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The role of dietary iron for intestinal immune responses in murine colitis models

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"Iron rusts from disuse; water loses its purity from stagnation... even so does inaction sap the vigor of the mind."

Leonardo da Vinci

TABLE OF CONTENTS

TABLE OF CONTENTS ..... **I**

INDEX OF FIGURES ..... **III**

INDEX OF TABLES ..... **V**

ABBREVIATIONS ..... **VI**

1. INTRODUCTION **1**

1.1. The importance of iron 1

1.2. Dietary iron bioavailability 1

1.2.1. Iron absorption and distribution 2

1.2.2. Iron utilization 3

1.2.3. Systemic iron homeostasis 5

1.2.4. Regulation of cellular iron metabolism 5

1.3. Gastrointestinal tract 6

1.4. Gut associated lymphoid tissue 7

1.5. Inflammatory bowel disease 12

1.5.1. Genetic factors driving IBD 12

1.5.2. Environmental factors 15

1.5.3. The role of intestinal immune system in IBD 16

1.6. Intestinal macrophages and dendritic cells in IBD 20

1.7. Iron as environmental factor influencing inflammation in IBD 24

2. AIMS OF THE STUDY **27**

3. MATERIAL AND METHODS **28**

3.1. Materials 28

3.1.1. Reagents 28

3.1.2. Kits and enzymes 29

3.1.3. Antibodies 29

3.1.4. Media and buffers 31

3.1.5. Equipment/Software 32

3.1.6. Mice 33

3.1.7. Diets 33

3.2. Methods 34

3.2.1. Dietary treatment and colitis induction 34

3.2.2. Colitis experiments 36

3.2.3. T cell isolation 37

3.2.4. Haematological analysis 39

3.2.5. Isolation of murine leukocytes from lymphoid tissue 39

3.2.6. Isolation of murine colon lamina propria and intraepithelial leukocytes ... 39
3.2.7. Leukocyte stimulation ... 40
3.2.8. Flow cytometry ... 40
3.2.9. Histology ... 41
3.2.10. RNA isolation and real time quantitative PCR (qPCR) ... 42
3.2.11. Statistical analysis ... 43
4. RESULTS ... 45
4.1. Effect of luminal iron depletion on intestinal immunity in the steady state ... 45
4.2. Dietary iron depletion fails to protect against T cell mediated colitis development ... 54
4.3. The effect of dietary iron depletion on colonic macrophages and DCs during experimental T cell colitis ... 59
4.4. Effect of extended luminal iron depletion on T cell mediated intestinal inflammation development ... 69
4.5. Effect of luminal iron depletion on T cell activation in T cell transfer colitis model ... 75
4.6. Luminal iron depletion fails to protect against DSS induced colitis ... 79
5. DISCUSSION ... 86
5.1. Experimental mouse colitis models ... 87
5.1.1. T cell transfer colitis model ... 88
5.1.2. Dextran sodium sulphate (DSS) induced colitis ... 89
5.2. The effect of luminal iron depletion in mouse experimental colitis models ... 90
5.3. Effect of dietary iron on colonic macrophages and DCs composition during intestinal inflammation ... 96
5.4. The effect of luminal iron depletion on T cell differentiation after T ceil induced colitis development ... 100
5.5. Conclusion ... 100
6. SUMMARY ... 102
7. REFERENCES ... 104
8. APPENDIX ... 125
8.1. Appendix 1 ... 125
8.1.1. Experimental diets ... 126
9. ACKNOWLEDGEMENTS ... 129
10. CURRICULUM VITAE ... 131

INDEX OF FIGURES

Figure 1 Cells regulating iron homeostasis **4**
Figure 2 Organisation of GALT in the large intestine **11**
Figure 3 Experimental setup of diet treatment and colitis induction **35**
Figure 4 Isolation of CD4+CD62L+ T cells from splenocytes **38**
Figure 5 Bodyweight course during dietary treatment **45**
Figure 6 Cytokine mRNA expression in steady state colon of *Rag1*$^{-/-}$ mice after diet treatment **47**
Figure 7 Leukocyte frequencies in the colon and mLNs after diet treatment in the steady state mice **48**
Figure 8 Effect of luminal iron change on colonic m in steady state *Rag1*$^{-/-}$ mice **49**
Figure 9 Comparison of intestinal MΦ frequencies and MΦ subset composition in resting *Rag1*$^{-/-}$ mice after diet treatment **50**
Figure 10 Phenotype characterization of colon dendritic cells on steady state *Rag1*$^{-/-}$ mice after diet treatment **51**
Figure 11 Effect of luminal iron depletion on intestinal DC frequencies in steady state *Rag1*$^{-/-}$ mice **53**
Figure 12 Development of T cell transfer colitis after iron depletion **55**
Figure 13 Cytokine induction in colon after 6-week diet treatment and colitis development **57**
Figure 14 Effect of luminal iron depletion on leukocyte infiltration in colon after T cell induced colitis development **60**
Figure 15 Gating strategy for characterization of colonic MΦ in T cell transfer colitis after luminal iron depletion **61**
Figure 16 Frequencies of colonic MΦ population after luminal iron depletion and colitis development **62**
Figure 17 Frequencies of colonic MΦ after diet treatment and colitis development **64**
Figure 18 Gating strategy for characterization of colonic DCs in *Rag1*$^{-/-}$ mice after diet treatment and colitis development **65**
Figure 19 Effect of luminal iron depletion on colonic DCs after T cell transfer induced colitis development **66**
Figure 20 Effect of luminal iron depletion on colonic DC composition after colitis development **67**
Figure 21 Development of T cell transfer induced colitis after extended dietary iron depletion **70**
Figure 22 Effect of extended dietary treatment on mRNA cytokine expression after T cell transfer colitis development **71**
Figure 23 Expansion of leukocytes during T cell transfer colitis after extended luminal iron depletion **72**
Figure 24 Effect of the extended luminal iron depletion on the colon macrophage compartment during T cell transfer colitis **73**
Figure 25 Effect of the extended luminal iron depletion on the colonic DC compartment after chronic experimental colitis development **74**
Figure 26 Effect of luminal iron depletion on the T cell infiltration after colitis development **76**
Figure 27 Effect of luminal iron depletion on T_{Reg} recruitment after T cell colitis development **77**

Figure 28 Effect of luminal iron depletion on IFN-γ and IL-17A secretion by T cells after colitis development 78
Figure 29 Survival curve during DSS induced colitis development in WT mice treated with experimental diets 80
Figure 30 Effect of luminal iron depletion on DSS colitis development in WT mice 81
Figure 31 Characterization of colonic MΦ after luminal iron depletion and DSS colitis development . 83
Figure 32 Characterisation of colonic DCs after luminal iron depletion and DSS mediated colitis development 84

INDEX OF TABLES

Table 1 Antibodies used in the study **30**
Table 2 Mouse strains used in the study **33**
Table 3 Diets used in the study **33**
Table 4 Clinical symptoms and score **37**
Table 5 Summary of hematological analysis after diet treatment in steady state mice **46**
Table 6 Monitoring of hematological parameters during 6-week diet treatment and T cell mediated colitis development **56**
Table 7 Effect of luminal iron depletion on mononuclear phagocytes after DSS colitis development **85**

ABBREVIATIONS

°C	Celsius
ACI/D	Anaemia of chronic inflammation / disease
APC	Antigen presenting cells
ARE	AU-rich elements
ATG16L1	Autophagy - related 16 - like 1 gene
Batf3	Basis leucin zipper transcription factor ATF like 3
BMP	Bone morphogenetic protein
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CD	Crohn's disease
CD45RB	Cluster of differentiation 45 receptor splice variant B
cDNA	Copy deoxyribonucleic acid
CRC	Colorectal cancer
CX_3CR1	CX_3C chemokine receptor 1
DC	Dendritic cells
DcytB	Duodenal cytochrome B
DMT1	Divalent metal transporter 1
DSS	Dextran sulphate sodium
DT	Diphtheria toxin
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmatic stress
EtOH	Ethanol
FACs	Fluorescence activated cell sort
FBXL5	F-box and leucine rich repeat protein 5
FCS	Fetal calf serum
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FITC	Fluorescein isothiocyanate
fL	Femmtolitre

FLVCR1	Feline leukaemia virus, subgroup C, cellular receptor
Foxp3	Forkhead box protein 3
Fpn1	Ferroportin 1
G	Gram
g/l	Gram per litre
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
GWAS	Genome-wide association study
H	Hour(s)
H&E	Haematoxylin and eosin
HBSS	Hank’s Balanced Salt Solution
HCT	Haematocrit
Hg	Hemoglobin
HH	Heredity hemochromatosis
HIF	Hypoxia inducible factor
HJV	Hemojuvelin
HO-1	Heme oxygenase -1
HPC-1	Heme carrier protein
i.p.	Intraperitoneal
IARC	International Agency for Research in Cancer
IBD	Inflammatory bowel disease
ID	Iron deficiency
IEC	Intestinal epithelial cells
IEL	Intraepithelial leukocytes
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRE	Iron responsive elements
IRF	Interferon regulatory factors
IRP	Iron regulatory protein
ISC	Iron sulphur clusters
JAK2	Janus kinase 2
LP	Lamina propria

LPS	Lipopolysaccharide
LysMcre	Cre recombinase expression under the control of murine M lysozyme
M cells	Microfold cells
M1	Macrophage 1
M2	Macrophage 2
MACs	Magnetic activated cell sort
MAMPs	Microbe-associated molecular pattern
MAP	Mitogen activated protein
MCH	Mean corpuscular haemoglobin
MCV	Mean corpuscular volume
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
Min	Minute(s)
mLNs	Mesenteric lymph nodes
MP	Mononuclear phagocytes
mRNA	Messenger ribonucleic acid
MUC2	Mucin 2
Myd88	Myeloid differentiation primary response protein
МΦ	Macrophages
NEAA	Nonessential amino acids
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NK	Natural killer cells
NLRs	Nucleotide binding oligomerization domain (NOD)-like binding receptors
NOD2/CARD15	Nucleotide-binding oligomerization domain 2 / caspase activation recruitment domain 15
Notch 2	Neurogenic locus notch homolog protein
o/n	Overnight
PAMPs	Pathogen-associated recognition receptors
PBS	Phosphate buffered saline
PE	Phycoerythrin
PE-Cy	Phycoerythrin – cyanine dye
PerCp	Peridinin-chlorophyll

Pg	Piccogram
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
PP	Peyer's patches
PRRs	Pathogen recognition receptors
PUFA's	Polysaturated fat
qPCR	Quantitative polymerase chain reaction
RA	Retinoic acid
Rag ½	Recombination activation gene
RBC	Red blood cell count
RIG-I	Retinoic acid-inducible gene
ROR-γt	Retinoic acid receptor – related orphan receptor gamma t
ROS	Reactive oxygen species
Rpm	Resolution per minute
RT	Room temperature
SCID	Severely combined immune deficiency
SD	Standard deviation
SFB	Segmented filamentous bacteria
SI	Small intestine
SMAD	Sma and Mad related proteins
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TEDs	Transepithelial dendrites
Tf	Transferrin
TfR1	Transferrin receptor 1
TfR2	Transferrin receptor 2
TGF-β	Transforming growth factor beta
T_H	T helper cells
TLRs	Toll like receptors
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF-α	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor alpha receptor 1
T_{Reg}	Regulatory T cells
TRUC	$Tbx2^{-/-}Rag2^{-/-}$ ulcerative colitis

TSLP	Thymic stromal lymphopoetin
UC	Ulcerative colitis
UPR	Unfold protein system
v/v	Volume per volume
WBC	White blood cell count
WT	Wild type
XBPI	X-box binding protein 1
$\alpha_4\beta_7$	Integrin subunit alpha 4 and beta 7
µM	Micro molar

1. INTRODUCTION

1.1. The importance of iron

Iron is essential mineral for almost all living organisms. Though unaware of its nutritive value, the ancient civilizations already used iron for medical purposes. Crucial knowledge about the immunobiological importance of iron was discovered during the last two centuries when its nutritive value was recognised in regard to states of anaemia and infection. During this time the first occurred the observations regarding iron absorption and its regulation were made and "mucosal block" theory was developed (Granick 1946). Although a long time has passed since these crucial observations were made, findings about molecular mechanisms of iron metabolism, its regulation and role in health and disease have recently been published (Beard JL 1996, Andrews and Schmidt 2007). Iron is a component of many metalloproteins and due to its high redox capacity it participates in numerous biochemical processes in living organisms. As a constituent of hemoglobin and myoglobin, it facilitates oxygen delivery from the lungs to other tissues. It contributes to energy metabolism, electron transport, DNA and RNA synthesis and gene regulation, normal cell functioning, growth and development (Beard 2001, Lieu, Heiskala et al. 2001). On the other hand, most environmental iron exists in free ferric form (Fe^{3+}), which is almost insoluble in extracellular fluids at neutral pH, and when in excess it can catalyse the generation of harmful reactive oxygen species (ROS) (Papanikolaou and Pantopoulos 2005). In order to keep the iron balance and avoid free iron related toxicity, organisms were challenged to develop robust, tightly regulated systems orchestrating iron intake and transport as well as cellular and systemic regulation of these processes.

1.2. Dietary iron bioavailability

The total body iron content of an average adult man or woman is about 3-5 g (35 – 45 mg/kg of bodyweight). Two thirds of this are captured within developing erythroid precursors and mature red blood cells and are constantly being recycled by macrophages of the reticuloendothelial system. The remaining iron is stored in the liver, spleen, bone marrow and muscle tissue. The basal daily iron loss via urine, feces, gastrointestinal tract and skin is around 0.5 - 2 mg and it has to be compensated by equivalent dietary iron intake (Finch 1994). The body iron status depends on the amount of dietary iron, its bioavailability and the extent of iron losses. Dietary iron comes in one of two forms inorganic and heme iron. Inorganic iron is present in both plant and animal foods and it is predominant in a standard

diet. Meat, seafood and poultry are rich sources of heme iron (Briat, Curie et al. 2007). Although representing only 10% of dietary iron, because of its highly efficient absorption, heme iron accounts for more than 50% of the iron that enters the body (Hooda, Shah et al. 2014). In contrast to heme, the total inorganic iron intake is more affected by other nutrients. Phytate, polyphenols, calcium and some proteins like albumin inhibit non-heme iron absorption whereas formation of iron complexes with ascorbic acid enhances inorganic iron intake (van Dokkum 1992, Teucher, Olivares et al. 2004). In general, the non-heme iron absorption is found to be 2-3 fold higher in mixed than in vegetarian diets (Hurrell and Egli 2010).

1.2.1. Iron absorption and distribution

Since it is not actively secreted, the iron amount in the body can only be regulated at the point of its absorption, that usually takes place in duodenum and jejunum, though small amounts can also be absorbed in stomach, ileum and colon (Frazer and Anderson 2005, Gulec, Anderson et al. 2014). Iron is transferred from the lumen through differentiated enterocytes. Iron transport through the epithelial barrier requires specific transporters and enzymes that can change the oxidative state of iron (Evstatiev and Gasche 2012).

Inorganic iron enters enterocytes via the divalent metal transporter 1 (DMT 1) across the brush border membrane. Inorganic ferric (Fe^{3+}) iron must be first reduced to ferrous (Fe^{2+}) iron by duodenal cytochrome B (DcytB) because of the higher binding affinity of DMT 1 for Fe^{2+} (Atanasova, Li et al. 2005, Latunde-Dada, Xiang et al. 2011).

Heme iron enters enterocytes intact via heme carrier protein (HCP-1). Internalized heme may be directly exported by feline leukemia virus, subgroup C, cellular receptor 1 (FLVCR1). Alternatively, heme oxygenase-1 (HO-1) catalyses heme degradation and iron release (Shayeghi, Latunde-Dada et al. 2005, West and Oates 2008, Laftah, Latunde-Dada et al. 2009). Once inside enterocytes, inorganic and heme derived iron become part of a common iron pool. Export across the basolateral membrane requires the membrane protein ferroportin 1 (Fpn), the only identified iron exporter so far (Abboud and Haile 2000, Donovan, Brownlie et al. 2000). Before being loaded on transferrin (Tf), Fe^{2+} iron is oxidised to Fe^{3+} by hephaestin (Chen, Huang et al. 2006). Tf-bound iron is then delivered to different tissues by binding to transferrin receptors (TfR1 and TfR2) (Uchida, Akitsuki et al. 1983).

1.2.2. Iron utilization

Because of its high reactivity and toxicity, cells have developed mechanisms that provide fast processing of iron. Once imported inside the cell, iron is used either for metabolic processes, or is stored or exported. The delivery of iron bound to Tf from endosomes to other cell organelles is not fully understood. It is most likely that iron binds to small chelating molecules or chaperons that carry it to the mitochondria (Lane, Merlot et al. 2015). Translocation of Fe^{2+} across mitochondrial membrane is mediated by mitoferrin 1 and mitoferrin 2, members of mitochondrial solute carrier family (Paradkar, Zumbrennen et al. 2009). Once inside the mitochondria, iron can participate in iron sulphur clusters (ISC) (Stehling, Smith et al. 2007). When in excess inside the cell, iron is converted into inert form by binding with ferritin. Ferritin bound iron is also found in plasma and it is the most useful clinical parameter to estimate body iron stores (Hintze and Theil 2006).

Iron plays an important role in many metabolic processes. Red blood cells represent the biggest reservoir of iron that is constantly being recycled. During erythropoiesis, iron is used for cellular requirements of erythroblasts and for formation of hemoglobin, a constituent of mature erythrocytes. Erythrocyte precursors take up iron bound to transferrin via Tf-TfR1 mediated endocytosis (Hentze, Muckenthaler et al. 2004). Mitoferrin then transports iron to the mitochondria where heme synthesis occurs (Ponka 1997, Shaw, Cope et al. 2006). At the end of their lifespan, senescent and damaged erythrocytes are taken up by reticuloendothelial macrophages via phagocytosis. This process mainly happens in spleen. Erythrocytes are lysed within the phagosomes at low pH and ferrous iron is liberated from hemoglobin by heme oxygenase-1 (HO-1). Macrophages can store released iron with ferritin. Erythropoiesis or other iron signals might stimulate macrophages to release iron via Fpn into the circulation when needed (Gammella, Buratti et al. 2014). Before being loaded on Tf, ferrous iron is oxidised into ferric form by multi-copper ferroxidase ceruloplasmin (Potdar, Sarkar et al. 2014).

Hepatocytes also have a large capacity for iron storage. These cells have different mechanisms for acquiring iron that are thought to be independent of the Tf-TfR axis. Most stored iron is in ferritin form, and it can be released at any time when needed (Randell, Parkes et al. 1994, Barisani, Berg et al. 1995)

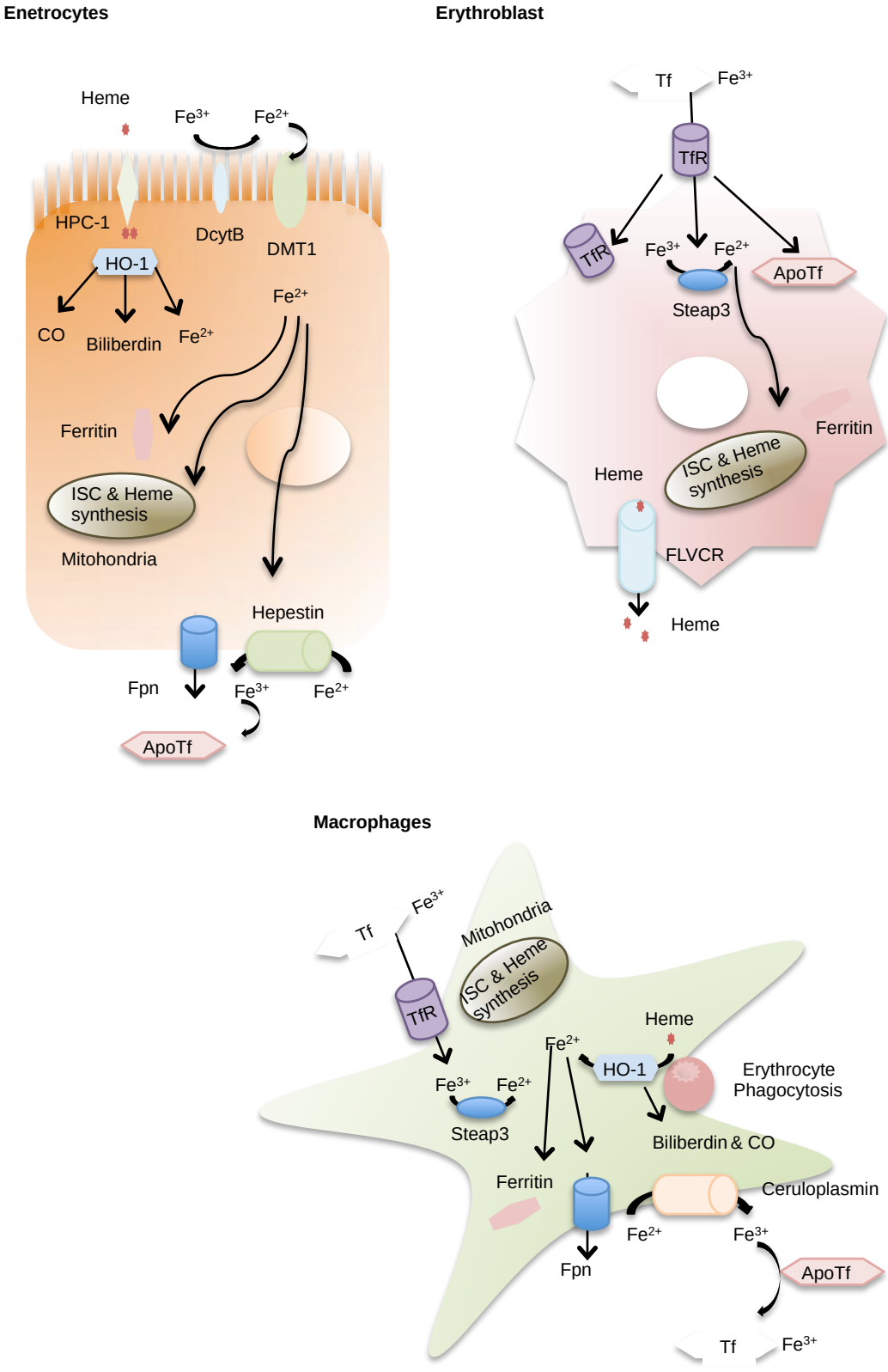

Figure 1 Cells regulating iron homeostasis
ApoTf – apotransferrin, **CO** – carbon monoxide, **DcytB** – duodenal cytochrome B, **DMT1** – divalent metal transporter 1, **FLVCR** - feline leukemia virus subgroup C receptor-related protein 1, **Fpn** – ferroportin, **HPC-1** – heme protein carrier 1, **HO -1** – heme oxygenase 1, **ISC** – iron – sulphur cluster, **Tf** – transferrin, **TfR** – transferrin receptor.

