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MEMORIES AND RETROSPECTIVES

Gnotobiotic mouse model's contribution to understanding host–pathogen interactions

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Abstract This brief review is dedicated to the legacy of Prof. Jaroslav Šterzl and his colleagues, who laid the foundation for gnotobiology in the former Czechoslovakia 55 years. Prof. Sterzl became one of the founders of modern Czechoslovak immunology, which was characterized by work on a wide range of problems needing to be solved. While examining the mechanisms of innate immunity, he focused his studies on the induction of antibody production by immunocompetent cells involved in adaptive immune transmission while using the model of pig fetuses and germ-free piglets and characterizing immunoglobulins in the sera of these piglets. Although not fully appreciated to this day, his experimental proof of the hypothesis focused on the common precursor of cell–forming antibodies of different isotypes was later confirmed in experiments at the gene level. Prof. Sterzl's work represented a true milestone in the development of not solely Czechoslovak but also European and global immunology. He collaborated closely with the World Health Organization for many years, serving there as leader of the Reference Laboratory for Factors of Innate Immunity.

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Introduction

The eukaryotic host–microbe interrelationship accompanies multicellular organisms from the origin of eukaryotic cells as a consequence of endosymbiosis. Several hundred billion microorganisms, such as viruses, bacteria, and fungi, populate hosts and are collectively named microbiota. Many species are beneficial to their hosts and keep their immune systems in a state of readiness. To study host–microbe interactions and microbes' influence on host fitness, the concept of the gnotobiotic animal was introduced. The words "gnotobiont" and "gnotobiotic" were derived from the Greek words "gnostos" and "biota" meaning known living flora and fauna in association with the host. In practice, the gnotobiotic animal is a germ-free or originally germ-free organism artificially populated by a known strain of microorganism. Germ-free organisms (axenic animals) can be obtained by Caesarean section, hysterectomy from their mothers, or by sterile embryo transfer into germ-free foster mothers. Depending on species, precocial animals can thereafter be reared in sterile isolators without their mothers, while altricial animals need germ-free foster mothers. The terms gnotobiotic, germ-free, and mono-associated models often overlap, because all have known microbial status (for gnotobiotic terminology and criteria, see, for example, Gordon and Pesti [1]).

Over the decades, the studies of gnotobiotic animals have demonstrated the appropriateness of differentially mono-associated gnotobionts for analyzing organism's protective functions. These studies have focused mainly on

the immune system [2, 3], structural and metabolic functions of the organism [4–6], and developmental aspects of vertebrate functional systems [7–10]. Furthermore, microbiome models have been adopted for studies on carcinogenesis [11–15]. One of the areas attracting considerable attention and utilizing the gnotobiotic animal model is that of the host–pathogen interrelationship, with studies covering the basic microbiological and immunological questions, vaccine development, and general questions on health–disease transition. The appeal of these gnotobiotic models is that they actually constitute "biologically pure" models, and the influence of external signals on the studied organism can be monitored without "interior noise" caused by that organism's microbiome.

Such bacterial intracellular pathogens as mycobacteria, brucellae, listeriae, francisellae, and salmonellae are studied assiduously, because their impact within the health care system or safety and security field can be considerable, and especially so if they were to be used for military or terroristic purposes. This brief compendium is, therefore, devoted to the issue of host–pathogen relationship studies conducted using gnotobiotic models, and particularly with reference to intracellular bacterial pathogens.

Historical perspective

The gnotobiotic concept originated in the requirement to work with strictly defined systems in biological experimentation. Various reviews on germ-free studies have reported the original concepts from the 19. Century and credited the ideas of J. B. Boussingault, E. Duclaux, L. Pasteur, and M. Schottelius [1, 16–18]. The initial purpose of using germ-free animals was to prove Pasteur's assertion that life of an animal host would become impossible in the context of a germ-free experiment and his view that "microbial associates have become synergists which are indispensable in the life of the host" [1]. Meanwhile, Nencki [19] and Metchnikoff [20] had asserted just the opposite and that microbes are antagonistic to the well-being of their host [1]. Actual proof that vertebrates' normal life is possible in the absence of microorganisms was found in Reyniers's studies during the 1940s (presented in a summary at the Eighth Annual Meeting of the Animal Care Panel, November 7–9, 1957 in San Francisco, CA, USA) and Gustafsson's group in the 1950s [21–23].

The technical ins and outs of germ-free technology during its history are famously presented in the paper "Life in a Germ-Free World": Isolating Life from the Laboratory Animal to the Bubble Boy by Robert G. W. Kirk [24]. This article describes the original ideas and concepts of germ-free technology and the basic principles of germ-free isolator creation. The applications of germ-free technology in the laboratory for the production of laboratory animals, in agriculture for the efficient production of farm animals, and in hospitals for the control and prevention of cross-infection and the protection of individuals from infection are discussed in detail.

Subsequently, to utilize the advantages of gnotobionts for biological experimentation, several gnotobiological laboratories were founded in Europe, the USA, and Japan during the 20 century. A substantial contribution to understanding the nature of physiological differences between germ-free and microbiota-colonized animals was made by studies carried out at the gnotobiological laboratory of the Czechoslovak Academy of Sciences in Novy Hradek.

