Aus dem Institut für Molekulare Medizin
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Die Rolle des β-catenin Signalweges in $CD11c^+$ myeloiden Zellen bei der Regulierung der intestinalen Immunhomöostase

Role of β-catenin signaling in $CD11c^+$ myeloid cells in regulating intestinal immune homeostasis

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Für alle, die mich immer unterstützen, insbesondere meine Familie, meine Freundin und meine Freunde.

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Table of abbreviations

APCs	Antigen-presenting cells
Bp	base pair
°C	Temperature in degrees Celsius
CCR7	C-C chemokine receptor 7
CD	Crohn's disease
CK1	Casein kinase 1
CNT	Control
CO_2	Carbon dioxide
DC	Dendritic cells
Del	Deletion
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
EDTA	Ethylenediaminetetraacetic acid
e.g.	for example
EtBr	Ethidium bromide
EtOh	Ethanol
FACS	Fluorescence-activated cell sorting
FBS/FCS	Fetal bovine serum/ Fetal calf serum
FoxP3	Forkhead box protein P3
FSC	Forward scatter
g	gram
GALT	Gut associated lymphoid tissue
GSK3	Glycogen synthase kinase 3
h	hours
HDAC	Histone deacetylase
IBD	Inflammatory bowel disease
IECs	Intestinal epithelial cells
IELs	Intraepithelial lymphocytes
IFN	Interferon
IL	Interleukin
kDA	Kilodalton
LEF	Lymphoid enhancer-binding factor
LI	Large intestine
LN	Lymph node
LP	Lamina propria

LPL	Lamina propria leukocytes
LPS	Lipopolysaccharide
LRP5/6	LDL-receptor related proteins 5 and 5
m	Meter
M	Molar
M-cells	Microfold cells
MALT	Mucosa-associated lymphoid tissue
MFI	Median of fluorescence intensity
MHC	Major Histocompatibility Complex
min	Minute
ml	Milliliter
mLN	Mesenteric lymph nodes
mM	Millimolar
MQ	Milli-Q ultrapure water
NaCl	Natrium chloride
NF-κB	Nuclear factor κB
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PP	Peyer's Patches
RA	Retinoic acid
RALDH2	Retinaldehyde dehydrogenase type 2
rpm	Rounds per minute
sec	Seconds
SI	small intestine
SSC	sideward scatter
Taq	Polymerase from Thermus aquaticus
TCF	T cell factor
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TLE	Transducing-like enhancer
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulfonic acid
TNF	Tumor necrosis factor
Treg	Regulatory T cell

UC	Ulcerative colitis
WT	Wild type
Wnt	Wingless-Int
μl	Microliter
μM	Micromolar

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Zusammenfassung

Der Darm ist als Barriere-Organ ständig Millionen von Mikroben und Umweltantigenen ausgesetzt. Dabei ist es von zentraler Bedeutung, dass das körpereigene Immunsystem harmlose Antigene, wie zum Beispiel kommensale Bakterien und Nahrungsmittelantigene, von pathogenen Krankheitserregern unterscheiden kann.

Folglich stellt die Regulierung des Gleichgewichts zwischen der Induktion einer Immunreaktion gegen schädliche Krankheitserreger und der Aufrechterhaltung der immunologischen Toleranz gegenüber harmlosen Antigenen eine der wichtigsten Herausforderungen für das intestinale Immunsystem dar. Eine Störung dieses Gleichgewichts kann zur Entwicklung von entzündlichen Darmerkrankungen (Inflammatory bowel diseases, IBD) beitragen.

Dendritische Zellen (DC) sind in der Lage, das Immunsystem des Darms so zu regulieren, dass es entweder Immunreaktionen gegen schädliche Krankheitserreger auslöst oder die Toleranz gegenüber harmlosen Antigenen aufrechterhält. Daher spielen sie eine entscheidende Rolle für das Gleichgewicht des intestinalen Immunsystems. Aus diesem Grund enthält die Darmschleimhaut zahlreiche DC, die in ihrer Rolle als professionelle antigenpräsentierende Zellen ständig Antigene aus dem Darmlumen aufnehmen und diese an T-Zellen präsentieren. Abhängig vom präsentierten Antigen und kostimulatorischen Signalen aus der Umgebung können DC entweder eine immunogene Funktion ausüben, indem sie proinflammatorische T-Zellen aktivieren, was zur Induktion einer Immunantwort führt, oder DC üben eine tolerogene Funktion aus, indem sie antiinflammatorische T-Zellen, wie zum Beispiel regulatorische T-Zellen (Tregs), induzieren, welche die Auslösung einer Immunantwort verhindern. Nun stellt sich die Frage, ob und in welchem Ausmaß verschiedene Signalwege und externe Faktoren das DC-vermittelte Gleichgewicht in diesem System beeinflussen können. Gibt es Mechanismen oder Faktoren, die dieses Gleichgewicht entweder in Richtung Immunität oder in Richtung Toleranz beeinflussen können? In diesem Zusammenhang ist β-catenin in den letzten Jahren zunehmend in den Mittelpunkt der Forschung gerückt. β-catenin ist ein Signalmolekül und eine zentrale Komponente des Wnt/ β-catenin-Signalwegs. Dieser Signalweg ist für die Regulierung von zellulären Entwicklungsprozessen wie Proliferation, Differenzierung und Migration von wesentlicher Bedeutung. Bisherige experimentelle Studien haben gezeigt, dass Mäuse, denen β-catenin in $CD11c^+$ Zellen, also unter anderem DC, fehlt, weniger regulatorische T-Zellen in der Lamina propria des Darms haben und dass diese DC ohne β-catenin auch weniger entzündungshemmende Zytokine produzieren. Aus diesen Ergebnissen wurde gefolgert, dass β-catenin einen tolerogenen DC-Phänotyp fördert, was den β-catenin-Signalweg in DC zu einem wichtigen Faktor für das Gleichgewicht zwischen Immunität und Toleranz im Immunsystem des Darms machen würde.

Das Ziel meiner Dissertation war es, diese Hypothese zu überprüfen und den Einfluss des β-catenin Signalweges in DC auf das Immunsystem des Darms genauer zu untersuchen. So haben die bisher veröffentlichten Studien nur die Auswirkungen einer Deletion von β-catenin in DC untersucht. Das Hauptziel meiner Arbeit war es nun, die Folgen einer Stabilisierung von β-catenin in DC zu untersuchen, d.h. welche Auswirkungen ein Überangebot von β-catenin in DC hat. Dafür wurden drei verschiedene Mauslinien gezüchtet: eine mit einer β-catenin -Deletion in $CD11c^+$ -Zellen **(CD11c-βcat^{DEL})**, um die zuvor veröffentlichten Ergebnisse zu verifizieren, eine mit einer stabilen Form von β-catenin in $CD11c^+$ -Zellen **(CD11c-βcat^{EX3})**, um die Auswirkungen eines Überangebots von β-catenin in DC zu untersuchen, und eine T-Zell-defiziente Variante der CD11c-βcat^{EX3}-Maus **(TCRβδ CD11c-βcat^{EX3})**, die wir für Studien zur Rolle von β-catenin in einer Umgebung ohne T-Zellen verwendeten.
Um den genauen Einfluss von β-catenin auf die Homöostase von DC und T-Zellen im Darm zu analysieren, haben wir die Zusammensetzung und Anzahl von diesen Zellen im Darm dieser Mauslinien unter steady-state und unter entzündlichen Bedingungen untersucht. Zu diesem Zweck wurden verschiedene Methoden wie die Polymerase-Kettenreaktion, die Durchflusszytometrie und diverse experimentelle Colitis-Modelle eingesetzt.

Im Gegensatz zu bisher veröffentlichten Ergebnissen zeigten unsere Experimente mit CD11c-βcat^{DEL} Mäusen keine Unterschiede in Bezug auf die DC- und T-Zell-Homöostase sowohl unter steady-state als auch unter entzündlichen Bedingungen. Hingegen zeigten unsere Experimente mit CD11c-βcat^{EX3}-Mäusen eine erhöhte Anzahl von Tregs im Darm im Vergleich zu Kontrollmäusen im steady-state und unter Entzündungsbedingungen. Weiterhin ergaben unsere Experimente mit T-Zell-defizienten CD11c-βcat^{EX3}-Mäusen (TCRβδ CD11c-βcat^{EX3} Mäusen), dass sie im Vergleich zu Kontrollmäusen weniger anfällig für eine spezifische Form der Kolitis, der T-Zell-Transfer-Kolitis, waren.
Insgesamt deuten unsere Ergebnisse darauf hin, dass der Wnt/ β-catenin -Signalweg in DC über seinen Einfluss auf die DC- und T-Zell-Homöostase eine wichtige Rolle bei der Regulierung von Toleranz und Immunität im Darm spielt. Dennoch sind weitere Experimente und Studien erforderlich, um ein besseres Verständnis der detaillierten Auswirkungen des Wnt/ β-catenin -Signalwegs in DC zu erlangen und die genauen Mechanismen zu untersuchen, durch die β-catenin zu einem tolerogenen DC-Phänotyp beiträgt.

Abstract

The intestine constitutes a critical barrier organ, permanently exposed to millions of microbes and environmental antigens. Hereby, it is of central importance that the host immune system can distinguish innocuous antigens, including commensal bacteria and food antigens from harmful pathogens.

Consequently, regulating the balance between initiating an immune response against harmful pathogens and maintaining immunological tolerance to harmless antigens represents one of the most important challenges of the mucosal immune system. Dysregulation of this balance may contribute to the development of inflammatory bowel diseases (IBD).

With the ability of orchestrating the mucosal immune system into initiating immune responses against harmful pathogens or to maintain tolerance to innocuous antigens, dendritic cells (DC) play a crucial role in balancing the intestinal immune system. For this reason, the intestinal mucosa contains numerous DC, which, through their role as professional antigen-presenting cells, constantly take up antigens from the lumen of the intestine and present them to T cells. Depending on the presented antigen and environmental costimulatory signals, DC can either exert an immunogenic function by activating proinflammatory T cells resulting in the induction of an immune response or DC perform a tolerogenic function by inducing anti-inflammatory T cells, such as regulatory T cells (Tregs), which prevent the initiation of an immune response. The question now arises whether and to what extent various signaling pathways and external factors can influence the DC-mediated balance in this system. Are there mechanisms or factors that can affect this balance either toward immunity or toward tolerance? Regarding this issue, β-catenin has increasingly become the focus of research in recent years. β-catenin is a signaling molecule and a central component of the Wnt/ β-catenin signaling pathway. This signaling pathway is essential for the regulation of cellular developmental processes such as proliferation, differentiation, and migration. Previous experimental studies have shown that mice lacking β-catenin in $CD11^+$ cells, i.e., among others DC, have fewer regulatory T cells in the intestinal lamina propria and that these DC lacking β-catenin also produce fewer anti-inflammatory cytokines. From these results, it was concluded that β-catenin promotes a tolerogenic DC phenotype, which would make β-catenin signaling in DC a key contributor to the balance between immunity and tolerance in the intestinal immune system.

The aim of my thesis was to verify this hypothesis and to further investigate the influence of β-catenin signaling in DC in the intestinal immune system. Thus, the previously published studies have only examined the impact of a deletion of β-catenin in DC. However, while we also aimed to reproduce these published results, the main objective of this thesis was now to investigate the consequences of β-catenin stabilization in DC, i.e., what would be the effects of an oversupply of β-catenin in DC. For this purpose, three different mouse lines were bred: one

with a β-catenin deletion in CD11c$^+$ cells **(CD11c-βcat^{DEL})** to verify the previously published results, one with a stable form of β-catenin in CD11c$^+$ cells **(CD11c-βcat^{EX3})** to investigate the effect of an oversupply of β-catenin in DC and a T cell deficient variant of the CD11c-βcat^{EX3} mouse **(TCRβδ CD11c-βcat^{EX3})**, which we used for studies regarding the role of β-catenin in an environment without T cells.

To determine the detailed impact of β-catenin on intestinal DC and T cell homeostasis, our experimental approach was to analyze intestinal DC and T cell composition and numbers in these mouse lines under steady state and inflammatory conditions. For this purpose, various methods such as polymerase chain reaction, flow cytometry and different colitis experimental models were used.

Contrary to previously published results, our experiments with CD11c-βcat^{DEL} mice displayed no differences regarding DC and T cell homeostasis in both, steady state and colitis experiments. In contrast, our experiments with CD11c-βcat^{EX3} mice revealed an increased number of Tregs in the intestine compared to control mice in steady state and under inflammatory conditions. Additionally, our experiments with T cell deficient CD11c-βcat^{EX3} mice (TCRβδ CD11c-βcat^{EX3} mice) displayed that they were less susceptible to T cell transfer colitis, a specific form of colitis, compared to control mice.

Overall, our results indicate, that via its impact on DC and T cell homeostasis, the Wnt/ β-catenin signaling pathway in DC plays an important role in the regulation of tolerance and immunity in the intestine. Nevertheless, further studies are required to get a better understanding of the detailed effects of the Wnt/ β-catenin signaling pathway in DC and to investigate the mechanisms by which β-catenin contributes to a tolerogenic DC phenotype.

1 Introduction

1.1 Anatomy of the intestine

From a macroscopic view the intestine consists of two main parts. The first one is the small intestine (SI), which succeeds the stomach and comprises duodenum, jejunum and ileum. Following the SI is the large intestine (LI), consisting of the caecum with the appendix connected to it, the ascending (proximal), transverse, descending (distal) and sigmoid colon, the rectum and the anus.

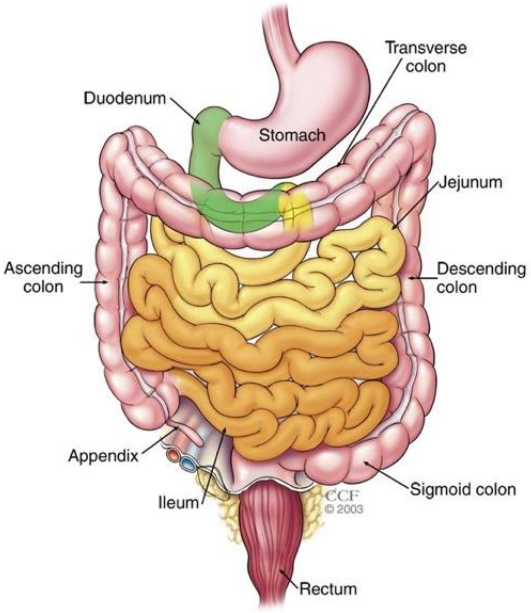

Figure 1 Representation of the macroscopic intestinal structure

Illustration displaying the structure of the gastrointestinal tract. After ingestion, the food pulp first enters the stomach. Here, the food pulp is predigested and then enters the small intestine with the sections duodenum, jejunum and ileum for further processing and digestion. After absorption of the important components of the food pulp, the non-digestible portion enters the large intestine. There, further thickening of the pulp takes place by removing water resulting in the storing of the remaining pulp as feces until defecation (1).

In terms of the general histological structure, the small and large intestine are quite similar. Therefore, this section will explain the rough histological composition, while the organ specific details will be discussed in the following sections.

The wall structure of the gastrointestinal tract consists of 4 layers. The innermost layer is the tunica mucosa. This is in turn divided into the lamina epithelialis mucosa, the lamina propria mucosa and the lamina muscularis mucosa. The next layer is the tela submucosa. It contains blood and lymph vessels as well as nerves to supply the mucosa. The tela submucosa is then followed by the tunica muscularis. This layer consists of a longitudinal and a circular muscle layer, which are responsible for the peristalsis of the intestine. The outermost layer is the tunica serosa (for intraperitoneal organs) or tunica adventitia (for extraperitoneal or retroperitoneal organs). This layer encloses the gut like a skin and acts like a fixture. Even though the individual cell components of these layers differ to some extent in the small intestine and colon, the rough function is the same in both organs (2, 3) (Figure 2).

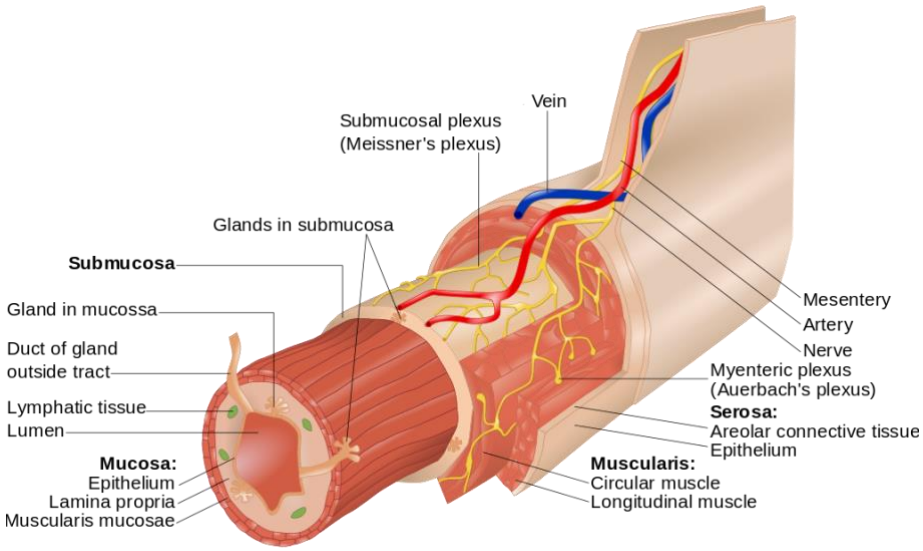

Figure 2 Histological structure of the intestine

Representation of the characteristic intestinal wall layers: on the very inside, the mucosa consisting of the lamina propria and muscle tissue, followed by the submucosa and muscularis propria (consisting of circular and longitudinal muscle tissue), and on the very outside, the serosa (4).

1.1.1 The small intestine

The main task of the small intestine is the absorption of water and the enzymatic disruption and resorption of food components. The structure of the small intestine provides optimal conditions here. One contributing factor is the 3-6 meter long, tortuous course of the small intestine, which provides a large surface area and thus facilitates the absorption of nutrients (Figure 1). The histological structure of the small intestinal layers also contributes to this increase in surface area (Figure 3). Thus, the mucosa in the small intestine forms an undulating course with villi (finger-like extensions) and crypts (at the base of the villi) and therefore the mucosa has contact with the food pulp over a wide area. In addition, microvilli sit on top of the villi, further increasing the surface area of the epithelium. This allows efficient digestion and absorption of nutrients by the enzymes of the mucosa epithelium (5, 6).

However, the mucosa is not only important for the absorption of nutrients, but also for the defense against pathogens and handling with the microbiota. It is the first surface to get into contact with pathogens from the food pulp and therefore must prevent them from being absorbed. This is the task of mucosal immune cells, which are mainly located in the lamina propria. These include B cells, T cells and many innate immune cells like DC, macrophages, eosinophils, mast cells and innate lymphoid cells (ILCs). Due to their location, these immune cells are also referred to as lamina propria leukocytes (LPL). Furthermore, there is a smaller group of immune cells in the epithelium that assist the LPL in the defense against pathogens. Those cells consist predominantly of T cells and are called intraepithelial lymphocytes (IEL). Together, they are essential for defense against pathogens.

After water and all crucial nutrients are removed from the food pulp in the small intestine, the food pulp is then transported further into the large intestine (5, 7).

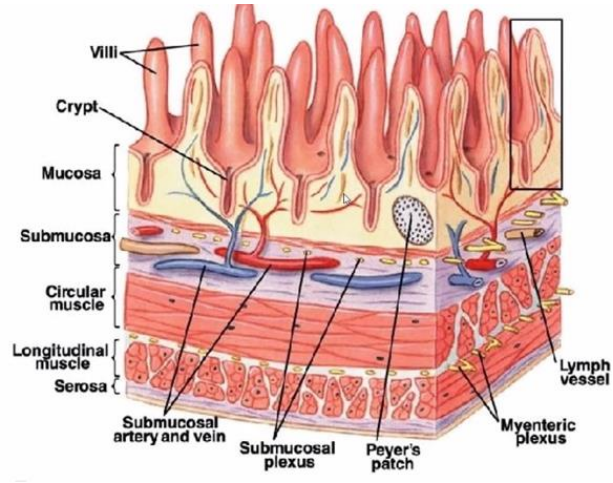

Figure 3 Histology of the small intestine

Representation of the histological structure of the small intestine. The mucosa displays the villi and crypts typical for the small intestine, which facilitate the absorption of food components due to their large surface area (8).

1.1.2 **The large intestine**

The main function of the large intestine is to transport and store stool. The colon also continues to extract water and electrolytes from the stool, thereby thickening the stool. The structure of the colon is adapted to the functions. At 1.2-1.3 meters in length, it is significantly shorter than the small intestine, but has a wider caliber and therefore offers plenty of storage space for stool (Figure 1). In addition, the large intestine displays some further characteristics ensuring the stability of the intestinal wall and facilitating the peristalsis in the gut. Those characteristics are the taenia coli (three longitudinally sloping muscle bunches), the plicae semilunaris (cross folds of the colon wall) and haustrae coli (bulges of the colon wall between the plicae semilunaris)(2).

Histologically, the large intestine also displays the typical layer structure of the whole digestive tract, consisting of the mucosa, the submucosa, the muscularis propria and the serosa respectively the adventitia. Compared to the small intestine, however, there are also differences here in terms of the exact structure of the layers, especially in the mucosa. Thus, the mucosa of the colon only contains crypts, while villi are absent. The crypts contain numerous Goblet cells, which produce mucus. This mucus in turn is important for the protection of the intestine against the local microbiota since the colon displays the highest microbial load in the whole intestine (10^9 to 10^{12} microorganisms per ml). Comparable to the small intestine, on the top of the crypts is also a short brush border of microvilli, which is important for the reabsorption of water and crucial electrolytes, like sodium and chlorine (2, 5) (Figure 4).

The distribution of immune cells in the colon is also comparable to the distribution in the small intestine. Thus, the lamina propria also contains the majority of immune cells in the colon. This

population of immune cells in the lamina propria, which is again called LPL, consists of macrophages, DC, T cells, Plasma cells, ILCs and mast cells. Besides LPL and as in the small intestine, there is a second population of immune cells in the colonic mucosa, which resides in the epithelium and is therefore called IEL again. In the colon, this population also consists predominantly of T cells. However, it should be considered that the proportions of the different cell types in IEL and LPL differ in small intestine and colon. This also applies to the population of DC, whose distribution during the gastrointestinal tract will be discussed later (5, 7).

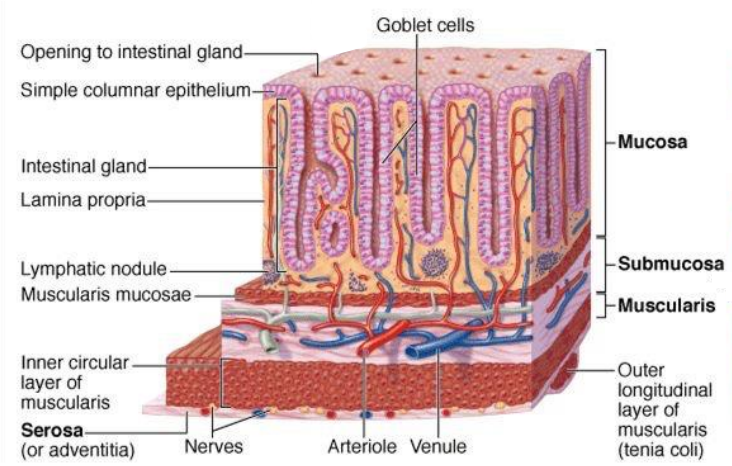

Figure 4 Histology of the large intestine

Illustration of the histological structure of the large intestine. The mucosa is characterized primarily by deep crypts containing mucus producing Goblet cells. The mucus is important in protecting the mucosa of the colon from pathogenic germs and local microbiota (9).

1.1.3 Anatomical Structure of the intestinal immune system

The gut is colonized by approximately 10^{14} commensal microorganisms, which live in symbiosis with their host and are necessary for the homeostasis of the gut. In addition, the gut is continuously exposed to antigens from the diet, resulting in a massive exposition of antigens and microorganisms to the intestinal immune system. Therefore, the intestine represents the tissue of the body with the largest number of immune cells, which is required for maintaining the balance between immune tolerance against self-antigens/ symbiotic microorganisms and a defensive immune response against harmful pathogens. It is therefore one of the most important parts of the human immune system.

The intestinal immune system is part of the mucosa associated lymphoid tissue (MALT). It is residing along the digestive tract and is subdivided into different compartments. These anatomically defined compartments include among others Peyer's patches, mesenteric lymph nodes (mLN) and the lamina propria.

1.1.3.1 Lamina propria

The lamina propria is part of the gastrointestinal mucosa and consists of loosely packed connective tissue, which contains blood and lymph vessels, nerves and a broad diversity of intestinal immune cells. Among those immune cells, which are called lamina propria leukocytes

(LPL) are T cells and antigen presenting cells like DC, macrophages and B cells (Figure 5), which sample up antigens from their environment screening for invading pathogens from the intestinal lumen. Depending on whether it is a harmless antigen or a harmful pathogen, antigen presentation results either in immune tolerance or, in the case of a harmful pathogen, to an immune response. In this case, numerous immune cells migrate into the LP, leading to a massive expansion of the LP (10).

As the LP is one of the first barriers getting in contact with invading luminal antigens and therefore contains several types of immune cells, it plays a pivotal role in the intestinal immune system.

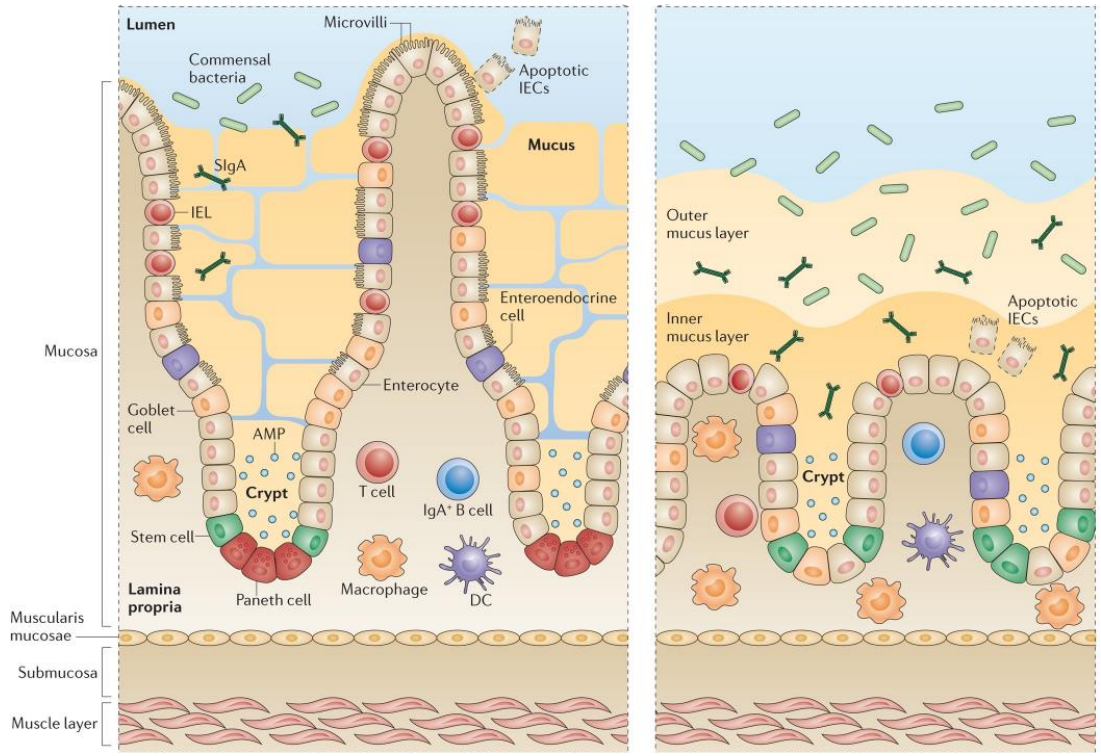

Figure 5 Illustration of the immune apparatus of the lamina propria

The figure displays the distribution of immune cells in the mucosa of the small intestine (left) and the colon (right). The lamina propria in the small intestine as well as in the colon contains a broad diversity of immune cells such as T cells, DC, macrophages and B cells. Thus, it forms the first barrier of the intestinal immune system.

1.1.3.2 Lymph nodes

Lymph nodes (LN, Nodus lymphoideus) are highly organized oval-shaped lymphoid organs. They are part of the lymphatic system and are located at the points of convergence of lymphatic vessels. Those vessels collect extracellular fluid, called lymph, from the tissues. Thus, LN are working as a collecting point for lymph. The lymph is a product of blood filtration, which carries pathogens and antigen-bearing cells, for instance migrating DC from the LP to the LN. While the free antigens simply diffuse through the extracellular fluid to the lymph node, the antigen-

bearing cells are actively migrating to the lymph node, attracted by chemokines.

A LN consists of an outermost cortex and an inner medulla. The outer cortex of the lymph node consists of B lymphocytes organized in lymphoid follicles, whereas the paracortical/ deep area is made up mostly of T cells and DC and is therefore called T cell zone. The medulla consists of strings of macrophages and antibody-secreting plasma cells, which are called the medullary cords. The afferent lymphatic vessels containing the free antigens and the antigen-bearing cells conclude in the marginal and cortical sinus of the LN, so the lymph flows from the outside to the inside of the LN. In addition, naïve lymphocytes from blood enter the LN via high endothelial venules and B cells and T cells spread to their areas.

Free antigens and antigen-bearing cells migrate directly from the sinuses into the cellular parts of the lymph node. There, they can interact with naïve T cells, which thereby become activated. Subsequently, those activated T cells interact with passing B cells, which thus can become activated as well. This can result in the induction of an immune response with the development of so-called germinal centers. In these germinal centers, a massive proliferation of some B cell follicles and the differentiation into plasma cells takes place.

Together with other activated immune cells, those plasma cells exit the LN via the efferent lymphatic vessels and subsequently enter the bloodstream to execute their function in the target tissue.

Since the large and the small intestine are both drained by the mesenteric lymph nodes (mLN), those are the most important LN for the mucosal immune system (7).

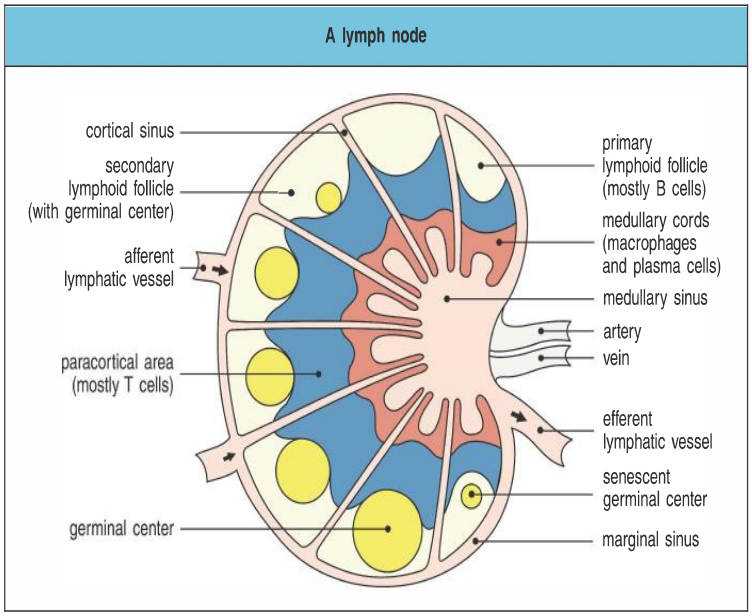

Figure 6 Structural organization of a lymph node

Illustration of a lymph node in longitudinal section. The outer cortex of the LN consists of lymphoid follicles containing B cells: primary lymphoid follicles without germinal centers (pale yellow) and secondary lymphoid follicles with germinal centers (yellow). In the paracortical area (blue) reside predominantly T cells and the medullary cord (red) contains DC and macrophages. Lymphatic fluid enters the LN through afferent vessels and exits through efferent vessels (7).

1.1.3.3 Peyer's Patches

Peyer's patches (PP or Noduli lymphoidei aggregati) are aggregations of organized lymphoid follicles, which are important for the induction of an adaptive immune response in the small intestine. They are mainly located in the mucosa and submucosa of the distal jejunum and ileum, as well as in the appendix. The lymphoid follicles of the PP are predominantly composed of B cells, whereas the space between the follicles is populated with T cells and APCs like DC. PP possess dome-like extensions with which they have direct contact to the intestinal lumen. Via these extensions, they can take up antigens from the intestinal lumen, which are then transported to antigen-presenting cells in the PP. Subsequently, the ingested antigens are presented to lymphocytes. Depending on the type of the presented antigen, this either results in the induction of tolerance towards the presented antigen, or it triggers the activation of T and B Lymphocytes causing an immune response in the intestine to eliminate the presented antigen. In contrast to the small intestine, the LP and submucosa of the large intestine contains no PP, but so-called colonic patches, which appear to be equivalent to PP (5, 7, 11).

1.2 Innate and adaptive immunity in the intestine

How can our intestinal immune system distinguish between pathogens and native or harmless proteins or microorganisms? This is a question that is still not completely resolved. Considering the vast number of proteins, chemicals or microorganisms we are in contact with e.g., in food, clothes or air, this feature of the immune system is very impressive and absolutely crucial for a healthy survival.

The human immune system consists of two subsystems: The unspecific innate and the adaptive immune system. Both systems consist of humoral (protein) and cellular components. The cellular part of the innate immune system consists of cells like monocytes, DC, macrophages, granulocytes and natural killer cells, whereas the humoral part is composed of the complement system and chemokines/ cytokines. The innate immune system exists congenitally and forms the first defense mechanism against pathogens. Contact between the innate immune system and a pathogen results in an immediate, non-antigen specific immune response, for example the induction of inflammation in the affected tissues to aggregate immune cells like macrophages, granulocyte etc. to defeat the pathogen.

The adaptive immune system includes antibodies as humoral components and lymphocytes as cellular parts. It develops over lifetime and is based on an antigen-specific immune response. Therefore, antigen-specific lymphocytes are activated by antigen-presenting cells from the innate immune system (such as DC, macrophages and B Lymphocytes), resulting in the production of specific antibodies and the induction of an adequate immune response. Due to its high specialization, the immune response of the adaptive immune system drags behind the immune response of the innate immune system, but it is more effective in defeating pathogens.

1.2.1 Innate immune cells

The cells of the innate immune system in the intestine play a central role in regulating the balance between immunity and tolerance in the intestine. Hereby especially professional antigen-presenting cells, i.e., DC, Macrophages and B cells are important for the distinction of antigens in the gut. Via their capability of presenting antigen fragments to adaptive immune cells they can guide the immune response in a certain direction. DC have the broadest range of antigen presentation and present antigen to helper and cytotoxic T cells as well as B cells. Macrophages can present phagocytosed peptide fragments to helper T cells. B cells, which actually belong to the adaptive immune cells, can internalize antigen which binds to their specific B cell receptor, and which is subsequently presented to helper T cells. The cells of the innate immune system also include granulocytes, mast cells and natural killer cells, which are responsible for implementing the induced immune response (7).