1.2.3. Systemic iron homeostasis

Iron balance has to be tightly regulated in order to provide sufficient amounts of iron when needed and to prevent iron overload because of its toxic effect. Signals about body iron status are transmitted by plasma factors like Tf, iron bound Tf, ferritin and hepcidin to the organs of iron absorption, storage and utilization. Hepcidin, a peptide hormone primarily produced by hepatocytes in the liver, plays a key role in systemic iron regulation. Hepcidin regulates the expression of iron transporters DMT1 and Fpn1. Direct binding of hepcidin to Fpn, followed by Fpn internalization and degradation, interrupts iron secretion in the epithelial layer and in macrophages (Nemeth, Tuttle et al. 2004, Nemeth 2008, Ganz and Nemeth 2012). Mice deficient for hepcidin develop a severe iron overloading phenotype, while the overexpression of hepcidin leads to development of iron deficiency (Nicolas, Bennoun et al. 2002, Viatte, Lesbordes-Brion et al. 2005). Since the function of hepcidin is of great importance, mammalians developed different mechanisms that enable fine-tuning of its expression and activity. Hepcidin signalling is directly regulated by erythropoiesis, inflammation, iron storage and hypoxia (Nicolas, Bennoun et al. 2001, Pigeon, Ilyin et al. 2001). Iron regulates its own metabolism through a negative feedback loop mediated by TfR2 and human hemochromatosis proteins HFE. When in excess, iron bound Tf binds to TfR2 and HFE, which induces hepcidin signalling via BMP/SMAD axis (Goswami and Andrews 2006, Johnson, Chen et al. 2007, Bhatt, Horgan et al. 2009)' (Wang, Li et al. 2005, Babitt, Huang et al. 2006). IL-6 (Nemeth, Rivera et al. 2004) and IL-1 (Lee, Peng et al. 2005) were shown induce hepcidin expression via signal transducer and activator of transcription (STAT)3 signalling pathway in inflammation and block iron export pathways as a host defence mechanism against pathogens (Wrighting and Andrews 2006, Verga Falzacappa, Vujic Spasic et al. 2007).

1.2.4. Regulation of cellular iron metabolism

The cellular iron pool is regulated by the iron responsive element (IRE)/iron regulatory protein (IRP) system that works together with proteins of the hypoxia inducible factor (HIF) signalling pathway (Wilkinson and Pantopoulos 2014).

The IRE/IRP regulatory apparatus controls synthesis of major proteins of iron transport and utilization at the translational level in response to cellular iron content. The result of IRP binding to mRNA stem-loop element IRE is dependent on IRE localisation. In iron replete

state, IRP1 bound to iron-sulphur clusters acts like an aconitase and participates in gluconeogenesis and ketone synthesis (Aziz and Munro 1987). The IRP2 protein is ubiquitinylated by FBXL5 and degraded. In iron deficiency, IRP1 is nitrated and it abolishes its aconitase activity while IRP2 is stabilized since FBXL5 is degraded (Kaptain, Downey et al. 1991, Rouault, Stout et al. 1991). Both proteins can bind to IRE elements. The position of IRE where IRPs bind, defines the destiny of the protein synthesis. IRP binding to the IRE situated in the 5'-untranslated region (UTR) results in repression of ribosome binding and decreases translation, like in the case of ferritin and Fpn. In contrast, IRP binding to 3'-UTR element of Tf mRNA protects the transcript from endonucleases and stabilises the mRNA, resulting in higher Tf expression (Pantopoulos 2004). The capacity of the two IRPs to regulate iron metabolism is slightly different due to their different binding affinity for IRE elements. Reactive oxygen species can induce binding of IRP1 to IRE while at tissue oxygen level IRP1 has a lower affinity for IRE then IRP2, and acts like aconitase in ISCs (Theil and Eisenstein 2000). On the other hand, IRP2 doesn't have any alternative function. Since IRP2 knockout induces microcytic anemia and neurodegeneration, it has been suggested that IRP2 plays a pivotal role in IRP/IRE mediated iron regulation (Cooperman, Meyron-Holtz et al. 2005). IRE/IRP regulation is a subject of other regulatory mechanisms like hypoxia (Hanson and Leibold 1998), phosphorylation (Fillebeen, Caltagirone et al. 2005) and IRP degradation (Fillebeen, Chahine et al. 2003).

mRNAs of other proteins that are not involved in iron metabolism like HIF2-α (Sanchez, Galy et al. 2007), cell cycle phosphatase cdc14A (Sanchez, Galy et al. 2006) and many others, are regulated by IRE/IRP system. Deletion of both IRPs is lethal during embryogenesis (Galy, Ferring-Appel et al. 2013).

1.3. Gastrointestinal tract

The gastrointestinal (GI) tract is the largest internal organ system in the body, having a surface area 200 times greater then skin (Garside and Mowat 2001). Mouth, esophagus and stomach belong to the upper part of the tract while its lower part consists of small intestine, cecum and colon. The main function of the GI tract is to ensure food intake and effective absorption of dietary nutrients and water, which mainly occurs in the small intestine and requires support of commensal microbiota (Mowat and Agace 2014). This makes the GI tract a site of constant exposure to complex mixtures of environmental and dietary antigens as well

as intestinal commensals and parasitic pathogens. To cope with this, the GI tract harbours the biggest immune cell reservoir in the body. It is the task of the intestinal immune system to discriminate between harmful and harmless antigens and initiate adequate immune response to each of them respectively (Mowat and Viney 1997, Hooper and Macpherson 2010). A potential failure in “self/non-self “ recognition might lead to excessive tissue damage and development of inflammatory bowel diseases (IBD) (Swiatczak, Rescigno et al. 2011).

1.4. Gut associated lymphoid tissue

A precisely regulated collaboration between the commensal microbiota, the underlying epithelium and the intestinal immune system plays a critical role in intestinal homeostasis. The epithelial monolayer and the mucus layer represent the first line of defence in the intestine. Although the majority of the cells bordering the intestinal lumen are enterocytes, which have a major role in nutrient absorption and digestion, stem cells at the bottom of Lieberkuhn crypts give rise to several other types of intestinal epithelial cells (IEC), which have digestive but also protective functions (Rescigno 2011). Highly glycosylated mucus, produced by goblet cells at the top of the villi, coats the epithelium of both intestines, reaching its highest thickness in the colon. Mucus forms a viscous, charged gel-like glycocalyx that traps bacteria, which are then exposed to the antimicrobial peptides leukotrienes, defensins and lysosomes produced by Paneth cells at the base of the crypts (Salzman, Underwood et al. 2007, Turner 2009). Apart from providing a physical and biochemical barrier, epithelial cells can sense environmental and microbial antigens via a wide range of pathogen recognition receptors (PRRs), like toll like receptors (TLRs) and NOD-like binding receptors (NLRs), while their polarized structure enables them to discriminate between commensal microbiota and invading pathogens (Fritz and Girardin 2005). Depending on the nature of the microbial antigen and the location of its binding site, epithelial cells are able to coordinate adequate immune responses ranging from tolerance to effector immunity via direct interactions with immune cells and/or production of immune mediators. Indeed, differential responsiveness of IECs to various TLR ligands seems to be predefined by the apical vs. basolateral stimulation (Gewirtz, Navas et al. 2001, Rhee, Im et al. 2005). For example, engagement of TLR9 on the apical side of IECs leads to partial activation of NF-kB without proinflammatory cytokine induction, while binding of TLR9 ligand on the basolateral side will induce robust inflammatory response (Lee, Mo et al. 2006). Furthermore, thymic stromal lymphopoetin (TSLP) and TGF-β secreted by IECs have been

shown to condition intestinal mononuclear phagocytes, including dendritic cells (DC) and macrophages M(Φ), which is essential for establishment of gut homeostasis (Rimoldi, Chieppa et al. 2005, Zeuthen, Fink et al. 2008).

Although the above mentioned physical and biochemical mechanisms form a first line of defence that is impermeable to most of the microbial antigens, some of them may cross the epithelium and tolerance or immunity must be established. The GI immune system, also known as gut-associated lymphoid tissue (GALT), includes large organized lymphoid structures, like Peyer's patches (PPs), found in small intestine (SI) and mesenteric lymph nodes (mLNs), as well as small lymphoid follicles scattered throughout the mucosa (Garrett, Gordon et al. 2010). The main task of the GALT is to mount an adequate immune response in order to protect the body against invasive pathogens, but also to prevent unnecessary inflammatory responses leading to destruction of the epithelium.

Intestinal innate immune cells like mast cells, natural killer cells (NK), eosinophils, dendritic cells (DCs) and macrophages MΦ are the first to encounter a broad spectrum of bacteria among which are also potential pathogens. Apart from neutralization of pathogens via phagocytosis, these cells produce immunoregulators like TGF-β, retinoic acid (RA), IL-10 and IL-22 that are essential for maintenance of intestinal homeostasis (Harrison and Maloy 2011).

The organized structures of GALT and draining LNs are the principal locations where priming of the adaptive immune response takes place. Specialized epithelial cells known as microfold cells (M cells) are scattered throughout the surface of PPs in the small intestine and lymphoid follicles in the colon, where they take up antigens from the lumen and deliver them to the underlying DC-enriched subepithelial zone (Kyd and Cripps 2008). These antigens can be directly presented to the T and B cells in the PP's or loaded on DCs that migrate to mLNs, where lymphocyte priming takes place (Steinman 2007). DCs recognize and respond to components of bacteria and other microorganisms via various PRRs, like TLRs, NLRs and retinoic acid inducible gene (RIG)-I-like receptors (Lavelle, Murphy et al. 2010). Rescigno et al. discovered the existence of lamina propria resident cells that were able to project their transepithelial dendrites (TEDs) into the gut lumen and sample the antigens (Rescigno, Urbano et al. 2001). At first these cells were thought to be DCs but subsequent studies showed that the majority of these cells belong to the CX3CR1^{high} macrophage population that

take up soluble antigens and transfer them to migratory $CD103^+$DCs (Medina-Contreras, Geem et al. 2011, Rivollier, He et al. 2012).

Binding of pathogen associated molecular patterns (PAMPs) or microbe associated molecular pattern (MAMPs) to their corresponding PRRs on the surface of DCs results in migration of DCs, upregulation of costimulatory molecules and cytokine secretion (Dudda, Lembo et al. 2005) (Schulz, Jaensson et al. 2009, Bogunovic, Mortha et al. 2012). The chemokine receptor CCR7, expressed on the surface of migratory DCs, directly interacts with chemokines CCL19/21 produced by stromal cells in the T cell zone in mLNs, directing antigen-loaded DCs to the T cell rich zone (Ohl, Mohaupt et al. 2004). Migratory DCs upregulate the expression of the gut-homing molecules CCR9 and $\alpha_4\beta_7$ on T lymphocytes and support forkhead box P3 $(Foxp3)^+$ regulatory T cells (T_{Reg}) differentiation from naïve T cells by secretion of retinoic acid (RA), TGF-β and IL-10 in the steady state (Coombes, Siddiqui et al. 2007, Sun, Hall et al. 2007). Mature IL-10 secreting T_{Reg} cells migrate constantly from PP's or mLNs back to the lamina propria (LP) and intraepithelial areas where they play an important role in maintenance of oral tolerance towards food and commensal microbiota antigens. While migratory $CD103^+$ DCs are important for the induction of T_{Regs} in mLNs, $CD103^-CD11b^+CX3CR1^+F4/80^+$ lamina propria macrophages control T_{Regs} expansion in the LP in a RA and TGF-β dependent manner (Hadis, Wahl et al. 2011, Medina-Contreras, Geem et al. 2011).

In contrast to the commensal microbiota, some pathogens possess virulence factors that can create a proinflammatory environment, which leads to the recruitment of neutrophils, MΦs and DCs that secrete proinflammatory cytokines and chemokines. Migratory $CD103^+$ DCs in mLNs can generate potent effector immune responses. Moreover, it has been shown that inflammation dampens the tolerogenic potential of migratory $CD103^+$ DCs by impairing their ability to induce T_{Reg}. T_H17 cells are quite rare in steady state GALT and they are find mainly in the lamina propria of the small intestine. IL-6, secreted mainly by $CD103^+CD11b^+$ DCs, triggers T_H17 polarization from $CD4^+$ T cells, while production of IL-6 together with IL-12p40 by $CD103^+CD11b^-CD8\alpha^+$ DCs contributes to the T_H1 differentiation (Denning, Norris et al. 2011, Fujimoto, Karuppuchamy et al. 2011). Specific commensals like segmented filamentous bacteria (SFB) shape the prevalence of distinct helper and regulatory T cell populations in the intestine (Denning, Wang et al. 2007, Atarashi, Nishimura et al. 2008, Farkas, Panea et al. 2015).

Upon antigen priming, T cells acquire some gut homing properties including RA dependent upregulation of adhesion receptors CCR9 and α4β7 integrin. In the small intestine CCR9 expressing antigen experienced T cells interact with $CCL25^+$ epithelial cells, which enables their homing to the small intestine (Iwata, Hirakiyama et al. 2004). Mucosal T cells are classified in two major subsets based on T cell receptor (TCR) and coreceptor expression. In general, in the intraepithelial (IEL) fraction unconventional or Type b T cells expressing γδ or αβ TCR in combination with CD8αα coreceptor are predominant. It has been estimated that there are 10-20 IELs per 100 IECs in the human gut (Ferguson 1977). Although being cytotoxic, in the absence of a strong stimulus these cells have an 'activated yet resting' phenotype. Their primary function is to regulate epithelial integrity (Komano, Fujiura et al. 1995). Both $CD4^+$ and $CD8\alpha\beta^+$ type a T cells are present in the lamina propria at a 2:1 ratio. The $CD4^+$ helper T cell compartment comprises different populations with distinct functions. The prevalence of both IL-10 secreting T_{Reg} and regulatory T_H17 cells in the lamina propria in the steady state is crucial for keeping the intestinal immune system attenuated in order to avoid excessive responses against the gut flora but yet being ready to eliminate pathogenic fungal and bacterial attacks (Izcue, Coombes et al. 2006, Veldhoen, Hocking et al. 2006). The LP lymphocytes produce various cytokines, including IFN-γ, IL-17 and IL-10. Recent studies highlight the ability of differentiated T cells to switch their function depending on the immune environment in the effector tissue. For example, it has been shown that most helper T cells (including T_H1 and T_H17) can produce certain amount of IL-10 cytokine giving them some regulatory potential (van Wijk and Cheroutre 2010).

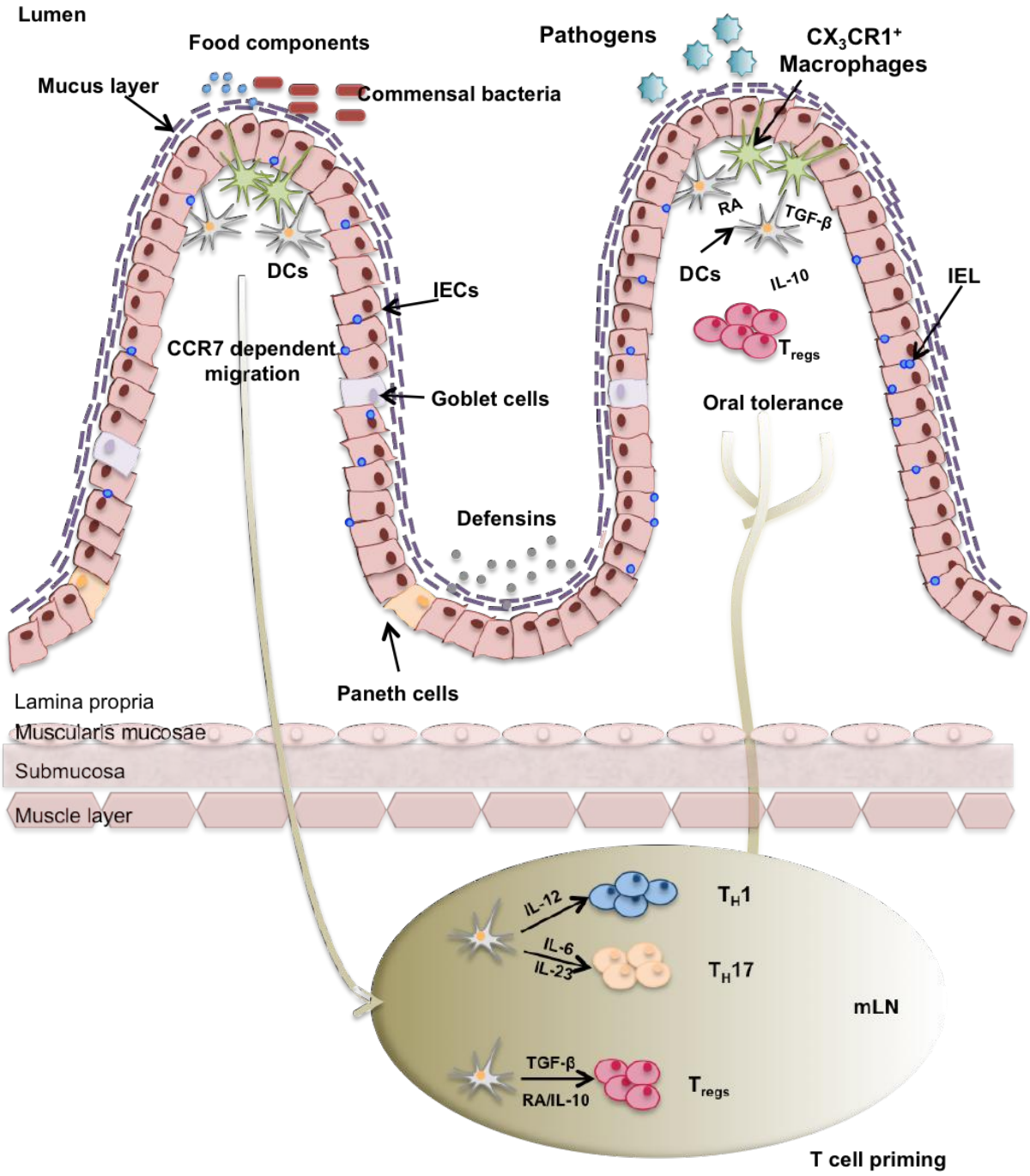

Figure 2 Organisation of GALT in the large intestine
DC – dendritic cells, IEC – intestinal epithelial cells, IEL – intraepithelial leukocytes, mLN – mesenteric lymph node

Immunoglobulin A (IgA), the most abundant intestinal antibody subtype, is produced by intestinal plasma cells. IgA neutralizes pathogens and toxins and prevents adhesion of commensal microbiota to IECs. Epithelial cells help IgA to reach its effector site by expressing Ig receptors and acting as a shuttle for IgA oligomers enabling their translocation from LP onto the mucosal surface (Cerutti 2008).

1.5. Inflammatory bowel disease

Inflammatory bowel disease (IBD) comprises the chronic, relapsing and remitting gastrointestinal disorders Crohn's disease (CD) and ulcerative colitis (UC). IBD is mainly seen in developed, urbanized countries with a peak incidence in early adult life. The ethiology of the disease is not fully understood, but it is believed to be multifactorial. The pathophysiology of IBD is characterized by intestinal inflammation and tissue damage as a result of excessive immune responses to gut bacteria secondary to environmental factors in a genetically susceptible host (Kaser, Zeissig et al. 2010). Although having similar pathological features, CD and UC are quite distinct in terms of clinical manifestation, underlying genetic background and immunological features. UC is characterized by continuous inflammation that is restricted to colon. Histopathology features of UC include neutrophil infiltration within lamina propria, which causes micro-abscesses formation in the crypts and superficial mucosal ulceration. UC is considered a T_H2 like disease associated with IL-5, IL-13 and TGF-β (Strober and Fuss 2011) although this may be true only for a subgroup of UC patients. In contrast, CD can appear at any site along the gastrointestinal tract, though terminal ileum is most likely to be affected with early mucosal lesions often appearing over PPs. CD is patchy and segmental with transmural leukocyte infiltration characterized granulomas formed by macrophage. CD is dominated by T_H1 responses on the basis of evidence reporting increased IFN-γ production (Xavier and Podolsky 2007).

1.5.1. Genetic factors driving IBD

Genetic alterations in the normal homeostatic processes that regulate interactions between host and microbiota, are considered to be one of the main triggers of the exaggerated immune response in IBD patients. In the last few years, genome-wide association studies (GWAS) have identified 163 IBD risk loci among which 30% are common for CD and UC, but most of which are CD specific (Zanello, Kevans et al. 2014). Analysis of IBD-associated gene loci revealed their involvement in several pathways that are crucial for intestinal homeostasis, including barrier functions and epithelial regeneration, microbial defence, reactive oxygen species (ROS) generation, autophagy, innate and adaptive immune regulation. One of the most common genetic associations to CD is the loss-of-function polymorphism in the bacterial sensing gene *NOD2/CARD15* (nucleotide-binding oligomerization domain 2/caspase activation recruitment domain 15) encountered in 30% of CD patients with disease manifestation in the small intestine (Kobayashi, Chamaillard et al. 2005). The dysfunction of

NOD2/CARD15 has been reported in Paneth cells and monocyte-derived cells (Maeda, Hsu et al. 2005) and it seems to result in impaired detection and responses to enteric bacteria by epithelial and innate immune cells. A reduction in Paneth cell α-defensin production has been reported in CD patients and NOD2 KO mice (Wehkamp, Harder et al. 2004, Voss, Wehkamp et al. 2006). The correlation between NOD2/CARD15 deficiency and intestinal inflammation in humans is not clear. Interestingly mice with the same gene mutation do not develop colitis spontaneously (Watanabe, Kitani et al. 2005).

Patients suffering from IBD show a compromised mucus layer and increased mucolytic bacterial load and *MUC2*$^{-/-}$ mice develop spontaneous colitis(Fu, Wei et al. 2011).

Ineffective bacterial clearing due to disruption of autophagy is another feature in CD. Autophagy is a "self-eating" process that plays an important role in protection of mammalian cells against invasive pathogens and cytotoxic effects of bacterial toxins. Autophagy is thought to be one of the primary attempts of the organism to establish homeostasis upon pathogen stimulation. Recent studies have implicated the correlation between dysfunction of autophagocytic genes *ATG16LI* and *IRGM* with increased risk for CD. ATG16LI is broadly expressed in IECs, APCs, T and B cells. Homozygosis for the *ATG16LI* risk allele leads to Paneth cell dysfunction and enhanced production of IL-1β by LPS stimulated Atg16l1$^{-/-}$ macrophages. ATG16LI deficiency in bone marrow derived cells increased susceptibility to DSS induced colitis in mice (Saitoh, Fujita et al. 2008). *IRGM*$^{-/-}$ KO mice exhibit increased susceptibility to various bacterial infections due to low killing efficiency by *IRGM*$^{-/-}$ macrophages in these mice (MacMicking, Taylor et al. 2003).

Abnormalities in the unfold protein response (UPR) in case of endoplasmatic reticulum (ER) stress are linked to IBD pathogenesis. Accumulation of misfolded proteins induces ER stress, which activates the UPR system whose task is to damper the stress (Walter and Ron 2011). Deletion of *XBP1* (X box binding protein 1), a transcription factor in the UPR system, results in imbalanced ER stress in the epithelium, development of SI enteritis and higher susceptibility to DSS colitis (Kaser, Lee et al. 2008). Because of its high capacity to generate ROS, cellular iron has been considered as a potential trigger of ER stress and tissue damage in context of various inflammatory disorders. Recent findings revealed that the activation of UPR is common in iron overload-hereditary hemochromatosis (HH), a disease that is a result of dysfunction in the iron regulator protein HFE. Tan et al. showed that iron toxicity

modulates ER stress signalling pathways in a mouse model of alcohol and high-fat diet - induced liver injury in *HFE*$^{-/-}$ null mice leading to UPR activation and failure to induce autophagy related genes (Tan, Crawford et al. 2013).

Although dysfunctional innate immune responses seem to be a prerequisite for the excessive activation of the adaptive immunity, the latter also seems to be a more prominent driver of tissue damage in IBD patients. For example, genome-wide association studies linked polymorphisms in *IL-23R* and *IL-12B* genes to IBD (Duerr, Taylor et al. 2006, Van Limbergen, Wilson et al. 2009, Pidasheva, Trifari et al. 2011). Both IL-12 and IL-23 are produced by monocytes, macrophages and DCs secondary to PRR-derived signals. IL-12p70 is composed of two covalent subunits (p35 and 40) and it drives T_H1 immune responses. On the other hand, IL-23 consists of the p40 subunit linked to the unique p19 subunit, and it regulates expansion and maintenance of IL-17 producing T cells (Trinchieri 1994, Vignali and Kuchroo 2012). Murine colitis models have supported the potential pathogenic role of both cytokines. Treatment of mice with neutralizing p40 antibody reduced IFN-γ production and attenuated ongoing inflammation in trinitrobenzen (TNBS) induced colitis (Neurath, Fuss et al. 1995). Furthermore, T_H17 cells were increased in CD patients and a specific *IL-23R* coding variant (*IL23R*^{G11421A}) was shown to be protective since it impairs the IL-23-induced T_H17 effector function while other noncoding *IL-23R* variants were shown to confer risk in both CD and UC (Duerr, Taylor et al. 2006) (Parkes, Barrett et al. 2007, Franke, Balschun et al. 2008). Blockade of p40 by ustekinumab (mab) was shown to be effective in some CD cases especially in patients previously treated with infliximab (Sandborn, Feagan et al. 2008).

