Addendum to: Critical Role for the Microbiota in CX3CR1+ Intestinal Mononuclear Phagocyte Regulation of Intestinal T Cell Responses.

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Abstract

Intestinal damage driven by unrestricted immune responses against the intestinal microbiota can lead to the development of inflammatory diseases including inflammatory bowel disease. How such breakdown in tolerance occurs alongside the mechanisms to reinforce homeostasis with the microbiota are a focus of many studies. Our recent work demonstrates coordinated interactions between intact microbiota and CX3CR1 expressing intestinal antigen presenting cells (APCs) that limits T helper 1 cell responses and promotes differentiation of regulatory T cells (Treg) against intestinal antigens including pathogens, soluble proteins and the microbiota itself. We find a microbial attachment to intestinal epithelial cells is necessary to support these anti-inflammatory immune functions. In this addendum, we discuss how our findings enhance understanding of microbiota-directed homeostatic functions of the intestinal immune system and implications of modulating this interaction in ameliorating inflammatory disease.

Introduction

In the human body, dynamic interactions between the host and microbiota underlie many critical homeostatic functions. The human intestine provides a hospitable environment for the microbes while the microbiota provides numerous benefits to the host, including facilitating nutrient breakdown and absorption.1,2 Interactions between the host and microbiota shapes the development and responsiveness of both the mucosal and systemic immune systems, allowing for the induction of protective immunity against pathogens while also limiting aberrant inflammatory responses against the microbiota and self-antigens. Studies in germ-free mice demonstrate the importance of the microbiota in shaping host immunity as germ-free mice have reduced immune cellularity as well as a lack of organized structures such as B cell germinal centers.1,3 Further, these mice display reductions in effector molecules such as antimicrobial peptides and IgA that lead to reduced barrier function.4 Thus germ-free mice are more susceptible to opportunistic infections, consistent with loss of colonization resistance and reduced mucosal barrier function.4

It has become increasingly clear that alterations in the microbiota, also known as dysbiosis, develop along with inflammatory diseases in both humans and mouse models. Compositional changes in the microbiota are observed in inflammatory bowel disease (IBD),5 including Crohn’s disease and ulcerative colitis,6,7 as well as other diseases such as diabetes,8 asthma,9 and rheumatoid arthritis.10 In IBD patients, reductions in potentially anti-inflammatory microbes such as Bacteroidetes, Lachnospiraceae,6 and Faecalibacterium prausnitzii7,11 have been observed alongside increases in potentially inflammatory microbes such as Proteobacteria.12-14 Further, mucosa-associated bacteria is increased including Enterobacteriaceae, Pasteurellaceae, Veillonellaceae, and Fusobacteriaceae.5,12-16 This includes finding of increased adherent-invasive E. coli (AIEC). Similar changes have been observed in mouse colitis models.17,18 Given the importance of the microbiota...
in directing development and functions of the immune system, it is likely that these compositional changes contribute to the pathogenesis of associated disease. Supporting a role for the microbiota in inflammatory disease, germ-free mice are resistant to a number of models of colitis, including in IL-10 deficient mice\textsuperscript{19} and T cell transfer colitis.\textsuperscript{20} These results are consistent with studies demonstrating that mice defective in pathways of microbial recognition have reduced sensitivity in colitis models.\textsuperscript{21,22}

While it is clear that the microbiota helps shape local and systemic immunity it is not completely understood how it does this. Further, it is unclear the impact of select members of the microbiota and how they can regulate distinct immunological outcomes.

**Microbiota limits pro-inflammatory Th1 responses against intestinal pathogens**

In our recent work,\textsuperscript{23} we set out to understand how microbiota shapes the balance between pro- and anti-inflammatory T cell responses. We utilized the enteric pathogen, *Salmonella* Typhimurium to determine the role of the microbiota in the regulation of pathogen-specific T cell responses. T helper 1 (Th1) cell responses are required to protect from *Salmonella* infection.\textsuperscript{24,25} While Th1 responses are crucial for clearance of intracellular pathogens, they are also associated with inflammatory disorders and autoimmune disease.\textsuperscript{26} Interferon-γ (IFN-γ), the prototypic Th1 cell effector cytokine, can induce tissue pathology associated with infectious disease.\textsuperscript{27} IFN-γ directly increases epithelial permeability both in vivo and in vitro\textsuperscript{28-30} resulting in the potential for increased food antigen, bacteria and bacterial products entering the mucosa alongside elevated local immune responses.\textsuperscript{31} Therefore, IFN-γ must be tightly regulated to mediate pathogen clearance while limiting unintended tissue damage.

To understand the impact of the microbiota on induction of Th1 cell responses against *Salmonella*, we utilized antibiotic treatment that could reduce the intestinal microbial load to the limit of detection. We utilized antibiotics instead of performing our analysis in germ-free mice because germ-free mice have severe intestinal and immune defects including altered intestinal villus structure, loss of immune cellularity and organization as well as loss of intestinal antigen presenting cells.\textsuperscript{3,32} When we compared the *Salmonella*-specific T cell response in the mesenteric (MLN) from animals with an intact or antibiotic-depleted microbiota, we found an increase in IFN-γ producing, *Salmonella*-specific T cells in animals with a disrupted microbiota. Importantly, we did not find increased *Salmonella* in the MLN that could be driving the enhanced response. Further, we did not find global alterations in T cell responses further supporting the antigen specificity of this regulation. These findings strongly suggest that signals from the microbiota regulates host immunity to control intestinal inflammation after pathogen infection.