The Institute of Biology of the Czechoslovak Academy of Sciences in Novy Hradek, Czech Republic was founded in 1953. From its beginning, the laboratory campus had a special security regime and was protected by armed guards. The reasons lay in its studies on anthrax. For these studies, there were developed special isolators (in fact a version of Hansen's sterile box) connected to a special air-conditioning system equipped with bacteriological filters. From 1958 to 1968, the Institute of Biology was used by the Czechoslovak army as a center for pathogenetic and immunogenetic studies on highly infectious microorganisms. After a lecture tour in the USA, Jaroslav Sterzl, a leading immunologist in the Czech Republic, rebuilt one of the infection laboratories into a gnotobiotic lab in 1960. The Laboratory of Gnotobiology was especially engaged in breeding of germ-free animals [25, 26] and began to utilize a unique experimental animal model allowing to distinguish the innate immune mechanisms of immune reaction that resulted from the interactions with microbiota as well as certain factors determining the course of antibody formation and development of immune response [27, 28]. Over the years, studies were carried out by Jaroslav Sterzl and fellow immunologists Helena Tlaskalova-Hogenova, Ivo Miler, Miloslav Pospisil, and Lydie Jaroskova; the chemists Jiri Rejnek and Jiří Zikan; and the microbiologist Vladimir Dlabac.

The dominant experimental models were rats, pigs, and mice. The model of colostrum-free and germ-free piglets came to be a suitable one for studying effects of the bacterial microbiota and examining innate as well as acquired immunity, where the development of antibodies under the influence of commensal bacteria was distinguished in comparison with a mother's passively transferred antibodies [28].

Some original studies were devoted to developmental aspects of antibody formation and the processes occurring during differentiation of immunologically competent cells, as well as the role of antigens in the development of humoral immune responses [28–32]; to induction and

regulation of secondary immune response to antigen (re-vaccination) [33, 34]; or to modeling of general aspects of antibody response [33, 35]. Powered by this knowledge, Jaroslav Sterzl and his colleagues demonstrated during the 1980s that the frequencies of antigen-binding B cells are comparable in the model of germ-free and in conventional mice. They further showed that antigen stimulation plays a role in activating antibody production but not in the establishment of B cell repertoire [36–43].

Thus, it occurred that over time gnotobiotic models began to be widely exploited in various branches of biomedical research.

Gut microbiota and microbiome

The coevolution of eukaryota with prokaryota across the epochs brokered for each body a unique set of microorganisms known as its microbiota. Gut microbiota, the most abundant and well-studied microbiota, has also been termed a "forgotten organ" [44] and a "neglected endocrine organ" [45]. Bacterial density in the gut reaches 10^{11}–10^{12} cells/g in the distal human colon [46], and the sum of gut microbiota genomes, known as a microbiome, contains more than 100-fold more genes than are encoded in the human genome [47].

Gut microbiota has the capacity to influence the body's physiology and metabolism. A very well-known example is the dependency of normal immune system development and function on the presence of gut microbiota (for example, see [48–50]). Modulatory effects on epithelial cell proliferation, villus architecture, and angiogenesis within the intestine, influence on xenobiotic metabolism, bone mineral density, behavior, and alteration of several metabolic functions have also been documented [51, 52]. Emerging data from humans and mouse models suggest that the gut microbiota plays a role in the development of metabolic diseases [6]. The microbiota seems to be important also for the programming and presentation of normal social behaviors, including social motivation and preference for social novelty. The original gut–brain axis concept has been changed to the microbiota–gut–brain axis, which is an emerging concept in modern medicine [53, 54].

The complex of genes which encompass the genomes of enteric and other bacteria comprises a particular organism's unique microbiome. Knowledge of the microbiome and its changes, as they relate to the alteration of physiological, developmental, or metabolic processes in the organism offer the possibility to study the association of the microbiome with health and/or disease (i.e., the host–microbe relationship). For this reason, the Human Microbiome Project (HMP) was funded as an initiative of the NIH Roadmap for Biomedical Research (http://nihroadmap.nih.gov). HMP is focused on high-throughput technologies that can characterize the aforementioned changes in the human microbiome that can be associated with health or disease traits. A detailed analysis of molecular relationships between the microbiome and host can contribute to the development of new monitoring, diagnostic, and therapeutic procedures. Moreover, currently, accumulating data testify to the feasibility of manipulating the current health status of an organism through its microbiota [55–59]. Recently, comprehensive reviews have summarized the functions of microbiota in shaping the nutrient environment of the mammalian intestine by contributing to nutrient exchange, carbohydrate metabolism, and manipulation of the host's metabolic machinery [60]. Other reviews have underscored the importance of the colonic environment for gut immune maturation [61], fully functional innate and systemic immunity [62], and anti-cancer responses [15, 63–65]. Nevertheless, quite limited data exist regarding the mutual interaction of germ-free models or their cells and pathogens. In this respect, the majority of experiments have been carried out using per os application of microbes that exert their pathogenicity predominantly through the gastrointestinal tract.

Gnotobionts as a model organism for study of host–pathogen interaction

Very soon after implementation of germ-free technology, it became evident that gnotobiotic animals constitute excellent tools for investigating the initial interactions of microorganisms with their hosts and that those studies can elucidate host immune responses to infections.

Initial studies tested mono-associated animals' resistance to infection. Rapid colonization of the organs of germ-free animals infected with *Listeria monocytogenes (L. monocytogenes)* and higher sensitivity to this infection was obvious from these original trials. Nevertheless, in parallel, it was also reported that the conventionalization of *L. monocytogenes* mono-associated rats or di-association of mice with some representative of indigenous flora protects these animals against systemic *L. monocytogenes* infection [66, 67]. A similar conclusion concerning the importance of indigenous flora on the host's immune status was made based on models of *Salmonella typhimurium* and *Vibrio cholerae* (*V. cholerae*) model infections [68, 69]. Thus, the extreme sensitivity of gnotobionts to pathogenic bacteria is a result of rapidly invasive bacterial infection and the slow development of immune response.

The onset timing, intensity, and composition of gnotobionts' immune responses are critical for their resistance to

pathogenic bacteria. Using the model of *L. monocytogenes* infection, it was demonstrated that lack of an intestinal microbiota impairs early innate immunity but enhances activation of memory T cells [70]. Although the T cells of gnotobionts are primed normally by listerial antigens, their trafficking to inflamed sites is severely impaired, and this may lead to increased susceptibility to infection with *L. monocytogenes* [71].