Although recent GWAS studies identified key molecular pathways in the pathogenesis of IBD, the ethiology of the disease remains to be explained. The relatively low concordance rate in monozygotic twins of 10-15% in UC and 30-35% in CD indicates the importance of non-genetic factors in development of IBD (Franke, McGovern et al. 2010, Anderson, Boucher et al. 2011). Furthermore, several genetic mouse models of intestinal inflammation lose their disease phenotype in germ free conditions indicating an important role of the microbiota in the onset of IBD (Iqbal, Oliver et al. 2002). These observations imply that the effects of environmental factors on the relationship between intestinal microbiota and immune system are important modifiers of the risk for IBD development in genetically susceptible individuals.

1.5.2. Environmental factors

The adoption of a Western lifestyle including dietary habits, food accessibility and scarce physical activity is one of the major explanations for the increasing incidence of IBD cases in some developing countries (Ng 2015). Earlier studies have reported higher rates of proinflammatory cytokines as well as increased gut permeability in obese patients, but convincing data about linkage between obesity and IBD is lacking (Hass, Brensinger et al. 2006, Long, Crandall et al. 2011). On the other hand, modern western diet contains processed food that is enriched in fat and refined carbohydrates and contains much less fibre and vegetables. The bowel lumen is continuously exposed to the food antigens as well as the macro- and micronutrients and additives. Proposed factors by which the diet could influence the incidence of IBD include direct dietary antigens, microbiota changes, gene expression alternations and gastrointestinal permeability dysfunction (Shah, Parian et al. 2015). Studies investigating the role of dietary patterns and the risk for IBD revealed that high dietary intake of total fats, polyunsaturated fat (PUFAs), omega-6 fatty acids, refined sugars and meat increase the risk for IBD, while a high fibre, vegetables and fruit enriched diet decreased the disease incidence (Shoda, Matsueda et al. 1996, Amre, D'Souza et al. 2007, Hou, Abraham et al. 2011). In 2015, The International Agency for Research in Cancer (IARC) ranked consumption of processed meat alongside smoking as one of the major causes of cancer development (Bernstein, Song et al. 2015, Nagle, Wilson et al. 2015). Colorectal cancer (CRC) development has been associated with increased dietary intake of iron porphyrin pigment – heme - that is present in red meat. Ijssennagger *et al.* reported that dietary heme induces acute oxidative stress and delayed cytotoxicity resulting in intestinal epithelial injury, which is compensated by hyper proliferation of crypt cells (Ijssennagger, Rijnierse et al. 2013, Ijssennagger, Belzer et al. 2015).

Smoking is one of the best-studied environmental factors in IBD that has an opposite effect on CD and UC. Active smokers have increased predisposition for CD development and worse outcome in case of surgical procedure than non-smokers. In contrast, former smokers are more susceptible to develop UC then smokers or people who never smoked (Fuss, Neurath et al. 1996). This can be explained by the fact that chronic nicotine stimulation of α7 nicotine receptor expressing T cells induces T_H1 expansion that is related to CD(Kikuchi, Itoh et al. 2008). Furthermore, smoking and smoking cessation leads to alternations of intestinal microbiota (Biedermann, Brulisauer et al. 2014).

Human and mouse studies support the role of commensal microbiota in IBD. In humans, IBD usually occurs with higher frequencies in the areas with the highest bacteria concentration like colon and ileum. Antibiotics can be beneficial in the early stages of the CD as they were shown to have a dramatic effect on the microbiota and subsequently on the underlying innate and adaptive immune system (Rahimi, Nikfar et al. 2006, Rahimi, Nikfar et al. 2007). In mouse models the microbiota seems to be the primary immunological driver of the intestinal inflammation as various genetic models fail to reproduce an IBD phenotype in absence of intestinal microbiota (Strober, Fuss et al. 2002). Most of animal IBD models either induced by pathogens (*Citrobacter rodentium*), chemicals (dextran sodium sulphate-DSS) or genetically (*IL-10* deficient mice) display similar changes in the phyla composition within the commensal microbiota that manifests in a reduction of Firmicutes (predominantly Gram-positive) and Bacteriodetes (predominantly Gram-negative) species and the prevalence of entero-adherent *E.coli* (Lupp, Robertson et al. 2007). This observation was also reported in human IBD cases where one third of both CD and UC patients showed similar shift in microbiota composition (Frank and Pace 2001, Sartor 2008)' (Mazmanian, Round et al. 2008). Another hint that microbiota drives inflammation in human CD is that deviation of fecal stream by surgery can heal disease in that portion of the intestine (Longman, Diehl et al. 2014).

1.5.3. The role of intestinal immune system in IBD

Animal models, established to resemble the pathogenesis of human IBD, cannot fully recapitulate the clinical and histopathological characteristics of the human disease, but rather serve as a powerful tool for studying the immunologic bases of this disease. Several models of mucosal inflammation have been developed including chemical induction, immune cell transfer and genetic manipulation. In human IBD as well as in the animal models, the mucosal inflammation that develops is always channelled through common pathways of innate and adaptive immunity eventually resulting in excessive inflammatory effector T_H - cell response (Strober, Fuss et al. 2007). Indeed, identification of cellular and molecular key players in these immunological pathways allowed development of newly applied or yet to be applied therapeutic strategies (de Mattos, Garcia et al. 2015).

Since increased epithelial permeability has been reported in CD patients as well as in their first relatives who have no evidence of disease, it was proposed by some studies to be one of the primary etiologic features of CD. In accordance with this were the studies from murine

DSS mediated colitis, where mice that are given DSS polymers in drinking water develop acute intestinal inflammation due to the direct toxic effect of DSS to gut epithelial cells of the basal crypts, resulting in increased cellular exposure to commensal microbiota. The disruption of epithelium leads to overexposure of mononuclear phagocytes to the bacterial components resulting in increased secretion of TNF-α and IL-12 by these cells (Dieleman, Palmen et al. 1998). Subsequently bacterial antigens are presented and trigger the differentiation of proinflammatory effector T cells recognizing antigens derived from commensal bacteria.

IL-6, IL-1β and TNF-α produced by DCs and MΦ are associated with both forms of IBD to lesser or greater degree. Each of them activates NF-κB and the mitogen activated protein (MAP) kinases and thereby induces various downstream proinflammatory responses that mediate tissue pathology in IBD (Ng, Benjamin et al. 2011). For example, IL-6/IL-6R complex exerts pro-inflammatory functions by activating multiple target cells like APC and T cells via STAT1 or STAT3 signalling pathway in experimental colitis and IBD patients (Atreya, Mudter et al. 2000, Kai, Takahashi et al. 2005). Neutralization of IL-6 with monoclonal antibody in mouse models induced T cell apoptosis, reduced production of IFN-γ and TNF-α and therefore suppressed chronic intestinal inflammation (Atreya, Mudter et al. 2000). TNF-α is another cytokine that mediates various proinflammatory functions in colitis by binding to its receptors TNFR1 and TNFR2. Neutralization of MΦ (and also T cell) derived TNF-α is one of the best-established clinical approaches in IBD (Atreya, Zimmer et al. 2011). The molecular and cellular mechanisms of TNF-α pathogenic action are still unclear. In $Tnf^{\Delta ARE}$ mice gene targeted deletion of regulatory AU-rich elements (ARE) in untranslated mRNA results in TNF-α overexpression leading to polyarthritis and human CD-like chronic intestinal inflammation mostly affecting terminal ileum. Transmural inflammation in $Tnf^{\Delta ARE}$ mice requires $CD8^+$ T cells, is dependent on IL-12/23p40 and is regulated by $CD4^+$ T cells producing IFN-γ. $Rag1^{-/-}Tnf^{\Delta ARE}$ mice exhibit only mild inflammation, which suggests that TNF can inflict superficial inflammation in the absence of B and T cells. On the other hand, it was shown that the disease can be transferred by adoptive transfer of the $Tnf^{\Delta ARE}$ derived T cells into a new recipient (Kontoyiannis, Pasparakis et al. 1999).

Whereas activation of innate immune cells is a common feature of IBD, different subpopulations of mucosal $CD4^+$ T cells play a central role in induction and persistence of intestinal inflammation in UC and CD. When stimulated *in vitro,* $CD4^+$ T cells isolated from

mucosa of CD patients are able to produce large amounts of T_H1/T_H17 associated proinflammatory cytokines (*e.g.*, IFN-γ, IL-17, TNF-α) while $CD4^+$ T cells from UC inflamed mucosa produce T_H2 cytokines (*e.g.*, IL-4, IL-13) as well as IL-17 (Baumgart and Sandborn 2012, Ordas, Eckmann et al. 2012). For instance, T-bet, the transcriptional regulator of T_H1 effector T cells, was upregulated in CD patients and the transfer of T-bet deficient T cells in animal transfer colitis models failed to induce intestinal inflammation while the transfer of T-bet overexpressing T cells caused colitis development (Neurath, Weigmann et al. 2002). The mouse studies first identified IL-12 as a potential therapeutic target for CD patient since IL-12 is the master cytokine driving the T_H1 differentiation from undifferentiated T cells leading to the development of humanized anti IL-12p40 antibody for CD treatment (Neurath, Fuss et al. 1995, Langrish, McKenzie et al. 2004). However, IL-23, a cytokine sharing the p40 subunit with IL-12 is also neutralized by anti p40 antibody. IL-23 receptor binding engages JAK2 and STAT3 that are both known to be risk loci associated with IBD, indicating a pivotal role of IL-23R downstream signalling for pathogenesis of IBD (Jostins, Ripke et al. 2012). Together with IL-6 and TGF-β, IL-23 induces differentiation of highly pathogenic T_H17 cells that produce high levels of IL-17, IL-22 and TNF-α (Ahern, Schiering et al. 2010). Yen et al. showed that incidence of colitis in T cell transfer model in *IL-10*$^{-/-}$ mice was suppressed by depletion of IL-23, but not IL-12 (Yen, Cheung et al. 2006). In contrast, in DSS and TNBS models *IL-23p19*$^{-/-}$ mice were not rescued from colitis development (Cox, Kljavin et al. 2012). In addition, Mangan et al. found that functional IL-23/IL-17 axis is required for induction of potent T_H17 response associated with host protection in *Citrobacter rodentium* colitis model (Mangan, Harrington et al. 2006). IL-17 is a pleotropic cytokine that functions through adaptive and innate immune responses. The main sources of IL-17 in intestinal mucosa are T_H17 cells originated from naïve $CD4^+$ T cells in presence of TGF-β and IL-6. Transcription factor RORγt is identified, as a master regulator of T_H17 differentiation while IL-21 with previously mentioned IL-23 is necessary for T_H17 amplification and stabilization. IL-17 expressing T cells are found in the healthy mucosa where they fulfil important homeostatic function (Bettelli, Oukka et al. 2007, Ivanov, Atarashi et al. 2009), but these cells are also infiltrating the inflamed areas of ileum and colon in colitic mice (Kobayashi, Okamoto et al. 2008, Rovedatti, Kudo et al. 2009). This distinguishes intestine from some other organs like brain, joints or skin where presence of T_H17 cells is connected to pathology. Furthermore, T_H17 cells were shown to have a protective role in some colitis models like DSS induced colitis and clinical trials with neutralizing IL-17A antibody aggravated the inflammation in CD patients (McGeachy, Bak-Jensen et al. 2007, Hundorfean, Neurath et al.

2012). This suggests that other cytokines produced by T_H17 cells promote inflammation in CD, whereas IL-17A is important for maintenance of the mucosal barrier and defense mechanism.

The importance of regulatory T cells for the maintenance of intestinal tolerance against intestinal antigens has been revealed in T cell transfer mediated colitis models. Adoptive transfer of $CD4^+CD45RB^{Hi}$ T cells into syngenic immunodeficient SCID (severe combined immune deficiency) or *Rag1/2* (recombination activation gene) deficient mice that lack mature B and T cells, results in development of intestinal inflammation, primarily in colon several weeks after transfer, depending on the local bacterial flora of the animal facility (Leach, Bean et al. 1996, Read, Malmstrom et al. 2000). At first, it was shown that recipient mice repopulated with $CD4^+CD4RB^{Lo}$ population do not develop colitis. The protective effect was assigned to the $CD25^+Foxp3^+$ subpopulation of $CD4^+CD45RB^{Lo}$ cells since protection was abrogated upon $CD25^+$ cell depletion (Read, Malmstrom et al. 2000, Kjellev, Lundsgaard et al. 2006). $Foxp3^+$ regulatory T cells have been generated in vitro by activation of $CD4^+CD25^-$ T cells in presence of TGF-β, and the cotransfer of $Foxp3^+$ T_{Regs} ameliorated T_H1 mediated colitis development induced by adoptive transfer of $CD4^+CD62L^+$ T cells (Fantini, Becker et al. 2004) (Chen, Jin et al. 2003, Fantini, Becker et al. 2006). Moreover, loss of $Foxp3^+$ T_{Reg} as well as $Foxp3^-$ $IL\text{-}10^+$ $CD4^+$T cells was found in IBD patients and this causes disturbances in mucosal tolerance and contributes to tissue injury due to local inflammation. Several studies showed that contransfer of IL-10-producing $CD25^+Foxp3^+$ in the T cell transfer model prevented gut inflammation similar to administration of recombinant IL-10 or TGF-β does (Asseman, Mauze et al. 1999, Buckner 2010).

IL-10 is known as a suppressor of T_H1 expansion and macrophage functions like IL-12 and TNF-α secretion. It also inhibits T cell proliferation and mediates T_{Reg} differentiation (Barnes and Powrie 2009). Mice with targeted deletion of the *Il-10* gene develop severe enterocolitis characterized by leukocyte infiltration and excessive macrophage and neutrophil activation accompanied with T_H1 cytokine production (Kuhn, Lohler et al. 1993). In addition to this, mice with IL-10 deficiency specific only in T cell also developed intestinal inflammation indicating the importance of T cell derived IL-10 for maintenance of intestinal homeostasis (Roers, Siewe et al. 2004). Although administration of recombinant IL-10 was shown to be effective in animal models, systemic injections of IL-10 seem to be less potent in disease suppression in clinical trials. The limited bioavailability of IL-10 in intestinal mucosa could

be one of the reasons for the low therapeutic efficacy of IL-10 (Tilg, van Montfrans et al. 2002, Van Montfrans, Hooijberg et al. 2002, Kobayashi, Kweon et al. 2003, Huyghebaert, Vermeire et al. 2005). IL-10 signalling involves STAT3, a genetic risk factor for IBD. *Stat3*^{LysMcre} mice lacking STAT3 in MΦ and neutrophils develop spontaneous transmural enterocolitis accompanied by augmented IL-12/23 dependent expression of proinflammatory cytokines.

1.6. Intestinal macrophages and dendritic cells in IBD

Intestinal mononuclear phagocytes (MP), comprising MΦ and DCs, are essential for both intestinal immunity and homeostasis. These two main families of MPs have complementary yet distinct functions. DCs are able to translocate from the LP via lymphatics to draining mLNs where they prime naïve T cells and polarize them towards regulatory or effector function. MΦ, on the other hand, are resident in LP where they clear cellular debris, engulf bacteria from the tissue and secrete cytokines to maintain intestinal homeostasis. Since they play crucial roles in intestinal immunity, MΦ and DCs are promising targets for development of new therapies in IBD (Cerovic, Bain et al. 2014). Therefore, the identification of MP subsets and characterization of their corresponding functions in steady state and inflammation is crucial. However, this is not an easy task because of the very sensitive isolation procedure and the overlap of surface markers used to distinguish intestinal MΦ and DCs. Furthermore, in a state of inflammation a continuous influx of monocyte-derived cells in the intestine complicates phenotypic analysis.

In the past few years the number of surface markers used for classical phenotypic characterization of intestinal MΦ and DCs has increased and due to this the nomenclature used to define subsets is constantly being modified. CD11c and class II major histocompatibility complex (MHCII), routinely used for DCs identification in spleen and lymph nodes, were not sufficient for DC recognition in peripheral tissues since these markers were also expressed on MΦ (Pavli, Woodhams et al. 1990) and activated lymphocytes. At the very beginning of my project, most of the studies used surface markers CD103 (integrin αE) and CD11b to classify different subsets of DCs within $CD11c^{+}MHCII^{+}$ MPs. Soon after, application of CD64 (the high affinity FcRγ1), chemokine receptor CX_3CR1 and glycoprotein F4/80, suggested by several groups, made a major breakthrough in intestinal MΦ/DCs discrimination and understanding of their function (Schulz, Jaensson et al. 2009, Bain, Scott

et al. 2013, Schlitzer, McGovern et al. 2013). According to latest data, $CD11c^{+}MHCII^{+}$ MPs that lack expression of CD64 and F4/80 are defined as *bona fide* DCs in the intestine and several other organs. This has been confirmed by expression of DC-specific transcription factor Zbtb46 and their Flt3L dependent development. These migratory $CD64^{-}F4/80^{-}$ MPs can be further divided into four DC subsets based on their CD103 and CD11b expression (Persson, Scott et al. 2013). On the other hand, the $CD64^{+}F4/80^{+}$ MPs that are absent from afferent lymph and have high phagocytic activity are thought to be intestinal macrophages. The development of the CX_3CR1-GFP reporter mouse facilitated detection and isolation of intestinal MΦ, since they have been shown to express intermediate to high levels of CX_3CR1, and the most mature MΦ have the highest expression (Bain, Scott et al. 2013, Persson, Scott et al. 2013). This mouse model became even more attractive after the discovery CX_3CR1^{high} macrophages form dendritic projections (previously assigned to DCs) into the intestinal lumen allowing them to sense and sample luminal contents (Kim, Vallon-Eberhard et al. 2011).

Traditionally macrophages were thought to derive from blood monocytes as part of the MP system. This hypothesis has recently been challenged by studies supporting the concept that resident tissue macrophages arise from yolk sac and foetal liver precursors, which seed the tissue during embryonic development and retain self-renewal capacity (Coggle and Tarling 1984, Alliot, Godin et al. 1999, Hoeffel, Wang et al. 2012, Schulz, Gomez Perdiguero et al. 2012). However, Jung and colleagues showed that $Ly6C^{high}$ monocytes replenish MΦ in gut mucosa after repeated diphtheria toxin-mediated depletion of intestinal MΦ (Varol, Landsman et al. 2007). Mowat *et al.* described that blood recruited $Ly6C^{high}$ monocytes differentiate locally into CX_3CR1^{+} MΦ(Bain, Scott et al. 2013). This differentiation process is accompanied by the downregulation of Ly6C and upregulation of MHCII, F4/80 and CD64 and it is shown to be highly dependent on migration via CCL2-CCR2 and on the commensal microbiota (Takada, Hisamatsu et al. 2010, Tamoutounour, Henri et al. 2012). In the steady state, these cells acquire an anti-inflammatory gene expression profile and form the population of resident macrophages, which maintain tissue homeostasis. During intestinal inflammation, $Ly6C^{+}$ monocytes activated through PRRs like TLRs and NLRs differentiate into proinflammatory macrophages which produce high levels of IL-12, TNF-α, IL-23 and inducible nitric oxide synthase (iNOS) (Zigmond, Varol et al. 2012).

According to current models, conventional DCs (cDCs) derive from committed cDC progenitors that develop in bone marrow (BM) and travel via blood into lymphoid organs and non-lymphoid tissues, where they give rise to mature cDCs. Both subtypes of LP $CD103^+$ ($CD11b^+$ and $CD11b^-$) DCs derive from pre-cDC precursors but with distinct growth factor requirements for their development (Pulendran, Tang et al. 2008). In addition, transcription factors Notch-2 (Lewis, Caton et al. 2011) and IRF4 (Persson, Uronen-Hansson et al. 2013) are necessary for differentiation of $CD103^+CD11b^+$ DCs while the ontogeny of $CD103^+CD11b^-$ DCs is dependent on basic leucin zipper transcription factor ATF-like (Batf) 3(Edelson, Kc et al. 2010) and IRF8 (Klebanoff, Spencer et al. 2013) transcription factors. The ontogeny of intestinal *bona fide* $CD103^-CX_3CR1$ DCs remains unclear (Cerovic, Bain et al. 2014).

Intestinal macrophages have high phagocytic and bactericidal activity and therefore their primary function is to capture and eliminate any potential dangerous material that breaches the epithelium. However, resident intestinal macrophages exist in a state of "anergy" where they do not produce proinflammatory cytokines upon ingestion of bacteria or exposure to TLR stimuli(Smythies, Sellers et al. 2005). One of the possible explanations for this phenomenon is the fact that resident macrophages are shown to have downregulated expression of MyD88, TRAF6, TRIF, IRAK1 and IRAK4 adaptor molecules resulting in a failure of NF-κB activation (Smythies, Shen et al. 2010). Instead of proinflammatory cytokines, these cells constitutively produce high levels of immunoregulatory molecules like IL-10 and TGF-β (Kamada, Hisamatsu et al. 2005). IL-10 expressing intestinal MΦs maintain the functionality of LP $Foxp3^+$ T_{Reg} since CX_3CR1-deficient mice have reduced number of T_{Reg} in intestinal LP (Hadis, Wahl et al. 2011). Apart from their role in intestinal homeostasis, intestinal macrophages associated with the crypts of Lieberkuehn in the colon contribute, upon tissue damage, to epithelial proliferation and survival in an Myd88-dependent manner (Slavin, Nash et al. 1992). Intestinal macrophages lack CCR7 expression and are inferior to DCs in antigen presentation probably because of the rapid degradation of antigens. They seem to counteract T_H17 generation by $CD103^+CD11b^+$ DCs and interestingly the number of $CD103^+CD11b^+$ and T_H17 cells decreases along GI tract while macrophages and T_{Reg} cells are most abundant in colon (Denning, Norris et al. 2011).

Intestinal DCs are specialized to communicate with T cells and direct their functional polarization depending on the local environment. All three populations of *bona fide* DCs in

murine LP ($CD103^+CD11b^+$, $CD103^+CD11b^-$ and $CD103^-CD11b^+$) are shown to migrate to mLNs where they present antigenic peptides in MHC context to T cells, secrete cytokines that govern T_H polarization into effector or regulatory cells and imprint gut homing factors CCR9 and $\alpha_4\beta_7$ on differentiated T cells (Milling, Yrlid et al. 2010). Both intestinal $CD103^+$ DC subsets have the ability to induce the differentiation of peripheral $Foxp3^+$ T_{Reg} cells from naïve T cells in a RA and TGF-β dependent manner while $CD103^-$ DCs are not as effective. In addition, the tolerogenic ability of $CD103^+$ DCs appears to depend on various local factors including thymic stromal lymphopoietin (TSLP) and prostaglandin (PG)E2 produced by epithelial cells (Artis 2008). The ability of intestinal DCs to generate T_H17 responses is associated with $CD103^+CD11b^+$ DCs. This subset was initially thought to drive exclusively T_{Reg} polarization but mounting evidence from *in vitro* and *in vivo* studies shows that these cells play a role also in effector T_H1/T_H17 differentiation (Persson, Uronen-Hansson et al. 2013, Schlitzer, McGovern et al. 2013).

