Reciprocal interactions between *Helicobacter hepaticus* and the mouse immune system

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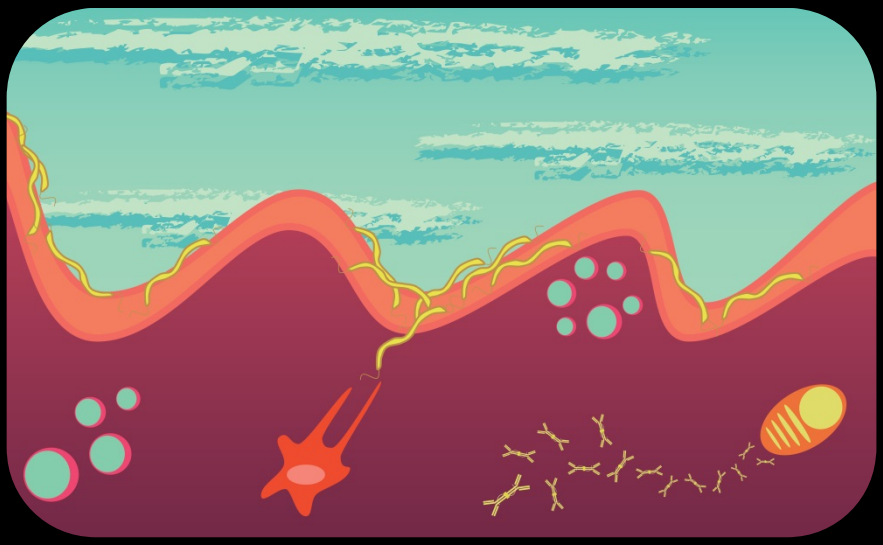

Dissertation presented to obtain the Ph.D degree in Immunology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
October, 2016

UNIVERSIDADE NOVA DE LISBOA

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Research work coordinated by:

FUNDAÇÃO CALOUSTE GULBENKIAN
Instituto Gulbenkian de Ciência

Oeiras, October, 2016

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

The work presented on this thesis was supported by Fundação para a Ciência e a Tecnologia – FCT, grant SFRH/BD/51883/2012

To Juliane and Antônio

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Acknowledgements

I would like to thank Jocelyne Demengeot, for your dedication and enthusiasm with the work, for being an example of commitment and determination, and for your guidance and patience. Your support and kindness were crucial in the difficult situations, both personal and work related, and your incredible ability to find solutions for complex problems was decisive for the outcome of this thesis.

I am very grateful to all the present and past members of the FL lab, especially Marie Louise Bergman, Ana Catarina Martins, Margarida Araújo, Vasco Correia and Inês Cabral, that were directly involved in this work. Thank you for the commitment and support! Thanks as well to Vânia Silva, for the most helpful discussions about work, science and the universe, to Francisca Fontes, for the great support, to Íris Caramalho, for your encouragement and the most clear-minded and objective opinions, and to everybody else for creating a great work environment.

Thanks to Ana Regalado, for sharing many of the sad and happy moments of this doctoral work. Thank you for your help with protocols, experiments and existential crisis. Your kindness and wise advice helped me get through everything.

I am very grateful to my thesis committee, Michael Parkhouse and Luis Teixeira, for helpful criticism and great advice. Thanks to Jorge Carneiro, who inspired me to use R and learn how to do a better job in data analysis. Thanks to Isabel Gordo, Karina Xavier and Miguel Soares, for helpful discussions in our “Mouse Microbiota Meetings”. Thanks as well to João Batista, Jorge Sousa, Jessica Thompson, Ana Rita Oliveira, Bahtiyar Yilmaz and Sofia Rebelo for all the help with experiments and discussions.

Thanks to Manuela Cordeiro, for all the help along the way, and to Élio Sucena, for the good advice and willingness to help.

I am in great debt to Manuel Rebelo, Joana Bom, Sofia Leocadio, Karen Berman, Ana Lúcia Ribeiro, Adérito Vieira, Lévi Pires and many other members of the IGC's animal house facility. Thanks for the patience and all the help structuring complex experiments along this project.

Thanks to Margarida Parente and Lígia Saraiva for help with the culture of *Helicobacter hepaticus* and antigen preparations.

Thanks to my friends Arnon Juberg, Tiago Macedo and Rodrigo Oliveira, for helping me keep in touch with Brazil, sharing many great moments of festivity and joy, and also for encouraging me in hard times. Thanks as well to Luciana Moraes, for the encouragement and all the hilarious discussions on the corridor, and to Thiago Carvalho, for good advice and for all the important papers you kept sending me over the years.

Thanks to all my colleagues of the PhD program, especially PIBS 2011, for the great discussions during the classes and the great fun in the AMeeGuS meetings. Thanks to the "bastardos" Özhan Özkaya, Jarek Surkont and Rafał Gumienny, for all the great times and also for the long conversations about the most random subjects. Thanks for your friendship and encouragement.

Obrigado a meus pais, Gildete e José Antônio, e a meu irmão Romero, pelo apoio nos momentos difíceis e entusiasmo nos momentos alegres.

Thanks to my wife, Juliane Menezes, for being my partner and my best friend for the last 8 years. Thanks for the great support and encouragement, for helping me on long weekend hours in the lab, for sharing the good and the bad moments, and for inspiring me to always give my best. And thanks to our son, Antônio, for helping me finish this thesis on time :)

Summary

Vertebrates are host for a very large number of bacteria, notably in the intestinal lumen. This complex microbiota encompasses microorganisms that can cause pathology in immunocompromised individuals, but not in healthy hosts who remain silent carriers. The reciprocal interactions between the host and such microbes must involve components of active immune tolerance maintaining at check protective immune responses of the ridding type. We addressed this hypothesis by studying mouse-*Helicobacter hepaticus* interactions. *H. hepaticus* is a gut bacteria commonly found in mouse facilities and in the wild. Mice can be persistently colonized with this microbe, even from the first days of life, without developing signs of pathology or decrease in breeding efficiency. However, immunocompromised animals can develop colitis when colonized with *H. hepaticus*. In this work, we sought to identify the immunological mechanisms triggered upon colonization that ensure a stable relationship between healthy mice and *H. hepaticus*.

Despite abundant evidence that *H. hepaticus* induces a strong adaptive immune response in mice, the impact of this response on the bacteria load is unknown. To directly address this issue, a reference *H. hepaticus* strain was used to colonize mice lacking various components of the adaptive immune system, and *H. hepaticus* load in the gut was monitored along time. We found that both B and T lymphocytes contribute to reduce the bacterial load, and that antibodies, notably of the IgA type, participate in this control, but do not account for the whole effect. We next evidence that IL-10 deficient animals mount an exacerbated immune response, promoting *H. hepaticus* elimination. We conclude that T and B cells limit the bacterial load in both an antibody-dependent and independent

manner and that regulatory mechanisms dampen this effect to promote the persistence of *H. hepaticus* in the gut.

H. hepaticus is transmitted through coprophagy in adults, and also from mother to pups around birth. Given the major physiological changes associated with post-natal maturation, we next asked whether and how the age of the host at primo-exposure affects its reciprocal interactions with *H. hepaticus*. We analyzed adult mice that were colonized with *H. hepaticus* either during the perinatal period or at 8-10 weeks of age. When compared with animals colonized at adult age, mice that were infected early in life present with increased load of *H. hepaticus* in the ileum and colonic mucosa. This increment associates with a total absence of fecal IgA specific to *H. hepaticus,* indicating that primo-exposure during the perinatal period leads to long lasting immune tolerance. Performing kinetic and limiting dilution analysis, and using loss-of-function approaches, we reveal that immunological tolerance to *H. hepaticus* is induced during the first two weeks of life by a mechanism that requires neither maternal Immunoglobulins nor the microbiota, and that its long lasting maintenance is mediated by $Foxp3^+$ regulatory T cells. Tolerant mice show normal serology for various physiological markers and no predisposition to induced gut pathology. Finally, 16S analysis of fecal bacterial content reveals that *H. hepaticus* shapes the microbiota composition both in a lymphocyte independent and dependent manner, and that in the latter case, age at primo-exposure matters.

In conclusion, our study provides new insights into how lymphocytes control bacteria in the microbiota and maintain intestinal homeostasis. Furthermore, they evidence a developmental time window favoring life-long symbiosis, through early education of the immune system.

Sumário

Os organismos vertebrados hospedam uma grande quantidade de bactérias, notavelmente no intestino. Esta microbiota complexa contém microorganismos que podem causar patologia em indivíduos imunocomprometidos mas não em hospedeiros saudáveis, que permanecem como portadores assintomáticos. As interações recíprocas entre o hospedeiro e tais microrganismos devem incluir componentes de tolerância imunológica que inibem respostas com vista à sua eliminação. Nós abordamos esta hipótese estudando as interações entre o ratinho e *Helicobacter hepaticus*. O *H. hepaticus* é uma bacteria intestinal encontrada comumente em biotérios e na natureza. Os ratinhos podem ser colonizados com este microrganismo, mesmo desde os primeiros dias de vida, sem apresentar sinais de patologia ou decréscimo de eficiência reprodutiva. No entanto, animais imunocomprometidos podem desenvolver colite quando colonizados com *H. hepaticus*. Neste tabalho procuramos identificar os mecanismos imunológicos ativados pela colonização que garantem uma relação estável entre o ratinho e *H. hepaticus*.

Várias evidências indicam que o *H. hepaticus* induz uma forte resposta imune adaptativa em ratinhos, porém o impacto desta resposta na carga bacteriana é desconhecido. Para testar este ponto diretamente, uma estirpe de referência de *H. hepaticus* foi usada para colonizar ratinhos deficientes em vários componentes do sistema imune adaptativo, e a quantidade desta bactéria no intestino foi medida ao longo do tempo. Os nossos resultados demonstram que tanto os Linfócitos B como os Linfócitos T contribuem para a redução da carga bacteriana com eficiência comparável. Apesar dos Anticorpos participarem neste mecanismo, estes não são responsáveis por todo o efeito, indicando que células T e B também contribuem para a eliminação de bactérias via um mecanismo independente de anticorpos. Em contrapartida, a ausência de IL-10 aumenta a resposta imune, levando à eliminação da bactéria. Assim, enquanto a citocina imunoregulatória IL-10 promove a persistência de *H.*

hepaticus no intestino, os Linfócitos T e B controlam o número de bactérias.

O *H. hepaticus* é transmitido por coprofagia em adultos e também de mães para filhos no período logo após o nascimento. Devido às grandes mudanças fisiológicas associadas à maturação pós-natal, nós testámos se e a idade aquando da primeira exposição afeta as relações recíprocas entre o ratinho e o *H. hepaticus*. Para este fim, foram analisados animais adultos previamente colonizados com uma estirpe de referência durante o período neonatal ou com 8 a 10 semanas de vida. Nós primeiro observamos que a quantidade de *H. hepaticus* estava elevada na mucosa do ileum e do colon de animais colonizados no período neonatal, quando comparados com animals colonizados quando adultos. Isto estava acompanhado de uma ausência de resposta de IgA fecal contra *H. hepaticus* em animals adultos colonizados em idade neonatal, contrastando com uma forte resposta de IgA em animais colonizados quando adultos. Portanto, a colonização com *H. hepaticus* no período neonatal promove uma tolerância de longa duração em ratinhos. Através de análise cinética e diluição limitante, e utilizando estratégias de perda de função, revelamos que a indução de tolerância imunológica a *H. hepaticus* durante o período neonatal é independente de imunoglobulinas maternas e da microbiota, sendo a sua manutenção mediada por células T reguladoras $Foxp3^{+}$. Demonstramos ainda que animais colonizados perinatalmente apresentam serologia normal e nenhuma predisposição a patologia intestinal. Por último, a análise de 16S em amostras de bactéria fecal revelou que o *H. hepaticus* molda a composição da microbiota de maneira independente e dependente de linfócitos, e neste último caso, a idade de exposição primária desempenha um efeito.

Em conclusão, os nossos resultados providenciam novos conhecimentos de como os linfócitos controlam bactérias da microbiota e mantêm a homeostase intestinal e revelam uma janela do desenvolvimento que favorece a simbiose vitalícia, via educação precoce do sistema imune.

List of Abbreviations

Adcol	Adult-colonized
AMP	Antimicrobial Peptide
CSR	Class Switch Recombination
DCs	Dendritic Cells
DEREG	mice expressing DTR under the promoter of Foxp3
DT	Diphtheria Toxin
DTR	Diphtheria Toxin Receptor
GALT	Gut Associated Lymphoid Tissue
GF	germfree
Hh	*Helicobacter hepaticus*
i.p.	intraperitoneal
IgA	Immunoglobulin A
IL	Interleukin
ILF	Isolated Lymphoid Follicles
MDS	Multidimensional scaling
MLN	Mesenteric Lymph Nodes
Nbcol	Newborn-colonized
OTU	Operational Taxonomic Unit
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
PP	Peyer's Patches
Q-PCR	Quantitative PCR
SPF	Specific Pathogen Free
TLR	Toll Like Receptor
Treg	Regulatory T cell

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Chapter 1 : Introduction

Helicobacter hepaticus is a gut bacterium commonly found in animal facilities and in the wild. This bacterium can cause pathology in immunocompromised animals, however *Wild Type* (WT) strains are not affected, and can be persistently colonized with this microbe, even from the first days of life, without signs of pathology or decrease in breeding efficiency. The goal of this doctoral work was to identify the immunological mechanisms that maintain such stable relationship between WT mice and *H. hepaticus*, and how each organism affects the other. In order to approach this problem, we explored different components of the mucosal immune system, and their relationship with the intestinal microbiota. The following chapter is an overview of the literature related to microbiota-immunity interactions relevant to this work.

Reciprocal interactions between the host and the microbiota

1 – Immunological impact of the host on the microbiota

The amazing diversity of bacteria in the mammalian gut poses a very particular challenge to the host, that of discriminating between beneficial and deleterious microbes, as some of the latter could even be pathogenic. During co-evolution of the host and the microbiota many mechanisms arose to control the bacteria in the gut (and other surfaces of the body), which guarantee the protection of the host and the stability of the bacterial community (Hooper et al., 2012).

In the gut, bacteria are stratified and isolated from the host tissue by redundant mechanisms. The epithelial cell layer is the ultimate barrier, above which there is the mucus layer, present in the small and large

intestine (Johansson et al., 2008; Vaishnava et al., 2011). Mucus proteins are secreted by intestinal goblet cells, and self-assemble to form a tightly packed inner layer in the colon, with a thicker and looser external layer above it. Only a loose layer is present in the small intestine, but in both places a 50μm region in the mucus, just above the epithelium, is found almost devoid of bacteria, in contrast to the bacteria-rich region above it. This inner layer is rich in antimicrobial peptides and IgA, which prevents bacterial contact with the epithelium (Vaishnava et al., 2011).

Antimicrobial peptides (AMPs) are effector molecules produced and secreted at the epithelial surface. They form a broad class of molecules, some constitutively expressed, like α-defensins and lysozyme, and others induced by microbial sensing, like cryptdin-related sequences (CRS)-peptides and RegIIIγ (Mukherjee and Hooper, 2015). Expression and secretion of RegIIIγ by epithelial cells is induced by microbial products via Toll-Like Receptors (TLR), and impairment of TLR signaling leads to reduced expression of RegIIIγ, which in turn abrogates the bacteria-free zone above the epithelium (Vaishnava et al., 2011). AMPs can be broadly expressed, or produced by specialized cell types, like Paneth cells, which are mostly found in the crypts of the small intestine, and release granules containing AMPs like lysozyme and α-defensins. Degranulation of Paneth cells is a highly controlled process, and occurs mostly through sensing of Interferon-γ (IFN-γ) (Farin et al., 2014).

Despite the mechanisms in place to confine bacteria above the epithelium, the intestinal tissue is not sterile. Bacteria that cross the epithelium are usually phagocytosed and killed by macrophages in the Lamina Propria (Kelsall, 2008). In addition, these bacteria can be engulfed by Dendritic Cells (DCs), which also sample microbes from the lumen (Farache et al., 2013). DCs carrying live bacteria can present their antigens to T and B cells in the Mesenteric Lymph Nodes (MLN), activated

lymphocytes will spread through all mucosal surfaces (Macpherson and Uhr, 2004). Microbes with higher propensity to cross the intestinal barrier, or living in close proximity to it, are more likely to get sampled and further controlled by activated T and B cells and targeted by IgA (Mirpuri et al., 2013).

IgA is the most common antibody in mucosal surfaces (Mantis et al., 2011). In newborns, before the Gut Associated Lymphoid Tissue (GALT) is mature, IgA in the maternal milk provides protection and helps shape the microbiota that colonizes the neonatal intestine (Rogier et al., 2014). After the maturation of the GALT, and in response to the microbiota, endogenous IgA is produced in great amounts, binding to bacteria inside the lumen and conferring protection against invasion (Bunker et al., 2015; Macpherson and Uhr, 2004; Mantis et al., 2011). IgA is secreted by plasma cells in the Lamina Propria, predominantly as dimers, which are transported through the Polymeric Immunoglobulin Receptor (pIgR) to the gut lumen (Kaetzel, 2014). There, IgA can bind to antigens and prevent their negative effects on the host, for example by neutralizing cholera toxin (Lycke et al., 1999). IgA can also affect the motility and invasiveness of pathogens (Forbes et al., 2008, 2011), for instance by binding to molecules on the bacteria surface used to attach to the mucus (Peterson et al., 2007). Indeed, it was found that the appearance of IgA that could bind to Segmented Filamentous Bacteria (SFB) in the gut correlated with a decrease in bacterial load (Jiang et al., 2001). Furthermore, absence of IgA results in expansion of opportunists contained in the microbiota (Mirpuri et al., 2013; Suzuki et al., 2004), which illustrates the role of this antibody in the maintenance of homeostasis in the gut.

The differentiation of B lymphocytes into IgA secreting cells (plasma cells) can occur with or without T cell help, as mice without T cells can produce IgA that bind to bacteria in the gut (Macpherson, 2000). More

potent immune responses occur though when both T and B cells are activated and help each other. Antigen presentation by B cells increases T cell responses (Merkenschlager et al., 2016), and T cell help in germinal centers allows B cells to undergo class-switch recombination (CSR) and somatic hypermutation. This, through the process of affinity maturation, will improve the binding of the secreted antibody to the inducing antigen (Fagarasan et al., 2010). Bacteria that induce strong responses in the host usually trigger T cell dependent IgA production, as is the case of SFB (Lécuyer et al., 2014).

T cells are major players in the immune system, and make up another layer of effector response. T cells can kill infected cells, improve antibody responses through the promotion of germinal center reactions, CSR and affinity maturation on B cells, and they can modify and regulate the innate immune response through the production of cytokines (Khader et al., 2009). Upon activation, T cells can differentiate into different profiles, that will dictate how they respond to further stimulation and where in the body they will migrate to (Bromley et al., 2008). Th2 cells produce Interleukin-4 (IL-4) and promote immunity against parasite infections. Th1 cells produce Interferon-γ (IFN-γ), which activates macrophages and promotes resistance to many infections (Khader et al., 2009), and can also induce the secretion of AMPs by Paneth Cells (Farin et al., 2014). IL-17 producing T cells (Th17) have been implicated in the protection against many pathogens and in the control of the microbiota composition, as the cytokines they produce can recruit neutrophils to the gut and also induce the production of AMPs by epithelial cells (Khader et al., 2009).

2 - Effects of the microbiota on the host

The gut microbiota also modifies and is essential for the normal development of many tissues in the host, including the immune system. Mice born and raised in the absence of microbes (germfree - GF) exhibit a series of defects when compared to conventionally raised animals. GF mice have enlarged ceca, reduced intestinal motility, longer villi and shorter crypts in the intestine, reduced amount of AMPs, absence of Isolated Lymphoid Follicles (ILF) in the Lamina Propria, reduced number of intraepithelial lymphocytes, and smaller Peyer's patches (PPs) and MLN (Gensollen et al., 2016). All these differences evidence the impact the microbiota has on the maturation of the host, and particularly of the GALT. Most of these problems can be reverted by colonization of GF mice with a conventional microbiota, but some cellular defects cannot be corrected once these mice age past a critical time window.

2.1 - Age independent effects of the microbiota on the host

Lymphocytes expressing αβ T cell receptors, $CD4^+$ and $CD8^+$, are greatly decreased in numbers in the Lamina Propria and Intraepithelial compartments in the intestines of GF compared to conventionally raised mice. This phenomenon can be reverted if adult GF mice are colonized with normal mouse microbiota, but not completely upon colonization with human or rat gut microbes, showing how the intricate relationship of the host immunity and microbiota was shaped by co-evolution (Chung et al., 2012).

Lamina Propria Lymphocytes produce less IL-10, IFN-γ, IL-4 and IL-17 in GF compared to conventional mice, which could be reverted by adult-colonization with conventional flora. Particularly Th17 cells, which are important for the production of IgA in the gut, can be restored to normal

levels in the Lamina Propria of adult GF mice with monocolonization with SFB (Gaboriau-Routhiau et al., 2009).

The homeostasis in the gut is dependent on effector mechanisms, which should be controlled to prevent exacerbated immune reactions. $CD4^+$ αβT cells expressing the transcription factor Foxp3 can suppress immune reactions and are designated Regulatory T cells (Treg). Treg are the major source of the immunoregulatory cytokine IL-10 in the gut (Rubtsov et al., 2008) and can respond to commensal antigens (Lathrop et al., 2011). The frequency of $Foxp3^+$ cells inside the $CD4^+$ αβT cell population reaches some of the highest levels in the colonic Lamina Propria of conventionally raised mice. Furthermore, Treg levels are reduced upon antibiotic treatment and are dramatically decreased in GF animals (Atarashi et al., 2011). Colonization of adult GF mice with conventional microbiota, or with a combination of commensals of the genus *Clostridium*, can restore Treg levels in the colonic Lamina Propria. Other groups of bacteria were also shown to induce Treg in mice, like *Bacteroides fragilis* (Round and Mazmanian, 2010), which is a human commensal, and *Lactobacillus murinus* (Tang et al., 2015).

The levels of IgA-producing B cells in the Lamina Propria of GF mice are also greatly reduced (Hapfelmeier et al., 2010). This phenotype can also be reverted by colonization of adult GF mice with a conventional microbiota (Gensollen et al., 2016), and could also be achieved by monocolonization, with the number of IgA^+ plasma cells being maintained increased in the Lamina Propria even after bacteria elimination (Hapfelmeier et al., 2010).

2.2 - Age dependent effects of the microbiota on the host

Strong immune responses to infection usually require Th1 differentiation and IFN-γ production. In neonates however, Th1 responses are usually impaired. Neonatal B cells produce high amounts of IL-10 upon TLR stimulation (Walker and Goldstein, 2007; Zhang et al., 2007), which prevents DC activation and IL-12 production (Sun et al., 2005). Murine neonatal T cells produce high levels of IL-4 and IL-13 upon activation, which favor Th2 polarization (Zaghouani et al., 2009). Th17 development is also impaired in neonates, since IL-6 stimulation is essential for this type of polarization and newly formed T cells (Recent Thymic Emigrants - RTEs) are less sensitive to IL-6 than mature T cells (Paiva et al., 2013). Additionally, neonatal T cells have a higher propensity to become Tregs than adult T cells, converting in high frequency to $Foxp3^+$ Tregs upon TCR stimulation (Wang et al., 2010). All these properties make neonates very susceptible to infections, but at the same time, they offer the best environment to forge symbiotic relationships through the development of tolerance. Therefore, the colonization with the microbiota in the neonatal stage shapes the immune system of the host in unique ways.

In GF mice, absence of microbiota causes accumulation of invariant Natural Killer T cells (iNKT) with a pro-inflammatory phenotype in the lung and intestine, leading to susceptibility to airway hyperresponsiveness and colitis. Colonization with a conventional microbiota during the first weeks of life, but not at adult age, could prevent iNKT accumulation and susceptibility to disease (An et al., 2014; Gensollen et al., 2016; Olszak et al., 2012). Similarly, disturbance of the microbiota by antibiotic treatment early in life causes increased susceptibility to Inflammatory Bowel Disease (IBD) in humans (Kronman et al., 2012; Shaw et al., 2010).

Another phenotype observed in GF mice is a high level of serum IgE and increased susceptibility to Antigen-Induced Oral Anaphylaxis. Colonization early in life with a complex microbiota could revert the hyper IgE phenotype in GF animals, yet this was not the case if colonization was performed in adults (Cahenzli et al., 2013).

Specific Pathogen Free (SPF) mice, which have a complex microbiota but are free of known pathogens, show accumulation of Helios$^-$ Foxp3$^+$ Treg (supposedly converted outside of the thymus) in the lungs in the first week of life, a phenotype that was not observed in GF animals. Treatment of SPF neonates for two weeks with anti-PD-L1 could prevent the Helios$^-$ Treg accumulation in the lungs and increased susceptibility to airway inflammation (Gollwitzer et al., 2014). This indicates that the lung microbiota induces the generation of Tregs early in life, which confers a long-lasting protective effect on the lungs. Furthermore, neonatal mice treated with antibiotics have increases susceptibility to allergic asthma later in life, which does not happen if treatment was performed in adult animals (Russell et al., 2012). A similar association between early life antibiotic treatment and the risk of asthma development was also reported in humans (Risnes et al., 2011).