The absence of intestinal microbiota is also accompanied by a state of inflammatory hyporesponsiveness actively mediated by IL-10. IL-10 restrains proinflammatory mediator production and neutrophil recruitment and favors *Klebsiella pneumoniae* growth and dissemination in gnotobiotic mice. A parallel study demonstrated that transient toll-like receptor (TLR) activation restores the inflammatory response and ability to control pulmonary bacterial infection [72]. All these studies conclusively confirmed the importance of microbiota for the immune system's effective response to pathogenic bacteria.

For the sake of completeness, it must be said that bacterial load of commensals alone is insufficient to protect against infection; rather, certain bacterial species correlate with protection [73]. Mice colonized with mouse microbiota, orally infected with *Salmonella enterica* (*S. enterica*) serovar *Typhimurium*, had a significantly lower *Salmonella* load in feces and less dissemination to the spleen in comparison to mice colonized with human microbiota. Moreover, ceca of mice colonized with mouse microbiota appeared healthy on histology, whereas ceca of mice colonized with human microbiota, similarly as original germ-free mice, had severe gross pathological changes characterized by thickening of the cecal wall, inflammation, and edema [61].

Gut microbiota have been demonstrated to be critical for mucosal protection from bacterial invasion and disease [74]. The precise role of microbiota in modulation of host immune response to pathogenic bacteria remains unknown, and however, comparative studies of host defence in gnotobionts and specific-pathogen-free (SPF) animals with normal microbial colonization are required.

The data demonstrated that the presence of commensal microbiota leads to competition for space and nutrients or regulates the production of intestinal mucins, which consequently inhibits the adherence of numerous pathogenic bacteria to intestinal epithelial cells [75–77]. The modulated immunological parameters have been demonstrated the induction of IgA as a means to reduce pathogen colonization and, by opsonizing the commensal bacteria, to interact with mucosal dendritic cells. This interaction is essential for the maintenance of intestinal homeostasis due to the tolerogenic profile of dendritic cells [78–80].

Commensal bacteria also influence the function of Th17 cells producing IL-17 and IL-22, which promote antibody class switching in B cells, induce production of anti-microbial peptides, and contribute to neutrophil recruitment [81, 82]. Another modulatory response can be the expression of pattern recognition receptors, including TLRs. One of these, TLR9, recognizes unmethylated CpG sequences in DNA. Its expression, along with the intracellular compartment of immune system cells, was demonstrated on the surface of intestinal epithelial cells. TLR9 was expressed on the colonic apical surface in wild-type mice but not in germ-free mice. Thus, intestinal epithelial cells responding to normal microflora colonization by surface expression of TLR9 may recognize pathogenic bacterial DNA and respond with an inflammatory response to pathogenic DNA [83]. Moreover, TLR9 engagement by commensal DNA promotes IL-17 and IFN-γ-producing T cells and reduces Treg frequency, which could facilitate immunity to pathogens [84].

Nevertheless, the data on the impact of changes induced by intestinal microbiota in the intestinal cell surfaces and, consequently, the impact on local immune responses to peripheral immune responses are ambiguous. On the one hand, commensals influence the mucosal and systemic immune responses to viruses and some bacteria [85–88]. On the other hand, there are data showing that germ-free mice intragastrically infected with *S. enterica* serovar *Typhimurium* have higher bacterial burden in the mesenteric lymph nodes compared to conventionally raised animals. *Salmonella* penetration into the lamina propria of the small intestine and splenic bacterial burden were not altered. Contrary to similar inflammatory phagocyte recruitment, intragastrically infected germ-free mice displayed a higher frequency of IFN-gamma-producing NK, NKT, CD4+, and CD8+ T cells in the mesenteric lymph nodes in comparison with conventionally raised mice. All these differences among germ-free and conventional mice were abrogated when the mice were infected intraperitoneally [89]. To explain the cellular and molecular consequences, infection caused by administrating bacteria to hosts via different routes will require further experiments.

Along with studies on immune response of gnotobionts to pathogens, germ-free animals have been used in analyzing bacterial pathogenicity. The pathogenicity of *Escherichia coli* [90], *Mycoplasma pneumoniae* [91], and *S. enterica* serovar *Typhimurium* [92] was demonstrated in germ-free animals. Moreover, gnotobionts seem to be useful for studies confirming individual bacterial components as virulence factors [93, 94], and they also have been used to monitor pathogen–host cell interactions [95]. The model of gnotobiotic animals does allow the evaluation of systemic and mucosal immune responses to antigens expressed by *V. cholerae* in vivo [69]. These data may be useful for designing a conjugate vaccine for

cholera. Thus, gnotobionts may be used in multiply focused studies within bacteriology, immunology, or infection biology.

Even as the contribution of commensal gut bacteria and their microbiomes has been well established over the decades, the gut also contains an enteric virome that can modulate the body's physiological parameters either alone or with a contribution from bacterial microbiota. The possible role of viruses present under homeostatic conditions in the gastrointestinal tract is not well understood [96–98]. Human virome tested on fecal samples from healthy children revealed a complex community of enteric viruses, including viruses of the families Picobirnaviridae, Adenoviridae, and Astroviridae and such species as bocaviruses, enteroviruses, rotaviruses, and sapoviruses [99]. Viruses that can create the gut virome include also pathogens, and these viruses are ubiquitously detected also in healthy individuals. Namely, these are members of the Anelloviridae that can cause chronic human viral infections or Caliciviridae, which cause viral gastroenteritis in humans. A substantial part of the gut virome is not characterized despite that there is limited sequence homology with known viruses [100–103].

