerance to fed antigens (Figure 4D). This is supported by other findings in the literature that tolerance to oral antigens is defective in animals lacking microbiota-derived signals (Belkaid and Hand, 2014). To understand if there was altered differentiation of OVA-specific T cells after microbiota depletion, we transferred OTII T cells into antibiotic treated OVA fed mice which were intact or depleted of CX_3CR1^+ MNPs. In animals with an intact microbiota, the vast majority of transferred T cells expressed FoxP3 in the absence of other T cell lineage specific transcription factors (Figure S4H, I). After microbiota depletion, a proportion of the OVA specific T cells in both the MLN and intestine expressed the Th17 cell lineage specific transcription factor RORγt alone or in combination with FoxP3 (Figure 4E, F, S4H). We observed loss of $FoxP3^+$, $ROR\gamma t^+$, and $FoxP3^+ROR\gamma t^+$ T cell populations after CX_3CR1^+ MNP depletion (Figure 4E, F). These findings suggest that the intact microbiota promotes Treg cells but suppresses inflammatory Th17 cell responses against food antigen and this regulation depends on CX_3CR1^+ MNPs.

CX_3CR1^+ MNP-dependent induction of Treg cells and inhibition of inflammatory T cell expansion limits intestinal pathology

To answer if CX_3CR1^+ MNPs also regulated microbiota-specific T cells and could thus limit microbiota directed inflammation-induced intestinal damage we performed T cell transfer colitis experiments in $RAG2^{-/-}$ mice that had intact CX_3CR1^+ cell populations, were depleted of CX_3CR1^+ MNPs, or depleted of all CX_3CR1^+ cells. Transfer of naïve $CD4^+CD25^-CD45RB^{high}$ T cells into *Helicobacter* colonized, immune-deficient mice gives rise to colitis mediated by T cells producing IFN-γ and IL-17A (Saleh and Elson, 2011). $RAG2^{-/-}$ mice in our colony exhibit minimal disease after transfer of naïve $CD4^+$ T cells (Figure 5A–C, S5A). This limited pathology is likely due to a lack of colonization with

Helicobacter or other potentially pro-inflammatory members of the microbiota (Cahill et al., 1997; Coombes et al., 2005). In contrast, transfer of naïve T cells into CX_3CR1^+ cell or CX_3CR1^+ MNP depleted *RAG2*$^{-/-}$ mice led to increased weight loss and pathology in the large intestine (Figure 5A-C, S5A). Using flow cytometry, we determined the effector phenotype of transferred T cells. We observed a significant reduction in Treg cells in mice depleted of all CX_3CR1^+ cells or CX_3CR1^+ MNPs (Figure 5D, S5B). We further observed increased Th1 cells and Th17 cells in colonic tissue in the absence of CX_3CR1^+ cells (Figure 5D). Increased Th1 cells and decreased Treg cells were also observed in the MLN (Figure S5C). To confirm sufficiency of monocytes or monocyte derived cells, we transferred wildtype bone marrow monocytes along with naive T cells into RAG2$^{-/-}$ CX_3CR1^+ cell depleted mice. Previous work demonstrated bone marrow monocyte transfer is sufficient to repopulate intestinal CX_3CR1^+ MNPs (Zigmond et al., 2012). Monocyte transfer rescued weight loss and colitis score (Figure S5D, E) as well as reduced expansion of colonic Th1 cells and restored colonic Treg cells (Figure S5F).

In the T cell transfer model of colitis, transfer of Treg cells can protect from or ameliorate disease (Coombes et al., 2005). As CX_3CR1^+ MNPs were required for Treg cell induction, we asked if Treg cells maintained their immunosuppressive impact in the absence of CX_3CR1^+ MNPs. We transferred naive T cells alone or with Treg cells into *RAG2*$^{-/-}$ CX_3CR1^+ cell depleted mice. Transferred Treg cells were able to fully protect from weight loss and histological damage as well as suppress enhanced Th1 and Th17 cell responses in the absence of CX_3CR1^+ cells (Figure 5E–G), supporting our findings that CX_3CR1^+ cells play an essential role in induction rather than maintenance of Treg cells. Together, these data support our findings that, in the presence of the normal microbiota, CX_3CR1^+ MNPs inhibit expansion of inflammatory Th1 and Th17 cells and promote expansion and differentiation of Treg cells to reduce intestinal inflammation.

Microbiota-induced IL-10 CX_3CR1^+ MNP production inhibits Th1 cell responses against intestinal pathogens and promotes Treg cell responses against food antigens

In the intestine, CX_3CR1^+ MNPs are the major producers of the anti-inflammatory cytokine IL-10 (Denning et al., 2007; Murai et al., 2009). To determine if the microbiota influenced IL-10 production, we isolated APCs from the intestine of untreated or antibiotic-treated animals and assessed IL-10 expression by qPCR and by flow cytometry of an *Il10* GFP reporter (Madan et al., 2009). As compared to other APC populations, CX_3CR1^+ MNPs from animals with an intact microbiota had high expression of IL-10 transcript and a high proportion were positive for *Il10*-GFP, and expression was reduced in CX_3CR1^+ MNPs from animals treated with antibiotics (Figure 6A, B, S6A). We did not observe altered expression of other cytokines by CX_3CR1^+ MNPs after antibiotic treatment (Figure S6B).

To test the in vivo role for CX_3CR1^+ MNP-produced IL-10 and determine if IL-10 production by CX_3CR1^+ cells was driving our observed effects, we bred *Cx3cr1*-creERT2 mice to mice harboring a conditional allele for IL-10 (Roers, 2004). We administered 4OHT to untreated or antibiotic-treated *Cx3cr1*-creERT2 *Il10*$^{flox/wt}$ (wildtype IL-10 expression by CX_3CR1^+ cells) and *Cx3cr1*-creERT2 *Il10*$^{flox/-}$ (CX_3CR1^+ cells deficient for IL-10, Figure S6C) before infecting with *Salmonella*. Loss of IL-10 did not alter the proportion of

CX_3CR1^+ MNPs in intestinal tissue (Figure S6D). In animals with antibiotic-disrupted microbiota, loss of IL-10 production by CX_3CR1^+ MNPs did not impact induction of a *Salmonella*-specific T cell response (Figure 6C). In contrast, loss of IL-10 production by CX_3CR1^+ MNPs in the presence of an intact microbiota led to an increased *Salmonella*-specific T cell response in the MLN and small intestine, mirroring what we found when we depleted CX_3CR1^+ MNPs (Figure 6C). To track expansion of *Salmonella*-specific T cells, we infected untreated or antibiotic treated animals with normal IL-10 or lacking IL-10 expression in CX_3CR1^+ cells with 2W1S expressing *Salmonella*. Loss of IL-10 production by CX_3CR1^+ cells led to increased number of *Salmonella* specific IFN-γ producing T cells in the presence of the intact microbiota, but no alteration in *Salmonella*-specific Th1 cells after microbiota depletion (Figure 6D). We did not observe alteration in T cell responses not specific for *Salmonella* in this system (not shown). We also do not find CX_3CR1^+ MNPs production of IL-10 regulates the function of CX_3CR1^+ MNPs themselves. Mice lacking IL-10 receptor on CX_3CR1^+ MNPs (IL-10 receptor flox mice (Pils et al., 2010) crossed to *Cx3cr1*-creERT2) showed no alteration in *Salmonella*-specific Th1 cell responses (Figure S6E).

To determine whether IL-10 production by CX_3CR1^+ MNPs induces Treg cells against soluble intestinal antigens, we transferred OTII T cells into OVA fed mice with normal IL-10 expression or lacking IL-10 expression by CX_3CR1^+ cells, and analyzed FoxP3 induction in transferred cells. We found decreased number of OVA-specific Treg cells from the MLN and intestine when CX_3CR1^+ MNPs lacked IL-10 (Figure 6E, F), but found no change in endogenous Treg cells (Figure S6F). Thus, IL-10 production by CX_3CR1^+ MNPs is required for the induction of antigen-specific Treg cells but not maintenance of intestinal Treg cells and this supports our findings for requirement of CX_3CR1^+ MNPs in the T cell transfer colitis model.

