"Oral vaccines: a safe and simple tool to eliminate *Salmonella* and other antibiotic-resistant gut bacteria"

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Whilst most of us are happy to think as little as possible about the contents of our intestines, we are all aware of the importance of this system to our health. Below is a small insight into my research revealing the mode of action of intestinal antibodies, and a flavor of how we can apply and extend these findings to tackle enteropathogens and the spread of antimicrobial resistance. I am very honored to be the 2017 recipient of the ETH Latsis prize and would like to thank the Latsis foundation, as well as the ETH Department of Biology and all of my mentors, collaborators and students who have made this work possible.

How does the immune system control bacteria in the intestine?

The large intestine of mammals contains one of the densest consortia of microorganisms found anywhere on the planet – the intestinal microbiota[1]. Health of the host critically depends on excluding food-bourn pathogens from the microbiota, but also on avoiding excessive pathological immunity against beneficial microbes and food[2]. This has to be achieved, despite the fact that relatively few genes can determine the difference between a bona fide pathogen and a common microbiota species[3]. The aim of my research is to unravel the mechanisms that maintain this "dynamic homeostasis" (Figure 1). Simultaneously, we aim to apply this knowledge to develop rational microbiota engineering techniques for application in human and veterinary medicine.

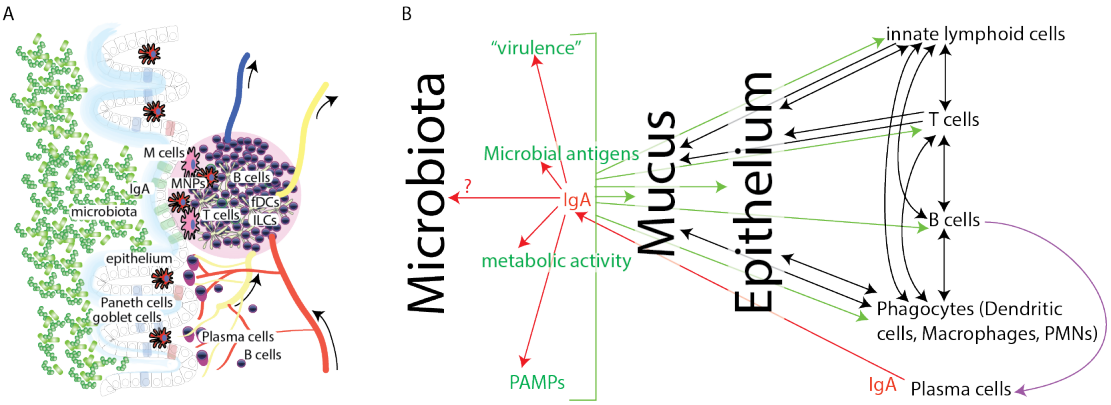

Figure 1: Mechanisms maintaining dynamic homeostasis between the host and its intestinal microbiota. A. location of major cell types involved. B. Network of homeostatic interactions between major cell types and the microbiota. Adapted from Moor et al. Antibodies 2015[4]

Why combine mathematical modeling with the study of the intestinal immune system?

As long as you are healthy, your blood and deep organs contain many resting immune cells and close to zero microorganisms. The immune system has therefore evolved a system of receptors (called "Pattern Recognition Receptors") that recognize highly conserved molecular signatures of microorganisms, such as bacterial cell walls[5]. During a serious infection, microbes are thus recognized and initiate activation of the immune system[5]. Such a system, coupled with the immune system's ability to tolerate or ignore

our own human cells, is sufficient to explain healthy immune system function in the blood. During my PhD I worked on elucidating the biochemistry used by the immune system to recognize yeast infections in the blood and spleen[6].

However, during this time it became clear to me that our elegant understanding of how the immune system functions in the blood could not be translated one-to-one to immune function at the body surfaces. In the intestine of a healthy human there are 10^{13} bacteria: a number that either does not change or actually drops during an intestinal infection[7]. Simply measuring bacterial density therefore carries very little information on whether an immune response is necessary. When I started my first postdoc with Prof. Andrew Macpherson at McMaster University in Hamilton, Ontario it had been speculated that these mechanisms are simply turned off in the intestine[8]. However, using highly simplified microbiotas we demonstrated that pattern recognition is essential to maintain a normal relationship with the microbiota[9]. In fact, the immune cells in the intestine, in contrast to the blood, are never truly resting. A low level of activity, driven by pattern recognition, is required to kill any bacteria that manage to enter the intestinal tissues[9].