As similarly true of bacteria, viruses can exert modulatory effects on an organism's structural, metabolic, and defence functions. For example, infection of germ-free or antibiotics-treated mice with murine norovirus restores intestinal morphology, induces transcriptional changes leading to the development of immune status, and modulates lymphocyte functions without inducing inflammation or disease [104]. The data suggest that eukaryotic viruses (of course those that do not harm their host) have the capacity to modulate intestinal homeostasis and mucosal immunity in a manner analogous to that of commensal bacteria.

In respect of viral and other nonbacterial infections, gnotobiotic models offer a useful tool for the study of mutual interrelationships between bacterial microbiota, other infection agents, and the host. The host's microbiota affects the replication and transmission of a diverse array of viral pathogens in both positive and negative senses. Changes in host microbiota have been suggested as a potential target for therapeutic intervention [105].

Nevertheless, the modulatory effect of microbiota on viral infections cannot be generalized. In contrast to orthomyxoviruses and arenaviruses, retrovirus-resistant mice control retroviral infection independently of commensal microbiota [106]. On the other hand, as was mentioned above, enteric viruses can replace the microbiota in supporting intestinal homeostasis and functioning of mucosal immunity, similarly as do commensal bacteria [104]. The gnotobiotic model also reveals a more complex interrelationship among bacterial, viral, and parasitic agents and their host [107].

Thus, the concept of germ-free animals has recently been exploited successfully in many branches of laboratory animal research [74–76]. Their almost naïve innate immune system actually provides a unique model for studying the cellular and molecular events that control host–pathogen interactions during the early stages of infection. These processes subsequently participate in the outcome of these interactions as seen in the emergence of states of health or disease.

Concluding remarks and open questions

Research utilizing gnotobiotic animal models has elucidated some questions concerning the influence of gut microbiota on the initiation and progression of such diseases as type 1 diabetes [108], metabolic syndromes [109, 110], obesity, autoimmune arthritis, inflammatory bowel disease, and irritable bowel syndrome [111–114], as well as the role of the microbiome in cancer [63, 64]. Other studies have investigated the effects of microbiota on the development and function of the immune system [6, 9, 10, 13, 115–117]. Studies on the basic problems of host–pathogen interaction utilizing gnotobiotic models are nevertheless only beginning. Although there have been studies concerning the interaction between mammals and bacteria, the majority of these have been focused on bacteria that colonize or infect the gastrointestinal tract [18]. Studies focused on pathogens that infect their host in a manner other than per os are practically nonexistent. According to our experience, germ-free mice infected subcutaneously with *Francisella tularensis* reacted differently to attenuated and virulent strains in comparison with specific-pathogen free mice. Thus, the ontogeny of these mice having no contact with bacteria can disclose the unique primary reaction of their immune system against pathogenic bacterial strains and can help in understanding the processes that lead to the establishment of full-fledged protective immunity. In the case of intracellular bacterial pathogens, the tools for inducing such effective immune reactions are still lacking.

Data collected from gnotobiotic as well as SPF animal models of microbial pathogenesis clearly demonstrate the decisive role of primary interaction between microorganism and the host cell that the microbe first encounters. In addition to the intrinsic characteristics of microorganism and host, a dominant role is played by the innate immune recognition process. The hierarchy of immune response functional modules based on the epigenetic reprogramming of innate immune cells after intercellular communication

by cytokines and chemokines is underscored by the spatiotemporal aspect of host–microbe interaction.

From this point of view, the cell, with its functional and secretion profile, rather than the host organism in its entirety, seems to be the primary microbe host. The signals generated by this primary host after interaction with a microbe comprise a signaling window that controls all consecutive interactions. The emerging concept of innate immune response based upon a hierarchy of signaling windows inside the social network of immune cells (first presented during Discussion Forum 2016—Host Pathogen Interaction [118]) slightly modifies the damage-response framework (DRF) of microbial pathogenesis [119] by redefining the host. DRF had postulated that: (1) microbial pathogenesis requires a microbe and a host, (2) the microbe and the host must interact, and (3) the relevant outcome of host–microbe interaction is damage within the host whereby damage results from microbial or host factors or both. The main impact of this framework was integration of host and microbe roles in microbial pathogenesis. A new problem emerged, however, in association with defining just what constitutes a host. The revised DRF of microbial pathogenesis defines a host as an entity which houses an associated microbiome/microbiota and interacts with microbes such that the outcome results in damage, benefit, or indifference, thus resulting in the states of symbiosis, colonization, commensalism, latency, and disease [120]. Moreover, the emerging concept of signaling windows does not necessarily assume that the outcome of host–microbe interaction will be damage to the cellular host. The interaction might influence the primary signaling pathways modulating gene expression, secretion profile, and, subsequently, expression of function in a microbe-specific way.

Thus, in contradiction to the unifying tendency of DRF regarding the definition of a host, we postulate the individual cell as host. This highlights the relevance of an individual interacting cell type and its functional profile in relation to an interacting microbe through the process of host–microbe interaction. This concept is more dynamic, is more useful in understanding the role of microenvironment on the host–microbe interaction in the compartmentalized immune system, and can better depict the dynamic processes during innate immune response against infection.

Germ-free animal models in combination with monoassociated gnotobionts and SPF animals can serve as a useful tool in analyzing the host–pathogen interaction, and that is in accordance with the original, pioneering work of Jaroslav Sterzl. A gnotobiont based on the interaction between a germ-free animal and pathogen can help to verify the existing models of innate immune responses induction and clarify the role of individual cell types and their receptors in intracellular and intercellular signaling during their mutual interaction which leads to expression of protective immunity against pathogenic microbes. The basic data obtained in using such an approach may influence the view as to the construction of effective and safe vaccines, especially in such cases as immunoprophylaxis fails or does not yet exist.