Epithelial adhesion by intestinal microbes is key to anti-inflammatory effects

Identification of microbiota regulation of CX_3CR1^+ MNP anti-inflammatory functions led us to address a critical question of what microbial factors are important for this regulatory loop. It has long been known that microbes such as *Escherichia coli (E. coli)* as well as microbial products such as lipopolysaccharide (LPS) can induce IL-10 production by macrophages in vitro (Figure 7A and (Dobrovolskaia and Vogel, 2002)). However, treatment of antibiotic treated mice with LPS or colonization with the K-12 lab adapted strain of *E. coli* did not induce in vivo IL-10 expression (Figure 7B). We previously identified that an adherent invasive *E. coli* (AIEC) strain 2A, isolated from a patient with Crohn's disease, induced intestinal inflammation in germ free mice deficient for IL-10 but not in wildtype germ-free mice (Viladomiu et al., 2017). Using this isolate, as well as an additional Crohn's disease AIEC strain (541-15) (Baumgart et al., 2007), we found that colonization with AIEC was sufficient to induce intestinal IL-10 expression in antibiotic treated mice which was lost in the absence of CX_3CR1^+ MNPs (Figure 7B). AIEC drove IL-10 expression specifically in CX_3CR1^+ MNPs as shown by IL-10 reporter mice (Figure 7C). A virulence factor, shared by pathogens such as AIEC and *Salmonella*, is the long polar fimbriae (LPF) which is used to mediate epithelial adhesion in vivo and in vitro (Baumler et al., 1996; Dogan et al., 2014). *lpfA* mutant AIEC 541-15 was unable to induce intestinal IL-10 expression although, they

had similar colonization (Figure 7B, C and not shown). Colonization with AIEC 2A or 541-15 further protected antibiotic treated mice from *Salmonella* mediated intestinal pathology whereas *E. coli* K-12 or *lpfA* mutant 541-15 did not (Figure 7D). No *E. coli* protection was observed in the absence of CX_3CR1^+ MNPs (not shown). Supporting the role of microbial adhesion in induction of IL-10 by CX_3CR1^+ MNPs, AIEC, but not *E. coli* K-12 or *lpfA* deleted 541-15 suppressed *Salmonella* specific Th1 cell responses after infection of antibiotic treated mice in the presence of CX_3CR1^+ MNPs (Figure 7E, F and S7A, B). Finally, AIEC did not suppress *Salmonella*-specific Th1 cell responses in mice where CX_3CR1^+ MNPs were unable to produce IL-10 (Figure 7G, S7C). These data demonstrate that intestinal microbial adherence offers crucial signals to limit tissue inflammation and pathology through activation of anti-inflammatory signals in CX_3CR1^+ MNPs.

Discussion

As regulating inflammation against intestinal microbes and dietary antigens is required for intestinal health, understanding how homeostasis is maintained remains a crucial question in the field of mucosal immunology. Using antibiotics to disrupt the microbiota we identified intestinal microbes as key to limiting intestinal inflammation through their impact on CX_3CR1^+ MNPs. In previous studies, we have demonstrated that CX_3CR1^+ MNPs promote barrier repair and the microbiota regulates their trafficking to the MLN (Longman et al., 2014, Diehl et al., 2013). However, the role for the microbiota in CX_3CR1^+ MNPs regulation of T cell responses remained unclear.

Using mice that allow for selective depletion of CX_3CR1^+ MNPs, we demonstrated these cells limit inflammatory T cells against intestinal microbes in the context of an intact microbiota. Upon depletion of CX_3CR1^+ MNPs or loss of their capacity for antigen presentation, oral infection with *Salmonella* or *Helicobacter* led to an enhanced bacteria-specific Th1 cell response. Utilizing the T cell transfer model of colitis, we demonstrated that CX_3CR1^+ MNPs also limit pro-inflammatory and promote anti-inflammatory T cell responses against the microbiota itself. Loss of CX_3CR1^+ cells resulted in increased pathology and corresponding reduced number of Treg cells and increased number of Th1 and Th17 cells. As the balance between T effector and Treg cells is critical to maintain intestinal homeostasis, this cellular pathway of regulation of CX_3CR1^+ MNPs likely limits the development of inflammatory conditions such as IBD (Shevach, 2009).

After microbiota disruption, loss of CX_3CR1^+ MNPs or CX_3CR1 mediated antigen presentation limited expansion of *Salmonella* specific T cell responses and reduced *Salmonella*-induced tissue pathology. Supporting our model of the importance of the microbiota in limiting inflammation, other groups have demonstrated increased IFN-γ-dependent intestinal pathology after *Salmonella* infection in antibiotic treated animals (Dolowschiak et al., 2016). Collectively, the anti-inflammatory role of CX_3CR1^+ MNPs depends on the microbiota.

Along with clearance of intestinal pathogens, the intestinal immune system must be tolerant to soluble food antigens. We were unable to induce oral tolerance in the absence of CX_3CR1^+ MNPs. Using transfer of antigen specific T cells, we observed corresponding

decreases in antigen-specific Treg cell induction. As with Th1 cell responses against pathogens, antigen presentation by CX_3CR1^+ MNPs is required for proper Treg cell expansion in response to soluble oral antigen. Further, similar to T cell responses against microbes, CX_3CR1^+ MNPs also limit the expansion of T cells specific for soluble intestinal antigens as we observed enhanced expansion of soluble antigen specific T cells in the absence of CX_3CR1^+ MNPs. In contrast to previous reports (Hadis et al., 2011), we did not observe a defect in oral tolerance in mice deficient in the chemokine receptor, CX_3CR1, only in mice lacking CX_3CR1^+ MNPs. As we observed CX_3CR1^+ MNPs function was dependent on the microbiota and individual animal facilities have differences in mouse microbiota composition, this could indicate differential dependence on the chemokine receptor CX_3CR1 in the presence of different microbes.

Moreover, we were unable to induce oral tolerance after antibiotic disruption of the microbiota. After OVA feeding of antibiotic treated animals, instead of observing induction of primarily OVA specific FoxP3 single positive Treg cells, we found induction of inflammatory OVA specific $ROR\gamma t^+$ Th17 cells. We also observed induction of $ROR\gamma t$ and FoxP3 double positive cells. A role for these cells in systemic tolerance to oral antigens is not defined. Within the colon, $ROR\gamma t^+FoxP3^+$ T cells represent a stable, microbiota specific Treg cell population (Sefik et al., 2015; Yang et al., 2016). In contrast, in human blood Th17 memory cells express both $ROR\gamma t$ and FoxP3 and produce IL-17 upon restimulation (Voo et al., 2009). These data indicate they might have differential function in the intestine and other body sites. We conclude that disruption of the microbiota corresponds to pro-inflammatory T cell differentiation in response to food antigens together with failure to induce oral tolerance.

IL-10 limits intestinal pathology (Moore et al., 2001) and we found CX_3CR1^+ MNPs to be a major producer of intestinal IL-10 with production strongly regulated by the microbiota. Microbiota mediated IL-10 production by CX_3CR1^+ MNPs limited Th1 cell expansion against *Salmonella* and also drove Treg cell differentiation against soluble oral antigens. Previous work has found that IL-10R on CX_3CR1^+ MNPs limits induction of colitis (Zigmond et al., 2014). We did not observe an impact of IL-10 autocrine signaling in CX_3CR1^+ MNPs in regulation of a pathogen specific Th1 cell response. In the previous work, IL-10R was lost constitutively however, in our system, IL-10 or IL-10 receptor is lost acutely revealing that IL-10 production by CX_3CR1^+ MNPs limits the induction of antigen-specific Th1 cell responses.

As there is increasing evidence that IBD results from a dysregulated interaction between the host immune system and its resident microbiota (Round and Mazmanian, 2009; Sommer and Bäckhed, 2013), our data support the hypothesis that the intestinal microbiota provides crucial signals to CX_3CR1^+ MNPs restraining the induction of inflammatory T cells and promoting induction of Treg cells. Understanding the microbial signals that regulate these anti-inflammatory signals is crucial to reestablish homeostasis in the context of inflammatory disease.

Critical questions remain as to which microbiota signals drive the anti-inflammatory functions of CX_3CR1^+ MNPs. While microbial signals such as LPS or *E. coli* K-12 are

sufficient to induce IL-10 production in vitro (Dobrovolskaia and Vogel, 2002), we found they were not sufficient to induce IL-10 or limit Th1 cell responses in vivo. In contrast, the capacity of intestinal microbes to adhere to the epithelium was critical for IL-10 induction and to limit Th1 cell responses against an intestinal pathogen. Intestinal microbe AIEC 2A is capable of inducing both Th17 cell responses and IL-10 expression and can induce pathology in the absence of IL-10 (Viladomiu et al., 2017). This demonstrates that microbial pathways that are thought to be markers of pathogenicity or drivers of pro-inflammatory responses can also activate cellular networks to reduce tissue inflammation. Thus, they can be pathogenic in the context of host genetic alterations such as impaired anti-inflammatory signals. The outcome of such interactions between intestinal microbes and host is likely further dictated by the specific nature of the microbe and association with other markers of pathogenicity. As an additional example, adhesion of *E. coli* O157 to intestinal epithelial cells is required for induction of pro-inflammatory Th17 cell responses (Atarashi et al., 2015). Along with our finding, this suggests microbial adhesion to epithelium is a critical requirement for both pro- and anti-inflammatory host immune cell responses. For pathogenic organisms, inducing this regulatory pathway could support their ability to colonize the host by limiting immune clearance. Of direct relevance to IBD, a number of groups have identified increased colonization by AIEC in patients as compared to controls (Baumgart et al., 2007; Gevers et al., 2014; Viladomiu et al., 2017). Our findings highlight the importance of integration of microbiota signals to balance between pro- and anti-inflammatory responses to intestinal antigens and pathogens.