1.2.1.1 Dendritic cells

Dendritic cells (DC) are phagocytic cells found in tissues that are in contact with the external environment, such as the intestine. Their main function is their task as professional antigen-presenting cells. They process phagocytized material and subsequently present the processed antigen on their surface to naïve T cells. This either results in the initiation of adaptive immune responses or in the induction of tolerance to the presented antigen. Since DC have the broadest range of antigen presentation, they are the most important cells for activation of naïve T cells. They therefore form a crucial link between the innate and adaptive immune system.

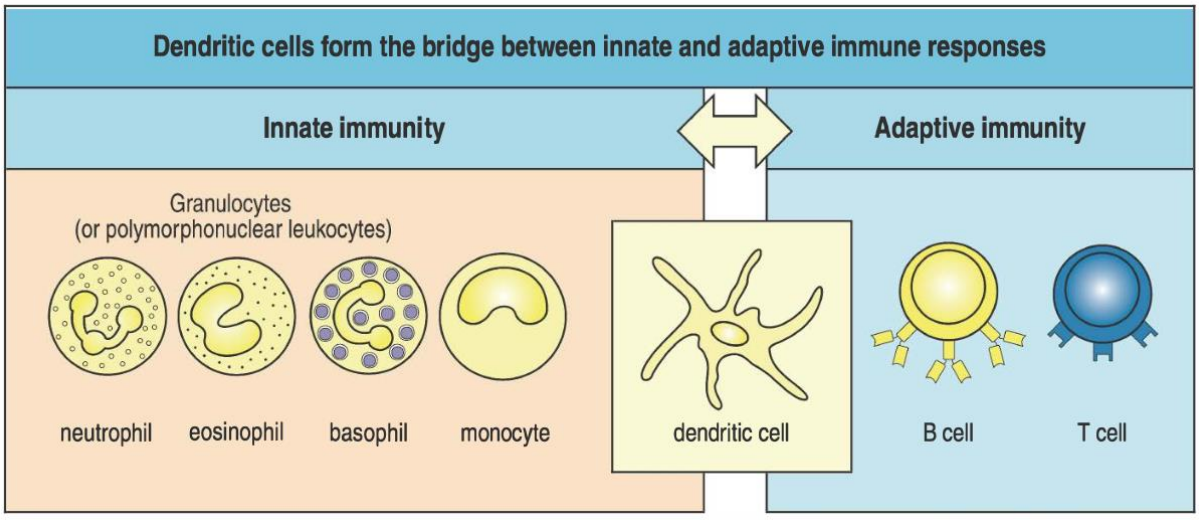

Figure 7 DC form a crucial link between the innate and adaptive immune system

With their capability of professional antigen presentation DC can take up pathogens and present them to cells of the adaptive immune system, resulting in their activation. DC therefore are a key player in the regulation of immunity and tolerance (7).

With their long, finger-like processes DC possess the ideal tool to scan their environment for potential antigens which can then be taken up for further processing.

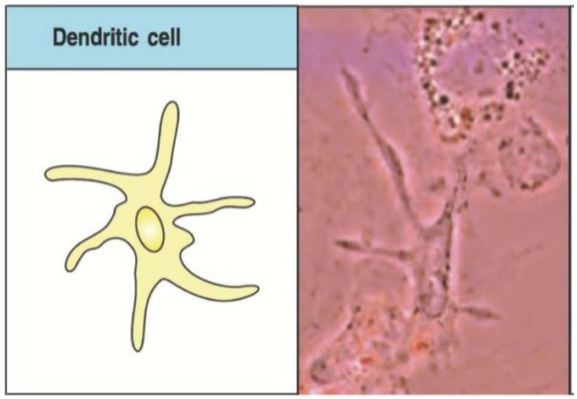

Figure 8 Schematic and photomicrographic representation of a dendritic cell

With their long finger-like processes DC can take up pathogens and present them to naïve T cells which is followed by their activation (7).

1.2.1.1.1 Development and residence

Dendritic cells (DC) arise from hematopoietic bone marrow progenitor cells and, like macrophages, belong to the group of mononuclear phagocytes. After emerging from the bone marrow as immature DC, they migrate via the blood to different tissues (for instance the intestine) and directly to peripheral lymphoid organs. Upon contact and uptake of a presentable antigen, immature DC become activated into mature dendritic cells and start migrating to the lymph node. Here they act as professional antigen-presenting cells and activate cells of the adaptive immune system resulting in the induction of an immune response or tolerance (7). In lymph nodes, DC can be divided into migratory and resident DC depending on their movement patterns and surface molecules. Migratory DC, which exhibit a high MHCII expression, are mainly responsible for antigen presentation to naïve T cells in lymph nodes. They possess long dendrites for taking up antigens in peripheral tissues, which is followed by their migration through lymphatics into lymph nodes. Therefore, migratory DC express the C-C chemokine receptor 7 (CCR7) on their cell surface, which is needed for migration. In lymph nodes, the digested antigenic peptides are presented on MHCII molecules to naïve $CD4^+$ T cells, resulting in their activation. In steady state, the migration frequency is low, so that a tolerance to self-antigens and the normal intestinal microbiota can be achieved without creating a massive immune response. During an intestinal infection with harmful pathogens, the migration frequency and therefore the antigen presentation activity of DC increases dramatically resulting in the activation of T cells and an intense inflammatory response.

Resident DC, which display a high expression of the surface molecule CD11c, are also involved in the presentation of digested antigenic peptides to naïve T cells, but to a lesser extent than migratory DC (12-14).

1.2.1.1.2 Immature vs mature DC

Immature DC are characterized by their high endocytic activity and their low T cell activation potential. When migrating through tissues, they constantly sample the surrounding environment for pathogens as bacteria or viruses. This is performed by toll-like receptors or pattern recognition receptors on the surface of immature DC. DC can take up, process and

present antigens from all types of sources. Contact of immature DC with presentable antigens results in the uptake of antigen by the DC as well as in the activation of those DC. The activation is followed by a program of functional maturation. Via this process of maturation, DC acquire several fundamental properties, such as antigen processing and presentation, migration and T cell co-stimulation. During this process, they also develop their finger-like extensions and upregulate cell-surface receptors which act as co-receptors in T cell activation. These include CD80, CD86 and CD40. Also, the expression of CCR7 is upregulated, a chemotactic receptor inducing the migration of DC to lymphatic tissues. After their maturation, DC can migrate to lymphoid tissues and activate T or B cells via antigen presentation (7).

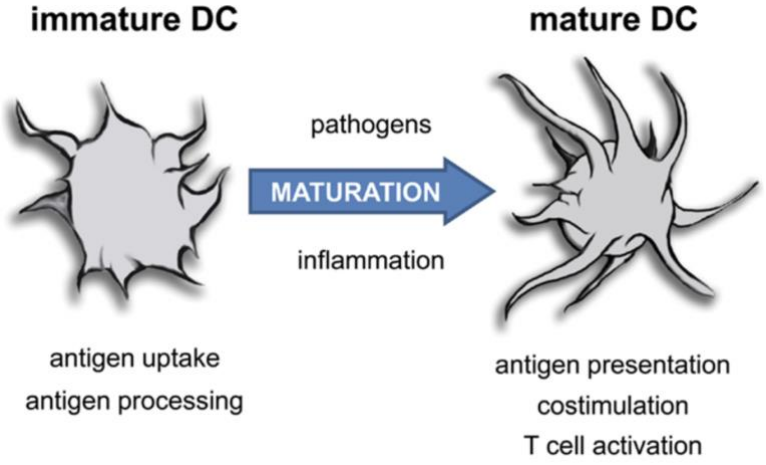

Figure 9 Change in appearance during maturation of DC.

While immature DC are specialized on antigen uptake and processing, mature DC with long dendrites are focused on antigen presentation and T cell activation (15).

1.2.1.1.3 Immunity vs tolerance

Following the maturation of DC, the ingested antigen is processed and then presented to T or B cells. In this process, DC can present the antigens to helper T cells (and thus also to regulatory T cells) as well as to cytotoxic T cells resulting in the activation and polarization of those cells. The process of antigen presentation will be elucidated in chapter 1.2.2. Depending on the presented antigen and co-stimulatory molecules presented by DC, antigen presentation by DC to T cells can either result in the induction of an immune response or in the promotion of tolerance. If the presented antigen turns out to be a harmful pathogen, the naïve T cell differentiates into a proinflammatory T cell inducing an immune reaction to defeat the pathogen. This immune reaction can be exerted by different types of helper T cells, for instance Th1 or Th17 cells (7).

In case the presented antigen is harmless, there are two ways of achieving tolerance to the antigen. On the one hand, presentation of antigen to a naïve T cell by DC can cause this T cell to differentiate into a regulatory T cell. This kind of T cell has anti-inflammatory effects and prevents the induction of an immune response (see chapter 1.2.3.1). On the other hand, the presentation of antigen by DC to naïve T cells can result in the induction of a tolerance mechanism called T cell anergy. In this mechanism, no priming of the T cell occurs. The naïve T cell is intrinsically functionally inactivated but remains alive for an extended period in a hyporesponsive state. This prevents the development of an immune reaction and induces

tolerance to the antigen.

Since DC, through antigen presentation, can direct the immune response in a specific direction, they play a crucial role in balancing the homeostasis between tolerance and immunity in the immune system (7, 16-18).

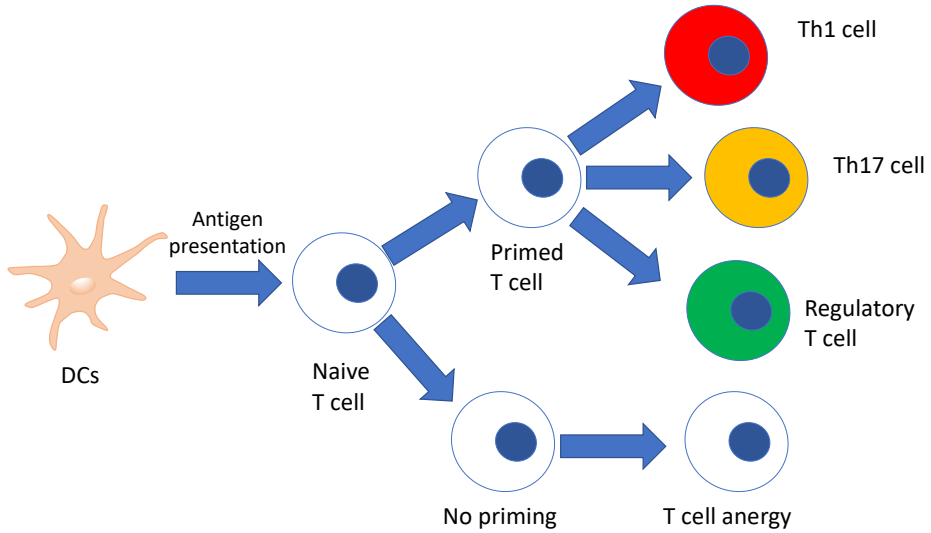

Figure 10 General scheme of helper T cell priming and polarization

After antigen presentation through DC naïve T cells either become primed T cells or no priming occurs. Primed T cells can differentiate into different helper T cells depending on the presented antigen and co-stimulatory signals. If no priming takes place, the T cell falls into a state of functional inactivity, resulting in the prevention of an immune response.

1.2.1.2 Macrophages

Macrophages belong to the innate immune system and develop from monocytes, which circulate in the blood and constantly migrate into tissues, where they differentiate. Therefore, macrophages are resident in nearly all tissues. Macrophages are characterized by their specific expression of the surface molecules F4/80 and CD64. However, they share the expression of the surface molecules CD11c, CD11b and MHCII with DC. Together with DC and granulocytes, monocytes and macrophages make up the three types of phagocytes in the immune system.

One of the main functions of macrophages is their phagocytotic activity. Therefore, they possess pattern recognition receptors, with whom they can bind pathogens. After binding of the pathogen, they engulf and eliminate the pathogen via phagocytosis. This is followed by presentation of pathogen fragments bound to MHCII on the surface of the macrophages. Thus, like DC, the antigen presentation capability of macrophages links the innate and adaptive immune system. In addition, macrophages also play an important role in orchestrating immune responses. Via their ability to secrete signaling proteins as for instance cytokines and chemokines, they are capable of initiating and regulating immune responses (7).

Since DC and macrophages share their activity as APCs and the expression of characteristic surface receptors, it is therefore important to distinguish DC from macrophages. DC as well as macrophages express CD11c (integrin αX), Major Histocompatibility Complex (MHC) class II and CD11b (integrin αM), but DC lack the expression of the macrophage associated markers F4/80 and CD64 (5). Furthermore, the expression of C-C chemokine receptor 7 (CCR7) allows intestinal DC migration into the lymph node, whereas intestinal macrophages remain in their primary tissues. Moreover, they also differ in their function as APCs. Whereas macrophages only act as APCs in certain situations, DC are the cells that are specialized in initiating adaptive immune responses (7).

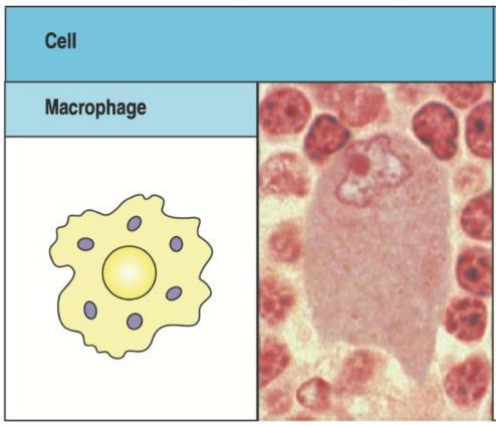

Figure 11 Schematic and photomicrographic representation of a macrophage

Macrophages perform phagocytosis and thus engulf and destroy pathogens, which are subsequently presented to T lymphocytes resulting in their activation (7).

1.2.2 T cell activation and polarization through antigen presenting cells

The process of antigen presentation is crucial for triggering a T cell immune response or the induction of tolerance. The activation of T cells requires the recognition of fragmented antigens bound to major histocompatibility complex (MHC) and displayed on cell surfaces. Before the antigen can be exhibited on the cell surface of the antigen presenting cell, the antigen needs to be processed and fragmented. Those antigens can derive from pathogens, such as viruses or bacteria, that have been ingested by cells, which then process those pathogens for antigen-presentation. But those antigens can also derive from harmless antigens, such as the microbiota in the gut. By antigen- presentation, the immune system can differ between pathogens and harmless antigens resulting in the according reaction, i.e., an immune response or the induction of tolerance. Depending on the MHC molecules and the involved cells, there are two different ways of antigen- presentation.

MHCI molecules are expressed on all nucleated cells in the body. Their main function is the presentation of intracellular antigens to $CD8^+$ T cells, i.e., cytotoxic T cells. Therefore, the antigen is disassembled in the proteasome, resulting in small antigen fragments. Subsequently, those fragments are transported into the endoplasmic reticulum, where they are bound to the MHCI molecule. Afterwards, the antigen can be presented to naïve $CD8^+$ T cells.

Following their activation, those T cells can eliminate the pathogen/ affected cells. This process enables the immune system to detect infected or transformed cells, that display peptides from mutated self or foreign proteins.

The expression of MHCII molecules is restricted to professional antigen-presenting cells, such as DC, macrophages or B cells. Of the professional APCs, DC contribute most to T cell activation. The main function of MHCII molecules is the presentation of extracellular antigens to $CD4^+$ T cells, i.e., helper T cells and regulatory T cells.
Extracellular organisms/ pathogens in the periphery, for instance bacteria trigger pattern recognition receptors (PRR) of professional APCs and activate them. Afterwards, professional APCs ingest those pathogens via phagocytosis. Following, APCs migrate into lymphatic tissue to get in contact with naïve $CD4^+$ T cells. Inside APCs, the ingested pathogens get disassembled into small fragments by the phagolysosome. Subsequently, those fragments are bound to MHCII molecules and presented to naïve $CD4^+$ T cells in lymphatic tissue. However, this process of activating $CD4^+$ T cells through professional APCs is mainly carried by DC, with macrophages and B cells playing a rather minor role.
Activation and polarization of those T cells requires in total three different DC derived signals. Presentation of antigen-derived peptides on MHCII molecules to T cell receptors on the surface of naïve $CD4^+$ T cells is the main signal. The second necessary signal is a co-stimulatory signal, namely engagement of the DC surface molecules CD80/86 to the T cell surface molecule CD28. Signal three, which gets activated by signal 1 and 2, is a feedback signal through T cells via binding of CD40 and CD40L (CD40 Ligand) and results in the release of polarizing factors from DC. As those polarizing factors determine T cell development into different subgroups, for instance Th1-, Th2-, Th17- or Treg cells, this signal is also called the polarization signal. The type of the polarization signal and consequently the subtype of the activated T cell depends on the presence or absence of certain cytokines, such as IL-6 or TGFβ, whose production in turn depends on the presence of pathogens. Thus, the presence of IL-6 promotes differentiation into pro-inflammatory Th17 cells, whereas the absence of IL-6 and presence of TGFβ promotes differentiation into anti-inflammatory Tregs. Depending on which subgroup the naïve T cell proliferates into, either an immune response or a tolerance induction takes place (7, 19).

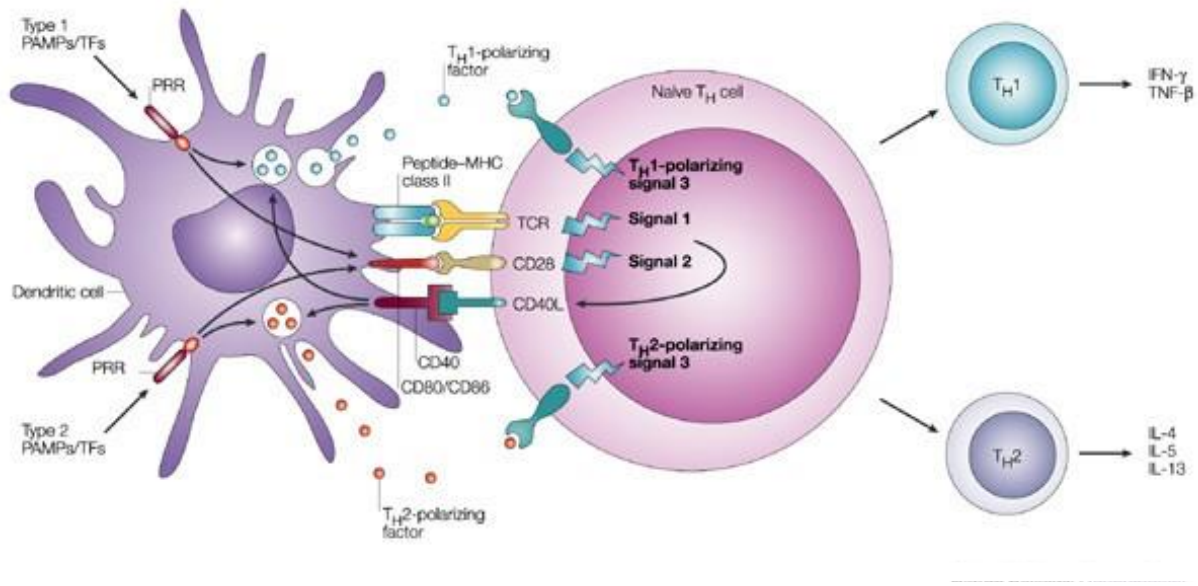

Figure 12 Activation of naive $CD4^+$ T cells through DC

Three signals are required for the activation of naïve T cells: 1. Presentation of the antigen peptide on MHCII to TCR on T cells; 2. Co-stimulatory signal CD80/86 binding to CD28; 3. Release of polarizing factors after a feedback signal through CD40/ CD40L binding. PAMPs: pathogen-associated molecular patterns, PRP: pattern recognition receptors, TFs: tissue factors (19).

1.2.3 Cells of the adaptive immune system

Cells of the adaptive immune system are specialized in carrying out the immune response initiated by the innate immune system, regardless of whether a tolerance or immune reaction is taking place. The adaptive immune system in the intestine consists exclusively of Lymphocytes. Lymphocytes originate from a lymphoid progenitor in the bone marrow and belong to the white blood cells. Lymphocytes can be further distinguished into two different types: T lymphocytes (T cells) and B lymphocytes (B cells)(7).

1.2.3.1 T Lymphocytes

T lymphocytes also originate from the bone marrow, but they mature in the Thymus (thus their name T cells) leaving as naïve T cells, that are tolerant to self- antigens. Subsequently, T cells patrol through the blood and secondary lymphoid organs searching for the suitable antigen for their specific T cell antigen receptor/ T cell receptor (TCR).

For the activation of T cells, the suitable antigen must be presented by antigen presenting cells (such as DC, macrophages or B cells). In addition, the presence of costimulatory molecules is necessary. The activation of a T cell is followed by their polarization and differentiation into one of several different functional types of effector T cells.

Those different types can be roughly subdivided into three groups: cytotoxic T cells, helper T cells and regulatory T cells (which also belong to helper T cells). Those groups can be distinguished by the different expression of cell surface molecules, which varies among the three different groups. In general, all T cells share the expression of CD3. Cytotoxic T cells are

characterized by their additional expression of CD8, while helper T cells in addition express CD4 on their surface. Regulatory T cells also express CD4 but are further characterized by their auxiliary expression of forkhead box P3 (FoxP3) in the nucleus and high levels of CD25 on their cell surface.

The main function of cytotoxic T cells is the elimination of cells, that are infected with viruses or other intracellular pathogens. In addition, they also play a pivotal role in the clearance of degenerated cells and thus they often prevent the formation of cancer cells. Therefore, cytotoxic T cells feature pro- apoptotic and cytotoxic substances (like Perforin and Granzyme), that induce apoptosis in their target cell.

Helper T cells play a pivotal role in inducing and regulating immune responses. Via their ability to secrete cytokines and chemokines they can regulate immune responses and recruit other immune cells. Helper T cells are further subdivided into several different groups. After their priming, the differentiation of naïve $CD4^+$ helper T cells into the different sub-groups is depending on the cytokine milieu and the antigen-presenting cell. The four main subtypes are: T helper cell 1 (Th1), Th2, Th17 and regulatory T cell (7).

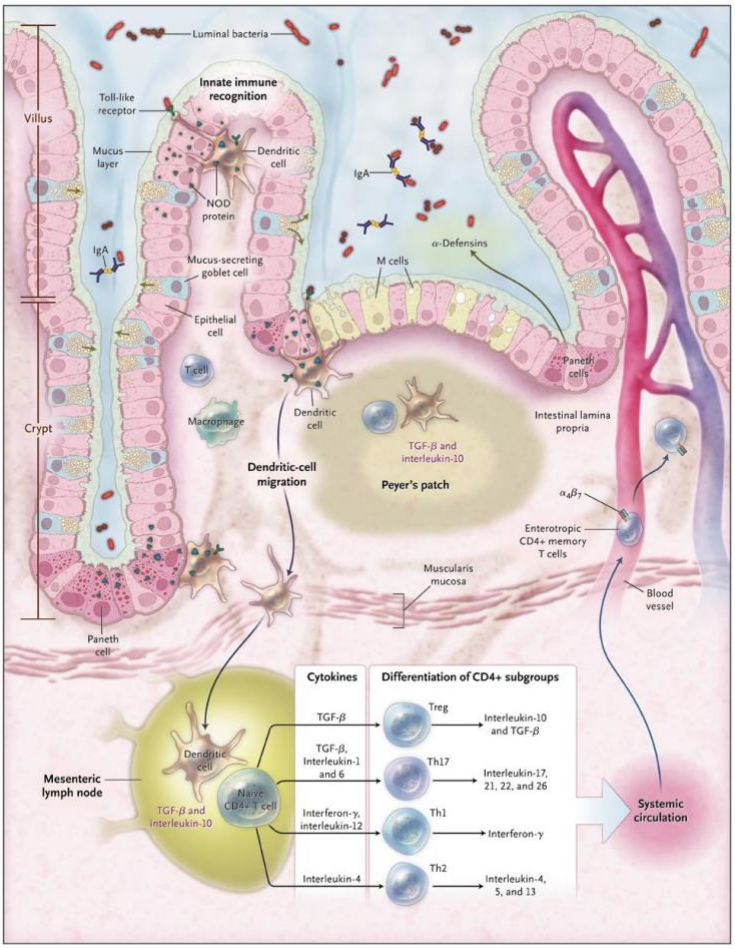

Figure 13 Differentiation of $CD4^+$ T cells into subgroups

DC sample up antigens from the intestinal lumen and process them for presentation. Subsequently, DC migrate to the mLN and present the antigens to naïve $CD4^+$ T cells, which differentiate, depending on the cytokine environment and APCs, into Tregs, Th17, Th1 or Th2 cells (10).

The main function of Th1 cells is the high-level production of the immune-modulating cytokines IL-2, IL-12, IFN- γ and TNF- α in case of immune responses.

Th2 cells play a central role in the defense against parasites via the production of IL-4, IL-5 and IL-13.

Th17 cells promote inflammation and the generation of immunity by producing IL-17, which is therefore increased during inflammations. Furthermore, they express IL-23 receptor (IL-23R) on their surface. The engagement of IL-23 (which is produced by APCs) with IL-23R results in the activation of the Janus-associated kinase-signal transducers and activation of transcription proteins (JAK-STAT). This pathway in turn regulates many genes, of which some are involved in the formation and development of inflammatory bowel diseases. Therefore, Th17 cells are thought to play an important role in inflammatory and autoimmune diseases (13).

Tregs can downregulate many types of immune responses. Therefore, they have multiple mechanisms at their disposal to mediate the suppressive effects. One of them is the production of anti- inflammatory cytokines like IL-10 and IL-35. In addition, they produce TGFβ, which is important for the generation of further Tregs and which also inhibits the differentiation of pro-inflammatory T cells. Tregs are also able to suppress pro-inflammatory cells either by performing cytolysis (with Granzyme A/B) or via cytokine-deprivation-mediated apoptosis of the targeted cells. Last, Tregs can modulate DC maturation or function resulting in a limited T cell immune response (20).

Via their ability of down-regulating all T cell- mediated immune responses, Tregs are crucial for gaining tolerance against harmless foreign antigens and self- antigens. Thus, they are essential for the prevention of autoimmune diseases.

There are three different ways of Treg development. Either they are developed in the thymus and then exported to peripheral tissues with their regulatory function already intact (tTregs), or they are generated from naïve $CD4^+$ T cells in the periphery through interactions with DC (pTregs) (13). However, Tregs can also be induced in cell culture in the presence of TGFβ; these Tregs are then called iTregs.

Although they differ in their development, the Treg sub-groups are thought to have the same functions. However, they differ in the way they perform their suppressive function. While tTregs primarily inhibit effector T cell trafficking to the target organ, antigen-specific pTregs prevent T cell priming by acting on antigen-presenting cells, mainly DC (21). Furthermore, it is worth considering, if the peripherally generated pTregs are functionally more specific for commensal bacteria or dietary antigens compared to tTregs, which developed in the thymus (7). iTregs are thought to have suppressive functions like pTregs, but this question is still the subject of current research (21).

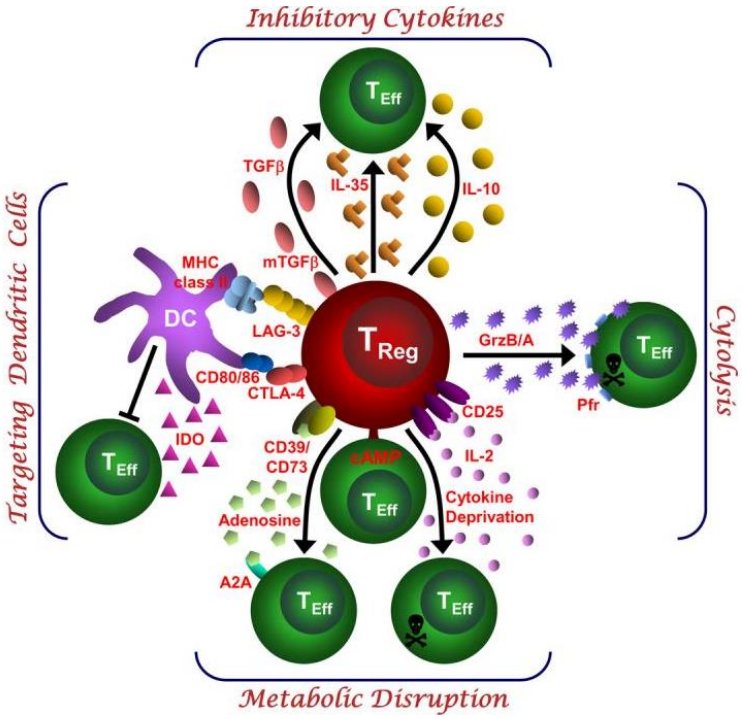

Figure 14 Basic immunosuppressive mechanisms used by Tregs

Tregs can limit immune responses through several pathways. This includes the release of inhibitory cytokines, Granzyme A/B induced cytolysis of the target cell, metabolic disruption of the target cell by cytokine deprivation and targeting dendritic cells. Thereby, Tregs can down-regulate all T cell-mediated immune responses.

1.2.3.2 B Lymphocytes

After their maturation, B Lymphocytes leave the Bone marrow (thus their name B cells) as naïve B cells, that are tolerant so self- antigens. Via the blood circulation, they migrate into secondary lymphoid organs as the spleen, LNs, PPs and mucosal tissues. There, on the one hand, they can function as antigen-presenting cells. Or, on the other hand, antigens directly bind to naïve B cells, as B cells are also able to identify and bind antigens themselves. Since the B cell antigen receptor (B cell receptor) is very specific, only the binding of a particular (foreign) antigen results in the activation and proliferation of B cells, which then differentiate into plasma cells. Plasma cells are the effector form of B Lymphocytes and produce specific antibodies, soluble secreted glycoproteins, which have an identical antigen specificity (7).

Hence the antigen, which activated a particular B cell, becomes the target of the specific antibodies produced by that cell's progeny, thus the plasma cell.

Antibodies are also called immunoglobulins and belong to the humoral part of the adaptive immune system. Depending on their function, they can be further subdivided into five different classes (IgA, IgD, IgE, IgG, IgM). The largest fraction of antibodies are IgG antibodies. Their main task is the defense against pathogens via the opsonization and neutralization of those pathogens (7) However, in mucosal secretions, for instance the intestine, secretory IgA is the dominant antibody class. Plasma cells producing IgA are in the mucosal membranes. The secreted IgA protects the mucosa against the adhesion of pathogens and their penetration through the intestinal barrier. Furthermore, IgA also regulates gut microbiota composition and therefore contributes to the intestinal homeostasis (22).

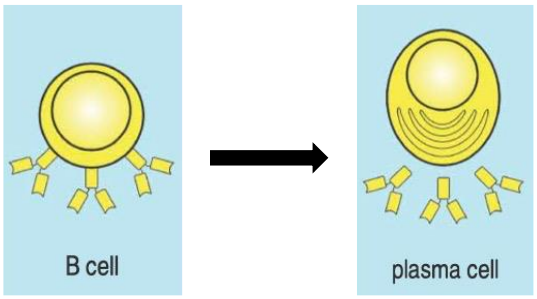

Figure 15 Schematic representation of a B lymphocyte and its development into a plasma cell

B cells exhibit the B cell receptor on their surface, which binds specifically to a particular antigen. After binding the B cell differentiates to a plasma cell and produces specific antibodies, that are directed against the targeted antigen (7).

1.2.4 Functional specification of DC subsets

DC can first be subdivided into two different subclasses: conventional DC (cDC) and plasmacytoide DC (pDC). cDC per se are also further subdivided into cDC1 and cDC2. Depending on the expression of surface markers, further subsets with different functions can be distinguished in the individual subgroups.

1.2.4.1 cDC vs pDC

cDC, previously called myeloid DC, orchestrate immune responses and therefore act as a bridge between innate and adaptive immunity. They comprise two major subsets: type-1 cDC and type-2 cDC. As professional APCs, type-1 cDC mainly contribute to the activation and polarization of naïve T cells, for instance the priming of naïve $CD8^+$ T cells or the polarization of naïve $CD4^+$ T cells into Th1 cells. On the other hand, type-2 cDC seem to comprise a heterogenous population with their main function being the presentation of exogenous antigens to $CD4^+$ T cells for the initiation of T helper differentiation into Th2 or Th17 cells (23). Type-1 cDC and type-2 cDC can be distinguished based on their surface markers, but in our experiments we examined the totality of cDC without differentiating between type-1 cDC and type-2 cDC. cDC are the main subject of this thesis and are therefore meant by DC.

Contrary to cDC, pDC have little or no antigen presentation activity, as they are less efficient in priming of naïve T cells. However, they express the intracellular receptors TLR (Toll-like receptor)-7 and TLR-9, which can sense viral infections. Those receptors combined with the ability of pDC to secrete large amounts of antiviral interferons give them a pivotal role in fighting viral infections (7).

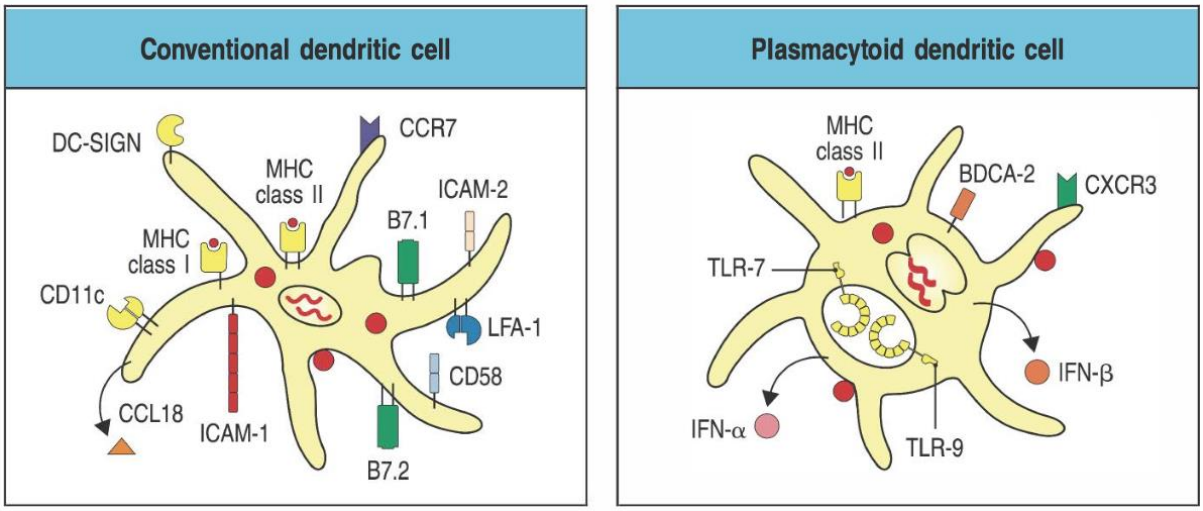

Figure 16 Schematic illustration of a conventional (cDC) and a plasmacytoid (pDC) dendritic cell

cDC and pDC have different roles in the immune response. cDC with their various subsets are primarily concerned with the priming of naïve T cells and can therefore process antigen efficiently for antigen presentation to naïve T cells. pDC are sentinels primarily for viral infections secreting large amounts of antiviral interferons. While they are less efficient in priming naïve T cells, pDC possess the Toll-like receptor 7 and 9 for sensing viral infections.(7)

1.2.4.1.1 cDC subsets and their functional specialization in the intestine

In the intestine, the subdivision into DC subsets is also based on surface markers of DC. Here, CD103 (integrin αE) and CD11b are of particular importance. Depending on the expression of CD103 and CD11b, DC in the intestine can be further subdivided into four different subsets: $CD103^+$/ $CD11b^+$ (double positive), $CD103^+$/ $CD11b^-$, $CD103^-$/ $CD11b^+$ and $CD103^-$/ $CD11b^-$ (double negative) DC. Those subsets all differ in their functions and their distribution alongside the intestine and the mLN. It is hypothesized, that the difference in the distribution is due to the presence of distinct microbiota at each site. In the LP of the small intestine, $CD103^+$/ $CD11b^+$ is the dominant cell subset, followed by $CD103^+$/ $CD11b^-$ and $CD103^-$/ $CD11b^+$, while double negative DC provide the smallest DC subset. In the LP of the colon, $CD103^+$/ $CD11b^-$ and $CD103^-$/ $CD11b^+$ are the major subsets, followed by $CD103^+$/ $CD11b^+$. Again, double negative DC provide the smallest subset (12, 24, 25).