The composition of murine intestinal MP is dramatically changed during inflammation, as seen in colitis models. Disruption of intestinal homeostasis during T cell transfer or DSS induced colitis results in the accumulation of monocyte-derived cells in the mucosa. Elicited $Ly6C^{high}$ monocytes do not mature into $CX_3CR1^{high}Ly6C^{low}$ anti-inflammatory macrophages as in steady state, but rather remain as CX_3CR1^{int} cells and retain the capacity to produce pro-inflammatory cytokines such as TNF-α, IL-6, IL-23 and IL-12 (Weber, Saurer et al. 2011, Zigmond, Varol et al. 2012). CCR2 dependent recruitment of pro-inflammatory $Ly6C^{high}$ monocytes has been associated with the pathogenesis of DSS induced colitis since CCR2 deficiency suppressed the inflammation. The resident anti-inflammatory macrophages keep their function despite the inflammatory environment (Bain, Scott et al. 2013).

$CX_3CR1^{int}Ly6C^{high}$ monocytes are reported to directly interact with T cells in the inflamed mucosa during *Citrobacter rodentium* infection where the IL-12 production by these cells was required for maintenance of IFN-γ single positive and IFN-γ IL-17 double positive T cells (Schreiber, Loschko et al. 2013). Moreover, $Ly6C^{high}$ monocytes are proposed to give rise to inflammatory DC like cells that migrate from mucosa to mLNs during T cell transfer or DSS colitis induced inflammation (Rivollier, He et al. 2012). Consistent with this CX_3CR1 expressing monocyte-derived cells have been shown to migrate to mLNs in response to pathogen infection like *Salmonella typhimurium* and *Toxoplasma gondii.* However, this concept has been challenged since it is assumed that monocytes give rise only to MΦ in

mucosa and any monocyte derived cell detected in lymphatics is probably coming from the bloodstream (Rivollier, He et al. 2012, Tamoutounour, Henri et al. 2012, Zigmond, Varol et al. 2012, Bain, Scott et al. 2013).

Individual DC subsets are shown to undergo a functional switch from immunoregulation to induction of adaptive immunity following appropriate stimulation. For example, $CD103^+$ DCs isolated from mLNs of colitic mice were shown to induce T_H1/T_H17 cells rather than T_{Reg} cells. There is evidence that DCs may play a protective or deleterious role in colitis pathology. In acute colitis model, diphtheria toxin mediated DC ablation during DSS administration in CD11c-DTR mice ameliorated disease while depletion of DCs prior to DSS administration exacerbated inflammation suggesting that DCs are protective in the initial phase of colitis, but play a pathogenic role during disease course (Abe, Nguyen et al. 2007).

1.7. Iron as environmental factor influencing inflammation in IBD

In a healthy organism, systemic and cellular iron concentrations are tightly regulated by hepcidin and the transcription of this liver derived hormone is regulated by systemic iron itself. This regulatory feedback loop is disrupted in infection and inflammation since elevated production of hepcidin in inflammation blocks primary iron release from iron storing macrophages as well as iron absorption, leading to systemic decrease of plasma iron levels. The described imbalance between iron intake and release resulting in impaired iron homeostasis is very often found in IBD where one third of patients develop either iron deficiency (ID) or anemia of chronic inflammation/disease. These patients therefore require iron supplementation therapy. Iron sulphate, ferrous gluconate and ferrous fumarate are most common oral iron supplements.

Oral iron therapy in form of iron salts like iron sulphate, ferrous fumarate and ferrous gluconate is commonly used in clinical treatments most probably because of their convenience and low price. However, in some clinical studies these preparations are reported to produce gastrointestinal side effects like abdominal pain and diarrhoea while intravenous iron does not (Kortman, Raffatellu et al. 2014). The effect of 1 week of oral iron therapy with ferrous fumarate resulted in increase in CD activity index and deterioration of the antioxidant status in IBD patients when compared to healthy controls. The significance of this study is limited by small sample size and short duration of the treatment (Erichsen, Hausken et al.

2003). In addition, oral iron is only partly absorbed in IBD patients because of the inflammation and non-absorbed iron could be toxic and worsen the disease activity as a consequence of oxidative stress, neutrophilic infiltration, increased cytokines and activation of NF-κB (Carrier, Aghdassi et al. 2006) as it was shown in DSS induced colitis in rats. However, the pro-inflammatory effect of oral iron treatment on IBD activity is still debated since the clinical trials so far show conflicting results. For instance, Powell et al. showed that iron sulphate supplementation was negatively associated with quality of life in IBD patients with mildly active disease (Powell, Cook et al. 2013). On the other hand, several studies reported decreased side effects with lower doses of elemental iron, which were still effective for haemoglobin reconstitution suggesting that high dose of iron, applied in oral treatments, could be the reason of intolerance (Cook 2005, Rimon, Kagansky et al. 2005).

Recent animal studies have supported the concept of proinflammatory effect of oral iron supplementation during intestinal inflammation. In rodent models with colitis induced by chemicals, such as DSS and TNBS, an increase in disease activity, inflammatory score and oxidative stress parameters like colonic and plasma lipid peroxides was seen (Reifen, Matas et al. 2000, Seril, Liao et al. 2002, Carrier, Aghdassi et al. 2006). Genetically predisposed $TNF^{\Delta ARE}$ mice held on iron deficient diet in combination with intravenous iron administration develop less severe distal ileitis (Werner, Wagner et al. 2011). In this study, Werner et al. showed that iron sulphate induces IEC apoptosis most likely through changes in the microbial environment. It is known that intestinal microbiota of IBD patients differs from that of healthy controls in favour of enteropathogenic strains. This change in composition of microbiota may be amplified in presence of iron. In their study, Werner et al. showed that dietary iron sulphate caused reduction of beneficial *Bifidobacteriaceae* and *Lactobacillaceae* and an increase of *Bacteroidaceae* in cecal microbiota. Indeed, dietary iron content has been shown in a number of animal and human studies to influence the composition of intestinal microbiota. Ettreiki et al. showed that juvenile treatment with ferric iron suppressed TNBS induced colitis accompanied with increase of *Enterobacteriaceae* in adult rodents (Ettreiki, Gadonna-Widehem et al. 2012). Since intestinal microbiota plays an important role in development and function of intestinal immune system, alteration of microbiota by luminal iron content might have an impact on the intestinal immune system.

The effects of luminal iron on intestinal immunity are still puzzling. On the one hand iron might support inflammation by supporting local generation of oxidative radicals. On the other

hand modulation of the activity of signalling pathways and transcription factors that mediate intestinal immune responses could also be involved. At first, experiments in animals and humans revealed that increased levels of pro-inflammatory cytokines, especially IL-6, upregulate hepcidin expression. Indeed, increased hepcidin expression was found in *Salmonella* enterocolitis and $TNF^{\Delta ARE}$ models as well as in the human IBD (Wang, Harrington et al. 2009, Oustamanolakis, Koutroubakis et al. 2011, Wang, Trebicka et al. 2012). Interestingly, disturbances of systemic iron metabolism in *Hfe* knockout mice that resembles human type I hematocromatosis influenced immune responses in the gut. The low hepcidin levels in Hfe knockout mice resulted in enhanced export of iron from enterocytes and macrophages via Fpn, which resulted in reduced severity of *Salmonella* enterocolitis in these mice. The attenuated intestinal inflammation was directly associated with impaired pro-inflammatory cytokine production by intestinal macrophages, as a result of low intracellular iron in these cells (Wang, Johnson et al. 2008, Wang, Harrington et al. 2009). Furthermore, TNF-α was shown to downregulate hepcidin expression during DSS induced colitis and spontaneous colitis development in *T-bet/Rag2*$^{-/-}$ deficient (TRUC) mice (Shanmugam, Ellenbogen et al. 2012). In vitro studies showed differential expression of genes of iron metabolism between pro-inflammatory M1 and alternative M2 polarized human macrophages (Recalcati, Locati et al. 2010). Corna et al. showed that cytokines that drive macrophage polarization probably also control iron handling since M1 macrophages were shown to have relatively sealed iron content while M2 subset was efficient in iron recycling (Corna, Campana et al. 2010). Therefore apart from altering composition of intestinal microbiota in IBD, luminal iron might directly sensitize innate and adaptive immune cells underlying in the already injured intestinal mucosa and further boost the inflammation.

2. AIMS OF THE STUDY

The main goal of this study was to investigate the potential protective role of luminal iron depletion on colitis development focusing on the impact of dietary intervention on innate immune responses and T cell activation.

With the exception of smoking, very few studies have been published concerning environmental factors contributing to the pathogenesis of IBD. Patients suffering from this disease are commonly found to develop iron deficiency anaemia and therefore are often recommended to consume iron rich foods and oral iron therapy. Due to the existing inflammation in the gut, iron is very poorly absorbed and because of its pro-oxidative capacity, it might induce increased ROS and iNOs production by innate immune cells in already damaged mucosa. According to current studies, the role of iron in inflammation in human IBD is still under the debate.

In this study, the influence of luminal iron deprivation on intestinal immune responses in homeostasis and inflammation was investigated. The first aim was to find out whether dietary iron depletion affects the health status of lymphopenic *Rag1*$^{-/-}$ steady state mice lacking T cells and B cells, with regard to systemic iron levels, clinical score including bodyweight and characterization of intestinal immune cell composition. The second aim was to examine the potential protective role of luminal iron depletion in the T cell transfer colitis model, by transferring naïve T cells into *Rag1*$^{-/-}$ mice. The third aim was to study the effect of luminal iron depletion on DSS-induced colitis as a second T-cell independent model. Colitis development was monitored by bodyweight and clinical colitis score assessment, intestinal cytokine expression at the mRNA level and tissue inflammation evaluated by histological scoring. To investigate the eventual effect of iron on the composition and frequency of the intestinal dendritic cell and macrophages, suitable cell isolation procedures and multiparameter flow cytometric analysis were developed. Furthermore, intestinal T cell infiltration and effector T_H1/T_H17 cell differentiation were studied.

3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Reagents

2-propanol	J.T. Baker (Deventer, Netherlands)
4 % (v/v) formaldehyde (Roti-Histofix)	Carl Roth (Karlsruhe, Germany)
Acetic acid	Merck (Darmstadt, Germany)
Agarose	Merck (Darmstadt, Germany)
β-mercaptoethanol	Sigma-Aldrich (Seelze, Germany)
Bovine serum albumin (BSA)	Sigma-Aldrich (Seelze, Germany)
Chloroform	J.T. Baker (Deventer, Netherlands)
Dextrane sulphate sodium salt (DSS) (30 000 – 50 000 M Wt. MP Grade)	MP Biomedicals (Eschwege, Germany)
Dithiothreitol (DTT)	Carl Roth (Karlsruhe, Germany)
dNTPs mix	Promega (Mannheim, Germany)
EDTA (0.5 M, pH 8.0)	Invitrogen (Karlsruhe, Germany)
Eosin	Carl Roth (Karlsruhe, Germany)
Ethanol absolute (for use in molecular biology)	Merck (Darmstadt, Germany)
Ethanol absolute (EtOH)	J.T. Baker (Deventer, Netherlands)
Ethidiumbromide (10 mg/ml)	Invitrogen (Karlsruhe, Germany)
Fc-block (mHB197 supernatant)	Own production
Fetal calf serum (FSC)	Biochrom (Berlin, Germany)
Glutamax-1 (100x)	Invitrogen (Karlsruhe, Germany)
Golgi Plug	BD Bioscience (Heidelberg, Germany)
Golgi Stop	BD Bioscience (Heidelberg, Germany)
Hank's Balanced Salt Solution (HBSS) (w/o Ca^{2+} and Mg^{2+})	Invitrogen (Karlsruhe, Germany)
Hank's Balanced Salt Solution (HBSS) (with Ca^{2+} and Mg^{2+})	Invitrogen (Karlsruhe, Germany)
Hematoxylin	Carl Roth (Karlsruhe, Germany)
Hemin	Sigma-Aldrich (Seelze, Germany)
Ionomycin	Sigma-Aldrich (Seelze, Germany)
Isolflurane (Forene (100 % (v/v))	Abbott (Wiesbaden, Germany)
Nonessential amino acids (NEAA) (100x)	PAA (Pasching, Austria)

Paraformaldehyde	Sigma-Aldrich (Seelze, Germany)
Penicillin/Streptomycin (100x)	PAA (Pasching, Austria)
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich (Seelze, Germany)
Phosphate Buffered Saline (PBS) (w/o Ca^{2+} and Mg^{2+})	PAA (Pasching, Austria)
Propidium Iodide	Sigma-Aldrich (Seelze, Germany)
Red Blood Cell Lysis Buffer	Sigma-Aldrich (Seelze, Germany)
RPMI 1640	Invitrogen (Karlsruhe, Germany)
Sodium pyruvate (100 mM)	Invitrogen (Karlsruhe, Germany)
TRIzol	Invitrogen (Karlsruhe, Germany)

3.1.2. Kits and enzymes

Intracellular Fixation & Permeabilizatin Buffer Set	eBioscience (San Diego, CA, USA)
Iron Stain kit	Sigma-Aldrich (Karlsruhe, Germany)
$CD4^+$ $CD62L^+$ T cell isolation kit II	Miltenyi Biotec (Bergisch Gladbach)
Collagenase V	Sigma-Aldrich (Seelze, Germany)
Collagenase D	Roche (Mannheim, Germany)
DNase I	Roche (Mannheim, Germany)
Fix & Perm Cell Permeabilization kit	Invitrogen (Karlsruhe, Germany)
Intracellular Foxp3 staining kit	eBioscience (San Diego, CA, USA)
Superscript III reverse transcriptase	Invitrogen (Karlsruhe, Germany)
Taq DNA Polymerase	Invitrogen (Karlsruhe, Germany)
TaqMan Gene Expression Master Mix	Applied Biosystems (Foster City, USA)

3.1.3. Antibodies

Antigens	**Clone**	**Fluorochrom**	**Manufacturer**
CD3e	145-2C11	APC 780	eBioscience (San Diego, CA, USA)
CD4	RM4-5	eFluor 450, FITC	eBioscience (San Diego, CA, USA)
CD8α	53-6.7	PE	BD Bioscience (Heidelberg, Germany)
CD11b	M1/70	PerCp-Cy5.5, APC-efluor780	eBioscience (San diego, CA,

			Germany)
CD11c	N418	PE-Cy7	BioLegend (San Diego, CA, USA)
CD62L	MEL-14	APC	BD Bioscience (Heidelberg, Germany)
CD64	X54-5/7.1	APC	BioLegend (San Diego, CA, USA)
CD45.2	104	APC-eFluor700, eFluor 450	eBioscience (San Diego, CA, USA)
CD103	M290	PE	BD Bioscience (Heidelberg, Germany)
F4/80	BM8	APC, eFluor 450	eBioscience (San Diego, CA, USA)
Foxp3	FJK-16s	PE, PE-Cy7	eBioscience, (San Diego, CA, USA)
IL-12p40	C17.8	PerCp-Cy5.5	eBioscience, (San Diego, CA, USA)
IL-17A	eBio17B7	APC	eBioscience (San Diego, CA, USA)
IFN-γ	XMG1.2	PE	eBioscience, (San Diego, CA, USA)
Ly6C	AL-21	FITC	BD Bioscience (Heidelberg, Germany)
MHCII ($I\text{-}A^b$)	M5/114.15.2	APC-eFluor780, eFluor450	eBioscience (San Diego, CA, USA)

Table 1 Antibodies used in the study

3.1.4. Media and buffers

3.1.4.1 Media

Name	Formulation
DC medium	RPMI 1640 10 % FCS (HI) 1 % NEAA 1 % Glutamax 1 % Penicillin/Streptomycin 1 % Sodium pyruvate 500 mM β-mercaptoethanol
Restimulation medium	DC medium 20 ng/ml Phorbol 12-myristate 13-acetate (PMA) 1 µg/ml Ionomycin 0.2 % v/v Golgi Plug 0.14 % v/v Golgi Stop
Digestion medium I	RPMI 1640 0.5 mg/ml Collagenase D 0.1 mg/ml DNase I grade II (D)
Digestion medium II (for colon tissue digestion)	RPMI 1640 5 % FCS 0.5 mg/ml Collagenase D 0.85 mg/ml Collagenase V 0.1 mg/ml DNase I grade II (D)
Epithelial cell dissociation medium	HBSS (w/o Ca^{2+} and Mg^{2+} 2 mM DTT 5 mM EDTA

3.1.4.2 Buffers

Name	Formulation
FACS Buffer / Staining buffer	PBS w/o Ca^{2+} and Mg^{2+} 2 % FCS
HBSS / Washing buffer	HBSS w/o Ca^{2+} and Mg^{2+}
***In vivo* injection buffer**	PBS w/o Ca^{2+} and Mg^{2+}
MACS Buffer / Sort Buffer	PBS w/o Ca^{2+} and Mg^{2+} 2 % FCS 2mM EDTA
Red Blood Cell Lysis Buffer	0.16 M NH_4Cl (4.28 g / 0.5 L) 0.17 M Tris (20,6 g Tris base / 900 ml water) Mix 10 ml 0.16 M NH_4Cl and 90 ml Tris buffer Adjust pH to 7.2 with HCl

3.1.5. Equipment/Software

Device	Manufacturer
C1000 Touch Thermo Cycler	Bio-Rad Laboratories Inc. (Hercules, CA, USA)
FlowJo Software	Tree Star (Olten, Switzerland)
Gallios Flow Cytometer	Beckman Coulter (Krefeld, Germany)
HM 355 S automatic microtome	Thermo Scientific (Langenselbold, Germany)
Kaluza Software	Beckman Coulter (Krefeld, Germany)
MACS Multi Stand	Miltenyi Biotec (Bergisch Gladbach, Germnay)
MACS Separation Columns (LS / MS)	Miltenyi Biotec (Bergisch Gladbach, Germany)
Nanodrop ND-1000 Spectrophotometer	Peqlab (Erlagen, Germany)
TaqMan StepOne Plus	Applied Biosystems (Carlsbad, CA, USA)

3.1.6. Mice

Wild type and *Rag1*$^{-/-}$ mice on the C57BL/6 background were bred under specific pathogen free conditions in the animal facility of the Institute of Microbiology at Technical University Munich. Sentinel testing was performed regularly according to FELASA guidelines. *Helicobacter spp*. were occasionally detected in sentinel mice.

Strain	Source	Application
Wild type C57BL/6	Institute of Microbiology, TUM, Munich, Germany Bred in SPF facility	DSS colitis titration Feeding experiments - DSS colitis T cell isolation
Rag1$^{-/-}$	Institute of Microbiology, TUM, Munich, Germnay Bred in SPF facility	Feeding experiments – steady state Feeding experiments (6 weeks) – T cell transfer Feeding experiments (9 weeks) – T cell transfer

Table 2 Mouse strains used in the study

3.1.7. Diets

Diet	Iron content	Manufacturer
Semi-synthetic experimental diet (C1000)	180 mg Fe/kg diet as Fe_2SO_4	Altromin (Lage, Germany)
Experimental diet (C1038)	< 10 mg Fe/kg diet	Altromin (Lage, Germany)
Experomental diet (C1038) fortified with Hemin	180 mg Fe/kg diet as Hemin	Altromin (Lage, Germany)
Harlan Global Rodent diet (2018)	200 mg Fe/kg diet	Harlan Winkelmann (Borchen, Germany)

Table 3 Diets used in the study

3.2. Methods

3.2.1. Dietary treatment and colitis induction

Wild type (WT) and *Rag1*$^{-/-}$ mice on the C57BL/6 background were raised under specific pathogen free conditions. Animals were fed with standard chow (Harlan Global rodent diet 2018, Harlan, Germany) *ad libitum.* At 10 – 12 weeks of age, mice were divided into three groups and were put on either iron depleted experimental diet (C1038 diet, containing < 10 mg Fe/kg) (Altromin, Lage, Germany) or iron adequate experimental diet containing 180 mg Fe/kg in form of $FeSO_4$ salt (C1000 diet) (Altromin, Lage, Germany) or Hemin (Sigma Aldrich, Seelze, Germany) added to the iron deficient experimental diet (C1038). Since preliminary experiments indicated poor tolerance of experimental diets, especially amongst *Rag1*$^{-/-}$mice, a two – week long adaptation phase was introduced prior to dietary treatment as shown in Figure 3**.** In short, instead of putting mice on the experimental diets at once, standard chow was gradually replaced with iron adequate C1000 diet before switching to the 3 different experimental diets, which improved animal survival during dietary treatment.

The effect of luminal iron depletion in intestinal homeostasis and inflammation was examined in the *Rag1*$^{-/-}$ mice. After 6 or 9 weeks of dietary treatment with experimental diets, *Rag1*$^{-/-}$ mice were sacrificed by CO_2 asphyxia and the organs were taken for further analysis or the mice were subjected to T cell transfer colitis.

Bodyweights and health states were evaluated once a week during feeding of experimental diet. Iron status was evaluated by collecting blood from the facial vein before and after feeding treatment. All research involving animal experiments were performed in accordance with German animal care and ethics legislation and have been approved by the local government authorities.

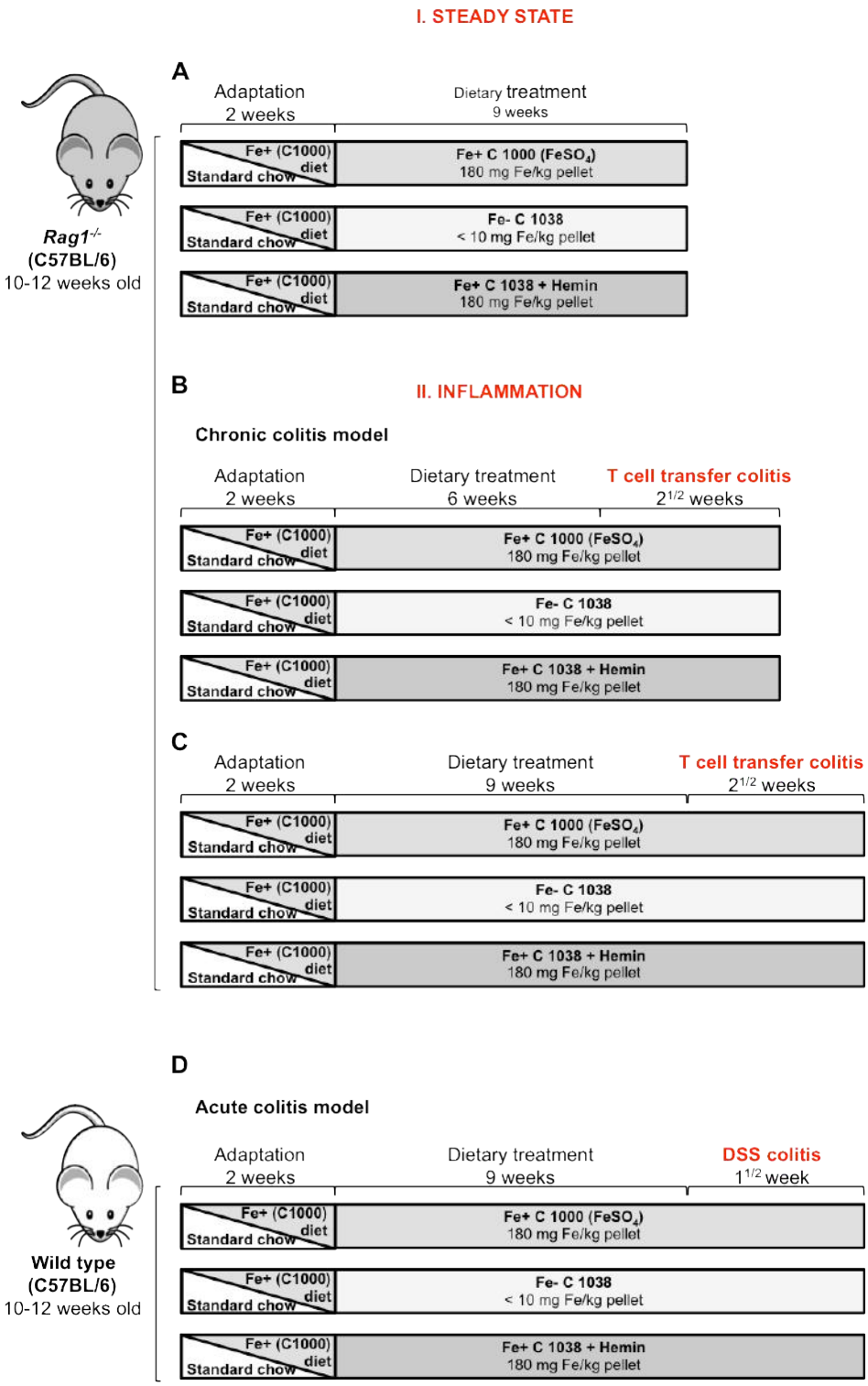

Figure 3 Experimental setup of diet treatment and colitis induction
10 - 12 weeks old *Rag1*$^{-/-}$ and wild type (C57BL/6) were adapted to the experimental diet by gradually replacing standard chow with iron adequate diet C1000 containing iron as $FeSO_4$ (180 mg Fe / kg). After 2 weeks, mice were divided into three groups and fed with iron depleted diet C1038 (< 10 mg Fe/kg) or iron adequate diets (Fe=180 mg/kg); C1000 and Hemin (C1038 + Hemin) for either 6 or 9 weeks (extended feeding treatment). After feeding treatments *Rag1*$^{-/-}$ mice were either sacrificed or used for T cell transfer experiments **(A) (B) (C)**. Wild type mice were administrated with 3 % DSS in drinking water for 5 days followed by water alone for 3 days **(D)**. In both colitis models mice were sacrificed when 20% bodyweight loss was reached.

