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The microbiome and rodent models of immune mediated diseases

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Abstract

Over the last six decades production of laboratory rodents have been refined with the aim of eliminating all pathogens, which could influence research results. This has, however, also created rodents with little diversity in their microbiota. Until 10 years ago the impact of the microbiota on the outcome of rodent studies was ignored, but today it is clear that the phenotype of rodent models differs essentially in relation to the environment of origin, i.e. different breeders or different rooms. In this review, we outline the mechanisms behind gut bacterial impact on rodent models of immune mediated diseases, and how differences in environment of origin leads to phenotypic model differences within research areas such as infectious diseases and vaccine development, the metabolic syndrome, gut immunity and inflammation, autoimmunity and allergy. Finally, we sum up some tools to handle this impact to increase reproducibility and translatability of rodent models.

Introduction

In the late fifties of the twentieth century, a new era of specific pathogen free (SPF) rodent breeding was initiated by the use of caesarian section and barrier protection (Foster 1958), at first attempting to eliminate strong pathogens such as *Citrobacter rodentium* (by then *C. freundii* type 4280) (Barthold et al. 1976) and zoonoses, such as *Streptobacillus moniliformis* (Kaspareit-Rittinghausen et al. 1990). In the coming decades, and especially enforced by the first set of common European standards for health monitoring issued by the Federation of European Laboratory Animal Associations (Kraft et al. 1994) in the mid-nineties, the process of pathogen eradication was speeded up, and now also included microorganisms with a more discrete impact on the research models, such as various *Helicobacter* spp. (Fox et al. 1994). In parallel to this development, the rearing of germ-free animals, so-called gnotobiotechnology, was developed from the thirties (Glimstedt 1936; Reyniers et al. 1946), which made it clear that bacteria played an essential role in many characteristics of the mammal host (Falk et al. 1998; Gustafsson and Norin 1977; O'Mahony et al. 2009). To minimize the phenomenon that germ-free rodents seem to be predisposed to opportunistic infections, when removed from their isolators and introduced into barrier rooms, the so-called Schaedler Flora was developed and later modified as the Altered Schaedler Flora (ASF) (Dewhirst et al. 1999; Schaedler et al. 1965a). Today, the starting point for most commercial breeding colonies of rodents will be rederivation by caesarian section or embryo transfer and subsequent association with the eight ASF bacteria, and such animals can be bred for generations still harbouring these bacteria (Alexander et al. 2006; Schaedler et al. 1965b; Stehr et al. 2009). Most likely, the remaining members of the microbiome of a barrier bred colony are transferred from either humans currently emitting several microbe-carrying particles (Whyte et al. 1983), or from the diet (Zhang et al. 2010), and the microbiome of laboratory rodents is, therefore, essentially different from that of feral or conventionally bred rodents (Itoh et al. 1983; Wilson et al. 2006). Moreover, establishment of the gut microbiota among genetically similar mice from different commercial vendors was already decades ago reported to be different (Hirayama et al. 1990a; O'Rourke et al. 1988). Even different colonies of the same strain within one vendor may differ (Hufeldt et al. 2010), and the same colony may differ over time (Fahey et al. 2017; O'Rourke et al. 1988). This can be problematic, as the microbiome with its more than 1 million genes is responsible for much of the phenotypic expression of an animal model (Bleich

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and Hansen 2012; Ellekilde et al. 2014; Hansen et al. 2014a; Holmes and Nicholson 2005), which also includes an essential impact on the immune system (Fig. 1). This includes stimulation of the innate immune system, when microbial molecules known as microbial-associated molecular patterns (MAMP), without any forms of previous memory-inducing stimulation, react with pattern-recognition receptors (PRR) in the host (Tlaskalova-Hogenova et al. 2004). It also includes stimulation of the adaptive immune system, as one single bacterial molecule may express several epitopes, which after presentation by antigen presenting cells, will turn naïve T cells, into specific $CD4^+$ T cells, including T helper cells of type 1, type 2, type 3 and type 17 as well as regulatory T cells, or $CD8^+$ cytotoxic T cells (D'Elios et al. 2011; Korn et al. 2009; Mosmann and Coffman 1989; Ohkura et al. 2013). In mice, the microbiota establishes itself around weaning, and this establishment to a certain extent guides the future immune responses of the host (Hansen et al. 2012b), as early life microbial encounters induce gut oral tolerance, which may counteract inflammatory or autoimmune disease later in life (Castro-Sanchez and Martin-Villa 2013; Gratz et al. 2013; Hsieh et al. 2012). Variation in microbiota stimulation at the breeding facility may therefore be a likely cause of phenotypic variation later in life, because many of the models are driven by specific T cell types (Bleich and Hansen 2012). Current microbial stimulation