DC in mLN are predominantly composed of $CD103^+$ DC, which consist of approximately equal numbers of $CD103^+$/ $CD11b^+$ and $CD103^+$/ $CD11b^-$ DC. Only a minor population of $CD103^-$ DC can be found in mLN, most of which are $CD11b^+$. In Peyer's patches $CD103^+$/ $CD11b^-$ is the major DC subset.

While all four different DC subsets have distinct functions within the intestine and the mLN, they all share the ability to acquire Antigen and migrate to the mLN via lymph in a CCR7-dependent manner to activate naïve T cells

In general, CD103$^+$ DC appear to have a predominantly tolerogenic function. After their migration to the mLN, the main function of CD103$^+$/ CD11b$^+$ DC is the retinoic acid (RA) and TGF-β dependent induction of regulatory FoxP3$^+$ CD4$^+$ T cells. Therefore, those DC take advantage of their expression of the enzyme retinaldehyde dehydrogenase type 2 (RALDH2), which converts retinal to retinoic acid (RA). CD103$^+$/ CD11b$^+$ DC also play a role in priming of Th17 cells and in the production of IL-6 and IL-23 (24, 26).

The main function of CD103$^+$/ CD11b$^-$ DC in the mLN is the cross-presentation of soluble antigens. Like double positive DC, they may also contribute to the induction of FoxP3$^+$ Tregs via their high-level expression of aldehyde dehydrogenase (ALDH), which allows them the metabolization of vitamin A into RA (24).

In contrast to CD103$^+$ DC, CD103$^-$ intestinal DC appear to have a more immunogenic phenotype. CD103$^-$/ CD11b$^+$ DC can prime the development of IFN-γ and IL-17 producing T cells (i.e., Th1/ Th17 cells), even in the absence of overt activation. In addition, they are also capable of producing proinflammatory cytokines like IL-6 (27). Meanwhile, the in vivo function of CD103$^-$/ CD11b$^-$ DC is currently unclear. However, those DC induce IL-17 production from CD4$^+$T cells in vitro.

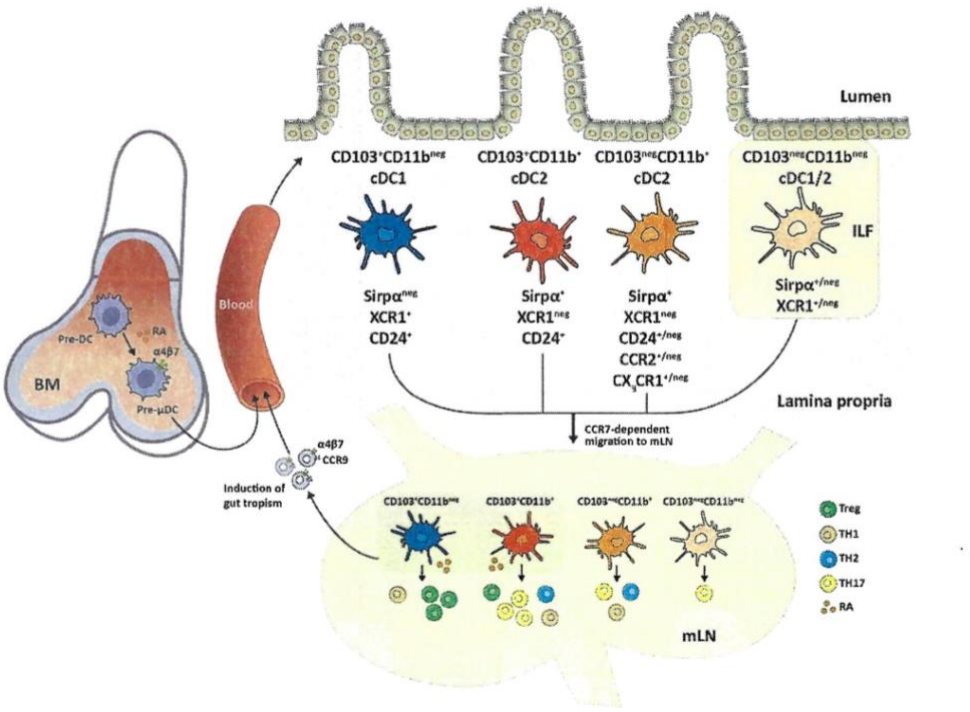

Figure 17 Evolution and function of intestinal DC subsets

After leaving the bone marrow as pre-DC, they migrate through the blood into the intestine. There they differentiate into one of four different subsets, which are distinguished by their expression of CD11b and CD103. These subsets all can take up antigen and subsequently migrate to the draining LN to activate naïve T cells. The different subsets differ in the T cells induced, with CD103$^+$ DC supporting the development of Tregs via the secretion of RA while CD103$^-$ DC rather activate effector T cells (24, 25, 28).

1.3 Intestinal inflammation

1.3.1 Inflammatory bowel diseases

Chronic inflammatory bowel diseases are characterized by recurring or permanent active inflammation in the small and large intestine. Those diseases are numbered as autoimmune diseases. The most frequently occurring inflammatory bowel diseases are Crohn's disease (CD) and ulcerative colitis (UC). In Germany, UC occurs with an incidence of ca. 4/100000 residents per year, whereas CD shows 7 cases per 100000 residents per year (29, 30).

UC and CD differ slightly in inflammatory pattern and disease symptoms: Whilst UC results in a non-interrupted mucosa restricted inflammation only of the large intestine and mostly the rectum, CD leads to a discontinuous transmural inflammation in the whole intestine and often becomes manifest in the terminal ileum. And whereas UC leads to bloody diarrhea, abdominal pain and fever, CD patients are rather suffering from non-bloody diarrhea, weight loss and abdominal pain (29, 30).

Both diseases also increase the risk of developing colonic carcinomas and other gastrointestinal diseases, such as fistulas or abscesses. The risk of contracting other autoimmune diseases, for example autoimmune arthritis, is also increased (10, 31).

Regarding therapy, in an acute episode, both diseases are treated with anti-inflammatory drugs like Prednisone, immune modulators like azathioprine or an anti-TNF-α antibody to induce remission. In the long term, UC can be curatively treated by an excision of the whole large intestine. However, there is currently no curative treatment available for CD (29, 30).

The aetiology of both diseases is unknown, but there are many genetic and non-genetic risk factors, which are discussed to play a role in the development of inflammatory bowel diseases. The most important risk factor for the development of UC and CD appears to be a positive family anamnesis. However, most of the IBD cases are not caused by familial disposition but are sporadic diseases. Those diseases appear to be precipitated by environmental trigger (diet changes, stress, antibiotic use) or alterations within the enteric immune system.(31)

The pathogenesis of IBD is characterized by a dysregulation of the intestinal immune system, resulting in the loss of tolerance and an inappropriate inflammatory response against common antigens and intestinal microbes. The dysregulation triggers a chain reaction, leading to the activation of immune cells, which subsequently invade the intestinal mucosa and increase their production of pro-inflammatory cytokines. This causes an inflammatory reaction in the mucosa, resulting in a massive expansion of the lamina propria and epithelial damage, increasing the permeability of the intestinal mucosa. Subsequently, significantly more pathogens can invade the mucosa, which again results in further activation of intestinal immune cells. This creates a vicious circle, leading to severe epithelial damage and massive intestinal inflammation (10, 31).

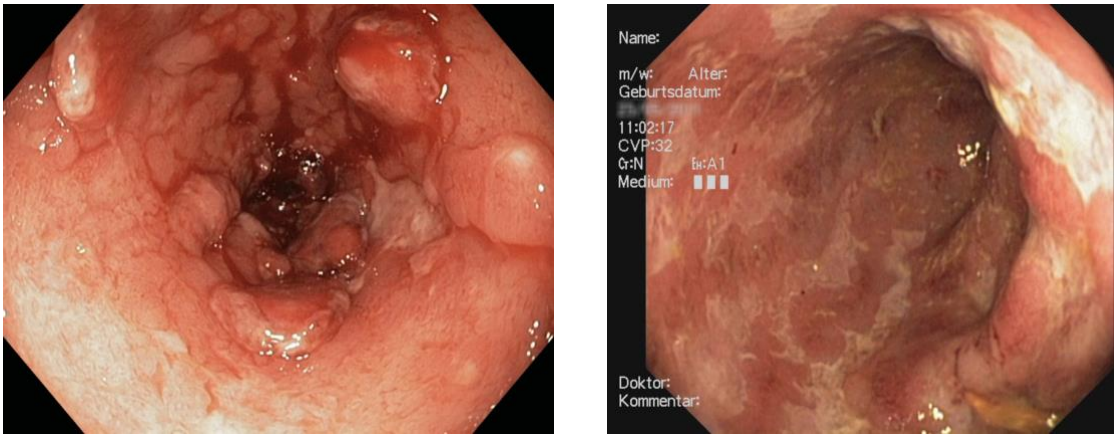

Figure 18 Endoscopic pictures of Ulcerative colitis (left) and Crohns disease (right)

On these endoscopic images of chronic inflammatory bowel diseases, the sections of the intestinal mucosa with inflammatory changes are particularly clearly visible. Traces of blood and fibrin deposits can also be seen on the intestinal mucosa (29, 30).

T cells are probably also involved in the initial dysregulation of the immune system, especially $CD4^+$ T cell subgroups. Thus, analysis of cytokine production by intestinal $CD4^+$ T cells in patients with IBD displayed increased production of pro-inflammatory cytokines such as IL-17 and IFN-γ. When the inflammatory response is triggered after the initial dysregulation, the differentiation of naïve T cells into Th1 and Th2 T cells also contributes to inflammation as they produce increased pro-inflammatory cytokines (10, 31).

Overall, much is still unknown about the detailed development and pathogenesis of IBD, and there are no good long-term treatment options yet, so a lot more research in this area is required.

1.3.2 **Murine models of intestinal inflammation**

To better study and understand the development and progression of IBDs, various experimental animal models for IBD have been established over the past decades. This yielded a progress in the understanding of mucosal immunity. Due to the complexity of the human disease, the results in the experimental animal models cannot be transferred to humans completely, but they are a valuable tool to investigate disease aspects, which are difficult to address in humans, for example, the pathophysiological mechanisms in the early stage of colitis (32).

Furthermore, mice do not only have a high genetic similarity with humans, but the functionality and structure of the murine immune system is quite like the human immune system.

In general, a good experimental model for IBD in mice should mimic human IBD and should exhibit similar characteristics as UC and CD. Additionally, it should be simple to use, reproducible, and not expansive. Expectedly, there is no murine model available, which would

fulfill all those criteria. But there are still several different models to induce IBD-like colitis in animals, especially in mice. Those mouse models all differ in their way of colitis induction, but most of them require the existence of commensal enteric bacteria to initiate and perpetuate colonic inflammation. For our experiments, we used three different paths to induce colitis in mice, which will be explained in more detail during this chapter. The first one is the DSS induced colitis, where administration of dextran sulfate sodium, an exogenous colitogenic chemical substance, causes damage to the epithelium of the intestine resulting in the development of colitis. Next, the intraperitoneal application of an anti CD40 antibody in T cell deficient mice can also induce colitis via the activation of CD40 signalling in macrophages and innate lymphoid cells resulting in a massive production of inflammatory cytokines. The third path that we used to induce colitis in mice is an adoptive T cell transfer model, where defective T cell mediated regulation results in intestinal inflammation (33-35).

1.3.2.1 DSS induced colitis

The induction of colitis with dextran sulfate sodium (a chemical colitogen) displays some similarities to human IBD in pathology, pathogenesis, etiology and therapeutic response. Furthermore, the induction of colitis via the administration of dextran sulfate sodium is cheap and the handling is quite simple (36, 37).

Oral administration of 2-10% DSS with the drinking water for several days is the most common way to induce colitis, even though DSS could also be administered rectally. Because DSS is directly toxic to gut epithelial cells of the basal crypts, it is reducing the integrity of the mucosal barrier resulting in an infiltration of pathogens into the intestinal mucosa and a massive immune response. This immune response develops based on cells of the innate immune system (among other macrophages and neutrophil granulocytes). Subsequently, those immune cells produce proinflammatory cytokines, which in turn results in the activation of T cells and acceleration of the immune reaction. However, the DSS model also works in T cell deficient mice, so the emergence of the immune response is not completely dependent on T cells. Although T cells contribute to the inflammatory process and can aggravate the inflammatory responses, they are not primarily responsible for inflammation (34-37). Depending on the given DSS dose and if DSS was given for one or for several cycles (e.g., five cycles of 7 days DSS, 14 days water), the mice develop active or chronic colitis. The colitis is characterized by bloody diarrhea, weight loss, ulcerations/erosions, decreased movements, shortening of the colon and infiltration of granulocytes (34, 36).

1.3.2.2 Anti CD40 colitis

This colitis model is quite useful to study the role of innate immunity in colon inflammation and IBD, as it is driven only by cells of the innate immune system. Furthermore, it is reproducible,

and the handling is quite simple (38). CD40 (Cluster of differentiation 40) is a costimulatory protein, which is highly expressed on antigen presenting cells in the colonic lamina propria and it is required for their activation. Under steady state conditions, naïve T cells bind to CD40 on APCs with their CD40L surface marker during the process of T cell activation in the LN. Stimulation of CD40 signaling with an agonist anti CD40 antibody initiates a complex innate immune cascade, resulting in the activation of innate immune cells and the development of colitis in T cell deficient mice. This is presumably driven by IL-23-producing DC, gut resident $CX3CR1^+$ macrophages and IL-22-producing group 3 innate lymphoid cells. The massive activation of those innate immune cells results in an increased level of proinflammatory cytokines in the proximal colon, an excessive infiltration of innate immune cells, loss of goblet cells, diarrhea and weight loss (38-40).

1.3.2.3 T cell transfer colitis

This model of colitis relies on the transfer of selected subsets of cells (mostly T cells), making it particularly useful for analyzing the function of the transferred subsets and how those subsets contribute to the pathogenesis of IBD. However, colitis takes 5-10 weeks to develop in this model, making it more elaborate than the other models.

The adoptive transfer of naïve $CD4^+$ CD45RBhigh T cells from healthy donor mice into histocompatible, T cell deficient mice results in a primarily colonic inflammation and a wasting disease 5-10 weeks after transfer. Mice receiving those naïve $CD4^+$ CD45RBhigh T cells develop transmural inflammation, diarrhea and weight loss as a result of transfer (32, 34, 37). The induction of colitis is presumably driven by the disruption of T cell homeostasis, as most of the transferred naïve T cells (lacking Tregs) differentiate to proinflammatory Th1 and Th17 cells, but not Tregs. Even if the naive T cells can differentiate into Tregs, this does not happen in time to prevent the expansion of effector T cells. Therefore, Tregs can only delay the progression of colitis, but not stop it completely.

In contrast, transfer of mature $CD4^+$ CD45RBlow T cells or cotransfer of naïve and mature T cells (containing Tregs) into the recipient did not result in colitis. Thus, Tregs appear to prevent onset of gut inflammation and antigen-specific immune responses when transferred together with pathogenic $CD4^+$ CD45RBhigh T cells. (32, 34, 35, 37)

1.4 β-Catenin

β-catenin is a central aspect of the canonical Wnt/ β-catenin signaling pathway that exerts a dual function in gene transcription and the regulation of cell- cell adhesions. Therefore, two different pools of β-catenin exist, one is part of the Wnt/ β-catenin signaling pathway, the other one is linked with E- Cadherin. Those pools will be elucidated later in this chapter.

1.4.1 Structure of β-Catenin

The β-catenin protein consists of 781 amino acids, which are split into three main parts: the first part is the NH2- terminal portion, consisting of approximately 130 amino acids. The second part is the central region, that consists of 550 amino acids containing 12 armadillo (arm) repeats. An armadillo repeat comprises a sequence of 42 amino acids.

The last part is the COOH- terminal region consisting of 100 amino acids (41). Those three regions all differ regarding their function: As the NH2- terminal domain contains several exons including the 3rd exon, which is the phosphorylation site for glycogen synthase kinase 3 (GSK-3), casein kinase I (CK1) and tyrosine kinases, this region is important for regulating cellular β-catenin-levels. The central region including the arm repeats is the binding site for many proteins, such as cadherins, adenomatous polyposis coli (APC) and Tcf- family transcription factors. The acidic COOH- terminal region serves as a transactivator, that is required for gene activation by β-catenin, when β-catenin is recruited into the nucleus to bind DNA and affect gene transcription. (41)

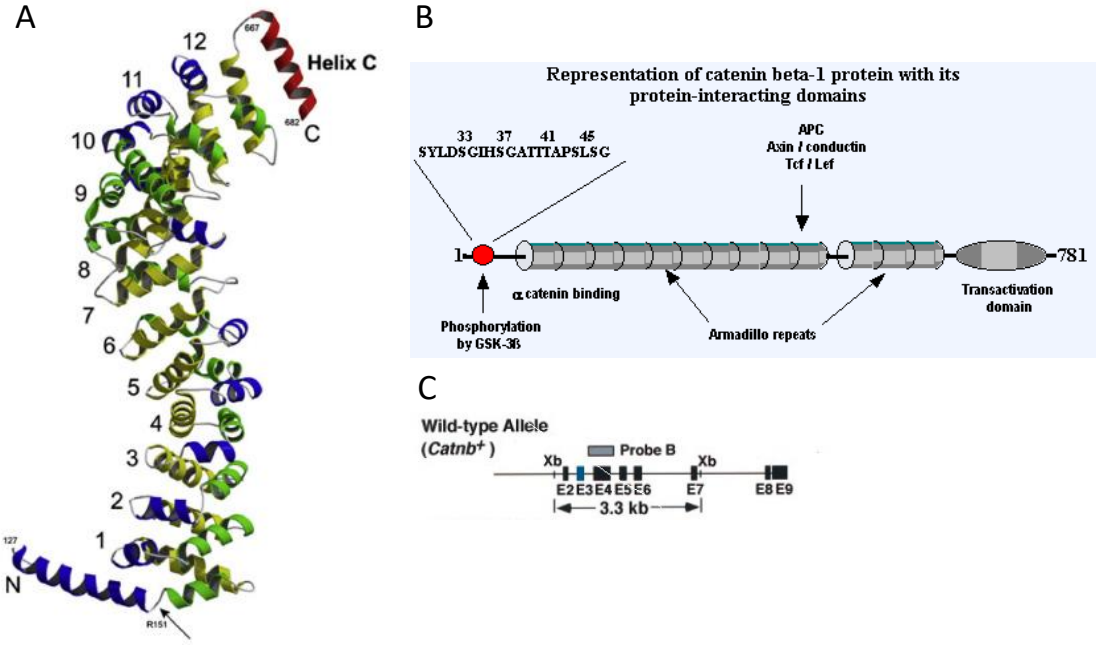

Figure 19 Structure of β-Catenin

(A) Crystal structure of full-length zebrafish β-catenin. N-terminal helix is colored in red, C-terminal helix is colored in blue. In between (Number 1-12) are armadillo repeats. (B) Interaction domains of the β-

catenin protein. On one side the N-terminal region with the phosphorylation site, on the other side the C-terminal region as transactivation domain. In between armadillo repeats. (C) Wild-type allele of the N-terminal region of β-catenin with exons as black boxes. Exon 3 was marked in blue because phosphorylation by GSK3 and CK1 occurs there A: (42), B: (43), C: (44).

1.4.2 The Wnt/ β-Catenin signaling pathway

One function of β-catenin is its involvement in gene transcription, as it is the central component of the canonical Wnt/ β-catenin signaling pathway, which is widely expressed in immune cells. The canonical Wnt/ β-catenin signaling pathway regulates developmental processes and key cellular functions such as proliferation, differentiation, migration and cell fate. Several cells secrete Wnts (wingless-related integration site), cysteine-rich glycoproteins, into the extracellular matrix, where those Wnts activate receptor-mediated signaling in cells of their direct environment. Wnts can not only activate the so called “canonical” β-catenin dependent pathway, but also several β-catenin independent pathways, which are therefore called “noncanonical” and play a role in the regulation of cellular polarization and migration (45-47).
In the absence of Wnt ligands, cytoplasmic β-catenin, which is produced constitutively, is continuously degraded. Therefore, it is recruited into a destruction complex, consisting of scaffolding protein Axin, APC, GSK-3 and CK1. This results in the phosphorylation of the amino terminal region of β-catenin by GSK-3 and CK1. Subsequently, β-Trcp, and ubiquitin ligase E3 subunit, recognizes the phosphorylated β-catenin, inducing the ubiquitylation and proteasomal degradation of β-catenin. (45, 48)
As the continuous degradation of β-catenin prevents β-catenin from reaching the nucleus, the Wnt target genes remain therefore repressed by the DNA-bound T cell factor (TCF) and lymphoid enhancer-binding factor (LEF). TCF and LEF are transcription factors, which are associated with corepressors, such as transducing-like enhancer (TLE)/ Groucho and histone deacetylases (HDAC). Summing up, in the absence of Wnt ligands, the cytoplasmic and nuclear levels of β-catenin remain low (45, 48).

The presence of Wnt ligands results in the activation of the Wnt/ β-catenin signaling pathway. Wnt ligands bind to the transmembrane Frizzled receptor and its coreceptors, low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6). This binding leads to the formation of a complex consisting of scaffolding protein Dishevelled (Dvl), the Wnt signal, the Frizzled receptor and LRP5/6. The formation of this complex results in phosphorylation and activation of LRP6, which recruits the Axin complex, which is usually responsible for β-catenin degradation. Subsequently, the Axin complex binds to LRP5/6 and gets degraded itself. This prevents β-catenin from Axin-mediated phosphorylation and degradation and leads to the stabilization of β-catenin. Following, β-catenin accumulates in the cytoplasm and migrates to

the nucleus, where it forms complexes with TCF/ LEF and activates the transcription of Wnt target genes (45, 46, 48).

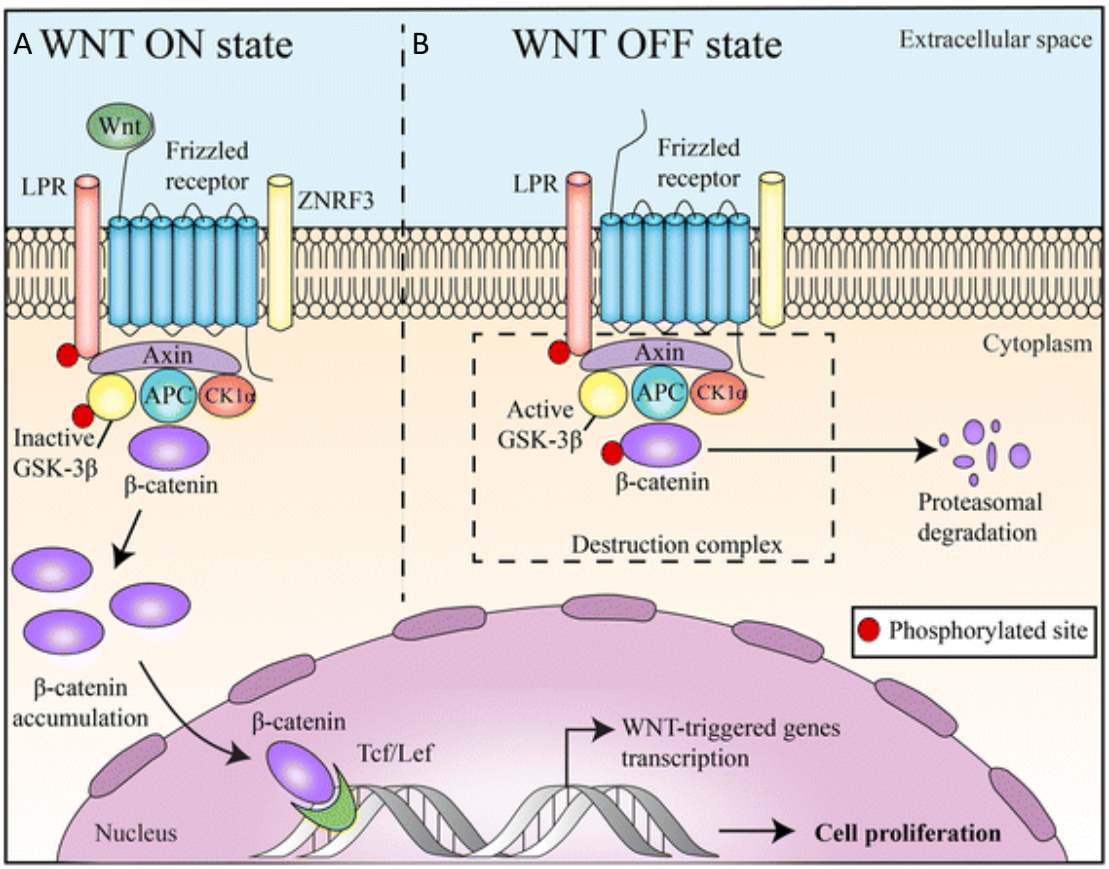

Figure 20 Wnt/ β-Catenin signaling pathway

(A) In presence of a Wnt signal, the complex containing Axin, APC, CK1 and GSK3, which usually degrades β-catenin, dissolves and β-catenin therefore accumulates. It then migrates to the nucleus, where it interacts with TCF/LEF and activates the transcription of the Wnt target genes. (B) In absence of Wnt signal, β-catenin is recruited into the destruction complex, where it gets phosphorylated. Subsequently, it gets ubiquitinated through β-Trcp and degraded. Thus, the Wnt target genes remain repressed by TCF/LEF and corepressors (46).

The Wnt/ β-catenin signaling pathway is also involved in the regulation of CD44. CD44 is not only important for cell adhesion and cell-cell interactions, but also for activation and function of T cells. The activation of T cells results in an increased expression of CD44 on their surface. In Tregs, the increased expression of CD44 is associated with an increased expression of the anti-inflammatory cytokine IL-10. This in turn suggests, that $CD44^{high}$ Tregs are more potent in suppressing effector T cell proliferation than $CD44^{low}$ Tregs (49, 50).

As the Wnt/ β-catenin signaling pathway is not only essential for a variety of developmental processes but also for the homeostasis of self- renewing adult tissues, the target genes of the Wnt/ β-catenin signaling pathway are cell- and context- specific and diverse. Therefore, the β-catenin concentration must be tightly controlled and regulated (48, 51).

1.4.3 Cell adhesion mediated by the E-Cadherin/ β-Catenin complex

The second function of β-catenin is its involvement in the regulation of epithelial cell- cell adhesions. Therein, β-catenin is part of a protein complex, that forms so-called adherens junctions, which are essential for the formation of complex tissues. This complex consists of β-catenin, E- Cadherin and α-catenin. E- cadherin is a trans membranous protein, which is capable of shaping cell- cell adhesions via forming extracellular homophilic bindings with E-Cadherin of other cells. In those so-called adherens junctions, E- Cadherin is pivotal for keeping epithelial cells together. For forming those adherens junctions, E- Cadherin recruits β-catenin to his intracellular binding site. β-catenin in turn, associates with α-catenin, a highly dynamic protein, which is connected to the actin filaments of the cell. In that way, E- Cadherin is linked with the actin cytoskeleton (52-54).

As β-catenin, which is linked to E- Cadherin cannot be phosphorylated and proteasomally degraded, the pool of β-catenin connected to E- Cadherin is protected from degradation. However, disruption of E- Cadherin mediated cell- cell contacts (for instance by mechanical trauma) destructs the connection between E- Cadherin and β-catenin, resulting in the release of β-catenin into the cytoplasm, where it accumulates. Although this source of β-catenin is not related to the Wnt/ β-catenin signaling pathway, it can still translocate into the nucleus and activate the transcription of Wnt target genes. In summary, there are two different pools/ sources of β-catenin: the Wnt/ β-catenin signaling pathway and E- Cadherin/ β-catenin cell-cell adhesions. Via both pathways, an accumulation of β-catenin in the cytoplasm is possible, resulting in the migration of β-catenin into the nucleus and transcription of Wnt target genes (53).

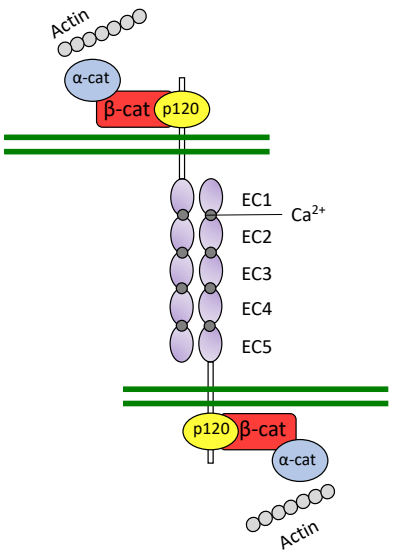

Figure 21 E-Cadherin/ β-Catenin complex

Together with E-Cadherin and α-catenin, β-catenin forms a protein complex. E-Cadherin in turn can form a homophilic binding with E-Cadherin from other cells, this bond is called adherens junction. These are important for the cohesion of tissues. If the intercellular bond between the E-Cadherins is destroyed, the β-catenin bound to E-Cadherin is released into the cytoplasm where it accumulates. Subsequently it migrates into the nucleus and activates the transcription of Wnt target genes (Image courtesy of Dr. A. Brand).

1.4.4 β-Catenin promotes a tolerogenic phenotype in DC

The maturation of DC after exposure to microbial products or foreign antigen plays a critical role in initiating the immune response or the induction of tolerance (55).

Jiang et al (53) found, that maturation of DC can also occur under steady state conditions, when it's triggered by alterations in E-Cadherin mediated DC-DC adhesion. Thus, in vitro selective physical disruption (cluster disruption, CD) of bone-marrow DC clusters and accordingly those adhesions resulted in a maturation of DC with upregulation of co-stimulatory molecules such as MHCII and chemokine receptors. This maturation process therefore seems comparable with DC maturation induced by microbial products. Jiang et al also exhibited, that disruption of E-Cadherin mediated adhesions by CD activates the Wnt/ β-catenin signaling pathway in DC, which thus at least partially contributes to these events and induces a mature DC phenotype. In further analysis, Jiang et al showed, that DC matured by CD exhibit a distinct transcriptional and functional profile compared to DC matured by contact with microbial products. Thus, these DC matured by CD could neither produce nor secrete proinflammatory cytokines in vitro. In addition, they also elicited distinct T cell responses in vivo compared to DC matured by TLR activation through contact with microbial peptides. While the DC matured by CD and TLR activation could generate IFN-γ-producing $CD4^+$ T cells, DC matured only by CD failed to prime $CD4^+$ T cells to produce immunostimulatory IFN-γ. However, DC matured only by CD generated high amounts of IL-10-producing regulatory T cells. They also failed to generate a substantial antibody response and therefore seem to be not immunogenic.

Thus, Jiang et al. hypothesized, that DC matured by CD could induce peripheral tolerance. To test this hypothesis, they asked if the injection of DC matured by CD could prevent the induction of experimental autoimmune encephalomyelitis (EAE, an animal model of multiple sclerosis) in mice. And in the mice, that received DC matured by CD, the development of EAE was prevented, suggesting that the DC had induced peripheral tolerance to the peptide causing the neuropathology. Therefore, the research group hypothesized, that maturation induced by the loss of E-Cadherin mediated adhesions results in DC exhibiting a functionally tolerogenic phenotype. And since CD activated the Wnt/ β-catenin signaling pathway in DC, Jiang et al. considered, if and to what extent β-catenin contributes (at least partially) to the generation of DC with a tolerogenic phenotype (53).

The study of Manicassamy et al. in 2010 (56) further promoted this consideration. They observed, that the Wnt/ β-catenin signaling pathway in intestinal DC is pivotal for regulating the balance between inflammatory and regulatory responses in the intestine. Manicassamy et al. demonstrated, that the β-catenin signaling pathway is constitutively active in intestinal DC. To further assess the role of β-catenin signaling in intestinal DC, they generated a mouse line with specifically abrogated β-catenin in $CD11c^+$ cells, thus in DC and macrophages. This

mouse line is called CD11c-βcat^{DEL} mice. Subsequently, they compared the frequencies of several T cell subsets from CD11c-βcat^{DEL} mice with wildtype (β-cat$^{fl/fl}$) mice and exhibited, that CD11c-βcat^{DEL} mice had lower frequencies of Tregs (FoxP3^{+} CD4^{+}) in the SI-LP, the LI-LP and the caecum. In contrast, the frequency of Th1 (IFN-γ^{+} CD4^{+}) and Th17 (IL-17^{+} CD4^{+}) cells in the SI-LP and LI-LP was increased in CD11c-βcat^{DEL} mice compared to wildtype mice. Furthermore, they showed, that LP-DC from CD11c-βcat^{DEL} mice produced higher amounts of the pro-inflammatory cytokines IL-6 and IL-23a and lower amounts of RA and the anti-inflammatory cytokines IL-10 and TGF-β in comparison with DC from wildtype mice (56).
While those pro-inflammatory cytokines promote the differentiation of Th1 and Th17 cells, RA and the anti-inflammatory cytokines are crucial for the induction of Tregs and the suppression of Th1 and Th17 responses. Thus, they estimated, that β-catenin signaling in intestinal DC is pivotal to produce anti-inflammatory cytokines and therefore for the induction of Tregs and suppression of Th1 and Th17 responses.