3.2.2. Colitis experiments

The effect of dietary treatment was studied in T cell dependent and T cell independent colitis models.

T cell mediated colitis was induced in *Rag1*$^{-/-}$ mice after receiving iron depleted or iron adequate diets containing $FeSO_4$) or Hemin for 6 and 9 weeks (extended feeding experiment). MACS enriched $CD4^+CD62L^+$ T cells were isolated from the wild type (C57BL/6) splenocytes and $3*10^5$ cells/animal were injected in 300 µl PBS into the peritoneum cavity using a 0.5 mL insulin syringe. The purity of enriched T cells was typically between 85-95% (Figure 4**B, Section 3.2.3.)**

Due to the variations in colitogenic potential of DSS between different mouse strains and experimental design, a titration experiment was performed to determine optimal DSS concentration that induces colitis development in a slow and steady fashion in WT (C57BL/6) mice receiving experimental diets. For titration experiments, WT mice were given 2.5%, 3.0%, 3.5% and 4% DSS in drinking water for 5 days after 9 weeks of treatment with iron adequate diet C1000. At day 5, DSS was replaced by drinking water alone. Based on the titration experiment, WT mice that were kept on iron depleted or adequate diets, received 3.0% DSS in drinking water for 5 days, followed by drinking water alone.

In both models, colitis activity was evaluated by bodyweight measurements and clinical scoring by giving a value from 0 absent symptoms to 3 (highest grade) for following parameters: inactivity, hunched posture, diarrhea, rectal prolapse and rectal bleeding (Table 4). After reaching 20% bodyweight loss, mice were sacrificed.

Clinical parameters	Description	Score – symptoms
Inactivity	- Fully mobile but more inactive than usual - Largely inactive without prompting - Moving only occasionally and largely unresponsive to prompting	**0 - None/equivocal** **0.5 - equivocal/mild** **1.0 - Mild** **1.5 - Mild to moderate** **2.0 - Modrate** **2.5 - Moderate to severe** **3.0 - Severe**
Hunched posture	- Small change in normal posture - Hunched over when not moving - Noticeably hunched over without regaining normal posture	
Ruffling of fur	- Some restricted ruffling of fur - Larger parts of fur ruffled - Obvious ruffling of most fur	
Diarrhea	- Stools relatively lose, stools in enrichment - Lose stools, stools in enrichment and at cage wall - Stools have completely liquid consistency	
Rectal bleeding	- Some blood in stool on close inspection - Blood visible at perianal region - Clear bleeding from rectum	
Rectal prolapse	Permanent prolapse	

Table 4 Clinical symptoms and score

3.2.3. T cell isolation

WT (C57BL/6) mice were sacrificed by cervical dislocation and spleens were excised. Attached fat tissue was removed and a single cell suspension was generated by passing the organs through 100 µm cell strainers (BD Falcon, Bedford, USA). Cells were centrifuged for 5 min at 1500 rpm at 4°C and resuspended in MACs buffer. The isolation of $CD4^+CD62L^+$ T cells was performed using the CD4 CD62L T cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) as shown Figure 4**A.** In the first step, non - $CD4^+$ T cells were depleted from the cell suspension by adding a biotinylated labelled antibodies (anti -CD8a, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC Class II, Ter-119, and TCRγ/δ). and anti-biotin microbeads and passing it over the MACS column. In the second step, $CD62L^+$ cells were positively selected using CD62L MicroBeads. After isolation, cells were stained with fluorescently labelled antibodies against CD4 and CD62L as described in section 3.2.8. and the purity of enriched $CD4^+CD62L^+$ was evaluated by flow cytometry. The percentage of $Foxp3^+$ cells within the $CD4^+CD62L^+$ T cells was also assessed by

intracellular staining and FACS analysis. The population routinely contained Foxp3^{+} cells (7-10%) as described before (Heiseke, Faul et al. 2012).

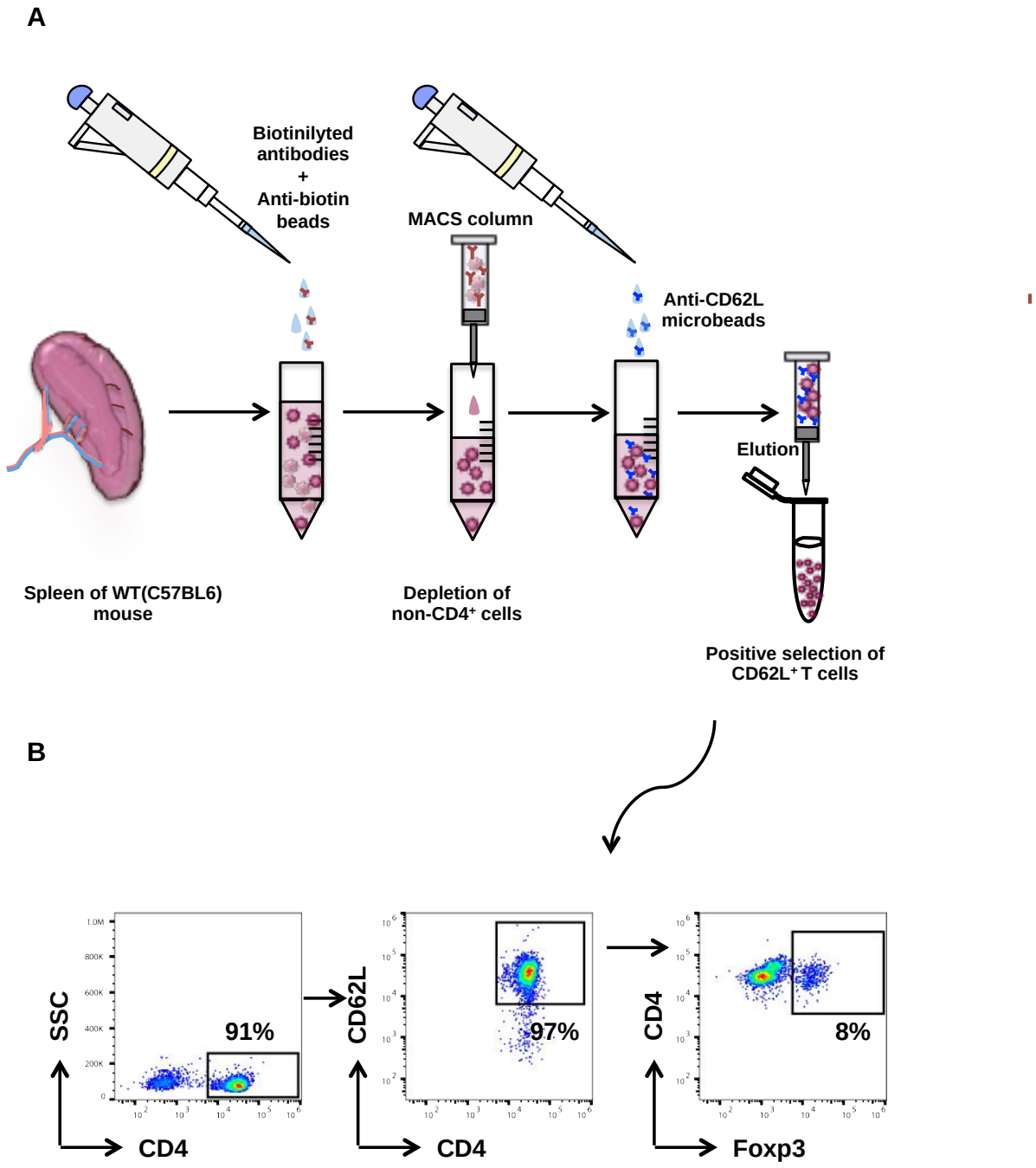

Figure 4 Isolation of CD4+CD62L+ T cells from splenocytes
(A) Two-step MACS enrichment procedure for CD4+CD62L+ T cells isolation from spleenocytes. **(B)** Purity check of enriched T cells by FACS analysis. Cells were stained against extracellular T cell markers CD4 and CD62L. Numbers indicate percentages of the gated cells.

3.2.4. Haematological analysis

Blood samples were collected from either facialral vein or postmortem cardiac puncture, depending on the time point of the sampling in the feeding experiments. For facial blood collection, mice were pinched behind the jawbone using a 5 mm lancet and blood was collected into EDTA containing collection tubes. Cardiac puncture was performed for terminal blood collection. Mice were sacrificed by CO_2 asphyxia. The blood was drawn from the heart using a 0.5 ml insulin syringe and transferred into EDTA containing collecting tubes. Blood samples were diluted in phosphate-buffered saline (1:3). Complete blood cell count (CBC) and other parameters (WBC, RBC, HGB, HCT, MCV, MCH and PLT) were measured by the Clinical Chemistry department using Sysmex XT-2000i-1 Hematology Analyzer (Sysmex Europe GmbH, Norderstadt, Germany).

3.2.5. Isolation of murine leukocytes from lymphoid tissue

Mice were sacrificed as described in **3.2.4.** Spleen and mLNs were removed and minced in 6 well plate supplied with 5 ml RPMI 1640. Tissues were digested with collagenase D (500 µg/ml) and DNase I (100 µg/ml) for 45 min at 37°C. After digestion, cells were passed through 100 µm cell strainer and strainers were washed two times with 10 ml RPMI 1640 in order to collect all the remaining cells. The cells suspensions were then centrifuged for 5 min at 1500 rpm at 4°C and the supernatants were removed. Splenocyte suspensions were incubated with 2 ml of RBC lysis buffer for 5 min at room temperature. The reaction was stopped by adding 10 ml of FACS buffer. Cells were centrifuged for 5 min at 1500 rpm at 4°C, supernatants were discarded and the cells were finally resuspended in 2mL FACS buffer.

3.2.6. Isolation of murine colon lamina propria and intraepithelial leukocytes

Mice were sacrificed as described in **3.2.4. .** Colons were excised and attached fat was carefully removed. Subsequently fecal content was flushed with ice cold HBSS. The whole colon was cut longitudinally twice and half of the colon was transferred into a 50 ml tube and washed three times with HBSS. Afterwards, the colon tissue was cut into 3 mm long pieces and incubated in 50 ml beaker containing 50 ml of HBSS (Ca-/Mg-), 2mM DTT and 5mM EDTA for 30 min at 37°C stirring in the incubator containing 5% CO_2. After the incubation, the buffer was passed over a 100 µm cell strainer (Greiner bio-one, Frickenhausen, Germany) and in order to isolate intra-epithelial leukocytes (IEL), the filtrate was further passed through

glass wool (Roth, Karlsruhe, Germany). and centrifuged for 7 min at 1500 rpm at 4°C. The cell pellets were resuspended in 1 mL FACS buffer and used for staining. For isolation of lamina propria leukocytes (LPL), the remaining colon pieces were incubated in digestion medium containing 5 ml of RPMI 1640, 100 µg/ml of DNase 1, 500 µg/ml of collagenase D and 850 µg/ml of collagenase V for 30 min at 37°C. After incubation, the tissue pieces were passed through 100 µm cell strainer using syringe plunger and the cell suspension was centrifuged at 1500 rpm for 7 min at 4°C and the supernatant was discarded. After centrifugation, the cell pellets were resuspended in FACS buffer or RPMI 1640 medium.

3.2.7. Leukocyte stimulation

Leukocytes were isolated as described in **3.2.3. 3.2.5. 3.2.6.** and $1*10^6$ cells/well were displaced into 96 well round bottom plate and 100 µl of stimulation medium containing 20 ng/ml Phorbol 12-myristate 13-acetate (PMA), 1 µg/ml Ionomycin, 0.2 % v/v Golgi Plug and 0.14 % v/v Golgi Stop was added. Cells were resuspended and cultured for 6 h at 37°C. After the incubation, cells were centrifuged for 3 min at 2100 rpm at 4°C and extracellular staining against T cell markers CD3e and CD4 was performed followed by intracellular staining against IFN-γ and IL-17A as described in section Error! Reference source not found.

3.2.8. Flow cytometry

Cells isolated as described in **3.2.3. 3.2.5. 3.2.6.** were passed through 50 µm Filcon filters (BD, Heidelberg, Germany) and then plated in 96-well round bottom plate and centrifuged for 3 min at 2100 rpm at 4°C. Supernatant were discarded and cells were stained in 200 µl of staining buffer containing an antibody cocktail for respective cell surface markers in final dilution of 1:200. In order to avoid unspecific binding of antibodies to Fc receptor, staining buffer contained supernatant of the mHB197 cell line, producing blocking antibodies (anti-CD16/anti-CD32), added to FACS buffer in 1:1 ratio. After the staining, cells were washed twice with ice cold FACS buffer and finally resuspended in 200 µl of FACS buffer. Propidium iodide (PI) was added to the cell suspension at 1:20 final dilution to exclude dead cells.

For intracellular cytokine staining, isolated splenocytes and leukocytes from mLNs and colon LPL and IEL fractions were stimulated as described before. After the incubation time, extracellular staining for CD4 and CD3 T cell surface markers was performed as described

above and the cells were washed with FACS buffer twice. Intracellular staining for IFN-γ and IL-17A was performed using Fix & Permeabilization kit following manufactor's instructions. In short, cells were fixed in 200 µl of fixation medium (Solution A) for 20 min in the dark at room temperature. Cells were centrifuged for 3 min at 2100 rpm at 4°C and the supernatants were discarded. The cells were resuspended in 100 µl of permeabilization medium (Solution B) and incubated in the dark for 15 min at room temperature. Afterwards, antibodies against respective cytokines were added directly into wells in final dilution of 1:100 and mixed gently. After 25 min incubation in the dark at room temperature, cells were washed once with permeabilization medium and twice with ice-cold FACS buffer. Finally cell were resuspended in 200 µl of FACS buffer and analysed.

Intracellular Foxp3 staining was performed using Foxp3 Transcription factor staining set (eBioscience, San Diegom CA, USA) according to the manufacturer instructions.

A Gallios flowcytometer was used for analysis. FlowJo software was used for data analysis.

3.2.9. Histology

Mice were sacrificed as described in **3.2.4.** Colon tissues were taken and flushed with ice – cold HBSS (Ca-/Mg-). Fat tissue was carefully removed and colon was cut longitudinally e in One half of the tissue was used for making "Swiss rolls" and directly fixed in 4 % formaldehyde (Roti-Histofix) o/n. Fixed tissues were then placed in 70 % ethanol, dehydrated and paraffin embedded. Tissue blocks were cut in 4 µm thick sections and left to air dry o/n. Colon tissue sections were stained with hematoxylin and eosin (H&E). Histological scoring was performed by blindly assessing the degree of leukocyte infiltration (0 for rare inflammatory cells in lamina propria, 1 for increased number of inflammatory cells in lamina propria, 2 for inflammatory cell aggregates reaching submucosa, 3 for transmural extention of inflammatory cells) and epithelial damage (0 – no mucosal damage present, 1 for focal lymphoepithelial lesions, 2 for mucosal erosion/ulceration, 3 for mucosal damage extended through deeper structures of bowel wall). When added together, the common histological score ranging from 0 – 6 was obtained as described by Siegmund et al (Siegmund, Rieder et al. 2001).

3.2.10. RNA isolation and real time quantitative PCR (qPCR)

RNA was isolated from proximal colon tissue samples. In short, 0.5 cm long tissue pieces were snap frozen in 1 mL TRIzol reagent (Invitrogen, Karlsruhe, Germany) and homogenized. After thawing, RNA isolation was performed according to the manufacturer's instructions. After 10 min of incubation at room temperature, 200 µl of chloroform was added and the samples were shaken vigorously for 15 sec. After 3 min incubation at room temperature, samples were centrifuged for 15 min at 12000 x g at 4°C. In the next step, the aqueous phase was transferred into a new tube and RNA was precipitated by adding 500 µl of isopropanol. Samples were incubated for 10 min at RT and centrifuged directly after for 10 min at 12000 x g at 4°C. After centrifugation, supernatants were carefully discarded and RNA pellets were washed with 1 ml 75 % ethanol. Samples were directly centrifuged (7500 x g, 5 min, 4°C) and supernatants were removed. Samples were air dried for 10 min and RNA was dissolved in 30 µl RNase free water. RNA concentrations and quality (A260/A280) were measured.

Superscript III kit was used to generate cDNA following the manufacturer`s instructions. 1 µg of total RNA was added in PCR tubes and mixed with master mix containing 1µl dNTPs and 1 µl oligo-dT and filled with 11 µl H_2O. Samples were incubated for 5 min at 65 °C and subsequently master mix containing 4 µl of 5x First Strand Buffer, 1 µl of (0.1M) DTT, 1 µl of RNase Out (40 U/µl) and 1 µl Superscript III reverse transcriptase was added into the PCR tubes. The mixture was incubated for 50 min at 50 °C, followed by heating at 70 °C for 15 min in the C1000 Touch^{TM} Thermo Cycler (BioRad),

Real-time PCR was performed using TaqMan StepOne Plus instrument applying TaqMan gene expression assay. TaqMan Gene expression assay is a 5`nuclease assay that uses fluorescently labelled MGB TaqMan probes which are designed to have high specificity for targeted sequence. Before PCR the signal is quenched since these probes are intact allowing the energy of emission from green fluorescent dye to be transferred to the red fluorescent dye (FRET). During PCR probes bind to correct target sequence, the polymerase 5`nuclease activity cleaves the probe releasing the reporter dye fragment from the quencher allowing detection of the reporter signal in form of fluorescent emission. The assays were performed according to the manufacturer`s instructions. In short, 9 µl diluted cDNA (1:3) was mixed with 10 µl TaqMan gene expression master mix and 1 µl of primer probe sets purchased from Applied Biosystems. *HPRT1* was used as a housekeeping gene for normalization and the fold

induction of mRNA expression was calculated according to Livak method using following formula: fold induction = $2^{-\Delta\Delta Ct} = 2^{-\Delta Ct(test) - \Delta Ct(control)}$ where ΔCt values refer to normalized Ct values of targeted gene to the Ct of the reference gene (*HPRT1*). In steady state feeding experiments, mRNA gene expression was calculated as fold change against mRNA gene expression measured in mice treated with standard chow using following formula: fold induction = $2^{\Delta Ct(tested\ diet) - \Delta Ct(standard\ chow)}$, while in colitis feeding experiments fold change was calculated against mRNA gene expression measured in steady state mice fed with respective diet (fold induction = $2^{\Delta Ct(tested\ diet\ (colitis)) - \Delta Ct(tested\ diet\ (steady\ state))}$).

The amplification efficiency tested by the manufacturer using bioinformatics pipeline was reported to be 100% (± 10%) when used in samples that are free of PCR inhibitors.

The following probes sets were used: *IL-6* (Mm00446190_M1), *IL-12p35* (Mm00434169_M1), *TNFα* (Mm00443260_g1), *IL-10* (Mm02188386_M1), *IL-17a* (Mm00439619_M1), *IFNγ* (Mm99999071_M1) and *HPRT1* (Mm00446968_M1). The data sheets for probes used in this study can be found on http://www.appliedbiosystems.com.

3.2.11. Statistical analysis

Results are presented as mean values ± standard deviations. For statistical analysis of the data presented in this thesis, one-way ANOVA test was used for comparison of multiple groups followed by Tukey`s post-hoc test for multiple comparisons. Kruskal-Wallis non-parametric test was used to evaluate the impact of iron depleted and iron adequate ($FeSO_4$ and hemin) diets on histology scores in T cell transfer induced colitis experiments. Statistical differences between iron depleted and $FeSO_4$ dietary groups in DSS colitis experiments presented in section **4.4,** were determined using unpaired t test. Histology scores between iron depleted and $FeSO_4$ diet treated groups after DSS colitis development were compared using Mann-Whitney non-parametric test.

Survival curves were compared by computing pair-wise comparison between the groups using the nonparametric Gehan-Breslow-Wilcoxon test since the hazard ratio (deaths per time) was not proportional. The significance level was adjusted using Bonferroni method for multiple comparisons ($P < 0.02$)

Normality test confirmed a Gaussian distribution of presented data. *P* values below 0.05 were considered to indicate statistically significant differences ($P^* < 0.05$, $P^{**} < 0.01$, $P^{***} < 0.001$). The statistical analysis was performed using Prism Software (GraphPad Software Inc).

4. RESULTS

4.1. Effect of luminal iron depletion on intestinal immunity in the steady state

Recent studies have shown that dietary iron supplementation contributes to the intestinal inflammation during colitis development in mice and rats. This finding imposed the question whether the reduction of dietary iron intake could have a protective effect on inflammation development in the T cell transfer colitis model. To study this, it was important to first investigate the effect of dietary intervention in *Rag1*$^{-/-}$ mice before induction of inflammation by T cell transfer. For this purpose, 10 - 12 weeks old *Rag1*$^{-/-}$ mice, previously kept on normal chow, were fed with iron adequate ($FeSO_4$ or Hemin) or iron deficient diets for 9 weeks. As described by Werner et al. the average daily iron intake during this dietary treatment was estimated to be 0.54 mg for the iron adequate groups and 0.03 mg for the iron depleted group (Werner, Wagner et al. 2011). A high mortality rate (10-20%) was observed among *Rag1*$^{-/-}$ mice in initial diet treatments most likely because of poor tolerance of the experimental diets independent of their iron content. For this reason, the normal chow was gradually replaced with the $FeSO_4$ supplemented experimental diet during a 2-week long adaptation phase, prior to experimental diet treatment, which was then well tolerated.

During the 9-week diet treatment bodyweight increase was similar between iron adequate ($FeSO_4$ and Hemin) and iron-depleted groups and mice gained 20-30% of bodyweight during this time period (Figure 5).

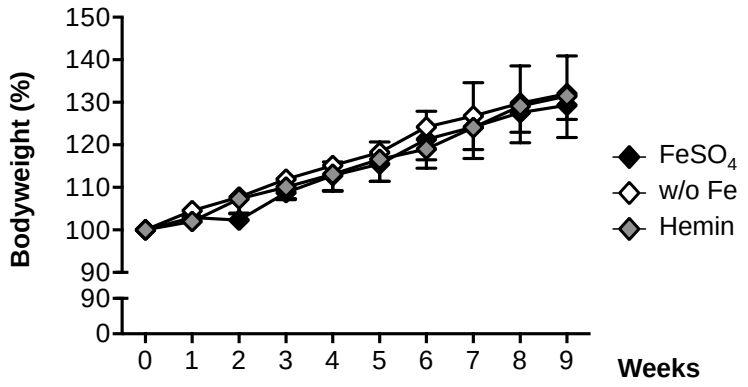

Figure 5 Bodyweight course during dietary treatment
Rag1$^{-/-}$ mice were treated with indicated experimental diets for 9 weeks and weekly bodyweight measurements are shown as mean values ± SD for each diet group. (n=5 mice per group) (One-way ANOVA followed by Tukey's post-hoc test)

The hematological profile of *Rag1*$^{-/-}$ mice after 9-week diet treatment is summarized **in** Table 5. The measured hemoglobin values in all three groups indicated no signs of anemia according to reference values for C57BL/6 mice published by Raabe et al. (Raabe, Artwohl et al. 2011). Furthermore, no significant differences were found in RBC, Hb, HCT and platelets count values between the dietary groups. Although the erythrocytes from the mice held on the iron depleted diet had lower mean corpuscular values and haemoglobin, these values were still in the normal range, indicating there was no severe systemic iron deficiency.