While this elegantly explained control of bacteria in the intestinal tissues, there are no immune cells present in the lumen of a healthy mammal, and therefore there can be no pattern recognition. How do we exclude unwanted bacterial species from the gut lumen?

This turns out to be an extremely complex problem. Bacteria in the intestinal lumen are actively growing and dying, and the population sizes are large: this permits rapid evolution. Further, the bacteria are flowing with the intestinal content. A major determinant of microbiota composition is actually the intestinal environment (nutrient availability, pH, motility etc), with dedicated immune system components such as antimicrobial peptides playing comparatively subtle roles. In contrast to the blood where immune mechanisms tend to immobilize and then kill microorganisms, the consequences of an immune response in the intestine are varied and dynamic. For this reason, I moved to the ETH Zürich to learn mathematical modeling and to apply this in situations where we induce intestinal immunity using oral vaccines.

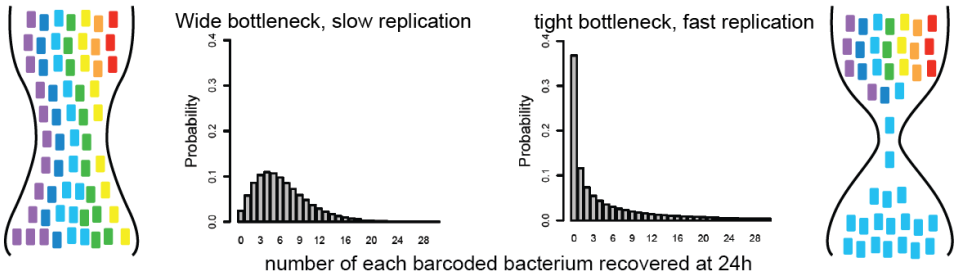

Figure 2. The principle of neutral tagging. By including traceable markers in a population and comparing the distribution of markers in your starting population and the final compartment of interest, it is possible to estimate the size of bottleneck encountered between the two compartments, as well as the net expansion. This allows you to distinguish two situations

which may give rise to populations with identical total size (i.e. indistinguishable by classical microbiology), but very different compositions.

At ETH Zürich I established a semi-independent group within the group of Prof. Wolf-Dietrich Hardt in the Institute of Microbiology and began to work with Prof. Roland Regös on neutral tagging techniques (Figure 2)[10]. Using *Salmonella* strains carrying detectable "barcodes" in their genome[11] we could measure how bacterial clones were distributed in different sites (e.g. in the gut lumen and in the tissues) during infection[10]. From this information, we can generate a best estimate of the growth, killing and migration of *Salmonella* in different parts of the intestine during a real infection[10]. These techniques can be adapted to study the dynamics of bacterial growth and death in any part of the body, in the presence and absence of immune responses. We can therefore quantify the effects of immunity over time and within different body compartments.

Inactivated oral vaccines

Secretory antibodies, also known as secretory IgA, are the only major immune components present in the healthy intestine that are capable of acting at the level of an individual species or strain[12]. When we began working on IgA in 2011, the current dogma was that low-level intestinal infection with live bacteria was necessary to induce a robust IgA response[13]. However, from earlier work, I knew that we could induce IgA against benign commensal bacteria, which do not cause disease[14]. It was only a small step from this point to realizing that oral delivery of high doses of inactivated bacteria could also work. We therefore developed a method to efficiently inactivate high densities of cultured bacteria, and concentrate these dead bacterial particles for oral delivery[15]. This method is attractively simple, and can be employed for any cultivable bacterial species tried so far.

We then needed to be able to quantify the strength of the vaccine-induced IgA response in complex fluids such as small intestinal lavages and fecal supernatant. As many bacterial intracellular proteins are very highly conserved across phyla, for example the ribosomal proteins, we wanted a method that excluded any burst or dead cells. For this purpose we developed a flow cytometry-based technique to detect antibodies bound to the surface of live bacteria from any body fluid[16].

Combining these two techniques demonstrated that our inactivated oral vaccines induced high levels of IgA specific for the target bacterium.

The mechanism of IgA function

When we now applied our within-host population dynamics techniques in vaccinated mice, we were surprised. IgA is a large molecule with four binding sites, ideal for sticking bacteria together. The prevailing model of IgA function was therefore that as bacteria collided with one another in the intestine, they

were cross-linked and clumped ("classical agglutination")[17]. Whilst we did indeed observe bacterial clumps in vaccinated mice, and these clumps were too large to approach the gut wall and initiate disease, the clumps were not being formed by collision[18].