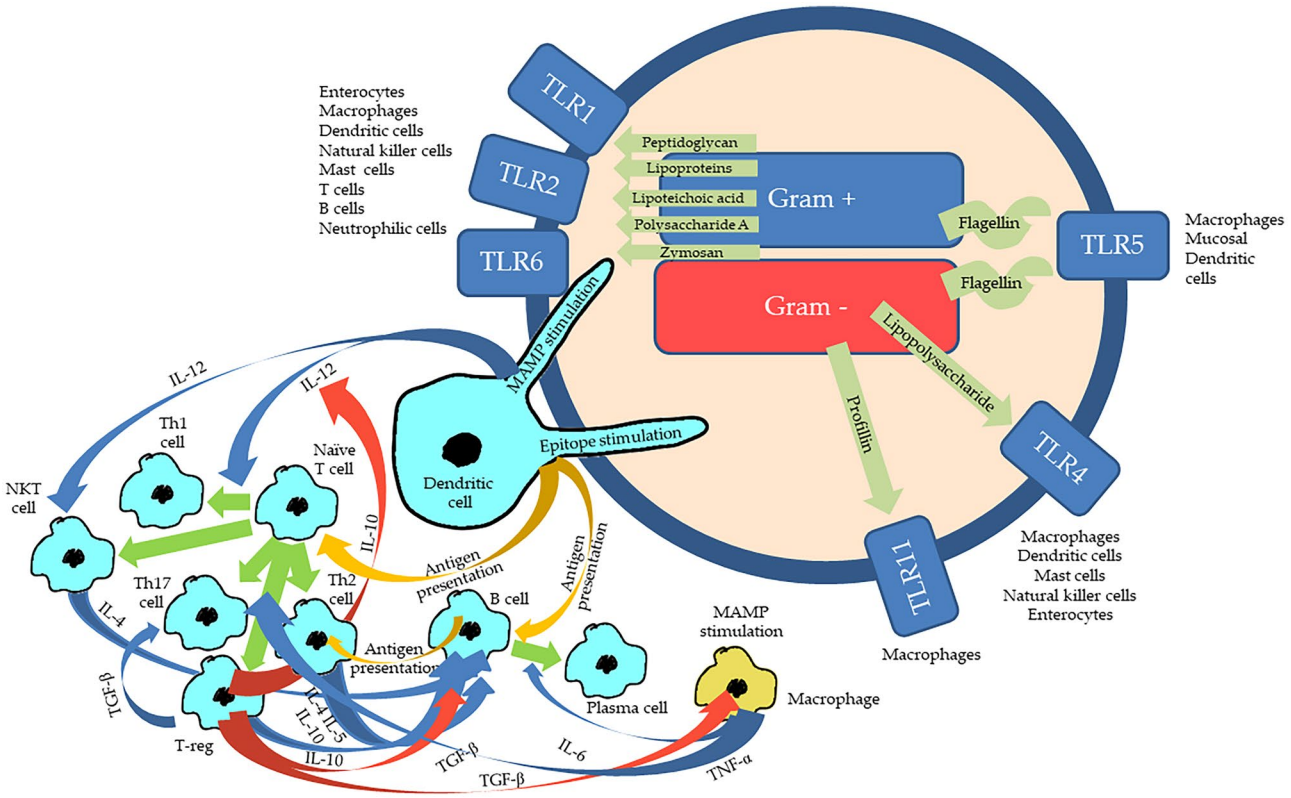

Fig. 1 Immune activation by bacteria in the gut. In the gut the immune cells and the enterocytes possess different types of Toll-like receptors (TLR), which can be stimulated by different types of microbial associated molecular patterns (MAMP), which dependent on cell type and TLR will cause different forms of innate immune activation and cytokine production. MAMPS are such as peptidoglycan, lipoproteins, lipoteichoic acid, polysaccharide A and zymosan from Gram positive bacteria, lipopolysaccharides and profillin from Gram negative bacteria, and flagellin from bacterial flagellas. Epitopes, which are different parts of bacterial molecules with an adaptive immune stimulatory potential, will be presented by antigen presenting cells (yellow arrows), such as the dendritic cells, to T and B cells. The cytokine production initiated by the innate MAMP-TLR stimulation will upscale (blue arrows) or downscale (red arrows) the differentiation (green arrows) of T and B cells into effector cells. Stimulation by IL-12 secreted by the dendritic cells as a response to MAMP stimulation will facilitate the differentiation of naïve T cells, into T helper cells type 1 (Th1) on the cost of T helper cells type 2 (Th2), while this process is downscaled by IL-10 from the regulatory T cells (T-reg). IL-12 will also stimulate the natural killer T (NKT) cells. The B cells will present the antigen for the Th2s, which will respond back with IL-4 and IL-5 to turn the B cell into an antigen producing plasma cell. This process is upscaled by IL-6 caused by the MAMP-TLR stimulation of the macrophages, and B cells will be stimulated by IL-10 from T-regs and Th2, while TGF-β from the T-regs will downscale activities of both B cells and macrophages. TGF-β will upscale the activity of Th17 cells. The activated macrophages will produce TNF-α, which will stimulate a number of different T cells. There are far more cytokines and cell types than this, there are other pattern recognition receptors than the TLRs, and in relation to gut virus infections cytotoxic T cells will also be relevant

of the gut lamina propria dendritic cells induces secretion of IL-12 from these, which will favour T helper cell type 1 differentiation on the cost of T helper cell type 2 differentiation (Hsieh et al. 1993) (Fig. 1). Therefore, animals with a low diversity or even being germ-free will everything else equal be dominated by T helper cells type 2 in their immune responses (Mazmanian and Kasper 2006).

The microbiota in addition to bacteria also includes protozoa and fungi, and the bacteria also have their own viruses, the so-called phages, which also can be shown to have an impact on animal model expression (Rasmussen et al. 2019, 2020). Therefore, the microbiota of genetically closely related strains from different vendors may be essentially different (Hirayama et al. 1990b; Hufeldt et al. 2010), and, therefore, in some (Rasmussen et al. 2019), but not in all cases (Siersbæk et al. 2020), be responsible for the lack or reproducibility of rodent studies. Recently, the SPF principles for rodent breeding have also been debated, as pathogens are important for the basic activation and the function of the immune system (Tao and Reese 2017). Therefore, it has been argued that especially within immunology one of the reasons for failures of translating results from the preclinical to the clinical phase of research is due to the naïve pathogen status of the animal models (Masopust et al. 2017). In relation to the abundances of cytotoxic T cells and T helper cells, a laboratory mouse unlike pet shop and wild mice more resemble the immune system of a newborn than that of an adult human being (Beura et al. 2016).

Therefore, the aim of this review is to elucidate how the commensal as well as the pathogenic microbiota impact the expression, translatability and reproducibility of rodent models on immune mediated diseases. In this review, we discuss major findings to date and challenges faced when using very clean SPF mice compared to the use of conventional, pet shop or feral mice for immunological research.

