Gut Microbes

ISSN: 1949-0976 (Print) 1949-0984 (Online) Journal homepage: https://www.tandfonline.com/loi/kgmi20

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To cite this article: Markus B. Geuking, Kathy D. McCoy & Andrew J. Macpherson (2011) The continuum of intestinal CD4$^+$ T cell adaptations in host-microbial mutualism, Gut Microbes, 2:6, 353-357, DOI: 10.4161/gmic.18604

To link to this article: https://doi.org/10.4161/gmic.18604

Published online: 01 Nov 2011.

Gut Microbes 2:6, 353–357; November/December 2011; © 2011 Landes Bioscience

The continuum of intestinal CD4+ T cell adaptations in host-microbial mutualism

Markus B. Geuking,* Kathy D. McCoy and Andrew J. Macpherson

Maurice Müller Laboratories, DKF; Universitätsklinik für Viszerale Chirurgie und Medizin; University Hospital (Inselspital); University of Bern; Bern, Switzerland

How a mutualistic relationship between the intestinal microbiota and intestinal T cell compartments is established is important, as a breakdown of intestinal T cell homeostasis may cause inflammatory bowel diseases. A number of studies have shown that different bacterial species modulate the intestinal CD4+ T cell compartment in different ways. We performed mechanistic in vivo studies that demonstrated the crucial requirement for regulatory T cells (Treg) and interleukin-10 (IL-10) in the induction of intestinal T cell homeostasis even following colonization with a completely benign microbiota. In the absence of a functional Treg response or IL-10 receptor signaling, the same bacteria that induced a Treg response in wild-type animals now induced T helper type 17 responses, without intestinal inflammation. Therefore, Treg, IL-10 and Th17 are crucial regulatory mechanisms in the intestine not only for controlling inflammation, but also to establish a continuum of CD4+ T cell homeostasis upon commensal colonization.

Keywords: germ-free, altered Schaedler flora, T cell homeostasis, regulatory T cells, interleukin-10, T helper type 17, segmented filamentous bacteria

Submitted: 10/06/11

Accepted: 11/01/11

http://dx.doi.org/10.4161/gmic.2.6.18604

*Correspondence to: Markus B. Geuking; Email: markus.geuking@dkf.unibe.ch

Addendum to: Geuking MB, Cahenzli J, Lawson MA, Ng DC, Slack E, Hapfelmeier S, et al. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. Immunity 2011; 34:794–806; PMID:21596591; http://dx.doi.org/10.1016/j.immuni.2011.03.021

Germ-Free and Gnotobiotic Mouse Models

The normal intestinal microbiota in humans and laboratory mice is extremely complex and harbors up to about a thousand different microbial species with considerable differences in their relative abundance in different individual hosts.[1,2] This variability makes it very difficult to study or understand immunological events occurring as mammals adapt to their commensal microbiota. To simplify the system and allow systematic studies we have used mice that are axenic (germ-free) or gnotobiotic (with a stable and defined simple microbiota). Germ-free mice were raised and housed in flexible-film isolators maintained under positive pressure by ventilation with HEPA-filtered air.[3,4] Importantly, one is not restricted to working with a single strain of wild-type mice for these studies, because almost any mouse strain can be made germ-free by transferring embryos from the strain of interest at the two-cell stage into germ-free pseudo-pregnant females. The surrogate mothers are carefully kept germ-free during and after surgery by aseptic techniques, so that they later deliver germ-free pups of the desired mouse strain. These germ-free pups can then be interbred to establish a colony of the new strain. This germ-free technology has allowed us not only to compare germ-free and colonized mice but also to control and modify the microbial status. Because a germ-free mouse is the best of good culture media, it is fairly straightforward to colonize the germ-free animals either by mono-association with a single species from pure bacterial culture, or with defined more complex microbial consortia consisting of several individual bacterial species. Mono-associated and gnotobiotic mice are also kept in isolators to avoid contamination or variability of the microbiota.