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References

1. Gordon HA, Pesti L (1971) The gnotobiotic animal as a tool in the study of host microbial relationships. Bacteriol Rev 35:390–429
2. Cebra JJ (1999) Influences of microbiota on intestinal immune system development. Am J Clin Nutr 69:1046S–1051S
3. Hooper LV, Littman DR, Macpherson AJ (2012) Interactions between the microbiota and the immune system. Science 336:1268–1273. doi:10.1126/science.1223490
4. Bäckhed F, Crawford PA (2010) Coordinated regulation of the metabolome and lipidome at the host-microbial interface. Biochim Biophys Acta 1801:240–245. doi:10.1016/j.bbalip.2009.09.009
5. Tilg H, Kaser A (2011) Gut microbiome, obesity, and metabolic dysfunction. J Clin Invest 121:2126–2132. doi:10.1172/JCI58109
6. Karlsson F, Tremaroli V, Nielsen J, Bäckhed F (2013) Assessing the human gut microbiota in metabolic diseases. Diabetes 62:3341–3349. doi:10.2337/db13-0844
7. Thompson GR, Trexler PC (1971) Gastrointestinal structure and function in germ-free or gnotobiotic animals. Gut 12:230–235
8. Butler JE, Lager KM, Splichal I et al (2009) The piglet as a model for B cell and immune system development. Vet Immunol Immunopathol 128:147–170. doi:10.1016/j.vetimm.2008.10.321
9. Grover M, Kashyap PC (2014) Germ free mice as a model to study effect of gut microbiota on host physiology. Neurogastroenterol Motil Off J Eur Gastrointest Motil Soc 26:745–748. doi:10.1111/nmo.12366
10. Umesaki Y (2014) Use of gnotobiotic mice to identify and characterize key microbes responsible for the development of the intestinal immune system. Proc Jpn Acad Ser B Phys Biol Sci 90:313–332. doi:10.2183/pjab.90.313
11. Schwabe RF, Jobin C (2013) The microbiome and cancer. Nat Rev Cancer 13:800–812. doi:10.1038/nrc3610
12. Francescone R, Hou V, Grivennikov SI (2014) Microbiome, inflammation and cancer. Cancer J Sudbury Mass 20:181–189. doi:10.1097/PPO.0000000000000048
13. Bultman SJ (2014) Emerging roles of the microbiome in cancer. Carcinogenesis 35:249–255. doi:10.1093/carcin/bgt392
14. Brawner KM, Morrow CD, Smith PD (2014) Gastric microbiome and gastric cancer. Cancer J Sudbury Mass 20:211–216. doi:10.1097/PPO.0000000000000043
15. Tlaskalova-Hogenova H, Vannucci L, Klimesova K et al (2014) Microbiome and colorectal carcinoma: insights from germ-free