Microbiota impact on reproducibility of various immunological mouse models

Infectious diseases and vaccine development

It is quite clear from the literature that the microbiota has a profound impact on disease severity and expression in infection models. For instance, it can be shown by antibiotic treatment that the microbiota enhances the severity of some gastrointestinal infections, such as human poliovirus (Kuss et al. 2011), murine norovirus (Jones et al. 2014) and mouse mammary tumour virus MMTV (Kane et al. 2011), while it alleviates severity for some respiratory infections, such as human influenza virus (Ichinohe et al. 2011; Steed et al. 2017; Wu et al. 2013), *Streptococcus pneumonia* (Brown et al. 2017), *Klebsiella pneumonia* (Brown et al. 2017) and *Mycobacterium tuberculosis* (Dumas et al. 2018; Khan et al. 2016). If mice infected with *C. rodentium* harbour a complex microbiota, they will at some time point of infection express some virulence genes (Kamada et al. 2012). This will relocate the agent to the gut lumen to be outcompeted by the commensal microbiota (Kamada et al. 2012). In contrast, germ-free mice are unable to eradicate *C. rodentium* (Kamada et al. 2012). A very nice example of the impact a vendor can have, was published by a group that noted that the source of C57BL/6 mice influenced the severity of an infection model of malaria (Villarino et al. 2016). The degree of parasitemia, weight loss and mortality were greater in mice from Envigo (Harlan) and Charles River compared to the more resistant mice from Jackson and Taconic, which harboured substantially different microbial communities in their gastrointestinal tract (Villarino et al. 2016). The causal role of the gut microbiota was nicely demonstrated by cecal transplant to germ-free mice (Villarino et al. 2016). Even mice from the same vendor, changed susceptibility to both malaria and *Salmonella* infection over time due to a change in the gut microbiota (Mandal et al. 2020), demonstrating the importance of not only considering the origin of the mice, but also the timing when comparing studies of the same character. In particular, vendor differences in *Escherichia coli* cause variation in host response to *Salmonella* infection by competing with *Salmonella* for resources (Velazquez et al. 2019). Moreover, transferring the clearly differing natural gut microbiome from a population of wild mice, which genetically were closely related to laboratory mice, made the recipients exhibit reduced inflammation and increased survival following an otherwise lethal influenza virus infection (Rosshart et al. 2017). In another study with 'dirty mice', in which laboratory mice caught pathogens when co-housed with pathogen infected pet shop mice those laboratory mice, which survived the high mortality that it induced, experienced lower severity of disease, when infected with *Plasmodium berghei* and *Listeria monocytogenes* (Beura et al. 2016). It has for the same reason been debated, whether it would be a good idea to test vaccines both in pathogen free and pathogen infected mice to improve validity of infection models (Masopust et al. 2017). Antibody responses induced by influenza vaccination in germ-free or antibiotic-treated TLR5 deficient mice were impaired, but restored by oral reconstitution with a flagellated, but not aflagellated, strain of *Eschericia coli* (Oh Jason et al. 2014). Antibiotic exposure in infant but not adult mice impaired antibody responses to vaccination against *Meningococcus* B (MenB) and C (MenC), *Pneumoccus* (PCV13), *Mycobacteria* (BCG) as well as against the polyvalent 6-in-1 Hexa vaccine, while restoring the commensal microbiota rescued the impaired antibody responses (Lynn et al. 2018). So, as microbiota differs substantially between different colonies of the same strain of mice (Ericsson et al. 2015; Hufeldt et al. 2010;

Rasmussen et al. 2019; Siersbæk et al. 2020), there are good reasons to assume that there is an impact of the microbiota on the disease expression in infection models, and that this also has an impact on vaccine efficiency.

Metabolic syndrome

