Chapter 6

General discussion
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The role of sex in many research fields is underappreciated, especially in immunology. Consequently, men and women are often treated in the same way, while they do show differences in the pathogenesis of several diseases, including intestinal diseases such as inflammatory bowel disease (IBD). Our gut microbiota plays a major role in health and disease and is in close contact with our intestinal immune system. Commensal bacteria contribute towards a balanced intestinal immune system, whereas pathogenic bacteria can lead to disorders, such as inflammatory bowel disease (IBD). As several intestinal related disorders also show a sex bias in prevalence, this suggests the existence of sex differences in both microbiota composition and immune responses. Insight into sex specific intestinal immune responses would contribute to the development of more effective and sex specific treatments for intestinal related disorders. Therefore, the aim of this thesis was to investigate the effect of sex on the intestinal immune system and microbiota composition. Additionally, we determined the effect of genetic background, age and reproductive condition, as these factors may interfere with the potential sex effects. In our studies, we used two mouse strains (C57B1/6OlaHsd (B6) and Balb/cOlaHsd (BALB/c)) with different genetic backgrounds and confirmed differences in intestinal immunity and microbiota. The effect of age was tested using young (3 months) and old (19 months) mice and in ovariectomized (ovx) mice (ovx at the age of 15 months) as a model for menopause. The effect of the reproductive condition was tested using pregnant mice.

The interaction between intestinal microbes and the intestinal immune system is reciprocal, both are able to influence each other. Several bacteria showed to induce the upregulation of immune cells, such as T regulatory cells (Tregs). On the other hand, the intestinal immune system is able to selectively promote specific bacteria that have beneficial properties (e.g. metabolic functions) by using specific mechanisms such as secretory immunoglobulin A (sIgA). In chapter 2 of this thesis, we studied the effect of sex on several intestinal immune cells (T cells, dendritic cells (DCs), macrophages and natural killer cells (NK) cells) in the Peyer’s patches (PP). We used the PP as a study site, since it is an important place for immune sampling of antigens from the gut lumen. We demonstrated that sex indeed influenced the intestinal immune populations in the PP. Overall, males showed an enhanced intestinal innate immune arm (DCs, macrophages, and NK cells) and a reduced adaptive immune arm (T cells) as compared to females. These results are in accordance with what is found...
in the peripheral immune system.\textsuperscript{(11,12)} Sex hormones are thought to underlie the peripheral immune differences between men and women,\textsuperscript{(13)} and this may also account for the intestinal immune differences we found in this thesis. This suggestion may be in line with the data in chapter 4 and 5 where we showed changes in intestinal immune responses and microbiota composition in pregnant (high levels of sex hormones) and ovariectomized mice (low levels of sex hormones). However, since intestinal microbes are able to modulate immune responses,\textsuperscript{(14)} it is tempting to speculate that microbiota composition also play a role in the peripheral and intestinal immune differences between males and females. This hypothesis is further supported in studies using non-obese diabetic (NOD) mice, who develop spontaneous type 1 diabetes (T1D) and display a sex bias in T1D phenotype.\textsuperscript{(15,16)} This sex bias disappeared when the mice were raised under germ free conditions, demonstrating the influence of microbiota communities on sex differences in immune responses. The same experiment showed that transfer of microbiota between male and female mice resulted in modulated sex hormone levels in the opposite sex,\textsuperscript{(16)} indicating the complexity of the interaction between microbiota, sex hormones and the immune system.