**Anti-inflammatory function of CX\textsubscript{3}CR1\textsuperscript{+} APCs depends on the microbiota**

We then sought to identify the intestinal antigen presenting cell (APC) that could drive this enhanced response. Within the intestine exist a number of APCs which can detect and respond to luminal antigens. APCs can be subdivided based on expression of CD103 or CX\textsubscript{3}CR1 (also known as fractalkine receptor), and each group has distinct roles in maintaining intestinal homeostasis.\textsuperscript{33} CX\textsubscript{3}CR1\textsuperscript{+} APCs arise from monocyte precursors\textsuperscript{34} which differentiate within the intestine into effector cell populations with characteristics of dendritic cells and macrophages.\textsuperscript{35-37} While the microbiota likely regulates a number of signaling pathways and immune cell populations, we previously found CX\textsubscript{3}CR1 expressing APCs are an essential target of regulation by the microbiota.\textsuperscript{37,38} Using mice where we could selectively deplete CX\textsubscript{3}CR1\textsuperscript{+} APCs\textsuperscript{37,38} we demonstrated the enhanced Th1 cell response after antibiotic treatment was lost after depletion of CX\textsubscript{3}CR1\textsuperscript{+} APCs. Further, antigen presentation by CX\textsubscript{3}CR1\textsuperscript{+} APCs was required for this enhanced *Salmonella*-specific T cell response. Interestingly, in the presence of the intact microbiota, depletion of CX\textsubscript{3}CR1\textsuperscript{+} APCs resulted in an enhanced Th1 cell response, as did loss of antigen presentation by CX\textsubscript{3}CR1\textsuperscript{+} APCs, indicating these cells normally function to limit inflammatory T cell responses. This indicates the requirement of intact microbiota for CX\textsubscript{3} CR1\textsuperscript{+} APCs to limit Th1 cell responses.

We next asked if CX\textsubscript{3}CR1\textsuperscript{+} APCs could promote anti-inflammatory responses to soluble antigens, an important mechanism to limit immunity against antigens derived from food. Using a model
of oral tolerance to ovalbumin, we found that tolerance could not be induced when CX3CR1+ APCs were depleted. This was due to reduced induction of ovalbumin-specific regulatory T cells (Tregs). As with the Salmonella responses, this also depended on antigen presentation by CX3CR1+ APCs. Interestingly, we found oral tolerance to ovalbumin was defective in animals treated with antibiotics. Similarly, it has been reported that germ-free mice have defective oral tolerance. 39 In the context of an intact microbiota, ovalbumin-specific T cells differentiated into FoxP3+ single-positive Treg cells. After antibiotic treatment, however, we additionally found differentiation to FoxP3+RORγt+ double-positive T cells or RORγt+ Th17 cells. These findings suggest that the intact microbiota promotes Treg cells while suppressing inflammatory Th17 cell responses against food antigen and this regulation depends on CX3CR1+ APCs.

Within the intestine, it is also important to limit T cell reactivity to the microbiota itself. To ask if CX3 CR1+ APCs also had an anti-inflammatory role in limiting microbiota-specific T cells, we depleted CX3 CR1+ APCs in the T cell transfer model of colitis. We found that animals depleted of CX3CR1+ APCs had increased weight loss and intestinal pathology alongside reduced numbers of Treg cells and increased Th1 and Th17 cells. Overall, our data show that CX3 CR1+ APCs, in the context of the normal microbiota function to limit inflammatory T cell responses and promote regulatory T cell responses.

One of the hallmark cytokines secreted by CX3 CR1+ APCs is the anti-inflammatory cytokine IL-10. 40,41 We found that CX3CR1+ APCs from animals with a disrupted microbiota expressed significantly reduced levels of IL-10. Further, when we infected mice whose CX3CR1+ APCs lack IL-10 production with Salmonella, we found an increased Salmonella specific Th1 response. If these mice were treated with antibiotics first, however, we did not observe an altered Salmonella specific Th1 cell response. These data suggest that the intact microbiota drives IL-10 production by CX3CR1+ APCs which provides a critical signal to suppress pathogen-induced inflammatory T cell responses. In line with significant role for IL-10 induced by the intact microbiota, we also found reduced generation of ovalbumin-specific Treg cells in ovalbumin fed mice who lacked IL-10 production by CX3CR1+ APCs. Overall, our data show that IL-10 production of CX3CR1+ APCs is a key molecular mechanism of microbiota regulation of anti-inflammatory T cell responses.