Microbiota-Dependent T Cell Responses

Several groups have studied the T cell response or modulation after intestinal colonization with different microbiotas or

single microbial species. It was initially thought that T helper type (Th) 17 responses, a subset of CD4+ T cells characterized by IL-17 expression, are normally present in the intestine of colonized mice.[5] It was then found that Th17 cells were very abundant in the intestinal lamina propria of mice sourced from the Jackson Laboratory but less so in animals bred by Taconic farms.[6] Follow-up studies then demonstrated that Jax mice contained intestinal segmented filamentous bacteria (SFB), which are very potent inducers of intestinal Th17 cells.[7,8] SFB have very tight interactions with intestinal epithelial cells: for example, in electron micrographs SFB indent epithelial cells so dramatically that the appearance is of stabbing with a sword.[9] Thus while SFB are widely present in mouse colonies (from some suppliers) their behavior is probably rather atypical for commensal bacteria. We therefore wanted to ask whether induction of Th17 cells in the intestinal mucosa is part of a general immune adaption to commensal bacteria, or just a specific immune response required to control the invasive behavior of SFB.

Other microbes can also modulate the intestinal CD4+ T cell population. For example, a very complex mixture of clostridial species has been shown to induce strong regulatory T cell (Treg) responses both in the intestinal mucosa and in the systemic immune system.[10] The very complexity of this mixture (because Clostridia are both commensals and pathogens) and the lack of compartmentalization of the immune responses makes it hard to know how far the system is representative of mechanisms seen with a benign commensal microbiota.[11-14]

Mutualistic Intestinal T Cell Adaptation

Our objective was to eliminate the possibility of pathogens or pathobionts in the intestine and understand how intestinal T cells adapt the host to the presence of a strictly benign commensal flora. To do this we decided to use the altered Schaedler flora (ASF) consisting of the following eight bacterial species: *Lactobacillus acidophilus* (*ASF 360*), *Lactobacillus murinus* (*ASF 361*), *Bacteroides distasonis* (*ASF 519*), *Mucispirillum schaedleri* (ASF 457), *Eubacterium plexicaudatum* (ASF 492), a Fusiform-shaped bacterium (ASF 356) and two Clostridium species (ASF 356, ASF 502). This combination of bacterial species provides a simple balanced physiological flora that colonizes the gut and limits the variability that is associated with more complex intestinal colonization.[15]

To define physiological intestinal CD4+ T cell adaptations following colonization with this ASF, we co-housed adult germ-free mice with an ASF-colonized sentinel for four weeks. Co-housing germ-free mice and ASF-colonized mice caused the germ-free animals to acquire the stable ASF flora reproducibly and rapidly without further manipulation. We then compared the trajectory of Th1, Th17 and Treg responses in the colonic lamina propria with those in the spleen, mesenteric lymph nodes (MLN) and Peyer's patches (PP). While ASF colonization consistently resulted in a 2-fold increase in the proportion of Foxp3+ Treg in the colonic lamina propria, Treg proportions were unchanged in other organs. These Treg increases are therefore selective for the mucosal immune system and were shown to be a general phenomenon, found in a series of different wild-type mouse strains.

The next question was whether Treg increases were coming from thymus-derived natural nTreg or from induced iTreg? It has recently become possible to distinguish these two different populations of Foxp3+ Treg based on differential expression of the transcription factor Helios.[16] Helios+ Treg are thymus-derived natural nTreg, whereas Helios- Treg are probably de novo peripherally-induced iTreg. We found that the colon lamina propria Treg population in germ-free mice was almost exclusively Helios+ while ASF colonization resulted in generation of a Helios- Treg population, indicating commensals induce de novo generation of iTreg. We did not see significant increases in Th1 or Th17 cells following ASF colonization in any of the wild-type mouse strains that we tested.

Some subsets of intestinal Treg express IL-10 and IL-10 deficiency is associated with intestinal inflammation in both mice and humans.[17,18] We therefore examined IL-10 expression in CD4+ T cells before and after ASF colonization. Just as the Treg increase following colonization was found only in the intestinal lamina propria, increased IL-10 expression post colonization was restricted to colonic lamina propria T cells and not seen in CD4+ T cell populations in other systemic lymphoid organs, as reported following colonization with clostridia.[10] The IL-10 induction following ASF colonization is functionally important. We showed this by blocking the IL-10 receptor (IL-10R) in vivo with a neutralising antibody: when we blocked the IL-10 response in this way during colonisation there was strong induction of intestinal Th17 cells and a weak intestinal Th1 induction. This finding was consistent with recent reports showing that Th17 cells are directly controlled through intrinsic IL-10R signaling.[19,20] Encouraged by the compartmentalized Treg response and the absence of Th1 and Th17 responses following ASF colonization we reasoned that this was a realistic model for intestinal T cell adaptation in response to truly benign commensal colonization without the occurrence of intestinal inflammation. We had shown that the IL-10 response is important in this setting to limit intestinal Th17 cell induction, but we were left with the issue of what happens if the Treg response as a whole is defective?

