

Role of Commensal Flora in Mucosal Immune Development

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Microbial species are able to colonize and populate an incredibly diverse range of different microenvironments on our planet. However, while the density of the microbiota found in soils or other geological or marine habitats typically reach up to 10^8 microbes/gram,¹ the mammalian lower intestine is home to at least 400 different microbial species, and densities reach up to 10^{12} microbes/gram of luminal contents.² Our commensal microflora therefore outnumbers the eukaryotic cells in our body by an order of magnitude. This demonstrates that our intestines constitute very good culture media. Mammals therefore coexist, mostly peacefully, with an extremely dense and diverse load of microbes in the lower intestine.³

Within the intestine, only a single layer of intestinal epithelial cells physically separates this massive load of bacteria from the inside of our body, which essentially remains sterile. Importantly, the mucosal immune system, which likely contains the majority of lymphocytes in the body,⁴ is strategically positioned directly opposing the luminal bacteria. Despite its close proximity to a multitude of bacterial antigens, in most human individuals and wild-type animals the host mucosal immune system enjoys peaceful mutual coexistence with the intestinal microbiota.

It is clear that the mucosal immune system is not ignorant of the presence of intestinal bacteria because a vigorous intestinal IgA response directed against the commensal microflora can be measured and a significant proportion of this IgA is induced through a T-independent pathway.⁵ Commensal organisms that penetrate past the epithelial barrier are efficiently killed by macrophages. In contrast, bacteria that are sampled by local intestinal dendritic cells are brought to the mesenteric lymph nodes (MLNs), where an efficient anticommensal IgA response is

induced. The MLNs are not absolutely required for induction of IgA, but they act as a barrier to restrict the commensal-laden dendritic cells from entering the systemic immune system, which in turn limits systemic priming and induction of systemic immunopathology.⁶

It is therefore clear that the mucosal immune system is neither ignorant nor tolerant of the intestinal microbiota. In neonates, however, the intestinal environment is sterile. Colonization of the intestine (and other body surfaces) begins only after the newborn is exposed to the external environment. The composition of the microflora is built up by successive waves of organisms that colonize the intestinal tract after birth⁷; once an individual is fully colonized, the composition of microbes remains reasonably stable.⁸ This successive colonization of the intestine occurs during the first few years of life, a window of time that also corresponds to a critical period of immune development and maturation. Unfortunately, investigation of the mechanisms by which early bacterial exposure influences immune development is very difficult to assess in humans because one cannot easily determine or manipulate microbial exposure. However, germ-free mice provide a powerful tool to investigate how the intestinal microflora can shape the developing immune system. Germ-free mice do not harbor any microorganisms in their intestines or other body surfaces, making it possible to use them to directly assess the impact of colonization in vivo. Deliberate colonization of germ-free mice allows the researcher to precisely manipulate the time, dose, and diversity of the bacterial exposure. Germ-free animals are bred and housed in flexible-film isolators that are maintained under positive pressure. Virtually any mouse strain of interest can be rederived to germ-free status through 2-cell embryo transfer into germ-free pseudopregnant recipient females.⁹

Analysis of germ-free animals has provided clear evidence that the absence of microbial stimulation has profound effects on development of the mucosal immune system.¹⁰ Immediate

downstream effects of the absence of intestinal microbiota include reduced numbers and size of Peyer's patches (PP), decreased intestinal IgA-secreting plasma cells, decreased lamina propria CD4⁺ T-cell numbers, and alterations in the T-cell content of the intraepithelial compartment.^{10,11} We have found that colonization of germ-free mice leads to a marked increase in CD3⁺ and B220⁺ cells in the intestinal lamina propria (Fig 1). The T-cell populations that enter the lamina propria are composed of both CD4⁺ and CD8⁺ T-cell subsets (data not shown).

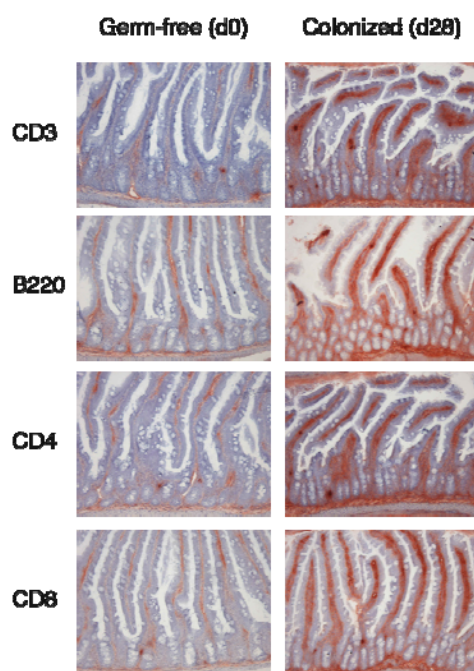


Fig 1. Colonization of germ-free mice leads to increased numbers of lamina propria B and T lymphocytes. The small intestine and colon (as indicated) were taken from germ-free C57BL/6 mice (left column) or from germ-free littermates 21 days postcolonization (right column) and analyzed for the presence of B cells (B220) and CD3⁺ T cells, as indicated. Photographs show sections at x10 magnification.