An imbalance of Treg, Th1 and Th17 responses and populations can result in the dysregulation of the intestinal immune system, which in turn contributes to the development of inflammatory bowel diseases (IBD). After Manicassamy et al. determined an impact of an CD11c specific β-catenin deletion on T cell responses under steady state conditions, the team also wanted to elucidate the impact of an CD11c specific β-catenin deletion under inflammatory conditions to further investigate a possible role of β-catenin in the development of IBDs. Therefore, they subjected CD11c-βcat^{DEL} mice to DSS induced colitis, a mouse model of IBD. In comparison with wildtype mice, CD11c-βcat^{DEL} mice lost significantly more weight in response to DSS treatment. In addition, comparable with the steady state, DSS treated CD11c-βcat^{DEL} mice also had lower frequencies of Tregs and increased frequencies of Th1 and Th17 cells compared with DSS treated wildtype mice. Coherent with this, the histological analysis of colon tissue displayed a considerable increase in inflammatory cell infiltration, edema, epithelial cell hyperplasia and loss of goblet cells in CD11c-βcat^{DEL} mice compared with wildtype mice.

Based on these results, Manicassamy hypothesized, that β-catenin signaling in intestinal DC promotes a regulatory DC phenotype and via its impact on T cell differentiation, β-catenin is important for balancing the intestinal T cell homeostasis. Therefore, β-catenin appears to play an essential role in the regulation of immunity and tolerance in the intestinal immune system (56).

Manicassamy's hypothesis on β-catenin is supported by results from other experiments using mice with CD11c specific (i.e., in DC and macrophages) alterations in the β-catenin expression.

For example, analysis of mice with a CD11c-specific stabilization of β-catenin displayed an increase in the number of Tregs in the thymus and lung in the steady state (Personal communication by Prof. Dr. B. Clausen). Furthermore, Manicassamy's hypothesis is also supported by results from experiments under inflammatory conditions. For instance, in EAE (experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis), DC-specific deletion of β-catenin led to exacerbation of the disease, whereas DC-specific stabilization of β-catenin appears to protect mice from severe EAE disease (57, 58). The same applies to an asthma animal model. Here, mice with a DC-specific β-catenin deletion exhibited fewer Tregs and increased asthma exacerbation, while mice with a CD11c-specific β-catenin stabilization displayed more Tregs in the lungs and an attenuated course of asthma (Personal communication by Prof. Dr. B. Clausen). The studies in a collagen-induced arthritis model in turn displayed rather inconsistent results. Even though the CD11c-specific deletion of β-catenin led to a reduced Treg number, this had no effect on disease progression (59). But since these disease models refer to other organs, Manicassamy's results from the intestine are most relevant to us and formed the basis for our hypothesis.

However, CD11c specific Cre-mediated recombination may not only induce the deletion of target alleles in DC, but also induce deletion of target alleles in other cells which express CD11c at low to intermediate levels, for instance macrophages. Thus, CD11c specific deletion of β-catenin also results in the deletion of β-catenin in a large percentage of macrophages. As it is still unclear, if reduced expression of β-catenin affects the function of macrophages, the role of β-catenin signaling in macrophages (in this scenario) needs to be further investigated. Until the role of β-catenin in macrophages is clearly determined, it is impossible to say with certainty that the results found by Manicassamy come only from the deletion of β-catenin in DC. Therefore, to what extent β-catenin stabilization can precisely alter T cell responses in vivo remains elusive (60).

1.5 Aim of the thesis

DC and Macrophages are important to maintain immune homeostasis in the intestine. Previous work suggested that β-catenin promotes a regulatory DC phenotype and mice with a CD11c specific β-catenin deletion exhibit an increased susceptibility to DSS-induced colitis (53, 56). Whether and to what extent enhanced β-catenin signaling in myeloid APCs can alternate inflammation in the intestine remains elusive.

Therefore, the aim of this thesis is to further elucidate the detailed role of β-catenin in the regulation of the intestinal DC and T cell homeostasis. Herein, we focused on how the deletion and stabilization of β-catenin impacts DC and their subsets in their generation of T cells, especially Tregs. For this analysis, our first aim was to investigate the DC and T cell homeostasis in mice with a CD11c specific stabilization and deletion of β-catenin under steady state conditions.

Building upon the steady state results, we then wanted to better characterize the role of β-catenin signaling under inflammatory conditions. Therefore, we used three different mouse models of intestinal inflammation. First, we subjected the same mouse lines as in the steady state experiments to DSS induced colitis, to see if alterations in the CD11c specific β-catenin expression would have an impact on the course of disease and on intestinal immune homeostasis. Next, we subjected T cell deficient mice with a CD11c specific stabilization of β-catenin to anti CD40 colitis. Since colitis in this model is driven only by cells of the innate immune system, this model is quite useful to analyze the impact of Wnt/ β-catenin signaling in $CD11c^+$ cells on innate immune cells under inflammatory conditions. The third model that we used was the T cell transfer colitis model. By subjecting T cell deficient mice with a CD11c specific stabilization of β-catenin to T cell transfer colitis we hoped to get further insights on the impact of Wnt/ β-catenin signaling in $CD11c^+$ cells on T cell homeostasis, with a special focus on Tregs.

By performing all these experiments, our aim was to get a better understanding of the role of Wnt/ β-catenin signaling in intestinal DC and its impact on regulating the intestinal immune homeostasis. In addition, we wanted to investigate if and to what extent Wnt/ β-catenin signaling in intestinal DC contributes to the generation of a tolerogenic DC phenotype.

2 Materials and Methods

2.1 Chemicals and reagents

2.1.1 Chemicals

Table 1: Chemicals

Chemical	Supplier
Acetone	Merck, Darmstadt, Germany
Agarose, electrophoresis grade	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Anti-CD40 antibody	BioXCell, West Lebanon, USA
BD Cytofix/ Cytoperm Fixation and Permeabilization solution	Thermo Fischer Scientific, Schwerte, Ger
Collagenase Typ IV	Worthington Biochemical, Lakewood, USA
Dextran sulfate sodium salt (DSS), colitis grade (36.000-50.000 M.Wt.)	MP Biomedicals, Santa Ana, USA
Dithiothreitol (DTT)	Sigma-Aldrich, Steinheim, Germany
100bp+ DNA marker	Fermentas, Thermo Fischer Scientific, Schwerte, Ger.
DNAse I (50U/ml)	Sigma-Aldrich, Steinheim, Germany
E Bioscience Fixation/ Permeabilization Concentrate	Thermo Fischer Scientific, Schwerte, Ger
Ethanol absolute	AppliChem, Darmstadt, Germany
Ethylendiaminetetraacetic acid (EDTA)	Fluka Chemie GmbH, Switzerland
Eye and nose ointment, Bepanthene	Bayer AG, Leverkusen, Germany
Fetal calf serum (FCS)	Thermo Fischer Scientific, Schwerte, Germany
Hank's buffered saline solution (HBSS) -/- (without Calcium and Magnesium)	Thermo Fischer Scientific, Schwerte, Germany
Hank's buffered saline solution (HBSS) +/+ (with Calcium and Magnesium)	Thermo Fischer Scientific, Schwerte, Germany
1M HEPES	Sigma-Aldrich, Steinheim, Germany
Isoflurane (Forene)	AbbVie, Ludwigshafen, Germany
Isopropyl alcohol	Hedinger, Stuttgart, Germany
10% Ketamine solution	Hameln Pharma plus GmbH, Hameln, Germany
Penicillin/Streptomycin	Thermo Fischer Scientific, Schwerte, Germany

Continuation Table: 1 Chemicals 1

Chemical	Supplier
Percoll	Sigma-Aldrich, Steinheim, Germany
Phosphat buffered saline (PBS) Dulbecco‘s -/- (-$CaCl_2$ –$MgCl_2$)	Sigma-Aldrich, Steinheim, Germany
Phosphat buffered saline (PBS) Dulbecco‘s +/+ (+$CaCl_2$ +$MgCl_2$)	Sigma-Aldrich, Steinheim, Germany
Protease Plus	Thermo Fischer Scientific, Schwerte, Germany
Roti Histofix 4%	Roth, Karlsruhe, Germany
RPMI-1640 Medium	Sigma-Aldrich, Steinheim, Germany
Trypan blue dye	Thermo Fischer Scientific, Schwerte, Germany
2% Xylazine solution (Rompun)	Bayer AG, Leverkusen, Germany
Percoll	Sigma-Aldrich, Steinheim, Germany
Phosphat buffered saline (PBS) Dulbecco‘s -/- (-$CaCl_2$ –$MgCl_2$)	Sigma-Aldrich, Steinheim, Germany

2.1.2 Buffers and solutions

Table 2: Buffers and solutions

Name	Ingredients
DNA isolation	Buffer L
	Protease Plus
Digestion mix (LN)	RPMI 1640 medium
	200U/ml Collagenase IV
	0.5U/ml DNAse
Digestion solution (Lamina propria dissociation)	1x HBSS +/+ containing 10mM HEPES
	5% FCS
FACS-buffer	2mM EDTA
	3% FCS
	1x PBS
MACS-buffer	1x PBS
	1% FCS
	2mM EDTA
PB Buffer	1x PBS
	0,5% FCS
PBS EDTA	1x PBS
	0,5mM EDTA
Percoll “100”	10% 10x PBS
	40% Percoll “100”
Percoll “40”	60% 1x PBS
	TAE buffer 50x

Continuation Table 2: Buffers and solutions

Name	Ingredients
Predigestion solution (Lamina propria dissociation)	1x HBSS -/- containing 10mM HEPES
	5mM EDTA
	5% FCS
	1mM EDTA
TAE buffer 1x	ddH_2O
	40mM Tris
TAE buffer 50x	20mM Acetic acid
	1m EDTA

2.1.3 Kits

Biotool™ Mouse Direct PCR Kit (supplier: Biotool, Munich, Germany):

- Buffer L: Lysis buffer
- Protease Plus
- 2x M-PCR OPTI Mix: Includes Taq DNA polymerase, dNTPs, $MgCl_2$, reaction buffer, loading dye (blue)

REDTaq ReadyMix (supplier: Sigma-Aldrich, Steinheim, Germany)

- REDTaq ReadyMix PCR Reaction Mix: $MgCl_2$, 20mM Tris-HCl, pH 8.3, with 100mM Kcl, 3mM $MgCl_2$, 0.002% gelatin, 0.4mM dNTP mix (dATP, dCTP, GTP, TTP), stabilizers, 0.06U/µL of TaqDNA Polymerase, red dye
- Water, PCR Reagent

FoxP3/Transcription Factor Staining Buffer Set (supplier: eBioscience, San Diego, USA)

- Fixation/Permeabilization Concentrate: is a 4x stock solution, must be diluted with Fixation/Permeabilization Diluent (1:3)
- Fixation/Permeabilization Diluent: intended to be used with Fixation/Permeabilization Concentrate.
- Permeabilization Buffer (PermWash)(10x): must be diluted to 1x with distilled water.

Lamina Propria Dissociation Kit_mouse (supplier: Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)

- Enzyme D
- Enzyme R
- Enzyme A
- Buffer A

CD11c Microbeads UltraPure (supplier: Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)

- 2 mL CD11c MicroBeads UltraPure, mouse: UltraPure MicroBeads conjugated to monoclonal anti-mouse CD11c antibodies. (Isotype hamster IgG)

CD4$^+$ T cell isolation kit mouse (supplier: Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)

- **1 mL CD4$^+$ T Cell Biotin-Antibody Cocktail, mouse:** Cocktail of biotin-conjugated monoclonal antibodies against CD8a, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC Class II, Ter-119, and TCRγ/δ.
- **2 mL Anti-Biotin MicroBeads:**
- MicroBeads conjugated to monoclonal anti- biotin antibodies (isotype: mouse IgG1).

Beads

- FACS CompBeads (BD Biosciences, Franklin Lakes, USA):
  - positive anti rat/hamster IG, κ
  - negative control anti rat/hamster
- MACS-beads: αCD11c (supplier: Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)

2.1.4 Antibodies

Table 3: Primary antibodies

Antigen	Fluorochrome	Clone	Origin	Isotype	Supplier
β-catenin	Alexa647	L54E2	Mouse	IgG1	New England Biolabs, Ipswich USA
CD3e	Alexa488	145-2C11	Hamster	IgG	Biolegend, San Diego, USA
CD3e	PE	145-2C11	Hamster	IgG	Biolegend, San Diego, USA
CD4	BV510	RM4-5	Rat	IgG2a, k	Biolegend, San Diego, USA
CD4	PE	GK 1.5	Rat	IgG2a, k	Biolegend, San Diego, USA
CD8	PE-Cy7	53-6.7	Rat	IgG2a, k	Biolegend, San Diego, USA
CD8	V500	53-6.7	Rat	IgG2a	Biolegend, San Diego, USA
CD11b	FITC	M1/70	Rat	IgG2b, k	eBioscience, San Diego, USA
CD11c	PE-Cy7	N419	Arm.Hamster	IgG	Biolegend, San Diego, USA
CD19	PE	PeCa1	Rat	IgG2a	Immunotools, Friesoythe, Germany
CD25	APC	PC61	Rat	IgG1	Biolegend, San Diego, USA
CD44	PE	IM7	Rat	IgG2b, k	eBioscience, San Diego, USA
CD44	FITC	IM7	Rat	IgG2b, k	eBioscience, San Diego, USA

Continuation Table 3: Primary antibodies

Antigen	Fluorochrome	Clone	Origin	Isotype	Supplier
CD45	BV510	30-F11	Rat	IgG2b, k	Biolegend, San Diego, USA
CD62L	APC	MEL-14	Rat	IgG2a, k	eBioscience, San Diego, USA
CD64	PE	X54-5/7.1	Mouse	IgG1, k	Biolegend, San Diego, USA
CD86	PE	GL-1	Rat	IgG2a, k	Biolegend, San Diego, USA
CD103	Alexa647	2E7	Arm.Hamster	IgG	Biolegend, San Diego, USA
CD103	PerCP-Cy5.5	2E7	Arm.Hamster	IgG	Biolegend, San Diego, USA
FoxP3	eFluor450	FJK-16s	Rat	IgG2a, k	eBioscience, San Diego, USA
MHC-II I-A/I-E	eFluor450	M5/114.15.2	Rat	IgG2b, k	eBioscience, San Diego, USA
CX3CR1	APC	SA011F11	Mouse	IgG2a, k	Biolegend, San Diego, USA
Ly6C	eFluor450	AL-21	Rat	IgM, k	BD Biosciences, Franklin Lakes, USA
B220	PE	RA3-6B2	Rat	IgG2a, k	BD Pharmingen, Germany
Fixable Life/ Dead staining					
Life/ Dead	ef780				eBioscience, San Diego, USA
FC Block			Rat		BioXCell, West Lebanon, USA

2.1.5 Consumables

Table 4: Consumables

Material	Supplier
Syringes and Needles	BD Becton Dickinson, Heidelberg, Germany
96 well plate	Thermo Fischer Scientific, Osterode am Harz, Germany
Cell culture plastic dish	Nalge Nunc International Corp., Rocherster, USA
	Greiner Bio-One GmbH, Frickenhausen, Germany
Cell strainer 70µm	Greiner Bio-One GmbH, Frickenhausen, Germany
Cell strainer 100µm	Greiner Bio-One GmbH, Frickenhausen, Germany
Eppendorf tubes 1.5ml/ 2ml	Eppendorf, Hamburg, Germany
Falcon tubes 15ml/50ml	Greiner Bio-One GmbH, Frickenhausen, Germany
GentleMACS C Tubes	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
MACS Columns	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
PCR 8 SoftStrips tubes	Biozym Scientific GmbH, Hessisch Oldendorf, Germany

2.1.6 **Primers**

Table 5: Primers

Gene	Primer name	Sequence	Base count	Product size (bp)
Actin	Actin 510 forward	5'-TGT TAC CAA CTG GGA CGA CA-3'	20	510
	Actin 510 reverse	5'-GAC ATG CAA GGA GTG CAA GA-3'	20	
β-catenin DEL	C-726	5'-AAG GTA GAG TGA TGA AAG TTG TT-3'	23	WT = 221
	C-727	5'-CAC CAT GTC CTC TGT CTA TCC-3'	21	Flox = 324
	C-728	5'-TAC ACT ATT GAA TCA CAG GGA CTT-3'	24	DEL = 500
β-catenin EX3	EX3 C-733	5'-GAC ACC GCT GCG TGG ACA ATG-3'	21	WT = 370
	EX3 C-734	5'-GTG CTG ACA GCA GCT TTT CTG-3'	21	Flox = 570
Cre -CD11c	CD11c-Cre sense/ oIMR7841	5'-ACT TGG CAG CTG TCT CCA AG-3'	20	Cre = 313
	CD11c-Cre antisense/ oIMR7842	5'-GCG AAC ATC TTC AGG TTC TG-3'	20	
Cre (general)	CRE5' new	5'-CCG GTC GAT GCA ACG AGT GA-3'	20	Cre = 235
	CRE3' new	5'-GGC CCA AAT GTT GCT GGA TA-3'	20	
TCRβ	TCRβ-12834 (TG for)	GCT ACT TCC ATT TGT CAC GTC C	24	WT TCRβ: 356bp
	TCRβ-15242 (WT for)	CCC CAC CCA GTA TAG GAC AG	20	Flox TCRβ: 460bp
	TCRβ-15243 (shared rev)	CCT CAA CCC AGA ATG ATC TTG	21	
TCRδ	TCRδ-15248 (WT rev)	AAA GAC AAA GAG GGG GCA CT	20	WT TCRδ 474bp
	TCRδ-15249 (shared for)	ACA TGG GCT TTC TGT TGT	18	Flox TCRδ 350bp
	TCRδ-oIMR2088 (TG rev)	AGA CTG CCT TGG GAA AAG CG	20	
JAX Internal controls	control for/ oIMR8744	5'-CAA ATG TTG CTT GTC TGG TG-3'	20	200
	control rev/ oIMR8745	5'-GTC AGT CGA GTG CAC AGT TT-3'	20	

2.2 Equipment

Table 6: Technical equipment

Equipment	Supplier
Advanced magnetic hotplate stirrer VMS-C7	VWR International, Radnor, USA
Aspiration system Vacusafe, Vacuboy	Integra Bioscience, Zizers, Switzerland
Cell counter CASY® Model TT	Roche Diagnostics, Rotkreuz, Switzerland
Centrifuge tabletop Mini-Star silverline	VWR International, Radnor, USA
Centrifuge Heraeus Fresco 21	Thermo Fischer Scientific, Schwerte, Germany
Centrifuge Heraeus Multifuge X1R	Thermo Fischer Scientific, Schwerte, Germany
Endoscope xenon 175	Karl Storz GmbH & Co. KG, Tuttlingen, Germany
FACS Canto II Flow Cytometer	BD Biosciences, Heidelberg, Germany
Gel-Imager: Gel Doc XR	Bio Rad, Munich, Germany
gentleMACS Octo Dissociator	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
MACS Seperators	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Microscope Olympus IX81	Olympus, Japan
Pipet ultra-high performance	VWR International, Radnor, USA
Pipetboy/Pipet filler S1	Thermo Fischer Scientific, Schwerte, Germany
Power Supply PowerPac 200 Basic	Bio Rad, Munich, Germany
Scale CS-series 200 g	Ohaus, Nänikon, Switzerland
Thermocycler T3000	Biometra, Göttingen, Germany
Thermocycler Biometra TRIO	Biometra, Göttingen, Germany
Thermomixer MKR 13 HLC by Ditabis	Ditabis AG, Pforzheim, Germany
Rotating incubator	Thermo Fischer Scientific, Schwerte, Germany
Vortexer tabletop	VWR International, Radnor, USA

2.3 Mice

I hereby confirm that I have taken note of the requirements of the Ethics Committee in accordance with the Animal Protection Act and the Genetic Engineering Act as amended and that its principles have been observed in the scientific work.

Hiermit bestätige ich, dass die Vorgaben der Ethik-Kommission gemäß Tierschutzgesetz und gemäß Gentechnikgesetz in ihrer jeweils geltenden Fassung zur Kenntnis genommen worden ist und ihre Grundsätze bei der wissenschaftlichen Arbeit eingehalten wurden.

2.3.1 The Cre/ loxP recombination system

The Cre/ loxP system is a site-specific recombinase technology, which facilitates targeted modification of chromosomal DNA in living organisms, and which is therefore very important for genetic research.

The process of recombination is characterized by the rearrangement of genetic material (DNA, RNA) in cells, resulting in the splitting and new conjunction of DNA/RNA. It is catalyzed by the enzyme recombinase and is ubiquitous in all organisms.

As the majority of the recombinase enzymes are derived from bacteriophages and yeasts, the Cre recombination recombinase (causes recombination/ cyclisation recombinase) stems from bacteriophages as well, namely the bacteriophage P1 (61).

The Cre protein is a 38-kDa recombinase enzyme consisting of four subunits and two domains. It catalyzes the recombination of DNA between specific sites in any kind of DNA molecule. Those specific sites consist of 34 bp and are called loxP sites (62, 63).

The Cre/ loxP recombination system allows the modification of different cell type specific genes in transgenic mice or just in particular tissues. As many total gene knockouts result in lethality, this is avoidable with the Cre/ loxP recombination system. Another great advantage of the Cre/ loxP recombination system is that there are no further co-factors or sequence elements required. The generation of mice with the Cre/ loxP recombination system only requires two genetically modified mouse lines. One of the transgenic mouse lines needs to express Cre either in all tissues or selectively in a special tissue, while the other transgenic mouse line needs to carry the loxP sites, which flank a target gene, but leave the gene fully functional. Crossing of both mouse lines results in mice that are deficient in the “floxed” gene (62-64).

To generate transgenic mice, there are different methods available. One method to generate transgenic mice, which is used for the creation of Cre- expressing transgenic mice, involves microinjection of the transgenic DNA construct into the pronucleus of a one-cell mouse embryo. Those embryos are then implanted into the oviduct of a pseudopregnant mouse. The transgenic founder offspring will then be bred to obtain a line of transgenic mice expressing Cre under the control of a promoter, which is specific for a particular cell or tissue type. Hence Cre is expressed only in specific tissues or cells. But additionally, one can also control the moment of recombination by ligand depending-activation of the Cre recombinase. In this case,

the Cre recombinase is only activated after application of a specific ligand (64). Another method used to introduce the loxP sites into mice is homologous recombination. In this process, the DNA target construct is inserted into embryonic stem cells, which are then implanted into normal blastocysts resulting in the birth of a chimeric offspring, which in turn will be bred to obtain a line of mice with the targeted flox gene (64).

loxP (locus of X over P1) is a site on the bacteriophage P1, which consists of 34bp. An 8bp asymmetric core of this nucleotide sequence is flanked on each side by 13bp of palindromic sequences. Because the 13bp sequences are palindromic, while the asymmetric 8bp sequence is not, the loxP sequence has a certain direction/ orientation.

One possible example for a loxP site would be:

Target sequences, which are flanked by two loxP sites are called “floxed”. After generating one mouse line with floxed genes, it can be crossed to any other mouse line expressing Cre under the control of a tissue or cell type specific promoter. In the tissue, where Cre recombinase is expressed, the DNA segment flanked by the loxP sites will be processed by the Cre recombinase (62-64).

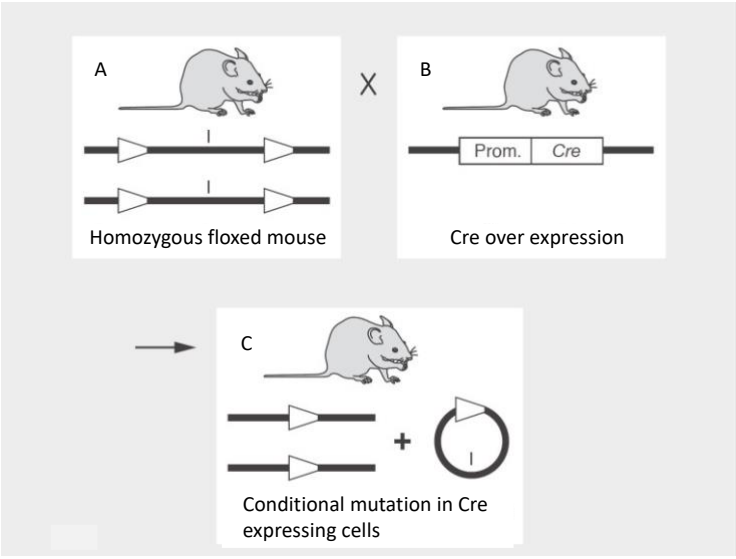

Figure 22 Conditional mutations with the Cre/ loxP recombination system

Crossing of a mouse carrying the floxed alleles (A) with a Cre expressing mouse line (B) for a stable conditional mutation. Mating of those mice should result in a mouse line that carries the targeted gene homozygously flanked by loxP sites and additionally expresses Cre (C) (65).

2.3.2 Experimental Setup

All experiments were performed with female or male mice in the age between 6-15 weeks. Furthermore, every experiment was conducted with at least 3 genetically mutated and 3 control mice. Regarding steady state experiments, we performed 3 independent experiments with CD11c-βcat^{DEL} mice (with a CD11c specific deletion of β-catenin) and 3 independent experiments with CD11c-βcat^{EX3} mice (with a CD11c specific stabilization of β-catenin). Regarding DSS colitis, we carried out two independent experiments with CD11c-βcat^{EX3} mice and four independent experiments with CD11c-βcat^{DEL} mice. The experiments with anti CD40 colitis were conducted 3 times with TCRβδ CD11c-βcat^{EX3} mice (T cell deficient CD11c-βcat^{EX3} mice). Last, the T cell transfer experiment was performed once with TCRβδ CD11c-βcat^{EX3} mice.

2.4 Methods

2.4.1 Genotyping

2.4.1.1 Deoxyribonucleic acid (DNA) Isolation

Toe or tail biopsies from the mice were used to isolate the DNA. After preparation of a digestion master mix containing 50µl of Lysis Buffer (Buffer L) and 1µl of Protease Plus per sample, 50µl of that mix was added to each biopsy into 1,5ml Eppendorf tubes. In the next step these tubes were then incubated for 55-60 minutes at 500rpm and 55°C in a thermoshaker. To inactivate the protease, the temperature of the thermoshaker was increased to 95°C after 55-60 minutes of incubation. Subsequently, the tubes were stored on ice for minimum 5 minutes to cool down.

2.4.1.2 Polymerase chain reaction (PCR)

The PCR was used to amplify the isolated DNA. Millions of copies of a specific DNA strand can be generated out of one single copy of DNA by using the PCR. For five different PCR programs five different PCR master mixes were prepared containing a ready-to-use PCR mix, DNAse free water and different primers, depending on the PCR program.

The REDTaq Ready-Mix was used for the analysis of the Cre recombinase, whereas the 2x M-PCR OPTI Mix (from the Biotool Mouse Direct PCR Kit) was used for the detection of the floxed β-catenin alleles and to determine the presence of T cell receptor alleles. The components of the different PCR mastermixes are shown in the following figure.

PCR- mix		**EX3**			
Samples:	1				
n=	1,1				
Reagent		per react.		**Mastermix**	
2x M-PCR OPTI Mix		10,0	µl	**11,0**	**µl**
100 ng/µl Primer		1,0	µl	**1,1**	**µl**
100 ng/µl Primer		1,0	µl	**1,1**	**µl**
JAX internal control for		0,3	µl	**0,3**	**µl**
JAX internal control rev		0,3	µl	**0,3**	**µl**
H2O		5,4	µl	**5,9**	**µl**
per reaction		18,0	µl	19,8	µl
DNA		2,0	µl		

PCR- mix		**bDEL**			
Samples:	1				
n=	1,1				
Reagent		per react.		**Mastermix**	
2x M-PCR OPTI Mix		10,0	µl	**11,0**	**µl**
100 ng/µl Primer		1,0	µl	**1,1**	**µl**
100 ng/µl Primer		1,0	µl	**1,1**	**µl**
100 ng/µl Primer		1,0	µl	**1,1**	**µl**
H2O		4,4	µl	**4,8**	**µl**
per reaction		18,0	µl	19,1	µl
DNA		2,0	µl		

PCR- mix		**TCRβ**			
Samples:	1				
n=	1,1				
Reagent		per react.		**Mastermix**	
2x M-PCR OPTI Mix		10,0	µl	**11,0**	**µl**
100 ng/µl Primer		1,0	µl	**1,1**	**µl**
100 ng/µl Primer 100 ng/µl Primer		1,0 1,0	µl µl	**1,1** **1,1**	**µl**
JAX internal control for		0,3	µl	**0,3**	**µl**
JAX internal control rev		0,3	µl	**0,3**	**µl**
H2O		4,4	µl	**4,8**	**µl**
per reaction		18,0	µl	19,8	µl
DNA		2,0	µl		

PCR- mix		TCRδ			
Samples:	1				
n=	1,1				
Reagent		per react.		**Mastermix**	
2x M-PCR OPTI Mix		10,0	µl	**11,0**	**µl**
100 ng/µl Primer		1,0	µl	**1,1**	**µl**
100 ng/µl Primer 100 ng/µl Primer		1,0 1,0	µl µl	**1,1** **1,1**	**µl**
JAX internal control for		0,3	µl	**0,3**	**µl**
JAX internal control rev		0,3	µl	**0,3**	**µl**
H2O		4,4	µl	**4,8**	µl
per reaction		18,0	µl	19,8	

PCR- mix		**CD11c Cre**			
Samples:	1				
n=	1,1				
Reagent		per react.		**Mastermix**	
Red Taq mix		10,0	µl	**11,0**	**µl**
100 ng/µl Primer		1,0	µl	**1,1**	**µl**
100 ng/µl Primer		1,0	µl	**1,1**	**µl**
β-actin for		1,0	µl	**1,1**	**µl**
β-actin rev		1,0	µl	**1,1**	**µl**
H2O		9,0	µl	**9,9**	**µl**
per reaction		23,0	µl	25,3	µl
DNA		2,0	µl		

Figure 23 PCR calculations

PCR master mix calculation table for β-catenin EX3, β-catenin DEL, TCRβ, TCRδ and CD11c Cre

For the identification of the mutations, different PCR programs were used. The CD11c primers and program were used to analyze, if the animals are Cre^+ oder Cre^-, whereas the B-DEL and the B-EX3 program and primers display the presence of floxed β-catenin alleles. The TCRβ and TCRδ program with the associated primers were used for the detection of T cell deficient mice (mice are only T cell deficient, if they are negative for both alleles of TCRβ and TCRδ).

PRG#	program name	pre heat lid	hold	cycle				end	hold	running time	PCR	Gel%
3	B-DEL	99	94 °C	38 cycles:	94 °C	58 °C	75 °C	72 °C	15 °C	2 h 21 min	β-catenin DEL	1,5%
			5 min		45 sec	45 sec	45 sec	5 min				
4	B-EX3	99	95 °C	38 cycles:	95 °C	62 °C	72 °C	72 °C	15 °C	2 h 02 min	β-catenin EX3	1,5%
			5 min		30 sec	30 sec	45 sec	10 min				
5	CD11C CRE	99	95 °C	40 cycles:	95 °C	63 °C	72 °C	72 °C	15 °C	2 h 13 min	CD11c Cre	1,5%
			5 min		30 sec	40 sec	45 sec	10 min				
11	TCRβ	99	95 °C 2 min	10 cycles: 30 cycles	95 °C 30 sec 95 °C 30 sec	64 °C* 30 sec 59 °C 30 sec	72 °C 45 sec 72 °C 45 sec	72 °C 5 min	15 °C	2 h 02 min	TCRβ	1,5%
12	TCRδ	99	95 °C 2 min	10 cycles: 33 cycles	95 °C 30 sec 95 °C 30 sec	64 °C* 30 sec 59 °C 30 sec	72 °C 45 sec 72 °C 45 sec	72 °C 5 min	15 °C	2h 11 min	TCRδ	1,5%
7	CRE NEW	99	95 °C	11 cycles:	95 °C	62 (-0,5)*	72 °C			3 h 08 min	CRE (general)	1,5%
			5 min		45 sec	45 sec	1 min					
				37 cycles:	95 °C	57 °C	72 °C	72 °C	15 °C			
					45 sec	45 sec	1 min	5 min				

Figure 24 PCR programs

Program (PRG#) 3 and 4 are PCR programs for β-catenin EX3 and β-catenin DEL. Program 5 is to identify the presence of CD11c Cre, while program 7 detects the general presence of Cre recombinase. Programs 11 and 12 are PCR programs for the detection of T cell deficiency.

* After each cycle the temperature decreases by -0,5 °C

2.4.1.3 Agarose gel electrophoresis

For displaying the results of the PCR, an agarose gel electrophoresis was performed with the samples amplified through the PCR. Therefore a 1,5% gel must be prepared by dissolving 4,5g Agarose in 300ml 1x TAE buffer by heating up in the microwave. After hardening, the gel was moved into a gel chamber filled with 1x TAE Buffer. Subsequently, 15µl of the PCR samples were loaded onto the gel plus a 100bp+ DNA marker, a positive control and a negative control (Milli-Q). The gel then ran for about 45 minutes at 135 volts. The gels were then analyzed under UV light with the gel doc system. Depending on the used PCR program, different bands were visible. While the CD11c Cre has a PCR product size of 313bp, the actin band has a product size of 510bp. The floxed allele of β-catenin DEL mutation displays a PCR product at 324bp, whereas the floxdel allele shows a band at 500bp and the wildtype allele at 221bp. The wildtype allele of the β-catenin EX3 mutation displays a PCR product at 370bp, whereas the floxed allele has a product size of 570bp.

Whereas the wildtype allele in the TCRβ PCR is at 356bp and the transgenic allele is at 460bp, the wildtype allele in the TCRδ PCR is at 474bp and the transgenic allele is visible at 350bp.

The band size of the Jax control is at 200bp. The genotyping results were then recorded in Excel and PyRat.

2.4.2 Identification of mutant and control mice by genomic PCR

Before the start of any experiment, genotyping is required to identify the mice into mutant mice and control mice. Control mice carry the floxed gene, but they are Cre negative ($^-$), which means, the floxed genes are not removed so the mice are considered to behave like wildtype mice. Mutated mice also carry the floxed gene, but they are Cre positive ($^+$), hence the floxed genes are removed leading to the targeted mutations, namely the CD11c specific deletion or stabilization of β-catenin. Regarding the classification of T cell deficient mice, the animals were analyzed if they were positive ($^+$) or negative ($^-$) in relation to the TCRβ and TCRδ alleles. Only TCRβ$^-$ and TCRδ$^-$ mice are T cell deficient. The process of genotyping was performed as described in chapter Genotyping.

Gel A exhibits the PCR product from samples from the CD11c-βcat^{EX3} line with a CD11c specific stabilization of β-catenin. The genotype of the mice is depending on the occurring band size. The respective PCR product for a floxP flanked allele (flanking the 3rd exon of β-catenin) has a size of 570bp, whereas the wildtype band has a size of 370bp. Hence all the shown samples on this gel are homozygously floxed. Gel B shows PCR products from samples from the CD11c-βcat^{DEL} line with a CD11c specific deletion of β-catenin. A band with the size of 324bp displays that the sample is homozygously floxed with the floxP sites flanking the β-catenin gene. The wildtype gene product would be visible at 221bp, while the floxdel allele would be visible at 500bp. Again, all the displayed examples are homozygously floxed.