Inflammation is a key element of the metabolic syndrome, as much of the peripheral insulin resistance is caused by cytokine interaction with the peripheral cell insulin receptors (Boucher et al. 2014). The most commonly applied animal model for this is the diet induced obese C57BL/6 mouse (Varga et al. 2010). This model can also be done on leptin or leptin receptor deficient mice (Varga et al. 2010). Much focus has been on microbiota impact on these models, since Turnbaugh et al. successfully transferred the obese phenotype to lean germ-free mice through a microbiota transplantation 15 years ago (Turnbaugh et al. 2006). The phenotype in that study was only characterized in relation to obesity and lipid parameters (Turnbaugh et al. 2006), but later Vrieze et al. showed that similar microbiota transplantation from lean to obese humans for a short period improved insulin resistance (Vrieze et al. 2012). In mice high levels of IL-10, IL-12, TNF-α as well as regulatory T cells correlate to impaired glucose tolerance (Ellekilde et al. 2014), as it does in humans (Claesson et al. 2012). One explanation may be that lipopolysaccharides (LPS) from the Gram negative bacteria of the gut or the diet enters the blood stream, known as metabolic endotoxinaemia, and by TLR4 stimulation induces cytokine secretion which interacts with the insulin receptor (Cani et al. 2007; Lindenberg et al. 2019). The gut microbiota of both C57BL/6N (Rasmussen et al. 2019) and C57BL/6J (Siersbæk et al. 2020) differ substantially between mice from different vendors. Differences in the microbiota, i.e. both bacteria and phages, of C57BL/6N mice could explain much of the phenotypic difference between mice from different vendors (Rasmussen et al. 2019). Even differences in microbiota between different facilities from the same vendor can affect the metabolic phenotype of high-fat diet induced obese CD-1 mice (Unger et al. 2020). The development of obesity and expression of the IFN-γ gene in the liver in these mice was reduced by phage transplantation in C57BL/6N mice (Rasmussen et al. 2020). In contrast, the microbiota seemed to have little impact on the phenotypic differences observed in C57BL/6J mice from different vendors (Siersbæk et al. 2020). Variation in important measures of barrier function, metabolic endotoxemia, and low-grade inflammation related to high-fat feeding is also strongly affected by the microbial differences observed between conventional and SPF housing. Several of the obesity-related metabolic dysfunctions disappear in too clean environments (Müller et al. 2016), including the inflammatory response of the liver, which is pivotal to develop a proper animal model for non-alcoholic fatty liver disease (Chen et al. 2020b). There does not seem to be any studies on the development of the cardiovascular part of the metabolic syndrome in mice in relation to how this differs between different environments, such as commercial vendors, but the involvement of the microbiota seems to become increasingly well documented. Hypertension seems to be influenced by the gut microbiota, as fecal microbiota transplantation to germ-free mice increases blood pressure (Li et al. 2017). As antibiotics and germ-free status influence the host differently, there are conflicting results between studies of germ-free and antibiotic treated mice (Bayer et al. 2019). The development of atherosclerosis is not altered in germ-free LDL receptor deficient mice (Kiouptsi et al. 2020), but microbiota depletion with antibiotics in mice has been shown to suppress the development of atherosclerosis in mice (Chen et al. 2020a; Man et al. 2020; Rune et al. 2016). This is not similarly evident in rats (Rune et al. 2018). Dietary lipid intake, gut microbiota and development of atherosclerosis is linked in mice, which seems to be mediated through the production of inducing metabolites, such as trimethylamine N-oxide (TMAO) (Wang et al. 2011). TMAO promotes the release of pro-inflammatory cytokines, such as IL-1β, IL-6 and IL-18 (Seldin et al. 2016; Yue et al. 2017), which may be mediated by stimulation of TLR5 by flagellin, which promotes hepatic production of ApoE (Yiu et al. 2020).