We have found that the number of intestinal CD4⁺ T cells increases over 100-fold in serial studies over 28 days after germ-free animals acquire a limited defined commensal flora. A smaller CD4⁺ increase and an oligoclonal expansion of CD8⁺ cells on colonization also have been observed in the intraepithelial compartment.^{12,13} The total number of B and T lymphocytes in the MLNs also increases dramatically following colonization. Exposure to commensal bacteria

also induces activation and differentiation of mucosal lymphocyte populations. In B cells there is strong induction of class-switch recombination to IgA, and IgA-secreting plasma cells increase dramatically following colonization of germ-free animals (Fig 2). Accordingly, the total level of SIgA found in the intestinal lumen also increases significantly.

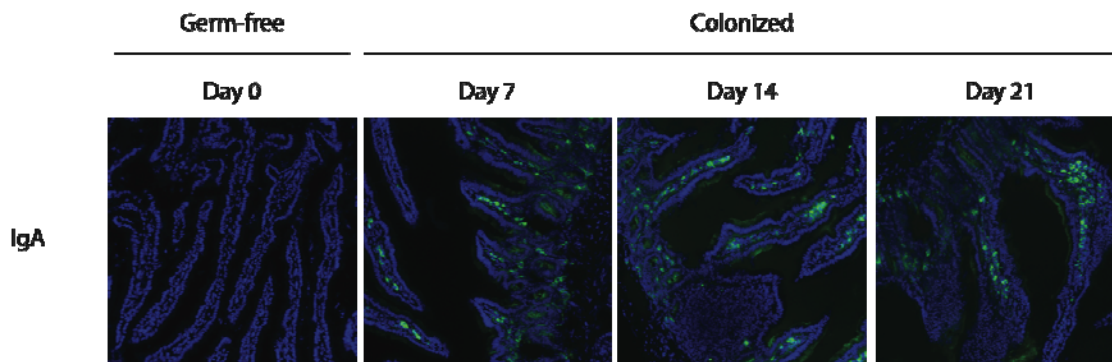


Fig 2. Colonization of germ-free mice leads to increased IgA in the intestinal lamina propria. The small intestine was taken from germ-free C57BL/6 mice (left panel) or from germ-free littermates 7, 14, and 21 days postcolonization, as indicated, and analyzed for the presence of IgA. Photographs show sections at x10 magnification.

We have developed a flow-cytometry-based method of measuring bacterial antibody binding that is specific for the immunizing strain, and extremely sensitive for all isotypes, with very small antibody and bacterial samples required. Using this method we have found that a specific IgA response is induced against the immunizing strain of commensal bacteria—colonization of germ-free mice with *Enterobacter cloacae* generated only IgA specific for *E cloacae* and not for *Escherichia coli* (Fig 3, upper panels). Conversely, monocolonization with *E coli* induces specific IgA that does not bind to *E cloacae* (Fig 3, lower panel). Colonization of the intestine with bacteria therefore induces maturation of the mucosal immune system and induction of specific anticomensal reactivity.