Gel C depicts PCR products from a CD11c Cre specific PCR. Samples with just one actin band (at 500bp) are Cre $^{-}$, whereas samples displaying two bands (one at 510bp and one at 235bp) are Cre $^{+}$.

Gel D and E demonstrate the PCR products regarding T cell deficiency. The TCRβ transgenic gene product has a band size of 460bp, whereas the wildtype gene product has a band size of 356bp. All tested mice on this gel carry the transgenic gene product. The TCRδ transgenic gene product shows a band with the size of 350bp while the wildtype gene product has a band size of 474bp. Like Gel D, all tested mice on Gel E carry the transgenic gene product. Those mice with the transgenic gene product in the TCRβ and TCRδ PCR are T cell deficient.

After each experiment samples from the wildtype and genetically modified mice were taken to confirm the correct genotype of the experimental animals.

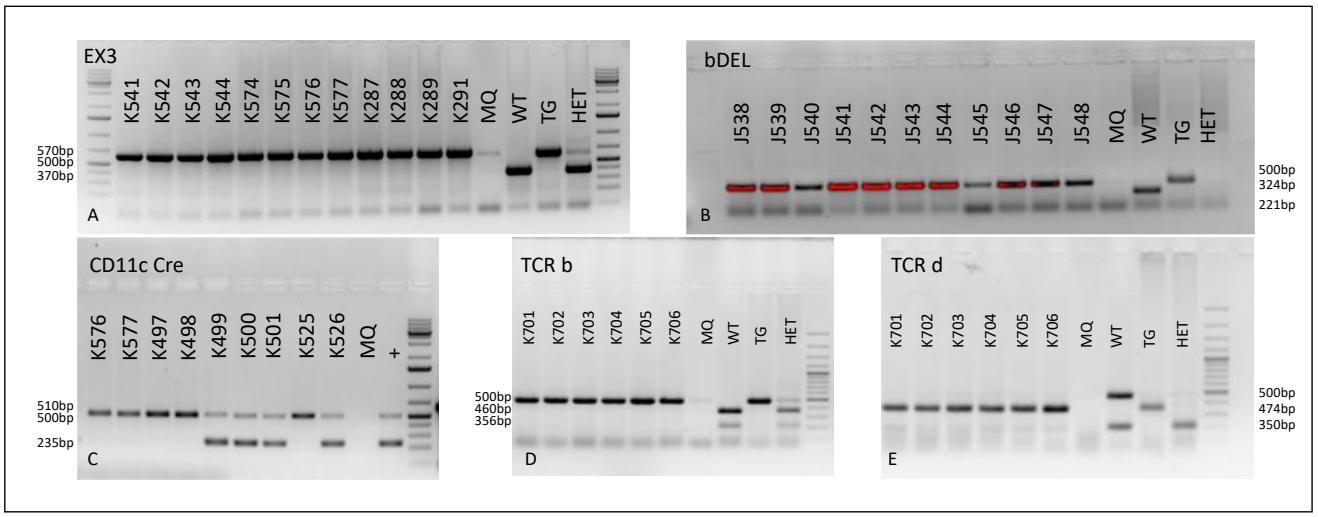

Figure 25 PCR results

(A) PCR to detect floxP sites flanking the 3rd exon of β-catenin. In this PCR all mice are homozygously floxed (band size 570bp). (B) PCR to detect floxP sites that flank the 2nd to 6th exon of β-catenin. Here all mice are homozygously floxed (band size 324bp). (C) CD11c specific Cre PCR to identify the expression of Cre under the control of the CD11c promoter. Mice K499, K500, K501 and K526 carry Cre recombinase and are thus Cre^{+}; the others are Cre- and can be used as control mice. (D) PCR to detect a TCRβ transgenic gene product. In this PCR all mice carry the TCRβ transgenic gene product. (E) PCR to identify a TCRδ transgenic gene product. Here, all mice carry the TCRδ transgenic gene product.

EX3 PCR used for identification of CD11c-βcat^{EX3} and TCRβδ CD11c-βcat^{EX3} mice, bDEL used for identification of CD11c-βcat^{DEL} mice, CD11c Cre used for identification of mutant mice, TCRb and d used for identification of TCRβδ CD11c-βcat^{EX3} mice.

MQ: Milli-Q negative control, WT: wildtype control, TG: transgene, HET: heterozygous control.

2.4.3 Isolation of primary cells

2.4.3.1 Isolation of lymph node cells

At the beginning of each experiment, mice are sacrificed by CO_2 asphyxiation. Subsequently, the target lymph nodes (mostly mesenteric lymph nodes) are isolated and put into a 1,5ml Eppendorf tube containing 20µl PBS Buffer. After collecting all the lymph nodes, 200µl of the digestion mix were added to the tubes and the lymph nodes were shred to small pieces by cutting them with small scissors. Afterwards, another 800µl of the digestion mix were added and the tubes were incubated on a thermoshaker with a temperature of 37°C at 1200rpm for 40 minutes.

Following, to separate the cell clusters 20µl of 500mM EDTA were added to the tubes incubating the cells for another 5 minutes at room temperature.

The next step is passing the cell suspension over a 70µm cell strainer into a 50ml falcon tube and washing with 10ml PBS/ 2mM EDTA.

The cells were then centrifuged for 5 minutes at 400g at 4°C and afterwards the supernatants were aspirated. After resolving the cell pellet in 1-4ml of PBS/ 2mM EDTA the cells are ready for counting. Therefore, two methods are available. First, the cells can be counted by hand with the microscope and secondly, they can be counted by the CASY (see below). Independent from the method, 10µl of the cell samples were used for counting the cells before staining for FACS analysis.

2.4.3.2 Isolation of lamina propria lymphocytes

After sacrificing the mice via CO_2 asphyxiation, the small or large intestine of the mice was removed and placed in HBSS Buffer in a petri dish. After that, the intestine was cleared of feces by washing it with HBSS Buffer and squeezing the feces out. Following the residual fat tissue and Peyer's patches are removed.

The next step is cutting the intestine first longitudinally and then laterally into small pieces, which are then transferred into a 50ml falcon tube containing 10ml of predigestion solution. The samples are then incubated for 20 minutes with a tube rotator at 37°C.

Subsequently the samples are mixed for 15 seconds with a vortex mixer and are then applied on a 100µm cell strainer, which is placed on a 50ml falcon tube.

The intestine pieces are then transferred into new 50ml falcon tubes containing predigestion solution and then incubation for 20 minutes at 37°C with the tube rotator is repeated.

Afterwards the samples are again mixed by a vortex mixer and applied on 100µm cell strainers which are placed on the same 50ml falcon tubes as in the step before.

This incubation step is repeated once again and the flow-through is once more collected in the 50ml falcon tube. During the time of the incubation, the enzyme mix can be prepared. It contains 100µl of Enzyme D, 50µl of Enzyme R, 12,5µl of Enzyme A and 2,35ml of the digestion solution per sample.

After the predigestion process, the intestinal tissue is transferred into a gentleMACS C Tube containing the enzyme mix. The samples are then incubated with the tube rotator at 37°C for 30 minutes and afterwards the tubes are placed on the gentleMACS dissociator. The next step is running the gentleMACS program m_intestine_01. After termination of this program, the tubes are shortly spun up to 300g and the supernatant after the centrifugation is aspirated. The samples are resuspended in 5 ml of PB buffer, the dispension is applied on 100µm cell strainer, which are placed on a 50ml falcon tube. After washing the strainers with 10ml of PB Buffer, the samples are centrifuged at 300g at room temperature for 10 minutes. After aspirating the supernatant and resolving the cells in an appropriate buffer, the cells (LPL) are ready to be counted.

The flow-through of the first incubation steps contain intraepithelial lymphocytes, which need to be stored on ice until further analysis. After applying the IELs to a 100µm cell strainer, the cell suspension is then ready for counting.

2.4.4 Cell counting

The isolated cells from the single cell suspension were counted either by hand with the microscope or by the CASY counter. For counting by hand 10µl of each sample was mixed with 20µl of Trypan blue and 70µl of FACS buffer. 10µl of that mix are then applied on to a specimen slide and the cells in the sample are counted by hand. The results are in millions per ml.

For counting with the CASY 10µl from the cell suspension were pipetted into a CASY cup and combined with 5ml PBS. The results from the counting are shown in cells per ml by the CASY.

2.4.5 Preparation for FACS analysis

2.4.5.1 Flow cytometry

50µl of the cell suspension with 1-2x10^6 cells of each sample were pipetted in a 96-well plate and combined with 10µl of a 1:10 diluted Fc-block solution per well. For each sample, two stainings were prepared. One for the staining of dendritic cells and their subsets and the other one for T cells and their subsets. Each staining master mix consisted of the desired antibodies and FACS-buffer. 40µl of the staining master mix were applied on the cell suspensions and then incubated for 40 minutes in the fridge at 4°C. Subsequently, the cell suspensions were spun down for 5 minutes at 4°C and then washed with FACS-buffer. Following, the cell suspensions need to be prepared for the intracellular staining (β-catenin in the DC staining panel, and FoxP3 in the T cell staining panel). For the intracellular staining of β-catenin, 100µl of the BD Cytofix/ Cytoperm Fixation and Permeabilization solution was added to the cell suspensions, which had received the DC staining master mix. After 40 minutes in the fridge at 4°C the cell suspensions were spun down and washed with FACS-buffer. During the waiting

period, the master mix for the intracellular staining of β-catenin was prepared. It consists of the β-catenin antibody and FACS-buffer. After the latency 40µl of the staining master mix was applied to the cell suspensions. This staining remained on the suspensions through the night and on the next day, the suspensions were again spun down and washed with FACS-buffer. Leaving the cells in 100µl of FACS-buffer, the cells are then ready for FACS analysis.

For the intracellular staining of FoxP3 in the T cell staining panel, the cell suspensions need to be fixed and stabilized. Therefore, a fixation master mix was generated consisting of Fixation/Permeabilization Concentrate and Fixation/Permeabilization Diluent at the rate of 1:3. 100µl of this master mix was then applied on the cell suspension samples, which had received the T cell staining master mix. After a waiting period of 30 minutes, the cells were spun down for 5 minutes at 4°C and washed with a 1:9 mix of Perm Wash Buffer and distilled H_2O. Subsequently, the intracellular staining for FoxP3 is applied on the cell suspensions, which then incubated during the night. On the next day, the samples are again spun down and washed with a 1:9 mix of Perm Wash Buffer and distilled H_2O. Afterwards, 100µl of FACS-buffer is applied on the cell suspensions, which are then ready for FACS analysis.

Until the FACS analysis, the cells are stored in the fridge at 4°C. In case of not staining intracellular, 100µl of Roti Histo Fix was applied on the cell suspensions. After an incubation for 20 minutes at room temperature, the cells were spun down for 5 minutes at 4°C and then washed with FACS-buffer. Following the cells were left in 100µl of FACS-buffer and were ready for FACS analysis. The used antibodies, which differ between the experiments, are shown in Table 3: Primary antibodies.

The FACS analysis was then performed with a FACS Canto II and the data was analyzed with the computer application FlowJo. Following, the statistical analysis was performed with Prism by GraphPad Software (see Statistical analysis).

2.5 Gating strategies for identification of the investigated cells

Gating strategies are required for the classification of cells after FACS sorting. Before FACS analysis, addition of staining antibodies to the analyzed cells results in binding of those staining antibodies to the cells depending on the expression of surface molecules. All antibodies used for FACS staining are recorded in 2.1.4. FACS analysis results in the presentation of several dots in the plot, each dot representing a cell encounter by the FACS laser. Yellow and red colored areas in the plot display an increased cell number. To group the analyzed cells into different subpopulations depending on their surface markers, gates are drawn. First, cells are gated regarding their size (FSC, forward scatter) and granularity (SSC, side scatter). For excluding cell duplets, the second step is to gate on FSC-H and FSC-A. By measuring the height (H) and the area under the curve (A) when a cell passes through the laser beam of the FACS, the FSC can differentiate between single cells and cell duplets. Hereafter, we used a

viability dye (eFluor780 L/D) to analyze the cell viability and segregate dead cells and living cells. The living cell population can then be further gated for the target cell population.

2.5.1 Identification of mLN and LPL DC

To analyze the DC homeostasis in mLN, following gating strategy was used: After pregating on single cells via SSC and FSC, we excluded dead cells via gating on living MHCII$^+$ cells. From this population, we gated on DC, which express high levels of CD11c and MHCII. Subsequently, DC were further subdivided based on their CD11b and CD103 expression. Hence four different subsets are possible: CD11b$^+$/ CD103$^-$, CD11b$^-$/ CD103$^+$, double positive and double negative.

The gating strategy for DC in LPL of the small and large intestine was quite similar: after pregating on single cells, we gated on living leukocytes (which are CD45$^+$). From this population, we then in turn gated on DC, which are CD11c$^+$ and MHCII$^+$. Subsequently, the DC population was further subdivided into the four subsets by their expression of CD11b and CD103.

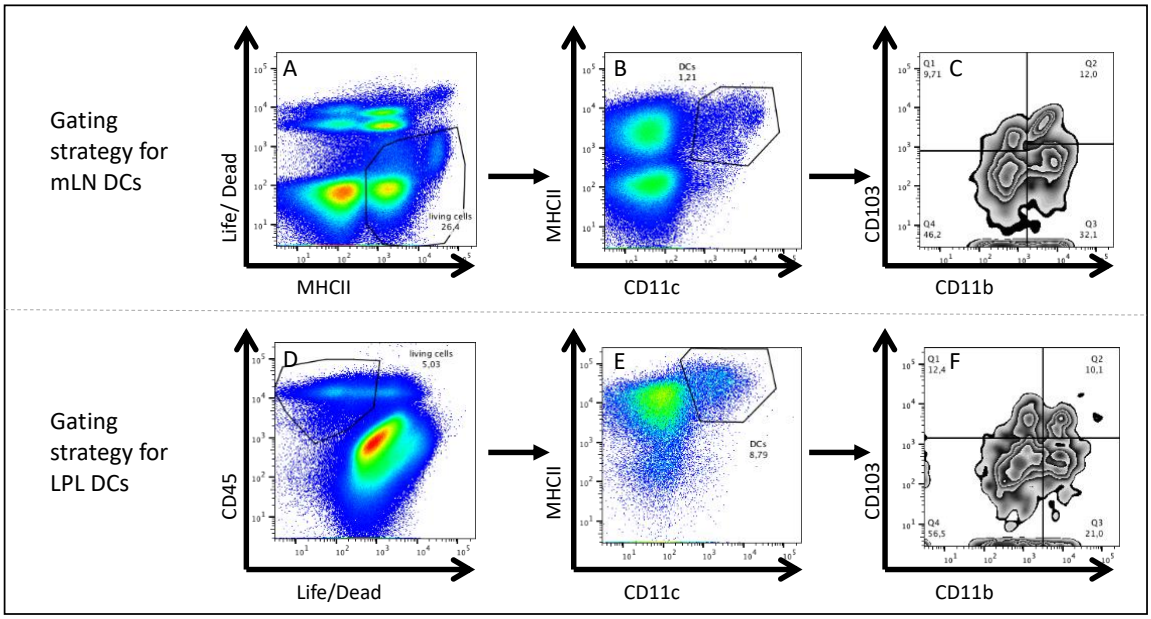

Figure 26 Gating strategy for DC and their subsets in mLN and LPL

DC were stained with fixable viability staining and indicated antibodies. Cells are pregated on single cells via SSC and FSC. (A-C) Gating strategy for mLN DC: (A) Gating on MHCII$^+$ living cells, followed by (B) gating on DC, as they are CD11c$^+$ and MHCII$^+$. (C) Depending on their CD11b and CD103 expression, DC are then further divided into different subsets (C). (D-F) Gating strategy for LPL DC: (D) Gating on CD45$^+$ living cells, followed again by (E) gating on DC and (F) their further division into different subsets.

2.5.2 Identification of mLN and LPL T cells

To investigate the T cell homeostasis in the mLN, the following gating strategy was used: After pregating on single cells via SSC and FSC, we excluded dead cells via gating on living T cells (which are $CD3^+$). From this population, we gated on $CD4^+$ and $CD8^+$ T cells to subdivide the T cell population. In addition, we also gated on CD4 and FoxP3 to separate Tregs - which are defined as $CD4^+$ and $FoxP3^+$ - from other T cell populations. In the steady state, we additionally investigated the expression of CD44 on Tregs in mLN.

The gating strategy for the analysis of the T cell homeostasis in LPL of the small and large intestine was quite similar. After pregating on single cells, we excluded dead cells via gating on living leukocytes (which are $CD45^+$). From this population, we gated on $CD4^+$ and $CD8^+$ T cells on the one hand and on Tregs, which are $CD4^+$ and $FoxP3^+$, on the other hand.

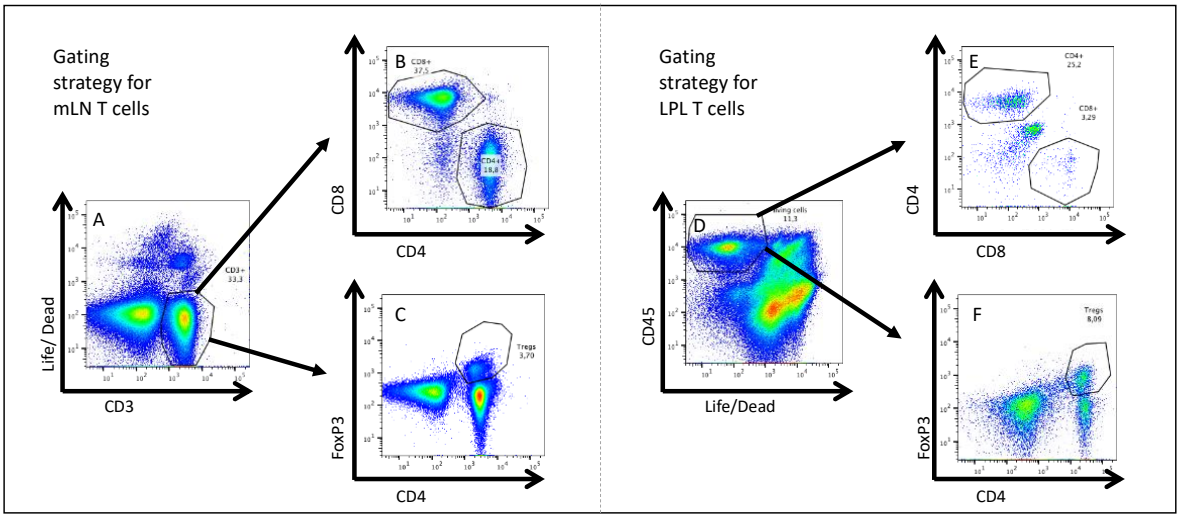

Figure 27 Gating strategy for T cells in mLN and LPL

T cells were stained with fixable viability staining and indicated antibodies. Cells are pregated on single cells via SSC and FSC. (A-C) Gating strategy for mLN T cells: (A) Gating on living T cells, as they are $CD3^+$. From this population, we gated on $CD4^+$ and $CD8^+$ T cells on the one hand (B) and on the other hand on Tregs (C). (D-F) Gating strategy for LPL T cells: (D) Gating on $CD45^+$ living cells. From this, we again gated on $CD4^+$ and $CD8^+$ T cells (E) and on Tregs (F).

2.6 In vivo mouse models of intestinal inflammation

2.6.1 DSS induced colitis

Dextran sulfate sodium (DSS) salt induces strong damage to the epithelium of the large intestine, which results in severe intestinal inflammation.

50-100ml (depending on the number of mice in the cage) of a 2-3% DSS solution were filled in the drinking bottles. Every third day the drinking water was replaced. From day 7 the mice received drinking water without DSS. During every experiment, mice were weighed daily at the same time and their course of bodyweight was documented and analyzed over a period of 10-12 days. While control mice received drinking water without DSS and should therefore gain

weight as usual, the weight loss of DSS treated mice usually occurs after 5-7 days. Following the maximum severity of disease on day 8-11, the mice start to recover slowly. On day 10-12 (depending on their weight loss) the mice were sacrificed for further analysis.

2.6.2 Anti CD40 colitis

The injection of an Anti-CD40 antibody induces a strong intestinal inflammation. This method was only applied to T cell deficient mice. Therefore, 300µl of the antibody are dissolved in 700µl of PBS. Subsequently 200µl of this mix were injected intraperitoneally into the mice. The weight of the mice was measured and documented daily around the same time. Usually, the mice develop colitis after 2 days, reaching the maximum severity after 4-5 days. Depending on the weight loss, the mice were sacrificed for further investigation on the maximum of the colitis severity, thus on day 4-5. T cell deficient control mice received an intraperitoneal injection of PBS.

2.6.3 T cell transfer colitis

Adoptive Transfer of naive $CD4^+$ T cells from donor mice into T cell deficient mice causes a wasting disease and a colonic inflammation developing 5 to 10 weeks after transfer. Therefore, donor mice were euthanized, and their spleens removed. Subsequently, the spleen cells were digested and washed. After determination of the cell number gained by the five spleens, the naive $CD4^+$ cells were isolated from $CD4^-$ cells by usage of the $CD4^+$ T cell isolation kit mouse by Miltenyi Biotec. Consecutively, the naïve $CD4^+$ T cells gained from the isolation were counted. Depending on the number of $CD4^+$ T cells isolated, a particular number of naïve $CD4^+$ T cells were injected intraperitoneally into the recipient mice. We injected $0{,}5*10^6$ $CD4^+$ naive T cells per mouse in T cell deficient mice. After 5-10 weeks, the recipient mice develop colonic inflammation and a wasting disease due to antigen-driven activation, polarization and homeostatic expansion of the naïve $CD4^+$ T cells.

During the first three weeks, the weight of the mice was measured twice a week, from week four on the frequency of weighing is increased to 3-5 times a week. Endoscopy is performed weekly from week 2 on.

Depending on their weight loss, mice are sacrificed after 5-10 weeks for further investigation. Control mice received an PBS injection. Though, the weight of the control mice, that received a PBS injection was not measured. Therefore, we used data from the Jackson Laboratory about the average weight development of C57BL/6J mice to generate a weight graph for untreated controls (66). However, this doesn't exclude the possibility of an unexpected weight loss of untreated control mice, but as we didn't see that in other experiments, the probability was estimated to be low.

2.6.4 **Endoscopy**

To estimate the severity of colitis an endoscopic examination of the colon of the mice was performed. Mice were therefore anesthetized with 120-150µl ketamine/xylazine anesthesia. When the mice were sleeping, the eyes were coated with Bepanthen eye ointment to avoid damage and drying out.

A low air flow was used to widen up the colon and to simplify the examination. After introducing the endoscope into the colon, the severity of colitis was assessed by five parameters: translucency, stool, granularity, fibrin and vascularity. Each parameter can get a score from 0 (minimum) to 3 (maximum) depending on their level of illness.

By addition of the scores of each parameter, the final score can be calculated and can vary from 0 (healthy) to 15 (sick).

2.7 **Statistical analysis**

The statistical analysis was performed with Prism Version 9.0b by GraphPad Software (Graphpad Software Inc., La Jolla, CA). An unpaired t-test with two tailed P values and a 95% confidence interval was used for the comparison of two statistical groups. Because a 2-way ANOVA test did not work due to software limitations, the statistical analysis of weight courses of DSS induced colitis was also performed with an unpaired t-test by comparing the weight of the two mouse groups on determined points in time, like day 1, day 2, etc.

Data were considered as significant with a P value less than 0.05.

*= $p<0.05$; **= $p<0.01$; ***= $p<0.001$.

Error bars were calculated with the standard error of the mean (SEM).

3 Results

3.1 Experimental approach

We used four different mouse lines for the experiments of this thesis. The genetic background for all of them is C57BL/6. Also, the cell specific Cre, the CD11c Cre, is identical in all four mouse lines. Thus, transgenic mice express the Cre recombinase under the control of the mouse integrin X alpha promotor, which is also called CD11c promotor. Hence, the Cre recombinase is expressed in all CD11c positive cells, which are mostly DC ($CD11c^{high}$) and secondary macrophages and monocytes.

The CD11c-Cre line was crossbred with two different transgenic mouse lines. For the generation of a conditional inactivation of the β-catenin gene (i.e., knockout), the CD11c-Cre line was crossbred with the β-$catenin^{floxdel/floxdel}$ line, which has two loxP sites flanking the exons 2 to 6 of the β-catenin gene (the mice are homozygous with two floxed alleles). Mating of these floxed mice with mice from the CD11c-Cre line results in mice, that just carry the floxed alleles without expressing the Cre recombinase as well as in mice, which comprise both. In the mice carrying the floxed alleles and express the Cre recombinase, the exons 2 to 6 of the β-catenin gene are deleted cell specifically in CD11c positive cells. Although the exons 7 to 9 remain in the gene, alone they are without function, resulting in a conditional inactivation of the β-catenin gene in $CD11c^{+}$ cells in those mice. In the following chapters of this thesis, those mice are labelled as CD11c-βcat^{DEL} mice (67).

For the generation of the other transgenic mouse line, which expresses a stable form of β-catenin in $CD11c^{+}$ cells, the CD11c-Cre line was crossbred with β-$catenin^{floxEX3/floxEX3}$ mice, which have two loxP sites flanking the 3rd exon of the murine β-catenin gene. Again, mating of these floxed mice with mice from the CD11c-Cre line results in mice carrying just the floxed alleles and in mice, which comprise both, the floxed alleles and the Cre recombinase. In those mice, that carry the floxed alleles and express the Cre recombinase, the 3rd exon of the β-catenin gene gets deleted in CD11c positive cells. This does not affect the production and function of β-catenin, but without the 3rd exon, β-catenin lacks the phosphorylation site and therefore can't get phosphorylated anymore. As this phosphorylation usually marks β-catenin for degradation, this results in a dominant-stable version of the β-catenin protein. Thus, mice carrying the floxed alleles and expressing the Cre recombinase have a cell specific constitutive active form of β-catenin in $CD11c^{+}$ cells. Those β-catenin overexpressing mice are further labelled as CD11c-βcat^{EX3} mice (44).

As a third mouse line, we used T cell deficient mice (TCR-βδ deletion) with an additional CD11c cell specific constitutive active form of β-catenin. Therefore, we crossed CD11c-βCat^{EX3} mice with T cell deficient mice, until the offspring was also T cell deficient. Thus, these mice are also

carrying the floxed alleles for β-catenin and express the Cre recombinase and in addition, they are T cell deficient. This results in T cell deficient mice which have a constitutive active form of β-catenin in CD11c expressing cells; these mice are further labelled as TCRβδ CD11c-βcat^{EX3} mice.

Furthermore, we also generated T cell deficient mice with a cell specific inactivation/ deletion of β-catenin in CD11c^{+} cells. Therefore, we also crossbred CD11c-βcat^{DEL} mice with T cell deficient mice, until the offspring was T cell deficient, too. Those mice, which are further labeled as TCRβδ CD11c-βcat^{DEL} mice are T cell deficient mice with a conditional inactivation of β-catenin in CD11c^{+} cells. But due to only little mouse numbers and breeding difficulties, we were not able to perform experiments with those mice.

Since we wanted to investigate the cell composition of DC and T cells in the mouse lines under different circumstances in each of our experiments, our analysis was always quite similar. After isolating the cells out of the mLN, small and large intestine, the cells were prepared for FACS analysis as described in chapter 2.4.3. After FACS analysis, we examined the cell number of DC and different T cell subset to figure out, if alterations in the CD11c specific expression of β-catenin would impact cell composition. Based on our findings and previously published results, we conducted experiments in different scenarios, namely steady state and under various inflammatory conditions.

3.1.1 Efficient ablation respectively accumulation of β-catenin in CD11c-βcat^{DEL} and CD11c-βcat^{EX3} mice

To see whether our gene targeting of β-catenin in CD11c^{+} cells had functioned efficiently (namely in the deletion of β-catenin in CD11c-βcat^{DEL} mice and the stabilization of β-catenin in CD11c-βcat^{EX3} mice), we analyzed the mean fluorescent intensity (MFI) of β-catenin in DC from CD11c-βcat^{DEL} and CD11c-βcat^{EX3} mice.

In CD11c-βcat^{DEL} mice, the in vivo analysis of the MFI of β-catenin in DC of mLN exhibited a significant decrease in comparison with controls confirming the deletion of β-catenin in resident DC in those mice.

Analysis of the β-catenin MFI in DC from CD11c-βcat^{EX3} mice displayed a significantly higher MFI of β-catenin in comparison with control mice, determining the stabilization of β-catenin in migratory DC in those mice.

This indicates that the gene targeting of β-catenin in CD11c^{+} cells had functioned properly in both mouse strains.

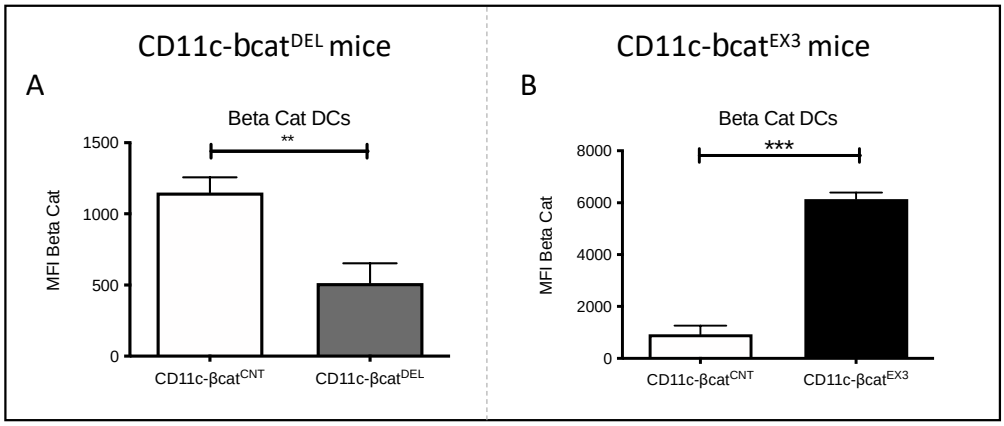

Figure 28 β-catenin expression in mLN DC of CD11c-βcat^{DEL} and CD11c-βcat^{EX3} mice

(A) Bar diagram depicts MFI of β-catenin in resident mLN DC of CD11c-βcat^{DEL} mice and controls +/- SEM. (B) Bar diagram depicts MFI of β-catenin in migratory mLN DC of CD11c-βcat^{EX3} mice and controls +/- SEM.

3.2 Role of β-Catenin in CD11c^{+} cells under steady state conditions

Manicassamy et al. suggested, that β-catenin signaling in intestinal DC enhances the stimulation of regulatory T cell induction while suppressing inflammatory effector T cells. In their experiments they displayed, that a CD11c specific deletion of β-catenin results in decreased Treg and increased effector T cell numbers in the lamina propria of the small and large intestine. They therefore hypothesized, that β-catenin programs DC into a tolerogenic state.

To test this hypothesis and to better characterize the impact of alterations in β-catenin expression on DC and T cell homeostasis in the intestine under steady state conditions, we analyzed the cell composition of DC and T cells in mesenteric lymph nodes (mLN) and large intestine lamina propria leukocytes (LI-LPL) of CD11c-βcat^{DEL} mice and CD11c-βcat^{EX3} mice. Since Manicassamy et al. did not investigate the cell composition in mLN, but only in LI-LPL, we also wanted to analyze if altered expression of β-catenin would have impacts on DC and T cells in mLN.

3.2.1 Previously published results do not show up in our experiments - limited influence of CD11c specific deletion of β-catenin on intestinal DC and T cell homeostasis

To determine the impact of a CD11c specific deletion of β-catenin on DC and T cell homeostasis, we first analyzed DC and T cell composition in mLN and LI-LPL of CD11c-βcat^{DEL} mice.

3.2.1.1 mLN DC and T cells of CD11c-βcat^{DEL} mice

The DC and T cell composition in mLN of CD11c-βcat^{DEL} mice has not been investigated yet. But since Manicassamy et al. displayed significant differences in the LI-LPL, we thought that this would also have an impact on the cell distribution in mLN.

However, the analysis of the absolute cell number of mLN DC in CD11c-βcat^{DEL} mice displayed no significant differences in comparison between CD11c-βcat^{DEL} and control mice. In both mouse groups, the largest subset population was the double negative subset, followed by the CD11b^{+}/ CD103^{-} subset. The CD11b^{-}/ CD103^{+} subset represented the 3rd largest population, whereas the double positive subset was the smallest one in the mLN (Figure 29; A, B).

We then investigated the absolute cell number of different T cell subsets in mLN. We analyzed the absolute cell number of Tregs, CD4^{+} FoxP3^{-} T cells and CD8^{+} T cells and we could not find any significant differences in those cell populations in comparison between CD11c-βcat^{DEL} mice and control mice (Figure 29; E).

3.2.1.2 LI-LPL DC and T cells of CD11c-βcat^{DEL} mice

Next, we wanted to reproduce the results of Manicassamy and compared the absolute cell numbers of DC and their subsets and T cells in LI- LPL between CD11c-βcat^{DEL} mice and control mice in the steady state. In their experiments, Manicassamy et al. displayed increased Treg numbers with decreased effector T cell numbers.

In our analysis of the absolute cell numbers of DC and their subsets in LI-LPL we observed no significant differences neither in the cell numbers of DC nor in the cell numbers of the different subsets. In mutant and wildtype mice, the CD11b^{+}/ CD103^{-} subset population displayed the highest amount of absolute cell numbers, followed by the double negative population. Next smallest population was the CD11b^{-}/ CD103^{+} population and the smallest population was the double positive one (Figure 29; C, D).

Regarding T cell homeostasis, as Manicassamy et al. displayed, that a CD11c specific deletion of β-catenin results in lower Treg and increased effector T cell numbers in LI-LPL under steady state conditions, we initially tried to reproduce their results. However, contrary to Manicassamy's results, in our experiments the comparison of the absolute cell number of T cells in LPL displayed no significant differences between CD11c-βcat^{DEL} mice and control mice, not only regarding Tregs, but also relating to CD4^{+} FoxP3^{-} and CD8^{+} T cells. Although the analysis of the different T cell subsets displayed a small tendency to decreased absolute cell

numbers in CD11c-βcat^{DEL} mice, the results remained non-significant (Figure 29; F).

In conclusion the influence of a CD11c specific deletion of β-catenin on DC and T cell homeostasis in mLN and LI-LPL of CD11c-βcat^{DEL} mice appears to be limited. This is contrary with previously published results and questions the hypothesis, that β-catenin signaling in DC is critical in the regulation of intestinal homeostasis and tolerance. Nevertheless, a small influence of β-catenin, which does not display in this scenario, cannot be completely excluded.