Accumulation of Tregs on the neonatal skin has been shown to be essential for the generation of immune tolerance to commensal at this site. Tregs specific for commensal antigens were found in high frequency in the skin of newborn-colonized mice, and shown to prevent experimentally induced skin inflammation (Scharschmidt et al., 2015). In addition, mice treated in the neonatal period with antibiotics displayed increased susceptibility to experimental psoriasis in adulthood (Zanvit et al., 2015). This could be partially reverted by co-housing the treated animals with non-treated controls, providing evidence that the disturbance in the microbiota caused the increased susceptibility to disease.

Helicobacter hepaticus

Helicobacter hepaticus was first isolated from the liver and mucosal scrapings of SCID/NCr and A/JCr mice (Fox et al., 1994), because it was causing liver pathology in these strains. It was also found later to cause colitis in B6.IL-10$^{-/-}$ and 129SvEv.Rag2$^{-/-}$ mice. Notwithstanding, *H. hepaticus* was found to colonize most *Wild Type* strains in animal facilities worldwide (Whary and Fox, 2006), without any sings of pathology or decrease in breeding efficiency (Solnick and Schauer, 2001). It was also found to be widespread in the wild (Wasimuddin et al., 2012), along with many other bacteria of the genus *Helicobacter*. The absence of disease in normal animals and the high prevalence in the wild suggest that *H. hepaticus* does not fit the normal classification of pathogen. Rather, the evidence suggests that most of the described pathologies associated with this microbe come from exacerbated immune responses and not from actual invasion and damage directly caused by the bacteria. Because of its resemblance to IBD in humans, colitis induced by *H. hepaticus* became a model to study pathological immune responses and immune regulation in the gut.

H. hepaticus triggers T cell-dependent colitis in IL-10$^{-/-}$ mice and in alymphatic mice transferred with naïve T cells (Cahill et al., 1997; Kullberg et al., 1998). This pathology can be reproduced by transfer of a CD4^{+} T cell clone specific for a bacterial epitope, and only *H. hepaticus*-colonized recipients develop the disease, suggesting that an uncontrolled T cell response to *H. hepaticus*, rather than a breach in tolerance to self, causes immunopathology in this model (Kullberg et al., 2003). Transfer of CD4^{+} CD45RB^{low} cells, which were previously shown to be enriched in T regulatory cells (Tregs) (Annacker et al., 2001; Read et al., 2000), can prevent colitis, but only when these cells come from colonized donors

(Kullberg et al., 2002). Tregs from *H. hepaticus*-colonized mice can suppress IFN-γ production, and secrete IL-10 when stimulated *in vitro* with *H. hepaticus* antigens (Kullberg et al., 2002). Taken together, these reports support the idea that *H. hepaticus* has the capacity to induce an antigen-specific regulatory T-cell response in mice, and when this regulatory component is absent or compromised, a strong T-cell mediated inflammatory response is mounted, mediated by IFN-γ and IL-17 (Morrison et al., 2013).

Despite the abundance of information on the immune response triggered by *H. hepaticus*, particularly in the T cell compartment of the gut, very few information is available on the actual impact of this response on the bacteria. Transfer of $CD4^+$ $CD45RB^{high}$ (predominantly naïve) T cells to $Rag^{-/-}$ mice colonized with *H. hepaticus* causes colitis, which can be suppressed by co-colonization with the human commensal *Bacteroides fragilis*. However, *H. hepaticus* intestinal load was found to be the same in both sick and healthy animals, suggesting that increased inflammation by itself is not enough to influence *H. hepaticus* numbers in the gut (Mazmanian et al., 2008). Similarly, gut pathology in 129SvEv.$Rag2^{-/-}$ mice colonized with *H. hepaticus* can be suppressed by the transfer of $CD4^+$ $CD25^+$ T cells (enriched in Treg), but the reduced inflammation also does not influence *H. hepaticus* intestinal load in this system (Maloy et al., 2002). On the other hand, in animals deficient for the innate immunity adaptor Nod2, which show decreased expression of α-defensins in the gut, *H. hepaticus* numbers were found to be elevated when compared to WT controls (Petnicki-Ocwieja et al., 2009). Additionally, the transgenic expression of the human α-defensin 5 in Nod2 deficient mice was sufficient to rescue this phenotype, reducing *H. hepaticus* load in the gut (Biswas et al., 2010). This suggests that innate immunity can control *H. hepaticus* in

the gut, but the role of the adaptive immune response in this control is largely unknown.

H. hepaticus transmission occurs easily between adults, mainly through coprophagy (Livingston et al., 1998). Possibly for that reason, *H. hepaticus* became widespread in animal facilities worldwide, which lead to the development of several methods for elimination of this bacteria from experimental mice. Since antibiotic treatments largely failed to eradicate infection (Solnick and Schauer, 2001), and rederivation techniques are often difficult to perform, fostering of pups born from *H. hepaticus* positive dams by *H. hepaticus*-free foster mothers was a great promise. However, in fostering attempts it was found that only transfers in the first day of life were successful, because if fostering was done from the second day of age onwards a large percentage of the pups was already positive for *H. hepaticus* (Singletary et al., 2003). This shows that *H. hepaticus* is transmitted from mothers to newborns, in the first few days of life, possibly through pups contact with maternal feces. Due to the intrinsic differences between the adult and neonate immune system, it is conceivable that mother to pup transmission shapes the interactions between the host and *H. hepaticus* differently than upon colonization at adult age.

Taken together, our analyses of the literature lead us to identify two sets of unanswered questions related to the interactions between the mouse and *H. hepaticus*, namely:

- whether the immune response in adult mice affects the bacteria, and which components of the immune system would be involved in this response;

- whether the age at colonization conditions the immune response to this bacteria and whether this would affect the health and the microbiota composition of the host.

In this work, we directly addressed these questions by analyzing various mice upon colonization with a reference strain of *H. hepaticus*.

Chapter 2 :

IL-10 promotes *Helicobacter hepaticus* persistence in the gut, while Antibodies, T and B cells contribute equally to bacteria elimination.

Preliminary notes

The author of the thesis participated in the planning, execution and analysis of all the experiments presented in this chapter

Abstract

Helicobacter hepaticus is a pathobiont that lives in close association with the mouse intestinal epithelium. Despite abundant evidence that *H. hepaticus* induces a strong adaptive immune response in the mice, the impact of this response on the bacteria living in the gut is largely unknown, as well as why this bacterium is not eliminated by this response. We tested if the adaptive immunity impacts *H. hepaticus* in the intestine by colonizing mice lacking different components of the adaptive immune system, and verifying the effect on the bacterial numbers in the gut. We found that B and T lymphocytes impact bacterial load in the gut, with a similar contribution. Antibodies participate in this effect, but do not account for the whole outcome, which indicates that T and B cells also contribute to bacteria elimination in an antibody-independent manner. Absence of IL-10 results in an increased response, which can lead to bacteria elimination. We conclude that IL-10 promotes the persistence of *H. hepaticus* in the gut, while T and B cells control bacterial numbers. Our study provides new insights into how lymphocytes control bacteria in the microbiota and maintain intestinal homeostasis, which could help in the understanding and prevention of gut immunophatologies.

Introduction

Some of the large amount of bacteria in the microbiota lives in close association with the host. One of those is *Helicobacter hepaticus*, which is broadly spread in the wild and animal facilities (Wasimuddin et al., 2012), and was found to be associated with the host epithelium (Chow and Mazmanian, 2010). It would be expected, hence, that the host would produce an immune response to avoid uncontrolled expansion of this bacterium, so close to the surface of the body. If this response exists, however, it is not strong enough to eliminate this microbe, since mice are positive for live upon colonization (Solnick and Schauer, 2001; Wasimuddin et al., 2012; Whary and Fox, 2006).

It is clear that *H. hepaticus* induces a T cell response in mice, particularly in the absence of immune regulation (Cahill et al., 1997; Kullberg et al., 1998), yet the information on the gut response to this bacterium remains limited, though it is known to induce a fecal IgA response (Whary et al., 1998).

Secretory IgA is thought to act as a barrier, regulating the microbiota and restricting the entrance of intestinal antigens into the blood (Pabst, 2012). Some reports have highlighted the importance of IgA on the host protection (Forbes et al., 2008, 2011; Lycke et al., 1999), and although there is evidence that T cell (particularly Tregs) are necessary for optimal IgA production and homeostasis of the microbiota (Kawamoto et al., 2014), others have claimed that only part of the IgA in the gut is T cell-dependent (Bunker et al., 2015) and that it only targets particular groups of bacteria in the gut (Mirpuri et al., 2013).

Here, we sought to understand the impact of the immune response on *H. hepaticus* load in the gut. We found that specific IgA to *H. hepaticus*

is T-cell dependent and negatively regulated by IL-10. In the absence of IgA, specific secretory IgM is found in the feces, but at a much lower absolute concentration. B and αβT lymphocytes, together with IgA, seem to contribute similarly to *H. hepaticus* load reduction, as the latter increases gradually with the removal of each component separately. These results evidence how complex and multilayered the immune response to bacteria is in the gut.

Results

Impact of the adaptive immunity on *H. hepaticus* in the gut

Mice are easily colonized with *H. hepaticus* through contact with contaminated feces, majorly through coprophagy (Livingston et al., 1998). To examine if colonization by this normal route results in a specific response in the gut, and to test if this response affects the bacteria inside the intestine, we colonized adult (10 weeks old) B6 WT and Rag2$^{-/-}$ mice by gavage with *H. hepaticus*-positive feces, and evaluated the bacterial load in the feces of these mice on the course of 16 weeks by qPCR. *H. hepaticus* colonization occurs in all animals, with a peak in bacterial load between 1 and 2 weeks post-colonization (**Fig. 2.1A**). However, while the bacterial load was considerably stable in Rag2$^{-/-}$ animals after this initial shift, a steady decrease in bacterial load was observed in WT mice, reaching levels almost 2 logs lower than those in Rag2$^{-/-}$ mice (**Fig. 2.1A**). This difference was not due to variations in the microbiota of WT and Rag2$^{-/-}$ mice, as co-housing of animals of these two genotypes from 4 to 9 weeks of age before colonization produced very similar results (**Fig. 2.1B**). These data indicate that the colonization with *H. hepaticus* induces an adaptive immune response in the mice, and that this response impacts the bacteria in the gut.

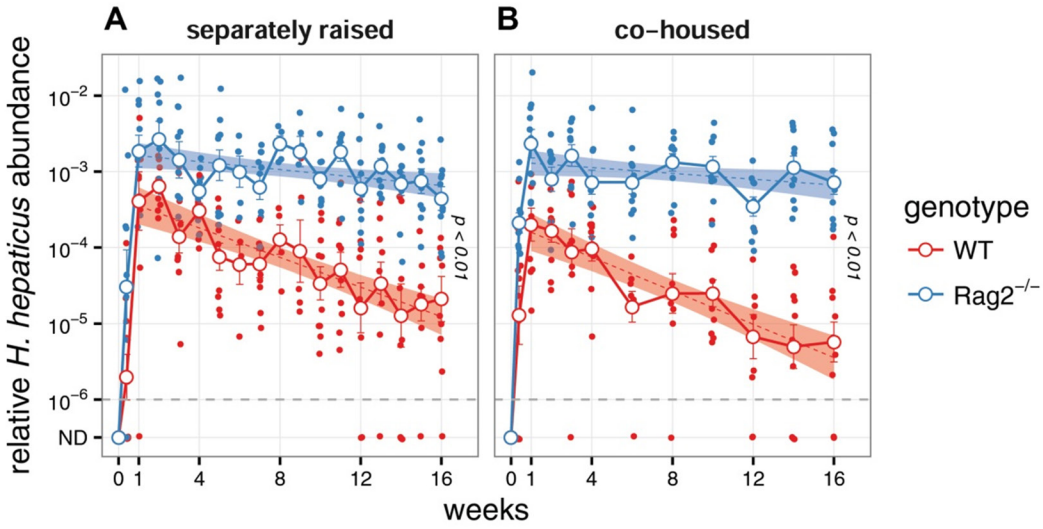

Figure 2.1 - Impact of the adaptive immunity on *H. hepaticus* in the gut

Adult mice (10 weeks old) WT and Rag2$^{-/-}$, separately raised (**A**) or co-housed for 5 weeks (**B**), were colonized with *H. hepaticus* by feces oral gavage. Bacterial load was measured on feces by qPCR up to 16 weeks post-colonization, and analyzed as relative abundance = (*H. hepaticus* 16S / eubacteria 16S). p < 001, linear regression (week 1 - 16). Colored dots = individual mice, white circles = mean, error bars = SEM, dotted line = linear regression from 1-16 weeks, shade = linear regression confidence interval. N = 10 per group, pooled from two independent experiments. ND = not detected.

Impact of T cells on the response to *H. hepaticus*

Because it is known that *H. hepaticus* induces a potent T cell response in the gut (Cahill et al., 1997), and that this response is even stronger in the absence of IL-10 (Kullberg et al., 1998), we next tested the impact of the T cell response on *H. hepaticus* in the intestine using mice deficient in either αβT cells (TCRβ$^{-/-}$) or IL-10 (IL-10$^{-/-}$). We observed a higher *H. hepaticus* load in the absence of T cells, compared to WT mice, but still lower than the levels in Rag2$^{-/-}$ mice (**Fig. 2.2A**). Accordingly, the response in IL-10$^{-/-}$ mice had a large effect on the *H. hepaticus* load, with levels dropping below the detection limit in most of the animals after 11 weeks of colonization (**Fig. 2.2A**). Taken together, these results evidence that T cells play a key role in the eliminating response to *H. hepaticus* in the gut.

WT mice displayed high titers of anti-*H. hepaticus* IgA on feces throughout the colonization (**Fig. 2.2B**), and this IgA was able to bind to the surface of live *H. hepaticus* (**Fig. S2.6**), hence suggesting that it can affect the bacteria in the gut. Meanwhile, IL-10$^{-/-}$ mice showed fecal IgA titers almost 2 logs higher than WT (**Fig. 2.2B**), which correlated with the *H. hepaticus* load reduction (**Fig. 2.2D**). Conversely, specific IgA was almost absent in animals lacking T cells, showing that the induction of anti-*H. hepaticus* mucosal IgA is T cell dependent (**Fig. 2.2B**). An increase of about 1 log in total fecal IgA production was seen in WT and IL-10$^{-/-}$ animals 1 week after colonization, but also in TCRβ$^{-/-}$ with a delay (**Fig. 2.2C**). This was correlated with the levels of lipocalin-2 (lcn-2), a biomarker of inflammation in the gut, in IL-10$^{-/-}$ and TCRβ$^{-/-}$ mice, but not in WT animals (**Fig. 2.2E and F** and **Fig. S2.5A**). Lcn2 levels did not correlate with the reduction in *H. hepaticus* levels in the gut (**Fig. S2.5C**) or with specific IgA levels (**Fig. S2.5B**), suggesting that the inflammation triggered by *H. hepaticus* is separated from the response targeting this bacteria.

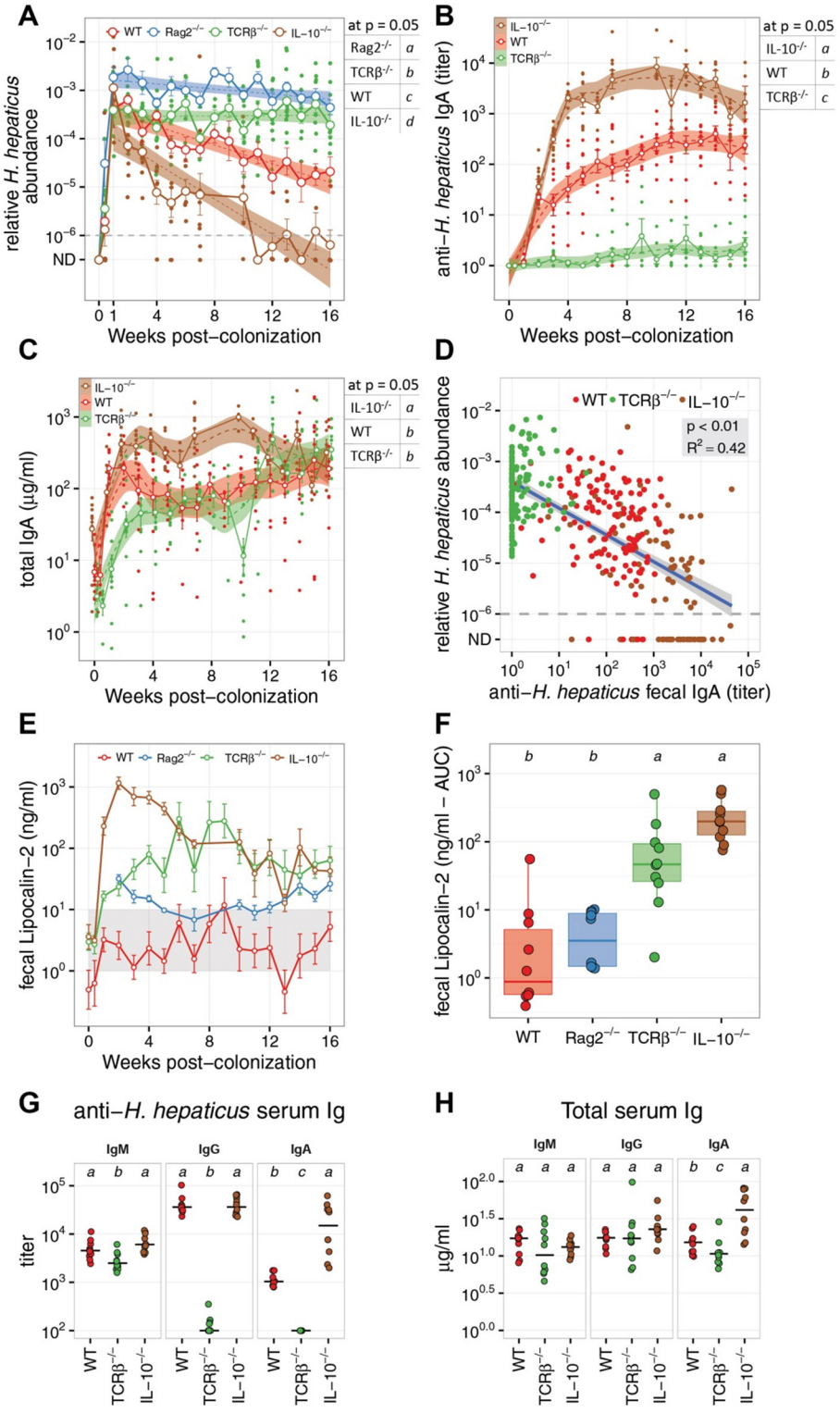

Figure 2.2 - Impact of αβT cells and IL-10 on the response to *H. hepaticus*

Fig.2.2 - Impact of αβT cells and IL-10 on the response to *H. hepaticus*

A) Adult mice (10 weeks old) TCRβ$^{-/-}$ and IL-10$^{-/-}$ were colonized with *H. hepaticus* by feces oral gavage. Bacterial load was measured on feces by qPCR as described in Fig.1. WT and Rag2$^{-/-}$ load (same as in Fig.1 A) is shown for comparison. Groups with the same letter are not significantly different at p = 0.05 (pairwise comparisons of linear regressions, p values corrected using single-step method). Colored dots = individual mice, white circles = mean, error bars = SEM, dotted line = linear regression from 1-16 weeks, shade = linear regression confidence interval. N = 10 per group, pooled from two independent experiments. **B** and **C**) Anti-*H. hepaticus* IgA titer (**B**) and total fecal IgA (**C**) were evaluated by ELISA on WT, TCRβ$^{-/-}$ and IL-10$^{-/-}$ mice shown in **A**, from 0 – 16 weeks post-colonization. Statistical comparison was performed using Area Under the Curve (AUC), calculated per mice with trapezoid method and averaged per week. Groups with the same letter are not significantly different at p = 0.05 (Wilcoxon test with BH correction). Colored dots = individual mice, white circles = mean, error bars = SEM, dotted line = local polynomial regression fitting (loess), shade = loess confidence interval. **D**) Comparison of specific IgA titers and bacterial load in mice shown in A. Colored dots = individual samples, line = linear regression, shade = linear regression confidence interval, p value and R^2 for the linear regression shown on the graph. **E** and **F**) Fecal Lipocalin-2 levels were evaluated by ELISA in the feces of the mice shown in **A**, from 0 – 16 weeks post-colonization (2 – 16 weeks for Rag2$^{-/-}$ mice). **E**) white circles = mean, error bars = SEM, lines connect the means. **F**) Dots represent individual mice. Groups with the same letter are not significantly different at p = 0.05 (Wilcoxon test with BH correction). AUC = Area Under the Curve, calculated per mice with trapezoid method and averaged per week. **G** and **H**) Anti-*H. hepaticus* serum Ig titer (**G**) and total serum Ig concentration (**H**) were measured in WT, TCRβ$^{-/-}$ and IL-10$^{-/-}$ mice shown in **A**, at 16 weeks post-colonization. Dots represent individual mice, groups with the same letter are not significantly different at p = 0.05 (Wilcoxon test with BH correction).

Analysis at 16 weeks post-colonization showed high titers of anti-*H. hepaticus* IgG and IgA in the serum of WT and IL-10$^{-/-}$ animals, suggesting that this bacterium crosses the intestinal barrier, eliciting a systemic immune response (**Fig. 2.2G**). Similarly to the mucosal response, specific IgA levels were higher in IL-10$^{-/-}$ animals compared to WT, but not IgG, suggesting that IL-10 could impact specifically the IgA production. Similar levels of specific IgM were found in WT, IL-10$^{-/-}$ and TCRβ$^{-/-}$ mice, but specific IgG and IgA were not found in the latter (**Fig. 2.2G**). Total Immunoglobulin levels were similar between the groups analyzed, with a small increase in total IgA levels in IL-10$^{-/-}$ mice (**Fig. 2.2H**).

Role of B cells and IgA on the response to *H. hepaticus*

Given the negative correlation between the IgA response and *H. hepaticus* load, we tested if the response to *H. hepaticus* would be affected by the removal of antibodies from the system, using mice devoid of B cells ($JhT^{-/-}$ mice), or by only removing IgA, using $AID^{-/-}$ mice (which can't perform class-switch recombination, hence only IgM can be produced). In the absence of B cells the *H. hepaticus* fecal load was higher than in WT animals, but lower than in $Rag2^{-/-}$ (**Fig. 2.3A**), suggesting that B cells impact the bacterial load, but do not account for the whole effect. Interestingly, the absence of IgA in $AID^{-/-}$ results in an intermediate phenotype between the B cell deficient and WT (**Fig. 2.3A**), indicating that IgA affects the bacterial load but also that B cells have other effects besides antibody production, since the IgM levels, specific and total, were very low in $AID^{-/-}$ after colonization (**Fig. 2.3B** and **C**). Fecal lcn-2 levels in $JhT^{-/-}$ and $AID^{-/-}$ (**Fig. 2.3D**) were very similar to the values found in WT mice (**Fig. 2.2E**), indicating no evident increase in gut inflammation.

To directly test the impact of secreted antibodies on the bacterial load, excluding any possibly effect of IgM in $AID^{-/-}$ mice, we used $AID^{-/-}uS^{-/-}$ animals, which have B cells but cannot secrete antibodies. We found that the *H. hepaticus* fecal levels were higher in $AID^{-/-}uS^{-/-}$ mice compared to WT, but lower than those in $Rag2^{-/-}$ mice (**Fig. 2.4A**). To confirm this effect, we analyzed the *H. hepaticus* mucosal load in the ileum and colon of the same mice, after 20 weeks of colonization. No difference between the groups was observed in the ileum, where *H. hepaticus* was found in lower frequencies (**Fig. 2.4B**). However, the bacterial load reached high levels in the colon of $Rag2^{-/-}$ animals, about 2 logs higher than in WT mice, with intermediate values in $AID^{-/-}uS^{-/-}$ (**Fig. 2.4B**). These data show that secreted antibodies can impact directly the bacterial load in the gut, but do not account for the whole effect.