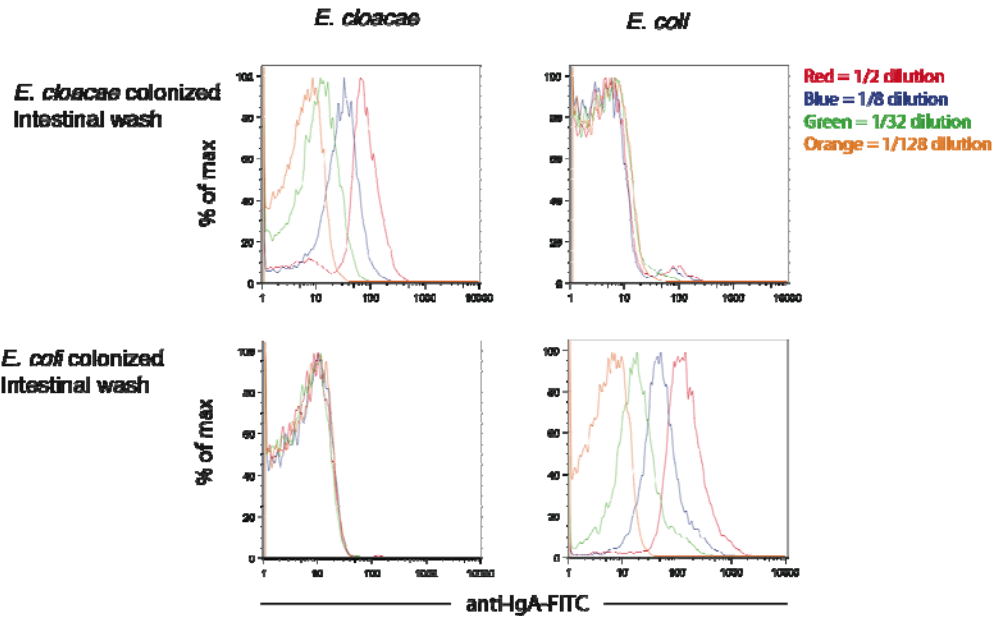


Fig 3. Bacterial surface staining using flow cytometry allows specific detection of antibacterial antibodies with minimal cross-reactivity. Germ-free C57BL/6 mice were monocolonized with *E. cloacae* or *E. coli*. Intestinal lavage was performed and the presence of anti-*E. cloacae*- (upper left panel) or anti-*E. coli*- (lower right panel) specific IgA was determined by flow cytometry using an anti-IgA-detecting antibody.

We currently are studying the functionality of the large intestinal CD4⁺ T-cell response generated by colonization. The function of normal CD4⁺ T cells in the lamina propria is especially important—while activated Th1 lamina propria lymphocytes have been traditionally seen as proinflammatory, it has been shown that CD4⁺ T cells are required to limit translocation of commensal bacteria,¹⁴ probably through the activation of biocidal activity in subepithelial macrophages.¹⁵ A further indirect line of evidence that the CD4 response is functional in maintaining the barrier against commensals is that the induction of lamina propria CD4 cells (germ free → colonized) is increased in strains with no protective antibody secretion, probably as a compensation to increased penetration of intestinal bacteria through the epithelial cell layer.¹⁰

The absence of microbial stimulation also has profound effects on development of the systemic immune system. Germ-free mice display systemic lymphopenia, hypoplastic secondary

lymphoid structures with reduced B- and T-cell content, and poorly formed high endothelial venules (as reviewed in reference 9), whereas colonization can increase CD4⁺ T-cell numbers and normalize the splenic architecture.¹⁶ Exposure to intestinal microbial stimulation also appears to greatly influence the background levels of different antibody isotypes, and germ-free mice display altered levels of spontaneously produced antibodies. While germ-free mice have normal serum levels of total IgM, they have greatly reduced levels of the class-switched antibodies IgA and IgG.¹⁰ The most striking differences in the number of antibody-secreting cells are in the MLNs, which have drastically reduced numbers of IgM-, IgG-, and IgA-secreting cells.¹⁷ In stark contrast, we have found that germ-free mice have elevated levels of natural IgE.¹⁸ Other groups have shown greatly increased IgE positive cells in the PP of germ-free rats,¹⁹ and feeding with certain bacteria or bacterial cell-wall components led to a decrease in the number of IgE-bearing PP cells.²⁰ These results suggest that in addition to shaping the developing mucosal immune system, intestinal microflora also can exert a major effect on the IgE response. It is unclear why, of all the class-switched antibody isotypes, only IgE is elevated in germ-free mice. This observation is potentially important, especially considering the epidemiological evidence in humans that the development of allergy is highly influenced by exposure to microbial stimulation early in life, which forms the basis for the hygiene hypothesis.²¹ It has been postulated that colonization of the intestine with specific bacterial species during immune development is an important factor in protection from allergic and autoimmune diseases.^{22,23}

It is clear that exposure to intestinal microflora can shape the developing immune system. Using colonization of germ-free mice to model the exposure to bacteria that occurs in all mammals after birth will provide valuable insight into the mechanisms by which the microflora

induces maturation and proper regulation of the mucosal, as well as the systemic immune system.

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Q & A

Q: I am not familiar with one technique you used in your IgA work. You said the western blot showed a lot of cross-reactivity. I am familiar with that technique, but not the flow-cytometry-based method of measuring antibacterial antibodies. It strikes me as a very powerful observation that has a lot of potential ramifications. Much of this research is done with western blots where we get a lot of denaturation, exposure of those binding sites, and cross-reactivity. In situ, however, that probably is not the case. What do you think this means?

Dr McCoy: When we looked through our bacterial flow cytometry method, IgA or even IgG anticomensal responses to the surface antigens were specific to each type of bacteria, and there was no cross-reactivity. In the western blots, where we did see cross-reactivity, we tried to determine what the antibodies were actually recognizing. We have not published this yet, but it looks as though when we systemically prime with commensal bacteria to try in a way to model a system in which we cannot handle our commensal flora and it becomes systemic all the time, we get antibodies to bacterial ribosomes. Here we see a lot of cross-reactivity, but probably because those structures are highly conserved between bacterial species. We think these antiribosomal antibodies are protective. So we did cecal puncture and ligation experiments. We punctured the cecum of the animals to release high levels of bacteria into the peritoneum, then ligated to close the punctured cecum. Anticomensal antibodies seemed to be protective and prolonged the life of the animal in response to the commensal bacteria.