In order to address the functions of the intestinal Treg response to colonization with commensal bacteria, we used a mouse strain where this response was defective. This was the SMARTA mouse, which expresses a transgenic CD4 T cell receptor (TCR) specific for a peptide of the lymphocytic choriomenigitis virus (LCMV) glycoprotein.[21] It may seem rather counterintuitive that we studied this strain at all during the commensal project, but about 10 y previously we had observed that some animals of this strain (maintained at the time in a vivarium with a rather diverse intestinal microbiota) had spontaneous rectal prolapse—indicative of likely intestinal inflammation.

In fact, in this study, we found that although the proportion of natural (Helios+) Treg in the lamina propria of germ-free SMARTA mice was equal to that in germ-free wild-type mice, SMARTA mice failed to expand and

generate Helios$^-$ Treg following ASF colonization. Thus the relevance of the LCMV specificity in this setting is that the transgenic TCR causes an extremely narrow T cell repertoire (to an extrinsic antigen that the mice never see) that is insufficient for the induced intestinal Treg response during commensal colonization.

Using the SMARTA model, we could now look at the consequences of commensal colonization on the mucosal immune system in the absence of Treg induction. Here we found that both intestinal mucosal CD4$^+$ Th1 and Th17 populations were strongly induced. Of course, this on its own did not prove that defective Treg induction was the cause of the CD4$^+$ subset disequilibrium, because the SMARTA mouse might have had other defective immune adaptations upon colonisation. However, we refined the experiment by transferring sorted wild-type Treg cells into germ-free SMARTA animals through intravenous injection prior to colonization: this showed that restoring Treg competence protected SMARTA mice from the induction of Th1 and Th17 responses as they became colonized with commensal bacteria.

The presence of potent Th1 and Th17 responses in the absence of Treg induction also demonstrated the powerful nature of the ASF species combination. It is not the case that the ASF fails to deliver the right stimulus to induce effector T cell responses and therefore by default induces a Treg response. The induction of Th1 and Th17 responses in SMARTA mice was also not due to differential microbial colonization in the absence of a functional Treg response. The abundance (determined by 16S rRNA sequencing) and the behavior (determined by electron microscopy) of the ASF species in wild-type and SMARTA mice was identical. This demonstrated that induction of T cell homeostasis is a fundamental Treg intrinsic effect and not extrinsically mediated by differences in the microbiota.

Altered Schaedler Flora (ASF) vs. Segmented Filamentous Bacteria (SFB)

As described earlier in this Addendum, SFB induces a strong Th17 response even in the presence of a functional and induced Treg compartment.[7] We verified this in our experiments, but have shown that the ASF colonisation only did so when the Treg response was defective. This does not necessarily mean that these Th17 responses are fundamentally different. The Th17 response in each situation may reflect a continuum of mucosal immune responses that ensure mutualism. It is possible that a pure Treg response is not enough to ensure mutualism with SFB, perhaps due to its intimate contact with the epithelium,[7,8] and that a Th17 response is useful to ensure mutualism. Similarly, where the Treg response is defective, ASF also induces Th17 (and Th1) responses, which may also be compensatory to achieve mutualism. Importantly, the intestinal Th17 (and Th1) responses observed in SFB-colonized wild-type or ASF-colonized SMARTA mice are not overtly pro-inflammatory since no intestinal inflammation was observed. Therefore, we hypothesize that intestinal CD4$^+$ T cell adaptations in response to bacterial colonization are a continuum ranging from pure Treg responses to a mixture of Treg and non-inflammatory Th17 (or Th1) effector responses depending on the bacterial species present.

What About IgA?

It has been suggested that the induction of Treg and intestinal IgA is a coordinated event.[22] One might therefore expect that the defective Treg response following colonization of SMARTA mice would result in a defective secretory IgA response. We did not detect any difference in the amount of total IgA secreted into the lumen of wild-type or SMARTA mice following ASF colonization (**Fig. 1**). Furthermore, we have previously demonstrated that the severe innate deficiency in *Myd88*$^{-/-}$*Ticam-1*$^{-/-}$ mice results in a breakdown of mucosal immune compartmentalization and systemic bacterial priming.[23] Interestingly, these mice also displayed a defect in Treg induction following ASF colonization[24] but they produce normal levels of intestinal IgA.[23] Exactly how a defective Treg response in the intestine influences the induction of secretory IgA therefore remains an open question.