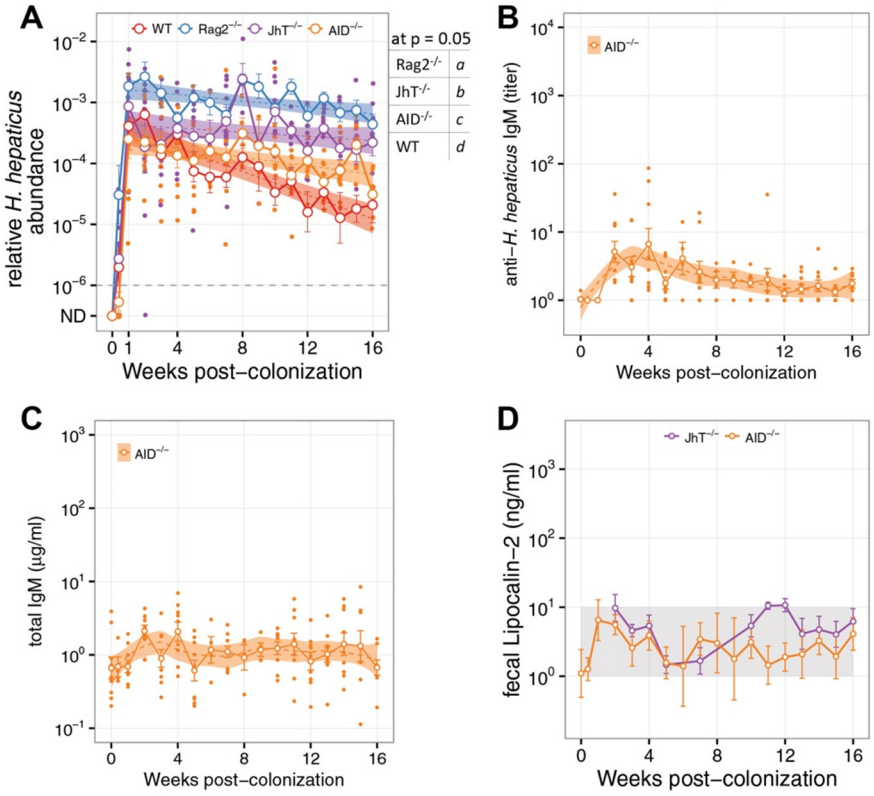

Figure 2.3 - Impact of B cells and IgA on the response to *H. hepaticus*

A) Adult mice (10 weeks old) JhT$^{-/-}$ and AID$^{-/-}$ were colonized with *H. hepaticus* by feces oral gavage. Bacterial load was measured on feces by qPCR. WT and Rag2$^{-/-}$ load (same as in Fig.1 A) is shown for comparison. Groups with the same letter are not significantly different at p = 0.05 (pairwise comparisons of linear regressions, p values corrected using single-step method). Colored dots = individual mice, white circles = mean, error bars = SEM, dotted line = linear regression from 1-16 weeks, shade = linear regression confidence interval. N = 9 per group, pooled from two independent experiments. **B** and **C**) Specific and Total fecal IgM in the AID$^{-/-}$ mice shown in **A**. **D**) Fecal Lipocalin-2 levels were evaluated by ELISA in the feces of the mice showed in **A**, from 0 – 16 weeks post-colonization (2 – 16 weeks for JhT$^{-/-}$ mice). White circles = mean, error bars = SEM, lines connect the means.

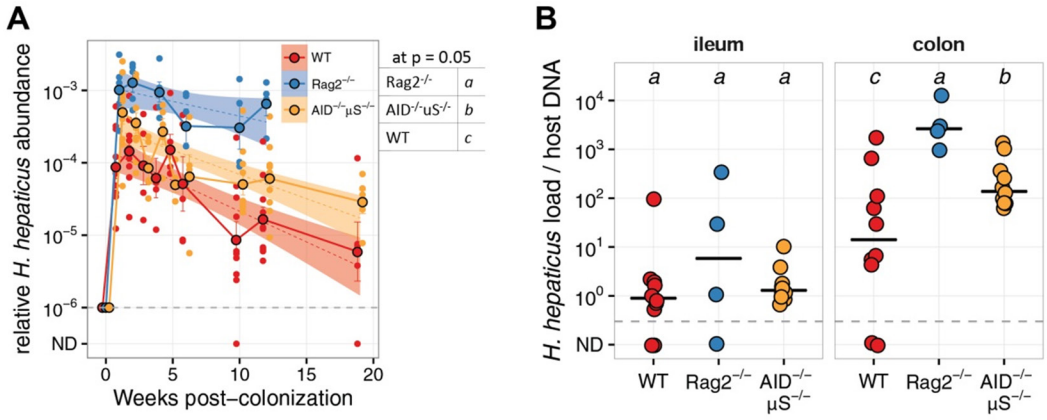

Figure 2.4 - Effect of secreted Immunoglobulin on *H. hepaticus* load in the gut

WT, Rag2$^{-/-}$ and AID$^{-/-}$µS$^{-/-}$ mice were colonized by feces gavage with *H. hepaticus*. **A**) Bacterial load was measured on feces by qPCR from 0 – 20 weeks post-colonization. Relative abundance = (*H. hepaticus* 16S / eubacteria 16S). Colored dots = individual mice, black circles = mean, error bars = SEM, dashed line = linear regression, shade = linear regression confidence interval. Groups with the same letter are not significantly different at p = 0.05 (pairwise comparisons of linear regressions, p values corrected using single-step method). **B**) *H. hepaticus* mucosal load, measured by qPCR, in the ileum and colon of the mice shown in **A**, >20 weeks post-colonization. Data was normalized to host DNA, quantified using 18S primers. Dots represent individual mice and groups with the same letter are not significantly different at p = 0.05 (Wilcoxon test with BH correction). WT and AID$^{-/-}$uS$^{-/-}$ n=10, Rag2$^{-/-}$ n=4, pooled from two independent experiments.

Discussion

The gut microbiota is composed of a great diversity of bacterial species, and the control of those, particularly the ones close to the epithelium, is a great challenge on the host immune system (Hooper and Macpherson, 2010). Using *Helicobacter hepaticus*, a pathobiont that lives in close association to the host epithelium (Chow and Mazmanian, 2010), we could explore how the host adaptive immunity influences the number of bacteria in the gut, exposing the essential components of this response.

We first showed that lymphocytes are essential to the optimal control of the bacterial load, as alymphatic ($Rag2^{-/-}$) mice present with higher levels of colonization (**Fig. 2.1**). Then, we demonstrated that αβT and B cells are both necessary for this effect, as mice without either one of these cell types presented with similar bacterial load, which was higher than observed in WT animals and lower than what was observed in $Rag2^{-/-}$ animals (**Fig. 2.2A** and **Fig. 2.3A**). As we verified that αβT cells are required for a sustained anti-*H. hepaticus* IgA production (**Fig. 2.2B**), and antibodies are absent in B cell-deficient mice, we tested if antibodies were solely responsible for the anti-bacterial effect, using mice deficient in IgA ($AID^{-/-}$) and in secreted antibodies ($AID^{-/-}uS^{-/-}$). With these models, we could verify that secreted antibodies do impact the bacterial load in the gut (**Fig. 2.4**) and that IgA makes the biggest difference (**Fig. 2.3A and B**), since a significant compensatory IgM response was not observed in the absence of IgA. However, antibodies do not seem to account for the whole effect of the adaptive immunity on *H. hepaticus* in the gut, since in their absence the bacterial levels do not reach the load observed in $Rag2^{-/-}$ mice (**Fig. 2.3A** and **Fig. 2.4**). Furthermore, although T and B cells cooperate to mount a robust antibody response, we found evidence of an antibody-independent effect from these two cell types on bacteria in the gut. First, a T cell effect

could explain the difference in load between JhT$^{-/-}$ and Rag2$^{-/-}$ mice (**Fig. 2.3A**). Second, the differences between the *H. hepaticus* load in TCRβ$^{-/-}$ and Rag2$^{-/-}$ (**Fig. 2.2A**) and between JhT$^{-/-}$ and AID$^{-/-}$ (**Fig. 2.3A**) could be explained by an antibody-independent B cell effect.

T cell derived cytokines could indirectly impact bacteria in the gut through the control of antimicrobial release at the epithelial level. As an example, Paneth cells, which are largely important for the maintenance of the homeostasis at the intestinal host-microbial interface (Vaishnava et al., 2008), release antimicrobial products only when stimulated by immune cell-derived IFN-γ, but not bacterial products (Farin et al., 2014). Antigen presentation at the epithelial level could then assure the specificity of the T cell response, guaranteeing that it would only occur if a particular group of bacteria were increased. B cells, on the other hand, have other ways to impact bacteria in the gut besides the production of antibodies. It was demonstrated that IgA^{+} plasma cells can acquire a multifunctional phenotype in the gut, with expression of iNOS and TNFα, which was important for bacterial control (Fritz et al., 2012). Moreover, B cells can facilitate the expansion of T cells (Merkenschlager et al., 2016), and possibly increase the T cell-mediated anti-bacterial response.

We also demonstrated that an exacerbated response, in the absence of IL-10, impact directly the bacteria in the gut (**Fig. 2.2A**), with a concomitant increase in specific intestinal IgA production (**Fig. 2.2B**). This response could even lead to elimination of this bacteria in some animals (**Fig. 2.2A**), which allow us to conclude that IL-10 promotes *H. hepaticus* persistence in the gut. Indeed, it was demonstrated that this bacterium induces a regulatory T cell (Treg) response, which produce IL-10 upon stimulation with *H. hepaticus* antigens (Kullberg et al., 2002). However, these Treg are not able to prevent the immune response to this bacterium, as we observed a steady decrease in *H. hepaticus* levels in WT mice,

which also reached undetected levels in some animals (**Fig. 2.1** and **Fig. 2.4A**). Still, the Treg response could avoid rapid elimination, allowing this bacteria to spread to other individuals and possibly reach the next generation, being also maintained later by coprophagy between different mice.

Collectively, our studies demonstrate that the adaptive immune response impacts *H. hepaticus* in the gut directly, through IgA but also by a mechanism involving B and T cells, possibly antibody-independent. Further analysis using antibody-deficient mice will help to understand this phenomenon. The exploration and understanding of these mechanisms are important for the comprehension of what maintains homeostasis in the gut, and how to preserve and restore it in pathological conditions.

Materials and Methods

Mice

All the mice were in a C57BL/6 background, bread in our specific pathogen free facility, which excludes *Helicobacter hepaticus*. All experiments involving *H. hepaticus* colonization were performed in a dedicated room. $AID^{-/-}uS^{-/-}$ mice were obtained by inter-crossing $AID^{-/-}$ and $uS^{-/-}$ mice in our facility, as described elsewhere (Lino et al., 2013).

Helicobacter hepaticus culture and mice colonization

Helicobacter hepaticus reference strain (CIP 104102) was obtained from the Biological resources center of the Pasteur Institute. Growth was performed in 10% horse blood-agar (HBA, Oxoid) plates, supplemented with 12.5 mg/L vancomycin, 0.3 mg/L polymyxin B, 6.3 mg/L trimethoprim and 5.0 mg/L amphotericin B, at 37°C, under microaerophilic conditions (6% O_2, 7% CO_2, 3.5% H_2 and 83.5% N_2) generated by an Anoxomat system (MART Microbiology). Bacteria were harvested after 5 days of cultured in PBS solution and numbers were estimated by OD quantification. Mice were colonized by oral gavage with 100µL of bacteria suspension at 10^9 CFU/mL in PBS, using an animal feeding needle with silicone tip, size 20G x 1.5'' (Cadence Science). Colonization was confirmed by PCR on fecal DNA using *H. hepaticus* 16S specific primers (Petnicki-Ocwieja et al., 2009). To reproduce colonization through coprophagy, mice were colonized using a preparation of *H. hepaticus* positive feces, from mice previously colonized with cultured *H. hepaticus*. Fecal pellets were homogenized in PBS at a ratio of 1 pellet to 400µL (~ 100mg/mL) and filtered in 100µm mesh. Each mouse received 100µL of this fecal preparation by oral gavage.

Helicobacter hepaticus quantification

The following primers were used in qPCR reactions: *H. hepaticus* 16S fwd: GCATTTGAAACTGTTACTCTG, *H. hepaticus* 16S rev: CTGTTTTCAAGCTCCCCGAAG, 417bp product (Petnicki-Ocwieja et al., 2009); Eubacteria 16S fwd: ACTCCTACGGGAGGCAGCAGT, Eubacteria 16S rev: ATTACCGCGGCTGCTGGC, ~180bp product, 18S fwd: CATTCGAACGTCTGCCCTAT, 18S rev: CCTGCTGCCTTCCTTGGA, 137bp product (Vaishnava et al., 2011). *H. hepaticus* (Hh) 16S copy number was estimated using plasmid standard curve, total bacteria (eubac) 16S copy number was estimated using a standard curve constructed with *E. coli* K12 DNA, and host DNA amount in ng was estimated using a standard curve constructed with intestinal tissue DNA. All reactions contained standard curves for absolute quantification. Fecal *H. hepaticus* amount is expressed as $\log_{10}$ of the ratio Hh 16S copies/ eubac 16S copy. Mucosal *H. hepaticus* amount is expressed as $\log_{10}$ of the ratio Hh 16S copies/ ng of host DNA. DNA from fecal pellets of ~40mg (average 40.2mg ± 10.3, measured from 100 samples) and from intestinal tissue (3 cm piece of ileum and colon, after washing luminal content with PBS) was extracted using NZYTech Tissue gDNA extraction kit (NZYTech), with an initial step at 95°C. For quantification, 2ng of fecal DNA and 10ng of mucosal DNA were used. Primers were used at 0.5μM in the reaction, containing 5μL of iTaq Universal SYBR Green Supermix (Bio-Rad), 2μL of sample and water to a final volume of 10μL. qPCR conditions: 95°C - 3min; 45 cycles of 95°C - 30s, 60°C - 30s, 72°C - 36s; followed by a melting curve of 65 - 95°C with 0.5°C increase every 5s. All reactions were prepared in 384 well hard shell plates (Bio-Rad), on ice, in less than one hour, to minimize primer dimer formation, and run immediately after preparation on a CFX384 real-time PCR detection system (Bio-Rad).

Total and specific Immunoglobulin quantification by ELISA

To quantify total and *H. hepaticus* specific fecal antibodies, fecal extracts were prepared from freshly collected or -80°C stored feces. Fecal pellets of ~50mg were collected from each mice (average 56.6mg ± 15.4, measured from 100 samples), and homogenized in 500µL of PBS containing a protease inhibitor cocktail (P8340, Sigma-Aldrich), for a solution of about 100mg/mL. Homogenates were centrifuged at 16,000G for 10min at room temperature, and supernatants were stored at -20°C until further use.

Soluble *H. hepaticus* antigens (SHelAg) were prepared as previously described (Kullberg et al., 1998). After cultured for 5 days in blood-agar plates, bacteria were harvested in PBS solution, washed and lysed in a French Press. The solution was centrifuged at 8,000G for 30 min at 4°C, the supernatant was filtered sterile with a 0.22µm pore size filter and protein content was determined using BCA protein assay. The preparation was stored at -80°C.

ELISA was performed using high binding 384 well ELISA plates (UltraCruz). For total Ig quantification, plates were coated with Goat anti-Mouse IgM, IgG or IgA polyclonal antibodies (Southern Biotech), and for anti-*H. hepaticus* Ig quantification, coating was done using 10µg/mL of SHelAg. Plates were coated overnight at 4°C, washed in PBS 0.05% Tween20 and blocked with 2% BSA solution in PBS. Fecal extracts were added undiluted (specific Ig) or 10 fold diluted (total Ig) to plates and diluted 7 times, 3 fold each time. For specific fecal IgA quantification, a reference sample pooled from 5 adult colonized B6 was used in all assays. For specific serum IgM, IgG and IgA, a reference serum sample of colonized IL-$10^{-/-}$ diluted 1:210 was used in all assays. After overnight incubation at 4°C, plates were washed in PBS 0.05% Tween20 and incubated with Goat anti-

Mouse IgM, IgG or IgA conjugated with HRP. Reactions were revealed with TMB (BD) and stopped with 0.1M H_2SO_4.

Fecal Lipocalin-2 quantification

Quantification of fecal Lipocalin-2 was performed on fecal extracts, obtained as described above, using the kit manufacturer instructions (Mouse Lipocalin-2/NGAL DuoSet ELISA, catalog number: DY1857, R&D).

IgA FACS on live *H. hepaticus*

H. hepaticus was cultured as described above, and 10^6 CFU were used per assay. Bacteria was washed once in PBS, pelleted (12,000G, 5min), resuspended in fecal extract (prepared as described for ELISA assay) from naïve or 16 weeks colonized Adult B6 mice, and incubated on ice for 30min. Bacteria were then washed in PBS and incubated for 15min on ice with Goat anti-Mouse IgA (Southern Biotech), labeled using Alexa647 Labeling Kit (Thermo Fisher Scientific). The bacteria was washed again and resuspended in a PBS solution containing 5µM of SYTO 9 (Molecular Probes). Events were acquired on a CyAn ADP Analyzer (Beckman Coulter) and analyzed on FlowJo Software (Tree Star). Analysis was performed gating on live bacteria, which were SYTO 9 Bright, as confirmed by counterstaining with 45µM of propidium iodide (Molecular Probes), and excluding events with high pulse width.

Data analysis

Data and statistical analysis were performed using R software version 3.2.5 (R Core Team, 2014). Multiple comparisons were done using Kruskal-Wallis and Mann–Whitney–Wilcoxon test with the Benjamini and Hochberg p value correction. Multiple comparisons of linear regressions were performed using linear model and the function glht (General linear hypotheses and multiple comparisons), using single-step procedure for p

value correction, in package multcomp (Hothorn et al., 2008). Graphs were made in R software using the package ggplot2 (Wickham, 2009).

Supplementary Material for Chapter 2

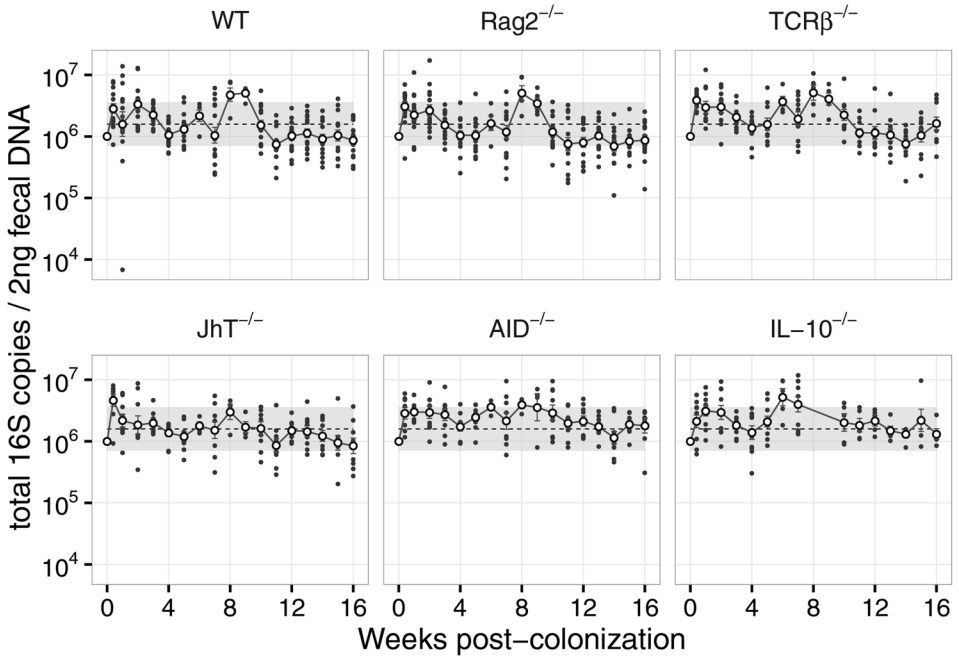

Figure S2.1 - Total 16S levels in fecal DNA after *H. hepaticus* colonization (complementary to Figures 2.1A, 2.2A and **2.3A).**

Total 16S (quantified using primers that recognize a conserved region in all bacteria 16S) in DNA extracted from feces of mice of the indicated genotype from 0 – 16 weeks post-colonization with *H. hepaticus*. Total 16S values were used to normalize the number of *H. hepaticus* 16S presented as relative abundance in figures **2.1A**, **2.2A** and **2.3A**. Dots = individual mice, white circles = mean, error bars = SEM, dotted line = overall mean, shade = overall standard deviation. N = 10 for WT, Rag2$^{-/-}$, TCRβ$^{-/-}$ and IL-10$^{-/-}$, N = 9 for JhT$^{-/-}$ and AID$^{-/-}$, pooled from two independent experiments.

Genotype	Intercept		slope		Significance codes* (p=0.05)
	estimate	SE	estimate	SE	
Rag2$^{-/-}$	-2.764	0.166	-0.026	0.017	*a*
TCRβ$^{-/-}$	-3.550	0.166	0.002	0.017	*b*
JhT$^{-/-}$	-3.351	0.171	-0.017	0.017	*b*
AID$^{-/-}$	-3.618	0.171	-0.035	0.017	*c*
WT	-3.355	0.118	-0.097	0.012	*d*
IL-10$^{-/-}$	-3.786	0.170	-0.183	0.019	*e*

Multiple R-squared: 0.53 ; Adjusted R-squared: 0.52

**groups with the same letter are not different at the significance level*

Table S2.1 - Linear regression coefficients (Fig 2.2A and Fig 2.3A)

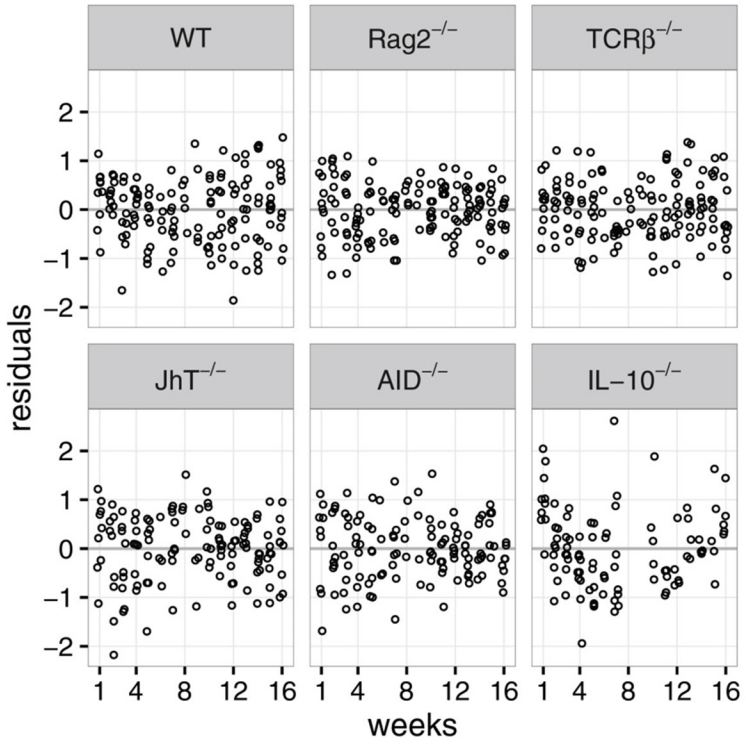

Figure S2.2 - Linear regression residuals plot (Fig 2.2A and Fig 2.3A)

Genotype	Intercept		slope		Significance codes* (p=0.05)
	estimate	SE	estimate	SE	
$Rag2^{-/-}$	-2.948	0.211	-0.041	0.030	*a*
$AID^{-/-}uS^{-/-}$	-3.492	0.156	-0.067	0.018	*b*
WT	-3.823	0.110	-0.085	0.013	*c*

Multiple R-squared: 0.52 ; Adjusted R-squared: 0.51

**groups with the same letter are not different at the significance level*

Table S2.2 - Linear regression coefficients (Fig 2.4A)

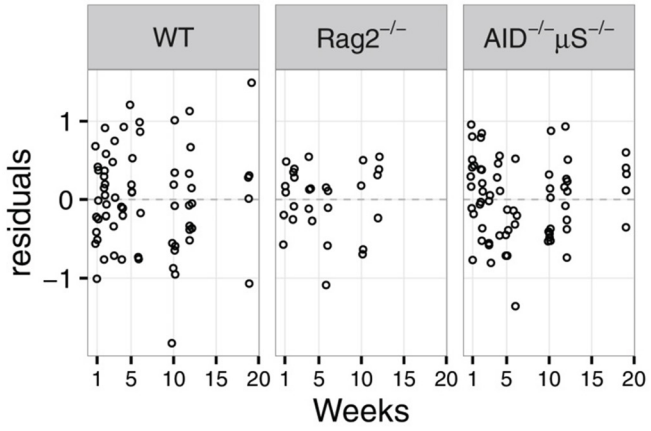

Figure S2.3 - Linear regression residuals plot (Fig 2.4A)

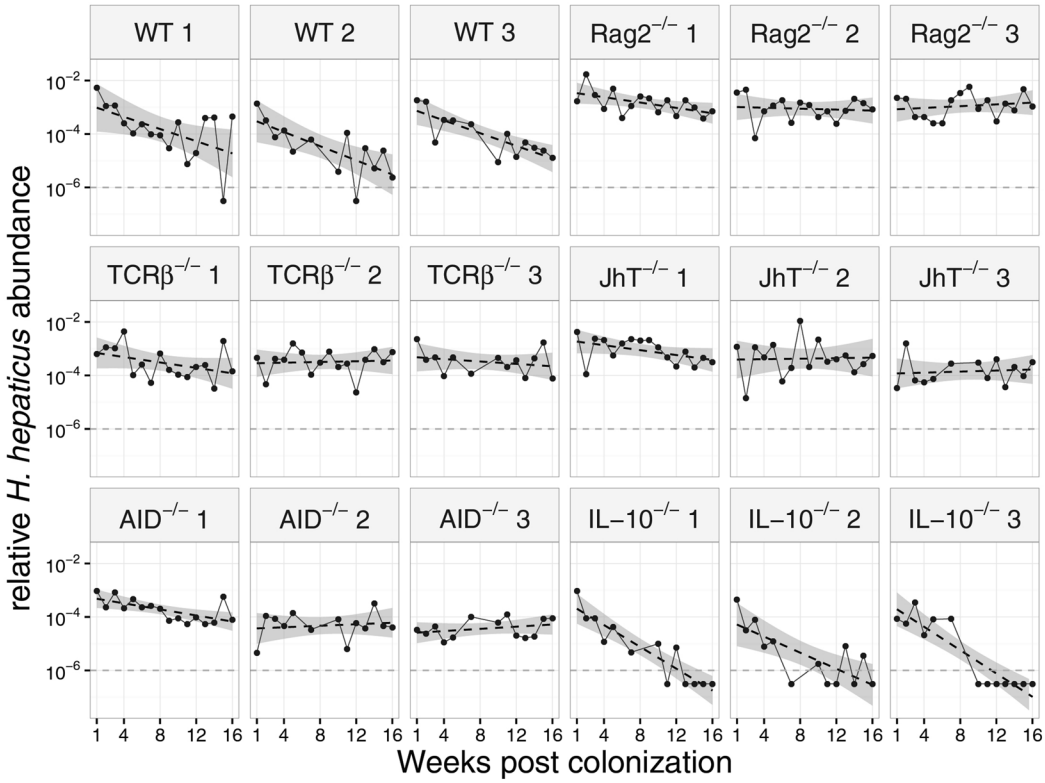

Figure S2.4 – Representative individual plots (Fig 2.2A and Fig 2.3A)

Individual plots of *H. hepaticus* fecal load from 1 – 16 weeks of colonization in 3 representative mice from each genotype in figures 2.2A and 2.3A. Dotted line = linear regression, shade = linear regression confidence interval.