**Microbial attachment can drive host anti-inflammatory response**

The intestine and intestinal immune system must detect and properly respond to complex microbiota-derived signals including a large number of pathogen-associated molecular patterns (PAMPs) and metabolites. Identifying specific microbiota signals with the capacity to limit intestinal inflammatory responses is a key for treating inflammatory diseases. PAMPs such as LPS can directly induce IL-10 production by macrophages but we found in vivo treatment with LPS was insufficient to induce IL-10 production by CX3CR1+ APCs in antibiotic-treated mice. Instead, we found that colonizing antibiotic-treated mice with human mucosa-derived AIEC 12,16 was sufficient to induce IL-10 production in vivo. In a model of Salmonella in antibiotic-treated animals, animals depleted of CX3CR1+ APCs lost this effect. Importantly, AIEC with reduced epithelial attachment 15 was unable to induce IL-10 production or reduce Th1 responses. Overall, our data show that microbial attachment to intestinal epithelium leads to anti-inflammatory function of CX3CR1+ APCs (Figure 1(a)).

**Concluding remarks**

Understanding the signals that limit intestinal inflammation and promote homeostasis are a major focus in the field of mucosal immunology. In our work, we have demonstrated that important anti-inflammatory signals are relayed through direct contact of the microbiota with the intestinal epithelium and highlight potential epithelial contribution to mucosal responses to intestinal microbes. These signals, which could be microbe or epithelial derived, are integrated by CX3CR1+ intestinal APCs. Defining these signals is an area of active investigation. CX3CR1+ APCs, through antigen presentation and cytokine secretion limit the expansion of antigen-specific Th1 cells and
promote the differentiation of antigen-specific Treg cells. As the balance between T effector and Treg cells supports intestinal homeostasis, this cellular pathway likely limits inflammatory conditions such as IBD. 43

Critical questions remain regarding how microbial signals are relayed between distinct immune populations in the intestine. For example, other intestinal APCs such as CD103+ dendritic cells (DCs) are also thought to promote Treg cell responses in the intestine. 44 It will be important to understand if the function of these cells is regulated by CX3CR1+ APCs or directly influenced by intestinal microbes (Figure 1(b)). It remains an open question which antigen presenting cell is driving the enhanced Th1 cell response after depletion of CX3CR1+ APCs. Further, other intestinal cell populations are likely part of a feedback loop within the intestine. Recent work has shown that intestinal LAG3+ Treg cells restrain inflammatory cytokine production by CX3CR1+ APCs during models of colitis. 45 It will be important to understand the role of microbial signals in this loop as well as the cellular network of communication between individual intestinal immune cell populations (Figure 1(b)).

Additional critical questions remain regarding the regulation of intestinal immunity by the microbiota. First, is microbial attachment to the epithelium sufficient to induce IL-10 and limit inflammatory responses to intestinal antigens? Further, how is the signal from attached microbes being relayed to the immune system (Figure 1(c)). Attachment could allow for increased local concentrations of microbial products or epithelial signaling could relay information about the attached microbe. We know intestinal epithelial cells secrete a number of mediators such as serum amyloid A (SAA) which activates inflammatory Th17 cell responses. 46 It will be important to define the epithelial mediators that activate anti-inflammatory pathways along with the upstream microbial drivers. One of the AIEC, we utilized to induce IL-10 enhances intestinal pathology in IL-10 deficient animals. 16 The balanced response to such pathogens will determine if a microbe can be contained within the lumen or induce tissue pathology. Such microbes could be more pathogenic depending on the genetic makeup of the host who might have defects in anti-inflammatory signals. This underscores the importance in host genetics or alterations in microbial responsiveness in linking the microbiota to intestinal disease. Additionally, for pathogenic organisms, the ability to limit inflammatory responses and clearance would enhance colonization. Of direct human relevance, increased colonization with AIEC has been found by a number of groups when comparing IBD patients with controls. 12,13,16 As we find epithelial adhesion is a critical for microbial induction of anti-inflammatory host immune cell responses, it will be interesting to
understand the selective pressures of microbes to adopt this phenotype.

In our system, we were only looking at outcomes after colonization with a single AIEC. A number of other microbes or microbial products have been identified which can also induce anti-inflammatory responses, including increased Treg cells. This includes microbes such as *Helicobacter hepaticus* and Clostridia species or microbial products or metabolites such as outer membrane proteins and short chain fatty acids. Integration of signals from multiple types of microorganisms and/or their metabolites likely occurs and dictates the overall immune tone of the tissue. It remains to be determined if the activated anti-inflammatory pathways overlap or are distinct. Further, the genetic makeup of the host will determine whether colonization with an organism increases or limits intestinal inflammation. Together, it will be important to assess the overall balance between pro- and anti-inflammatory immune responses after colonization with a more complex microbial community.

As IBD results from dysregulated interactions between the immune system and the microbiota, it is important to understand how normal homeostasis is achieved and maintained. By defining a global anti-inflammatory role for the microbiota in limiting inflammatory T cell responses and/or promoting regulatory T cells against pathogen, soluble antigens and the microbiota, our studies demonstrate how intestinal microbes set the stage for intestinal homeostasis. Understanding how these signals are relayed to underlying immune cells such as CX3CR1+ APCs will define numerous therapeutic opportunities to limit pathology in IBD.

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