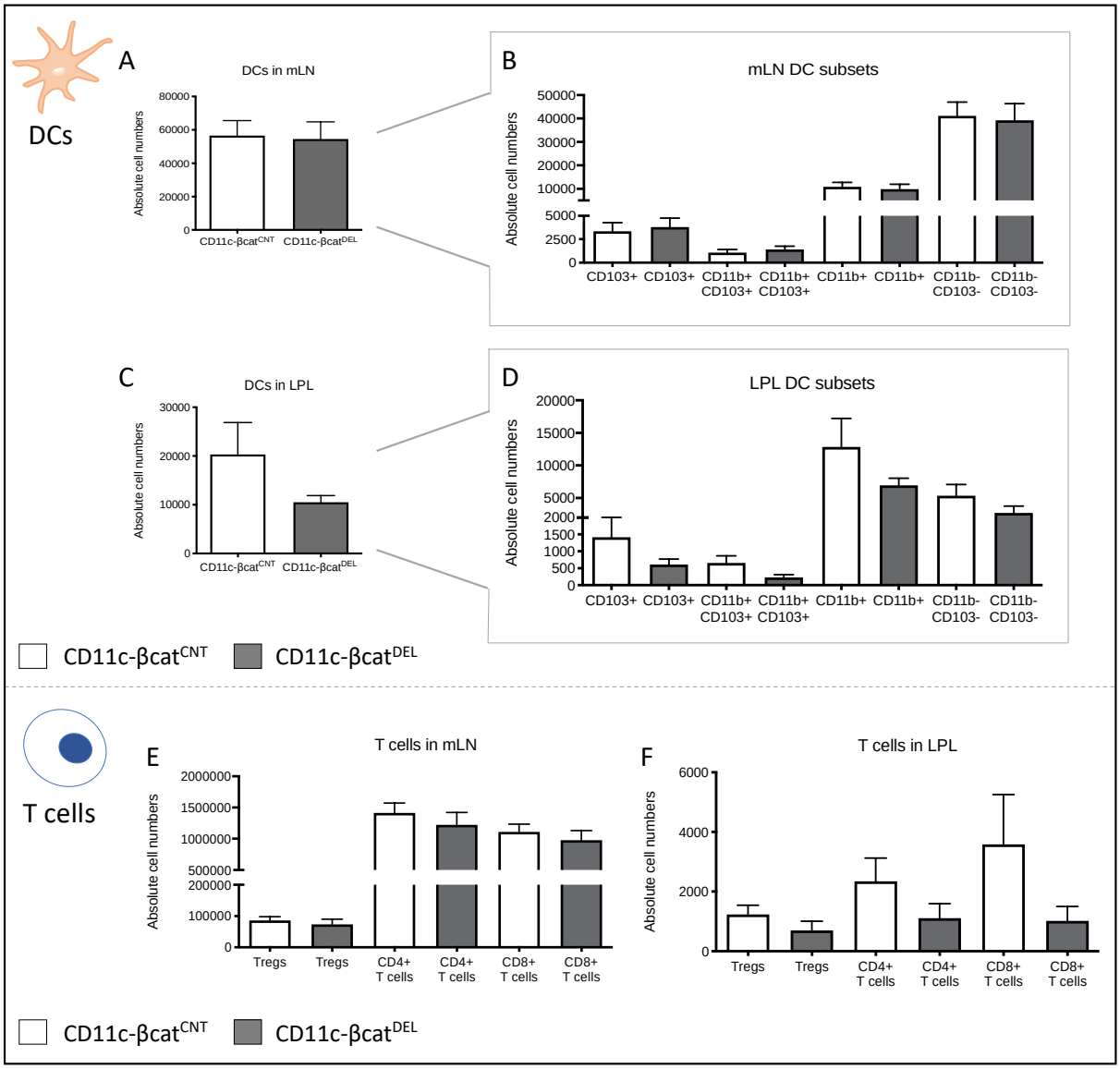

Figure 29 CD11c specific β-catenin deficiency exhibits no influence on intestinal DC and T cell homeostasis of CD11c-βcat^{DEL} mice

(A-B) Absolute cell number of mLN DC and their subsets in CD11c-βcat^{DEL} and control mice. (C-D) Absolute cell number of LI-LPL DC and their subsets in CD11c-βcat^{DEL} and control mice. (E-F) Absolute cell number of Tregs, CD4^{+} T cells and CD8^{+} T cells in mLN and LI-LPL of CD11c-βcat^{DEL} and control mice.

Bar diagrams depict absolute cell numbers +/- SEM.

3.2.2 CD11c specific stabilization of β-catenin results in increased DC and Treg numbers in mLN

After determining the impact of a CD11c specific β-catenin deletion as limited, we next wanted to characterize the influence of a stabilization of β-catenin in $CD11c^+$ cells on DC and T cell homeostasis in the intestine. This scenario has not been analyzed yet, since Manicassamy et al. only conducted their experiments with CD11c-βcat^{DEL} mice and not CD11c-βcat^{EX3} mice. Therefore, we investigated the absolute cell number of DC and T cells in mLN and LI-LPL of CD11c-βcat^{EX3} mice under steady state conditions. According to Manicassamy's hypothesis, a CD11c-specific stabilization of β-catenin should result in an increase in the number of Tregs and a reduction in the number of effector T cells. Though, based on our results we would expect no differences at all by the CD11c specific stabilization of β-catenin, since the CD11c specific deletion of β-catenin had no influence on DC and T cell homeostasis in mLN and LI-LPL.

3.2.2.1 mLN DC and T cells of CD11c-βcat^{EX3} mice

Interestingly, analysis of mLN DC of CD11c-βcat^{EX3} mice displayed a significantly increased absolute cell number of DC in mLN in comparison with control mice. Investigation of the four different DC subsets exhibited a significant increase in the absolute cell number of $CD11b^-$/ $CD103^-$ DC, which also provided the largest population, whereas the other subsets showed a similar level of absolute cell numbers in comparison between CD11c-βcat^{EX3} mice and control mice. The 2nd largest population was the $CD11b^+$/ $CD103^-$ DC subset followed by the double positive subset while the smallest population was the $CD11b^-$/ $CD103^+$ DC population (Figure 30; A; B).

To see whether the increased absolute cell number of mLN DC and especially $CD11b^-$/ $CD103^-$ DC in CD11c-βcat^{EX3} mice would impact T cell homeostasis, we next investigated the absolute cell number of T cells and their subsets in mLN of those mice. Mainly because of the large proportion of $CD11b^-$/ $CD103^-$ DC, which are considered to play a more immunogenic role, we wanted to investigate whether this immunogenic influence is reflected in T cell composition. The in vivo analysis of T cells in mLN of CD11c-βcat^{EX3} mice exhibited a significant increase in the absolute cell number of Tregs compared to control mice in the steady state. Also, Tregs from CD11c-βcat^{EX3} mice showed a higher expression of CD44 in comparison with Tregs in control mice. However, the absolute cell number of $CD4^+$ $FoxP3^-$ and $CD8^+$ T cells displayed no significant differences in the comparison between CD11c-βcat^{EX3} mice and control mice (Figure 30; E, G). The increased number of $CD11b^-$/ $CD103^-$ DC therefore does not appear to have an immunogenic influence, but rather a tolerogenic effect. This results in increased Treg numbers, however, how $CD11b^-$/ $CD103^-$ DC exert this influence remains unclear, perhaps β-

catenin is involved. Next, we wanted to investigate if the differences in mLN are also reflected in LI-LPL.

3.2.2.2 LI-LPL DC and T cells of CD11c-βcat^{EX3} mice

Since Manicassamy et al. found differences in the T cell composition only in LI-LPL and not in mLN and since we were wondering whether the differences in mLN would affect DC and T cell homeostasis in the large intestine, we analyzed the absolute cell number of DC and T cells in LI-LPL of CD11c-βcat^{EX3} mice. In contrast to our results in the mLN, we could not observe any differences in the in vivo analysis of the absolute cell number of DC and their subsets in LI-LPL of CD11c-βcat^{EX3} mice compared to the results in control mice. In LI- LPL of CD11c-βcat^{EX3} mice and control mice the largest subset population was the double negative subset, followed by the CD11b^{-}/ CD103^{+} population. The CD11b^{+}/ CD103^{-} population was the 3rd largest and the double positive one the smallest (Figure 30; C, D).

Regarding T cell homeostasis, in vivo analysis of the absolute cell number of Tregs in LI-LPL exhibited only a small tendency to increased Treg numbers in CD11c-βcat^{EX3} mice compared to control mice. However, the differences remained non-significant. In addition, comparison of the absolute cell number of CD4^{+} FoxP3^{-} and CD8^{+} T cells also displayed no significant differences between CD11c-βcat^{DEL} mice and control mice (Figure 30; F).

In summary, the CD11c specific stabilization of β-catenin appears to have a tolerogenic effect resulting in the increase of DC and Tregs in mLN under steady state conditions. This could be due to β-catenin programming DC into a tolerogenic state as Manicassamy hypothesized. This could mean that β-catenin at least partially contributes to the regulation of immunity and tolerance in the intestine. However, in contrast to Manicassamy's findings, the influence is only evident in mLN and only in mice with a CD11c specific stabilization and not deletion of β-catenin. Furthermore, it remains unclear how strong the effect of β-catenin is and how exactly β-catenin exerts this effect. Still, our data suggest an influence of a CD11c specific stabilization of β-catenin on DC and T cell homeostasis in the intestinal immune system under steady state conditions.

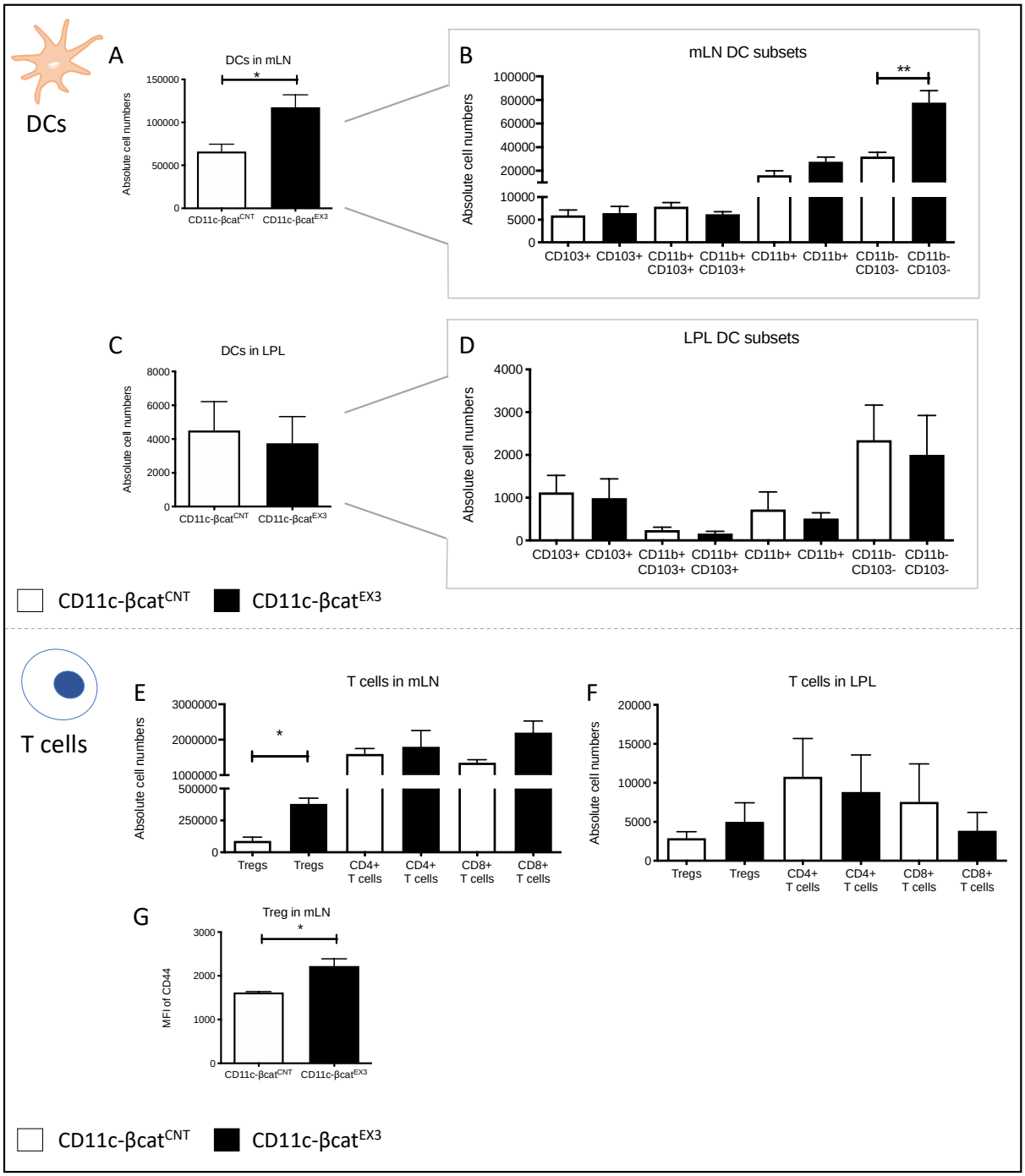

Figure 30 CD11c specific stabilization of β-catenin results in increased DC and Treg numbers in mLN of CD11c-βcat^{EX3} mice

(A-B) Absolute cell number of mLN DC and their subsets in CD11c-βcat^{EX3} and control mice. (C-D) Absolute cell number of LI-LPL DC and their subsets in CD11c-βcat^{EX3} and control mice. (E-F) Absolute cell number of Tregs, CD4^{+} T cells and CD8^{+} T cells in mLN and LI-LPL of CD11c-βcat^{EX3} and control mice. (G) Expression level of CD44 on Tregs in mLN of CD11c-βcat^{EX3} compared to control mice.

Bar diagrams depict absolute cell numbers and median fluorescence intensity +/- SEM.

3.3 Role of β-catenin in CD11c$^+$ cells under inflammatory conditions

Our steady state results displayed that especially CD11c specific stabilization of β-catenin could program DC into a tolerogenic state. To verify whether this effect of β-catenin is also confirmed under inflammatory conditions, we examined DC and T cell composition in mLN and LI-LPL of mice in which intestinal inflammation was previously induced by different mouse models of IBD. We therefore subjected CD11c-βcat^{DEL}, CD11c-βcat^{EX3} and TCRβδ CD11c-βcat^{EX3} mice to three different models of IBD: DSS induced colitis, anti CD40 induced colitis and T cell transfer induced colitis. Detailed explanations of the individual models and their mode of operation can be found in chapter 1.3.2.

3.3.1 Limited influence of alterations in β-catenin expression on disease progression in DSS induced colitis

In their experiments, Manicassamy et al. also investigated the effects of a CD11c-specific deletion of β-catenin on disease progression in intestinal inflammation. For this, they used DSS-induced colitis, a common mouse model for IBD. They exhibited that CD11c-βcat^{DEL} mice were significantly more susceptible to intestinal inflammation and lost significantly more weight than control mice. In addition, DSS-treated CD11c-βcat^{DEL} mice showed increased effector T cell and reduced Treg numbers in LI-LPL, as in the steady state. This would again make β-catenin an important regulator of the intestinal immune system. In our experiments, we wanted to reproduce and verify Manicassamy's results with CD11c-βcat^{DEL} mice. On the other hand, we also sought to determine the influence of a CD11c-specific stabilization of β-catenin on the course of DSS colitis. Especially after our steady state results with increased Treg numbers, we wanted to see if this could also be found under inflammatory conditions and whether this would affect disease progression.

Therefore, we subjected CD11c-βcat^{DEL} and CD11c-βcat^{EX3} mice to DSS induced colitis as illustrated in chapter 2.6.1. We analyzed the bodyweight courses and the cell composition of DC and T cells in mLN and LI-LPL of DSS treated CD11c-βcat^{DEL} mice and DSS treated CD11c-βcat^{EX3} mice.

3.3.1.1 Similar disease progression independent of alterations in β-catenin expression

First, as mice lost around 10-15% of their original weight, it can be determined, that the DSS induced colitis model is working and is causing an acute disease. By analyzing the bodyweight course during disease progression, we wanted to investigate, if alterations in the expression of β-catenin would affect the course of disease.

3.3.1.1.1 No differences in weight loss between CD11c-βcat^{DEL} mice and controls

Manicassamy et al. (56) published, that CD11c specific ablation of β-catenin expression enhances disease severity in a DSS induced colitis model. However, contrary to those results, we couldn't observe any tendencies or significant differences between DSS treated CD11c-

βcat^{DEL} mice and DSS treated control mice regarding their weight loss and severity of disease in all four DSS experiments (Figure 31; A).

3.3.1.1.2 Weight loss between CD11c-βcat^{EX3} mice and controls equal

Considering our previous results from steady state experiments and the increased absolute cell number of DC and Tregs in mLN in CD11c-βcat^{EX3} mice compared to the results in control mice, we assumed, that the disease severity of DSS induced colitis and hence the weight loss would be less intense in CD11c-βcat^{EX3} mice in comparison with control mice. But the analysis of the weight loss displayed no alterations between CD11c-βcat^{EX3} mice and control mice in both experiments with DSS induced colitis (Figure 31; B).

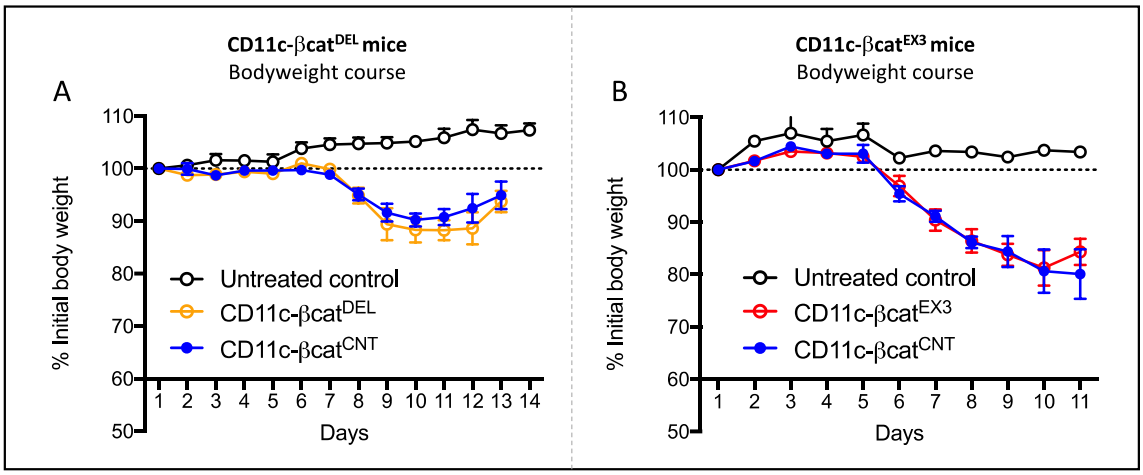

Figure 31 Alterations in β-catenin expression in CD11c^{+} cells exhibit no impact on course of disease in DSS treated CD11c-βcat^{DEL} and CD11c-βcat^{EX3} mice

(A) Bodyweight course of DSS treated CD11c-βcat^{DEL} mice and controls.

(B) Bodyweight course of DSS treated CD11c-βcat^{EX3} mice and controls.

Collectively, alterations in the expression of β-catenin in CD11c^{+} cells appear to have no influence on the course of disease in DSS treated mice. To further investigate the reasons for the limited influence of β-catenin in this scenario, we analyzed the DC and T cell composition in DSS-treated CD11c-βcat^{DEL} and CD11c-βcat^{EX3} mice.

3.3.1.2 Like in steady state - Limited influence by CD11c specific deletion of β-catenin on intestinal DC and T cell homeostasis in DSS treated mice

As in his steady state experiments, Manicassamy et al. found besides the increased susceptibility to colitis lower Treg and increased effector T cell frequencies in LI-LPL of DSS treated CD11c-βcat^{DEL} mice. However, in our steady state experiments we could not find any significant differences. Furthermore, our CD11c-βcat^{DEL} mice were not more susceptible to DSS induced colitis, making differences in Treg and effector T cell numbers unlikely. To determine, if the CD11c specific deletion of β-catenin still would impact intestinal DC or T cell homeostasis as hypothesized by Manicassamy, we analyzed DC and T cell composition in

mLN and LI-LPL of DSS treated CD11c-βcat^{DEL} mice.

3.3.1.2.1 mLN DC and T cells of DSS treated CD11c-βcat^{DEL} mice

Like our results in steady state, in vivo analysis of DC and their subsets in mLN of DSS-treated CD11c-βcat^{DEL} mice displayed no significant differences in the absolute cell numbers compared to DSS-treated control mice. Furthermore, there were no differences in the distribution of the DC subsets. The largest subset population was the CD11b^{+}/ CD103^{-} subset followed by the double negative subset. The 3rd largest population was the CD11b^{-}/ CD103^{+} subset whilst the double positive subset displayed the smallest population (Figure 32; A, B).

Regarding T cell homeostasis, the results under inflammatory conditions also looked alike the results in the steady state. So, the analysis of the absolute cell number of Tregs, CD4^{+} FoxP3^{-} T cells and CD8^{+} T cells in mLN of DSS treated CD11c-βcat^{DEL} mice exhibited no significant differences compared to DSS treated control mice (Figure 32; E).

3.3.1.2.2 LI-LPL DC and T cells of DSS treated CD11c-βcat^{DEL} mice

Next, we investigated DC homeostasis in LI-LPL of DSS treated CD11c-βcat^{DEL} mice. Herein, analysis of the absolute cell number of DC in DSS treated CD11c-βcat^{DEL} mice exhibited no significant differences compared to DSS treated controls. Those results are comparable to our findings in the steady state, where we also couldn't detect any differences. Additionally, there were no differences visible in the comparison of the subset distribution. In LI-LPL, the double negative subset provided the largest population followed by the CD11b^{+}/ CD103^{-} population. CD11b^{-}/ CD103^{+} DC displayed the 3rd largest population whilst the double positive subset was the smallest (Figure 32; C, D).

Regarding the T cell homeostasis, Manicassamy et al. found a decreased Treg and increased effector T cell frequency in LI-LPL of DSS treated CD11c-βcat^{DEL} mice. However, in our experiments the in vivo analysis of the absolute numbers of Tregs, CD4^{+} FoxP3^{-} and CD8^{+} T cells in LI-LPL from DSS treated CD11c-βcat^{DEL} mice displayed no alterations compared to the results in DSS treated control mice. This again fits with our results in the steady state and with the similar course of DSS colitis (Figure 32; F).

In conclusion, under inflammatory conditions, the effects of a CD11c specific deletion of β-catenin on DC and T cell homeostasis in mLN and LI-LPL of CD11c-βcat^{DEL} mice seems to be restricted. Yet, the non-existent differences in DC and T cell frequencies fit the similar disease course of DSS colitis in CD11c-βcat^{DEL} mice and control mice. Hereby it would have been surprising if, as in Manicassamy's experiments, a reduced number of Tregs had been

displayed, which would not have affected the course of disease.

Still, even though our results match with our steady state results from CD11c-βcat^{DEL} mice, they contrast with previously published results and further question the role of β-catenin as a key regulator in intestinal immunity.

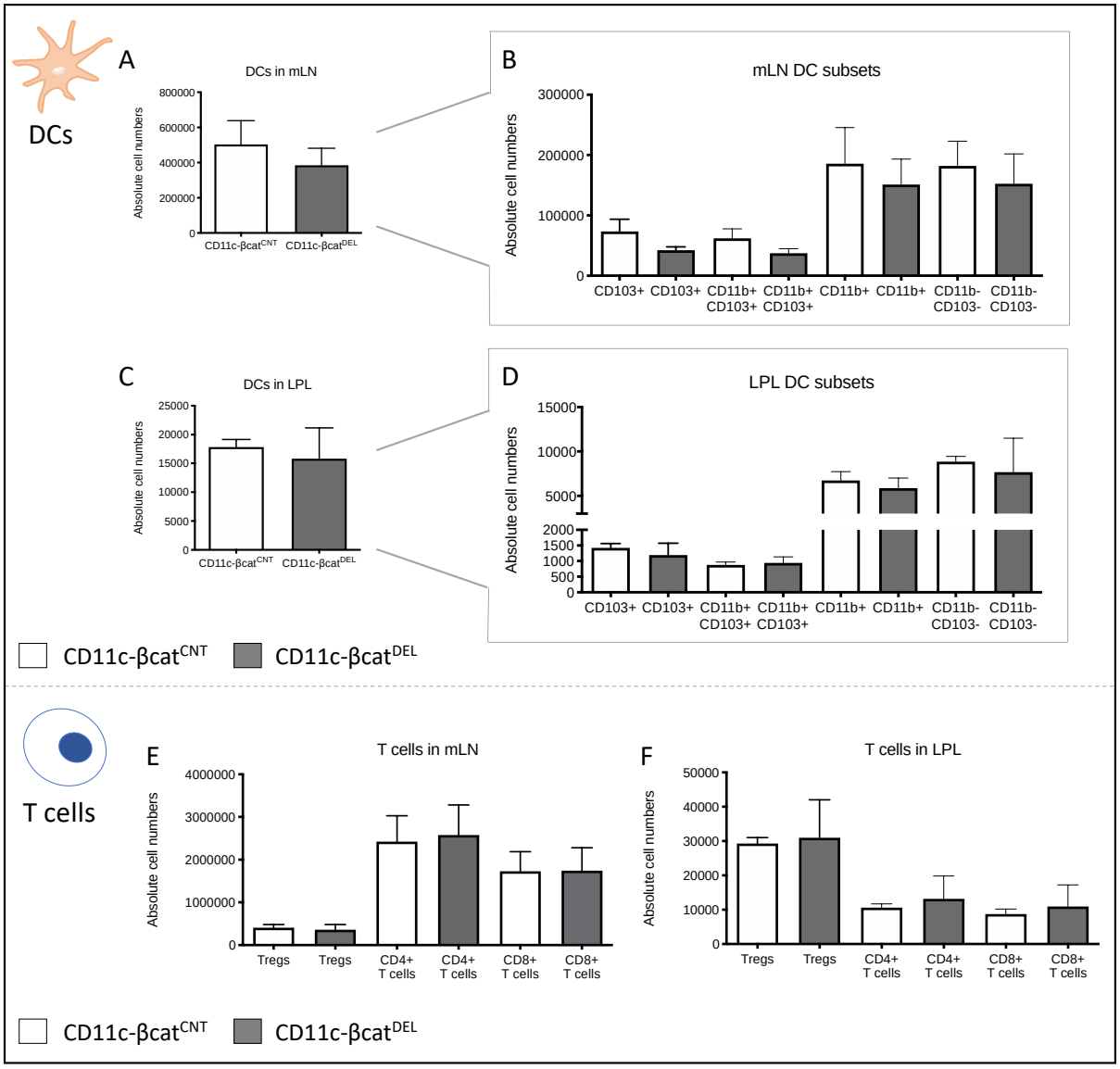

Figure 32 CD11c specific β-catenin deletion displays no impact on intestinal DC and T cell homeostasis of DSS treated CD11c-βcat^{DEL} mice

(A-B) Absolute cell number of mLN DC and their subsets in DSS treated CD11c-βcat^{DEL} and control mice. (C-D) Absolute cell number of LI-LPL DC and their subsets in DSS treated CD11c-βcat^{DEL} and control mice. (E-F) Absolute cell number of Tregs, CD4$^+$ T cells and CD8$^+$ T cells in mLN and LI-LPL of DSS treated CD11c-βcat^{DEL} and control mice.

Bar diagrams depict absolute cell numbers +/- SEM.

3.3.1.3 Increased Treg numbers in mLN of DSS treated CD11c-βcat^{EX3} mice without impact on disease severity

However, since our steady state results with CD11c-βcat^{EX3} mice gave an indication that a CD11c specific stabilization of β-catenin could have a tolerogenic impact in the intestinal immune system, we next investigated intestinal DC and T cell homeostasis in CD11c-βcat^{EX3} mice under inflammatory conditions. Therefore, we analyzed the absolute cell number of DC and T cells in mLN and LI-LPL of DSS treated CD11c-βcat^{EX3} mice.

3.3.1.3.1 mLN DC and T cells of DSS treated CD11c-βcat^{EX3} mice

Due to our results in steady state, we also expected an increased absolute cell number of DC in mLN of DSS treated CD11c-βcat^{EX3} mice. But controversially the in vivo analysis of mLN of DSS treated CD11c-βcat^{EX3} mice displayed no significant differences in the absolute cell number of DSS treated CD11c-βcat^{EX3} mice compared to DSS treated control mice. Besides the distribution of DC subsets also showed no significant differences. The double negative DC subset displayed the largest population, followed by the CD11b^{+}/ CD103^{-} DC subset. CD11b^{-}/ C103^{+} DC provided the 3rd largest population whilst the double positive subsets displayed the smallest population (Figure 33; A, B).

Regarding T cell homeostasis in mLN, in vivo analysis of mLN from DSS treated CD11c-βcat^{EX3} mice displayed a significantly increased absolute cell number of Tregs in comparison with the results in DSS treated control mice. This result matches with our findings in steady state experiments, where we also saw an increased absolute cell number of Tregs in mLN. But this result is inconsistent with the similar course of DSS colitis, as we expected the increased Treg number to attenuate the course of disease at least slightly. It also remains unclear where the increased number of Treg originates from, since comparison of the cell number of DC displayed no differences. Furthermore, comparison of the absolute cell numbers of CD4^{+} FoxP3^{-} and CD8^{+} T cells displayed a small tendency to increased numbers in CD11c-βcat^{EX3} mice, though the results remained non- significant (Figure 33; E).

3.3.1.3.2 LI-LPL DC and T cells of DSS treated CD11c-βcat^{EX3} mice

In terms of LPL DC homeostasis, the absolute cell number of DC in the LI-LPL of DSS treated CD11c-βcat^{EX3} mice displayed no significant differences in comparison with the results in DSS treated control mice, even though the analysis showed a small tendency to increased numbers in DSS treated CD11c-βcat^{EX3} mice.

But, regarding the distribution of DC subsets, the in vivo analysis of the four different DC subsets exhibited a significantly increased absolute cell number of double negative DC in DSS treated CD11c-βcat^{EX3} mice compared to control mice. This DC subset also provided the largest population of DC in both mouse lines. CD11b^{+}/ CD103^{-} DC were the 2nd largest

population, whilst the double positive and the CD11b$^-$/ CD103$^+$ DC provided the smallest populations (Figure 33; C, D). This increased double negative DC cell number matches our steady state results, where the double negative DC subset was also increased. However, the increase in our steady state experiments was in mLN, not in LI-LPL. Nevertheless, the double negative DC subset was affected in both cases, suggesting a possible correlation.

Regarding T cell homeostasis, we found no significant differences in the absolute cell number of T cells in LI-LPL of DSS treated CD11c-βcat^{EX3} mice compared to DSS treated control mice. But there was a clear trend toward increased absolute cell number of Tregs in LI-LPL of CD11c-βcat^{EX3} mice visible, yet the results remain non-significant (P-value: 0,0545). Still, as in the steady state experiments, there could be a correlation between increased numbers of double negative DC and increased Treg frequencies. And again, as in mLN, even though there is a strong tendency to increased Treg numbers, this appears to have no impact on disease severity. This could be due to a strong tendency to increased cell numbers of CD4$^+$ FoxP3$^-$ and CD8$^+$ T cells in CD11c-βcat^{EX3} mice compared to control mice, since these cells are thought to play an immunogenic role. They could offset the mitigating effect of Tregs on disease progression. However, this analysis also remained non-significant (Figure 33; F).

In conclusion, CD11c specific stabilization of β-catenin does not appear to have an attenuating effect on the disease course of DSS colitis. Still, it seems to affect DC and T cell homeostasis as under steady state conditions resulting in increased Treg numbers in mLN and LI-LPL, indicating a possible tolerogenic effect of β-catenin. This could also be related to the double negative DC subset, whose cell number was partly shown to be increased in the experiments. The exact role of β-catenin in increasing Treg numbers and why the increased Treg numbers do not have a mitigating effect on disease progression remains unclear.

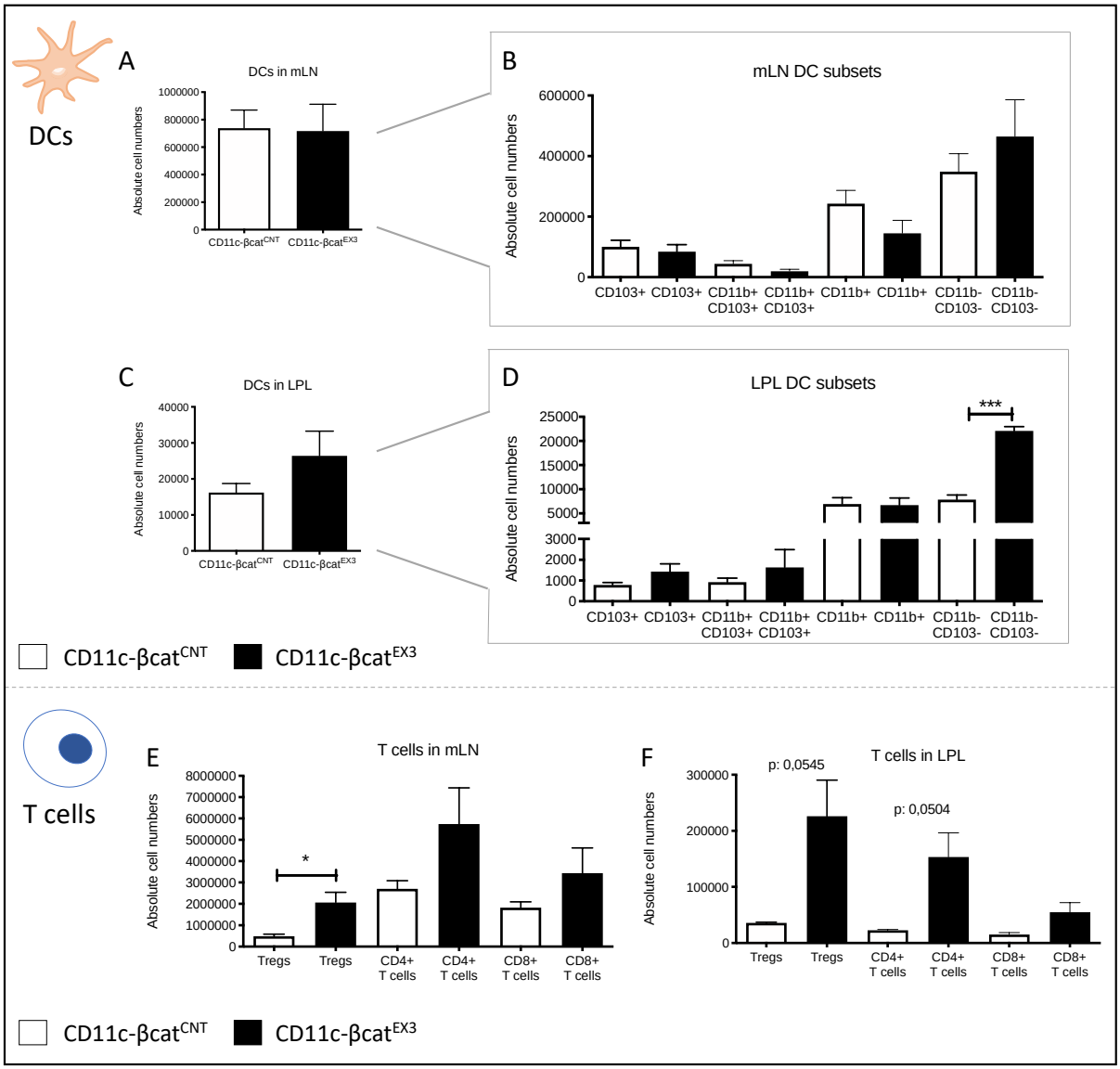

Figure 33 CD11c specific β-catenin stabilization results in increased double negative DC numbers in LI-LPL and increased Treg numbers in mLN of DSS treated CD11c-βcat^{EX3} mice

(A-B) Absolute cell number of mLN DC and their subsets in DSS treated CD11c-βcat^{EX3} and control mice. (C-D) Absolute cell number of LI-LPL DC and their subsets in DSS treated CD11c-βcat^{EX3} and control mice. (E-F) Absolute cell number of Tregs, CD4$^+$ T cells and CD8$^+$ T cells in mLN and LI-LPL of DSS treated CD11c-βcat^{EX3} and control mice.