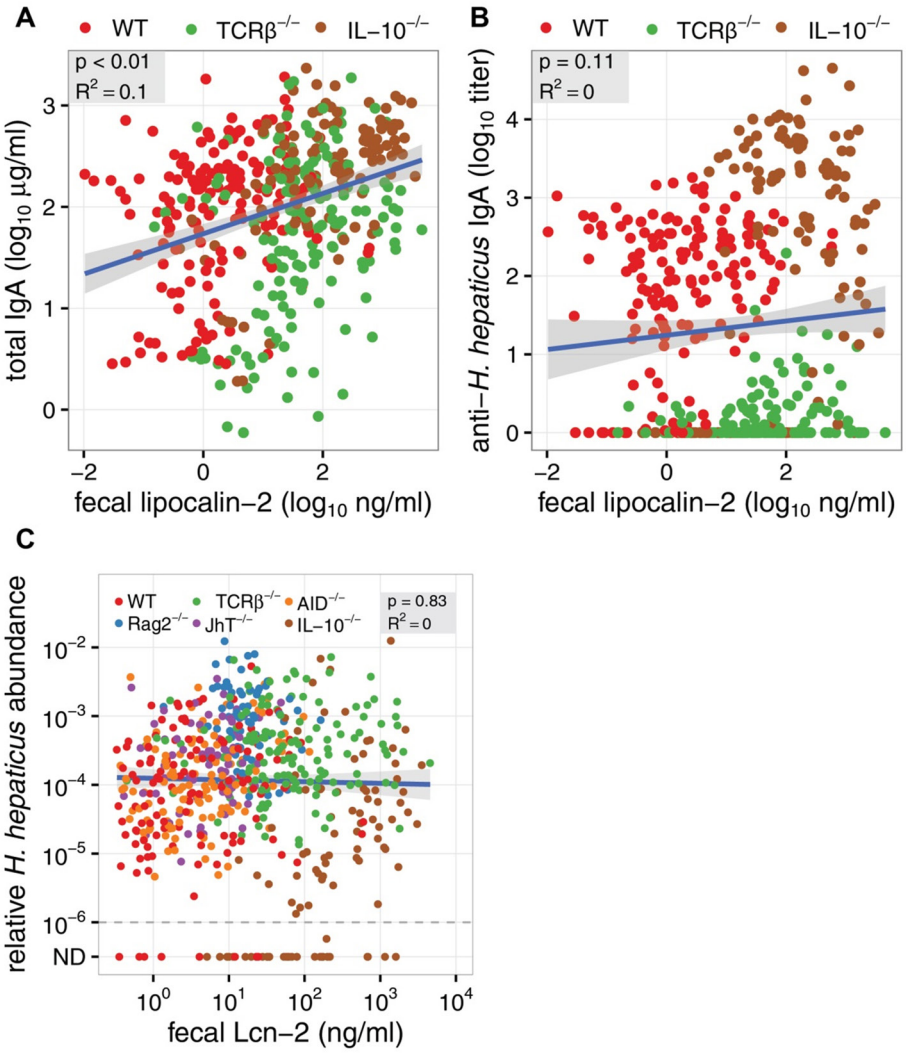

Figure S2.5 – Lipocalin-2 correlation to IgA and load (complementary to Fig. 2.2)
Individual samples from mice of the indicated genotypes from 0-16 weeks (**A** and **B**) and from 1-16 weeks (**C**). Blue line = linear regression, shade = linear regression confidence interval, p value and R^2 of the linear regression indicated on the graphs.

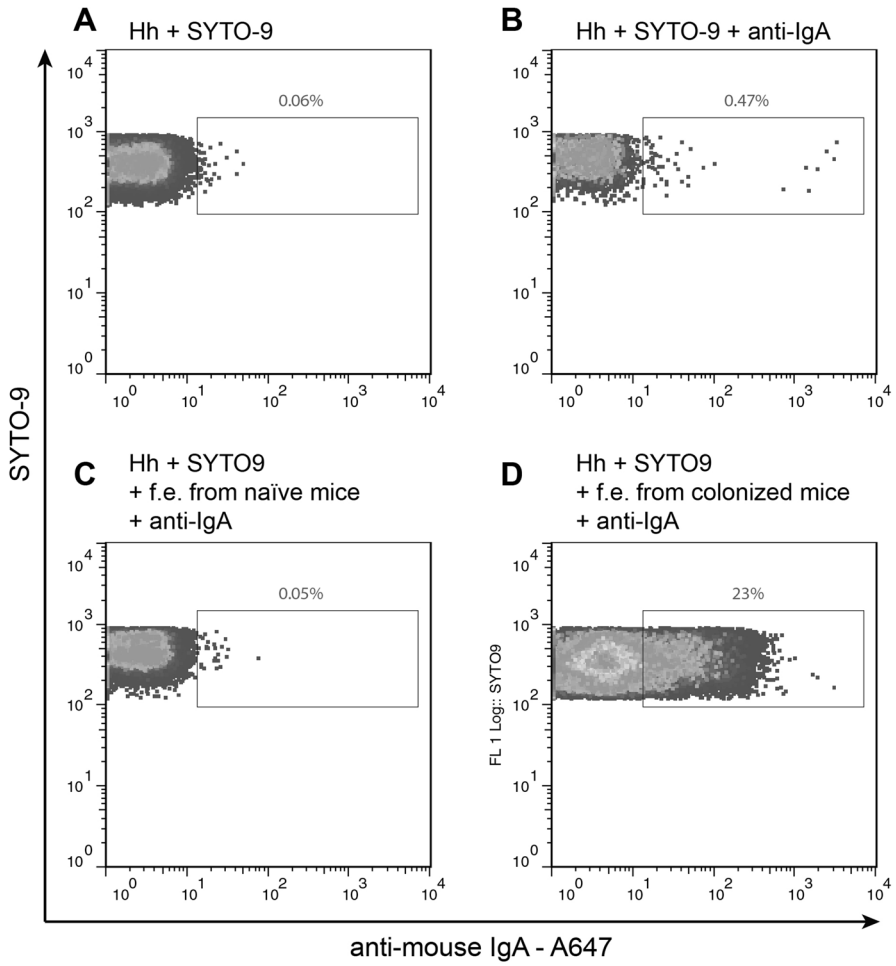

Figure S2.6 – IgA from colonized mice binds to the surface of *H. hepaticus*

FACS analysis of live *H. hepaticus* (from culture), stained with SYTO9 (nucleic acid staining, membrane permeable) (A) or SYTO9 + anti-mouse IgA (B), or pre-incubated with fecal extract (f.e.) from naïve (C) or 16 weeks colonized Adult B6 WT mice (D) before staining with SYTO9 + anti-mouse IgA. Events shown were gated on SYTO9 bright (live) events with low pulse width.

Chapter 3 :

Perinatal transmission of *Helicobacter hepaticus* consolidates symbiosis through induction of life-long immune tolerance mediated by Foxp3^{+} regulatory T cells

Preliminary notes

The author of the thesis participated in the planning, execution and analysis of all the experiments presented in this chapter.

Abstract

Helicobacter hepaticus (Hh), a frequent symbiont of both wild and laboratory mice, is transmitted from mother to pups at birth, and through coprophagy in adults. Given the major physiological changes associated with post-natal maturation, we asked whether and how the age of the host at primo-exposure affects mouse-Hh reciprocal interactions. We analyzed adult mice that had been colonized with a reference Hh strain either during the perinatal period, or at 8-10 weeks of age. We first observed that *H. hepaticus* numbers were increased in the mucosa of the ileum and colon of perinatally colonized mice, compared to adult colonized ones. This was accompanied by an absence of fecal IgA response to *H. hepaticus* in adult mice colonized on the perinatal period, contrasting with a strong IgA response in mice colonized as adults. Hence, *H. hepaticus* colonization in the perinatal period causes a long lasting tolerance in mice. Performing kinetic and limiting dilution analysis, and using loss-of-function approaches, we reveal that induction of immunological tolerance to Hh during perinatal life does not depend on maternal Immunoglobulins or microbiota, and that its long lasting maintenance is mediated by $Foxp3^+$ regulatory T cells. Moreover, perinatally colonized mice show normal serology and no predisposition to gut pathology. Finally, 16S analysis of fecal bacterial content reveals that Hh shapes the microbiota composition both in a lymphocyte independent and dependent manner, and that in the latter case, age at primo-exposure matters. Altogether, our study evidences a developmental time window favoring life-long symbiosis through early education of the immune system.

Introduction

Helicobacter hepaticus is a gram-negative, microaerophilic ε-proteobacteria highly prevalent in wild mice (Wasimuddin et al., 2012). It can persistently colonize the gut of most known commercial strains (Whary and Fox, 2006), triggering pathology in some immunocompromised (Kullberg et al., 1998; Whary et al., 1998), but no decrease in breeding efficiency and no clinical signs of disease is found in *Wild Type* (WT) animals (Solnick and Schauer, 2001). Colonization with *H. hepaticus* occurs easily through coprophagy in adults (Livingston et al., 1998), and newborns are colonized by their mothers in the first few days of life (Singletary et al., 2003).

The neonate gut microbiota, naturally seeded by the mother, is dominated by proteobacteria at the first week of life. By 3 weeks of age, however, this group is replaced by Bacteroidetes and Firmicutes, similar to what is found in adults, with proteobacteria levels dropping to about 1% of the total (Mirpuri et al., 2013). These changes are associated with the onset of a solid diet, coprophagy (that starts at 2 weeks of age), but also with the host immune system maturation and the presence of antibodies. Production of secretory IgA only starts in the gut by 3 – 4 weeks of age, but antibodies from the milk are abundantly found in the pup's intestine on the first weeks. Maternal antibodies prevent bacteria translocation from the neonatal gut into draining lymph nodes, and have long lasting consequences, shaping the microbiota and inflammatory genes expression in the adult's gut (Rogier et al., 2014). Additionally, antibodies from the mother are crucial in the protection to infections, particularly because immune responses occur in a different way in neonates (Adkins et al., 2004). For example, increased propensity for IL-10 production causes susceptibility to *Streptococcus* mediated sepsis in newborns (Andrade et al., 2013). These properties of

the neonatal immunity, however, help shape the adult immune system, particularly in the prevention of autoimmune responses late in life. Perinatally generated Regulatory T Cells (Treg) persist through adult life and show different properties from adult generated Tregs, like greater capacity for preventing autoimmunity (Yang et al., 2015).

It has been shown that *H. hepaticus* triggers a strong immune response upon adult colonization, which is dampened by IL-10-producing antigen-specific Tregs (Kullberg et al., 2002), but no information is available on newborn colonization by this bacteria. Taking into account the major developmental differences, microbiota composition and immune system maturation, we asked whether the symbiosis of *H. hepaticus* and the mouse depends on the age at first exposure. Surprisingly, and in opposite to what happens in adults, mice perinatally exposed to *H. hepaticus* do not mount a Secretory IgA response to these bacteria in the gut from young through adult life, allowing an expansion of this bacteria in the intestinal mucosa. This absence of specific secretory IgA was observed when the mice were colonized up to 2 weeks of age, was independent of maternal antibodies and the microbiota, and could be reverted by depletion of Tregs *in vivo* and by IL-10 deficiency, supporting the idea of dominant tolerance. We conclude that *H. hepaticus* is best adapted to newborn colonization, where it induces a robust and long lasting tolerogenic response, which guarantees persistence until reproductive age. Furthermore, our studies evidence the role of bacteria-induced Tregs in the suppression of the response to these bacteria, particularly specific secretory IgA.

RESULTS

Mother to pup transmission confers long-lasting tolerance that benefits *H. hepaticus*

To directly assess whether the age at colonization affects mouse-*H. hepaticus* symbiosis we compared adult animals that had been either inoculated naturally by their mother when newborn (Newborn colonized = Nbcol) or, to mimic natural exposure upon coprophagy, by feces gavage at 10 weeks of age (Adult colonized = Adcol) (**Fig. 3.1A**). We first analyzed the *H. hepaticus* mucosal load at the ileum and colon of Adcol and Nbcol mice colonized for a long time (>12 weeks), by Q-PCR using *H. hepaticus* 16S specific primers. We could observe a significant increase in *H. hepaticus* load in Nbcol compared to Adcol mice, with an increase of 2 logs in the ileum and 1 log in the colon (**Fig. 3.1B**), which suggests that perinatal colonization benefits *H. hepaticus*. We next monitored *H. hepaticus* specific IgA titers in feces, a reliable read-out of specific mucosal immune response. In mice colonized at adult age, IgA specific to *H. hepaticus* was readily detectable and remained at elevated titers over time (**Fig. 3.1C** and **E**). In contrast, and despite persistent and elevated load of *H. hepaticus* (**Fig. 3.1B**), IgA specific to *H. hepaticus* was not detected in the feces of mice that had been colonized in the perinatal period, irrespective of whether these were analyzed at 4 or 16 weeks of age (**Fig. 3.1D**). Nbcol mice analyzed at older ages (22w and 34w old) showed very low values of anti-*H. hepaticus* IgA, still more than 2 logs lower than Adcol mice. We further ascertained that no other *H. hepaticus* specific Ig isotype were found in Nbcol feces (**Table S3.1**), that gut IgA from Adcol animals bind live *H. hepaticus* (**Fig. S3.1**) and that Adcol, Nbcol and SPF mice have IgA coated bacteria in the gut (**Fig. S3.2**). Together, these results demonstrate that colonization with *H. hepaticus* in adults elicits a robust immune

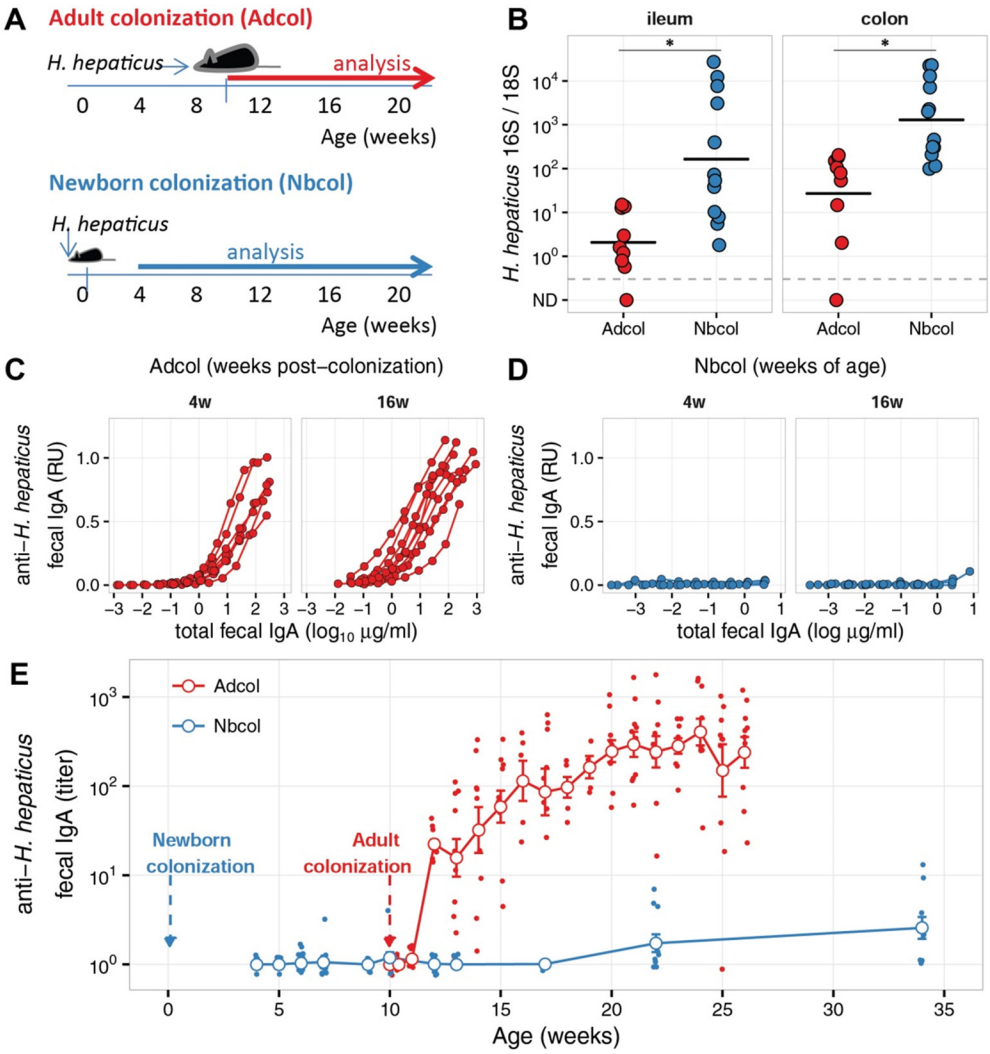

Figure 3.1 - Mother to pup transmission confers long-lasting tolerance to *H. hepaticus*

A) Adult B6 mice (10 weeks old) were colonized with *H. hepaticus* by gavage with positive feces. Newborn colonization was achieved naturally by mother to pup transmission, which happens in the first days of life. **B)** *H. hepaticus* mucosal load in the ileum and colon of Adcol and Nbcol mice was assessed by Q-PCR using *H. hepaticus* 16S specific primers, normalized to host DNA (measured using 18S specific primers). Group – N.(time of colonization): Adcol – 4.(13w), 5.(26w); Nbcol – 6.(13w), 4.(17w), 2.(18w). * $p < 0.05$ (Wilcoxon test). **C – D)** Anti-*H. hepaticus* and total fecal IgA were analyzed by ELISA in the feces of Adcol (**C**) and Nbcol (**D**) mice at 4 and 16 weeks post-colonization. Dots represent measurements and lines connect dilutions of the same sample. Adcol N = 10 in both points, Nbcol N = 6 at 4w and N = 5 at 16w. **E)** Anti-*H. hepaticus* fecal IgA titers in Adcol and Nbcol mice at indicated ages were estimated by ELISA. White symbol = mean. Error bar = SEM. Adcol: N = 10 from 10 - 26w of age. Nbcol - N.(age): 6.(4w), 11.(5w), 24.(6w), 16.(7w), 10.(9w), 10.(10w), 10.(12w), 3.(13w), 5.(16w), 10.(22w), 10.(34w)

response, and that this response is inhibited in animals colonized early in life.

The age but not the mode of primo-exposure to *H. hepaticus* conditions the host response

Low dose and chronic exposure to foreign antigens has been proposed to promote Immune tolerance, by inducing T cell anergy, exhaustion or differentiation into Treg. It is conceivable that pups of *H. hepaticus* colonized mothers are continuously ingesting low number of *H. hepaticus* organisms, while our protocol of gastric gavage would deliver suddenly a large number of *H. hepaticus* in adults. To address this discrepancy we first colonized adult mice with titrated amounts of *H. hepaticus*-positive fecal preparations. The inoculum used in figure 1 contained about 10^6 CFU (estimated by Q-PCR and culture) and serial dilutions revealed that as long as the inoculum contained an estimated 100 CFU (10,000x dilution), SPF mice could be colonized with *H. hepaticus* (**Fig. S3.3**), indicating absence of colonization resistance. More important for this study, administration of an estimated 100CFU by oral gavage to adult mice led to readily detectable *H. hepaticus* specific IgA in the feces with titers in the range of 10^1-10^2 at 4 weeks post-colonization (**Fig. 3.2A**), similar to those obtained with undiluted inoculum (**Fig. 3.1E**). Then, we performed gastric gavage in newborn SPF animals using inoculum estimated at $2x10^5$ CFU (20ul of an undiluted fecal preparation). To further gain insight on the time window when immune tolerance rather than response follows exposure to *H. hepaticus*, pups were inoculated at different ages, from 1 to 4 weeks of age (**Fig. 3.2B**). Successful colonization was confirmed by qPCR 10 weeks post-inoculation (**Fig. S3.4**). Strikingly, intra-gastric gavage reproduced mother transmission as pups inoculated before 3 weeks of age did not develop high levels of fecal *H. hepaticus*-specific IgA (**Fig. 3.2C** and **D**). In contrast, past this age,

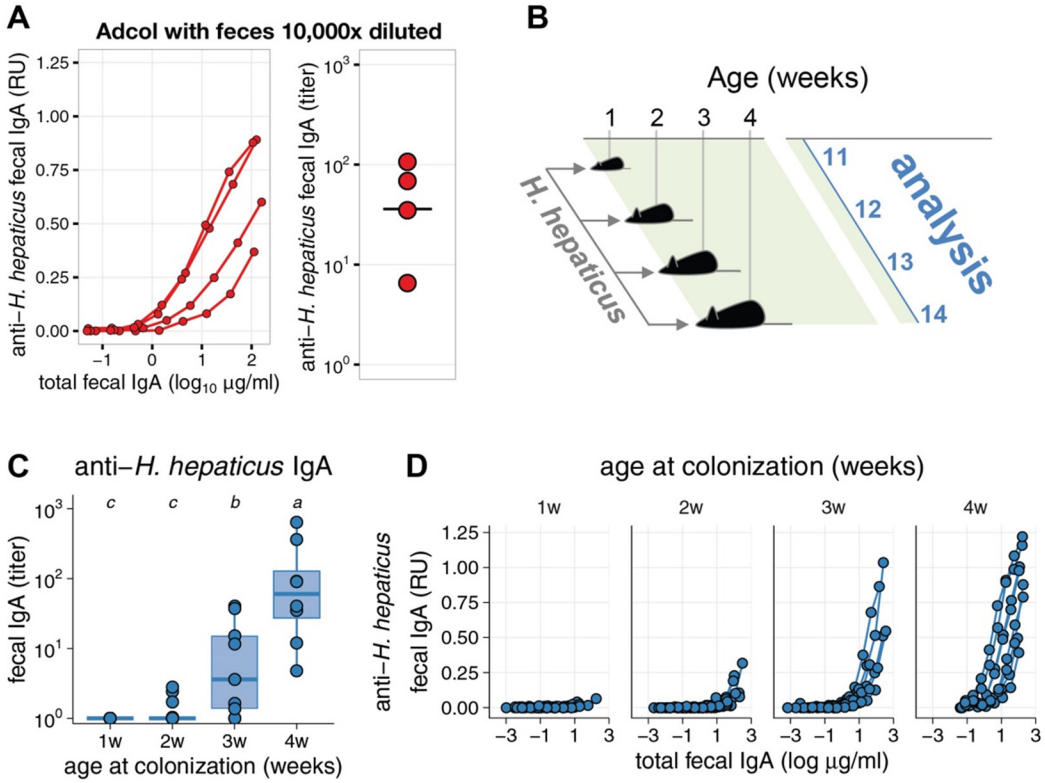

Figure 3.2 - The age but not the mode of primo-exposure to *H. hepaticus* conditions the host response

A) Adult (9w old) B6 were gavaged with a feced preparation from *H. hepaticus* positive animals, diluted 10,000x in PBS. Mice were confirmedly positive for *H. hepaticus* 7 days after gavage. 4 weeks after colonization, feces were analyzed for anti-*H. hepaticus* and Total IgA by ELISA. N = 4. **B - D**) Mice at 1-4 weeks of age were colonized with *H. hepaticus* by feces oral gavage. 10 weeks after colonization, feces were analyzed for anti-*H. hepaticus* and Total IgA by ELISA. N = 8, 13, 9 and 8 respectively. Groups with the same letter are not significantly different at p = 0.05

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inoculation led to readily detectable immune responses as indicated by *H. hepaticus* specific IgA titer approximating, albeit lower than, those obtained in Adcol animals (**Fig. 3.2C** and **3.1E**). The group of mice inoculated at 3 weeks of age was heterogeneous, with some animals unresponsive and others responsive (**Fig. 3.2C**). We conclude that irrespective of the mode of

transmission and the inoculum load, mice infected early in life develop long lasting immune tolerance to *H. hepaticus ,* and that the transition from non-responder to responder state occurs around weaning age, possibly lagging for several weeks before full competence.