Epidemiological studies suggest that increased antibiotic exposure is associated with an increased risk for developing inflammatory diseases including IBD, celiac disease, and allergy (Belkaid and Hand, 2014) and our data suggests this increased pathology could be due to a loss of microbiota driven anti-inflammatory function in CX_3CR1^+ MNPs. By combining analysis of both pro- and anti-inflammatory responses, our studies demonstrate a critical role for the microbiota and their capacity to interact with the intestinal epithelium in regulating the inflammatory potential of a potent intestinal antigen presenting cell, CX_3CR1^+ MNPs. Our work also demonstrates the potential therapeutic benefits of microbiota manipulation with the goal of amplifying or inhibiting intestinal T cell responses.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

KEY RESOURCE TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse MHCII (I-A/I-E), Pacific blue, clone M5/114.15.2	BioLegend	Cat# 107620
Anti-mouse CD11c, PEcy7, clone N418	BioLegend	Cat# 117317
Anti-mouse/human CD11b, APC/Cy7, clone M1/70	BioLegend	Cat# 101226
Anti-mouse CX_3CR1, FITC, clone SA011F11	BioLegend	Cat# 149020

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-mouse Ly6C, Percp/Cy5.5, clone AL-21	BD Pharmingen	Cat# 560525
Anti-mouse Ly6G, PE, clone 1A8	BioLegend	Cat# 127608
Anti-human/mouse CD44, Percp/Cy5.5, clone IM7	eBioscience	Cat# 45-0441-82
Anti-mouse CD62L, FITC, clone MEL-14	eBioscience	Cat# 11-0621-82
Anti-mouse CD4, FITC, clone RM4-5	BioLegend	Cat# 100510
Anti-human/mouse T-bet, PEcy7, clone 4B10	Invitrogen	Cat# 25-5825-82
Anti-mouse CD3, APC/Cy7, clone 145-2C11	BioLegend	Cat# 100330
Anti-mouse TCRβ, Percp/Cy5.5, clone H57-597	BioLegend	Cat# 109228
Anti-mouse Vα2 TCR, Biotin, clone B20.1	BioLegend	Cat# 13-5812-82
Anti-mouse/rat Foxp3, eFluor660, clone FJK-16s	eBioscience	Cat# 50-5773-82
Anti-mouse CD103, APC, clone 2E2	BioLegend	Cat# 121413
Anti-mouse RORγt, PE, clone BD2	eBioscience	Cat# 12-6981-82
Anti-mouse CD45.2, APC-eFluor780, clone 104	eBioscience	Cat# 47-0454-82
Anti-mouse CD45.1, Percp/Cy5.5, clone A20	eBioscience	Cat# 45-0453-82
Anti-mouse CD45RB, APC, clone C363.16A	eBioscience	Cat# 17-0455-81
Anti-mouse CD25, PEcy7, clone PC61.5	eBioscience	Cat# 25-0251-82
Anti-Gata3, BV421, clone 16E10A23	BioLegend	Cat# 653814
Anti-mouse IFN-γ, Percp/Cy5.5, clone XMG1.2	eBioscience	Cat# 45-7311-82
Anti-mouse IL-17A, Bv421, clone TC11-18H10.1	BioLegend	Cat# 506926
Anti-2W1S:I-A^b, APC	NIH tetramer core	Cat# 36906
Streptavidin-eFluor 450	eBioscience	Cat# 48-4317-82
Bacterial and Virus Strains		
Salmonella ΔAroA	Vazquez-Torres et al., 1999	N/A
Salmonella ΔAroA/ΔinvA	Vazquez-Torres et al., 1999	N/A
Salmonella ΔinvA	Vazquez-Torres et al., 1999	N/A
Salmonella 2W1S-expressing ΔAroA	Dr. M. Jenkins	N/A
E. coli K-12	ATCC	PTA-7555
E. coli 2A	Viladomiu et al., 2017	N/A
E. coli 541-15	Dogan et al., 2014	N/A
541-15Δlpf	Dogan et al., 2014	N/A
Chemicals, Peptides, and Recombinant Proteins		
Lipopolysaccharides from *Escherichia coli* O55:B5	Sigma-Aldrich	Cat# L6529
Phorbol myristate acetate	Sigma-Aldrich	Cat# P1585
Ionomycin calcium salt	Sigma-Aldrich	Cat# I0634
GolgiPlug	BD Bioscience	Cat# 51-2301KZ
SYBR Green Supermix	Roche	Cat# 4887352001
SuperScript III Reverse Transcriptase	Invitrogen	Cat# 18080-051
Trizol Reagent	Invitrogen	Cat# 15596018
0.5M EDTA pH8.0	Invitrogen	Cat# AM9261

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Collagenase 8	Sigma-Aldrich	Cat# C2139
DL-Dithiothreitol	Sigma-Aldrich	Cat# D9779
Deoxyribonuclease I from bovine pancreas	Sigma-Aldrich	Cat# DN25
Percoll	GE Health	Cat# 17-0891-01
Ampicillin sodium Salt	Sigma-Aldrich	Cat# A0166
Streptomycin sulfate	Sigma-Aldrich	Cat# S9137
Albumin from chicken egg white	Sigma-Aldrich	Cat# A5503
Diphtheria toxin	Sigma-Aldrich	Cat# D0564
(Z)-4- Hydroxytamoxifen, 98% Z isomer	Sigma-Aldrich	Cat# H7904
Freund's Adjuvant	Sigma-Aldrich	Cat# F5881
HEPES	HyClone	Cat# SH30237.01
Fetal Bovine Serum	Corning	Cat# MT35016CV
FoxP3 /Transcription Factor Staining Buffer set	eBioscience	Cat# 00-5523-00
Critical Commercial Assays		
Mouse IL-10 ELISA kit	BioLegend	Cat# 431414
Mouse IFN-γ ELISA kit	BD Bioscience	Cat# 551866
Experimental Models: Cell Lines		
RAW 264.7	ATCC	TIB-71
Experimental Models: Organisms/Strains		
Mouse: CX_3CR1-STOP-DTR	Diehl et al., 2013	JAX# 025629
Mouse: CX_3CR1-DTR	Longman et al., 2014	N/A
Mouse: CX_3CR1-CreERT2	Parkhurst et al., 2013	JAX# 021160
Mouse: CD11c-Cre	Jackson Laboratories	JAX# 008068
Mouse: $MHCII^{-/-}$	Jackson Laboratories	JAX# 003584
Mouse: MHCII-conditional	Jackson Laboratories	JAX# 013181
Mouse: $IL\text{-}10^{-/-}$	Jackson Laboratories	JAX# 003968
Mouse: VertX	Jackson Laboratories	JAX# 014530
Mouse: $RAG2^{-/-}$	Jackson Laboratories	JAX# 008449
Mouse IL-10 conditional	Roers, 2004	N/A
Mouse: IL-10 receptor conditional	Pils et al., 2010	N/A
Mouse: Ly5.1	Jackson Laboratories	JAX# 002014
Mouse: OTII-Tg	Jackson Laboratories	JAX# 004194
Mouse: C57BL/6	Jackson Laboratories	JAX# 000664
Mouse: CX_3CR1-GFP	Jackson Laboratories	JAX# 005582
Oligonucleotides		
Primers for quantitative PCR, see table S1	This paper	N/A
Software and Algorithms		
GraphPad Prim 5.0	GraphPad Software	N/A
FlowJo LLC version 10.1.	Becton Dickinson	

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Live/dead fixable Blue dead cell stain kit	Thermo Fisher	Cat# L34962
DAPI	Sigma-Aldrich	Cat# D9542
123count eBeads	Thermo Fisher	Cat# 01-1234
anti-B220 magnetic microbeads	Miltenyi Biotec	Cat# 130-049-501
anti-CD8 magnetic microbeads	Miltenyi Biotec	Cat# 130-117-044

CONTACT FOR REGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gretchen Diehl (Gretchen.diehl@bcm.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Experimental Animals—CX_3CR1-STOP-DTR, CX_3CR1-DTR and CX_3CR1-CreERT2 were previously described (Diehl et al., 2013; Longman et al., 2014; Parkhurst et al., 2013). The following strains are from Jackson Laboratories: CD11c-Cre (JAX # 008068), MHCII $^{-/-}$ (JAX # 003584), MHCII-conditional (JAX # 013181) and IL-$10^{-/-}$ (JAX # 003968), VertX (JAX # 014530), CX_3CR1-GFP (JAX # 005582), $RAG2^{-/-}$ (JAX # 008449), $Ly5.1^{+}$ (JAX # 002014), OTII-Tg^{+} (JAX # 004194), and C57BL/6 mice (JAX # 000664). IL-10 and IL-10 receptor conditional mice were from Dr. Werner Muller (Pils et al., 2010; Roers, 2004). All mice were kept in specific pathogen-free conditions and bred at the animal facility of the Skirball Institute or Baylor College of Medicine. Mouse experiments were performed with at least 3 mice per group and all mice were bred to generate littermate control animals for each experiment. Littermates were randomly assigned to experimental groups. Animals were used between 6 – 12 weeks of age with males and females used in approximately equal ratios. All animal experiments were performed in accordance with approved protocols for the NYU or BCM Institutional Animal Care and Usage Committee.

Diphtheria toxin (DT) and 4-hydroxy tamoxifen (4OHT) administration—Mice were intraperitoneally (I.P.) injected every other day with 200ng diphtheria toxin (DT, Sigma) in saline. (Z)-4- Hydroxytamoxifen, 98% Z isomer (4OHT, Sigma) was resuspended to 20mg/ml in ETOH with heating to 37°C. 4OHT was diluted to in corn oil (Sigma) and mice were injected every 3 days with 0.2mg by I.P.

Depletion of gut commensal microbiota—Animals were administered a single dose of 20 mg streptomycin (Sigma) per os (P.O.) and provided ampicillin (Sigma) at 1 g/L in drinking water for 1-2 weeks. Mice were switched to water without antibiotics two days before infecting or treating them as below. Bacterial DNA in feces was determined by qPCR after antibiotic treatment.