Bar diagrams depict absolute cell numbers +/- SEM.

3.3.2 Similar disease progression in anti CD40 colitis - Impact of CD11c specific β-catenin stabilization on innate intestinal immunity appears to be limited

As a next mouse model for IBD, we used anti CD40 colitis to better characterize the role of β-catenin under inflammatory conditions. Since the development of colitis in this model is driven only by cells of the innate immune system and independent from T cells, this model allows us to draw conclusions about the impact of β-catenin on innate intestinal immunity without the influence of T cells. However, based on our prior experiments and previously published results, we expected β-catenin to have an effect mainly in an environment with T cells, so we did not anticipate differences in disease progression in this scenario. Still, since these studies have not been conducted yet, we wanted to perform these experiments. Therefore, we subjected T cell deficient TCRβδ CD11c-βcat^{EX3} mice to anti CD40 colitis following the instructions given in chapter 2.6.2. Herein, we analyzed the course of disease and DC homeostasis in mLN, small (SI-LPL) and large intestine (LI-LPL) of TCRβδ CD11c-βcat^{EX3} mice.

3.3.2.1 Similar disease progression independent of CD11c specific stabilization of β-catenin in anti CD40 colitis

First, the weight graph displays, that the anti CD40 induced colitis model is working and is causing an acute disease, as the mice lost around 20% of their original weight.

To investigate, if a CD11c specific stabilization of β-catenin would affect the course of disease, we analyzed the bodyweight course during disease progression.

3.3.2.1.1 No differences in weight loss between TCRβδ CD11c-βcat^{EX3} mice and controls

However, regarding the course of disease, we could not see any significant differences in the weight loss of TCRβδ CD11c-βcat^{EX3} mice compared to TCRβδ control mice (Figure 34; A).

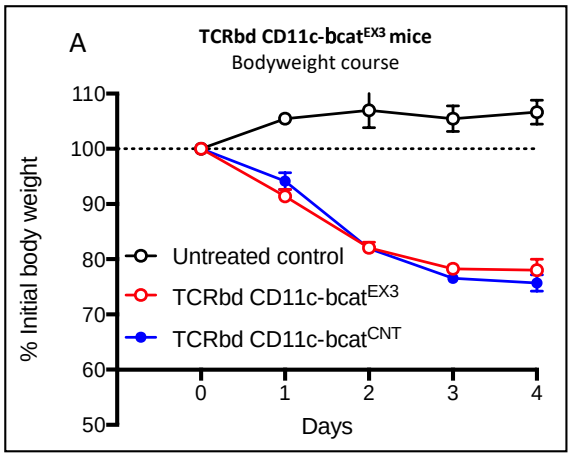

Figure 34 CD11c specific stabilization of β-catenin expression displays no impact on course of disease in anti CD40 treated TCRβδ CD11c-βcatEX3 mice

(A) Bodyweight course of anti CD40 treated TCRβδ CD11c-βcatEX3 mice and controls.

Collectively the CD11c specific constitutive expression of β-catenin appears to have no impact on the course of disease in anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice. To further analyze the reasons for the restricted impact of β-catenin in this scenario, we investigated the DC composition in anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice.

3.3.2.2 Decreased DC numbers in mLN of anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice

In steady state, we found an increased absolute cell number of DC in mLN of CD11c-βcat^{EX3} mice, which could not be detected in DSS induced colitis. To examine whether the CD11c specific β-catenin stabilization would have an impact on intestinal DC homeostasis in the context of anti CD40 colitis, we analyzed the absolute cell number of DC and their subsets in mLN, LI-LPL and SI-LPL of anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice.

3.3.2.2.1 mLN DC of anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice

In mLN of anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice, analysis of the absolute cell number of DC displayed a significant decrease in comparison with the results in anti CD40 treated TCRβδ control mice. Additionally, the distribution of DC subsets differed in anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice compared to anti CD40 treated TCRβδ control mice. Especially the absolute cell number of CD11b$^-$/ CD103$^+$ and double positive DC was significantly decreased in anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice compared to control mice. In TCRβδ CD11c-βcat^{EX3} mice CD11b$^+$/ CD103$^-$ DC provided the largest population, followed by double negative DC, double positive DC and CD11b$^-$/ CD103$^+$ DC. In anti CD40 treated TCRβδ control mice, the distribution of DC subsets was divided in almost equal parts (Figure 35; A, B). These results are in part unexpected, since the absolute cell number of DC in mLN of CD11c-βcat^{EX3} mice was increased in steady state experiments with a particularly increased frequency of double negative DC. Also, the decrease of CD11b$^-$/ CD103$^+$ and double positive DC in this anti CD40 colitis experiment is surprising, since we did not see any affections of those subsets in steady state. Furthermore, since those DC subsets are thought to play a tolerogenic role, their decrease would result in a more severe course of disease, which we did not observe in our analysis. Collectively, our experiments do not allow us to exactly determine where these differences come from, but the assumed tolerogenic effects of β-catenin from the steady state experiments on DC and T cell composition in mLN do not appear to occur in this scenario.

3.3.2.2.2 LI-LPL and SI-LPL DC of anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice

In vivo analysis of absolute cell numbers of DC and their subsets in the LI-LPL of anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice exhibited no significant differences compared to the results in anti CD40 treated TCRβδ control mice. In both mouse groups, subset distribution was as follows: the double negative subset provided the biggest population, followed by the CD11b$^+$/ CD103$^-$ subset. Subsequently, the CD11b$^-$/ CD103$^+$ subset displayed the 3rd largest population whilst the double positive subset was the smallest population (Figure 35; C, D). As in mLN of anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice, β-catenin does not appear to affect DC composition in LI-LPL in the way as in the steady state experiments.

Next, we investigated the absolute cell numbers of DC and their subsets in LPL from the small intestine. This analysis of DC in SI-LPL of anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice displayed no significant differences in comparison with the results in TCRβδ control mice. In both groups, the CD11b^{+}/ CD103^{-} subset provided the largest population, followed by the double negative subset. 3rd largest population was CD11b^{-}/ CD103^{+} DC whilst the double positive subset exhibited the smallest population (Figure 35; E, F). Again, the effect of CD11c-specific β-catenin stabilization in this case seems to be limited, but we did not study DC composition in SI-LPL in the steady state, so a comparison is difficult.

Since the inflammation in anti CD40 colitis is driven only by innate immune cells, macrophages are also involved in the disease process as part of innate immunity. And because CD11c-specific stabilization of β-catenin also affects macrophages, we wanted to investigate whether this change in β-catenin expression also affects the number of macrophages, which has not been investigated before. Therefore, we analyzed the absolute cell number of different macrophage subsets in LI-LPL and SI-LPL of anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice. However, we could not find any differences in comparison with anti CD40 treated TCRβδ control mice (data shown in the appendix (chapter 6.3)). The influence of β-catenin on macrophages in this scenario thus seems to be rather small, but the effect of β-catenin on macrophages should be investigated in more detail in further experiments.

Collectively, apart from the decreased DC number in mLN, the non-existent differences in cell numbers fit the similar course of disease in mutant and control mice. What causes the reduced DC number in mLN remains unclear. Still, it can be concluded that CD11c specific β-catenin stabilization does not seem to affect disease progression or cell composition of DC and macrophages in anti CD40 colitis. Thus, under inflammatory conditions, the impact of β-catenin on the intestinal innate immune system without the presence of T cells is rather small.

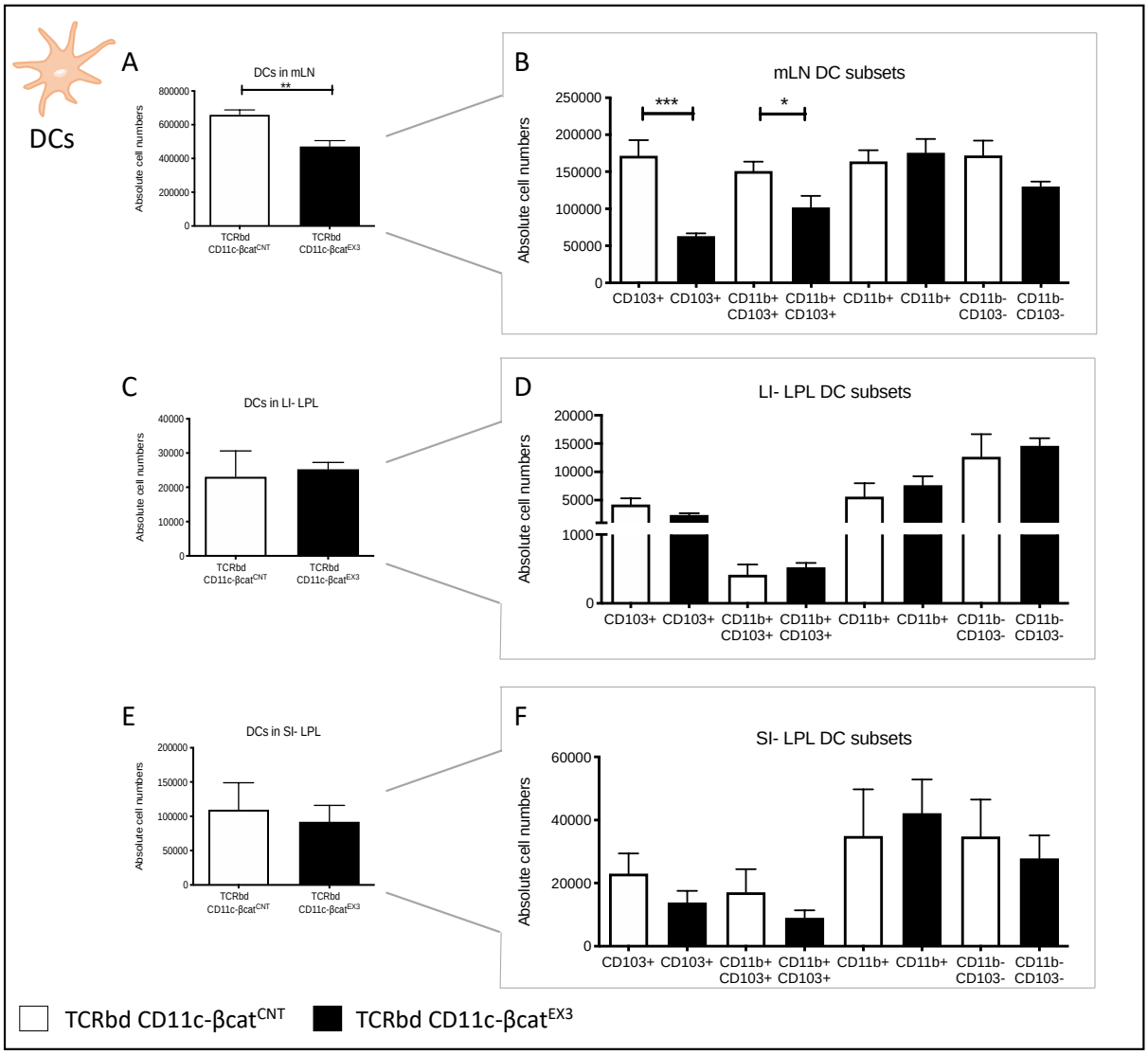

Figure 35 CD11c specific β-catenin stabilization results in decreased DC numbers in mLN of anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice

(A-B) Absolute cell number of mLN DC and their subsets in anti CD40 treated TCRβδ CD11c-βcat^{EX3} and control mice. (C-D) Absolute cell number of LI-LPL DC and their subsets in anti CD40 treated TCRβδ CD11c-βcat^{EX3} and control mice. (E-F) Absolute cell number of SI-LPL DC and their subsets in anti CD40 treated TCRβδ CD11c-βcat^{EX3} and control mice.

Bar diagrams depict absolute cell numbers +/- SEM.

3.3.3 β-catenin affects T cell mediated inflammation and attenuates course of disease in T cell transfer colitis

As our results in steady state and after DSS treatment displayed an increased absolute cell number of Tregs in mLN of CD11c-βcat^{EX3} mice, we wanted to further investigate the influence of a stabilization of β-catenin in CD11c^{+} cells on T cell homeostasis, especially Tregs, under inflammatory conditions. Therefore, we used the T cell transfer model, which is ideal to study the role of T cells and Tregs in intestinal inflammation and which has not been applied in previous studies on β-catenin. In this model, adoptive transfer of naïve CD4^{+} T cells from healthy donor mice results in their differentiation to proinflammatory T cells inducing intestinal inflammation. However, parts of the transferred naïve T cells can, depending on environmental influences, differentiate into Treg, which would prevent or delay onset of intestinal inflammation. So, we subjected TCRβδ CD11c-βcat^{EX3} mice (T cell deficient mice with a CD11c specific stabilization of β-catenin) to T cell transfer colitis based on the instructions given in chapter 2.6.3. Besides examining T cell homeostasis, we also studied the course of disease and DC homeostasis in T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice. Therefore, we analyzed the bodyweight course and the cell composition of DC and T cells in mLN, LI-LPL and SI-LPL of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice.

3.3.3.1 Attenuated disease progression through CD11c specific stabilization of β-catenin in T cell transfer colitis

To investigate, if CD11c specific stabilization of β-catenin would affect the course of disease and survival of the treated mice, we analyzed the survival and bodyweight course and performed endoscopy to monitor disease progression.

3.3.3.1.1 Less weight loss and better survival of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice

In contrast to our previous results under inflammatory conditions, analysis of the weight loss of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice and T cell transfer treated TCRβδ control mice displayed a significantly reduced weight loss in TCRβδ CD11c-βcat^{EX3} mice compared to TCRβδ control mice.

In the first two weeks, both mouse groups gained weight regularly. But while after 14 days TCRβδ CD11c-βcat^{EX3} mice continued to gain weight almost normally, TCRβδ control mice lost around 10% of their original body weight already between week 2 and 3 and not as expected in this model after 5-10 weeks. As the TCRβδ control mice lost weight dramatically and we wanted to analyze both mouse groups (TCRβδ CD11c-βcat^{EX3} mice and TCRβδ control mice) contemporaneously, we euthanized both groups on day 23 after T cell transfer, although TCRβδ CD11c-βcat^{EX3} mice hadn't lost weight (Figure 36; A).

However, we didn't only see differences in the weight loss of T cell transfer treated TCRβδ

CD11c-βcat^{EX3} mice and T cell transfer treated TCRβδ control mice, but also in the comparison of the survival of both mouse groups. While TCRβδ CD11c-βcat^{EX3} mice displayed no impairments due to colitis, two TCRβδ control mice had to be euthanized due to an extensive weight loss during week 3 (Figure 36; B).

3.3.3.1.2 Endoscopic analysis displays less inflammation in T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice compared to T cell transfer treated control mice

Besides analyzing the weight loss and survival of both mouse groups, we also subjected T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice, T cell transfer treated TCRβδ control mice and healthy TCRβδ control mice to an endoscopic analysis of the inflammation in the colon after 2 and after 3 weeks. This process is illustrated in chapter 2.6.4.
The endoscopic analysis of those mouse groups in week 2 displayed no signs of intestinal inflammation in all mouse groups. This is consistent with the course of bodyweight, as the mice gained weight regularly in the first two weeks (Figure 36; D).

However, after 3 weeks, the endoscopic analysis of T cell transfer treated TCRβδ control mice displayed several signs of intestinal inflammation i.e., severe diarrhea, increased intestinal vascularization, low intestinal translucency and a fibrin film on the surface of their intestine. Therefore, those mice were graded with a high endoscopy score. In contrast, endoscopic analysis of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice after 3 weeks exhibited considerably fewer signs of intestinal inflammation leading to a significantly lower endoscopic score. Though, T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice still showed some signs of intestinal inflammation, as their endoscopy score was significantly higher than the score of healthy TCRβδ control mice. Again, those endoscopic results are consistent with the attenuated weight loss and improved survival of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice, while T cell transfer treated TCRβδ control mice lost weight dramatically (Figure 36; C, E).

In conclusion, the course of T cell transfer colitis is considerably attenuated in TCRβδ CD11c-βcat^{EX3} mice compared to TCRβδ control mice. It is likely, that the CD11c specific constitutive active expression of β-catenin contributes at least partially to this attenuated course of disease in T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice. To further analyze the impact of β-catenin in this scenario, we investigated DC and T cell composition in T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice.

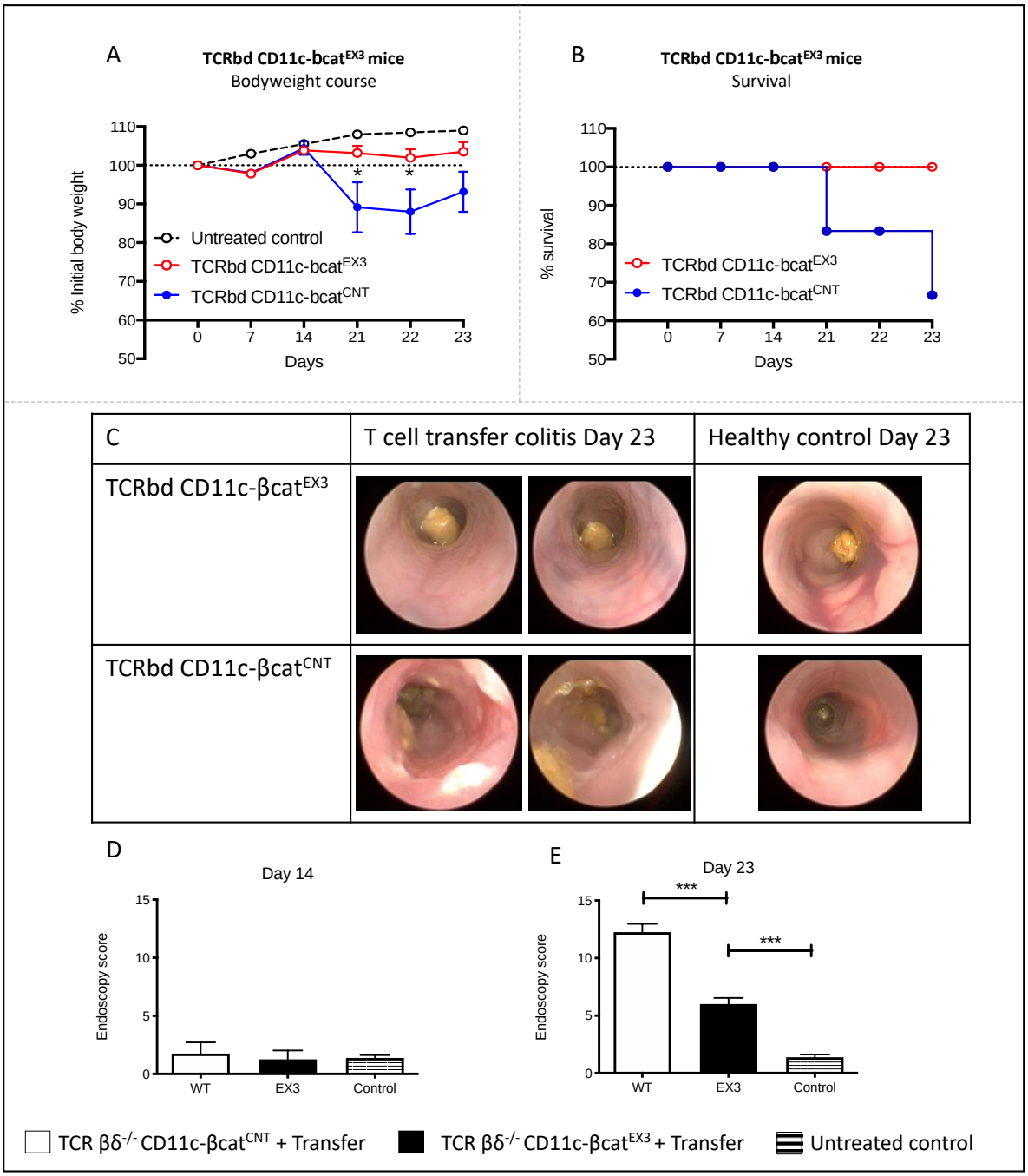

Figure 36 CD11c specific stabilization of β-catenin results in an attenuated course of disease in T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice

(A) Bodyweight course of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice and controls. (B) Survival of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice and controls. (C-E) Representative section of the mini- endoscopy on day 23 and bar diagrams that depict the average colitis score on day 14 and day 23 respectively.

3.3.3.2 Decreased DC numbers in mLN but no impact on T cell homeostasis through CD11c specific stabilization of β-catenin in T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice

First, before we start into analyzing the results, it can be determined, that the transfer of CD4$^+$ naïve T cells into T cell deficient TCRbd mice worked out well, as we detected T cells in the isolated organs.

Since we found significant alterations in the course of disease, we wanted to investigate DC and T cell homeostasis to search for explanations for the differences.

3.3.3.2.1 mLN DC and T cells of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice

In our steady state experiments, we found increased DC and Treg numbers in mLN of mice with CD11c specific stabilization of β-catenin. If this is also seen in mLN of T cell transfer treated mice, it could explain the attenuated disease course. However, analysis of the absolute cell number of DC in mLN of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice exhibited a significantly decreased absolute DC number in comparison with the results in T cell transfer treated TCRβδ control mice. Following, the absolute cell number of all four DC subsets is also decreased in TCRβδ CD11c-βcat^{EX3} mice compared to TCRβδ control mice. In both mouse lines, subset distribution was as follows: Double negative DC provide the largest subset, succeeded by CD11b$^+$/ CD103$^-$ DC. Whilst CD11b$^-$/ CD103$^+$ DC displayed the 3rd largest population, double positive DC provided the smallest population (Figure 37; A, B). These results are comparable to the results from anti CD40 colitis, where we also found decreased DC numbers in mLN. Yet β-catenin does not seem to be responsible for the lower DC cell number, which is probably due to the lower inflammation in T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice. In T cell transfer treated control mice severe inflammation leads to increased DC numbers. Still, our results do not explain the attenuated course of disease in T cell transfer colitis.

In our further analysis we investigated T cell homeostasis in mLN of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice. This analysis also displayed no significant differences in comparison with the results in TCRβδ control mice. We neither found alterations in the absolute cell number of Tregs, nor in the absolute cell number of CD8$^+$ T cells and CD4$^+$/ FoxP3$^-$ T cells in T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice compared to T cell transfer treated control mice (Figure 37; G). So, the number of Tregs in mLN does not seem to be responsible for the attenuated course, as we had assumed from our steady state results.

3.3.3.2.2 LI-LPL and SI-LPL DC and T cells of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice

Since we could not find differences in mLN, we next examined DC and T cell homeostasis in LI-LPL and SI-LPL of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice to search for reasons

for the attenuated course of disease. First, we analyzed the absolute cell number of DC in LI-LPL and SI-LPL of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice. Hereby, we couldn't find significant differences in LI-LPL as well as in SI-LPL compared to the results in T cell transfer treated TCRβδ control mice. But the comparison of the different DC subsets between both mouse groups displayed few differences. In LI-LPL, the absolute cell number of CD11b^{-}/ CD103^{+} DC was significantly decreased in TCRβδ CD11c-βcat^{EX3} mice compared to control mice. The distribution of DC subsets in LI-LPL was as follows: In both mouse groups, double negative DC provided the largest population followed by CD11b^{+}/ CD103^{-} DC. 3rd largest population was displayed by CD11b^{-}/ CD103^{+} DC and double positive DC provided the smallest population (Figure 37; C, D).

In SI-LPL, the absolute cell number of double positive DC was significantly decreased in comparison between T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice and T cell transfer treated TCRβδ control mice. All other DC subsets displayed a non-significant tendency to a decreased absolute cell number in TCRβδ CD11c-βcat^{EX3} mice compared to the results in TCRβδ control mice. In both mouse groups, double negative DC provided the largest population followed by CD11b^{+}/ CD103^{-} DC. CD11b^{-}/ CD103^{+} DC were the 3rd largest population and double positive DC the smallest (Figure 37; E, F). These results do not seem to be responsible for the attenuated course of disease. And again, the increased DC numbers in control mice probably result from the strong inflammation caused by the T cell transfer colitis, which was not so strong in mutant mice.

Regarding T cell homeostasis, in vivo analysis of the absolute cell number of Tregs in LI-LPL and SI-LPL of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice displayed no significant differences compared to the results in T cell transfer treated TCRβδ control mice. Additionally, analysis of the absolute cell number of CD8^{+} T cells and CD4^{+}/ FoxP3^{-} T cells in LI-LPL and SI-LPL also exhibited no significant differences in comparison between T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice and T cell transfer treated TCRβδ control mice (Figure 37; H, I). So, these results also do not provide an explanation for the differences in the course of disease.

In summary, the CD11c specific stabilization of β-catenin appears to have an attenuating impact on the course of T cell transfer colitis. However, this effect does not seem to be due to differences in the absolute cell number of DC or Treg, since we could not find any directional differences in our analyses. How exactly β-catenin achieves the attenuating effect on the disease progression therefore remains unclear and should be further investigated in future experiments.

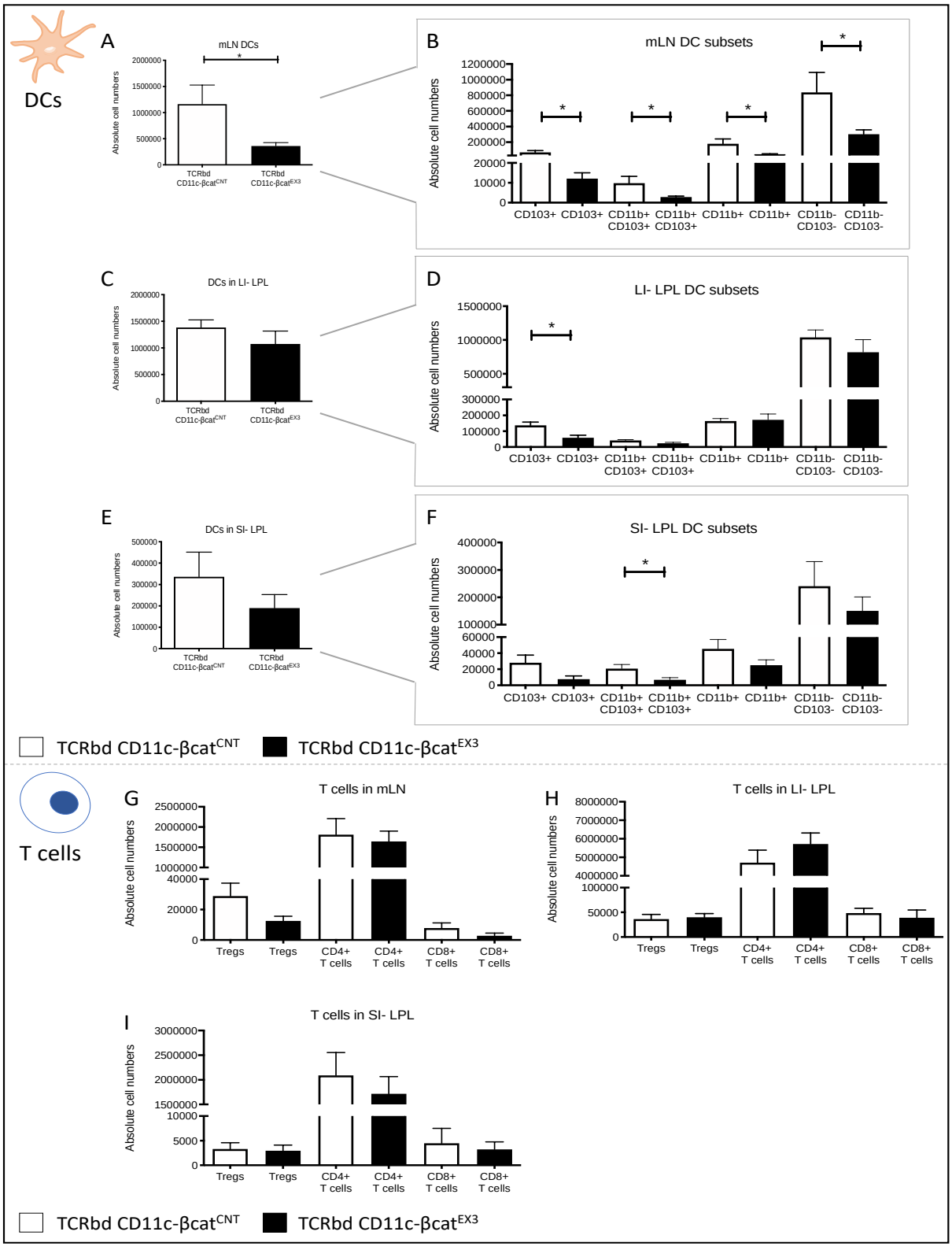

Figure 37 CD11c specific stabilization of β-catenin results in decreased DC in mLN but displays no differences on T cell homeostasis of T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice

(A-B) Absolute cell number of mLN DC and their subsets in T cell transfer treated TCRβδ CD11c-βcat^{EX3} and control mice. (C-D) Absolute cell number of LI-LPL DC and their subsets in T cell transfer treated TCRβδ CD11c-βcat^{EX3} and control mice. (E-F) Absolute cell number of SI-LPL DC and their subsets in T cell transfer treated TCRβδ CD11c-βcat^{EX3} and control mice. (G-I) Absolute cell number of Tregs, CD4^{+} T cells and CD8^{+} T cells in mLN, LI-LPL and SI-LPL of T cell transfer treated TCRβδ CD11c-βcat^{EX3} and control mice. Bar diagrams depict absolute cell numbers +/- SEM.

4 Discussion

DC are a central component of the immune system. By linking the adaptive and innate immune system, they are pivotal for the regulation of immune responses. This is particularly important in the intestine, as dysregulation of intestinal immune responses can result in inflammatory bowel diseases. Here, previous studies hypothesized, that β-catenin contributes to maintaining the balance of the intestinal immune system by regulating immunity versus tolerance. Jiang et al. (53) demonstrated, that activation of the β-catenin pathway via cluster disruption of E-Cadherin induces DC maturation. However, DC maturated by cluster disruption elicited different T cell responses in vivo, generating T cells with a regulatory instead of an effector phenotype. These DC induced tolerance in vivo indicating a possible contribution of β-catenin to a tolerogenic DC phenotype. Manicassamy et al. (56) further supported this hypothesis. They reported that β-catenin in intestinal dendritic cells is essential for the stimulation of Tregs while suppressing inflammatory effector T cells. In addition, they showed that ablation of β-catenin expression in $CD11^+$ cells enhances disease progression in a mouse model for IBD. They therefore summarized, that β-catenin signaling programs DC to a tolerogenic state, limiting inflammatory responses. With the here described experiments with three genetically transformed mouse lines in steady state and under inflammatory conditions, we further elucidated the role of β-catenin signaling in intestinal immunity. In addition to analyzing the effects of a CD11c-specific β-catenin deletion, we also investigated the impact of a CD11c-specific β-catenin stabilization for the first time.

4.1 β-catenin signaling in DC under steady state conditions

In previous studies, Manicassamy et al. demonstrated, that a CD11c specific deletion of β-catenin results in decreased frequencies of Tregs and increased effector T cells in LPL of the large intestine in steady state. They therefore ascribed β-catenin a central role in regulating the balance between inflammatory versus regulatory responses in the intestine. In our thesis we wanted to extend their experiments. We wanted to find out how a CD11c specific β-catenin deletion affects not only T cell homeostasis in LI-LPL, but also in mLN. In addition, we investigated DC homeostasis in those organs to see, if alterations in the expression of β-catenin would also impact DC homeostasis. However, after analyzing our results, there were no significant differences in any of the groups studied (Figure 29). This contradicts previously published results and suggests that the influence of β-catenin in this scenario may not be as strong as previously assumed. It remains unclear why our results differ so significantly from Manicassamy's. This could be due to a mixed background of the mice Manicassamy et al. used for the experiments. While our mice are completely backcrossed to the genetic background C57BL/6, CD11c-βcat^{DEL} mice from Manicassamy have a B6.129 background (56). This could influence/ intensify the effects of a β-catenin deletion and is therefore a

possible reason for the different results obtained by Manicassamy and by us. To rule out this confounder as a reason for the different results, it would be necessary to repeat the experiments again with mice with a B6.129 background to see what influence this has on DC and T cells. However, based on our results, we cannot verify Manicassamy's hypothesis. It thus appears that the influence of a CD11c-specific β-catenin deletion is rather small in the regulation of the intestinal immune system.

Despite lack of differences in our experiments with mice with a CD11c specific β-catenin deficiency, we wanted to investigate whether and to what extent a CD11c specific stabilization of β-catenin has an impact on intestinal DC and T cells homeostasis. Eventually, an oversupply of β-catenin may have a stronger effect on the intestinal immune system than absence of β-catenin. And indeed, we found a significant increase in DC and Treg numbers in mLN of CD11c-βcat^{EX3} mice (Figure 30). Furthermore, there was also a tendency for increased Tregs in LI-LPL of CD11c-βcat^{EX3} mice, but the differences remained non-significant. Still, these results support the hypothesis stated by Jiang et al. and Manicassamy et al., that β-catenin programs DC to a tolerogenic state stimulating the induction of Tregs. Now the question arises, exactly how β-catenin exerts this effect and thus regulates intestinal immunity. This cannot be determined completely from our experiments. One possible reason for the increased Treg numbers could be the increase in absolute cell number of DC that we found in mLN. If β-catenin were to reprogram DC to a tolerogenic state, this DC increase would subsequently lead to increased production of regulatory factors such as IL-10, RA or TGF-β, for example, which would then in turn increase the number of Tregs (68). This is especially true for CD103$^+$ DC possessing the ability to induce Tregs. However, in the more detailed analysis of mLN DC, we found mainly an increase in double negative DC, which typically exert more immunogenic functions (i.e., priming of CD4$^+$ and CD8$^+$ naïve T cells) rather than inducing Tregs (24, 69).