Gut immunity and inflammation

The development of inflammatory bowel disease (IBD), i.e. T helper cell type 2 dominated ulcerative colitis and the T helper cell type 1 dominated Crohn's disease, is obviously under strong impact of the microbiota (Nell et al. 2010), as the gut microbiota is a key element in the disease etiology (Nell et al. 2010). The ability of mouse models to develop IBD is, therefore, under strong influence of the presence or absence of specific bacteria. From the first attempts to turn conventional IBD prone mice into SPF mice, it has been realized that germ-free status eliminates or dramatically alleviates symptoms of IBD in mice, such as in senescence accelerated P1/Yit mice (Matsumoto et al. 1998), as well as in mice deficient of IL-10 (Sellon et al. 1998), IL-2 (Contractor et al. 1998), T-cell receptor-α (Dianda et al. 1997) and intestine specific transforming growth factor-β signalling (Hahm et al. 2001). IL-10 deficient mice spontaneously develop chronic colitis, when maintained in conventional facilities, but they fail to express a similar phenotype under SPF conditions. The presence of *Helicobacter hepaticus* is, however, sufficient for the SPF IL-10 deficient mice to develop a more severe intestinal inflammatory response (Kullberg et al. 1998). Another important example is *Faecalibacterium prausnitzii* (Carlsson et al. 2013), which also in humans is an important anti-inflammatory agent with

a potential to alleviate severity of IBD (Lopez-Siles et al. 2018). Yet another very important example is segmented filamentous bacteria (SFB or *Candidatus Savagella*), which strongly influences IgA production, T-cell response, and intestinal T helper cell type 17 induction (Ericsson et al. 2014; Gaboriau-Routhiau et al. 2009), as well as the functional specializations of antigen presenting cells able to induce regulatory T cells or T helper cell type 17 (Denning et al. 2011). The first descriptions of the T helper cell type 17 were published, when a research group discovered that C57BL/6 mice obtained from several university and commercial SPF sources had relatively high proportions of T helper cells type 17 (Ivanov et al. 2008). In contrast, C57BL/6 mice obtained from the Jackson Laboratory consistently had only very few, and, therefore, the adaptive transfer model of Crohn's disease could not be induced in Jackson mice (Ivanov et al. 2008). Another important factor is the properties of the intestinal mucus layer in preventing intestinal bacteria from penetrating and getting in contact with the intestinal epithelial lining. The mucus phenotype was found to differ between different rooms of the same facility which would be expected to have an essential impact on IBD models (Jakobsson et al. 2015). Wild mice have mucus impenetrable to bacteria and are also resistant to induction of IBD and colitis-associated tumorigenesis (Rosshart et al. 2017). So, it is beyond any discussion that the gut microbiota is of crucial importance for the reproducibility of IBD studies between mice from different commercial vendors, housing conditions and in comparison to wild or dirty mice. This would also be expectable in other animal models of intestinal inflammation. As an example, mice expressing the human DQ8 gene, which confers moderate susceptibility to gluten-induced immunopathology, i.e. celiac disease, develop increased response to dietary gluten in conventional conditions compared to SPF conditions with a more restricted microbial composition (Galipeau et al. 2015). The attenuated response in the SPF mice was reversed by introducing a *Proteobacterium* from a patient with celiac disease (Galipeau et al. 2015).