Infection with *S.* Typhimurium—For assessing T cell responses and tissue inflammation, mice were infected orally with 3×10^8 *Salmonella* ΔAroA colony forming unit (cfu) or 3×10^8 ΔAroA/ΔinvA cfu in 100 l of phosphate-buffered saline (PBS). For bacterial

titers animals were infected with 3×10^8 *Salmonella* cfu or 3×10^8 ΔinvA cfu in 100 l of PBS. For titers, organs were homogenized two days after infection, diluted in PBS and plated on LB agar with streptomycin (50 g/ml). Colony counts were expressed as cfu per organ. 2W1S-expressing ΔAroA *Salmonella* was a kind gift of Dr. M. Jenkins, University of Minnesota, Minneapolis, MN. For T cell analysis and histological analyses, mice were analyzed 10-12 days after infection.

Colonization of mice with *E. coli*—Before colonization, mice were treated with antibiotics as above. *E. coli* were cultured overnight in LB media with or without selective antibiotics. Antibiotic treated mice were colonized with *E. coli* isolate: lab adapted *E. coli* strain K-12 or human AIEC strains (2A and 541-15) and 541-15 mutant strain (541-15Δ*lpfA*) at 1×10^9 cfu p.o.. Colonization was confirmed by qRT-PCR with *E. coli* specific primers. For LPS treatment, antibiotic treated mice were injected every other day with 0.3 g LPS (Sigma).

OVA specific Immune responses—For transfer of OVA-specific OTII cells, T cells were isolated from the lymph nodes of Ly5.1$^+$OTII-Tg$^+$ mice. 2×10^6 cells were injected I.V. into Ly5.2 recipient animals. Animals were given 1.5% OVA in their drinking water (changed every other day) and T cell responses were measured 12 days after transfer. Oral tolerance studies were performed as described in (Hadis et al., 2011). Mice were tolerized with 25mg of ovalbumin (OVA, Type V, Sigma) P.O. every other day. 7d later, mice were immunized subcutaneously with 250ug OVA in a PBS and CFA emulsion. 7d later mice were challenged with injection of 50ug OVA in the footpad. PBS was injected into the opposite side for control purposes. Footpad thickness was measured in a blinded fashion prior to injection and 48 hr after injection. OVA-specific footpad swelling was calculated as the relative swelling as compared to t0 of the OVA injected footpad versus the PBS injected footpad. To determine the microbiota impact, mice were treated with antibiotics 1 month before and throughout oral tolerance experiment.

Adoptive transfer colitis—Induction of colitis by transfer of naïve T cells into a lymphopenic recipient was performed as described (Coombes et al., 2005). CD4$^+$CD45RB^{high}CD25$^-$ naïve or CD4$^+$CD45RB^{low}CD25$^+$ Treg cells were sorted from WT B6 mice. 4×10^5 naïve T cells alone or with 1×10^5 Treg cells were transferred into RAG2$^{-/-}$ mice with intact CX$_3$CR1$^+$ cells or depleted CX$_3$CR1$^+$ cells or RAG2$^{-/-}$ mice with intact CX$_3$CR1$^+$ MNPs or depleted CX$_3$CR1$^+$ MNPs. For some experiments, 1.5×10^6 FACS sorted bone marrow-monocytes (CD11b$^+$Ly6C^{high}Ly6G$^-$CX$_3$CR1$^+$) were co-transferred with naïve T cells and transferred again at 2 weeks post transfer into RAG2$^{-/-}$ mice with depleted CX$_3$CR1$^+$ cells. Mice were weighed weekly and sacrificed when reached 15% weight loss.

METHOD DETAILS

Ex vivo measurement of *Salmonella*-specific T cell responses—For antigen specific T-cell responses, single-cell suspensions from the indicated organ were first negatively depleted with anti-B220 and anti-CD8 magnetic microbeads (Miltenyi Biotec). The depleted fraction was stained and cell sorting was performed on an Aria flow cytometer (BD Biosciences) to obtain memory and/or effector TCRβ$^+$CD4$^+$CD44$^+$CD62L$^-$ T cells

(>99% purity). These cells were plated at 1×10^5 cells per well with 5×10^5 irradiated splenocytes and 1 μg of heat-killed *Salmonella*. At 24 h, IFN-γ in the supernatant was measured by ELISA (BD Biosciences).

Cell isolation—Small or large intestinal lamina propria cells were isolated as previously described (Diehl et al., 2013). Briefly, mouse intestines were washed in PBS, once with 1 mM DTT and twice with 30 mM EDTA, and then digested in collagenase 8 (Sigma-Aldrich) and DNase-containing media with 10% fetal bovine serum. Digested material was passed through a cell strainer and separated on a discontinuous 40%/80% Percoll gradient. MLN cells were isolated by mechanical grinding using frosted glass slides.

Antibodies, cell staining, and flow cytometry—Flow cytometric analysis was performed on a LSR II (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc.). Cell sorting was performed on an Aria Cell Sorter (BD Biosciences). Antibodies were from BD Pharmingen, eBiosciences or BioLegend. DAPI or UV live/dead fixable dead cell stain (ThermoFisher) was used to exclude dead cells. All samples were stained with CD16/32 (Fc receptor) to prevent non-specific antibody binding. Total cell counts were determined with123count eBeads (ThermoFisher). For intracellular staining of IFN-γ and IL-17A cells were activated in RPMI 1640 (10% fetal bovine serum) with phorbol myristate acetate (50 ng/ml), ionomycin (1 μM), and GolgiPlug (BD bioscience) for 4 h. Cells were then stained for surface antigens (CD3, TCRβ, and/or CD4). Cells were then fixed, permeabilized, and stained with antibodies to IL-17A, or IFN-γ. Cells were stained with an antibody to mouse FoxP3, T-bet, GATA-3, or RORγt according to the manufacturer's protocol (eBioscience). For antigen presenting cells staining, cells were stained with antibodies for CD11b, CD11c, MHCII, CD103, and CX_3CR1. For bone marrow monocyte staining, cells were stained with antibodies for CD11b, Ly6C, Ly6G, and CX_3CR1. For 2W1S tetramer staining, cells were stained with tetramer on ice for 1hr before staining with antibodies as above. Tetramer was a kind gift of Dr. M. Jenkins, University of Minnesota, Minneapolis, MN and from the NIH tetramer core.

RNA extraction and real-time RT-PCR—RNA from FACS sorted primary intestinal CX_3CR1^+ MNPs or total lamina propria cells was prepared with Trizol (Invitrogen). RNA was reverse transcribed into cDNA (SuperScript III; Invitrogen) and qPCR was performed with a Lightcycler with SYBR Green Supermix (Roche), 20 pmol forward and reverse primers, and 0.1 μg of cDNA. The thermocycling program was 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, with an initial cycle of 95°C for 2 min. Relative expression of target gene were determined by using the delta Ct value compared to delta Ct (GAPDH) using the following primer sets: mIL-10-F: CATCATGTATGCTTCTATGCAG; mIL-10-R: CCAGCTGGACAACATACTGCT, GAPDH-F: ACCACAGTCCATGCCATCAC; GAPDH-R: TCCACCACCCTGTTGCTGT, IL-6-F: CCAGAGATACAAAGAAATGATGG; IL-6-R: ACTCCAGAAGACCAGAGGAAAT, IL-12p35-F: AGACGGCCAGAGAAATGAA; IL-12p35-R: GTTTGGTCCCGTGTGATGTCTT, IL-12p40-F: TGTGGGAGAAGCAGACCCTTA; IL-12p40-R: GGGTGCTGAAGGCGTGAA, IL-23p19-F: CCAGCAGCTCTCTCGGAATC; IL-23p19-R: GATTCATATGTCCCGCTGGTG.

In vitro RAW cell culture—RAW 264.7 macrophage cells (ATCC) were plated in DMEM media containing 10% FBS at 5×10^5 cells per 24 well. Cells were treated with 5×10^6 of indicated *E. coli.* Plates and spun down for 10 min at 1,000g followed by a 1 hr incubation at 37°C. Cells were washed with sterile PBS and media was replaced with new media containing 100ug/ml gentamycin. Media was replaced one hour later with media containing 20ug/ml gentamycin. For LPS treatment, 1 μg/ml LPS was added. 24hr later, supernatants were harvested and IL-10 was assessed by ELISA (BioLegend).

Histopathology—Tissues were fixed in 10% neutral buffered formalin, routinely processed, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E) for light microscopic examination. Samples were assessed in a blinded fashion by a trained pathologist. Samples were scored 0 to 4 based on the criteria described in (Berg et al., 1996): (grade 0) no change from normal tissue; (grade 1) One or a few multifocal mononuclear cell infiltrates in lamina propria with minimal epithelial hyperplasia and slight to no depletion of mucus from goblet cells; (grade 2) More tissue involved or more frequent lesions than grade 1 including increased neutrophils along with mild epithelial hyperplasia and mucin depletion and occasional or small epithelial erosions with rare submucosal involvement; (grade 3) large area of mucosa involvement or more frequent lesions than grade 2 with moderate inflammation and submucosal involvement along with rare transmural and/or crypt abscesses and moderate epithelial hyperplasia, mucin depletion and ulcers; (grade 4) Involvement of most of the intestinal section and more severe than grade 3 including transmural lesions, marked epithelial hyperplasia, marked mucin depletion along with abscesses and ulcers.