and conventional animal models. Cancer J Sudbury Mass 20:217–224. doi:10.1097/PPO.0000000000000052
16. Luckey T (1963) Germfree life and gnotobiology. Academic Press Inc., New York
17. Luckey T (1965) Effects of microbes on germfree animals. Advan Appl Microbiol 7:169–223
18. Bibiloni R (2012) Rodent models to study the relationships between mammals and their bacterial inhabitants. Gut Microbes 3:536–543. doi:10.4161/gmic.21905
19. Nencki M (1886) Bemerkung zu einer Bemerkung Pasteur's. Arch Exp Pathol Pharmacol 20:385–388
20. Metchnikoff E (1903) Les microbes intestinaux. Bull Inst Pasteur 1:265–282
21. Gustafsson B (1948) Germfree rearing of rats. General technique. Acta Pathol Microbiol Scand Suppl 73:1–130
22. Gustafsson B (1959) Lightweight stainless steel systems for rearing germfree animals. Ann N Acad Sci 78:17–28
23. Gustafsson BE, Laurell C-B (1959) Gamma globulin production in germfree rats after bacterial contamination. J Exp Med 110:675–684
24. Kirk RG (2012) "Life in a germ-free world": isolating life from the laboratory animal to the bubble boy. Bull Hist Med 86:237–275. doi:10.1353/bhm.2012.0028
25. Trávnícek J, Mandel L, Kasal P et al (1977) Equipment for germ-free caesarean section and baby care. Folia Microbiol (Praha) 22:523–526
26. Trávnícek J, Mandel L (1979) Gnotobiotic techniques. Folia Microbiol (Praha) 24:6–10
27. Sterzl J, Silverstein AM (1967) Developmental aspects of immunity. Adv Immunol 6:337–459
28. Sterzl J (1967) Factors determining the differentiation pathways of immunocompetent cells. Cold Harb Symp Quant Biol 32:1–22
29. Sterzl J (1963) Quantitative and qualitative aspect of the inductive phase of antibody formation. J Hyg Epidemiol Microbiol Immunol 7:301–318
30. Sterzl J, Mandel L (1964) Estimation of the inductive phase of antibody formation by plaque technique. Folia Microbiol (Praha) 14:173–176
31. Sterzl J, Ríha I (1965) Detection of cells producing 7S antibodies by the plaque technique. Nature 208:858–859
32. Sterzl J, Rejnek J, Trávnícek J (1966) Impermeability of pig placenta for antibodies. Folia Microbiol (Praha) 11:7–10
33. Jílek M, Sterzl J (1967) Influence of the amount of antigen and interval on the secondary reaction. Folia Microbiol (Praha) 12:6–20
34. Sterzl J, Jílek M (1967) Number of antibody-forming cells in primary and secondary reactions after administration of antigen. Nature 216:1233–1235
35. Klein P, Sterzl J, Dolezal J (1981) A mathematical model of B lymphocyte differentiation: control by antigen. J Math Biol 13:67–86
36. Kovářů F, Štěpánková R, Kruml J et al (1979) Development of lymphatic and haemopoietic organs in germfree models. Folia Microbiol Praha 24:32–43
37. Tlaskalová-Hogenová H, Štěpánková R (1980) Development of antibody formation in germ-free and conventionally reared rabbits: the role of intestinal lymphoid tissue in antibody formation to *E. coli* antigens. Folia Biol Praha 26:81–93
38. Tlaskalova-Hogenova H, Vetvicka V, Sterzl J, Stepankova R (1982) Development of immune potential and migration pattern of cells from germfree (GF) and conventionally (CONV) reared rats. Adv Exp Med Biol 149:515–520
39. Tlaskalova-Hogenova H, Sterzl J, Stepankova R et al (1983) Development of immunological capacity under germfree and conventional conditions. Ann N Acad Sci 409:96–113
40. Větvička V, Tlaskalová-Hogenová H, Štěpánková R (1983) Effects of microflora antigens on lymphocyte migration patterns in germfree and conventional rats. Folia Biol Praha 29:412–418
41. Fornůsek L, Větvička V, Jaroskova L, Stěpánková R (1983) Some properties of the plasma membrane of macrophages from germ-free rats. J Reticuloendothel Soc 34:331–340
42. Stěpánková R, Kovárů F (1985) Immunoglobulin-producing cells in lymphatic tissues of germfree and conventional rabbits as detected by an immunofluorescence method. Folia Microbiol Praha 30:291–294
43. Sterzl J, Mandel L, Stepankova R (1987) The use of gnotobiological models for the studies of immune mechanisms. Nahrung 31:599–608
44. O'Hara AM, Shanahan F (2006) The gut flora as a forgotten organ. EMBO Rep 7:688–693. doi:10.1038/sj.embor.7400731
45. Clarke G, Stilling RM, Kennedy PJ et al (2014) Minireview: gut microbiota: the neglected endocrine organ. Mol Endocrinol Baltim Md 28:1221–1238. doi:10.1210/me.2014-1108
46. Bäckhed F, Ley RE, Sonnenburg JL et al (2005) Host-bacterial mutualism in the human intestine. Science 307:1915–1920. doi:10.1126/science.1104816
47. Qin J, Li R, Raes J et al (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464:59–65. doi:10.1038/nature08821
48. Arrieta M-C, Stiemsma LT, Amenyogbe N et al (2014) The intestinal microbiome in early life: health and disease. Front Immunol 5:427. doi:10.3389/fimmu.2014.00427
49. Maranduba CM, De Castro SBR, de Souza GT et al (2015) Intestinal microbiota as modulators of the immune system and neuroimmune system: impact on the host health and homeostasis. J Immunol Res 2015:931574. doi:10.1155/2015/931574
50. Bermon S, Petriz B, Kajėnienė A et al (2015) The microbiota: an exercise immunology perspective. Exerc Immunol Rev 21:70–79
51. Tremaroli V, Bäckhed F (2012) Functional interactions between the gut microbiota and host metabolism. Nature 489:242–249. doi:10.1038/nature11552
52. Sommer F, Bäckhed F (2013) The gut microbiota—masters of host development and physiology. Nat Rev Microbiol 11:227–238. doi:10.1038/nrmicro2974
53. Grenham S, Clarke G, Cryan JF, Dinan TG (2011) Brain-gut-microbe communication in health and disease. Front Physiol 2:94. doi:10.3389/fphys.2011.00094
54. Clarke G, Grenham S, Scully P et al (2013) The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. Mol Psychiatry 18:666–673. doi:10.1038/mp.2012.77
55. Thomas LV, Ockhuizen T, Suzuki K (2014) Exploring the influence of the gut microbiota and probiotics on health: a symposium report. Br J Nutr 112(Suppl 1):S1–S18. doi:10.1017/S0007114514001275
56. Patel R, DuPont HL (2015) New approaches for bacteriotherapy: prebiotics, new-generation probiotics, and synbiotics. Clin Infect Dis Off Publ Infect Dis Soc Am 60(Suppl 2):S108–S121. doi:10.1093/cid/civ177
57. Meehan CJ, Langille MGI, Beiko RG (2015) Frailty and the microbiome. Interdiscip Top Gerontol Geriatr 41:54–65. doi:10.1159/000381162
58. Le Barz M, Anhê FF, Varin TV et al (2015) Probiotics as complementary treatment for metabolic disorders. Diabetes Metab J 39:291–303. doi:10.4093/dmj.2015.39.4.291
59. Malnick S, Melzer E (2015) Human microbiome: from the bathroom to the bedside. World J Gastrointest Pathophysiol 6:79–85. doi:10.4291/wjgp.v6.i3.79
60. Hooper LV, Midtvedt T, Gordon JI (2002) How host-microbial interactions shape the nutrient environment of the mammalian