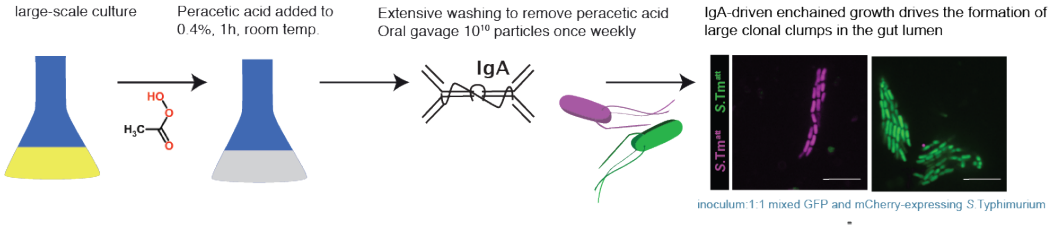

Figure 3: Inactivated oral vaccines induce high-avidity IgA driving enchained growth in the gut lumen. Schematic of vaccine production and function. This protocol works with all so-far tested cultivable bacteria, without additional adjuvants. *S.* Typhimurium-vaccinated mice were ampicillin pre-treated and infected with a mixture of ampicillin-resistant *S.* Typhimurium constitutively expressing GFP (green-fluorescent) or mCherry.(red-fluorescent). At 5h post-infection cecal content was harvested and imaged live by confocal microscopy. Representative images show clumps of a single colour, inconsistent with random collisions; adapted from Moor et al. Nature 2017[18].

In fact pathogenic bacteria, such as *Salmonella* are typically swallowed in low numbers compared to the resident intestinal microbiota, so the chance of two *Salmonella* colliding in the gut lumen is rather like the chance of blindly bumping into a friend at a very large music festival - possible, but rare[18]. However, there is one situation where two *Salmonella* do contact each other, and that is as the bacteria grow and divide. We could demonstrate that bacterial "enchained-growth" was actually driving IgA-mediated clumping and protection *in vivo*[18].

Preventing the spread of antibiotic resistance

Once we saw how IgA was actually functioning in the intestine, something became immediately obvious: these clumps were the product of a single bacterium and were therefore genetically uniform. Plasmids, for example carrying antibiotic resistances, require cell contact to spread. Therefore a plasmid could be isolated in one clump and would be unable to transfer further. Indeed we could demonstrate that vaccination not only prevented disease, but also prevented spread of antimicrobial resistance within the intestinal *Salmonella* population[18].

A second mode by which bacteria can acquire new virulence mechanisms and resistances is via bacterial viruses, called temperate phages, which sit dormant in the bacterial genome. When the host bacteria become stressed, these viruses are reactivated and can infect new hosts, transferring genetic material. Interestingly, IgA-mediated protection actually prevents bacterial stress and therefore avoids release of these viruses, blocking a second aspect of horizontal gene transfer[19].

The future: Understanding the dynamics of immunity and bacterial evolution at mucosal surfaces and translating oral vaccination into veterinary and medical practice.

There remain a large number of unanswered questions in how the mucosal immune system enables us to maintain a dense, complex and healthy intestinal ecosystem. For example, it remains unclear how we differentiate between benign and pathogenic bacteria; how we avoid pathological cross-reactivity to food antigens; and how the immune system drives bacterial evolution to favor beneficial behaviors of gut bacteria. We will continue to expand our available tools and models to provide answers to these questions. In particular, we hope to assemble a computational model for mucosal immune system function that will permit simulations to test complex hypotheses and aid in experimental design and data interpretation.

The other arm of our research will aim first to optimize our oral vaccinations to tackle *Salmonella* infections in domestic pigs. *Salmonella* remains prevalent on pig farms and causes serious morbidity in young animals. We will then develop the technique to carry out microbiota engineering, targeting antibiotic-resistant bacteria commonly carried in the microbiota of domestic pigs. This will remove a major reservoir of antibiotic resistance genes that can spread to important human pathogens. If successful, this will also generate the preliminary large-animal model data for translation into human medicine.

Thus we hope to rapidly introduce inactivated oral vaccines as a safe and cheap tool in the fight against the spread of antibiotic resistance, and in the control of enteric and microbiota-associated diseases. As our understanding of mucosal immunity improves, we hope to develop further novel tools and approaches to pressing medical problems.