Autoimmunity

Environmental factors are at play in defining susceptibility to type 1 diabetes in both humans and animal models. Disease incidence in non-obese diabetic (NOD) mice and Bio-Breeding (BB) rats, which spontaneously develop hyperglycemia, vary substantially depending on barrier status in different facilities (Klöting et al. 1984; Pozzilli et al. 1993; van der Werf et al. 2007). One study transplanted microbiota from a low-incidence NOD colony to a high-incidence colony but failed to transfer protection due to a few bacterial taxa not successfully transferred, including *Akkermansia muciniphila* (Hanninen et al. 2018). However, upon weekly gavage of the high incidence colony with *A. muciniphila*, the authors found a delay in diabetes onset along with an improvement in gut barrier function and regulatory immunity, demonstrating the significance of harboring this specific taxon in NOD mouse facilities (Hanninen et al. 2018). *A. muciniphila* was first associated with a reduced incidence of type 1 diabetes in NOD mice that were treated with vancomycin, which eliminates most taxa and, thereby, favours growth of *A. muciniphila* (Hansen et al. 2012a). Interestingly, another study with vancomycin treated NOD mice shortly after reported a similar increase in the abundance of *A. muciniphila*, but the diabetes incidence of this population was higher compared to untreated mice (Brown et al. 2016). The contradicting results may likely be explained by the remaining microbiota composition, as the mice in the first study from Taconic were not colonized by SFB unlike the Jackson mice in the study by Brown et al. The presence of SFB was already a decade ago shown to be associated with the diabetes incidence of a NOD colony (Barfod et al. 2015), and antibiotic treatment of the NOD mice colonies at Jackson eliminated SFB from their colony which caused a noteworthy increase in the diabetes incidence in their otherwise low incidence colony (Fahey et al. 2017). Importantly, *A. muciniphila* and SFB have no effect on diabetes incidence in mono-colonized NOD mice (Hansen et al. 2016; Yurkovetskiy et al. 2013), and thus appear to exert their protective properties through interaction with other commensals. The gut microbiota is also a strong mediator of dietary interventions, and it is not surprising that the result of a dietary change depends on the host's microbial composition. A gluten-free diet, for example, had an alleviating effect in NOD mice, in which *A. muciniphila* was propagated by the diet (Hansen et al. 2014b; Marietta et al. 2013), but failed to reduce diabetes incidence in NOD mice, in which *A. muciniphila,* for unknown reason, was reduced (Liu et al. 2016). Treatment with the antibiotic fusidic acid reduces the incidence of type 1 diabetes in BB rats (Kaas et al. 2001) probably by preventing the impact of IL-1 and IL-6 on the islets (Bendtzen et al. 1992).

Susceptibility and severity of arthritis in a variety of rodent strains also depends on the gut microbiota environment, which often exhibit reduced bacterial diversity in laboratory animal facilities. In the collagen induced mouse model for rheumatoid arthritis (RA), susceptibility is highest in mice enriched with *Lactobacillus*, and transplantation of feces from mice with high susceptibility was shown to increase the frequency of arthritis induction compared to recipients of feces from arthritis-resistant mice (Liu et al. 2016). Also for the SKG mouse model, disease severity depends on the microbial environment; arthritis develops spontaneously in conventional facilities, while it must be induced in SPF facilities (Sakaguchi et al. 2003). This phenotype seems to be mainly depending on a single species *Prevotella copri*, which is sufficient to induce arthritis, when

mono-colonized in germ-free SKG mice by induction of intestinal Th17 cells (Maeda et al. 2016). The other well-known Th17-inducing bacteria, SFB, also drives autoimmune arthritis, as shown in SFB mono-colonized K/BxN mice (Wu et al. 2010). At first, the authors noticed low autoantibody titers and attenuated arthritis development in K/BxN mice from Jackson, which typically lack this bacterium and a proper Th17 response (Wu et al. 2010). Therefore, they tested the importance of SFB by transferring germ-free K/BxN mice to a SFB-negative SPF facility, while treating some of them with oral gavage with feces from SFB-monocolonized mice (Wu et al. 2010). The SFB-treated mice showed accelerated arthritis development and appearance of gut Th17 cells, which migrated systemically to trigger the autoimmune response (Wu et al. 2010).