Q: The results you described are different from those originally described by Cebra's group [Talham GL et al: *Infect Immunol* 1999;67:1992-2000; Keilbaugh SA et al: *Gut* 2005;54:623-629]. As you know, they compared segmented filamentous bacterium to *Morganella morganii* and also to a Schaedler's Cocktail, which is a defined blend of bacteria used to standardize the

microbiota used to colonize germ-free rodents. Is it possible that you have simply chosen an organism in which the response is directed wholly against the IgA class, and if you used another organism, it is not?

Dr McCoy: Yes, but we are now trying to isolate as many different commensal organisms as we can in the population. We have done about 10 different isolates, and all the IgA in the gut seems to be specific.

Q: Can you account for the difference between your results and those of Cebra's group? Better methodology?

Dr McCoy: Maybe. Certainly a different methodology was used in the research you refer to.

Q: Cebra and the Japanese researchers before him [Umesaki Y et al: *Microbiol Immunol* 1995;39:555-562; Umesaki Y et al: *Infect Immunol* 1999;67:3504-3511] showed that the segmented filamentous bacterium has extraordinary capacity. Just a simple monoassociation with that particular organism that is unculturable at the moment, except in germ-free animals, was capable of extending the whole immune repertoire. To me, this is an extraordinary finding. Do you have a similar understanding about the *E cloacae* you are using?

Dr McCoy: Mazmanian and colleagues used polysaccharide A and got good architectural correction in the spleen [*Cell* 2005;122:107-118]. However, we have not yet done enough monocolonizations to precisely determine what each bacteria may induce. We have not examined enough different bacteria to say whether they all induce the same response or whether different bacteria possess different abilities to mature the immune system. Probably many different bacteria act in concert. If we select one of bacteria, maybe we would see a big response in germ-free mice, but it is hard to say how much that translates into in a real colonization.

Q: The question about western blot vs flow cytometry was interesting. I think it is important to distinguish these two methods. I assume you use a cocktail and sonicate when you do a western blot. Is that correct?

Dr McCoy: Yes. We sonicate to lyse the bacteria, centrifuge to get rid of the membrane fractions, then ultracentrifuge to collect the ribosomes.

Dr Brandtzaeg: In inflammatory bowel disease, the normal, healthy immune response against the bacterial cytosolic antigens shifts more to bacterial surface antigens. The IgG response—IgG1 in ulcerative colitis, for example—is predominately against the surface antigen. Obviously, the immune system sees various things, in various conditions. If there is invasion of a particular organism, the characteristics of the immune response may vary [Furrie E et al: *Gut* 2004;53:91-98].

Q: You mentioned only live commensal flora were triggering and inducing the IgA response, and the dead probiotics did not do that. How were they killed?

Dr McCoy: They were heat killed.

Q: Have you tried any other method of killing?

Dr McCoy: We are trying to kill the bacteria using peracetic acid. That seems to fix the structure and does not destroy it. We are looking at that now to see whether that method of killing can have inductive effects. It seems to be a bit in between, depending on what immune parameters we look at. It was able to induce some IgA, but it does not induce a big T-cell response. We have a new model in which we colonize germ-free mice, and they become germ-free again because the bacteria can no longer propagate. We are using that model to look at bacteria that are alive, dead, or killed in different ways, and probably how intact the bacterial structure is might make a difference.

Q: Some experiments have been conducted with toll-like receptor 9 (TLR9). Do you know whether the germ-free mouse expresses TLR9 in a polarized fashion?

Dr McCoy: We have not looked at that.

Q: If the mesenteric lymph node acts as a fire wall in humans—I know this is debated—that could preclude the entry of probiotics into breast milk through the circulation. If the mesenteric lymph node does act as a fire wall, and it is blocking the entry of commensal flora and pathogens into the circulation, how do probiotics enter the breast milk? Is it possible that there is a unique resident microbiota lining the mammary gland?

Dr McCoy: The mesenteric lymph nodes act as a fire wall for live bacteria, so, of course, microbial products become systemic. When we colonize mice, the live bacteria do not affect the systemic immune system; the microbial products probably produce the effect.

Dr Bienenstock: This area is controversial. There is no question that the breast can push out viruses such as cytomegalovirus. Whether or not commensal probiotic organisms appear in human breast milk still remains an important question that has important implications for this discussion.

Dr McCoy: We should look at lactating germ-free or monocolonized mice where it is easier and cleaner to collect the milk.