Indeed we found that male and female mice had different microbiota profiles (chapter 3). Females had an increased microbiota diversity as compared to male mice, which is positively correlated to beneficial health effects.\textsuperscript{(17)} In addition, several bacteria that were enriched in female mice (such as Clostridium leptum et rel., Parabacteroides distasonis et rel., and Lactobacillus plantarum et rel.) can be linked to regulatory immune responses.\textsuperscript{(8,18,19)} These findings support the work of Bábíčková et al. (2015) who found that male mice (B6) have a higher sensitivity to develop DSS induced colitis (used as IBD model) than female mice (B6).\textsuperscript{(20)} Although female mice did not have a higher percentage of Tregs in the PP in baseline conditions, these cells might be upregulated during a challenge of infection. The absence of sex differences in several human microbiota analyses\textsuperscript{(21-23)} may be caused by the fact that factors like reproductive condition of females (e.g. menstrual cycle, the use of oral contraceptives and menopause) is often not taken into account. Such factors can interfere with immune responses,\textsuperscript{(24,25)} and perhaps also with microbiota composition, modulating the sex effects. In addition, we found that the sex effects in microbiota composition were mouse strain dependent, indicating that genetic background also influence the sex effects. As in humans the genetic variation is enormous, this may also explain the absence of sex differences in human studies. It can also be argued that men and women
have a different microbiota composition because of different exposure to environmental factors such as diet, smoking or drugs. However, all mice in our study were housed under the same conditions and received a standardized diet. When correlating our microbiota data to our colonic transcriptomics data we found that several bacteria enriched in B6 female mice positively correlated to genes involved in humoral and cell-mediated immune responses, while bacteria enriched in BALB/c male mice showed the opposite correlation. This is in accordance with the results we found in the PP, where females had a lower percentage of innate immune cells (DCs, macrophages, and NK cells) and a higher percentage of adaptive immune cells (T cells). Our data suggest that host-microbe interactions are more complex than assumed and that more than one strategy of microbiota adaptations might lead to the same immunological outcome in the host.

As mentioned above, genetic background was found to have a large impact on both immune responses and microbiota composition. BALB/c and B6 mice are well known for their different immune responses during infection; the BALB/c is skewed towards a Th2 responses, while the B6 strain is more skewed towards a Th1 response. In addition, both strains have been shown to have different IgA production in the intestines. These immune differences may be associated to the different microbiota profiles that have been found between the two mouse strains. Therefore, we used these two different mouse strains (C57BL/6 and BALB/c) in this thesis, with two difference genetic backgrounds and different immune responses and microbiota profiles, to validate the sex effects (chapter 1-4). We found that the genetic background of the mice had a larger impact on both the intestinal immune system and microbiota composition than sex, which is in line with the results of Kovacs et al. (2011) who also found that genotype is a stronger determinant than sex on microbiota composition. Interestingly, we found that the sex effects on microbiota composition were mouse strain dependent (i.e. the effect of sex was not similar in both mouse strains), whereas the effect of sex on the immune populations in the MLN were mainly similar in the two mouse strains. This suggests strain specific strategies in microbiota composition to induce sex specific differences in immune responses. Overall, female mice appeared to have intestinal immune populations and microbiota species which could be correlated to adaptive immune responses and regulatory responses. However, this was more pronounced in females of the B6 strain as compared to females of the BALB/c strain. The interaction between sex and genetic background seemed to be in line with the results of Zelinkova et al. (2014), who found
that the sex bias in susceptibility to IBD is also related to geographical factors (e.g. genetic background and environmental factors). This finding suggests that is important to consider genetic background when comparing males and females in the field of immunology and microbiota, as this may interfere with the results.