Allergy

Atopic individuals with a predisposition to developing allergic asthma, food allergies, anaphylaxis and childhood eczema, i.e. atopic dermatitis, are becoming increasingly prevalent, and in industrialized countries they represent 20–25% of the population (Al-Herz 2018). There is evidence that the skin and gut microbiomes contribute to allergic reactions of the skin, which is usually the first allergic response in IgE sensitized children, which is then followed by allergic rhinitis and asthma later in life (Bantz et al. 2014). Overgrowth of skin colonizing *Staphylococcus aureus* is well known to precede onset of disease in atopic dermatitis patients (Meylan et al. 2017), and its abundance correlates with severity of dermal symptoms (Tauber et al. 2016). Perhaps for the same reason, it is not strange that in SPF facilities, in which *S. aureus* is often notoriously eliminated, certain spontaneous animal models of atopic dermatitis, such as the NC/Nga and DS-*Nh* mice, fail to display skin lesions and IgE levels (Hikita et al. 2002; Suto et al. 1999). This is comparable to human atopic dermatitis, and to mice raised in conventional facilities (Hikita et al. 2002; Suto et al. 1999). In contrast, a diverse gut microbiota early in life has been shown to prevent high IgE levels and risk of allergy later in life (Cahenzli et al. 2013). However, besides the presence in conventional mice of fur mites and *S. aureus*, both strongly involved in the induction of dermal symptoms, mechanistic pathways underlying gut microbial influences on IgE levels and development of allergies are likely more complex and species specific. As an example, intestinal *Prevotella* spp. were found to be strongly associated with low IgE levels and a low-responding phenotype in mice with oxazolone-induced dermatitis, and prebiotic treatment of the mice propagated *Prevotella* spp. and reduced the number of high-responders (Laigaard et al. 2020). In addition, the high-and low-responding phenotypes of mice with oxazolone-induced dermatitis were transferable with the gut microbiome to germ-free mice, strongly indicating the importance of its composition (Zachariassen et al. 2017). Of note, wild mice share the majority of their skin microbial taxa with laboratory mice which therefore would not be expected to influence the outcome on atopic dermatitis models drastically (Belheouane et al. 2020). This is however in contrast to the more diverse gut microbiota evident in wild life mice (Ericsson et al. 2017), which unlike the skin microbiota may play an essential role comparing wild mice with laboratory mice.

Not only the gut and skin microbiota, but also the respiratory microbiome vary with vendor, and it is strongly correlated with the local immune response in the lung (Dickson et al. 2018). As such, a large variation exists in airway responsiveness among mice from different vendors used as asthma models (Chang et al. 2012). Also, the gut microbiota contributes to development of pulmonary inflammation. Germ-free mice have more NKT cells, both systemically and in the lung, compared to SPF mice, and they are more prone to allergic asthma (Hansen et al. 2012b; Olszak et al. 2012). Abundance of invariant natural killer T (iNKT) cells was especially dependent on stimulation with a glycosphingolipid from *Bacteroides fragilis* in the very early life (An et al. 2014), which pinpoints that in the early time window before the animals are shipped to the research facilities, the gut microbiota compositions associated with vendor's facilities are important. Specifically, vendor differences strongly affect iNKT cell phenotypes and functions, and, for example, the percentage of iNKT cells in the spleen is significantly lower in Taconic compared Jackson mice (Wingender et al. 2012). It is, however, important to note that the vendor effect is model specific, as for example an LPS induced lung inflammation model was unaffected by vendor despite clear differences in gut microbiota and pulmonary TNF-α levels (Wolff et al. 2020).

Strategies for handling impact on the mouse models of immune diseases

Having obtained a basic understanding on how essential the microbiota is for immune-mediated disease models, the next steps will be to handle this in biomedical research.