intestine. Annu Rev Nutr 22:283–307. doi:10.1146/annurev.nutr.22.011602.092259
61. Chung H, Pamp SJ, Hill JA et al (2012) Gut immune maturation depends on colonization with a host-specific microbiota. Cell 149:1578–1593. doi:10.1016/j.cell.2012.04.037
62. Round JL, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol 9:313–323. doi:10.1038/nri2515
63. Vannucci L, Stepankova R, Kozakova H et al (2008) Colorectal carcinogenesis in germ-free and conventionally reared rats: different intestinal environments affect the systemic immunity. Int J Oncol 32:609–617
64. Vannucci L, Stepankova R, Grobarova V et al (2009) Colorectal carcinoma: importance of colonic environment for anti-cancer response and systemic immunity. J Immunotoxicol 6:217–226. doi:10.3109/15476910903334343
65. Tlaskalová-Hogenová H, Štěpánková R, Kozáková H et al (2011) The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. Cell Mol Immunol 8:110–120. doi:10.1038/cmi.2010.67
66. Zachar Z, Savage DC (1979) Microbial interference and colonization of the murine gastrointestinal tract by *Listeria monocytogenes*. Infect Immun 23:168–174
67. Czuprynski CJ, Balish E (1981) Pathogenesis of *Listeria monocytogenes* for gnotobiotic rats. Infect Immun 32:323–331
68. Nardi RM, Vieira EC, Crocco-Afonso LC et al (1991) Bacteriological and immunological aspects of conventional and germfree mice infected with *Salmonella typhimurium*. Rev Latinoam Microbiol 33:239–243
69. Butterton JR, Ryan ET, Shahin RA, Calderwood SB (1996) Development of a germfree mouse model of *Vibrio cholerae* infection. Infect Immun 64:4373–4377
70. Mittrücker H-W, Seidel D, Bland PW et al (2014) Lack of microbiota reduces innate responses and enhances adaptive immunity against *Listeria monocytogenes* infection. Eur J Immunol 44:1710–1715. doi:10.1002/eji.201343927
71. Inagaki H, Suzuki T, Nomoto K, Yoshikai Y (1996) Increased susceptibility to primary infection with *Listeria monocytogenes* in germfree mice may be due to lack of accumulation of L-selectin+ CD44+ T cells in sites of inflammation. Infect Immun 64:3280–3287
72. Fagundes CT, Amaral FA, Vieira AT et al (2012) Transient TLR activation restores inflammatory response and ability to control pulmonary bacterial infection in germfree mice. J Immunol Baltim Md 1950(188):1411–1420. doi:10.4049/jimmunol.1101682
73. Croswell A, Amir E, Teggatz P et al (2009) Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric Salmonella infection. Infect Immun 77:2741–2753. doi:10.1128/IAI.00006-09
74. Ferreira RBR, Gill N, Willing BP et al (2011) The intestinal microbiota plays a role in Salmonella-induced colitis independent of pathogen colonization. PLoS One 6:e20338. doi:10.1371/journal.pone.0020338
75. Mack DR, Michail S, Wei S et al (1999) Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. Am J Physiol 276:G941–G950
76. Mack DR, Ahrne S, Hyde L et al (2003) Extracellular MUC3 mucin secretion follows adherence of Lactobacillus strains to intestinal epithelial cells in vitro. Gut 52:827–833
77. Srikanth CV, McCormick BA (2008) Interactions of the intestinal epithelium with the pathogen and the indigenous microbiota: a three-way crosstalk. Interdiscip Perspect Infect Dis 2008:626827. doi:10.1155/2008/626827
78. Fagarasan S (2006) Intestinal IgA synthesis: a primitive form of adaptive immunity that regulates microbial communities in the gut. Curr Top Microbiol Immunol 308:137–153
79. Macpherson AJ, Geuking MB, McCoy KD (2011) Immunoglobulin A: a bridge between innate and adaptive immunity. Curr Opin Gastroenterol 27:529–533. doi:10.1097/MOG.0b013e32834bb805
80. Gutzeit C, Magri G, Cerutti A (2014) Intestinal IgA production and its role in host-microbe interaction. Immunol Rev 260:76–85. doi:10.1111/imr.12189
81. Jarchum I, Pamer EG (2011) Regulation of innate and adaptive immunity by the commensal microbiota. Curr Opin Immunol 23:353–360. doi:10.1016/j.coi.2011.03.001
82. Maynard CL, Elson CO, Hatton RD, Weaver CT (2012) Reciprocal interactions of the intestinal microbiota and immune system. Nature 489:231–241. doi:10.1038/nature11551
83. Ewaschuk JB, Backer JL, Churchill TA et al (2007) Surface expression of Toll-like receptor 9 is upregulated on intestinal epithelial cells in response to pathogenic bacterial DNA. Infect Immun 75:2572–2579. doi:10.1128/IAI.01662-06
84. Hall JA, Bouladoux N, Sun CM et al (2008) Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. Immunity 29:637–649. doi:10.1016/j.immuni.2008.08.009
85. Abt MC, Osborne LC, Monticelli LA et al (2012) Commensal bacteria calibrate the activation threshold of innate antiviral immunity. Immunity 37:158–170. doi:10.1016/j.immuni.2012.04.011
86. Abt MC, Pamer EG (2014) Commensal bacteria mediated defenses against pathogens. Curr Opin Immunol 29:16–22. doi:10.1016/j.coi.2014.03.003
87. Clarke TB, Davis KM, Lysenko ES et al (2010) Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat Med 16:228–231. doi:10.1038/nm.2087
88. Denny JE, Powell WL, Schmidt NW (2016) Local and long-distance calling: conversations between the gut microbiota and intra- and extra-gastrointestinal tract infections. Front Cell Infect Microbiol 6:41. doi:10.3389/fcimb.2016.00041
89. Fernández-Santoscoy M, Wenzel UA, Yrlid U et al (2015) The gut microbiota reduces colonization of the mesenteric lymph nodes and IL-12-independent IFN-γ production during salmonella infection. Front Cell Infect Microbiol 5:93. doi:10.3389/fcimb.2015.00093
90. Fournout S, Dozois CM, Odin M et al (2000) Lack of a role of cytotoxic necrotizing factor 1 toxin from Escherichia coli in bacterial pathogenicity and host cytokine response in infected germfree piglets. Infect Immun 68:839–847
91. Hayakawa M, Taguchi H, Kamiya S et al (2002) Animal model of *Mycoplasma pneumoniae* infection using germfree mice. Clin Diagn Lab Immunol 9:669–676
92. Stecher B, Macpherson AJ, Hapfelmeier S et al (2005) Comparison of *Salmonella enterica* serovar *Typhimurium colitis* in germfree mice and mice pretreated with streptomycin. Infect Immun 73:3228–3241. doi:10.1128/IAI.73.6.3228-3241.2005
93. Vecht U, Wisselink HJ, Jellema ML, Smith HE (1991) Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infect Immun 59:3156–3162
94. Vecht U, Wisselink HJ, van Dijk JE, Smith HE (1992) Virulence of *Streptococcus suis* type 2 strains in newborn germfree pigs depends on phenotype. Infect Immun 60:550–556
95. Havell EA, Beretich GR, Carter PB (1999) The mucosal phase of Listeria infection. Immunobiology 201:164–177. doi:10.1016/S0171-2985(99)80056-4
96. Duerkop BA, Hooper LV (2013) Resident viruses and their interactions with the immune system. Nat Immunol 14:654–659. doi:10.1038/ni.2614