Since we found sex differences in intestinal immune populations and microbiota composition and these differences are thought to be related to sex hormone levels, we investigated the effect of increased female sex hormone levels (progesterone and estrogen) during pregnancy on intestinal immune populations and microbiota composition (chapter 4). Progesterone and estrogen, mainly produced by the placenta and the fetus, are necessary for a healthy pregnancy, but also interfere with the maternal immune system. Pregnancy is associated with immunological adaptations of the mother in order to tolerate and support development of the semi-allogeneic fetus. One of the most important changes is the increased numbers of peripheral Tregs. Both progesterone and estrogen have been linked to the generation of Tregs, however, since certain bacteria in the microbiome are also linked to the generation of Tregs, we hypothesized that the intestinal microbiota is altered during pregnancy and that this is associated with the changes in the peripheral immune responses. In addition we expected to find the intestinal immune system to be changed as well during pregnancy. Indeed we found that during pregnancy microbiota species related to a regulatory immune phenotype seemed to be increased. However, our data also demonstrated that the two mouse strains seem to respond differently to pregnancy. BALB/c mice showed the largest changes in microbiota composition during pregnancy. Some of the enriched bacterial species in pregnant BALB/c mice (such as Lactobacillus paracasei et rel., Roseburia intestinalis et rel. and Eubacterium hallii et rel.) are associated with anti-inflammatory and Treg inducing properties. B6 mice hardly changed their microbiota species during pregnancy. However, several of the bacteria already present in non-pregnant mice of this strain (such as bacteria from the Lactobacillus and Bifidobacterium genus) are associated with anti-inflammatory and Treg inducing properties. Our data suggest that different mouse strains have different strategies to obtain similar regulatory immune responses, i.e. BALB/c mice increased bacteria that are associated with induction of a tolerance and anti-inflammatory immune response, while B6 mice already had bacteria that induce such an immune milieu and therefore do not have to adapt their microbiome to pregnancy. Additionally, we found that, similar to what is found peripherally, pathways...
involved in intestinal regulatory immune responses were upregulated during pregnancy. We speculate that microbiota during pregnancy may be involved in regulating the placenta induced pro-inflammatory changes during pregnancy.

Generally, with ageing there is a functional change of the peripheral immune system called ‘immunosenescence’. This includes ‘inflammaging’, which is chronic, low-grade systemic inflammation in the absence of a clear infection,\(^{41}\) but also a decrease in naïve T cells and an increase in memory T cells.\(^{42}\) Interestingly, the changes related to immunosenescence were found to be more apparent in males than in females,\(^{43}\) suggesting a sex bias in immunosenescence. As ageing showed to deteriorate the symptoms of IBD,\(^{44}\) we hypothesized that the intestinal immune response also changes with age and also in a sex dependent fashion. Because of the complex interactions between host-microbes, the intestinal barrier and immunity, we also studied, beside intestinal immunity, the effect of age and sex on the gut microbiome and mucus integrity (chapter 5). We found that young (3 months) males and females had a different microbiota composition, similar to what was found in chapter 3. However, these sex differences were not seen in old mice (19 months). We demonstrated that female mice had a higher microbial diversity as compared to male mice at an age of 3, 8 and 13 months, but this difference disappeared at an age of 15 months, which suggests it might be a sex hormone dependent phenomenon, since sex hormone differences are more apparent at younger age than at old age.\(^{45,46}\) Indeed a recent study showed differences in microbiota diversity between males and females in NOD mice, which disappeared after castration of the males.\(^{16}\) Our results suggest that the sex hormone levels of females of 19 months old are reduced as compared to an age of 13 months. Some studies showed that only 25% of the normal ageing female mice go into anestrus, including low levels of sex hormones.\(^{6}\) Therefore, we ovariectomized (ovx) half of our female mice at an age of 15 months. This was done to investigate the effect of a loss of females sex hormone levels, and to mimic human menopause. Old ovx females developed a similar microbiota diversity as old females, supporting the suggestion that sex hormones are involved in microbiota diversity.

Our studies collectively showed that host-microbe interactions were different in males and females with different strategies in immunity to maintain intestinal homeostasis. Females may have more bacteria species related to regulatory responses (chapter 3), which were found to be reduced in old ovx females, who had a lower abundance of bacteria species involved in butyrate
production and therefore regulatory immunity (chapter 5). Our data and that of others\(^{(16)}\) suggest that sex hormones are involved in modulating the microbiome, but other factors like genetics also play a role. As levels of sex hormones reduce with ageing, this may explain the absence of sex differences in microbiota composition in old mice. However, when studying intestinal immune populations, we found that sex differences in young mice still existed in old mice, suggesting that the sex differences in immune populations induced in young mice are permanent and not affected by changes in the microbiome associated with ageing.