Diet	$FeSO_4$	w/o Fe	Hemin	*P* values
WBC (10^9/l)	0.9 ± 0.3	1.4 ± 1.3	1.2 ± 0.5	0.6504
RBC (10^{12}/l)	9.6 ± 0.9	12.0 ± 2.0	10.3 ± 1.4	0.0882
Hb (g/dcl)	14.9 ± 1.4	16.6 ± 3.2	15.3 ± 2.3	0.5395
HCT (%)	60.0 ± 6.6	57.5 ± 10.8	52.1 ± 7.0	0.3751
MCV (fl) *	62.0 ± 1.5	47.4 ± 3.5	51.0 ± 0.5	< 0.0001
MCH (pg) *	15.5 ± 0.2	13.9 ± 1.0	14.9 ± 0.3	< 0.01
Platelets (10^9/l)	1.0 ± 0.1	1.5 ± 0.5	1.3 ± 0.7	0.2303

Table 5 Summary of hematological analysis after diet treatment in steady state mice
Rag1$^{-/-}$ mice treated with iron-depleted or iron adequate (FeSO4 and hemin) experimental diets for 9 weeks. Hematology parameters were determined on blood samples collected from facial vein at the end of diet treatment. Mean values and standard deviations are shown (n=5 per group). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test were performed to compare differences between dietary groups. Comparisons were made between all dietary groups. Statistically significant differences were found in following parameters; MCV ($FeSO_4$ vs. w/o Fe; $FeSO_4$ vs. Hemin) and MCH ($FeSO_4$ vs. w/o Fe) (**WBC**, white blood cells; **RBC**, red blood cells; **Hb**, hemoglobin; **HCT**, hematocrit; **MCV**, mean corpuscular volume; **MCH**, mean corpuscular hemoglobin).

To get an insight into the effect of luminal iron depletion on the intestinal immune system during homeostasis, cytokine expression in proximal colon tissue has been assessed by qRT-PCR 9 weeks after diet treatment. As shown in Figure 6, no differences were detectable among the diet groups in the mRNA expression levels of *Il-6, Il-12p35, Il-10, Ifn-γ* and *Il-17a* in the colon tissues. Induction of *Tnf-α* mRNA expression was reduced in colon tissue of hemin treated mice compared to mice held on iron-depleted diet.

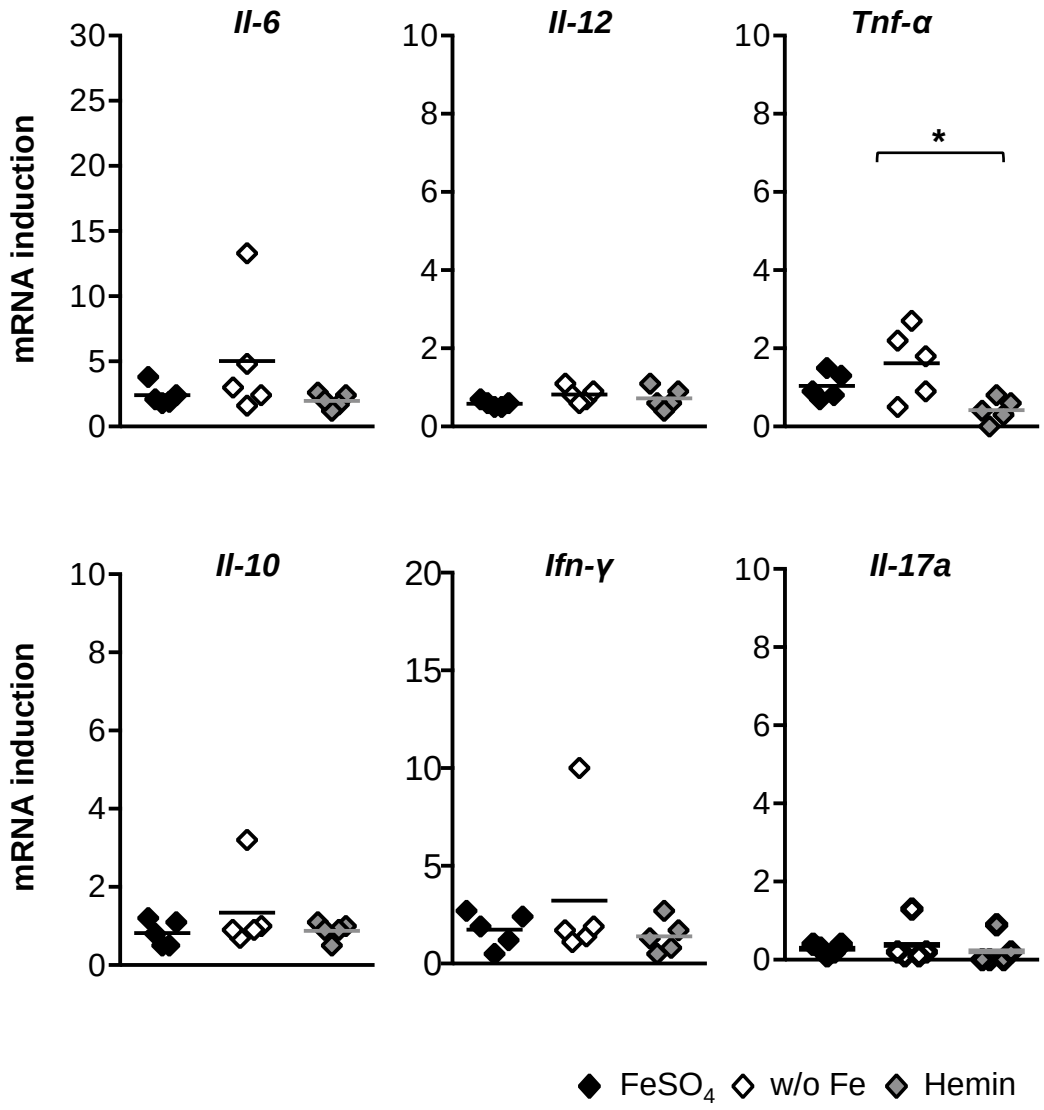

Figure 6 Cytokine mRNA expression in steady state colon of *Rag1*$^{-/-}$ mice after diet treatment
RNA was isolated from proximal colon tissue of *Rag1*$^{-/-}$ mice that were held on iron depleted or iron adequate (FeSO4 or hemin) experimental diets for 9 weeks and qRT–PCR was performed. The expression of indicated cytokines relative to the expression in *Rag1*$^{-/-}$ mice fed with conventional chow is shown as fold induction ($2^{-\Delta\Delta CT}$) values. Results for one individual mouse were shown. Crossbars indicate the mean values for each group (n=5) (one-way ANOVA followed by Tukey's post-hoc test, $P^* < 0.05$)

In the next step, the effect of the diet treatment on leukocyte infiltration was evaluated in the mLNs and colon of steady state *Rag1*$^{-/-}$ mice. Therefore, cells from mLNs and colon LPL and IEL fractions were isolated after 9 weeks diet treatment and live leukocytes were identified by gating on the CD45.2$^+$ PI$^-$ population. There were no significant differences between diet groups in the frequency of leukocytes infiltrating mLNs and colon IEL fraction. However, a significant increase in the proportion of CD45$^+$ cells was detected in colon LPL of *Rag1*$^{-/-}$ mice on iron depleted diet compared to iron adequate dietary groups (Figure 7).

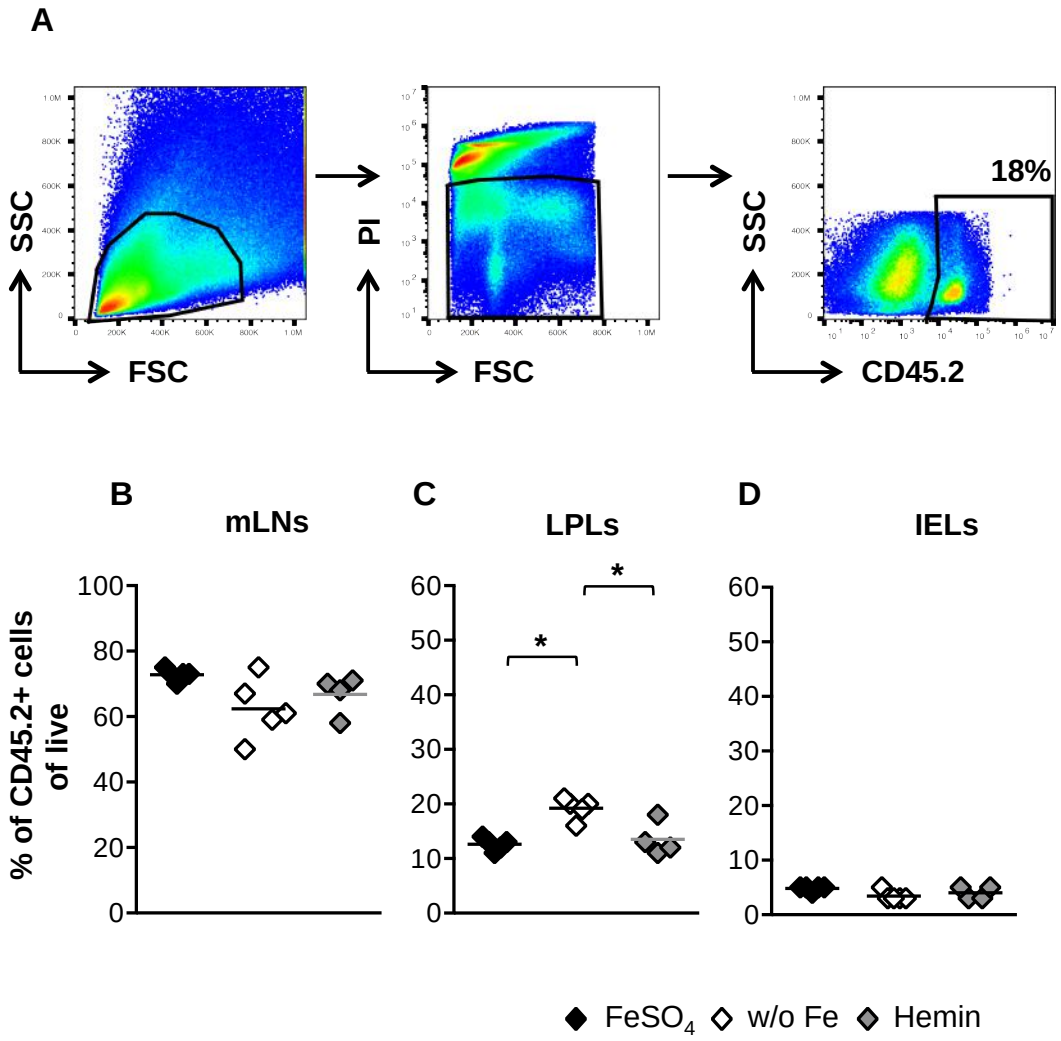

Figure 7 Leukocyte frequencies in the colon and mLNs after diet treatment in the steady state mice
Colon LPL and IEL cells were isolated from resting *Rag1*$^{-/-}$ mice after 9-week diet treatment. **(A)** Representative FACS plots of CD45.2 expression within live-gated colon LPL fraction **(B) (C)** and **(D)** Percentages of CD45.2^{+} cells in mLNs and colon lamina propria (LPL) and intraepithelial leukocytes (IEL) fractions. Dots represent values obtained from individual mice and the mean values of each group are shown as black crossbars. (n=5) (one-way ANOVA followed by Tukey's post-hoc test, $P^{*} < 0.05$).

The next step was to analyse the effect of diet treatment on the intestinal mononuclear phagocytes including MΦ and DCs in resting *Rag1*$^{-/-}$ mice after 9-week diet treatment. To assess this, a 10-colour staining panel was established to identify colonic MΦ and DC subpopulations. First, total CD11b^{+} cells were identified within the CD45.2^{+} live LPL fractions and SSC^{high} cells were gated out to exclude eosinophils and neutrophils. Further, CD64 expression was analysed within CD11b^{+}SSC^{low} LP leukocytes and CD45^{+}CD11b^{+}SSC^{low}CD64^{+} cells were defined as colonic MΦ as shown in Figure 8 **(Left panel)**. The expressions of Ly6C and MHC class II were then analysed within colonic MΦ and this revealed three distinct populations; Ly6C^{low}MHCII^{+}, Ly6C^{high}MHCII^{+} and Ly6C^{high}MHCII^{-} cells as shown in Figure 8 **(Right panel)**.

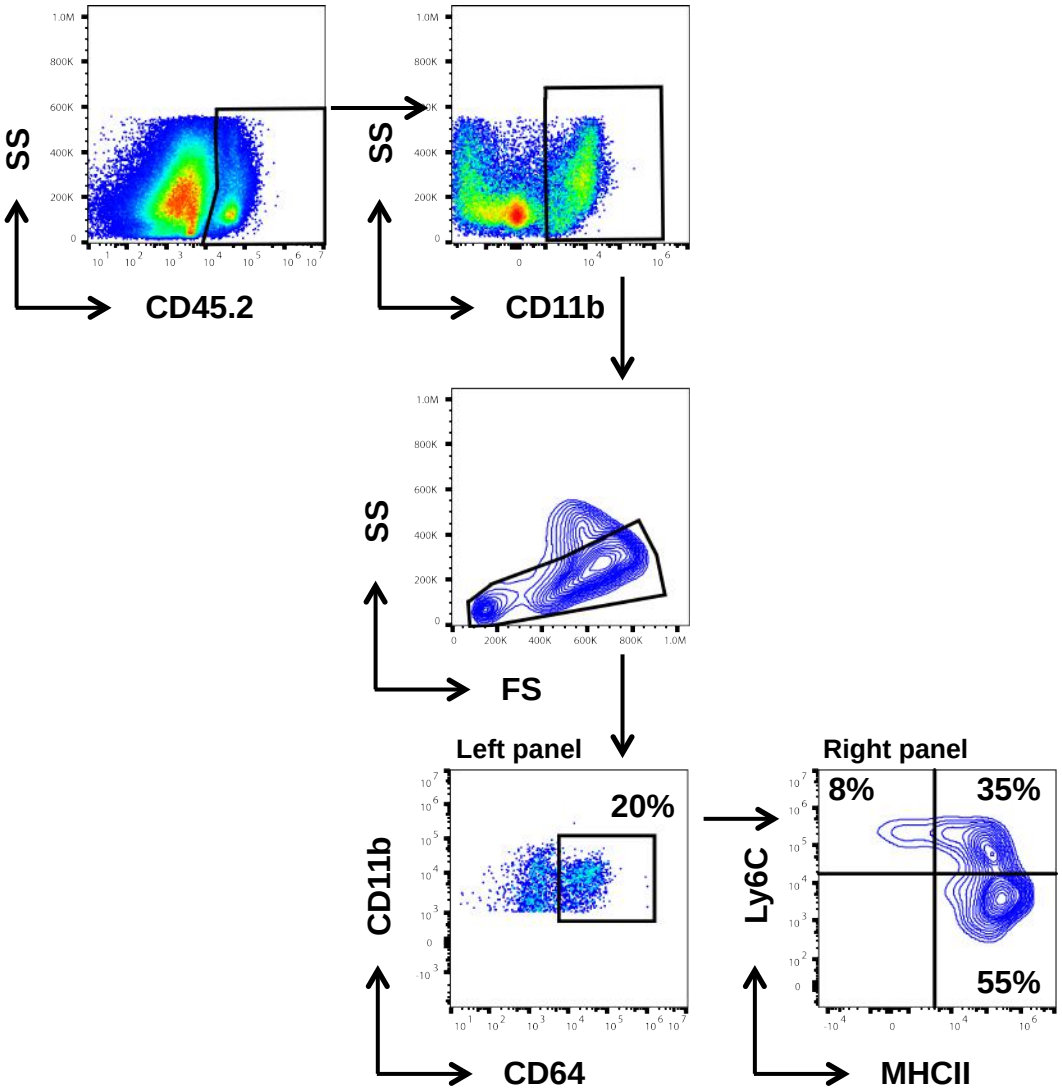

Figure 8 Effect of luminal iron change on colonic m in steady state *Rag1*$^{-/-}$ mice
Dot plots showing representative gating strategy for identification of the colonic macrophage population and its subsets after dietary intervention in resting *Rag1*$^{-/-}$ mice. Numbers indicate percentages of live-gated CD45.2^{+}CD64^{+}CD11b^{+} macrophages (MΦ) within colonic LPL **(Left panel)** and the percentages of subsets within the MΦ population **(Right panel**) between groups (n=5).

Changes in frequencies of colonic MΦ and MΦ subsets after 9-week diet treatment were investigated. An increased proportion of CD11b^{+}CD64^{+} MΦ within LP leukocytes was found in colons of *Rag1*$^{-/-}$ mice on iron depleted diet when compared to both iron adequate diet groups (mean ± SD; $FeSO_4$: 4.2 ± 1.3%; w/o Fe: 14.0 ± 6.9%; hemin: 3.0 ± 1.4%) (Figure 9**A**) However, no significant difference could be detected in MΦ subset composition between diet groups as indicated in Figure 9**B**.

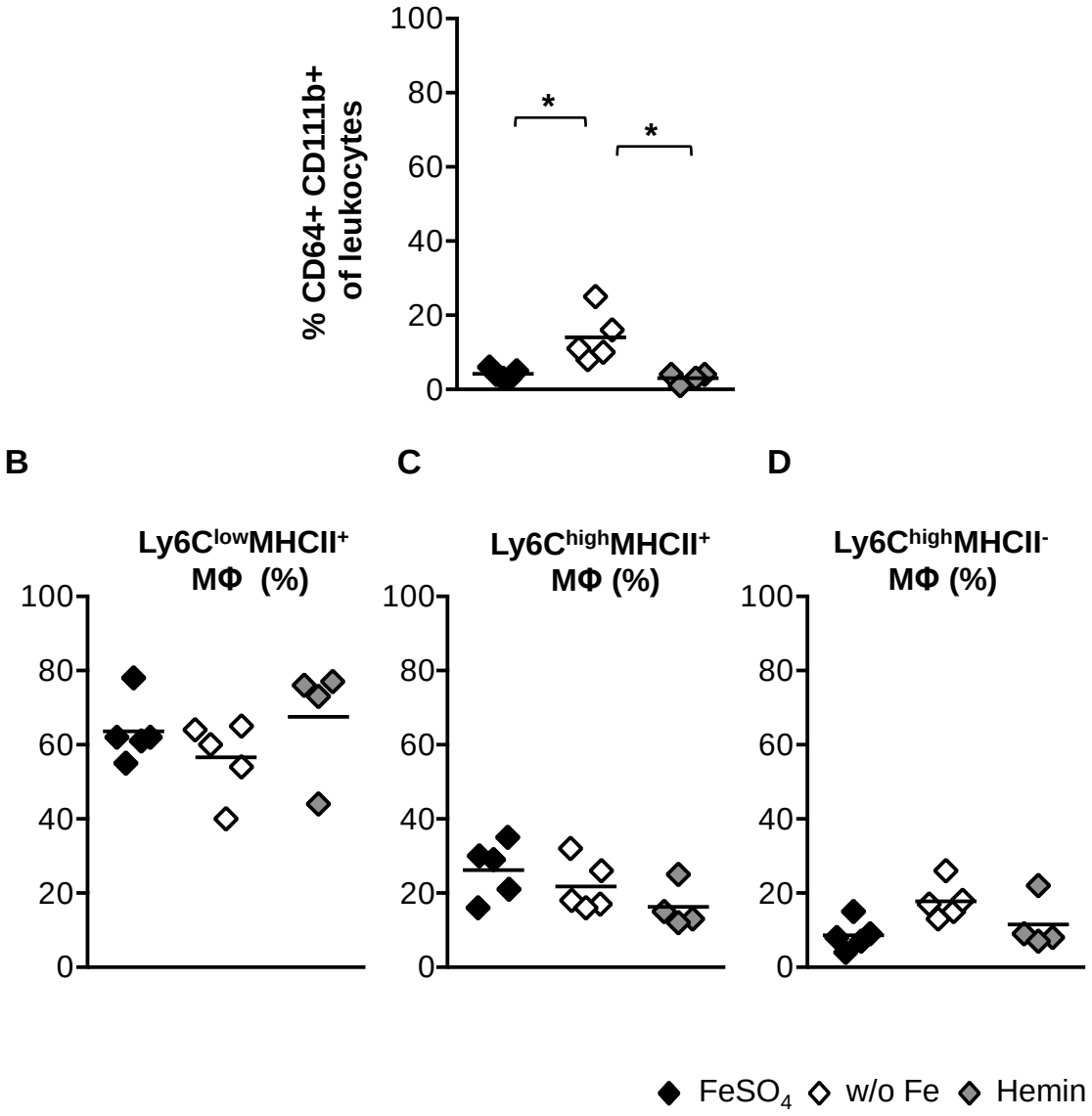

Figure 9 Comparison of intestinal MΦ frequencies and MΦ subset composition in resting *Rag1*$^{-/-}$ mice after diet treatment
(A) Frequencies of MΦ population within colon LPL fraction. **(B) (C)** and **(D)** Data showing percentages of three major MΦ subsets within live-gated CD45.2^{+}CD11b^{+}SSC^{low}CD64^{+} cell population in colon samples from mice that went through 9 week diet treatment. Dots represent the data obtained from individual mice and the crossbars indicate mean values. (One-way ANOVA followed by Tukey's post-hoc test, $P^{*} < 0.05$) (n=5)

To examine the effect of diet treatment on intestinal DCs, the CD64^{-} population was gated within live-gated CD45.2^{+} population isolated from colon LP and mLNs and the expression of CD11c and MHC class II was analysed within this population. Within CD64^{-} CD11c^{+}MHCII^{high} cells, identified as DCs, three subsets were revealed based on their CD103 and CD11b expression: CD103^{+}CD11b^{-}, CD103^{+}CD11b^{+} and CD103^{-}CD11b^{+} cells Figure 10 A population of CD103^{-}CD11b^{-} cells which is not clearly defined was also observed (as seen by others (Cerovic, Bain et al. 2014).