QUANTIFICATION AND STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) with Bonferroni's posttest, Mann-Whitney U-test (2-tails), or unpaired t-test was performed using a 95% confidence interval. Weight loss was compared using linear regression. All analyses were performed using GraphPad Prism version 5.0. Differences were considered to be significant at P values of <0.05. Statistical significance is indicated as follows: ns: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Microbiota limits Th1 and supports Treg responses against intestinal antigens.
- Microbiota dependent anti-inflammatory functions depend on CX_3CR1^+ MNPs.
- Microbiota supports CX_3CR1^+ MNPs IL-10 production which directs Th1 and Treg balance.
- CX_3CR1^+ MNPs IL-10 production requires microbial epithelial attachment.

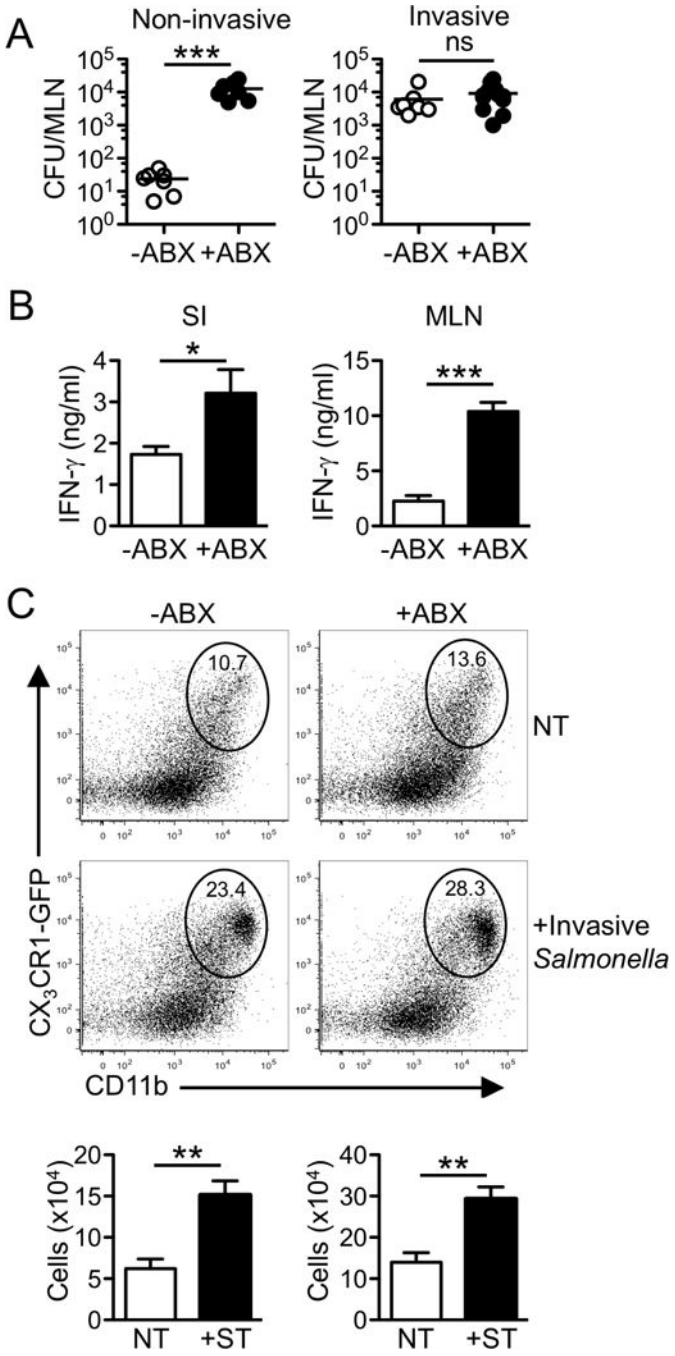

Figure 1. Enhanced *Salmonella* specific Th1 cell responses after microbiota disruption

(A) C57BL/6 mice were left untreated (−ABX) or treated with antibiotics (+ABX) for 1 week before infection with invasive or non-invasive (ΔinvA) *Salmonella*. Bacterial titers in MLN were assessed by plating 3d post infection. (B) Antibiotic (ABX) treated or untreated animals were infected with invasive *Salmonella* (ST). 10d post infection, FACS sorted $CD4^+$ T cells from mesenteric lymph node (MLN) or small intestinal lamina propria (SI) were cultured with irradiated splenocytes and *Salmonella* antigen for 24hr. *Salmonella*-specific IFN-γ production was measured by ELISA. (C) Flow cytometric analysis of MNPs in the

MLN of uninfected (NT) or 3d post invasive *Salmonella* infection of ABX treated or untreated B6 mice. Representative dot plots shown is gated on $Live^{+}MHCII^{+}CD11c^{+}$ cells (left). Absolute numbers of $Live^{+}$ $MHCII^{+}CD11c^{+}CD11b^{+}CX_3CR1\text{-}GFP^{+}$ cells are shown (right). Data points represent a single mouse, bar represents geometric mean (A). Error bars represent the SEM (B, C). Data shown is representative of at least 2 independent experiments with n=5/group. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. Two-tailed Student's t test. Please see also Figure S1.

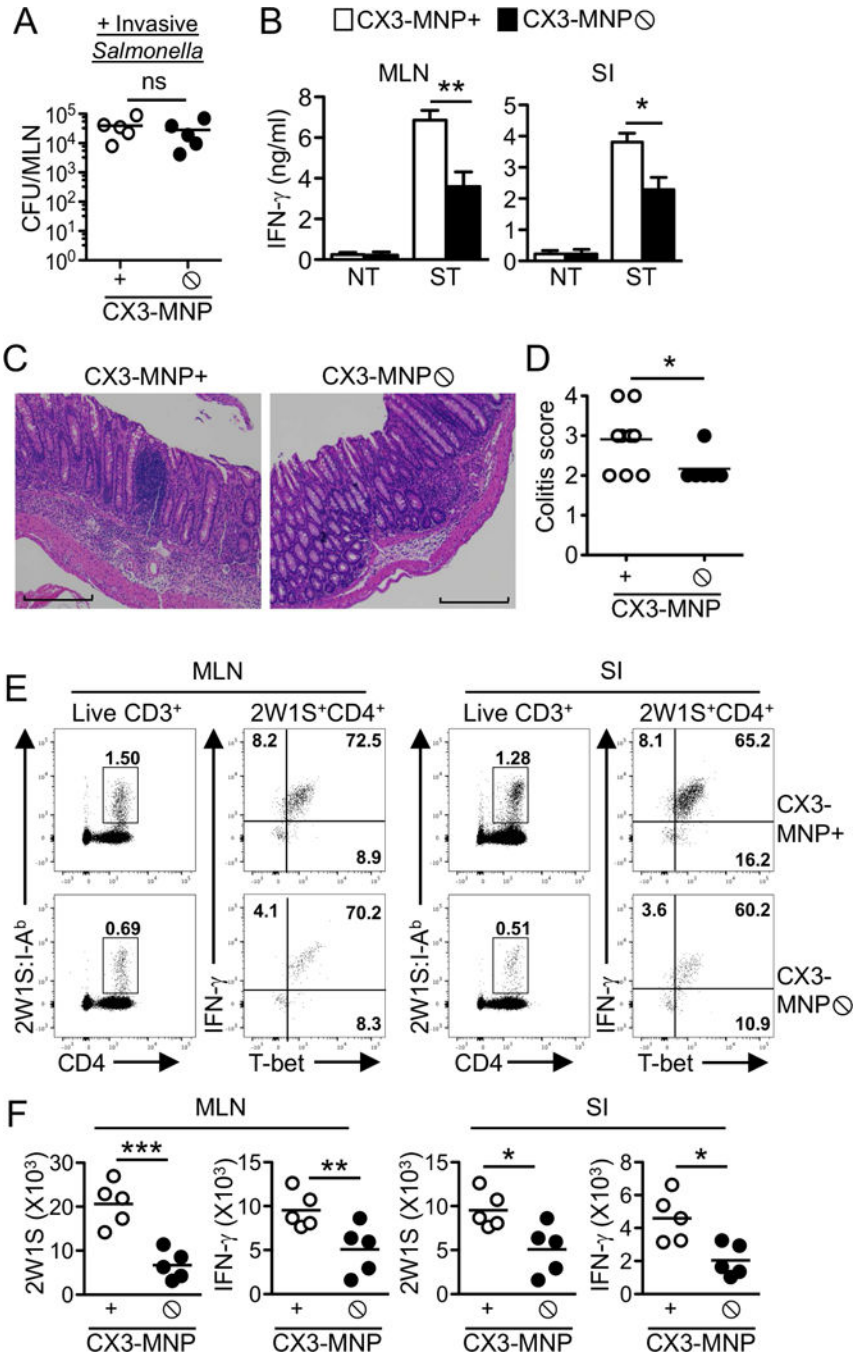

Figure 2. CX_3CR1^+ MNPs drive increased Th1 cell response after microbiota disruption

Littermate CX_3CR1-STOP-DTR mice without (CX3-MNP+, intact CX_3CR1^+ MNPs) and with CD11c-Cre (CX3-MNP⊘, depletion of CX_3CR1^+ MNPs) were treated with antibiotics (ABX) and diphtheria toxin (DT) before and during infection with invasive ST. 3d later bacterial titers in the MLN were analyzed by plating (A). *Salmonella* specific Th1 cell responses were measured as in Figure 1B. (B). Representative H&E stained images (C) and blinded colitis score (D) for colon sections from animals as described above. Scale bar indicates 200μm. Littermate CX3-MNP+ and CX3-MNP⊘ mice were treated with ABX and

DT before and during infection with *Salmonella* expressing the 2W1S:I-A^b T cell epitope. 10d later, 2W1S recognizing T cells were analyzed by flow cytometry (E). Dot plots from a representative animal is shown. Absolute numbers are shown in (F) with each dot representing a single animal. Data points represent a single mouse, bar represents geometric mean (A, D, F). Error bars represent the SEM (B). Data is representative of 2 independent experiments. ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***,$P \leq 0.001$. Two-tailed Student's t test. Please see also Figure S2 and S3.