This contrasts with previously published results, in which double negative DC accounted for only a small proportion of DC in mLN and CD103$^+$ DC dominated (69). But, in our results, the absolute cell number of CD103$^+$ DC, whose function is the induction of Tregs, were not increased in CD11c-βcat^{EX3} mice compared to controls. Therefore, we thought that perhaps β-catenin reprogrammed the function of double-negative DC so that they now exerted a more tolerogenic function instead of an immunogenic function. That β-catenin turns double negative DC into direct Treg inducers seems unlikely, since Treg induction by DC requires the expression of CD103 (13, 27) and β-catenin is not able to cause a change in the expression of surface markers. Still β-catenin appears to affect the function of double negative DC as they normally induce immunogenic T cells, and we did not detect an increase in these T cells in our

studies, although the absolute cell number of double negative DC was significantly increased. So perhaps β-catenin limits the production of proinflammatory cytokines like IL-6 by double negative DC making the function of $CD103^+$ DC more prominent. This in turn would indirectly result in an increased induction of Tregs. To investigate this hypothesis, cytokine production of the DC subsets could be analyzed individually. This would show whether this is changed by the CD11c-specific stabilization of β-catenin and whether this could affect the induction of Tregs. For example, evidence of lower production of proinflammatory IL-6 by double negative DC would raise the possibility that the effect of anti-inflammatory cytokines like TGF-β or IL-10 produced by $CD103^+$ DC is more prominent. Or the analysis of cytokine production exhibits an increased secretion of anti-inflammatory cytokines such as TGF-β or IL-10. Both approaches would lead to an increase in the number of Treg. Furthermore, an analysis of the cytokine production of Tregs would be interesting. Perhaps Tregs activated by DC that express a constitutive active form of β-catenin produce more anti-inflammatory cytokines (TGF-β or IL-10). This in turn would lead to a further increase in Tregs, which would contribute to maintaining the immune homeostasis.
Thus, for elucidating the detailed impact of β-catenin on DC and T cell homeostasis in the intestinal immune system under steady state conditions further experiments are required. Still, our data show, that a CD11c specific β-catenin stabilization appears to have a tolerogenic impact on the regulation of intestinal immunity.

We further investigated the expression of the surface molecule CD44 on Tregs and found a significant increase in CD11c-βcat^{EX3} mice compared to control mice (Figure 30). CD44 is a marker for T cell activation (70) and is also important for cell survival and cell motility (50). The CD11c specific constitutive expression of β-catenin not only increases the number of Tregs, but also appears to enhance the ability of DC to activate naïve T cells to Tregs, which subsequently express increased CD44. And as the expression of CD44 positively correlates with FoxP3 expression, CD44 may be an additional marker for the maturation of Tregs. Furthermore, Tregs with a high expression of CD44 ($CD44^{high}$Tregs) produce increased amounts of IL-10 and TCR-β compared to $CD44^{low}$Tregs (50). Both molecules are important for further Treg generation and thus for regulating immune responses. Therefore, the increased expression of CD44 on Tregs results in a more mature Treg phenotype with improved regulatory and suppressive functions compared to $CD44^{low}$Tregs. Thus, a preponderance of $CD44^{high}$Tregs over $CD44^{low}$Tregs in turn would lead to increased tolerance (50). Whether and how exactly β-catenin affects the expression of CD44 on Tregs needs to be investigated in more detail in further experiments. To figure this out, we would have to analyze which factors lead to an increase in CD44 expression on Tregs. Various influences come into question, for instance an increased production of anti-inflammatory cytokines. Here, the extent

to which concentration changes of anti-inflammatory cytokines such as TGF-β or IL-10 affect CD44 expression on Tregs could be investigated. However, until further research, the influence of β-catenin in this scenario remains unclear.

Conclusively, although we could not confirm Manicassamy's results in CD11c-βcat^{DEL} mice, stabilization of β-catenin results in increased intestinal DC and Treg numbers in CD11c-βcat^{EX3} mice. Thus, it seems possible, that β-catenin programs DC to a tolerogenic state and thereby promotes Treg generation. Even though the exact processes of how β-catenin affects DC function need to be determined, this would make β-catenin signaling in DC an important regulator of the balance between inflammatory versus regulatory responses in the intestine.

4.2 β-catenin signaling in DC under inflammatory conditions

Next, we sought to determine what effect alterations in CD11c specific β-catenin expression might have under inflammatory conditions. If β-catenin were to program DC into a tolerogenic state, then changes in CD11c specific β-catenin expression would affect the development of inflammation in the intestine. To examine this question, we subjected mice to three different murine models of intestinal inflammation. As a first model we used DSS colitis to be able to compare our data to previous published studies, as they had also used DSS colitis. Secondly, we performed experiments with anti CD40 colitis, since colitis in this model is only driven by cells of the innate immune system allowing us to draw conclusions about the effects of β-catenin on innate intestinal immunity. Lastly, we used T cell transfer colitis, as this model is ideal to study the impact of β-catenin on T cells, especially Tregs under inflammatory conditions.

Administration of DSS to mice in their drinking water for a short period results in the destruction of the mucosal barrier and the infiltration of pathogens. This leads to the induction of acute inflammation. In this model, inflammation is predominantly triggered by cells of the innate immune system such as granulocytes and macrophages and to a small extent by T cells (36, 37). Manicassamy et al. (56) exhibited, that DSS treated mice with a CD11c specific deletion of β-catenin display a decrease in Treg numbers and are more susceptible to DSS colitis. This was to some extent remarkable, since the inflammatory response in the DSS colitis model is only partially dependent on T cells and it is therefore questionable whether the decrease in Treg numbers can lead to such a significant attenuation of disease progression. Nevertheless, we tried to reproduce Manicassamy's studies and subjected CD11c-βcat^{DEL} mice to DSS colitis. And in contrast to Manicassamy's results, our analysis of DSS treated CD11c-βcat^{DEL} mice displayed no significant differences from DSS treated control mice in terms of both weight progression and absolute cell numbers of DC and T cells (Figure 32, Figure 32). In addition,

we could not find any tendency to reduced Treg numbers in LI-LPL either, as we had found in steady state. There are various approaches to explain the differences between Manicassamy's and our results. As in steady state, one possible explanation could be the mixed background of the mice Manicassamy et al. used for their experiments. As mentioned before, their mice have a B6.129 background while our mice are completely backcrossed to the genetic background C57BL/6. This could affect the impact of a β-catenin deletion. This confounding factor could be eliminated by repeating the experiments with mice with a B6.129 background to check, if this confirms Manicassamy's results. Another explanation for the differences in the experiments could be the varying microbiome between their mice and ours. Simply because of the different animal facilities, our mice have a distinct microbiota than Manicassamy's. These discrepancies can become even more pronounced because of small differences in the nutrition of the mice. And since administration of DSS results in the destruction of the mucosal barrier, it allows the intestinal microbiome to invade the tissue inducing an immune response. This immune response depends on the invading pathogens and can be stronger or weaker accordingly. Perhaps the microbiome in Manicassamy's mice has a higher pathogenicity compared to the microbiome in our mice, resulting in an enhanced immune response and therefore more severe course of disease. Or maybe the effect of β-catenin is also dependent on the microbiome and thus works differently in Manicassamy's than in our experiments. Hence, the Wnt/ β-catenin signaling pathway is an important target of some virulence factors produced by bacteria such as Escherichia coli, Bacteroides fragilis or Helicobacter pylori. These can interfere the pathway via various mechanisms (71). To further determine the influence of these possible confounders, the experiments would have to be repeated with mice with different microbiota. For instance, the targeted use of antibiotics could eliminate certain bacteria and then investigate how this affects the influence of β-catenin. It would also be possible to eradicate the entire microbiome with a cocktail of antibiotics to analyze whether this changes the effect of β-catenin or whether it is independent of the microbiome.
But apart from the differences to Manicassamy's studies, the results from our analyses of DC and T cell homeostasis (which displayed no differences) fit the similar disease course of DSS colitis in our experiment and our steady state results. Here, a changed DC or Treg number would have been remarkable.

Based on these results, the picture of the influence of β-catenin on the regulation of intestinal immune homeostasis does not seem to be as simple black and white as Manicassamy et al. suggest. Our results do not confirm the hypothesis of Manicassamy et al. that β-catenin plays a central role in the regulation of immune homeostasis in the intestine. It rather seems, that the effect of the Wnt/ β-catenin signaling pathway is not robust and can be easily influenced by changes in external factors. Still, the Wnt/ β-catenin signaling pathway could be important

for the fine regulation of immune homeostasis. To better characterize the role of β-catenin in this scenario it would be interesting to investigate what changes a CD11c specific deletion of β-catenin causes at the molecular level. One method that could be used for this analysis would be RNA sequencing, for example. This enables precise observation of the molecular changes caused by altered β-catenin expression. However, until further experiments, the detailed role of β-catenin under these circumstances remains unknown.

We next subjected CD11c-βcat^{EX3} mice to DSS colitis. Since our steady state experiments with CD11c-βcat^{EX3} mice exhibited a significant increase in the absolute cell numbers of DC and Tregs in mLN and a tendency to increased Treg numbers in LI-LPL we wanted to analyze, if those results could be confirmed under inflammatory conditions. And, if so, whether this would affect the course of disease.

Our analyses of DSS-treated CD11c-βcat^{EX3} mice exhibited significantly increased Treg numbers in mLN and LI-LPL (Figure 33). Nevertheless, we could not detect any differences in disease progression when comparing DSS-treated CD11c-βcat^{EX3} mice and DSS-treated control mice (Figure 31). This contrasts with the results of Manicassamy et al (56). who, as already mentioned, observed a more severe course of the disease with a lower Treg number in LI-LPL. According to this, an increased Treg number would result in a weakened course of colitis, but this does not seem to be confirmed in our experiments. One reason for this could be that the course of DSS colitis depends only to a small extent on T cells and mainly on cells of the innate immune system (36, 37). But since there is little evidence to date that changes in β-catenin expression also affect the innate immune system, a CD11c-specific deletion or stabilization of β-catenin would have little or no effect on the course of DSS colitis. While this questions the results of Manicassamy et al., it would explain the disease progression in our experiment. Thus, to investigate the influence of alterations in β-catenin expression under inflammatory conditions in more detail, an inflammation model would have to be used in which mainly T cells contribute to the development of inflammation. This will be elucidated later.

Our results regarding cell numbers of DC and Tregs from the DSS colitis studies are comparable to the results from the steady state experiments with CD11c-βcat^{EX3} mice. In both examinations we found increased numbers of DC and Tregs in both mLN and LI-LPL. This indicates that CD11c specific stabilization of β-catenin has a tolerogenic influence on cell composition in the intestinal immune system. How exactly β-catenin exerts this effect remains unclear here as well. As in the steady state, it would be interesting to analyze the cytokine production of the individual DC subsets, but also of the Tregs, to see how this is changed by the CD11c-specific stabilization of β-catenin.

Regarding the increased Treg numbers in steady state experiments and under inflammatory

conditions, it would be interesting to distinguish between tTregs and pTregs in the Treg population. The transcription factor Helios can be used for this purpose since it is expressed by tTregs but not by pTregs. Distinguishing both Treg subgroups would allow us to analyze whether the change in β-catenin expression particularly affects a subgroup of Tregs. These exert their suppressive function in different ways. While one prominent mechanism of tTregs is to inhibit the trafficking of effector T cells to the target organ, antigen specific pTregs primarily prevent T cell priming by acting on antigen-presenting cells such as DC and disable their capacity to present antigen (21). By distinguishing between the different subgroups, one could analyze whether β-catenin particularly supports one subgroup and thus one mode of functioning.

In summary, the results from the steady state experiments with CD11c-βcat^{EX3} mice also seem to be confirmed under inflammatory conditions. Even though it had no influence on the course of DSS colitis, the CD11c specific stabilization of β-catenin led to an increased Treg number in mLN and LI-LPL. Here, too, the exact function and effect of β-catenin on Tregs would have to be investigated in further experiments. Like the steady state, an analysis of the cytokine production of the individual DC subsets as well as of Tregs would provide information on how the CD11c-specific stabilization of β-catenin affects the cells. RNA sequencing would also be interesting in this context to investigate the changes triggered by a constitutively active β-catenin at the molecular level. Perhaps important genes for the regulation of T cell homeostasis are activated by constitutively active β-catenin and others are thereby switched off.
Thus, the precise effects of a CD11c-specific stabilization of β-catenin remain unclear. Still, our results suggest that β-catenin signaling in DC stimulates Treg induction and thus contributes to the balance between tolerance and immunity in the intestinal immune system.

In our next murine model of intestinal inflammation, we wanted to address the question, of what role β-catenin plays in the innate immune system of the intestine. Therefore, we used the anti CD40 colitis model in T cell deficient mice, since inflammation in this model is driven only by cells of the innate immune system and is therefore independent of T cells. Analysis of the weight graph of anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice displayed no significant differences compared to anti CD40 treated control mice (Figure 34). Analysis of DC homeostasis displayed a significant decrease in the absolute cell number of mLN DC, especially the number of CD103^{+} and double positive DC were significantly reduced (Figure 35). Investigation of DC cell numbers in LPL of the small and large intestine exhibited no significant differences. The analysis of the absolute cell number of macrophages in mLN, LI-LPL and SI-LPL also displayed no significant differences (Figure 38). These results suggest that CD11c specific stabilization of β-catenin does not appear to influence the cells of the innate

immune system in the intestine. Perhaps the tolerogenic function of the β-catenin signaling pathway, that we had attributed to β-catenin after our experiments with CD11c-βcat^{EX3} mice is dependent on the presence of T cells. Maybe the presence of T cells contributes to the functioning of the pathway with a feedback mechanism to DC. Since these experiments were performed with T cell deficient mice and the anti CD40 colitis model is T cell independent, the absence of T cells together with the inflammatory response could lead to a dysfunction and dysregulation of the β-catenin pathway and thus of DC homeostasis resulting in similar courses of disease and maybe also decreased DC numbers.

For further analysis of this question, it would be interesting to analyze the DC and macrophage composition in anti CD40 treated TCRβδ CD11c-βcat^{DEL} mice. And an investigation of the cell composition of DC and macrophages from TCRβδ CD11c-βcat^{EX3} mice and TCRβδ CD11c-βcat^{DEL} mice in steady state would also be of interest so that we could compare the findings with the results from CD11c-βcat^{EX3} mice and CD11c-βcat^{DEL} mice in steady state. Thus, until further analysis, it appears that alterations in the CD11c specific β-catenin expression have no effect on the innate immune system of the gut.

As mentioned before and based on our previous steady state and DSS experiments (displaying an increase in Treg numbers), we hypothesized that β-catenin promotes a tolerogenic DC phenotype, leading to an increase in Tregs. However, we further hypothesized, that this effect would be dependent on the presence and influence of T cells. Therefore, we wanted to investigate the impact of a CD11c specific stabilization of β-catenin on course of disease, on DC and on T cells in an inflammation model, which is dependent on T cells, namely the T cell transfer colitis model. The T cell transfer colitis model is based on the transfer of naïve T cells (that do not contain Tregs) into T cell deficient mice (TCRβδ CD11c-βcat^{EX3} mice). The T cells then usually differentiate into proinflammatory Th1 and Th17 cells, triggering inflammation and the development of colitis. Though, the transferred naïve T cells can also differentiate into Tregs, which would then delay or prevent the onset of gut inflammation. Here, it is worth mentioning, that the Tregs arising from the transferred naïve T cells are mainly pTregs, which are induced in the periphery. Yet, differentiation from naïve T cells into Tregs tends to occur too late to prevent expansion of effector T cells and the development of colitis. But the direction of T cell differentiation is dependent on external influences, for example by DC. Thus, it is possible that under the influence of tolerogenic DC, so many Tregs are induced that the development of colitis can be prevented.

If our hypotheses were correct, then β-catenin would also lead to a tolerogenic DC phenotype here, which would result in an increase in Treg numbers. Thus, since this inflammatory model

is more T cell dependent in contrast to DSS colitis (where we didn't find any differences despite an increase in Tregs), an increase in Tregs would attenuate the course of disease. It should be considered in the analysis of the data, that we were only able to use and analyze this model once due to time constraints.

In the analysis of our results, we observed that TCRβδ CD11c-βcat^{EX3} mice displayed a significantly attenuated course of disease and overall better survival compared to control mice. The endoscopy score of TCRβδ CD11c-βcat^{EX3} mice was also significantly lower than that of control mice (Figure 36). Anyhow, analysis of T cell numbers in TCRβδ CD11c-βcat^{EX3} mice exhibited no significant differences compared with control mice. Furthermore, the absolute cell number of DC was rather reduced in TCRβδ CD11c-βcat^{EX3} mice (Figure 37). These results naturally raise the question of what is responsible for the differences in disease progression.

But before addressing this question, we should elaborate on one striking aspect, namely the early onset of the disease in control mice. Usually, colitis develops after 5-6 weeks. However, our control mice lost a lot of weight after only 3 weeks. Furthermore, the TCRβδ CD11c-βcat^{EX3} mice also showed endoscopic signs of inflammation after 3 weeks, even though their weight development was normal. This early onset of disease is surprising, as we used a well-known protocol (72). One possible reason could be the microbiome of our mice. Perhaps it contained microorganisms or bacteria that accelerate the onset and progression of the disease. Still, the exact reason for the early onset of the disease remains unclear.

When interpreting the results, the first thing that stands out is that the absolute cell number of DC in all organs examined is lower in TCRβδ CD11c-βcat^{EX3} mice than in control mice. This is not particularly astonishing, as the increased inflammation in control mice compared with TCRβδ CD11c-βcat^{EX3} mice results in a higher infiltration of inflammatory cells in the affected organs, so also from DC. Then again, it is even more surprising that the analysis of absolute cell numbers of T cells did not reveal any major differences, not only in anti-inflammatory Tregs, but also in proinflammatory CD4$^+$ T cells. Accordingly, relative to the number of DC, TCRβδ CD11c-βcat^{EX3} mice have an increased number of Tregs, but also an increased number of CD4$^+$ T cells. That despite the low DC numbers, the number of Tregs in TCRβδ CD11c-βcat^{EX3} mice is like the Treg numbers in control mice could be due to an effect of β-catenin. We also found this effect already in our steady state and DSS experiments. There, too, we found an increase in Treg number (Figure 30, Figure 33), suggesting that DC from mice with CD11c-specific stabilization of β-catenin are more tolerogenic and produce higher amounts of regulatory factors, such as IL-10, RA and TGF-β than DC from control mice. This would result in a higher absolute cell number of Tregs. Where the high numbers of CD4$^+$ T cells in relation to the DC number come from remains unclear.

Nevertheless, the question remains why TCRβδ CD11c-βcat^{EX3} mice have an attenuated course of colitis compared with control mice, even though both have the same absolute cell number of T cells, especially Tregs (which are even slightly significantly reduced in mLN of TCRβδ CD11c-βcat^{EX3} mice). One possible reason for this could be that constitutively activated β-catenin signaling in DC enhances the function of Tregs, for example, by increasing the release of anti-inflammatory cytokines, such as TGF-β, IL-10 or RA. This would mainly affect pTregs, as these represent the majority of Tregs in the T cell transfer colitis model. To investigate this question in more detail, one could perform an immunoassay to analyze the cytokine production of Tregs from TCRβδ CD11c-βcat^{EX3} mice and control mice, with a special focus on the production of anti-inflammatory cytokines. In case of an increased production of those anti-inflammatory cytokines by Tregs from TCRβδ CD11c-βcat^{EX3} mice an equal Treg number compared to control mice would be sufficient to mitigate the progression of the disease, as the course of disease is T cell dependent. This would also fit the endoscopy findings of the TCRβδ CD11c-βcat^{EX3} mice: Here, the mice showed signs of mild to moderate inflammation, while no abnormalities were seen in the weight curve. The inflammatory signs visible in endoscopy arise from the T cell transfer and result in infiltration of the tissue by immune cells, including Tregs. The β-catenin mediated enhanced function of Tregs would then in turn reduce the strength of the immune response by secretion of anti-inflammatory cytokines, which would in turn prevent weight loss. This would explain why the TCRβδ CD11c-βcat^{EX3} mice show signs of inflammation but do not lose weight. However, this hypothesis then raises the question of why we did not see any differences in our DSS experiments, where we even saw an increase in Tregs in CD11c-βcat^{EX3} mice. As the DSS model also works in T cell deficient mice and is not completely dependent on T cells, it is likely that the influence of T cells in the DSS colitis model is not as great as in T cell transfer colitis. Hence, the differences regarding Tregs result in attenuation of disease progression in T cell transfer colitis but not in DSS colitis. In this context, an analysis of the course of T cell transfer colitis in TCRβδ CD11c-βcat^{DEL} mice with a CD11c-specific deletion of β-catenin would also be of interest. Following our results with a CD11c-specific stabilization of β-catenin, which led to an attenuated disease course, a CD11c-specific deletion of β-catenin would then be expected to lead to a more severe disease course. And here, too, an analysis of the cytokine production of Tregs would be interesting with the question of whether they produce fewer anti-inflammatory cytokines than Tregs from control mice.

One question that could be further explored is whether altering CD11c-specific β-catenin expression changes the induction of T cell anergy and thus initiates tolerance. T cell anergy is a mechanism induced by antigen-presenting cells as DC, where no priming of the T cell occurs, and the naïve T cell remains in a hyporesponsive state for an extended period. This prevents

the development of an immune reaction and induces tolerance to the antigen. To test, if differences in the CD11c specific expression of β-catenin changes the induction of T cell anergy, one could analyze the absolute cell number of naïve $CD4^+$ T cells in T cell transfer treated TCRβδ CD11c-βcat^{EX3} mice and control mice. If T cell anergy were a mechanism by which CD11c-specific stabilization of β-catenin would induce a tolerogenic effect, the number of $CD4^+$ naïve T cells in TCRβδ CD11c-βcat^{EX3} mice would be higher than in control mice. This needs to be elucidated in further experiments.

After analysis of the results from the T cell transfer colitis experiments, it further appears possible that β-catenin signaling in DC promotes a tolerogenic DC phenotype and thus influences the number and function of Tregs. Hence, constitutive active expression of β-catenin in $CD11c^+$ cells would result in an increase in Treg number and an enhancement of the immunosuppressive function of Tregs (with an increased secretion of anti-inflammatory cytokines). This would in turn attenuate the disease course of colitis, but only in a disease model in which inflammation is strongly dependent on T cell homeostasis. Furthermore, these results support the hypothesis from the antiCD40 colitis experiments that the β-catenin signaling pathway requires the presence of T cells for exerting its function. However, further experiments are necessary for the exact confirmation of these hypotheses.

The functions of β-catenin under inflammatory conditions have also been studied in other animal disease models. For instance, Suryawanshi et al. (58) displayed that DC-specific deletion of β-catenin exacerbated disease progression in an EAE (experimental autoimmune encephalomyelitis) model, whereas activation of the β-catenin signaling pathway attenuated EAE progression (58). This fits with the hypothesis we made based on our results, as inflammation in the EAE model is largely mediated by T cells (73). Results from experiments with an asthma model also support our hypothesis: thus, mice with a CD11c-specific deletion of β-catenin were more prone to severe asthma progression, whereas disease progression was attenuated in mice with a CD11c-specific stabilization of β-catenin (Personal communication by Prof. Dr. B. Clausen). This also fits our hypothesis, as inflammation in asthma is also predominantly mediated by T cells. Thus, β-catenin would also play a tolerogenic role here. On the other hand, studies in a collagen-induced arthritis (CIA) model exhibited no differences in disease progression between mice with a CD11c-specific β-catenin deletion and control mice, even though mice with a CD11c-specific β-catenin deletion displayed fewer Tregs (59). But since inflammation in the CIA model depends only to a small extent on T cells but is mediated mainly by a CII-specific antibody, these results would also fit our findings and hypothesis (73).

The synopsis of these data further supports the hypothesis that β-catenin signaling in DC under certain conditions has a tolerogenic impact on the regulation of the balance between tolerance and immunity.

Still, the role of macrophages in this scenario should not be neglected in the interpretation of the results. Indeed, the CD11c specific change in β-catenin expression does not only affect DC, but also macrophages, as they are also $CD11c^+$. Therefore, we cannot say with certainty that the results and changes in our experiments are entirely because of β-catenin signaling in DC, because β-catenin signaling in macrophages may also play a role here. However, the changes in T cells, specifically in Tregs, found in our results, tend to argue that this difference is caused by DC, since T cells and especially Tregs are predominantly induced by DC and not by macrophages. Even though macrophages can induce T cells, they tend to play a minor role in the activation and induction of T cells compared to DC and they mainly interact with already primed effector CD4 T cells. While DC drive the initial clonal expansion and differentiation of naïve T cells into T helper and T effector cells, macrophages do not present antigen mainly to activate naïve T cells, but rather to make use of the effector functions of T cells that have been previously primed by DC. These effector functions of the activated T cell then help the macrophages to exert the immune response, which was initially induced by DC activating naïve T cells (7). Thus, it seems more likely that the differences in our experiments are caused by β-catenin signaling in DC. To completely exclude an influence of macrophages under these circumstances, further experiments are required. To address this question, β-$catenin^{floxdel/floxdel}$ mice or β-$catenin^{floxEX3/floxEX3}$ mice could be crossed with a CX3CR1-Cre to obtain a macrophage-specific deletion or stabilization of β-catenin expression. Repeating our experiments in these mice under steady state and under inflammatory conditions may provide further insights on the contribution of β-catenin signaling in macrophages to our results.

5 Summary

Conclusively our results widely support the hypothesis stated at the beginning, that β-catenin signaling promotes a tolerogenic DC phenotype and Treg induction and is therefore pivotal for regulating the balance between inflammatory and tolerogenic responses in the intestinal immune system in steady state and under inflammatory conditions. Based on our results, however, it appears that the impact of the tolerogenic β-catenin effect on intestinal inflammation is apparent only under certain conditions, depending on, for example, the type of inflammation. To verify our data and the resulting hypothesis, further experiments such as those mentioned in the discussion are required.

Furthermore, to better study the effects of alterations in β-catenin expression at the molecular level and to discover the mechanisms by which β-catenin affects DC function, DNA microarray analysis or RNA sequencing of DC/ a particular DC subset could be performed. And, as stated earlier, an investigation of the cytokine production of DC and DC subsets would be interesting to analyze, whether and to what extent β-catenin changes the cytokine production by DC and how this impacts Treg induction or function, since we suspected this as one of the mechanisms by which β-catenin exerts its tolerogenic effect. Also, to better determine the impact of β-catenin specifically on $CD103^+$ DC, one could cross β-$catenin^{floxdel/floxdel}$ mice or β-$catenin^{floxEX3/floxEX3}$ mice with an XCR1-Cre to obtain a $CD103^+$ DC specific β-catenin deletion/ stabilization. Experiments with this mouse line could then reveal what role the change in β-catenin expression plays in $CD103^+$ DC.

Still, the problem remains that the CD11c-specific change in β-catenin expression also affects macrophages and therefore we cannot be completely sure whether the differences in the experiments were really induced by β-catenin signaling in DC. This question could be addressed with the previously mentioned experiments with a macrophage-specific deletion or stabilization of β-catenin.

In conclusion our data indicate, that via influencing DC and T cell homeostasis β-catenin signaling in DC plays a central role in the regulation and balancing of intestinal immune homeostasis. However, after our experiments some questions remain unanswered and, consequently, further research is required to clarify the detailed role of β-catenin in this scenario. Once the role of β-catenin in this process has been further identified, the results could potentially be transferred to the immune system in the human intestine and eventually to the treatment of IBD.

6 Appendix

6.1 Mice as model organisms

The mouse is the most common research mammalian in the world. Studies on mice have contributed enormously to our knowledge of the human biology. This is not surprising, as mice are phylogenetically related to humans and physiologically like humans. Furthermore, maintaining and breeding of mice in laboratories is relatively easy and many inbred strains are available, simplifying research on mice.

Still, mice and humans have evolved in different environments, to which they adapted, and they therefore became different organisms. They thus differ in several characteristics, for instance their size, their lifespan, their microbiome and their anatomy of the gastrointestinal track (only rough differences in the intestine and the immune system are elucidated here.) Although both mice and humans are omnivores, the nutrition of mice and humans markedly differs resulting in different microbiomes. Furthermore, the length of the SI in relation to the colon is greater in mice than in humans. Mice also have a prominent cecum and lack the appendix. Hence the complete transfer of results from experiments with mouse intestine to humans is difficult (74).

The general structure of the immune system and the immune cells in mice and humans are relatively similar. Research on mice has contributed tremendously to our understanding of the immune system. However, since mice and humans are confronted with different pathogens and microbiomes, it is not surprising, that there are also many significant differences between the mouse and the human immune system. Those differences include for instance alterations in the balance of leukocyte subsets and the function and expression of costimulatory molecules. Therefore, the response to experimental interventions often differs considerably between mice and humans, making it difficult to transfer much research on immunological diseases in mice to humans (74, 75).

Regarding the intestinal immune system, most of the immune cell subsets are similar in the human and murine intestine, though their relative proportions may differ. However, subdividing intestinal DC subsets differs between mice and human. While murine intestinal DC subsets are grouped depending on their expression of CD103 and CD11b, human intestinal DC subsets are grouped depending on their expression of CD103 and signal regulatory protein α (SIRPα). $CD103^+$ $SIRP\alpha^+$ DC likely represent the human equivalent of $CD103^+CD11b^+$ murine DC, whereas $CD103^+$ $SIRP\alpha^-$ DC seem to be the human counterpart of $CD103^+$ $CD11b^-$ murine DC (12). It remains to be determined, if an equivalent to murine $CD103^-$ DC exists in the human intestine. Furthermore, it is currently unclear, whether those human intestinal DC subsets function in a similar way to their murine counterparts. Although determining the function of

human intestinal DC subsets would be crucial for understanding the development of IBD, experiments with human tissue are understandably very difficult to conduct compared to experiments with mouse tissue. As experiments with mouse tissue are more feasible and provide informative hints about the role of DC in the intestinal immune system, research on mice is crucial for understanding the role of DC in the development of human IBD (12, 76).
In conclusion it can be said, that even though mice and humans are different organisms, they display several similarities in their general composition and therefore, research on mice is essential for a better understanding of the human organism. However, all those differences mentioned above should be considered before translating research on mice to humans (12, 76).

6.2 Prospective therapeutic strategies involving Wnt/ β-Catenin signaling

Besides its role in the regulation of the immune system, the Wnt/ β-catenin signaling pathway is also involved in the development of several malignancies. Mutations of the Wnt/ β-catenin signaling pathway are frequently observed in carcinomas, for instance in colorectal cancer.
As most of the mutations result in the permanent activation of the Wnt/ β-catenin pathway and correspondingly strong growth, several therapeutic strategies have been developed aiming on the inhibition of this pathway. This would decrease the proliferation stimulus by Wnt/ β-catenin signaling resulting in reduced growth of the tumor.

As of 2017, several therapeutic strategies aiming on inhibition of the Wnt/ β-catenin pathway were in clinical development. One of those strategies involves the specific blocking of Porcupine, the enzyme which catalyzes the activation of Wnt Proteins. As the activation of Wnt proteins by Porcupine is essential for Wnt transport, inhibition of Porcupine results in blocking of Wnt secretion. Lack of Wnt signals in turn would decrease the activation of the Wnt/ β-catenin pathway eliminating the proliferation stimulus triggered by this pathway (46, 77).

Another therapeutic strategy, which has already been tested in preclinical studies, but not in clinical trials, involves the stabilization of the β-catenin destruction complex. Tankyrase is an enzyme, which regulates the stability of Axin and induces its proteasomal degradation. As Axin is a central component of the β-catenin destruction complex, inhibition of Tankyrase results in the stabilization of the destruction complex and therefore in lower levels of intracellular β-catenin (46, 77).

However, in certain diseases, an activation of the β-catenin pathway could also be useful. For example, in IBDs, dysregulation of intestinal immune cells contributes to their development. As

β-catenin signaling in DC is suggested to play an important role in the prevention of this dysregulation, therapeutic strategies involving the activation of β-catenin signaling specifically in DC could be a potential target in the prospective treatment of IBD. And if an absence of β-catenin in DC leads to a worse course of colitis in mouse experiments, activation of the β-catenin signaling pathway in DC in turn could have an attenuating effect on the disease course of colitis. Perhaps this approach can also be applied to IBD in humans.

Taken together, affecting the Wnt/ β-catenin signaling pathway as a potential therapeutic strategy is still at an early stage and requires further investigations. However, with several clinical trials running, there are promising hints, that interference with the Wnt/ β-catenin signaling pathway may soon be part of a regular clinical therapy.

6.3 Limited impact of CD11c specific β-catenin stabilization on macrophage subsets in LI-LPL and SI-LPL of anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice

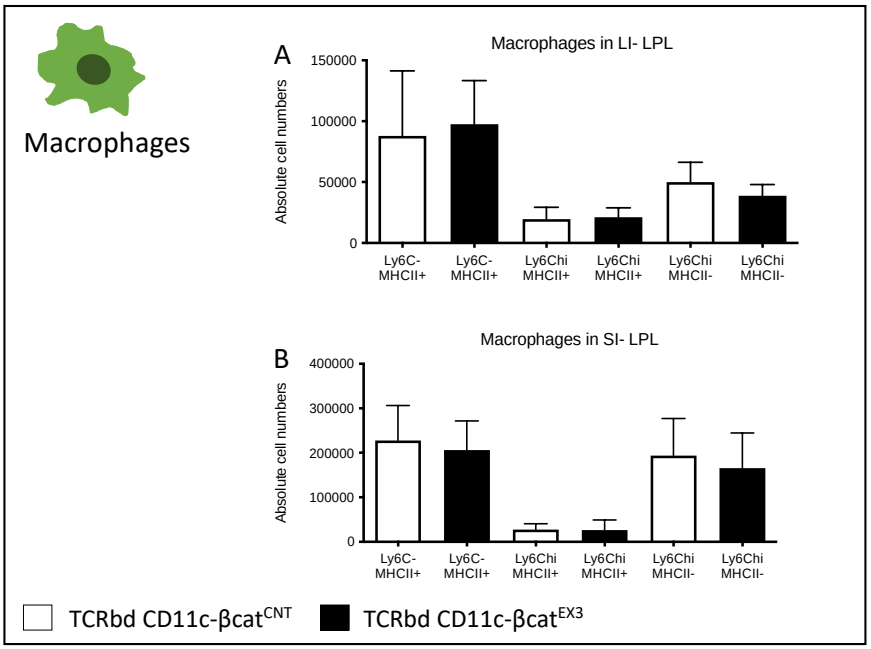

Figure 38 CD11c specific β-catenin stabilization appears to have no impact on macrophage subsets in LI-LPL and SI-LPL of anti CD40 treated TCRβδ CD11c-βcat^{EX3} mice

(A) Absolute cell number of different macrophage subsets in LI-LPL of anti CD40 treated TCRβδ CD11c-βcat^{EX3} and control mice. (B) Absolute cell number of different macrophage subsets in SI-LPL of anti CD40 treated TCRβδ CD11c-βcat^{EX3} and control mice.

Bar diagrams depict absolute cell numbers +/- SEM.

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