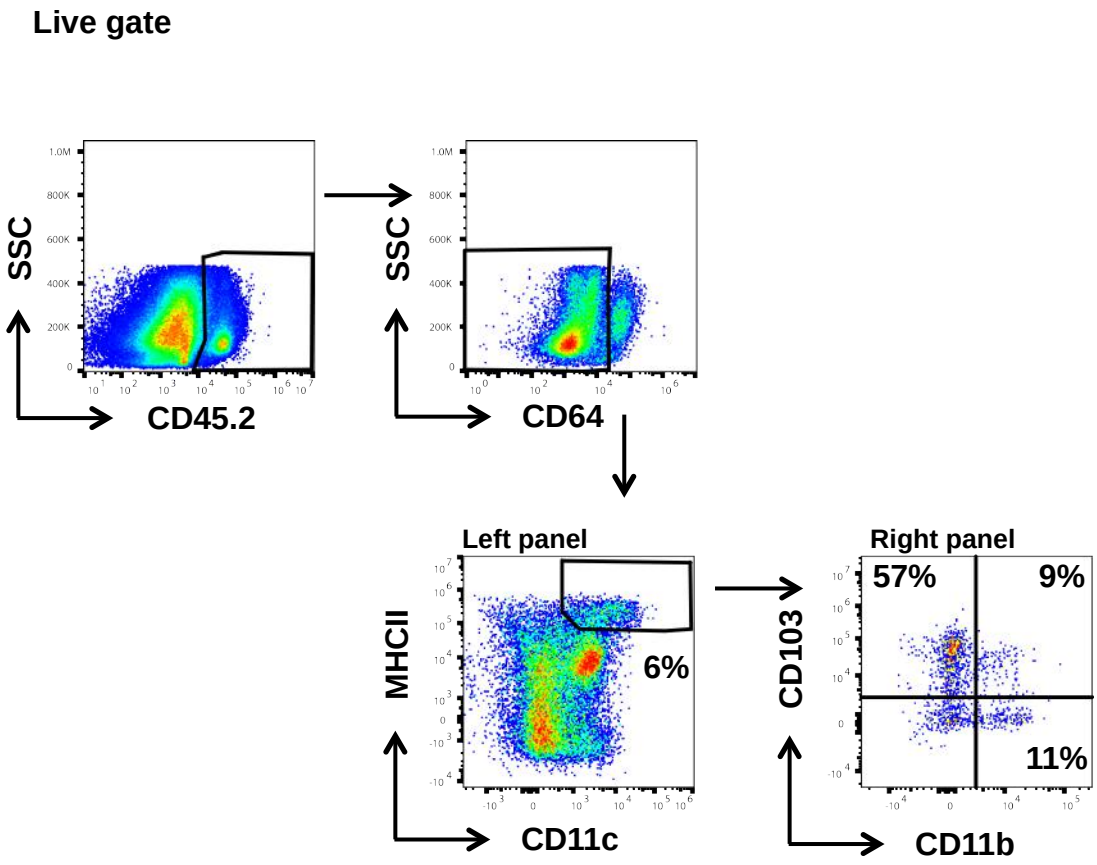

Figure 10 Phenotype characterization of colon dendritic cells on steady state *Rag1*$^{-/-}$ mice after diet treatment
Colon LPL and IEL were isolated from resting *Rag1*$^{-/-}$ mice after 9-week diet treatment and live-gated CD45.2^{+} cells were analysed for DC populations by flow cytometry. FACS plots representing gating strategy used to define the DC population and DC subsets within colon LPL after diet treatment. Numbers indicate representative percentages of DCs amongst LPL (Left panel) and percentages of DC subsets within LP DCs (Right panel) in steady state colon (n=5).

When looking at the frequencies of DCs isolated from mLNs and colon LP, we could only observe a decrease of CD64^{-}CD11c^{+}MHCII^{high} cells isolated from mLNs in iron deficient fed mice when compared with hemin fed group. Iron depletion had no effect on the percentage of colon LP DCs (Figure 10**A**). Further, we found that DC composition was affected by dietary iron depletion. First, the percentage of both CD103^{+} DC subsets was increased in mLNs of mice treated with iron-depleted diet when compared to both iron adequate diets (CD103^{+}CD11b^{-}: $FeSO_4$ 31.6 ± 6.3 vs. w/o Fe 43.8 vs. 5.6 vs. hemin 30.4 ± 2.1; CD103^{-} CD11b^{+}: $FeSO_4$ 36.8 ± 7.3 vs. w/o Fe 49.2 ± 4.1 vs. hemin 33.2 ± 1.8) (Figure 11**B** and **C**). In contrast the percentage of CD11b single positive DCs was significantly lower in mLNs of iron-depleted group in comparison to both iron adequate groups (CD103^{-}CD11b^{+}: $FeSO_4$ 18.4 ± 11.0 vs w/o Fe 5.2 ± 5.0 vs. hemin 32.7 ± 6.1) (Figure 11**D**). The CD103^{-}CD11b^{+} DC population was also found to be less frequent in colon LP of iron depleted group in comparison to both iron adequate groups (CD103^{+}CD11b^{-}: $FeSO_4$ 19.4 ± 7.5 vs. w/o Fe 16.6

± 5.0 vs. hemin 32.8 ± 6.1) (Figure 11**D**). Iron depletion had no effect on the other two DC subsets in colon LP. Because of the low numbers of DCs and MΦ in the steady state, the IEL fraction was not analysed.

Taken together, the feeding experiments demonstrated that 9-week dietary iron depletion had no effect on bodyweight course and haematological profile in resting *Rag1*$^{-/-}$ mice. Apart from slightly higher expression of *Tnf-α* in the iron-depleted group, the cytokine profile in colon tissue of steady state *Rag1*$^{-/-}$ mice was not affected by iron content in the diet. Characterization of antigen presenting cells in colon and mLNs after diet treatment revealed increased frequencies of CD45.2^{+} leukocytes and colonic MΦ in colon LP of *Rag1*$^{-/-}$ on iron-depleted diet. The frequency of DCs was found to be decreased in mLNs of *Rag1*$^{-/-}$ mice fed with iron depleted diet with similar trend in colon LP. While the colonic MΦ composition was not affected by iron depletion, both CD103^{+} subsets were found to be more prominent in mLNs of the iron depleted group in comparison to iron adequate groups with similar trend in colon LP. In contrast the percentage of CD11b single positive DCs was significantly lower in iron-depleted group when compared with iron adequate groups. These results suggest that the reduction of iron content in the food may shift the composition of DCs towards the CD103^{+} DCs, which exert regulatory function by producing TGF-β and RA, and inducing regulatory T cells.

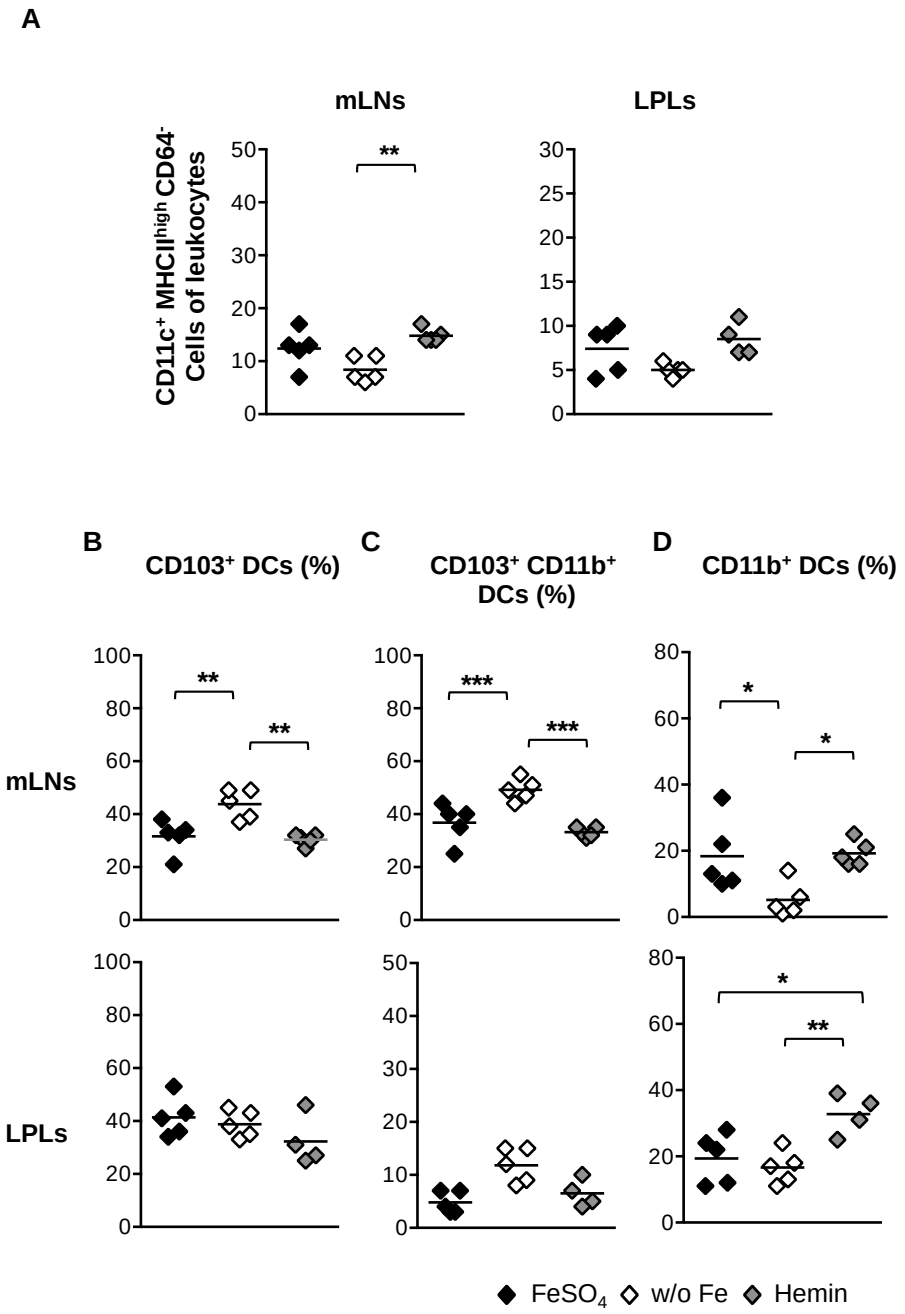

Figure 11 Effect of luminal iron depletion on intestinal DC frequencies in steady state *Rag1*$^{-/-}$ mice
Rag1$^{-/-}$ mice were treated with indicated experimental diets for 9 weeks and the colon LPL and IEL were isolated at the end point for FACS analysis **(A)** Percentages of DCs defined as live-gated CD45.2^{+}CD64^{-} CD11c^{+}MHCII^{high} within leukocyte populations isolated from mLNs and colon. **(B) (C)** and **(D)** Percentages of CD103^{+}CD11b^{-}, CD103^{+}CD11b^{+} and CD103^{-}CD11b^{+} DC subsets within DC populations isolated from mLNs and colon LPL fraction in *Rag1*$^{-/-}$ mice fed with experimental diets. Dots represent one individual mouse and crossbars indicate mean values of each group (n=5) $P < 0.05$, one-way ANOVA followed by Tukey's post-hoc test ($P^{*} < 0.05$, $P^{**} < 0.01$, $P^{***} < 0.001$).

4.2. Dietary iron depletion fails to protect against T cell mediated colitis development

To investigate a potential protective role of dietary iron reduction in T cell mediated colitis, $CD4^+CD62L^+$ T cells were isolated from WT splenocytes and adoptively transferred into *Rag1*$^{-/-}$ mice after 6 weeks dietary treatment as shown in (Figure 3)

Colitis development was monitored by bodyweight measurements and clinical scoring in *Rag1*$^{-/-}$ mice fed with iron-depleted diet (w/o Fe) compared to *Rag1*$^{-/-}$ mice on iron adequate diets ($FeSO_4$ vs. hemin). As shown in the Figure 12**A** bodyweight records indicate that dietary iron depletion had no effect on colitis related bodyweight loss since more then 50% of mice in each dietary group lost between 15 – 20 % of their starting bodyweight by day 17 after T cell transfer. (The percentage of starting weight was for $FeSO_4$: 85.8 ± 5.5; w/o Fe: 90.1 ± 6.1; hemin: 92.4 ± 7.7 on day 17). Bodyweight loss was accompanied by increasing disease severity regarding diarrhea, hunched posture and inactivity present in iron depleted as well as in iron adequate groups (Figure 12**B**). *Rag1*$^{-/-}$ mice fed with hemin diet developed less sever diarrhea and had only small changes in normal posture. Histological examination of colon tissue sections showed that dietary iron depletion had no effect on histological signs of colitis. Representative H&E stainings shown in Figure 12**C** demonstrate that the disrupted architecture, epithelial damage with extensive transmural ulceration and massive inflammatory leukocyte infiltration were present in all dietary groups. This was confirmed by a comparable histological score in the three dietary groups (Figure 12**D**).

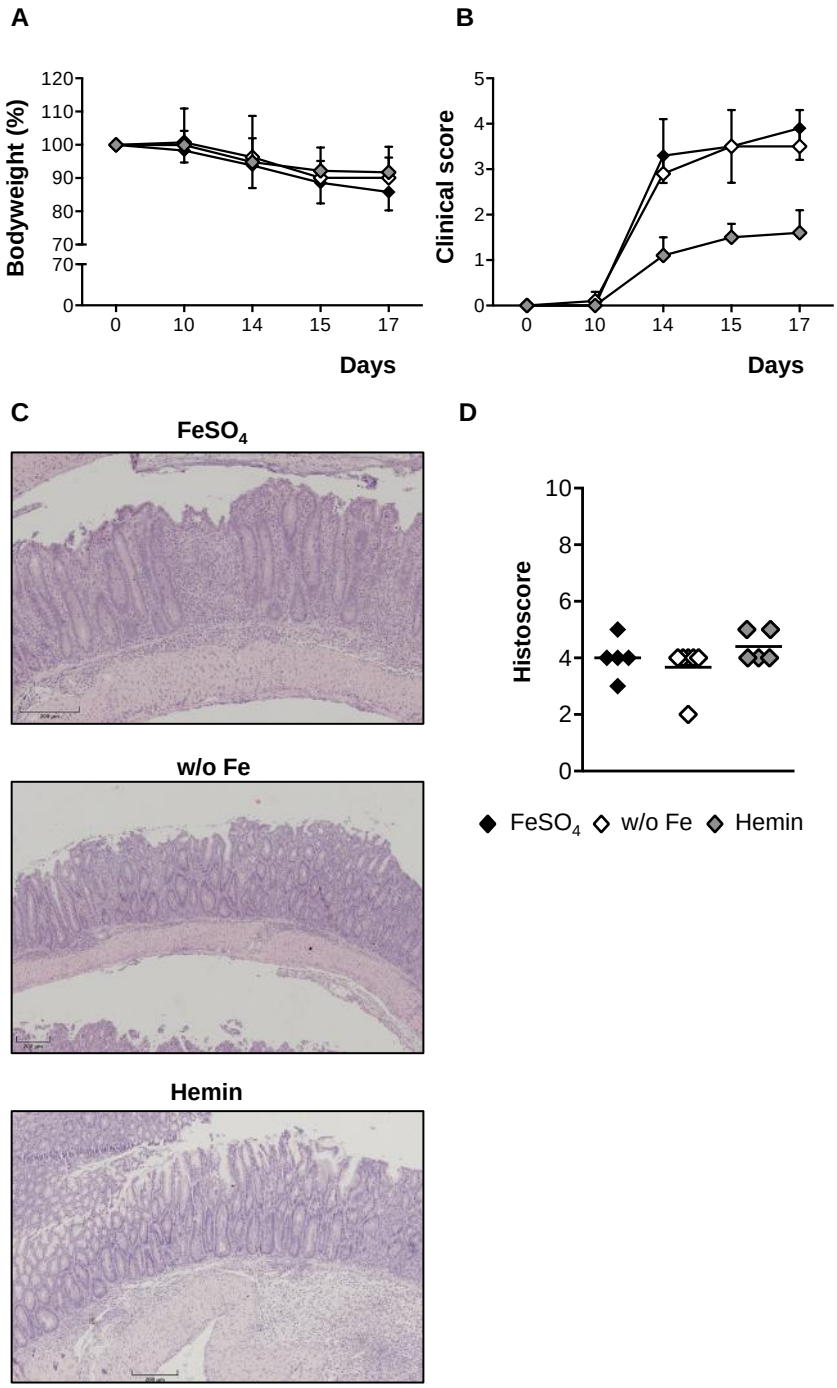

Figure 12 Development of T cell transfer colitis after iron depletion
Rag1$^{-/-}$ mice were treated with iron-depleted or iron adequate ($FeSO_4$ or hemin) experimental diets. After 6 weeks colitis was induced by transferring $3*10^5$ $CD4^+CD62L^+$ splenic T cells. On day 17 after T cell transfer mice were sacrificed as the majority of them had lost 15-20% of their starting weight. (A) Bodyweight in percentage of starting weight measured at the indicated time points. (B) Colitis activity (score 0 - 15). Results of one representative experiment are shown (means ± SD) (n=6-7 mice per group). (C) On day 17 (+ 3) after T cell transfer colon tissues were fixed in 4% formalin, embedded in paraffin and stained with H&E for histological analysis. Representative images are shown (magnification 40x). (D) The histological score was determined. Dots represent values obtained from individual mice and crossbars indicate mean values for each dietary group (one-way ANOVA with Tukey's post-hoc test or Kruskal-Wallis test) (n=6-7 mice per group).

To investigate if dietary iron depletion during T cell transfer colitis leads to development of anemia in *Rag1*$^{-/-}$ mice, blood Hb and HCT measurements were evaluated prior to 6-week dietary treatment, before T cell colitis induction and at day 17 after T cell transfer as shown in Table 6**.** No significant difference in Hb level between dietary treatments could be found at the indicated time points. Transfer of *Rag1*$^{-/-}$ mice from standard chow to $FeSO_4$ supplemented experimental diet increased systemic Hb level. In contrast, there was no significant change in Hb levels in iron depleted or hemin dietary groups between indicated time points. These results show that dietary iron depletion did not lead to development of anemia in *Rag1*$^{-/-}$ during T cell transfer colitis.

The WBC count values significantly increased in all treatment groups after colitis development due to the systemic inflammatory response. However no significant differences were found in WBC counts between dietary groups at the indicated time points (Table 6).

	Diet	$FeSO_4$	w/o Fe	Hemin	*P* values
Before diet treatment	WBC (10^9/l)	2.0 ± 0.6	4.6 ± 2.8	5.9 ± 4.7	0.1372
	Hb (g/dcl)	14.1 ± 6.3	15.5 ± 5.3	16.8 ± 4.7	0.3662
	HCT (%)	55.7 ±13.5	72.7 ± 12.7	59.5 ± 8.1	0.2072
After diet treatment	WBC (10^9/l)	3.1 ± 1.9	2.2 ± 1.4	2.2 ± 0.4	0.8231
	Hb (g/dcl)	20.7 ± 4.2	17.8 ± 2.0	17.0 ± 6.6	0.5266
	HCT (%) *	54.0 ± 19.6	63.8 ± 4.2	60.7 ± 10.2	0.0152
T cell induced colitis	WBC (10^9/l)	9.2 ± 2.6	7.1 ± 1.5	6.9 ± 3.4	0.3011
	Hb (g/dcl)	17.0 ± 3.9	19.6 ± 4.0	19.3 ± 2.2	0.3819
	HCT (%)	58.1 ± 14.9	46.4 ± 16.7	61.6 ± 6.9	0.9747

Table 6 Monitoring of hematological parameters during 6-week diet treatment and T cell mediated colitis development

Colitis was induced by transfer of $CD4^+CD62L^+$ T cells in *Rag1*$^{-/-}$ mice treated with iron-depleted or adequate ($FeSO_4$ or hemin) diets. Blood samples were taken from facial vein at indicated time points and white blood cell count (WBC); hemoglobin (Hb) and hematocrit (HCT) were measured. Mean ± standard deviations are shown (n= 5-7 mice per group). One-way ANOVA followed by Tukey's post-hoc test was performed.

To investigate the role of dietary iron depletion for inflammatory cytokine production in the colon during colitis, qRT-PCR was performed to analyse mRNA expression levels of cytokines in proximal colon tissue of *Rag1*$^{-/-}$ mice 17 days after T cell transfer. Cytokine mRNA fold induction for each dietary group was shown relative to cytokine expression in the steady state mice treated with the same diet. As shown in Figure 13, all cytokines, which were measured, were induced during colitis, but *Ifn-γ* and *Il-17* were most strongly induced. However, no significant differences in cytokine induction between dietary treatments were observed.

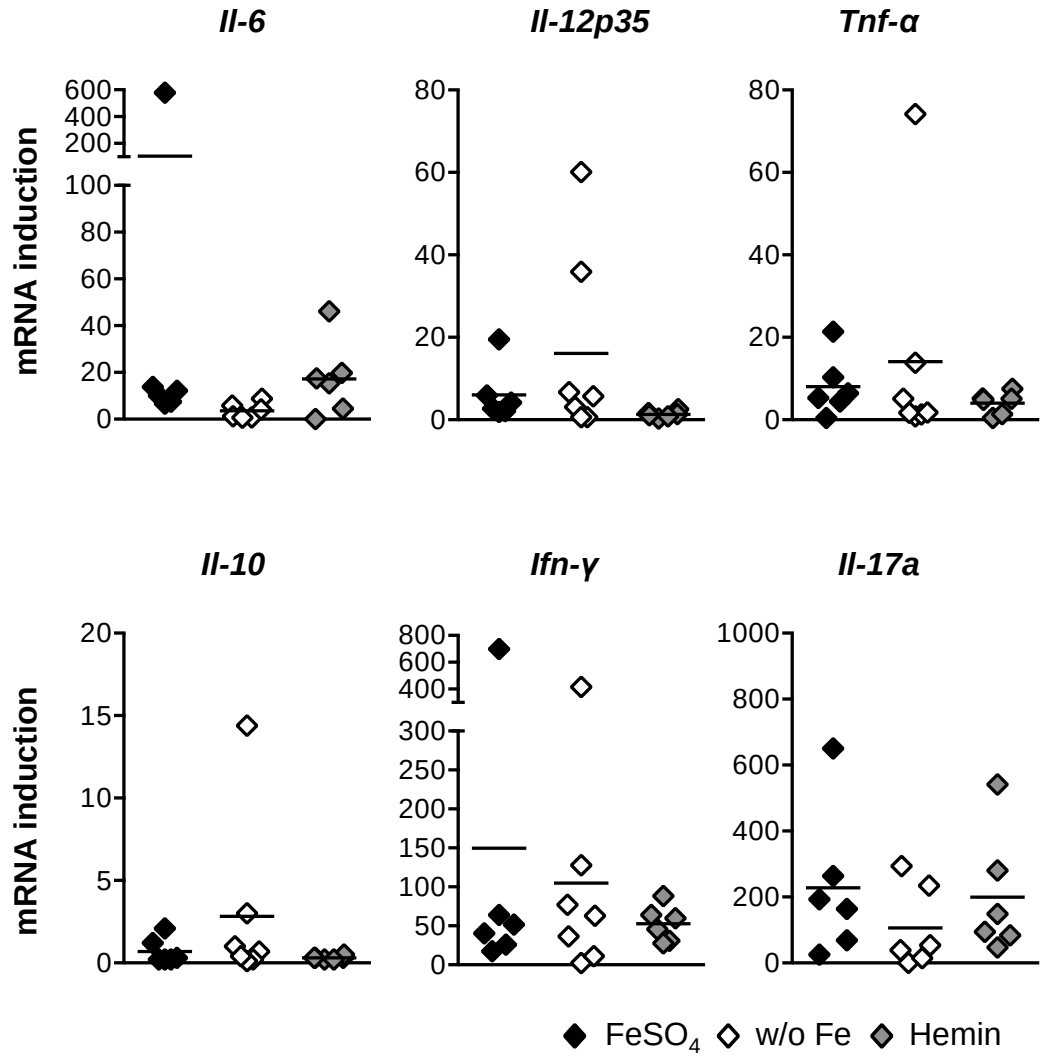

Figure 13 Cytokine induction in colon after 6-week diet treatment and colitis development
Colitis was induced by T cell transfer in *Rag1*$^{-/-}$ mice treated with iron-depleted or iron adequate ($FeSO_4$ and hemin) experimental diets for 6 weeks. RNA from the whole colon tissue was isolated on 17 days after T cell transfer and qRT PCR was performed. Induction of mRNA for indicated cytokines in each dietary group is calculated as relative to the expression in steady state mice given the same type of diet. Dots represent values obtained from individual mice and crossbars indicate mean values. (n=6-7 mice per group) One-way ANOVA test followed by Tukey's post hoc test was performed.

These results support our findings of comparable clinical and histological colitis activity in the three experimental groups.