As the intestinal immune system not only comprise immune cells, but also involves a mucus layer, which covers and protects the intestinal epithelial cells from direct contact with microbes, we also studied the effect of age and sex on mucus integrity (chapter 5). The focus was on mucus as mucus has become recognized as an essential protective layer preventing contact between microbiota and intestinal epithelial cells. We found that sex only had an influence on mucus integrity at an old age. Both old male and female mice had a lower mucus thickness than young mice and we found several spots of contact between the epithelium and microbiota in the old mice, illustrating inadequacies in the mucus layer in both sexes. Although the mucus layer was thinner, it was not necessarily only the amount of mucus that changed during ageing. We found many changes in genes involved in the biosynthesis of mucus in old mice. Mucus forms a network on epithelial cells. Its functionality not only depends on the amount of mucus produced, but also on its interconnectivity and viscosity. Mucus has been shown to be highly glycosylated and to be decorated with a wide variety of O-linked oligosaccharide side chains that make up more than 70% of the weight of the molecules.\(^{(47)}\) Also the sulfonation determines the thickness and the adequacy of the mucus layer. Our data suggest that in all these decoration changes occur during ageing, but in a sex dependent fashion. With ageing in males more, but also different genes involved in the process of mucus biosynthesis were downregulated as compared to old females (both ovx and non-ovx). Just like shown for young animals with a knock-out in one of the Muc2 genes,\(^{(48)}\) this was associated with dysbiosis and increased colonization by pathobionts. More studies have to be done to unravel the cause and effect of this observation.
Implications & Future perspectives

Overall we conclude that sex in mice indeed impacts intestinal immunity and microbiota composition. Young healthy male and female mice seemed to have different microbiomes and associated intestinal immune responses that may benefit their sex specific needs. However, several factors such as reproductive condition, age and genetic background interfered with the sex effects (Figure 1). Our studies demonstrated that interactions between host immunity and the microbiome are more complex than assumed. In mice studies this should be considered when choosing a mouse model. In addition, the current search for specific biomarkers for health and disease should also take into account the effect of sex and all the factors influencing these sex effects. For each condition their might be a different adapted microbiome and associated immune responses. In order to gain more insight in the implications of the sex differences in microbiome and intestinal immunity, future studies are required. This could include studies investigating the effect of sex on intestinal immune populations in challenge models like colitis (IBD) or oral infections, such as salmonella. Salmonella would be interesting, since it can enter the body via the PP.\(^{[49]}\) Additionally, future research should focus on the functions of the different microbiota species between the two sexes under several conditions (e.g. genetic background and age) to develop more tailored made treatment

Figure 1. In this thesis we determined the impact of sex on the intestinal microbiota composition and immune cells. In addition we determined the effect of reproductive condition, age and genetic background on the sex effects.
strategies. Microbiota transfer studies in germ-free mice already substantially contributed to elucidate the interaction between sex hormones and the microbiome. These studies may further help to understand the functions of the sex specific microbiota on the immune system. Strategies to improve the aged dependent impairment of mucus integrity may include administration of galacto-oligosaccharides (GOS), which have been shown to enhance the intestinal barrier function through the modulation of mucus producing goblet cells.\(^{50}\) In addition, supplementation with probiotic strains may be a promising tool for treatment therapies for age related intestinal disorders, as supplementation of *Lactobacillus plantarum* to old mice, restored the age related decline in mucus barrier.\(^{51}\)

**Conclusions**

This thesis demonstrated sex differences in intestinal immunity and microbiome, however these differences were dependent on mouse strain and age. Young healthy male and female mice had different microbiomes and associated intestinal immune responses that may benefit their sex specific needs. Although our studies suggest a relation between the intestinal microbiota and immune system at a young age, the sex differences in microbiota composition disappeared at an old age, while most sex differences intestinal immunity were maintained in old mice. This suggests that immune differences develop at a young age and are permanent, and that the age induced changes in the microbiome have no effect on the intestinal immunity. These findings may contribute to the development of more sex specific treatments for intestinal related disorders.

**References**