Mucosal Immune Mutualism during Intestinal Damage

We also addressed the function of ASF-induced intestinal Treg in a pathological model of dextran sulfate sodium (DSS)-induced damage of the colon epithelium. ASF-colonized wild-type C57BL/6 and SMARTA mice were treated with 2% DSS in drinking water for seven days and allowed to recover for three days without DSS. This treatment induced a massive Treg response in the colon lamina propria of wild-type mice with no induction of Th1 or Th17 cells. In contrast, SMARTA mice again failed to increase their Treg population in the colon lamina propria following DSS treatment and Th17 responses were highly induced. With a very limited benign microbiota, these Th17 responses were not pro-inflammatory since no overt intestinal inflammation could be

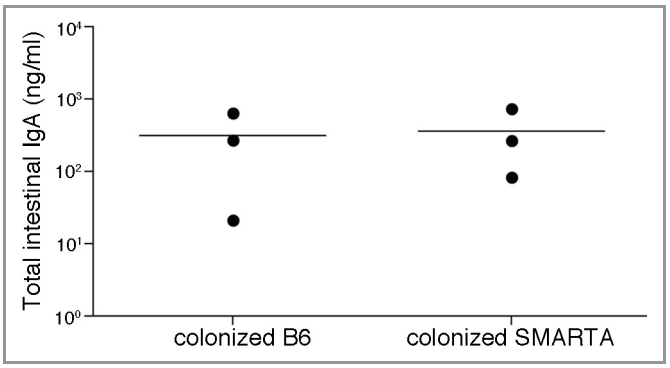

Figure 1. Germ-free wild-type C57BL/6 and Treg-defective SMARTA mice were colonized with ASF for 28 d. The level of total IgA in intestinal washes of both groups of mice was measured by ELISA.

detected. Therefore this Th17 response might again reflect a compensatory response in the absence of Treg induction to ensure mutualism and protect the mucosa. Since the DSS model causes colitis in specific pathogen free (SPF) mice, this indicated that in the presence of a complex SPF flora inflammation, such as neutrophil recruitment, might be the next compensatory mechanism to attempt and re-establish mutualism. The idea of Th17 cells being tissue protective is not new since IL-22, a Th17-derived cytokine, has been previously demonstrated to protect mice from inflammatory bowel disease.[25] In addition, although intestinal Th17 responses have been suggested to promote experimental autoimmune encephalomyelitis (EAE),[26] they have also been demonstrated to be protective following infection with *Citrobacter rodentium*.[8] Similar to our findings, Clostridia-mediated Treg responses have been shown to ameliorate DSS-induced colitis in SPF mice that contained Clostridia compared with SPF mice not harboring a significant amount of Clostridia.[10]

Conclusion

Taken together, intestinal and systemic $CD4^+$ T cell adaptations to intestinal colonization with specific bacterial species have been studied in a variety of systems.[7,8,10,24,27,28] Depending on the model used, different subsets of the $CD4^+$ T cell compartment seemed to provide the dominant response following colonization. We have to keep in mind that bacterial species in very complex real-life microbiotas range from truly mutualistic through pathobionts to frank pathogens. Therefore a variety of different innate and adaptive immune mechanisms need to act in concert to control such a complex microbiota. We propose a model whereby there is a continuum of different $CD4^+$ T cell responses that ensure mutualism. The intestinal Treg response is the early fundamental component but if the Treg response is dysfunctional or is over-ridden by slightly more aggressive species (such as SFB) additional non-inflammatory Th17 (and/or Th1) responses are required. In the case of severe bacterial translocation or intestinal damage these responses must sacrifice intestinal function with inflammation to clear the pathogen.

Germ-free and gnotobiotic mouse models have been crucial in identifying discrete immune mechanisms in response to individual species, or more important, in response to different more or less complex consortia of bacterial species. We believe that these models will continue to allow us to dissect the immunological adaptations in response to commensal colonization in increasing detail to understand and treat the different consequences of our intestinal microbiota on our health.

Acknowledgments

This work was funded by grants from the Swiss National Science Foundation, Canadian Institute for Health Research, Crohn's and Colitis Foundation Canada and the Canada Research Chairs program. We would also like to acknowledge the Farncombe family, the Chefärzte of the Inselspital and the Genaxen foundation for their generous support.

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