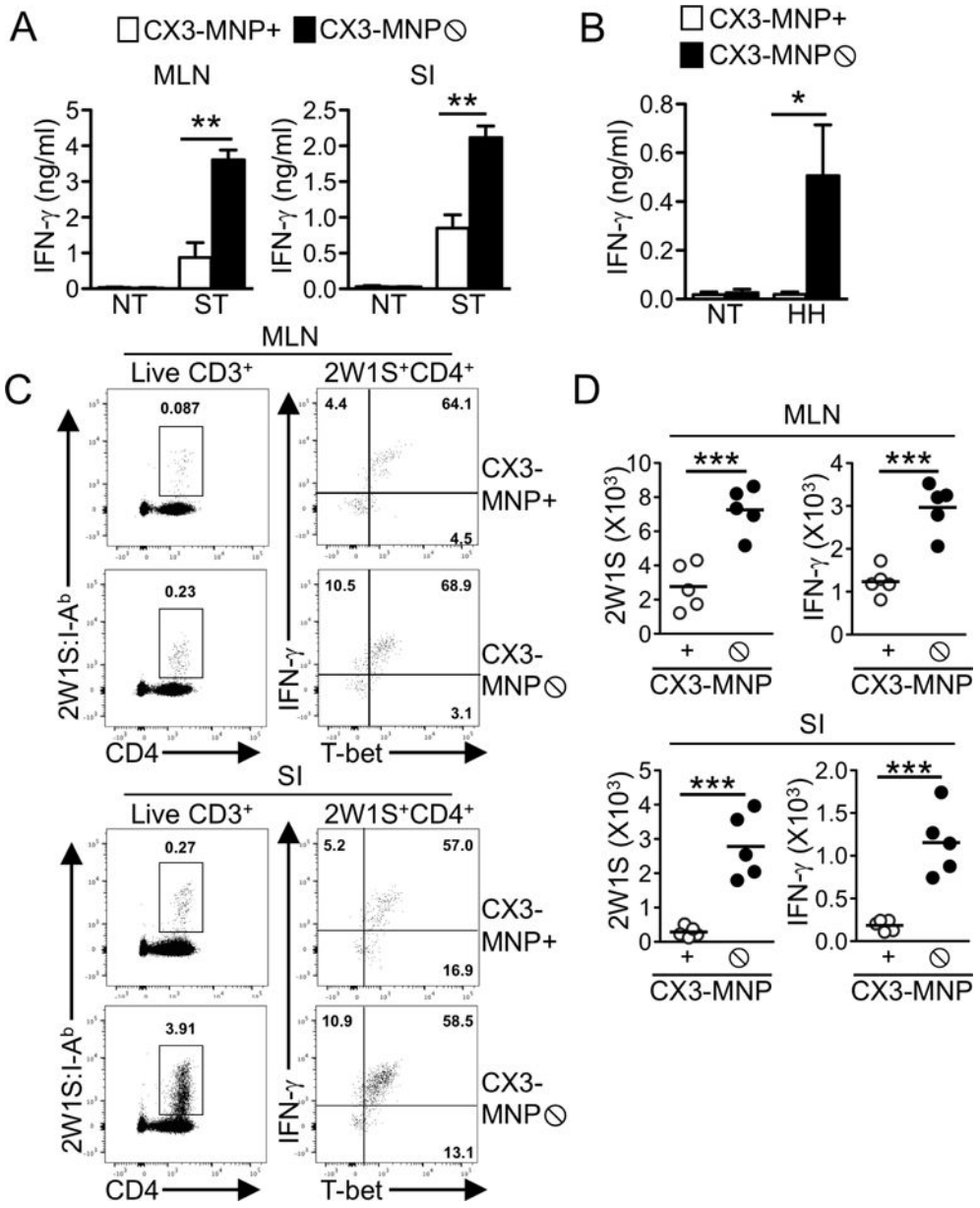

Figure 3. CX_3CR1^+ MNPs limit Th1 cell responses against intestinal pathogens in the presence of the intact microbiota

Littermate CX3-MNP+ (intact CX_3CR1^+ MNPs) and CX3-MNP⊘ (depletion of CX_3CR1^+ MNPs) were treated with DT and infected with *Salmonella* (A) or *Helicobacter hepaticus* (HH) (B). 10d later, *Salmonella* (A) or *Helicobacter* (B) specific IFN-γ production was measured by ELISA as in Figure 1B. (C, D) Littermate CX3-MNP+ and CX3-MNP⊘ mice were treated with DT and infected with 2W1S-*Salmonella*. 10d later, 2W1S recognizing T cells were analyzed by flow cytometry. Dot plots shown are from a representative animal (C). Absolute numbers are shown in (D). Error bars represent the SEM (A, B). Data points represent a single mouse, bar represents geometric mean (D). Data is representative of 2 independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. Two-tailed Student's t test. Please see also Figure S3 and S4.

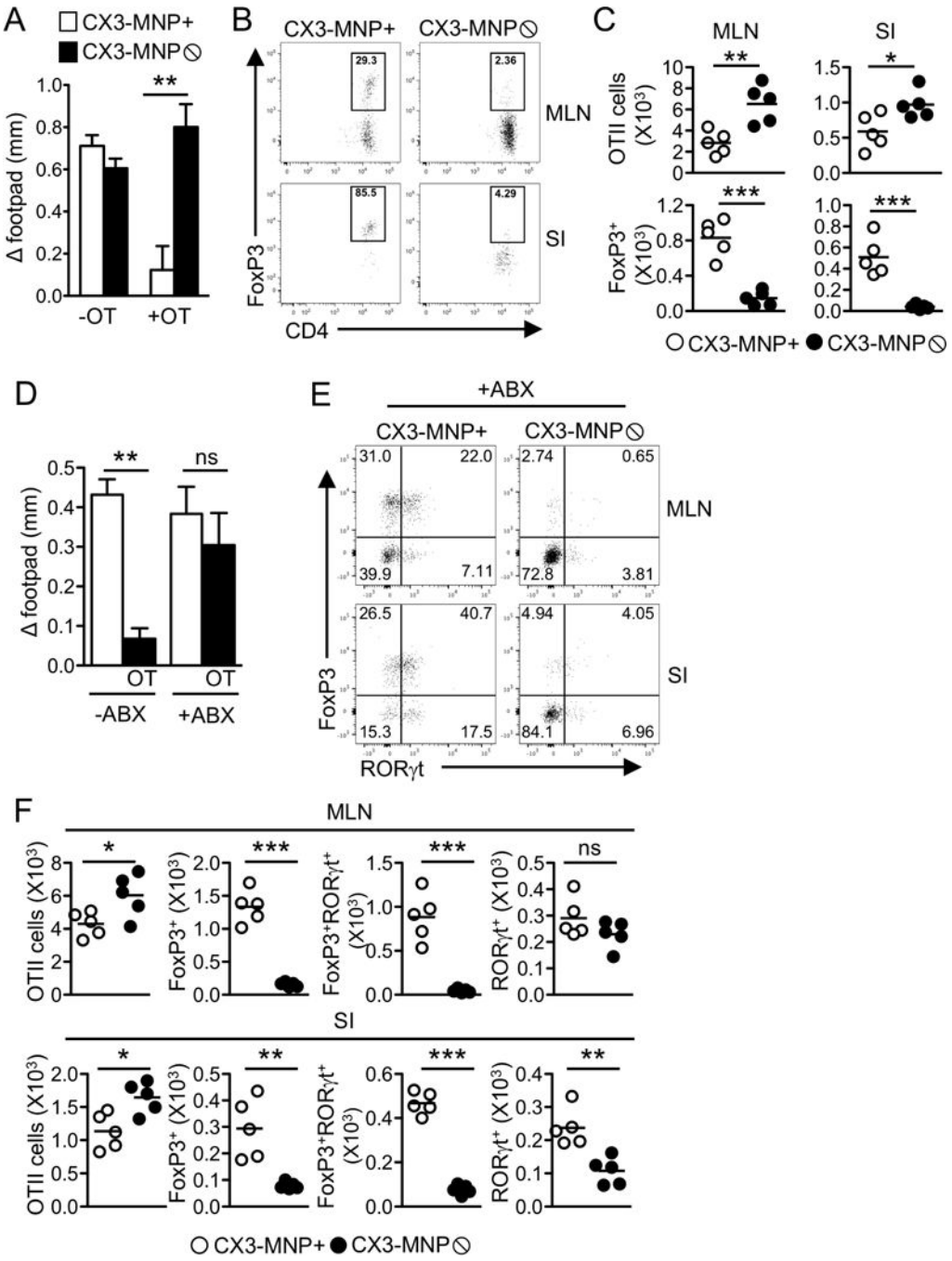

Figure 4. The intact microbiota and CX_3CR1^+ MNPs are required to induce Treg cells against soluble intestinal antigens