First step will be simply to be aware that rodents from different environments, such as vendors and rooms, have different microbiota, and that this most likely will lead to differences in phenotypes. Based upon the huge amount of literature available scientists should carefully consider in the study planning, which animals of which origin would be most feasible for a specific study. Also, they should carefully report the exact origin of the animals applied in manuscripts, as guided by the ARRIVE guidelines (Kilkenny et al. 2010). This also includes precision in the description of the diet supplied, as diet is a very strong microbiota modifier

(Moreno-Indias et al. 2020). Also, the vendors must soon respond to this new situation and be willing to supply information of the status of their animals; at least for a short list of bacteria generally relevant for many research areas (Hansen et al. 2019). With a rapid development and reduction in costs of microbiota sequencing these methods have become generally available, and much more cost efficient than the bacterial pathogen cultivation used by the vendors ever since the introduction of the SPF concept. In the longer run, routine sequencing will also lead to experience in accounting for the microbiota impact on animal models. Sequencing data will allow incorporation of the microbiota composition as a factor in the statistical analyses. Thereby, we will get even more information on specific bacterial species relevant for disease in the animals, which is also highly relevant for developing microbiota-mediated therapeutics or personalized medicine for human patients. A special discussion topic will be, whether we should start testing vaccines and immune interventions on 'dirty' animals, i.e. animals infected by the well-known pathogens. This has been excellently reviewed by Masopust et al. who argues that this should be seen as a supplement rather than an alternative to the use of SPF mice (Masopust et al. 2017). It will be a major challenge if we were to re-introduce eradicated pathogens in breeding colonies, and this does not seem very likely to happen. Also, it should be considered that captivity bred mice, such as a pet shop mice, although infected with all the well-known pathogens do not have an immune system comparable to a wild mouse, which for instance has a much lower abundance of $CD8^+$ cells; probably due to a lower viral exposure (Beura et al. 2016).

Next step could be to develop rodents tailor-made with microbiota specific for certain research purposes. This may not necessarily be attractive as a general trend, as the diversity among rodents used would, if combined with a thorough microbiota characterization as described above, give detailed information on microbiota impact on disease development. On the other side, it does not make much sense to use animals, which cannot develop specific disease phenotypes or respond to certain interventions, because they lack specific bacteria. A simple approach is, therefore, to ensure that at least these are harboured by the animal. This could for example be SFB's in mice used for IBD research (Ivanov et al. 2009), *A. muciniphila* in mice used for type 1 diabetes research (Hanninen et al. 2018; Hansen et al. 2012a), or *Bifidobacterium* spp. in mice used to test oligosaccharides (Hansen et al. 2013). This is not necessarily a simple task, and Hanninen et al. had to pass over germ-free status, mono-association and then fecal matter transplants to associate NOD mice with *A. muciniphila* (Hanninen et al. 2018). In this package of tools also belongs the opportunity to modify the microbiota into a desirable state through the diet, e.g. by using diets with a more humanized profile to optimize human microbiota transplantation studies (Moreno-Indias et al. 2020). The more advanced step will be to give the animals specific complex microbiotas for specific studies. In the present world, this can be difficult as too little is known about what would be the perfect phenotype for a specific disease phenotype in rodents, and whether this would be relevant for the human patients. However, it might within certain research types, such as atopic dermatitis, for which it is known that there is a strong correlation between microbiota and phenotype (Lundberg et al. 2012), be possible to conserve and transplant high responder microbiotas (Zachariassen et al. 2017), as these can be shown to respond to interventions (Debes et al. 2020). The transfer is not necessarily an easy task, as the colonization rate from mice to mice is approximately only 60—80%, and the transplanted mice seem to have a Firmicutes overgrowth (Lundberg et al. 2017). On the other hand, over generations the microbiota seems to be quite stable independent of housing systems (Lundberg et al. 2020). Microbiota transfer can be used to give mice a human microbiota, although the colonization rate will be even lower and key bacteria, such as *F. prausnitzii* and *Bifidobacterium* spp., fail to colonize (Lundberg et al. 2020). Another attractive approach could be to transfer a wild mouse microbiota to laboratory mice (Rosshart et al. 2017); a technique that has been further improved by the creation of so-called 'wildlings', i.e. C57BL/6 mice born by wild mouse mothers after embryo transfer (Rosshart et al. 2019). One problem is that not only will the commensal microbiota be transplanted, but the pathogens will come as well.

Conclusion

The microbiota has a substantial impact on immune models in rodents and may be the cause of essential phenotypic variation due to differences in the originating environment of the animals. In the handling of the reproducibility crisis, this is a factor not to be ignored.

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Compliance with ethical standards

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