Neither maternal antibodies nor the microbiota are required for perinatally induced tolerance to *H. hepaticus*

Weaning is associated with the end of maternal antibody supply and a diet change leading to major alterations in the microbiome composition. We next addressed whether these changes were related to the physiological switch we evidenced above. To produce immune-competent animals born to antibody-deficient dams, young adult Rag2$^{-/-}$ females were colonized with *H. hepaticus* by intra-gastric gavage*,* and mated with WT males. Controls were born to WT mothers also colonized as adults. The progenies were monitored when adults, from 6 to 18 weeks of age. Analysis of feces extract confirmed animals were *H. hepaticus* positive and showed that while they displayed normal total IgA concentration, none produced *H. hepaticus* specific IgA (**Fig. 3.3A**), demonstrating that maternal antibodies are dispensable for the induction of long lasting tolerance to *H. hepaticus* in newborns. To test the contribution of the microbiota to the host responsiveness to *H. hepaticus,* adult WT germ-free mice were mono-colonized with cultured *H. hepaticus*, confirmed positive, and either left to age, or set in breeding to produce progenies mono-colonized at birth. Analysis at 9 weeks post colonization revealed that the microbiota is dispensable for robust anti-*H. hepaticus* specific IgA production in animals that had been monocolonized when adults, and for long lasting tolerance induction and maintenance in animals that had been mono-colonized perinatally (**Fig. 3.3B** - **C**). We conclude that induction of immune tolerance upon primo-infection with *H. hepaticus* is a newborn intrinsic feature. In turn, this finding suggests that the maintenance of such

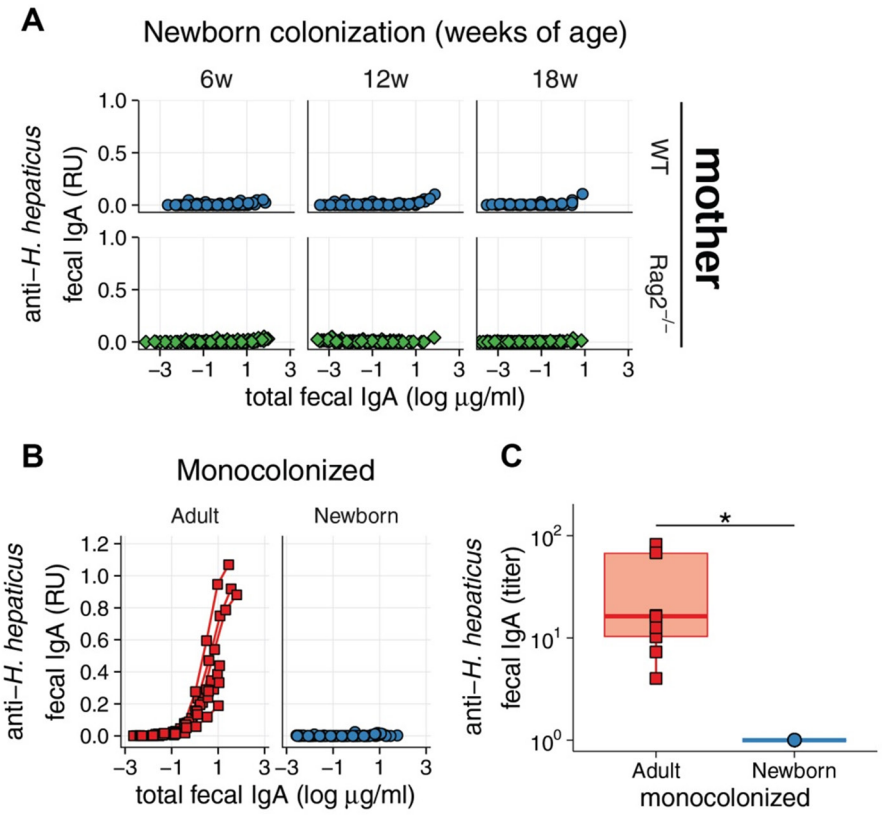

Figure 3.3 - Neither maternal antibodies nor the microbiota are required for perinatally induced tolerance to *H. hepaticus*

A) Adult WT and Rag2$^{-/-}$ females were colonized with *H. hepaticus* and bred to WT males. Pups from these breedings were analyzed at 6, 12 and 18 weeks of age for anti-*H. hepaticus* and Total IgA by ELISA. N=10, 10 and 5 for WT mother at the indicated ages, and N=12 for Rag2$^{-/-}$ mother at all the time points. **B - C**) Adult germ-free mice were colonized with pure *H. hepaticus* culture and analyzed 9 weeks later. Monocolonized mice were bred to produce Newborn-colonized pups, which were analyzed at 9 weeks of age. Anti-*H. hepaticus* and Total IgA were assessed by ELISA. Adult: n = 9; Newborn: n = 15. * $p < 0.05$ (Wilcoxon test).

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immune tolerance into adult age is an intrinsic property of the immune system.

Long lasting immune tolerance to *H. hepaticus* upon mother to pup transmission is Treg mediated

The results above, indicating that the very long lasting immune tolerance to the persistent pathobiont *H. hepaticus* upon perinatal exposure is a host intrinsic property, evoked either immunological ignorance or robustly controlled immunity. Both the immunosuppressive cytokine IL-10 and Treg cells are triggered upon adult exposure to *H. hepaticus* (Berg et al., 1996; Kullberg et al., 2002; Rubtsov et al., 2008), yet as shown here, they do not prevent an immune response to this bacteria. We reasoned these pathways may be more robustly recruited during perinatal life, and tested the role of these components by loss of function approaches. IL10$^{-/-}$ newborn-colonized mice were analyzed from 4 to 15 weeks of age. Anti-*H. hepaticus* IgA was evident in feces of Nbcol IL-10$^{-/-}$ mice already at 5 weeks of age, with a continuous increase after that (**Fig. 3.4A**). Total fecal IgA was also increased In Nbcol IL-10$^{-/-}$ mice with age (**Fig. S3.5A**). Analysis of fecal *H. hepaticus* load in those mice showed a continuous decrease in *H. hepaticus* numbers with age (**Fig. S3.5B**), and although they had diarrhea through the course of the analysis, their weight increased continuously (**Fig. S3.5C**). These results indicate that IL-10 is required for the induction and/or maintenance of immune tolerance to *H. hepaticus*. In fact, we found that 10 weeks old Nbcol WT mice that received 4 weekly 1mg doses of anti-IL-10R antibody started producing low levels of anti-*H. hepaticus* IgA 10 weeks after the beginning of treatment, unlike untreated littermate controls (**Fig. S3.11**), corroborating the idea that IL-10 is involved in the maintenance of this tolerance. Additionally, these findings exclude negative selection as a mechanism for explaining the lack of response in perinatally colonized mice, indicating that active tolerance rather than ignorance mediates this effect.

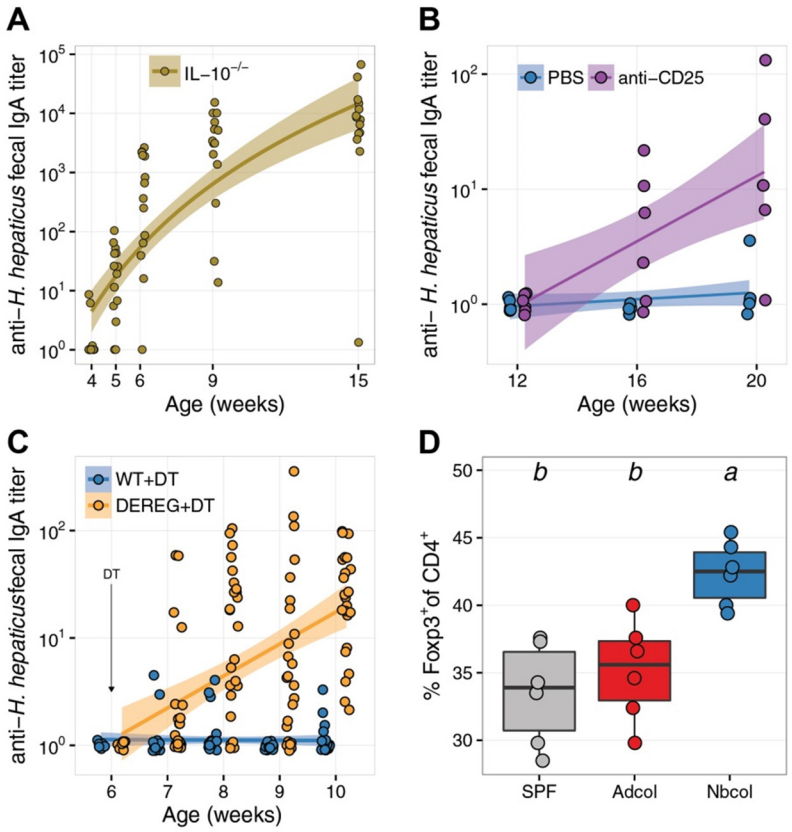

Figure 3.4 - Long lasting immune tolerance to *H. hepaticus* upon mother to pup transmission is Treg mediated

A) Anti-*H hepaticus* fecal IgA was analyzed by ELISA from 4 to 15 weeks of age in IL10$^{-/-}$ newborn colonized mice. N=14. Linear model: y~log(x), line = regression, shade = confidence interval; regression coefficient: $p < 0.01$; $R^2 = 0.63$; F statistic: $p < 0.01$. **B**) Adult WT mice, newborn colonized with *H. hepaticus*, were injected one time *i.p.* with PBS or 500µg of anti-CD25 (PC61 clone). Anti. *H. hepaticus* fecal IgA was assessed at 0, 4 and 8 weeks post-treatment (12, 16 and 20 weeks of age). PBS: n=5; anti-CD25: n=6. Line = linear regression, shade = confidence interval; group comparison: $p < 0.01$; multiple $R^2 = 0.58$; F statistic: $p < 0.01$. Representative of two independent experiments. **C**) B6 WT and DEREG (Foxp3-DTR) mice colonized at birth received 1µg of Diphtheria Toxin (DT) *i.p.* at 6 weeks of age, and fecal IgA titers were measured by ELISA on the indicated ages. N(weeks): WT+DT = 6(6), 19(9-10); DEREG+DT = 9(6), 24(9-10), pooled from 2 independent experiments. Line = linear regression, shade = confidence interval; group comparison: $p < 0.01$; multiple $R^2 = 0.49$; F statistic: $p < 0.01$. **D**) Frequency of TCRβ$^+$ CD4$^+$ Foxp3$^+$ cells in the large intestine lamina propria of adult WT mice either SPF, or colonized with *H. hepaticus* when adult or at birth. n=6 per group. Groups with the same letter are not significantly different at $p = 0.05$ (Wilcoxon test).

To address the role of CD4 regulatory T cells in the maintenance of immune tolerance to *H. hepaticus*, we first administrated, in a single injection, the depleting anti-CD25 antibody (PC61) to young adult mice that had been colonized at birth. This procedure depletes more than 50% of Tregs in the intestine and lymphoid tissues (Wang et al., 2015; Zelenay and Demengeot, 2006). Anti-*H. hepaticus* specific IgA could be detected in the feces of the treated mice at 4 and 8 weeks post-injection, but not in untreated controls (**Fig. 3.4B**). These results indicate that immune responses in animals that had been colonized at birth are maintained at check by a CD25 expressing cellular subset. As CD25 is expressed by a large subset of Treg but also by recently activated T cells and some B cells, we next analyzed animals genetically engineered to express the diphtheria toxin (DT) receptor specifically in $Foxp3^+$ cells (Foxp3-DTR / DEREG). Newborn-colonized WT and DEREG mice were treated with one injection of 1µg DT at 6 weeks of age. This treatment leads to a transient but substantial decrease in $Foxp3^+$ cell numbers in the mucosal compartment already at 2 days post-injection (**Fig. S3.7**), and caused a rapid appearance of anti-*H. hepaticus* IgA in feces of DEREG mice from 1 to 4 weeks post treatment, but not in WT littermate controls (**Fig. 3.4C**). Total fecal IgA was transiently increased in DEREG mice 1 week after treatment (**Fig. S3.6A**), and *H. hepaticus* load was slightly but significantly decreased in the mucosa of the ileum and colon of DEREG mice 9 weeks after the single treatment (**Fig. S3.6B**). Together these data indicate that primo-exposure during perinatal life leads to a very robust tolerogenic immune response, ensured by the recruitment of $Foxp3^+$ Treg that suppress, in an IL-10 dependent manner, otherwise fully competent effectors cells. Indeed, $Foxp3^+$ cell frequency was significantly increased in the colonic Lamina Propria of Nbcol mice, compared to SPF and Adcol mice, all analyzed at adult age (**Fig. 3.4D**). Finally, we tested if TLR2 was involved in the *H. hepaticus* induced tolerance, as it has been shown to be involved in

microbial induced Tregs in the gut (Round et al., 2011). TLR2$^{-/-}$ mice Newborn-colonized with *H. hepaticus* did not mount a detectable anti-*H. hepaticus* IgA response when adults, indicating *H. hepaticus* promotes Treg in a TLR2 independent manner (**Fig. S3.8**). Taken together, these results establish that *H. hepaticus* immune evasion relies on the induction of Treg, a mechanism robustly favored by the newborn physiological state and self-perpetuated along host maturation, beyond reproductive age.

Tolerance to *H. hepaticus* does not affect the health of the host

Because *H. hepaticus* was found in higher frequency in the colon of Nbcol mice (**Fig. 3.1B**), which does not seem to mount a response to this bacteria, we checked if there was indication of *H. hepaticus* translocation to host tissues, analyzing the number of *H. hepaticus* 16S copies recovered in the Mesenteric Lymph nodes (MLN) and gallbladder DNA extracts from Adcol and Nbcol mice. This analysis indicated that *H. hepaticus* seems to translocate at a similar frequency to the MLN of Adcol and Nbcol mice, but *H. hepaticus* DNA was found more frequently in the gallbladder of Nbcol mice (**Fig. 3.5A**). Additional analysis revealed high titers of anti-*H. hepaticus* IgM, IgG and IgA in the serum of Adcol mice, with increased levels of total IgM and IgG (**Fig. 3.5B** and **C**). In contrast, Nbcol mice had levels of specific IgM similar to those of naïve mice (SPF), which suggest these were not induced but rather natural IgM (**Fig. 3.5B**). Anti-*H. hepaticus* IgA was not detected in the serum of Nbcol mice, as wells as in naïve mice, in accordance to what was found for IgA in the gut (**Fig. 3.1E**). Anti-*H.* hepaticus IgG was found in the serum of Nbcol mice, albeit at levels more than 10 fold lower than those of Adcol mice. Collectively, these results suggest that perinatal colonization with *H. hepaticus* induces not only mucosal but also systemic tolerance. Nevertheless, despite increased *H. hepaticus* accumulation, Nbcol mice did not present a serological state indicative of pathology, with normal levels of AST and ALT (**Fig. 3.5D**),

similar to Adcol and SPF mice, with the same being true for other serological parameters (**Fig. S3.9A**). Likewise, fecal values of Lipocalin-2, which are increased in the face of gut pathology, were normal in Nbcol mice, similarly to SPF and Adcol mice (**Fig. 3.5E**). Also, induction of colitis using DSS showed not difference between these groups in susceptibility to gut inflammation (**Fig. 3.5F** and **Fig. S3.9B**). Taken together, these studies allow us to conclude that tolerance to *H. hepaticus* does not affect negatively the host.

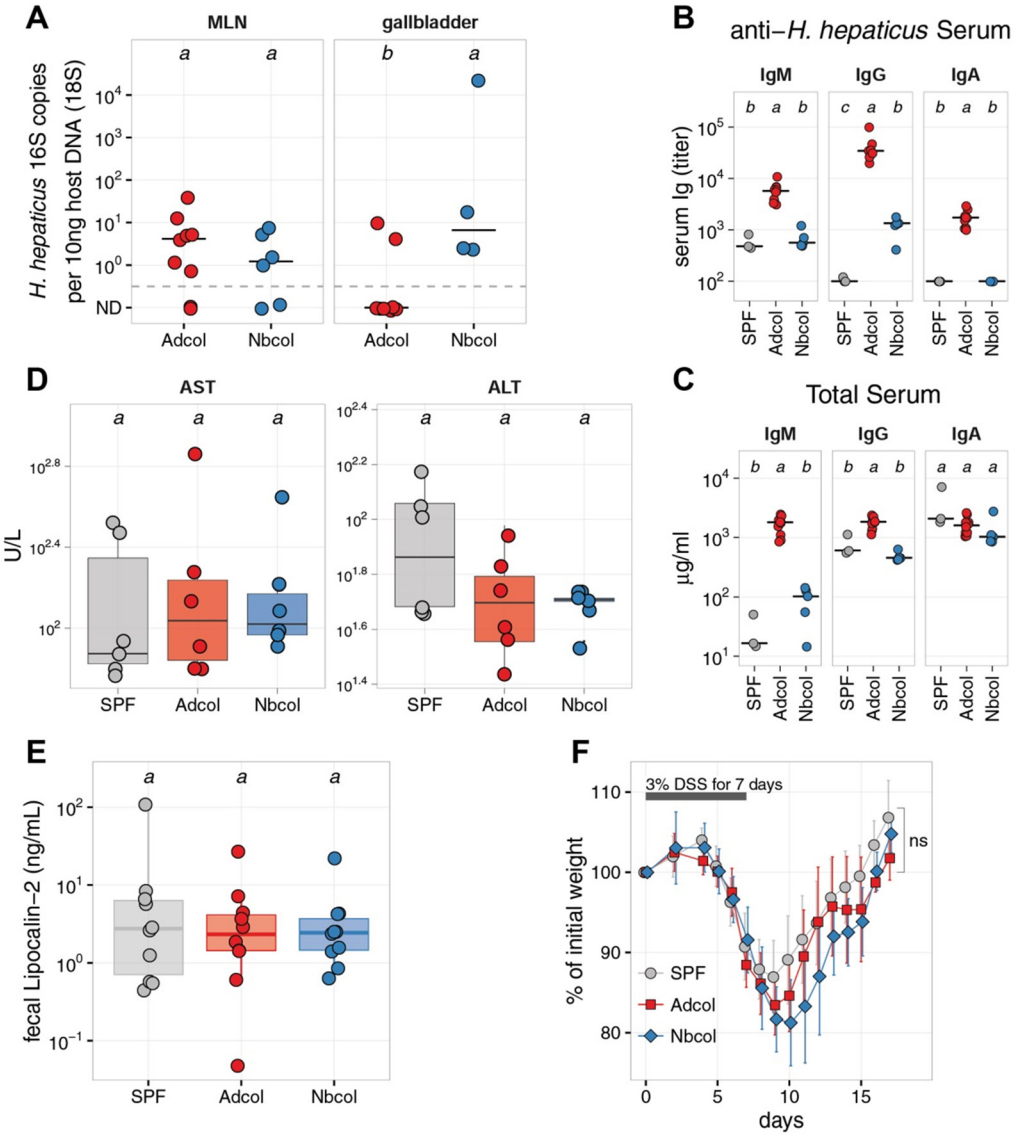

Figure 3.5 – Tolerance to *H. hepaticus* does not affect the health of the host

Figure 3.5 – Tolerance to *H. hepaticus* does not affect the health of the host

A) Estimated *H. hepaticus* 16S copies per 10ng of host DNA extracted from Mesenteric Lymph Nodes (MLN) or gallbladder of Adult WT mice, Adult-colonized (Adcol) or Newborn-colonized (Nbcol), colonized for more than 12 weeks. Host DNA amount was ascertained using 18S specific primers. N respectively for Adcol and Nbcol: MLN = 9, 6; gallbladder = 8, 4. **B** - **C**) Adult-colonized (Adcol), Newborn-colonized (Nbcol) and SPF B6 mice were analyzed for anti-*H. hepaticus* and total serum IgM, IgG and IgA by ELISA. SPF: n = 3, 14 weeks old. Adcol: n = 10, 16 weeks post-colonization. Nbcol: n = 5, 8 weeks old/colonized. **D**) Serologic analysis on SPF, Adcol and Nbcol mice. SPF: n = 6, 11 weeks old. Adcol: n = 6, 12 weeks colonized, colonization at 10 weeks of age. Nbcol: n = 6, 11 weeks old/colonized. Groups with the same letter are not significantly different at p = 0.05 (Wilcoxon test with BH correction). **E**) Lipocalin-2 concentration was measured by ELISA on the feces of SPF, Adcol and Nbcol mice. SPF: n = 10, 12 weeks old. Adcol: n = 10, 14 weeks old, 4 weeks colonized. Nbcol: n = 10, 12 weeks old/colonized. **F**) The same mice shown in **E** were treated for 7 days with 3% DSS in drinking water. Weight was scored for 17 days. ns = non-significant (linear mixed effects analysis).

Perinatal transmission of *H. hepaticus* shapes the microbiota of the host in immuno-dependent and independent ways

We next evaluated if *H. hepaticus* would alter the microbiota of the host, and if the adaptive immunity and the age at primo-exposure would modify this effect. Amplification of 16S rRNA variable region 4 was carried out on DNA extracted from feces of SPF, Adcol and Nbcol mice, either WT or Rag2$^{-/-}$. Analysis of species richness (**Fig. S13B**) and diversity (**Fig. S13C**) showed no major differences between the groups analyzed, however analysis of microbiota composition on those mice showed that *H. hepaticus* does affect the microbiota of the host, since colonized mice have a different community structure compared to SPF mice, regardless of the genotype (**Fig. 3.6A**). These alterations were not enough to fill the gap between WT and Rag2$^{-/-}$ though, as mice of these two genotypes remained well separated in the first component of the PCoA plot (**Fig. 3.6A**). Nonetheless, the age of colonization did not affect how *H. hepaticus* shaped the microbiota in Rag2$^{-/-}$ animals, while WT Adcol and Nbcol mice had community structures very well separated in the second component of the PCoA (**Fig. 3.6A**). These results suggest that the age at primo-exposure affects how *H. hepaticus* shape the host microbiota, but this phenomenon is dependent on the adaptive immunity.

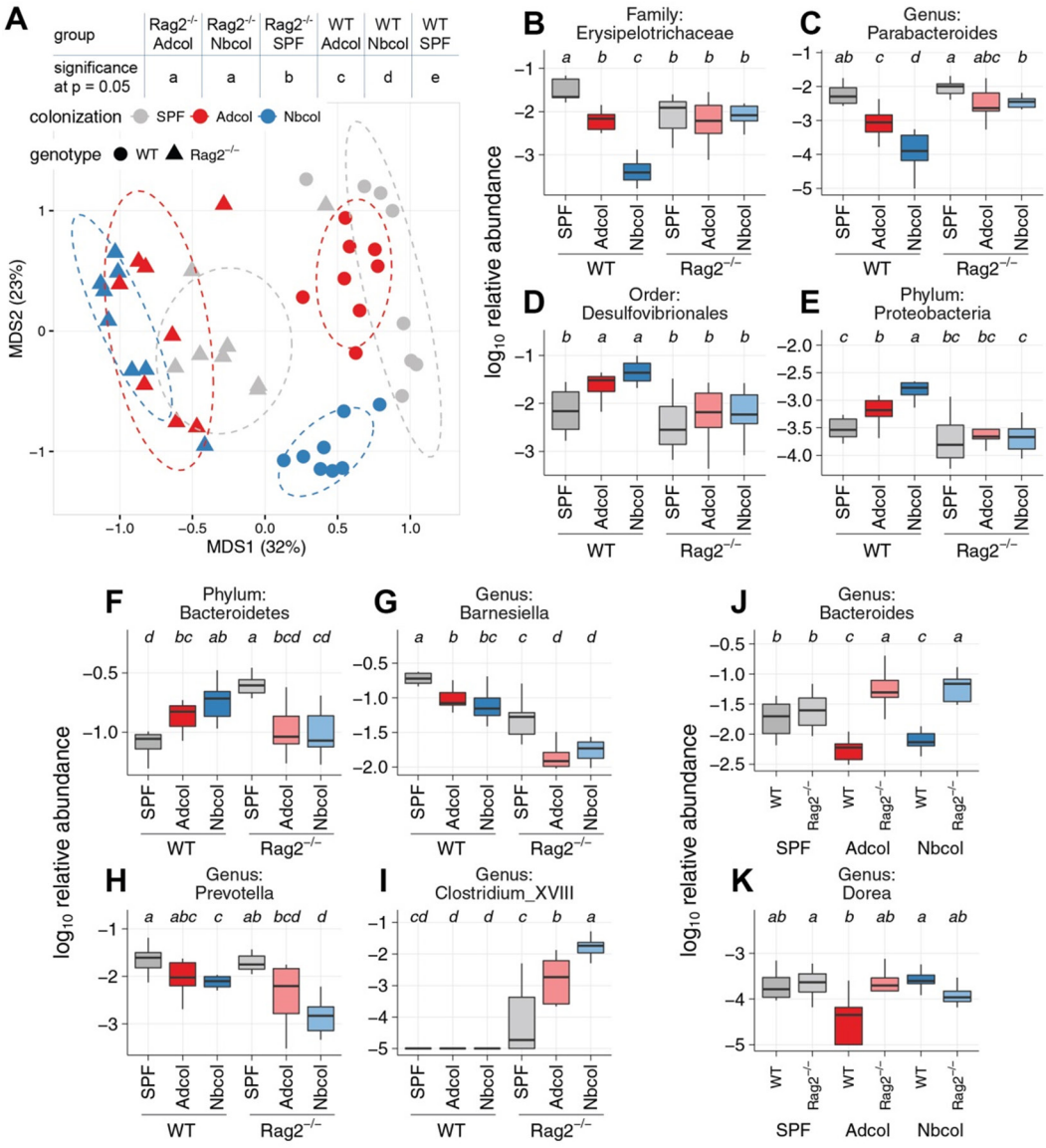

Figure 3.6 - Perinatal transmission of *H. hepaticus* shapes the microbiota of the host in immuno-dependent and independent ways

16S rRNA variable region 4 (V4) analysis on fecal DNA from WT and Rag2$^{-/-}$ animals, either SPF, Adcol or Nbcol, 4 Males and 4 Females per group. SPF and Nbcol mice were 8-12 weeks old; Adcol mice were colonized at 10 weeks of age and analyzed at 10 weeks post-colonization. **A**) Principal Coordinates Analysis (PCoA) on dissimilarity matrix using Yue & Clayton index. Statistical analysis was performed using AMOVA. **B** – **K**) Relative abundance of different taxa between the groups analyzed. Lowest classification level achieved in the analysis shown above the graph. Statistical analysis was performed using Wilcoxon test, with Benjamini and Hochberg correction. Groups with the same letter are not significantly different at p = 0.05.