97. Virgin HW (2014) The virome in mammalian physiology and disease. Cell 157:142–150. doi:10.1016/j.cell.2014.02.032
98. Norman JM, Handley SA, Virgin HW (2014) Kingdom-agnostic metagenomics and the importance of complete characterization of enteric microbial communities. Gastroenterology 146:1459–1469. doi:10.1053/j.gastro.2014.02.001
99. Kapusinszky B, Minor P, Delwart E (2012) Nearly constant shedding of diverse enteric viruses by two healthy infants. J Clin Microbiol 50:3427–3434. doi:10.1128/JCM.01589-12
100. Donaldson EF, Lindesmith LC, Lobue AD, Baric RS (2008) Norovirus pathogenesis: mechanisms of persistence and immune evasion in human populations. Immunol Rev 225:190–211. doi:10.1111/j.1600-065X.2008.00680.x
101. Minot S, Sinha R, Chen J et al (2011) The human gut virome: inter-individual variation and dynamic response to diet. Genome Res 21:1616–1625. doi:10.1101/gr.122705.111
102. Popgeorgiev N, Temmam S, Raoult D, Desnues C (2013) Describing the silent human virome with an emphasis on giant viruses. Intervirology 56:395–412. doi:10.1159/000354561
103. Spandole S, Cimponeriu D, Berca LM, Mihăescu G (2015) Human anelloviruses: an update of molecular, epidemiological and clinical aspects. Arch Virol 160:893–908. doi:10.1007/s00705-015-2363-9
104. Kernbauer E, Ding Y, Cadwell K (2014) An enteric virus can replace the beneficial function of commensal bacteria. Nature 516:94–98. doi:10.1038/nature13960
105. Wilks J, Beilinson H, Golovkina TV (2013) Dual role of commensal bacteria in viral infections. Immunol Rev 255:222–229. doi:10.1111/imr.12097
106. Wilks J, Beilinson H, Theriault B et al (2014) Antibody-mediated immune control of a retrovirus does not require the microbiota. J Virol 88:6524–6527. doi:10.1128/JVI.00251-14
107. Osborne LC, Monticelli LA, Nice TJ et al (2014) Coinfection. Virus-helminth coinfection reveals a microbiota-independent mechanism of immunomodulation. Science 345:578–582. doi:10.1126/science.1256942
108. Knip M, Siljander H (2016) The role of the intestinal microbiota in type 1 diabetes mellitus. Nat Rev Endocrinol 12:154–167. doi:10.1038/nrendo.2015.218
109. Mondot S, Lepage P (2016) The human gut microbiome and its dysfunctions through the meta-omics prism. Ann N Y Acad Sci. doi:10.1111/nyas.13033
110. Mazidi M, Rezaie P, Kengne AP et al (2016) Gut microbiome and metabolic syndrome. Diabetes Metab Syndr. doi:10.1016/j.dsx.2016.01.024
111. Li Y, Liu Y, Chu C-Q (2015) Th17 cells in type 1 diabetes: role in the pathogenesis and regulation by gut microbiome. Med Inflamm 2015:638470. doi:10.1155/2015/638470
112. Lerner A, Matthias T (2015) Rheumatoid arthritis-celiac disease relationship: joints get that gut feeling. Autoimmun Rev 14:1038–1047. doi:10.1016/j.autrev.2015.07.007
113. Rogier R, Koenders MI, Abdollahi-Roodsaz S (2015) Toll-like receptor mediated modulation of T cell response by commensal intestinal microbiota as a trigger for autoimmune arthritis. J Immunol Res 2015:527696. doi:10.1155/2015/527696
114. Block KE, Zheng Z, Dent AL et al (2016) Gut microbiota regulates K/BxN autoimmune arthritis through follicular helper T but not Th17 cells. J Immunol Baltim Md 1950(196):1550–1557. doi:10.4049/jimmunol.1501904
115. Vieira SM, Pagovich OE, Kriegel MA (2014) Diet, microbiota and autoimmune diseases. Lupus 23:518–526. doi:10.1177/0961203313501401
116. Hansen CHF, Nielsen DS, Kverka M et al (2012) Patterns of early gut colonization shape future immune responses of the host. PLoS One. doi:10.1371/journal.pone.0034043
117. Hansen AK, Hansen CHF, Krych L, Nielsen DS (2014) Impact of the gut microbiota on rodent models of human disease. World J Gastroenterol WJG 20:17727–17736. doi:10.3748/wjg.v20.i47.17727
118. Macela A, Kubelkova K (2016) Innate immune recognition: Lesson from Francisella models. Discussion Forum 2016 - Host Pathogen Interaction conference, Broumov, Czech Republic
119. Casadevall A, Pirofski LA (1999) Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. Infect Immun 67:3703–3713
120. Casadevall A, Pirofski L-A (2015) What is a host? Incorporating the microbiota into the damage-response framework. Infect Immun 83:2–7. doi:10.1128/IAI.02627-14