Induction of oral tolerance was assessed in littermate CX3-MNP+ (intact CX_3CR1^+ MNPs) and CX3-MNP⊘ mice (depletion of CX_3CR1^+ MNPs) (A). (B, C) Ovalbumin (OVA) specific $CD4^+$ T cells (OTII) were transferred to littermate DT treated CX3-MNP+ and CX3-MNP⊘ mice. 10d later, induction of $FoxP3^+CD4^+$ T cells in OTII cells was assessed by flow cytometry. Dot plots from representative animal gated on $Live^+Ly5.1^+V\alpha2^+CD3^+$ cells (B). Absolute numbers are shown (C). Induction of oral tolerance (OT) was compared in wildtype mice with or without ABX treatment (D). (E, F) OTII cells were transferred to

littermate CX3-MNP+ and CX3-MNP⊘ mice with ABX treatment. 10d later, induction of FoxP3 and RORγt in OTII cells was assessed by flow cytometry. Dot plots shown are gated on Live$^+$ Ly5.1$^+$Vα2$^+$CD3$^+$CD4$^+$ cells in MLN and SI from a representative animal for each group (E). Absolute numbers are shown in (F). Error bars represent the SEM (A, D). Data points represent a single mouse, bar represents geometric mean (C, F). Data is representative of two independent experiments. ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. One-way analysis of variance (ANOVA) with Bonferroni's posttest correction or Two-tailed Student's t test. Please see also Figure S4.

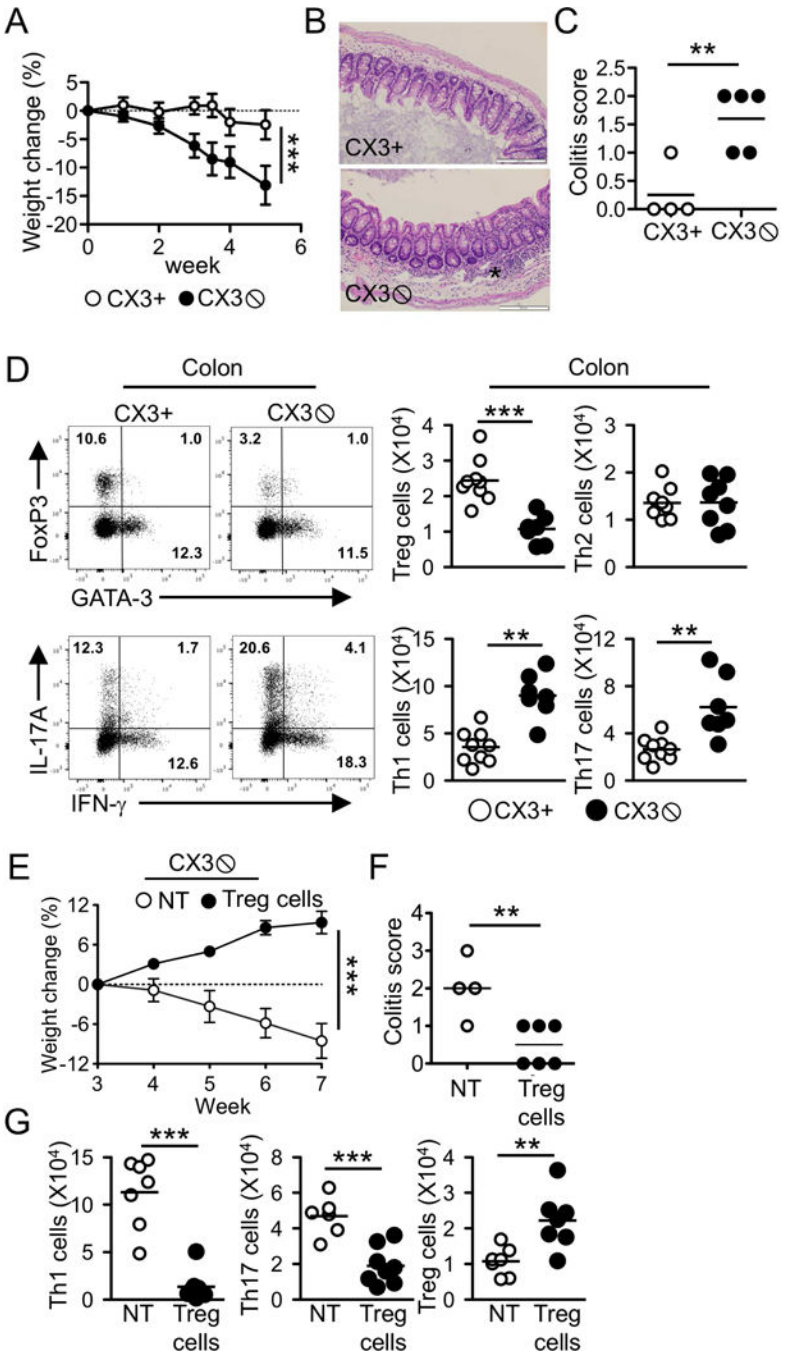

Figure 5. CX_3CR1^+ MNPs induce Treg cells and suppress inflammatory T cells against the microbiota, limiting intestinal inflammation

Naïve $CD4^+$ T cells were transferred into littermate *RAG2*$^{-/-}$ CX3+ (intact CX_3CR1^+ cells) and CX3⊘ (depletion of CX_3CR1^+ cells) mice. Body weight was measured weekly (A). 5 weeks post transfer, animals were analyzed for extent of colitis and T cell differentiation. Representative H&E images of intestinal sections shown in (B) with blinded scoring for colitis (C). Asterisks show area of submucosal involvement with infiltrating neutrophils. (D) Flow cytometry analysis of colonic T cell populations. Dot plots shown are gated on Live $^+CD3^+CD4^+TCR\beta^+$ cells from a representative animal (left). Absolute numbers of T cell

populations are also shown (right). (E-G) Naïve $CD4^+$ T cells and Treg cells were co-transferred into littermate $RAG2^{-/-}$ CX3∅ mice. Body weight was measured weekly (E). 7 weeks post transfer, animals were analyzed for extent of colitis (F) and absolute numbers of T cell populations in the colon was determined by flow cytometry analysis as in E (G). Error bars represent the SEM (A, E). Data points represent a single mouse, bar represents geometric mean (C, D, F, G). Data is representative of 2 independent experiments. **P ≤ 0.01; ***P ≤ 0.001. Significance was determined through two-tailed Student's t test or linear regression. Please see also Figure S5.