Analysis of frequency of OTUs grouped by taxa revealed many examples where the adaptive immunity and age at primo-exposure modified the way *H. hepaticus* shaped the host microbiota. Bacteria of the Family Erysipelotrichaceae and also *Parabacteroides* were dramatically decreased in frequency by Newborn colonization with *H. hepaticus*, but only in WT mice (**Fig. 3.6B** and **C**), while Desulfovibronales and an unclassified Proteobacteria were increased by colonization in WT but not in Rag2$^{-/-}$ (**Fig. 3.6D** and **E**), indicating that changes in the adaptive immunity provoked by the colonization with *H. hepaticus* could favor some groups of bacteria while negatively impacting others. An unclassified Bacteroidetes and *Odoribacter* were increased in frequency in Nbcol WT but the opposite happened in Nbcol Rag2$^{-/-}$ (**Fig. 3.6F** and **Fig. S3.12M**), suggesting that newborn-colonization could create some favoring conditions for some groups of bacteria in an immune dependent way, but these could turn into disadvantages for the same groups in absence of adaptive immunity. *Barnesiella* and *Prevotella* were decreased in Nbcol WT and Rag2$^{-/-}$ (**Fig. 3.6G** and **H**), while *Clostridium* of the group XVIII were only present in the Rag2$^{-/-}$ mice, and dramatically increased with newborn colonization (**Fig. 3.6I**), suggesting that these groups are affected by *H. hepaticus* in an adaptive immunity independent way. *Bacteroides* had the same frequency in SPF WT and Rag$^{-/-}$, but *H. hepaticus* colonization created opposite effects on these genotypes, leading to decrease of this bacteria in WT but increase in Rag2$^{-/-}$ (**Fig. 3.6J**). *Dorea* was only affected, with decreasing in frequency, in WT Adcol mice (**Fig. 3.6K**), which suggests an adaptive immunity dependent effect that is inhibited by newborn colonization. Taken together, these analyses illustrate how the adaptive immunity can affect the way the microbiota is shaped, and demonstrate that the age at primo-exposure to *H. hepaticus* change the way this bacteria modifies the bacterial composition of the host's gut in an immune dependent and independent way.

Discussion

The control of intestinal bacteria is important to maintain homeostasis in the gut, and secretory IgA is an important component of this control (Macpherson et al., 2012; Pabst, 2012). Absence of IgA results in expansion of dangerous groups of microbes, resulting in sustained inflammation in the gut (Mirpuri et al., 2013), and also permits the expansion of Segmented Filamentous Bacteria (SFB) in the small intestine, showing that this antibody is a significant pressure on the intestinal levels of these bacteria (Jiang et al., 2001; Suzuki et al., 2004). Hence, it is striking to find no specific IgA in the gut of mice Newborn-colonized with *H. hepaticus* (**Fig. 3.1D**), even though they have contact with these bacteria all their life, mainly because Adult-colonization results in high levels of specific IgA secretion in mice (**Fig. 3.1C**). This indicates an absence of immune response to *H. hepaticus* in Nbcol mice, which has a direct impact in the load of these bacteria, permitting its expansion in the mucosa of ileum and colon (**Fig. 3.1B**).

H. hepaticus DNA was also found in the MLN of Adcol and Nbcol mice, and was found more frequently in the gallbladder of Nbcol mice, suggesting accumulation of these bacteria (**Fig. 3.5A**). Furthermore, high levels of anti-*H. hepaticus* antibodies were found in the serum of Adcol mice, which supports the idea that these bacteria cross the intestinal barrier (**Fig. 3.5B**). *H. hepaticus* was first isolated from the liver of A/J mice, where it causes a persistent T-cell mediated inflammatory reaction that ultimately leads to liver cancer (Whary et al., 1998). A/J mice are deficient in the C5 component of complement (Rhodes et al., 1980), which raises the possibility that antibody-mediated responses could have a role in preventing an exacerbated immune response to these bacteria in the liver. We found very low levels of anti-*H. hepaticus* antibodies in the serum of

Nbcol mice, but these mice had no evident signs of liver or other pathology (**Fig. 3.5D** and **Fig. S3.9A**). These data suggest that the tolerance to *H. hepaticus* happens also in a systemic level, preventing exacerbated T-cell reactions inside the body.

The capacity of the mice to become tolerant to *H. hepaticus* decreases with age, as colonization from 3 weeks onwards results in specific IgA production in adulthood (**Fig. 3.2C** and **D**). This could be explained by reports that neonatal T cells are more prone to become Tregs, a feature that decreases after 2 weeks of age (Wang et al., 2010). Furthermore, Recent Thymic Emigrants (RTEs), which are the preferential precursors of Tregs differentiated in the periphery (Paiva et al., 2013), are increased in the lymphoid organs of neonates and decrease progressively with age (Hale et al., 2006). Therefore, *H. hepaticus* seems to explore an intrinsic propensity of neonatal mice to become tolerant, inducing early high levels of Tregs that will later suppress responses to these bacteria. Likewise, it was found that lung microbiota induce Tregs in the first 2 weeks of age in mice, with implications for allergen tolerance (Gollwitzer et al., 2014), that Tregs specific for microbial antigens can be induced in the skin by bacterial colonization in 7 days old mice (Scharschmidt et al., 2015), and that *Helicobacter pylori* colonization in newborns results in absence of gastric inflammation later in life (Arnold et al., 2011).

Despite the particular propensities for tolerance generation in the neonate system, not all perinatal gut bacteria colonization results in tolerance. For example, mice develop a mucosal IgA response to SFB even when colonized perinatally with it (Jiang et al., 2001), and newborn mice develop strong inflammatory responses when infected with *Yersinia enterocolitica* (Siefker et al., 2014). A great proportion of bacteria are found coated with IgA in the gut of adult mice, in T-cell dependent and independent manners (Bunker et al., 2015), which suggests that the

generation of antigen-specific Tregs and suppression of IgA responses in the gut are particular features of a select group of microbes. Nevertheless, the role of Tregs on the IgA generation in the gut remains controversial. Some reports indicate that Tregs promote the generation of Intestinal IgA (Cong et al., 2009; Kawamoto et al., 2014; Tsuji et al., 2009), while others argue that Tregs prevent germinal center formation (Chung et al., 2011; Linterman et al., 2011). As some studies have identified T-cell dependent and independent IgA responses in the gut, with proposed differences in the role of the IgA generated in each, the Treg role as promoter or suppressor of gut IgA could as well be differentiated by the type of IgA generated. As T-cell dependent IgA would require differentiated, antigen-specific T cells, most likely Th17 (Hirota et al., 2013), Tregs could act as suppressors of antigen-specific IgA by suppressing the specific T-cell response. In our model, anti-*H. hepaticus* fecal IgA responses were T cell dependent (**Fig. S3.10**), which supports the idea that Tregs could prevent these responses upstream of germinal center reactions.

We found that the natural method of colonization by *H. hepaticus* in the mice, from mother to newborns, results in the generation of Tregs, that suppress the immune responses to this bacteria. This results in an absence of specific IgA, and also allows this bacteria to reach higher levels in the intestinal mucosa, hence ensuring higher probability of dissemination and survival of this microbe. This mechanism would explain the high incidence of *H. hepaticus* infection in animal facilities and in the wild, where the *Helicobacter* genus is highly prevalent (Wasimuddin et al., 2012). A parallel model was also proposed for *H. pylori*, which was until recently highly prevalent in human populations (Blaser et al., 2008). *H. pylori* infection would be harmless, or even beneficial, when occurring very early in the development, but harmful when happening at later stages, for the most

problems associated with this infection come from the uncontrolled immune response to the bacteria (Arnold et al., 2011).

In the understanding of how the mucosal immune system works, one should take into account how this system evolved and was shaped by the microbial world interacting with it. As studies with wild mice suggest, *Helicobacter* spp. are part of the indigenous flora of the mice, which probably co-evolved with this organism. As it happens, due to problems with immunodeficient mice these bacteria were purged from the microbiota of experimental animal facilities, which could have a great effect into all evaluations of the mechanisms existing in the gut to control and benefit from bacteria. Indeed, it was found that free-living mice have a thicker, better developed mucus layer in the colon, with a better separation of bacteria from the epithelium (Jakobsson et al., 2015). Furthermore, we observed that perinatal colonization with *H. hepaticus* shapes the microbiota of the mice in a particular way, which could even be more favorable to the host. For example, we found that, in WT mice, *H. hepaticus* perinatal colonization promotes a dramatic decrease in the frequency of the family Erysipelotrichaceae (**Fig. 3.6B**), which was found in higher frequencies in mice with a poor metabolic profile in the intestine (Fleissner et al., 2010; Turnbaugh et al., 2009). Additionally, we found that perinatally colonized mice had increased levels of Tregs in the large intestine Lamina Propria (**Fig. 3.4D**), which also points to a benefit for the host. Therefore, perinatal colonization with *H. hepaticus* guarantees an early education of the immune system through generation of Tregs and ensures a life-long symbiosis between this two species. In this light, a more comprehensive analysis of the indigenous microbiota of mice and humans may help to identify other groups of bacteria with similar properties and the capacity to maintain and improve the robustness of homeostasis in the gut.

Materials and Methods

Mice

Wild type (WT), Rag2$^{-/-}$, IL10$^{-/-}$, TLR2$^{-/-}$, TCRβ$^{-/-}$ and DEREG (Lahl et al., 2007) mice were all in C57BL/6 background, bread in our specific pathogen free (SPF) facility, which excludes *Helicobacter hepaticus*. All experiments involving *H. hepaticus* colonization were performed in a dedicated room. Germfree (GF) C57BL/6 mice were raised in the IGC gnotobiology facility in axenic isolators (La Calhene/ORM). Adults GF mice were transferred to sterile ISOcages (Tecniplast) for use in experiments, and their microbiological status was confirmed multiple times along the study.

Helicobacter hepaticus culture and mice colonization

Helicobacter hepaticus reference strain (CIP 104102) was obtained from the Biological resources center of the Pasteur Institute. Growth was performed in 10% horse blood-agar (HBA, Oxoid) plates, supplemented with 12.5 mg/L vancomycin, 0.3 mg/L polymyxin B, 6.3 mg/L trimethoprim and 5.0 mg/L amphotericin B, at 37°C, under microaerophilic conditions (6% O_2, 7% CO_2, 3.5% H_2 and 83.5% N_2) generated by an Anoxomat system (MART Microbiology). Bacteria were harvested after 5 days of cultured in PBS solution and numbers were estimated by OD quantification. Mice were colonized by oral gavage with 100µL of bacteria suspension at 10^9 CFU/mL in PBS, using an animal feeding needle with silicone tip, size 20G x 1.5'' (Cadence Science). Colonization was confirmed by PCR on fecal DNA using *H. hepaticus* 16S specific primers: fwd: GCATTTGAAACTGTTACTCTG, rev: CTGTTTTCAAGCTCCCCGAAG, 417bp product (Petnicki-Ocwieja et al., 2009). Fecal DNA was prepared either with NZYtech tissue gDNA kit (NZYtech), with a pre-step of 95°C,

using 5ng for reactions, or by a simple boiling method, as described elsewhere (Truett et al., 2000). Briefly, to one fecal pellet was added 700ul of a 25mM NaOH, 0.2mM EDTA solution with pH=12, and followed the procedure: 95°C, 3min, vortex, 95°C, 7min, vortex, centrifugation 16,000G 2min, take 50µl of supernatant and add to 50µl of 40mM Tris HCl solution, with pH=5. 2µl of this final sample was used in PCR reactions. PCR was performed in 25µl, with the final concentrations: 1.25mM $MgCl_2$, 0.2mM dNTP, 0.4µM of each primer, 1.5U of GOTaq and 2µl of template (Promega). Reactions were performed on a MyCycler (BioRad) with the following conditions: 95°C 5min, followed by 36 cycles of 95°C - 30s, 60°C - 45s, 72°C - 1min, with a final extension of 72°C - 10min. Products were visualized on a 2% agarose gel (Lonza).

To reproduce colonization through coprophagy, mice were colonized using a preparation of *H. hepaticus* positive feces, from mice previously colonized with cultured *H. hepaticus*. Fecal pellets were homogenized in PBS at a ratio of 1 pellet to 400µL (~ 100mg/mL) and filtered in 100µm mesh. Each adult mouse received 100µL of this fecal preparation by oral gavage. 1, 2 and 3 weeks old mice were colonized with 20ul of fecal suspension using a 24G x 25mm long feeding needle (Fine Science Tools), bent ~30° to facilitate insertion into the oral cavity (Butchbach et al., 2007). Adult GF were colonized with cultured *H. hepaticus* by oral gavage, and confirmed positive using PCR on fecal DNA. Monocolonized mice were bred in ISOcages to produce pups newborn-monocolonized with *H. hepaticus*, which were kept in ISOcages after weaning until 9 weeks of age.

Helicobacter hepaticus quantification

The following primers were used in qPCR reactions: *H. hepaticus* 16S fwd: GCATTTGAAACTGTTACTCTG, *H. hepaticus* 16S rev: CTGTTTTCAAGCTCCCCGAAG, 417bp product (Petnicki-Ocwieja et al.,

2009); 18S fwd: CATTCGAACGTCTGCCCTAT, 18S rev: CCTGCTGCCTTCCTTGGA, 137bp product (Vaishnava et al., 2011). *H. hepaticus* (Hh) 16S copy number was estimated using plasmid standard curve, and host DNA amount in ng was estimated using a standard curve constructed with intestinal tissue DNA, measured using Qubit dsDNA Assay (Molecular Probes). All reactions contained standard curves for absolute quantification. *H. hepaticus* amount is expressed as $\log_{10}$ of the ratio Hh 16S copies/ ng of host DNA, unless otherwise stated. Intestinal mucosa, MLN and gallbladder DNA was extracted using NZYTech Tissue gDNA extraction kit (NZYTech), with an initial step at 95°C. For quantification, 10ng of DNA was used. Primers were used at 0.5μM in the reaction, containing 5μL of iTaq Universal SYBR Green Supermix (Bio-Rad), 2μL of sample and water to a final volume of 10μL. qPCR conditions: 95°C - 3min; 45 cycles of 95°C - 30s, 60°C - 30s, 72°C - 36s; followed by a melting curve of 65 - 95°C with 0.5°C increase every 5s. All reactions were prepared in 384 well hard shell plates (Bio-Rad), on ice, in less than one hour, to minimize primer dimer formation, and run immediately after preparation on a CFX384 real-time PCR detection system (Bio-Rad).

Total and specific Immunoglobulin quantification by ELISA

To quantify total and *H. hepaticus* specific fecal antibodies, fecal extracts were prepared from freshly collected or -80°C stored feces. Fecal pellets of ~50mg were collected from each mice (average 56.6mg ± 15.4, measured from 100 samples), and homogenized in 500μL of PBS containing a protease inhibitor cocktail (P8340, Sigma-Aldrich), for a solution of about 100mg/mL. Homogenates were centrifuged at 16,000G for 10min at room temperature, and supernatants were stored at -20°C until further use.

Soluble *H. hepaticus* antigens (SHelAg) were prepared as previously described (Kullberg et al., 1998). After cultured for 5 days in blood-agar plates, bacteria were harvested in PBS solution, washed and lysed in a French Press. The solution was centrifuged at 8,000G for 30 min at 4°C, the supernatant was filtered sterile with a 0.22µm pore size filter and protein content was determined using BCA protein assay. The preparation was stored at -80°C.

ELISA was performed using high binding 384 well ELISA plates (UltraCruz). For total Ig quantification, plates were coated with Goat anti-Mouse IgM, IgG or IgA polyclonal antibodies (Southern Biotech), and for anti-*H. hepaticus* Ig quantification, coating was done using 10µg/mL of SHelAg. Plates were coated overnight at 4°C, washed in PBS 0.05% Tween20 and blocked with 2% BSA solution in PBS. Fecal extracts were added undiluted (specific Ig) or 10 fold diluted (total Ig) to plates and diluted 7 times, 3 fold each time. Serum samples were assayed starting from a dilution of 1:200. For specific fecal IgA quantification, a reference sample pooled from 5 adult colonized B6 was used in all assays. For specific serum IgM, IgG and IgA, a reference serum sample of colonized IL-10$^{-/-}$ diluted 1:210 was used in all assays. After overnight incubation at 4°C, plates were washed in PBS 0.05% Tween20 and incubated with Goat anti-Mouse IgM, IgG or IgA conjugated with HRP. Reactions were revealed with TMB (BD) and stopped with 0.1M H_2SO_4, and read at 450nm in a Multiskan GO Microplate Spectrophotometer (Thermo Scientific). Titers were calculated as the dilution at 10% of the maximum signal (normalized to a reference in all samples), using interpolation from a 4-parameter logistic regression fitting.

Induction of acute colitis with DSS

Mice were treated with Dextran Sulfate Sodium (DSS) (36,000-50,000 Da, MP Biomedicals), diluted in drinking water, for 7 days. DSS solution was changed for a new one every 2 days. Weight was scored for 18 days.

Serological Analysis

Mice were euthanized with CO_2 and blood was collected from the infrahepatic vena cava into heparine-coated collection tubes. Assays were performed on plasma, on the same day of extraction. Serological analyses were performed on a Siemens Advia 1200 Chemistry Analyzer by DNAtech, Portugal, on the following parameters: Total, Direct and Indirect Bilirubin, Alanine transaminase (ALT), Aspartate transaminase (AST), Alcaline Phosphatase, Lactic Dehydrogenase (LDH), Total Protein and Albumin.

Fecal Lipocalin-2 quantification

Quantification of fecal Lipocalin-2 was performed on fecal extracts, obtained as described above, using the kit manufacturer instructions (Mouse Lipocalin-2/NGAL DuoSet ELISA, catalog number: DY1857, R&D).

Drug Treatments

Mice were treated with anti-CD25 (PC61 clone, IGC's Antibody Facility) with one injection of a 500µg dose intraperitoneally (i.p.); with anti-IL-10R (1B1.2 clone, IGC's Antibody Facility) with 4 injections, weekly, of 1mg i.p.; and with Diphtheria Toxin from *Corynebacterium diphtheriae* (Sigma) with one injection of a 1µg dose i.p..

Lamina Propria Lymphocytes Isolation

Lymphocyte isolation from Lamina Propria of the large intestine was performed by extracting the intestines, cutting in 6 cm pieces, opening longitudinally and washing vigorously in PBS in a petri dish. Pieces were then cut into 1cm fragments, collected in 20ml of PBS with 4% Fetal Bovine Serum (FBS, Gibco) and EDTA (Gibco) was added to a final concentration of 5mM. After 15min of shaking at 2000rpm, 37°C in an orbital shaker, and a final vortex for 5s, pieces were collected in steel mesh and this EDTA procedure was repeated one more time. Pieces were then collected into a 2ml eppendorf tube with 1ml HBSS 4% FBS and minced with scissors. Fragments were then collected into a 20ml solution of HBSS with 4% FBS and 1mg/mL Colagenase Tye VIII and 0.1mg/mL DNAse I (Sigma) and digested for 30min at 2000rpm, 37°C in an orbital shaker. The solution was filtered with a 70µm cell strainer (BD), centrifuged at 450G, 5min at 4°C, resuspended in 8mL of a 40% Percoll (Sigma) solution in HBSS (Percoll previously corrected to 310mOsm/kg, pH 7) and layered onto 4mL of a 80% Percoll solution. After centrifugation at 620G, 20min, 20°C, with no brake or acceleration, cells were collected from the interface of the two Percoll layers and washed once before use for FACS staining. Cells were pre-incubated with Fc-block (anti-CD16/CD32, IGC-AbService), and then incubated with anti-mouse TCRβ (H57-597, eBioscience), CD4 (GK1.5, eBioscience), CD8 (53-6.7, eBioscience), CD25 (PC61, IGC-AbService), Foxp3 (FJK-16S, eBioscience). Events were acquired on an LSRFortessa X-20 cell analyzer (BD) and analyzed on FlowJo Software (Tree Star).

IgA FACS on live *H. hepaticus*

H. hepaticus was cultured as described above, and 10^6 CFU were used per assay. Bacteria were washed once in PBS, pelleted (12,000G, 5min), resuspended in fecal extract (prepared as described for ELISA

assay) from SPF, Adcol or Nbcol mice and incubated on ice for 30min. Bacteria were then washed in PBS and incubated for 15min on ice with Goat anti-Mouse IgA (Southern Biotech), labeled using Alexa647 Labeling Kit (Thermo Fisher Scientific). Bacteria were washed again and resuspended in a PBS solution containing 5μM of SYTO 9 (Molecular Probes). Events were acquired on a CyAn ADP Analyzer (Beckman Coulter) and analyzed on FlowJo Software (Tree Star). Analysis was performed gating on live bacteria, which were SYTO 9 Bright, as confirmed by counterstaining with 45μM of propidium iodide (Molecular Probes), and excluding events with high pulse width.

16S rRNA analysis

48 samples were analyzed, from WT and Rag2$^{-/-}$ animals, either SPF, Adcol or Nbcol, 4 Males and 4 Females per group. SPF and Nbcol mice were 8-12 weeks old; Adcol mice were colonized at 10 weeks of age and analyzed at 10 weeks post-colonization. DNA from fecal pellets was extracted using NZYTech Tissue gDNA extraction kit (NZYTech), with a pre-step of 95°C, and used for 16S rRNA gene sequencing as previously described (Walters et al., 2016). Sample processing and sequencing were performed by the IGC Genomics Unit. The 515f-806r bacterial/archaeal primer pair was used, which targets the 16S rRNA gene variable region 4 (V4), with the following sequences: 515f - GTGYCAGCMGCCGCGGTAA; 806r – GGACTACNVGGGTWTCTAAT; bar codes were added in the 5' end of the 515f primer, and Illumina adapters were present in both primers. Sample processing and PCRs were performed as described in the Earth Microbiome Project (EMP; http://www.earthmicrobiome.org/emp-standard-protocols/16s/, 16S rRNA Amplification Protocol version 6_15). Pair-end sequencing was performed on an Illumina MiSeq Benchtop Sequencer, following manufacturer's instructions. Reads were processed and analyzed using Mothur Software version 1.36.1 (Schloss et al., 2009), following the

MiSeq SOP (Kozich et al., 2013) on the Mothur wiki (http://www.mothur.org/wiki/MiSeq_SOP, accessed on May 2016), using SILVA database release 102 for alignment (Pruesse et al., 2007). Samples had an average of 55,789 high quality bacteria sequences (min: 32,681, max: 81,489, SD: 10,707), which were clustered into 17,903 OTUs at 97% similarity, classified to 203 unique taxa, 142 classified until Genus, 35 until Family, 14 until Order, 8 until Class, 3 only to Phylum and 1 only as Bacteria. OTU based analysis of alpha and beta diversity were performed on Mothur and R software, with Shannon index used to estimate diversity (Shannon, 1948), Chao index used to estimate richness (Chao, 1984), and dissimilarity between the structures of two communities measured using Yue & Clayton index (Yue and Clayton, 2005), which was used to build the PCoA plot. For taxa based analysis, OTUs were clustered into taxa using the 'classify.otu' command in Mothur (using 'basis = sequence'), each were filtered to have a minimum of 5 sequences in at least one sample, samples with less than 2 sequences of a given taxa were considered to have 0 sequences, and sequence numbers were then converted to frequencies (relative abundance). Taxa that passed the filtering were then tested between groups with Kruskal-Wallis test, p-values were adjusted using the Benjamini and Hochberg correction, and taxa with a p-value lower than 0.05 were selected for multiple comparison. Pairwise Mann–Whitney–Wilcoxon test, with BH correction, was then performed in the selected taxa. For plotting, log10 transformation was applied on the frequencies, with zeros given a log value of -5.

Data analysis

Data and statistical analysis were performed using R software version 3.2.5 (R Core Team, 2014). Multiple comparisons were done using Kruskal-Wallis and Mann–Whitney–Wilcoxon test with the Benjamini and Hochberg p value correction. Multiple comparisons of linear regressions were

performed using linear model and the function glht (General linear hypotheses and multiple comparisons), using single-step procedure for p value correction, in packaged multcomp (Hothorn et al., 2008). Graphs were made in R software using the package ggplot2 (Wickham, 2009).

Supplementary Material for Chapter 3

Colonization	total IgM µg/mL	anti-Hh IgM	Total IgG µg/mL	anti-Hh IgG
SPF	ND	ND	0.03 ± 0.52 [b]	ND
Adcol	ND	ND	0.54 ± 0.41 [a]	ND
Nbcol	ND	ND	0.06 ± 0.23 [b]	ND

groups with the same letter are not different at p=0.05 (pairwise t test); ND = not detected

Table S3.1 – IgM and IgG analysis by ELISA on fecal extracts

Analysis of IgM and IgG total and specific to *H. hepaticus* by ELISA on fecal extracts from 12 weeks old mice, Adult colonized (Adcol, 8 weeks at colonization, 4 weeks colonized), Newborn colonized (Nbcol) and not colonized (SPF).

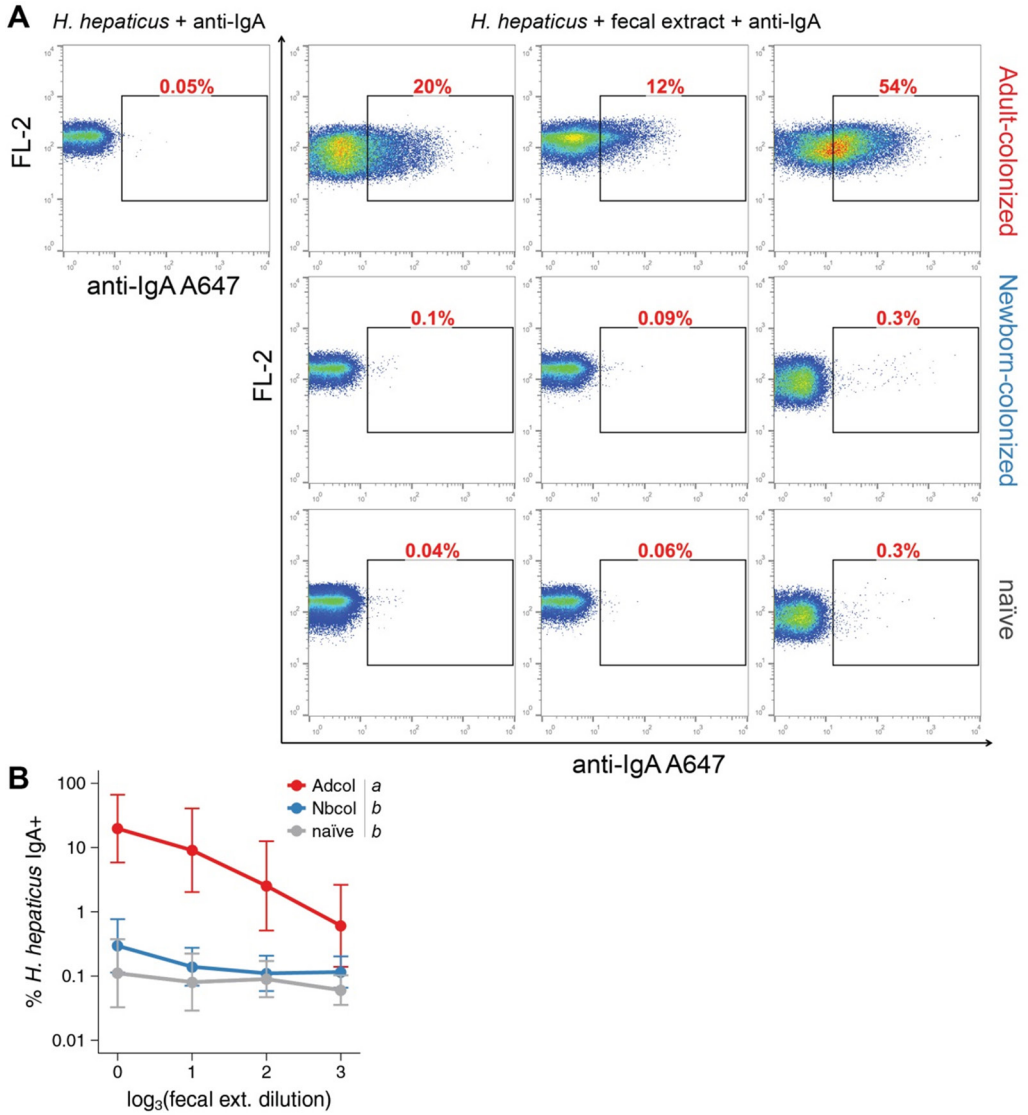

Figure S3.1 – fecal IgA from Adcol mice binds live *H. hepaticus*

A) Cultured *H. hepaticus* was pre-incubated with serial dilutions of fecal extract from naïve (SPF), Adult colonized (Adcol) and Newborn colonized (Nbcol) mice, washed and then stained with anti-mouse IgA-A647 antibody. Events shown were gated on live bacteria using SYTO-9, from the undiluted fecal extract samples. **B**) Percentage of *H. hepaticus* coated with mouse IgA when incubated with fecal extracts from the indicated mice. Lines connect means, error bar represent standard deviation. Groups with the same letter are not different at p = 0.05 (multiple comparison of linear regressions, using Tukey contrasts and BH p-value correction).

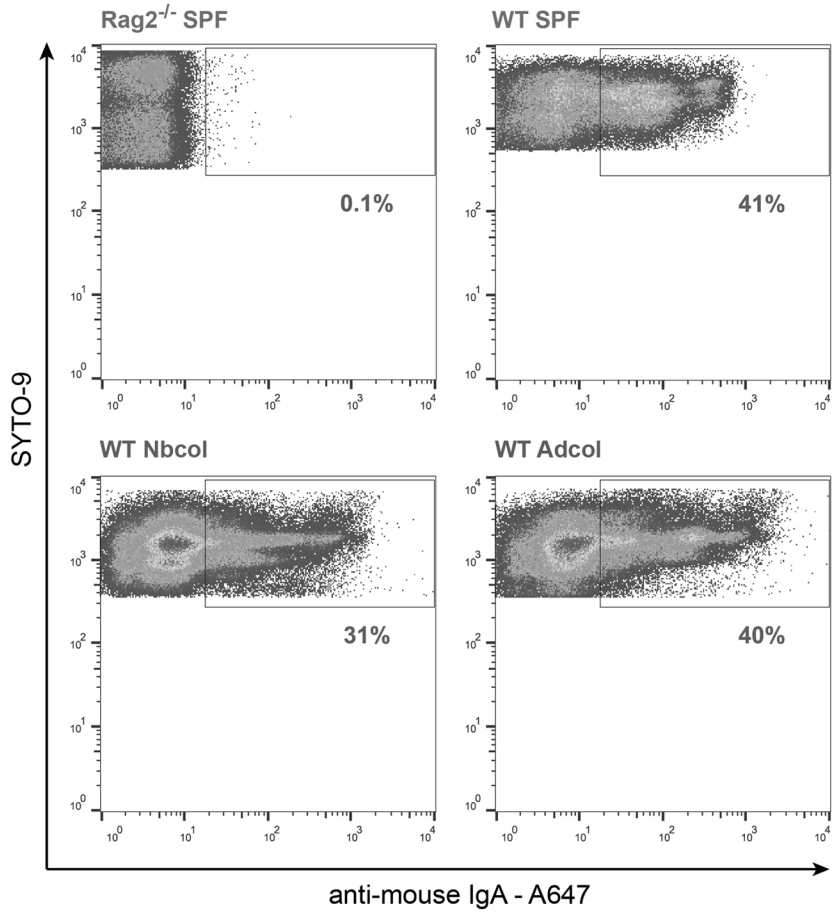

Figure S3.2 – IgA coated bacteria in the feces of SPF, Nbcol and Adcol mice

Feces from Rag2$^{-/-}$ SPF and WT SPF, Adult colonized (Adcol) and Newborn colonized (Nbcol) mice were collected in PBS, homogenized, centrifuged to remove large particles and stained with anti-mouse-IgA-A647 and SYTO-9. Events shown were gated in the live population using SYTO-9.

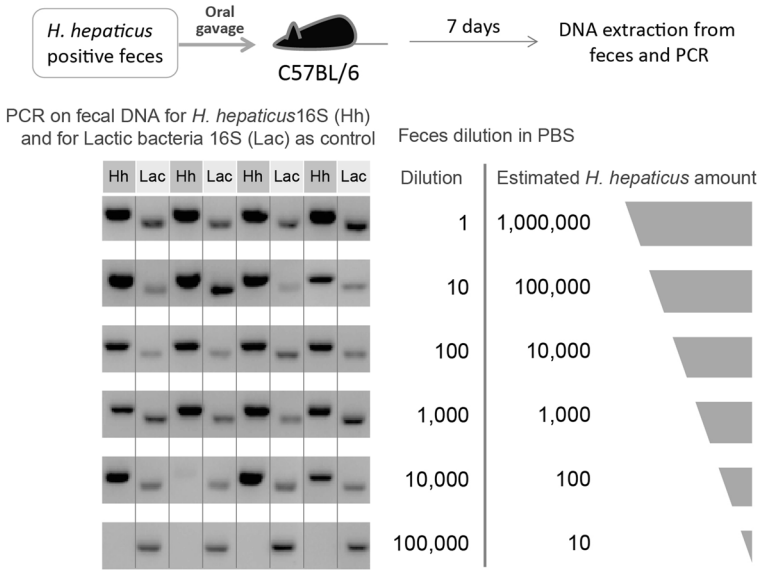

Figure S3.3 – Very low amount of *H. hepaticus* from feces is able to colonize SPF WT mice

Feces preparation from *H. hepaticus* positive mice (Adult colonized) were diluted from 1 to 100,000 times in PBS and used to colonize SPF WT mice. DNA was extracted from feces of those mice 7 days later and PCR using *H. hepaticus* 16S specific primers was performed, using Lactic bacteria 16S primers as positive control. Amount of *H. hepaticus* in feces preparation from Adult colonized mice was estimated by culture and qPCR in separate experiments, not from the same donor mice in this experiment.

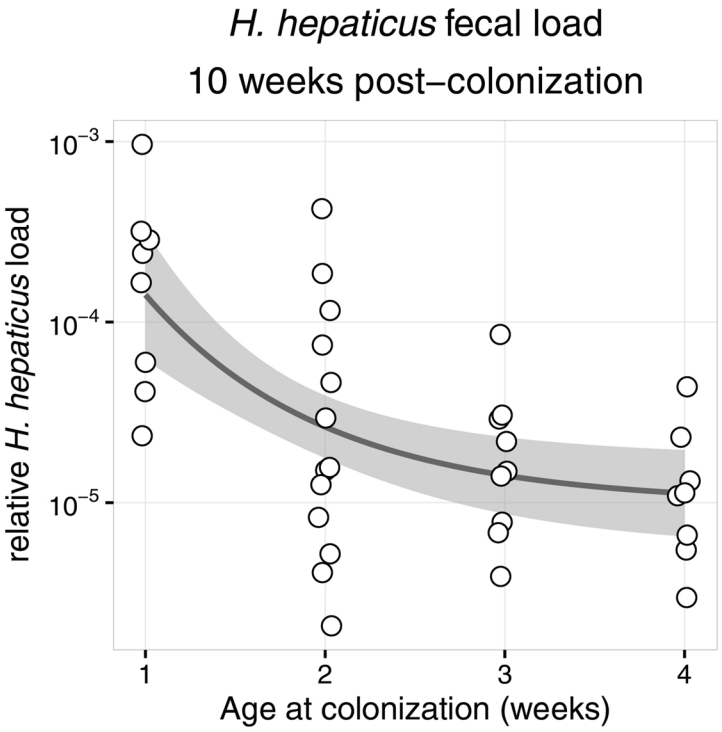

Figure S3.4 – *H. hepaticus* fecal load 10 weeks post-colonization (Figure 3.2 B - D)
Mice at 1-4 weeks of age were colonized with *H. hepaticus* by feces oral gavage. 10 weeks after colonization, feces were analyzed for anti-*H. hepaticus* and Total IgA by ELISA. N = 8, 5, 6 and 8 respectively. Linear model: y~exp(-x), line = regression, shade = confidence interval; regression coefficient: $p < 0.01$; $R^2 = 0.30$; F statistic: $p < 0.01$

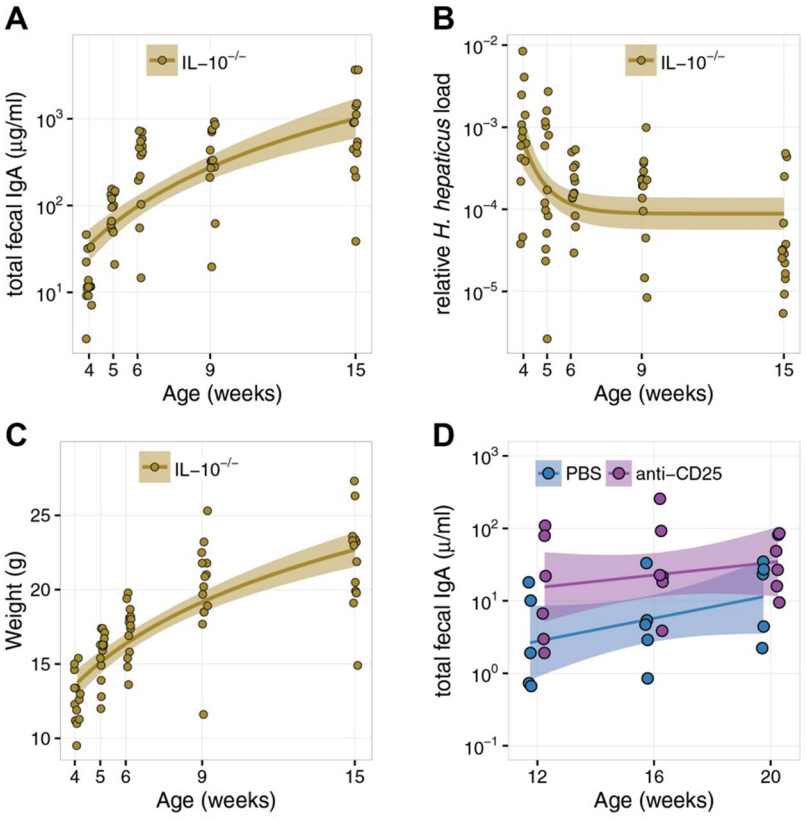

Figure S3.5 – Complementary to Figure 3.4 A and B

A - C) Total fecal IgA, *H. hepaticus* fecal load and weight of the mice shown in figure **3.4A**. **A**) Linear model: y~log(x), regression coefficient: $p < 0.01$; $R^2 = 0.52$; F statistic: $p < 0.01$; **B**) Linear model: y~exp(-x), regression coefficient: $p < 0.01$; $R^2 = 0.21$; F statistic: $p < 0.01$; **C**) Linear model: y~log(x), regression coefficient: $p < 0.01$; $R^2 = 0.62$; F statistic: $p < 0.01$. **D**) Total fecal IgA measured on the mice from figure **3.4B**; Linear regression, group comparison: $p = 0.57$; multiple $R^2 = 0.31$; F statistic: $p < 0.05$. Line = regression, shade = confidence interval.

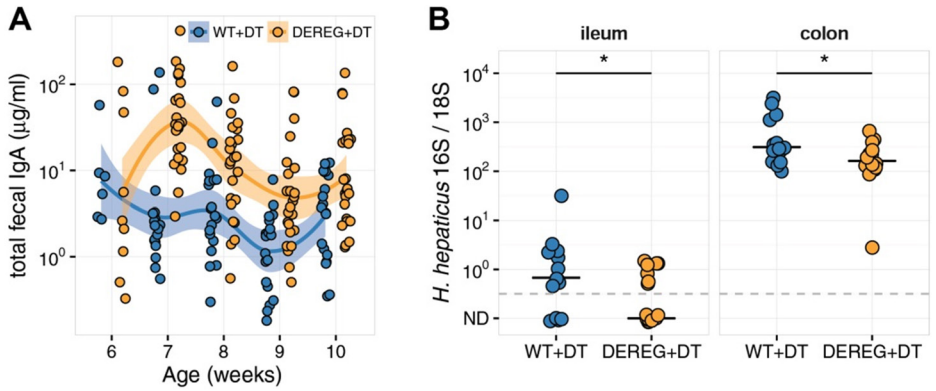

Figure S3.6 – Complementary to Figure 3.4 C

A) Total IgA measured on the feces of the mice presented in Figure **3.4C**. Line = local polinomial regression, shade = confidence interval. **B**) *H. hepaticus* load in the mucosa of the ileum and colon, normalized to host 18S, in part of the mice shown in Figure **3.4C**, 9 weeks after DT treatment. N per group: WT+DT = 14; DEREG+DT = 15. Points = individual mice, bar = median. * $p < 0.05$ (Wilcoxon test).

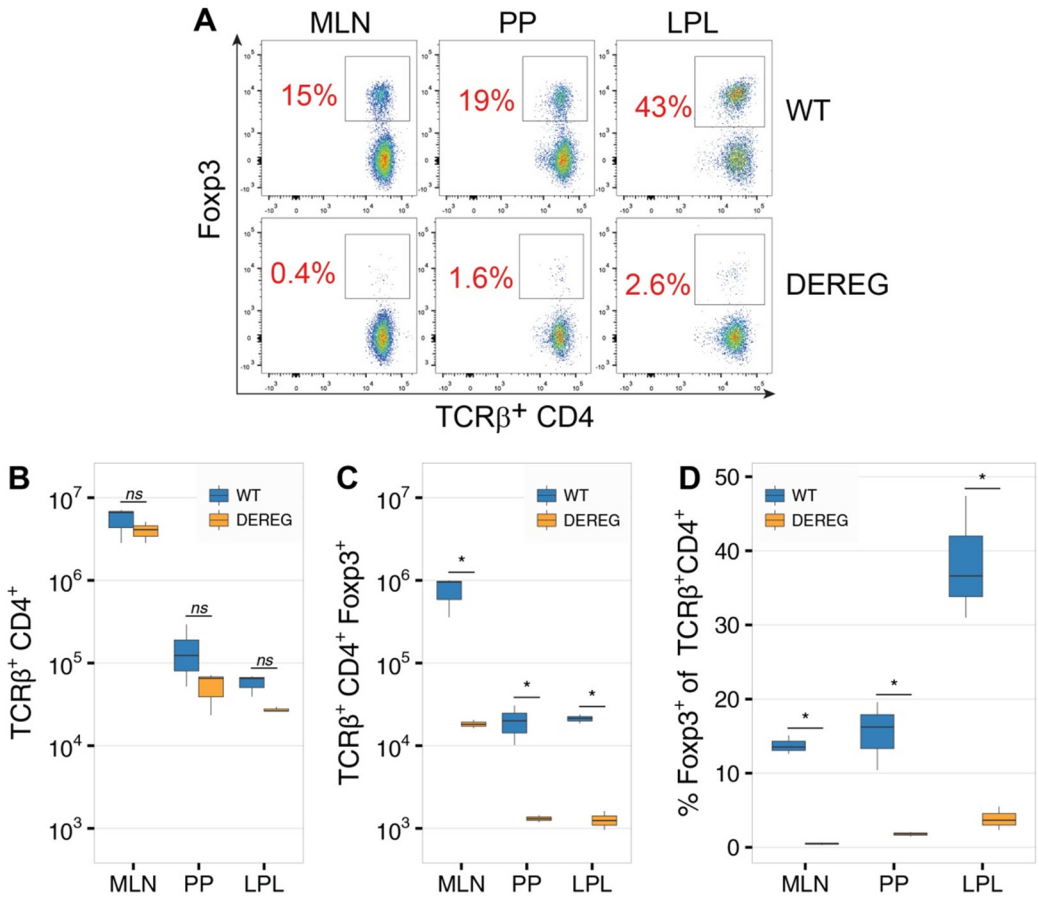

Figure S3.7 – Tregs are depleted in MLN, PP and Large Intestine Lamina Propria upon DT treatment in DEREG mice

Newborn colonized littermate WT and DEREG mice were treated with 1µg of DT i.p. at 7 weeks of age. Analysis of frequency and total numbers of TCRβ$^+$ CD4$^+$ Foxp3$^+$ cells was performed 2 days later on MLN, PP and Large Intestine Lamina Propria. **A**) Representative FACS plots of the Foxp3$^+$ cells frequency in the TCRβ$^+$ CD4$^+$ population, at the indicated organs. **B**) Number of TCRβ$^+$ CD4$^+$ cells. **C**) Number of TCRβ$^+$ CD4$^+$ Foxp3$^+$ cells. **D**) Percentage of TCRβ$^+$ CD4$^+$ Foxp3$^+$ cells. N = 3 per group, * $p < 0.05$ (t test), ns = not significant. MLN = Mesenteric Lymph Nodes; PP = Peyer's Patches; LPL = Large Intestine Lamina Propria; DT = Diphtheria Toxin.

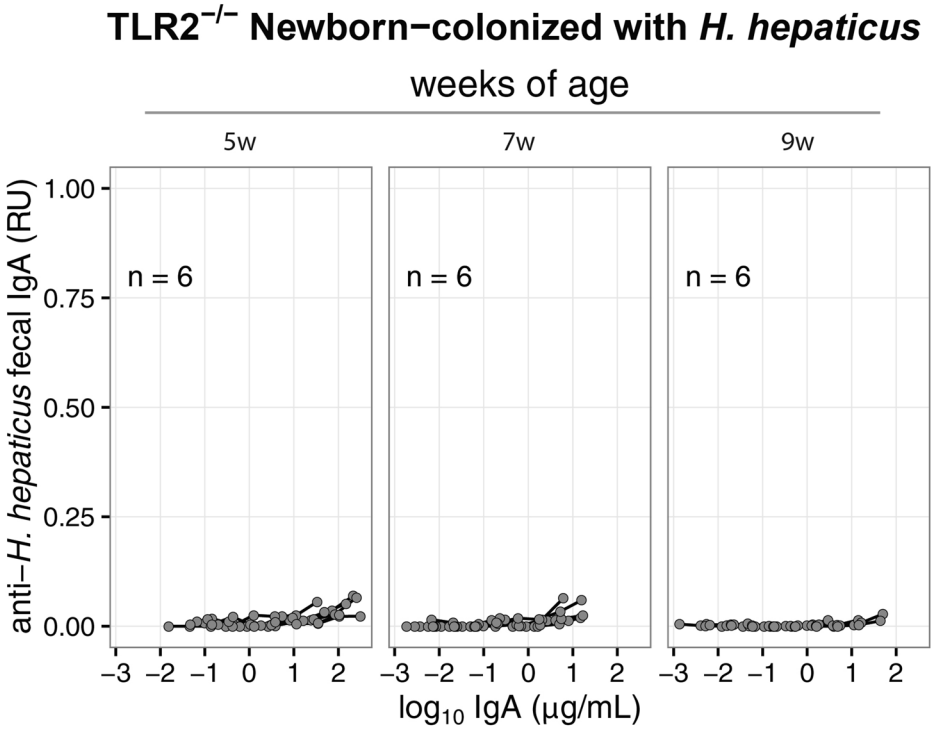

Figure S3.8 – Newborn tolerance to *H. hepaticus* is TLR2 independent

Young Adult TLR$^{-/-}$ males and females were colonized with *H. hepaticus* by feces gavage and bred to produce Newborn-colonized pups. Total and anti-*H. hepaticus* IgA were analyzed by ELISA on the feces of Newborn-colonized TLR2$^{-/-}$ mice at 5, 7 and 9 weeks of age. N = 6 at all points.

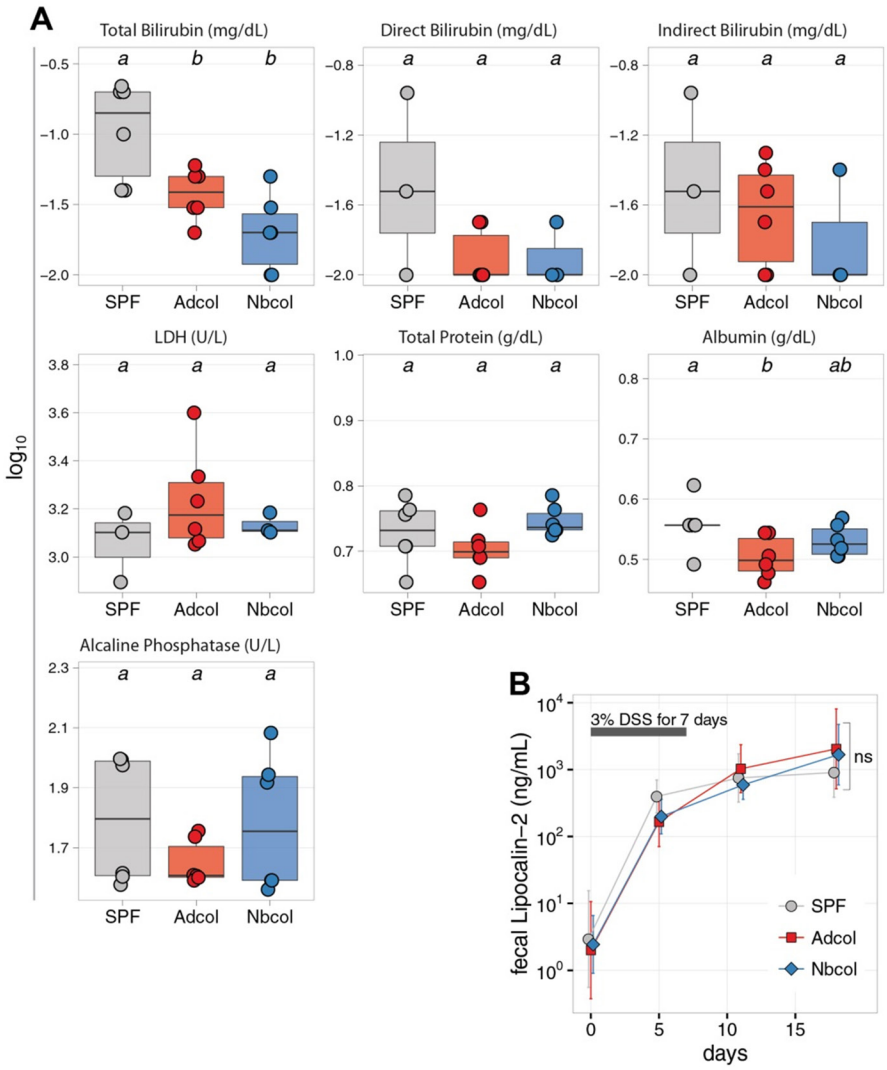

Figure S3.9 – Complementary to Figure 3.5 D and F

A) Serologic analysis on SPF, Adcol and Nbcol mice. SPF: n = 6, 11 weeks old. Adcol: n = 6, 12 weeks colonized, colonization at 10 weeks of age. Nbcol: n = 6, 11 weeks old/colonized. Groups with the same letter are not significantly different at p = 0.05 (Wilcoxon test with BH correction). **B**) Lipocalin-2 concentration was measured by ELISA on the feces of SPF, Adcol and Nbcol mice at days 0, 5, 11 and 18 after treatment with 3% DSS in drinking water for 7 days. SPF: n = 10, 12 weeks old. Adcol: n = 10, 14 weeks old, 4 weeks colonized. Nbcol: n = 10, 12 weeks old/colonized. ns = non-significant (linear mixed effects analysis).