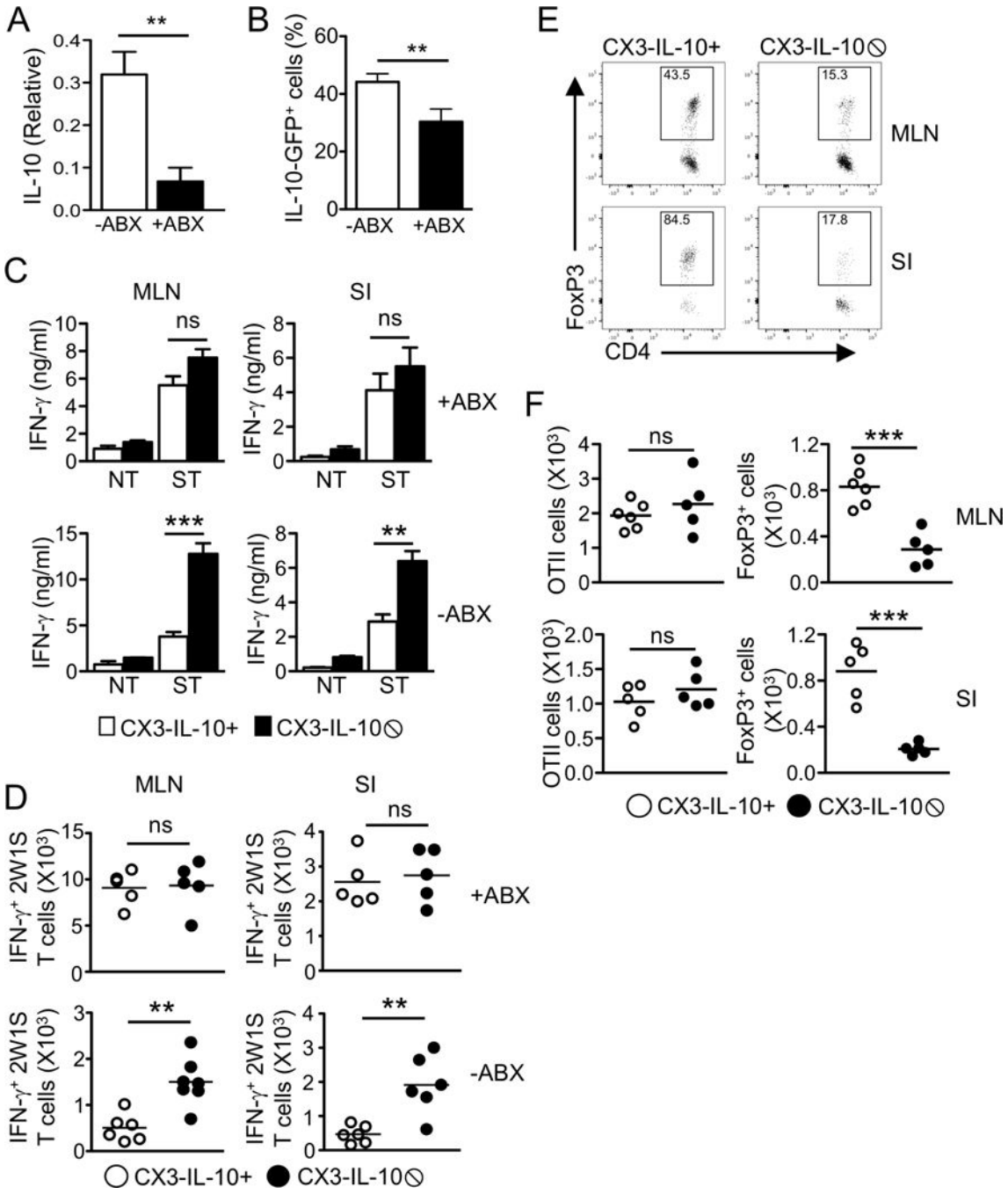

Figure 6. Anti-inflammatory role of the intact microbiota depends on IL-10 production by CX_3CR1^+ MNPs

(A) IL-10 expression by CX_3CR1^+ MNPs of untreated or antibiotic treated animals. CX_3CR1^+ MNPs were sorted from the small intestinal lamina propria of CX_3CR1-GFP^+ mice. qPCR was used to quantify relative IL-10 mRNA (A). IL-10 expression in CX_3CR1^+ MNPs in IL-10-VertX mice was assessed by flow cytometry. Percentage of $IL\text{-}10^+$ cells among CX_3CR1^+ MNPs ($CD11b^+CX_3CR1^+$ $MHCII^+$ cells) shown (B). (C) Littermate *Cx3cr1*-creERT2 $Il10^{flox/+}$ (CX3-IL-10+, normal IL-10 expression by CX_3CR1^+ cells) and $Il10^{flox/-}$ (CX3-IL-10⊘, lacking IL-10 expression by CX_3CR1^+ cells) mice were left

untreated or treated with ABX before treatment with 4OHT and infection with *Salmonella*. 10d later *Salmonella*-specific IFN-γ production by T cells was analyzed as in Figure 1B. (D) Littermate CX3-IL-10+ and CX3-IL-10⊘ mice were treated as above before infection with *Salmonella* expressing the 2W1S T cell epitope. 10d later, expansion of 2W1S recognizing Th1 cells was analyzed by flow cytometry. Absolute numbers are shown. (E, F) OTII T cells were transferred into 4OHT treated CX3-IL-10+ and CX3-IL-10⊘ mice. OVA was provided in the drinking water. 10d later, induction of FoxP3 in OTII cells was assessed by flow cytometry. Representative dot plots shown is gated on $Live^{+}Ly5.1^{+}V\alpha2^{+}CD3^{+}$ cells (E) and absolute numbers (F) are shown. Data shown is representative of 2 independent experiments with n=5/group. Error bars represent the SEM (A–C). Data points represent a single mouse, bar represents geometric mean (D, F). ns, $P > 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. Two-tailed Student's t test. Please see also Figure S6.

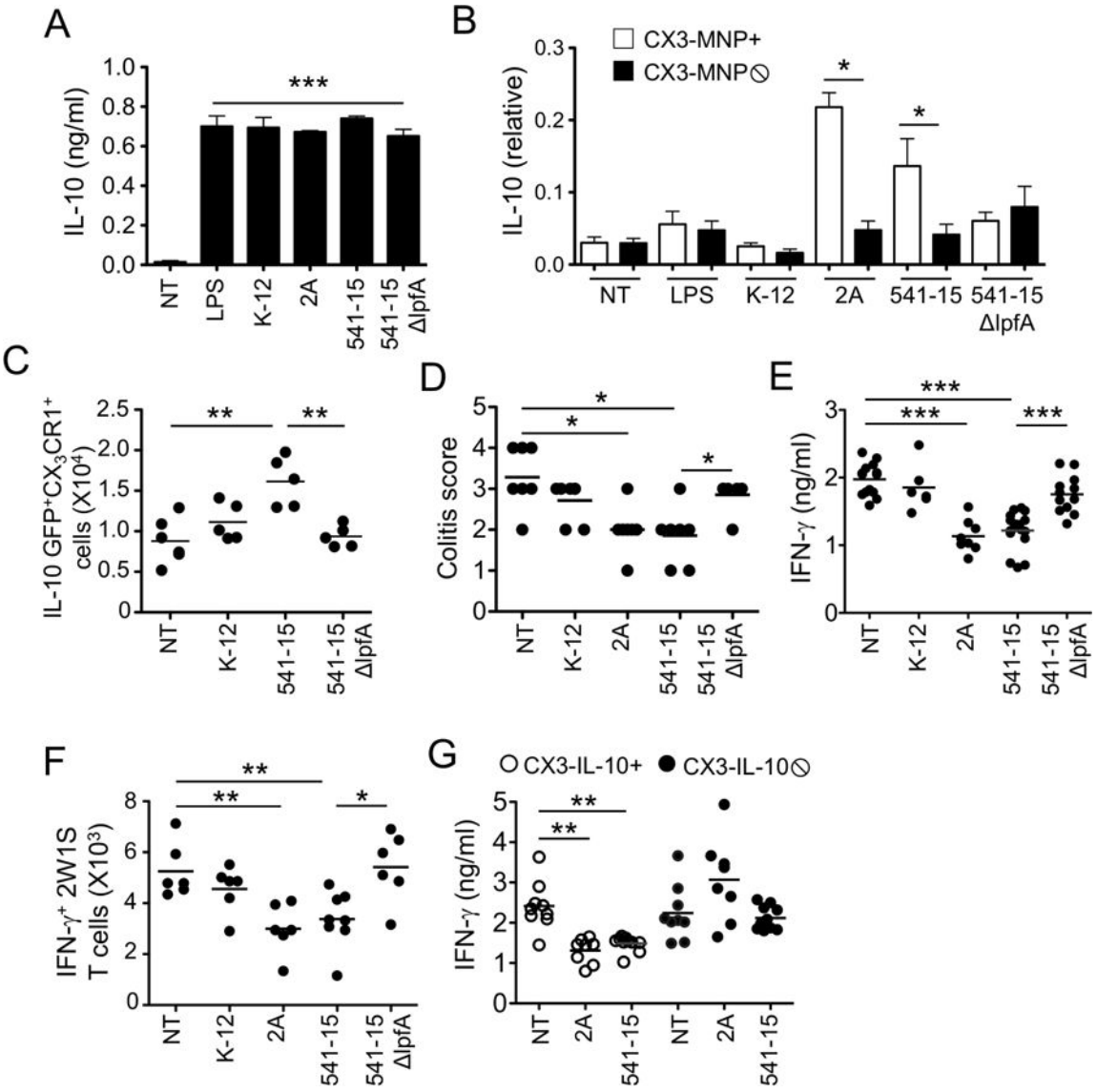

Figure 7. Microbial adhesion to epithelium is critical for induction of anti-inflammatory function of CX_3CR1^+ MNPs

(A) RAW macrophages were cultured with LPS or different *E. coli* strains and IL-10 was measured by ELISA 24hr later. (B) Littermate ABX and DT treated CX3-MNP+ (intact CX_3CR1^+ MNPs) and CX3-MNP⊘ (depletion of CX_3CR1^+ MNPs) mice were colonized with indicated *E. coli* strains or treated with LPS. 5d later, IL-10 expression by isolated total lamina propria cells was analyzed by qPCR. (C) Absolute numbers of IL-10 expressing CX_3CR1^+ MNPs ($CD11b^+MHCII^+CX_3CR1^+$) in ABX-treated IL-10 reporter (VertX) mice colonized with indicated *E. coli* strains were analyzed by flow cytometry. (D, E) Antibiotic treated CX3-MNP+ mice colonized with indicated *E. coli* strains were infected with *Salmonella*. 12d later, blinded H&E stained colons were scored for colitis (D) and *Salmonella* specific IFN-γ production was measured as in Figure 1B (E). (F) CX3-MNP+ mice were treated as above before infection with *Salmonella* expressing the 2W1S T cell epitope. 12d later, 2W1S recognizing T cells was analyzed by flow cytometry. Absolute cells

numbers are shown. (G) Littermate ABX and 4OH treated CX3-IL-10+ (normal IL-10 expression by CX_3CR1^+ cells) and CX3-IL-10⊘ mice (lacking IL-10 expression by CX_3CR1^+ cells) colonized with indicated *E. coli* strains were infected with *Salmonella*. *Salmonella* specific IFN-γ production by $CD4^+$ T cells was measured as in Figure 1B. Error bars represent the SEM (A, B). Data points represent a single mouse, bar represents geometric mean (C-G). Data shown is representative of 2-3 independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. Mann-Whitney U-test or ANOVA with Bonferroni posttest correction. Asterisks indicate significant differences from NT group (A) or indicated comparison (B–G). Please see also Figure S7.