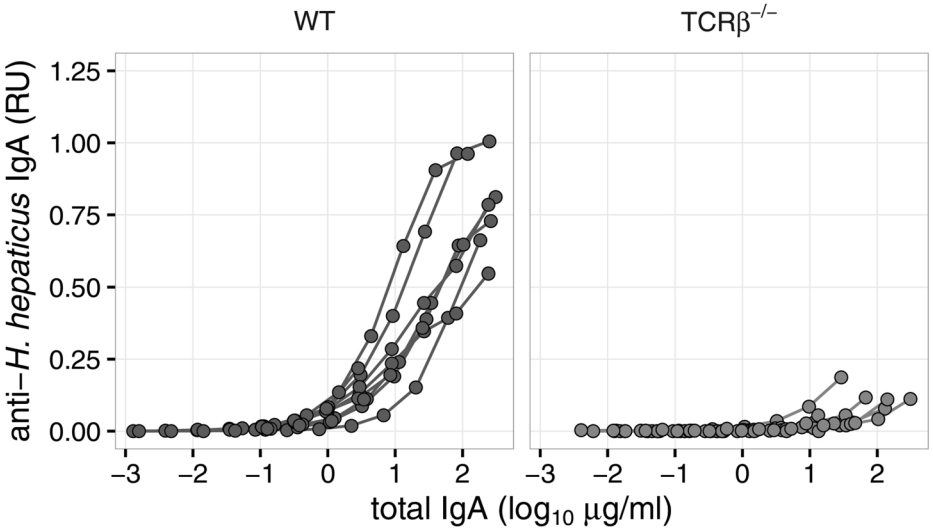

Figure S3.10 – Anti-*H. hepaticus* mucosal IgA is T-cell dependent

Adut (10 weeks old) WT and TCRβ$^{-/-}$ mice were colonized with *H. hepaticus* by feces gavage and analyzed 4 weeks post-colonization for total and anti-*H. hepaticus* IgA by ELISA. N= 10 in each group.

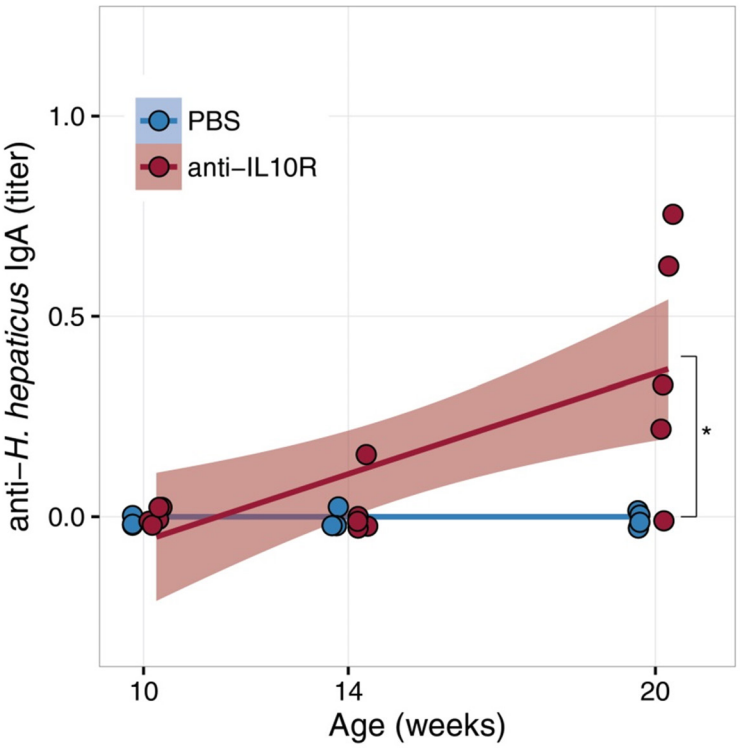

Figure S3.11 – anti-IL10R treatment is able to break tolerance to *H. hepaticus*

10 weeks old Nbcol mice received 4 weekly 1mg doses of anti-IL-10R antibody (clone 1B1.2) or PBS. Total and anti-*H. hepaticus* IgA were analyzed on feces on 0, 4 and 10 weeks post-treatment. Anti-IL10R: n=5; PBS: n=4. Lines = linear regression, shades = confidence interval, Group comparison: $p < 0.01$, Multiple $R^2 = 0.55$; F statistic: $p < 0.01$.

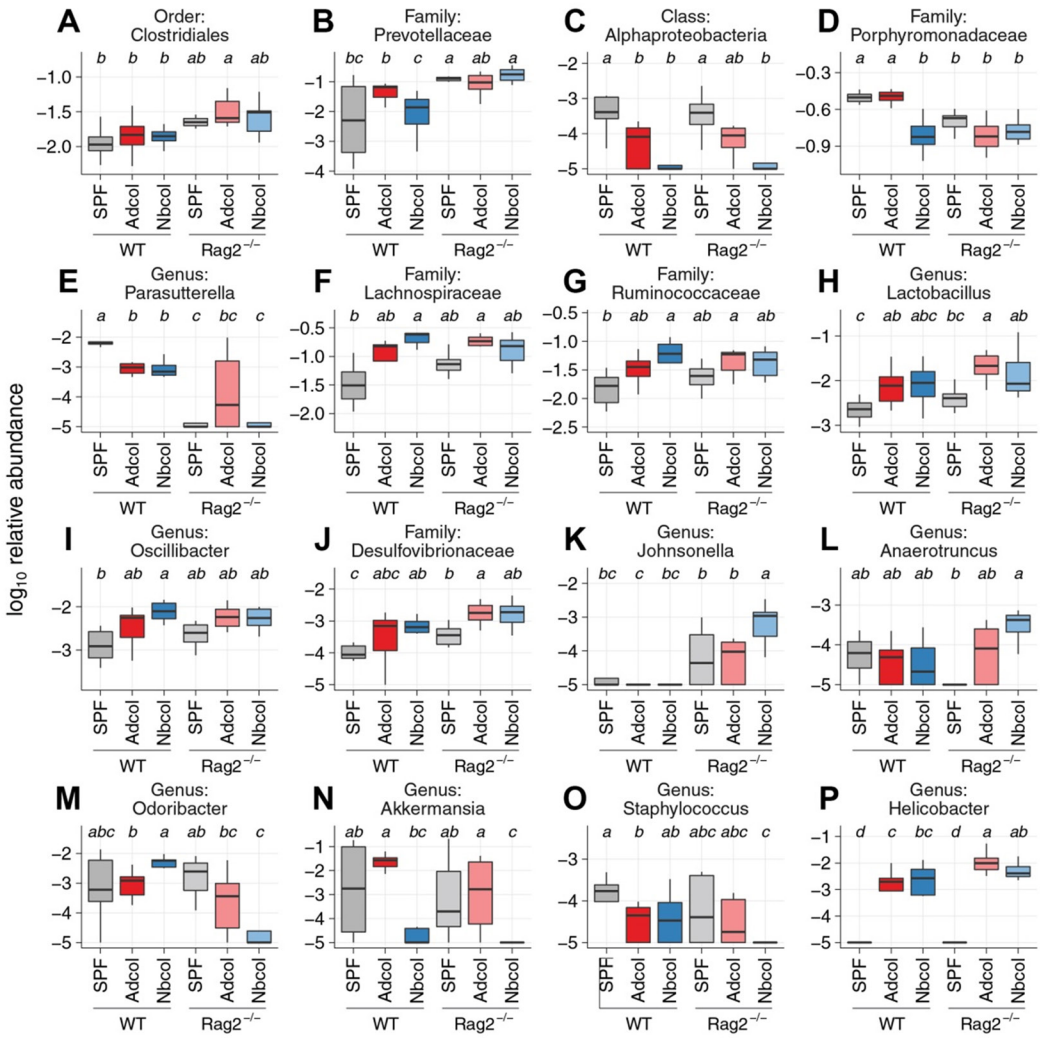

Figure S3.12 – Complementary to Figure 3.6

16S rRNA variable region 4 (V4) analysis on fecal DNA from WT and Rag2$^{-/-}$ animals, either SPF, Adcol or Nbcol, 4 Males and 4 Females per group. SPF and Nbcol mice were 8-12 weeks old; Adcol mice were colonized at 10 weeks of age and analyzed at 10 weeks post-colonization. Relative abundance of different taxa between the groups analyzed. Lowest classification level achieved in the analysis shown above the graph. Statistical analysis was performed using Wilcoxon test, with Benjamini and Hochberg correction. Groups with the same letter are not significantly different at p = 0.05.

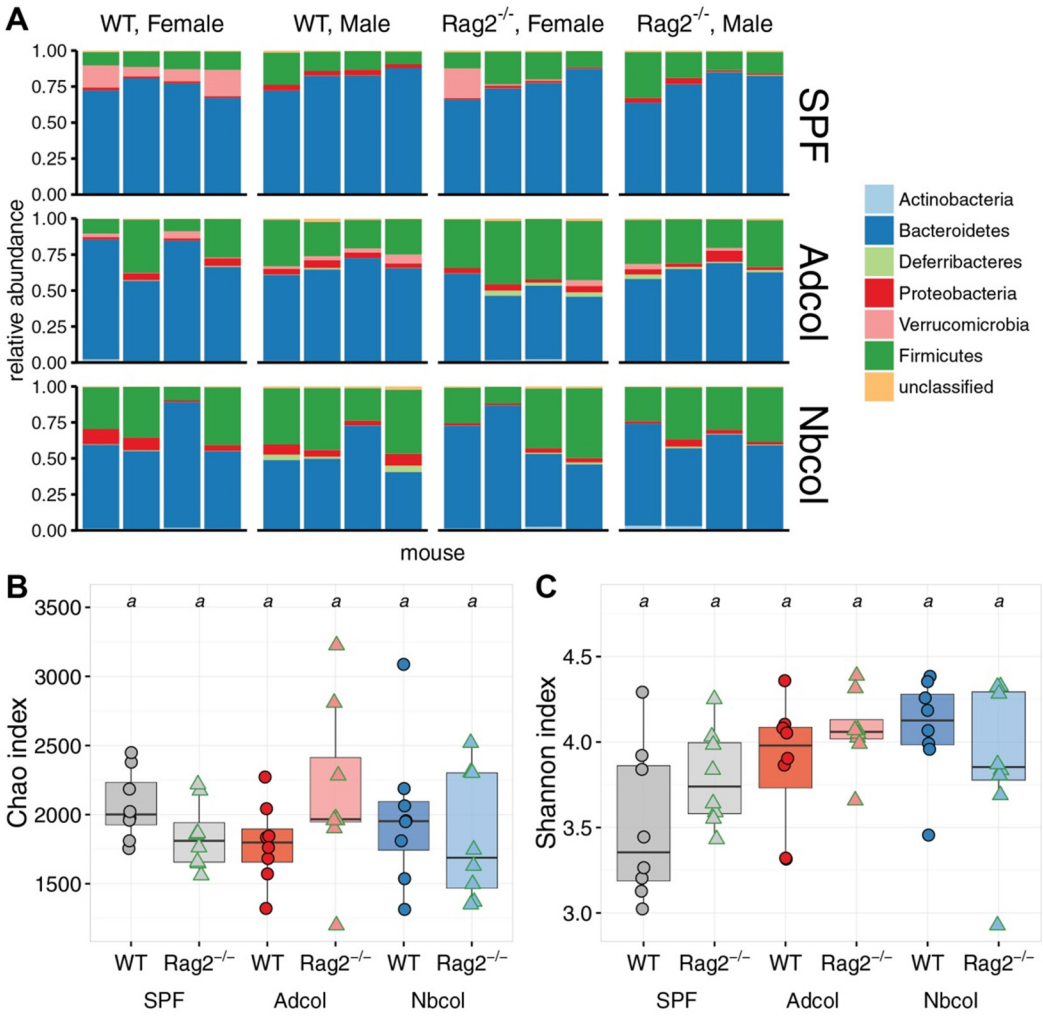

Figure S3.13 – Complementary to Figure 3.6

16S rRNA variable region 4 (V4) analysis on fecal DNA from WT and Rag2$^{-/-}$ animals, either SPF, Adcol or Nbcol, 4 Males and 4 Females per group. SPF and Nbcol mice were 8-12 weeks old; Adcol mice were colonized at 10 weeks of age and analyzed at 10 weeks post-colonization. **A**) Plots of relative abundance of the indicated taxa on individual mice of the indicated groups. Taxa with relative abundance above 0.1% are shown. **B**) Species richness, estimated with Chao index. **C**) Species diversity, estimated with Shannon index. Groups with the same letter are not significantly different at p = 0.05 (Wilcoxon test with BH correction).

Chapter 4 : Discussion

The aim of this doctoral work was to study the relationship between the mouse adaptive Immune System and the gut pathobiont *Helicobacter hepaticus*. More specifically, we wanted to assess whether and how this bacteria was affected by the host adaptive immunity, and how host immunity was affected by colonization at different stages of development.

Our results indicate that *H. hepaticus* is very well adapted to the colonization of mice, being able to establish itself in the gut even from few initial numbers (**Fig. S3.3**). Upon Adult colonization, which could happen through coprophagy, a strong response is mounted against *H. hepaticus* in the gut, with even the possibility of elimination of this bacteria in some mice (**Fig. 2.1A**). However, through the induction of Tregs producing IL-10 (**Fig. 2.1A**), *H. hepaticus* is able to persist for a longer time in the mouse, and as it is easily dispersed through coprophagy, it could rapidly colonize a whole colony, increasing the probability of reaching the next generation. Then, upon newborn colonization, a potent Treg component is induced by *H. hepaticus* (**Fig. 3.4**), which suppress the response to this bacteria, to the point that no specific IgA are detected even at later ages (**Fig. 3.1D**). This tolerant state appears to benefit the bacteria directly, for its load in the gut is greatly increased (**Fig. 3.1B**). In this way, *H. hepaticus* establishes itself in the microbiota of the host and has increased chance to colonize other groups of mice. This mechanism would explain the high prevalence of *H. hepaticus* in the wild, and is probably conserved within the genus (Wasimuddin et al., 2012).

Interestingly, having a higher amount of *H. hepaticus* in the gut does not cause harm to the mouse, for we observed a normal serological pattern and no increased susceptibility to gut inflammation in Newborn compared

to Adult colonized mice (**Fig. 3.5D – F** and **Fig. S3.9**), even though we have indications that this bacteria crosses the intestinal barrier and can travel to other sites of the body (**Fig. 3.5A** and **B**). Furthermore, the pathology caused by *H. hepaticus* in mouse models is in great extent explained by the lack of immune regulation, which results in an increased immune response that ultimately leads to immunopathology (Cahill et al., 1997). Therefore, the induction of Tregs by *H. hepaticus* upon newborn colonization, and the maintenance of this regulatory potential through life, ensures a lifelong peaceful relationship between the host and this bacteria, regardless of the bacterial numbers in the gut and eventual transit through the body.

Conversely, we found indications that the host might benefit from this relationship as well. We found that $Foxp3^+$ regulatory T cells were increased in frequency in the large intestine Lamina Propria of mice perinatally colonized with *H. hepaticus* (**Fig. 3.4D**), which might indicate this animals could be less susceptible to T-cell mediated inflammatory conditions in the gut. In our studies, we did not find evidence of a decreased susceptibility to acute gut inflammation induced by DSS on Nbcol mice (**Fig. 3.5F**), however this model is known to be T-cell independent, as it induces acute colitis in Rag deficient animals (Wirtz et al., 2007). Further analysis using T-cell dependent models of gut inflammation, like the chronic TNBS colitis model, will help to clarify the role of the increased numbers of Treg in the gut of Nbcol mice.

Additionally, we observed reduced levels of members of the Erysipelotrichaceae Family in the microbiota of Newborn colonized mice (**Fig. 3.6B**), which hints on a better metabolic profile in the gut, since this group of bacteria was found to be increased in mice where metabolic homeostasis was disrupted through a high fat and high sugar diet (Fleissner et al., 2010; Turnbaugh et al., 2009). Erysipelotrichaceae was

increased in SPF WT compared to Rag2$^{-/-}$ mice, and decreased upon *H. hepaticus* colonization only in WT animals, indicating that this effect was immune-dependent (**Fig. 3.6B**). In fact, immune activation in the epithelium has been associated with metabolic dysfunction in the gut (Shulzhenko et al., 2011), which suggests that *H. hepaticus* might affect the metabolic profile of the gut by inhibiting immune activation mediated by adaptive immunity at the epithelial level. This could be mediated either by the Tregs induced by *H. hepaticus* or by a different IgA profile in the gut. The presence of IgA indeed was shown to inhibit immune activation and metabolic dysfunction in the gut (Shulzhenko et al., 2011). Based on this, it would be also interesting to determine how *H. hepaticus* newborn colonization affects the IgA production to other members of the microbiota. Finally, as *H. hepaticus* colonization results in pathology only in immunocompromised hosts, causing not harm to individuals containing a healthy immune system, this bacteria also acts as a purifying selective pressure, as it would result in great disadvantage for hosts with poor immune regulation, which would in turn help to prevent the spread of autoimmunity susceptibility alleles in the population.

We found that Tregs can suppress the immune response to a bacteria of the microbiota, which results in suppression of the specific IgA response (**Fig. 3.1D**). Indeed, Tregs with TCRs recognizing commensal bacteria antigens were found in mice (Lathrop et al., 2011), which could be part of a general mechanism to suppress immune responses to a select group of bacteria, those with the ability to induce Tregs. It would be important to better define which bacteria have these properties, and if they confer benefits to the host or just explore a mechanism for survival.

It was shown that Tregs are important as mediators of the response in germinal centers, favoring a more diverse IgA profile, which in turn creates more diversity in the microbiota (Kawamoto et al., 2014). Our

analysis, on the other hand, highlights the impact of Tregs on a strong T cell dependent IgA response, where a complete suppressive capacity is obtained. It might be important to verify differences on Treg cells in these two systems, searching for surface markers and cytokines that could explain these discrepancies and improve the understanding of the immune control in the gut.

Fig. 4.1 summarizes our findings on the reciprocal interactions between *H. hepaticus* and the host immune system at different developmental stages. The mechanism through which *H. hepaticus* induces Tregs in newborn-colonized mice is still to be defined. However, our results in monocolonized mice (**Fig. 3.3B**) indicate that it is a property of *H. hepaticus* and not an indirect effect from other bacteria of the microbiota. As other bacteria do not have this property, for instance SFB (Jiang et al., 2001), this phenomenon cannot be explained just by the developmental state of the host, for something on the bacteria exerts this effect, which is maximum upon perinatal colonization. One possibility is that newborn dendritic cells, known to be more immature (Zaghouani et al., 2009), present *H. hepaticus* antigens in a tolerogenic manner, generating Tregs that suppress the generation of an effector T cell response. Another nonexclusive scenario would involve neonatal T cells, that were shown to be more prone to become Tregs than adult T cells (Wang et al., 2010). Additionally, newborns have an increased amount of Recent Thymic Emigrants (RTEs) in the periphery, which are also more prone to become Tregs (Paiva et al., 2013). A combination of an intrinsic Treg-inducing property of *H. hepaticus*, the maturation state of newborn DCs, and an increased amount of RTEs in the periphery of newborns could explain the tolerogenic phenomenon of the Newborn-colonization with *H. hepaticus* compared to Adult colonization.

Furthermore, it remains to be determined whether newborn colonization results in the generation of Tregs specific to *H. hepaticus* without resulting as well in the generation of effector T cells and B cells. We found evidence that T and B cells can affect *H. hepaticus* in the gut upon adult colonization in antibody dependent and independent manners, and the mechanism underlying these latter effects require further investigation.

Finally, we found that the Treg fraction is expanded in the large intestine Lamina Propria of newborn colonized mice, which could have implications for the physiology of the host. It is unclear whether this expansion happened upon *H. hepaticus* colonization in newborns and was maintained until adulthood or if it happened progressively along the maturation of the mice.

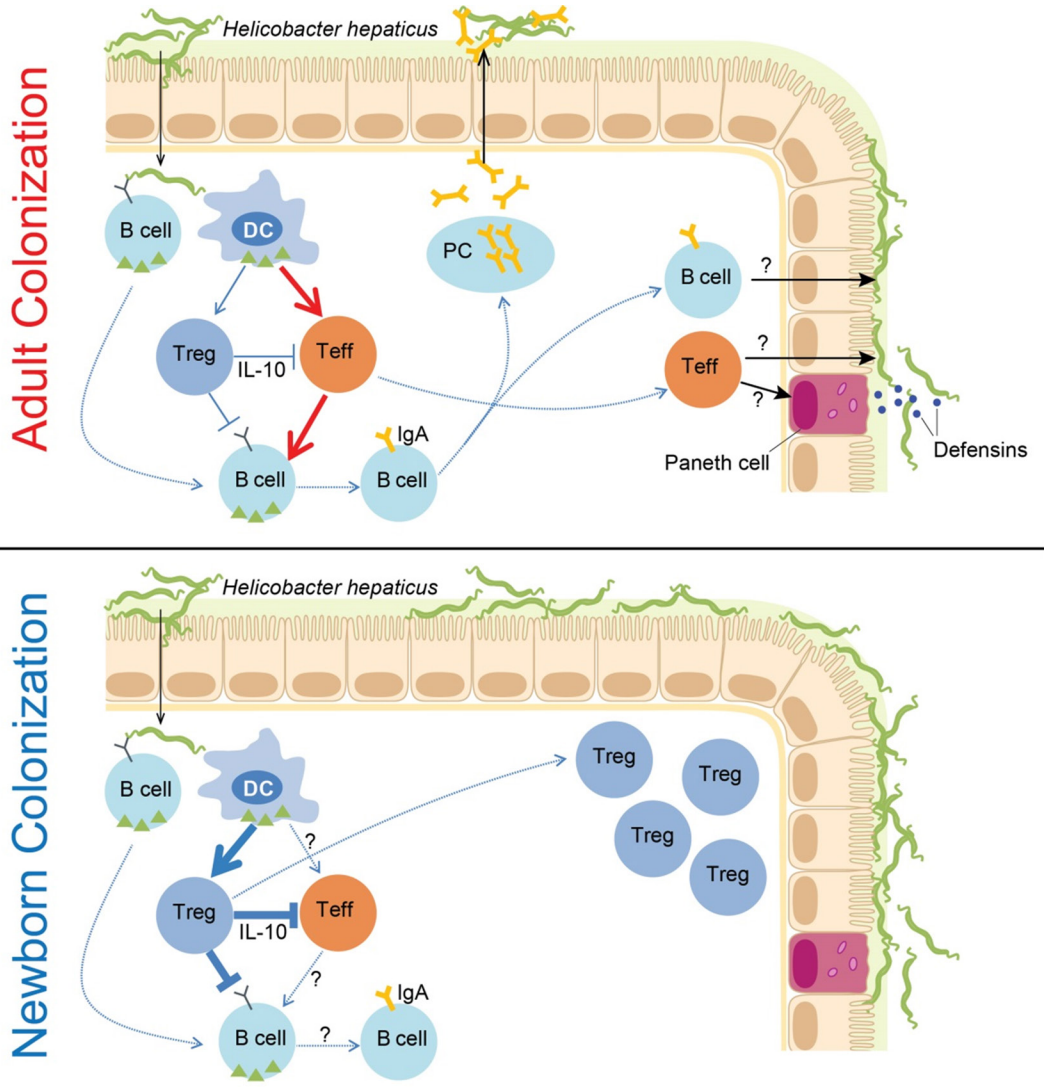

Figure 4.1 - Reciprocal interactions between *H. hepaticus* and the host immune system on different developmental stages

DC: Dendritic Cell; Teff: Effector T cell; Treg: Regulatory T cell; PC: Plasma Cell

Conclusion

Our studies of the reciprocal interactions between *H. hepaticus* and the mouse immune system provide novel insights into how the host adaptive immunity controls the microbiota and how it is affected by it. Furthermore, they highlight the importance of revising the list of bacteria excluded from the microbiota on animal studies, for some of those, like *H. hepaticus*, are an important component of this system that probably evolved with this host to compose a balanced structure. Therefore, more investigations on natural populations are necessary to search for other key components of the microbiota of mice and humans, which could help to understand how the gut associated immune system evolved and provide new tools to restore homeostasis in pathological conditions.

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