Taken together, the 6-week dietary iron deprivation had no effect on intestinal inflammation development during T cell induced colitis as confirmed by bodyweight loss, clinical score and histological analysis as well as cytokine profiling in colon tissue.

4.3. The effect of dietary iron depletion on colonic macrophages and DCs during experimental T cell colitis

It is well established that the composition of the intestinal immune system dramatically changes during inflammation due to the influx of innate and adaptive immune cells into the intestinal mucosa (Kamada, Hisamatsu et al. 2008, Platt, Bain et al. 2010). For example, increased migration of blood monocytes into the intestine is a hallmark of the inflammation. To explore the effect of luminal iron deprivation on the mononuclear phagocyte system in the intestine during inflammation, the frequency and composition of colonic macrophages and DCs was analysed by flow cytometry during T cell transfer colitis.

Next, T cells were transferred into *Rag1*$^{-/-}$ mice that had received iron depleted or iron adequate experimental diets for 6 weeks. The frequency of live-gated CD45.2^{+} isolated from colon LP and intraepithelial fraction was analysed by FACS on day 17 after T cell transfer. As shown in Figure 14**A** and **B** iron depleted diet had no significant effect on leukocyte infiltration in colon.

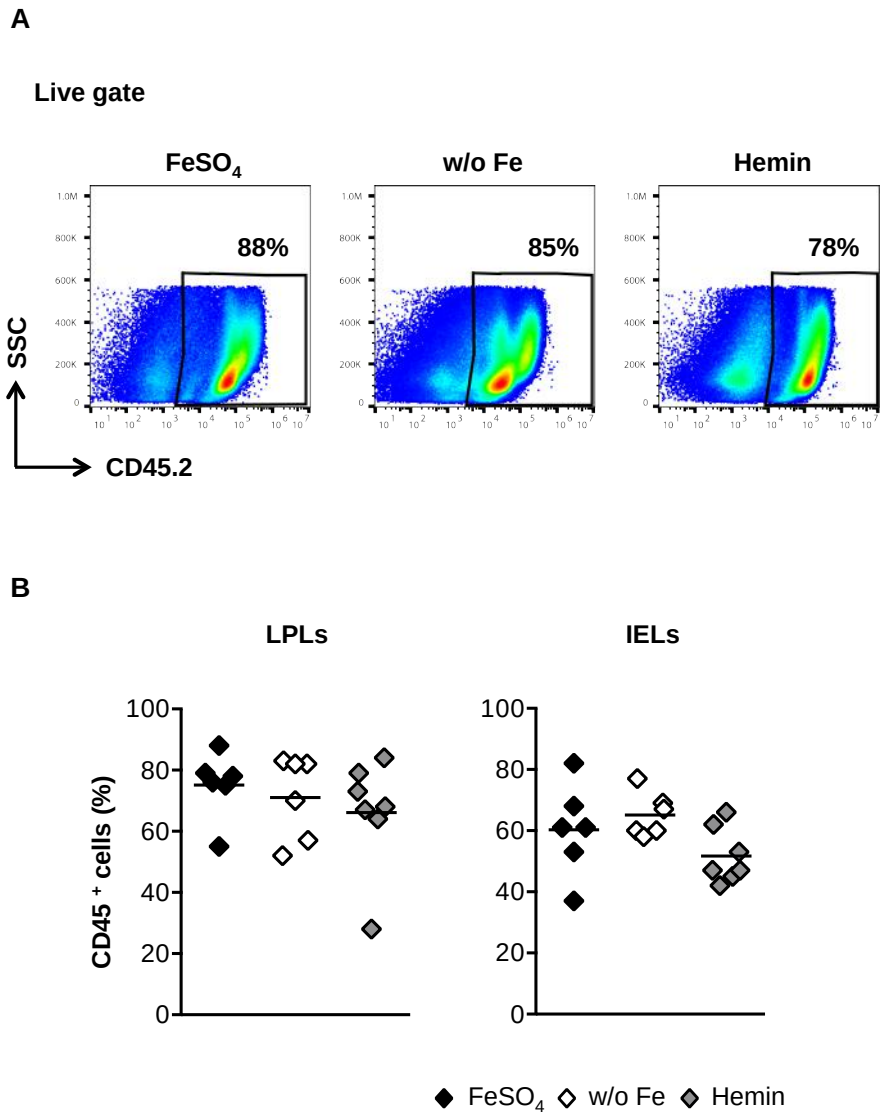

Figure 14 Effect of luminal iron depletion on leukocyte infiltration in colon after T cell induced colitis development
Rag1$^{-/-}$ mice were given iron depleted or adequate ($FeSO_4$ or hemin) experimental diets for 6 weeks followed by T cell induced colitis. Colon LPL and IEL fraction were analysed by flow cytometry 17 days after T cell transfer. **(A)** Representative FACS plots and **(B)** frequencies of $CD45^+$ cells in colon LPL and IEL. Each dot represents the value of an individual mouse and crossbars indicate mean values (n=6-7 mice per group). One-way ANOVA was used followed by Tukey's post-hoc test to determine statistical difference.

Further we analysed the effect of luminal iron depletion on the mononuclear phagocyte system during intestinal inflammation. First, the FSC and SSC profile of $CD45^+CD11b^+$ cells was examined and to exclude granulocytes SSC^{high} cells were gated out. As demonstrated in Figure 15**,** transfer of $CD4^+CD62L^+$ T cells resulted in a 3-5 fold increase of $CD11b^+CD64^+$ MΦ in the colon LPL fraction compared to steady state mice fed with indicated experimental diets. However, the only significant variation in MΦ frequency due to the iron content was found between $FeSO_4$ and hemin supplemeted diets (42.7 ± 5.0 vs. 33.6 ± 6.3) (Figure 16).

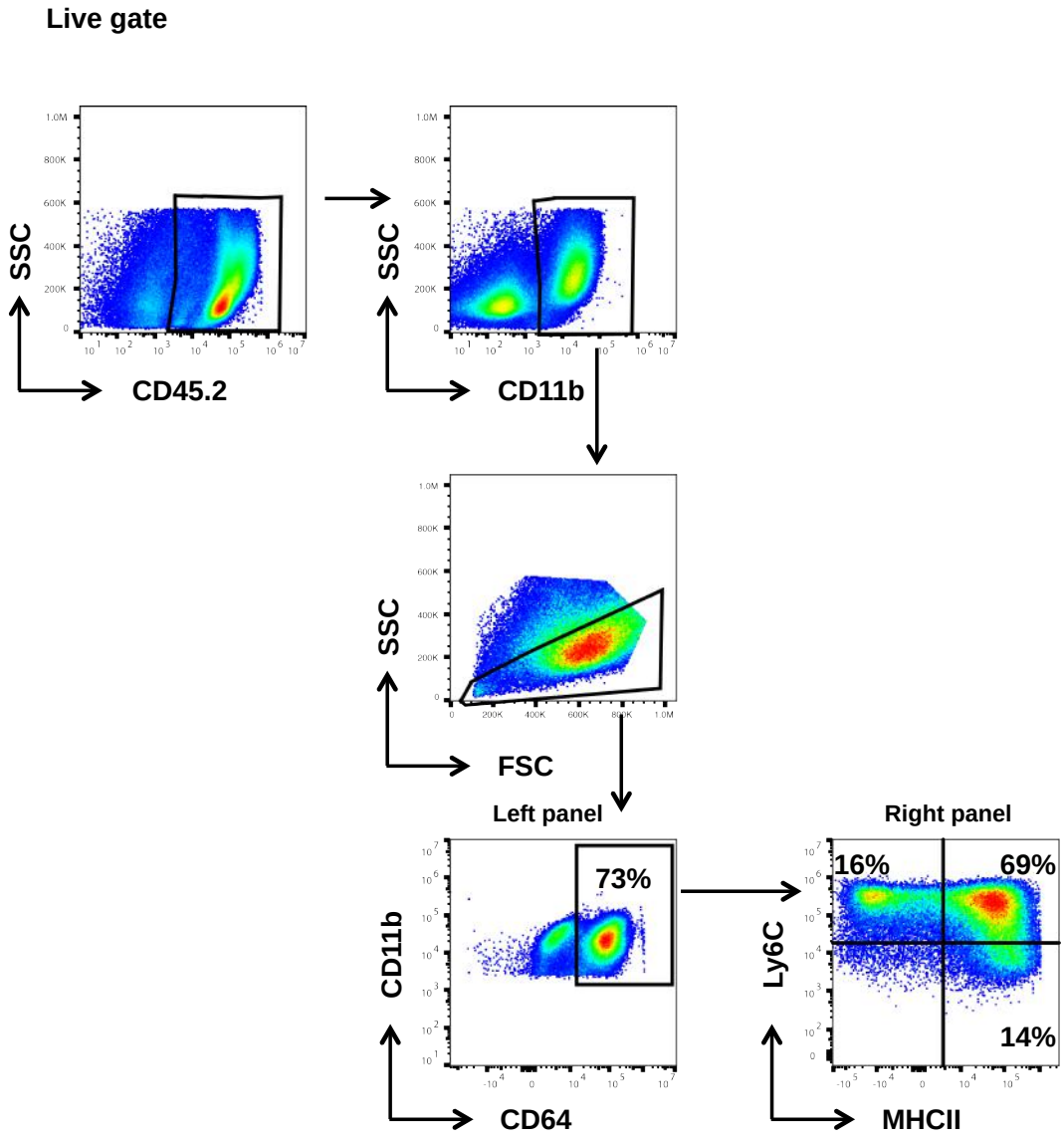

Figure 15 Gating strategy for characterization of colonic MΦ in T cell transfer colitis after luminal iron depletion

Colonic LPL cells were isolated from *Rag1*$^{-/-}$ mice after 6-week diet treatment followed by T cell transfer colitis. Live-gated CD45^{+} were analysed for the expression of CD11b, CD64, Ly6C and MHC class II on day 17 after transfer. Colonic MΦ were identified based on their expression of CD11b and CD64 within the leukocyte population **(Left panel)**. CD11b^{+}CD64^{+} MΦs were then assessed for the expression of MHC class II and Ly6C to define MΦ subsets in colon **(Right panel)**. One representative staining is shown and numbers indicate frequencies of MΦ and MΦ subsets representative for each diet group.

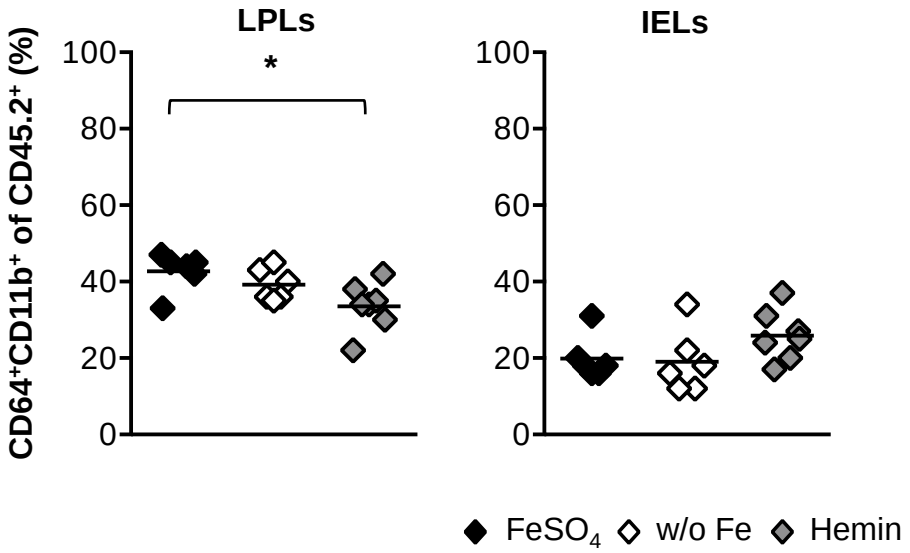

Figure 16 Frequencies of colonic MΦ population after luminal iron depletion and colitis development
The proportions of $CD64^{+}CD11b^{+}$ colonic MΦ within LPL and IEL population isolated from *Rag1*$^{-/-}$ mice, treated with iron depleted or adequate (FeSO4 and hemin) experimental diets, 17 days after T cell transfer. The dots represent the percentage of infiltrated MΦ in individual mice and the crossbars indicate mean values (n=6-7 mice per group). One-way ANOVA followed by Tukey's post-hoc test was used to determine statistically significant differences ($P^{*} < 0.05$).

As discussed in section 4.1. , the antiinflammatory $Ly6C^{low}MHCII^{+}$ cells were the most abundant macrophage subset in colon LP of the resting *Rag1*$^{-/-}$ whereas proinflammatory $Ly6C^{high}MHCII^{+}$ and $Ly6C^{high}MHCII^{-}$ cells were less abundant. The development of inflammation after T cell transfer in *Rag1*$^{-/-}$ mice fed with experimental diets resulted in dramatic shift between three MΦ subsets in colon, with 2-4 fold decrease in $Ly6C^{low}MHCII^{+}$ MΦ and 2-3 fold increase in $Ly6C^{high}MHCII^{+}$ and $Ly6C^{high}MHCII^{-}$ subsets compared to the steady state mice **(**Figure 15 **Right panel** and Figure 17). Significant differences in the frequencies of macrophage subsets in colon LP were found only between iron depleted and $FeSO_4$ fed mice with higher proportions of both antiinflammatory $Ly6C^{low}MHCII^{+}$ and proinflammatory $Ly6C^{high}MHCII^{+}$ cells in iron-depleted group ($Ly6C^{low}MHCII^{+}$: $FeSO_4$ 16.1 ± 8.0 vs. w/o Fe 29.1 ± 5.9; $Ly6C^{high}MHCII^{+}$ $FeSO_4$ 43.9 ± 5.9 vs. w/o Fe 62.2 ± 12.3) while the percentage of $Ly6C^{high}MHCII^{-}$ cells was decreased within iron depleted LP MΦ compartment ($Ly6C^{high}MHCII^{-}$: ($FeSO_4$) 28.5 ± 7.5 vs. (w/o Fe) 6.5 ± 12.0). As shown by work of others, these $Ly6C^{high}MHCII^{-}$ blood derived monocytes infiltrate the small and large intestine in steady state and regulate their expression of MHCII and Ly6C depending on the environmental milieu in the intestine (Bain, Scott et al. 2013). Monocyte recruitment to the colon is greatly increased during inflammation and recruited monocytes upregulate MHC class II (Bain, Scott et al. 2013). Therefore the remarkable reduction of $Ly6C^{high}MHCII^{-}$ cells in the iron depleted group could be the result of the upregulation of MHCII by these cells and

their shift to the Ly6C MHCII double positive compartment that was found to be the most abundant in colon LP of iron depleted group. The same effects of luminal iron depletion on proinflammatory MΦ subsets were found in the IEL fraction but this time differences were found in comparison to both iron adequate diets ($Ly6C^{high}MHC^{+}$ $FeSO_4$ with 59.3 ± 8.9, w/o Fe with 75.5 ± 10.2, hemin with 48.6 ± 9.3; $Ly6C^{high}MHCII^{-}$: $FeSO_4$ with 23.3 ± 3.7, w/o Fe with 6.2 ± 9.7, hemin with 29.0 ± 6.0). The antiinflammatory $Ly6C^{low}MHCII^{+}$ MΦ in IEL fraction were increased in iron depleted diet in comparison to $FeSO_4$ experimental diet ($Ly6C^{-}$ $MHCII^{+}$: with $FeSO_4$ 11.2 ± 2.3, with w/o Fe 17.8 ± 1.9).

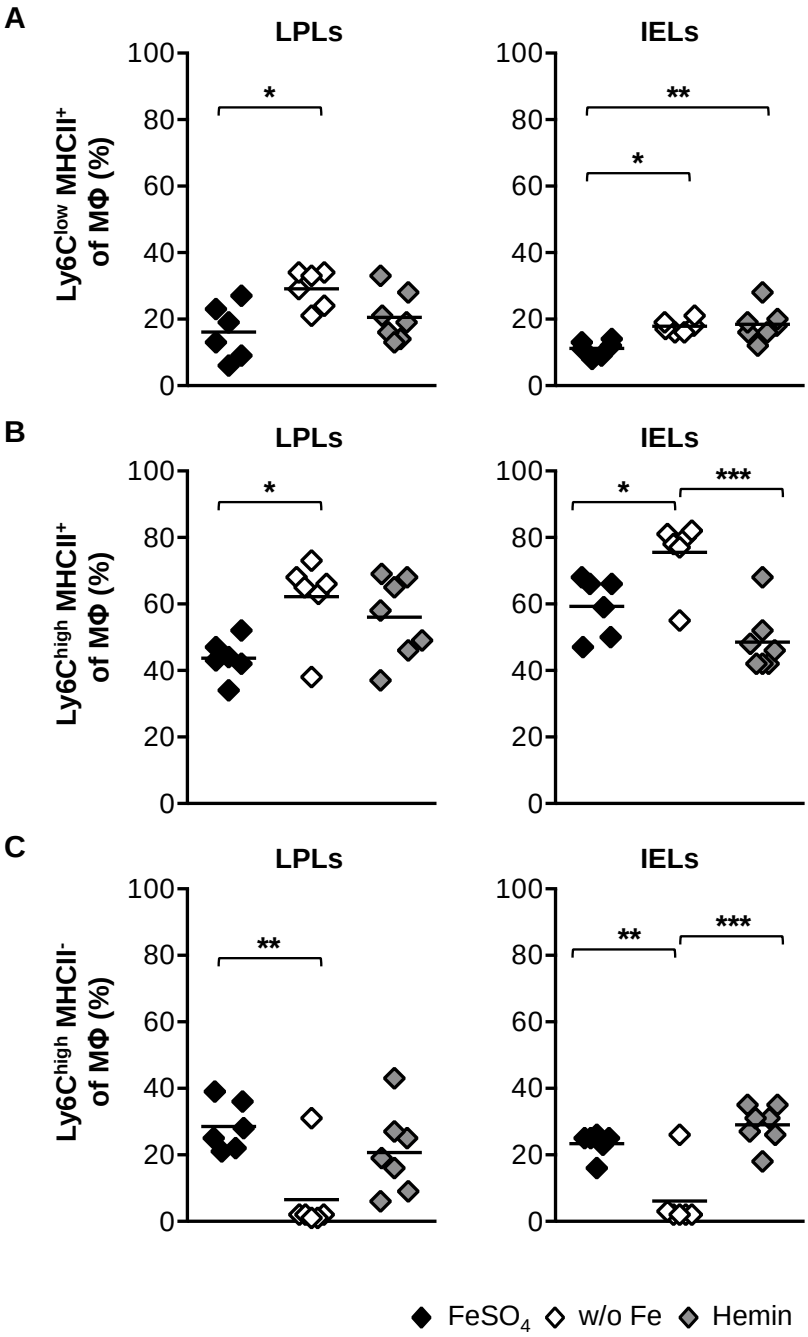

Figure 17 Frequencies of colonic MΦ after diet treatment and colitis development
Rag1$^{-/-}$ mice were treated with indicated experimental diets followed by T cell induced colitis. Macrophage composition based on Ly6C and MHC class II expression was examined within colonic live-gated CD45.2^{+}CD11b^{+}CD64^{+} MΦ by FACS on day 17 after T cell transfer. Graphs showing proportions of **(A)** Ly6C^{low}MHCII^{+}, **(B)** Ly6C^{high}MHCII^{+} and **(C)** Ly6C^{high}MHCII^{-} within colon LPL and IEL MΦ populations. Dots represent percentages of respective MΦ subsets in individual mice and crossbars indicate the mean values for each diet group (n=6-7 mice per group). One-way ANOVA followed by Tukey's test was performed to determine statistically significant differences ($P^{*} < 0.05$, $P^{**} < 0.01$, $P^{***} < 0.001$).

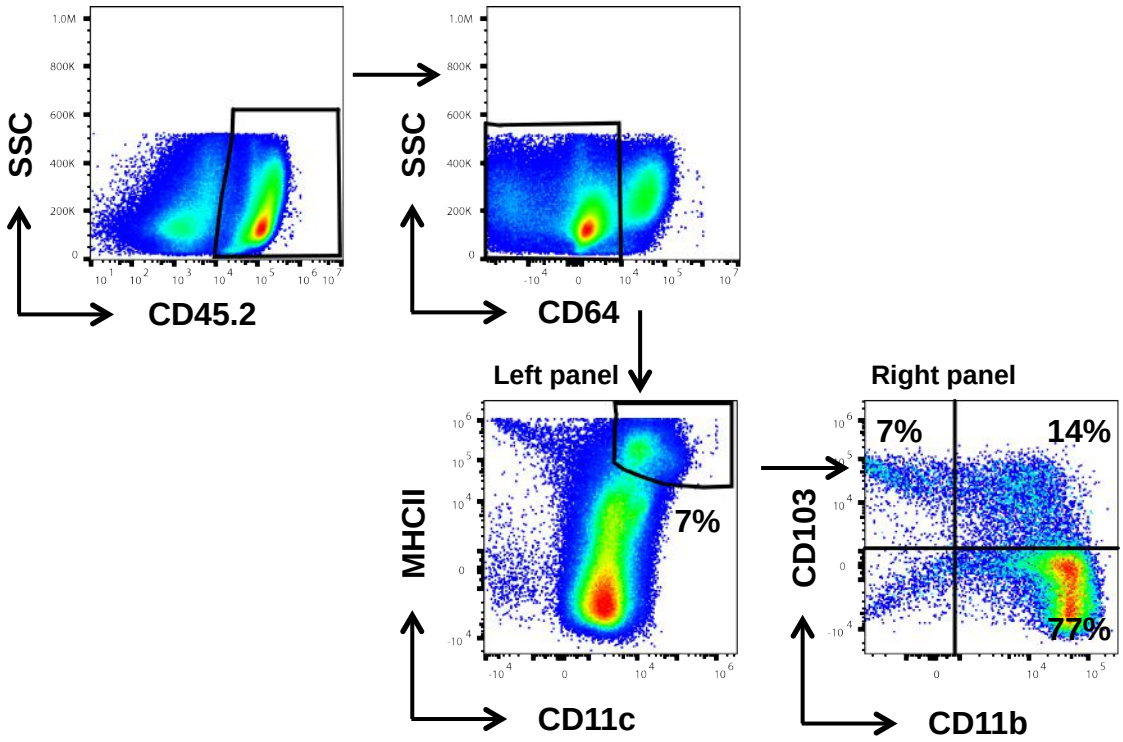

Figure 18 Gating strategy for characterization of colonic DCs in *Rag1*$^{-/-}$ mice after diet treatment and colitis development
Rag1-/- mice were treated with iron-depleted or adequate ($FeSO_4$ and hemin) experimental diets. After 6 weeks colitis was induced by T cell transfer and colon LPL and IEL were isolated on day 17 after transfer. Live-gated $CD45.2^+$ cells were analysed for the expression of CD11c, CD64, MHCII, CD103 and CD11b. Colonic $CD64^-$ $CD11c^+MHCII^{high}$ DC were identified within live-gated $CD45.2^+$ population **(left panel)** and further assessed for the expression of CD103 and CD11b to define DC subsets **(right panel)**. Result of one representative staining of colon LPL is shown and the numbers indicate percentages of DC and DC subsets in this example.

The staining panel for intestinal DCs, previously established in steady state dietary treatment, was used to analyse the effect of iron deprivation on DC compositions in Rag1-/- mice at day 17 after T cell transfer. CD64-CD11c+MHCIIhigh DCs were gated within colon LPL and IEL fractions and DC subsets were further defined according to the expression of CD103 and CD11b as shown in Figure 18.

Luminal iron depletion had no major impact on overall DC percentage in colon LP after colitis development while the DC abundance in colon IEL fraction was lower in iron depleted diet compared to both $FeSO_4$ and hemin diets ($CD64^-CD11c^+MHCII^{high}$ in IEL: $FeSO_4$ with 4.7 ± 1.2, w/o Fe with 2.5 ± 0.8, hemin with5.6 ± 1.7) **(**Figure 19**).**

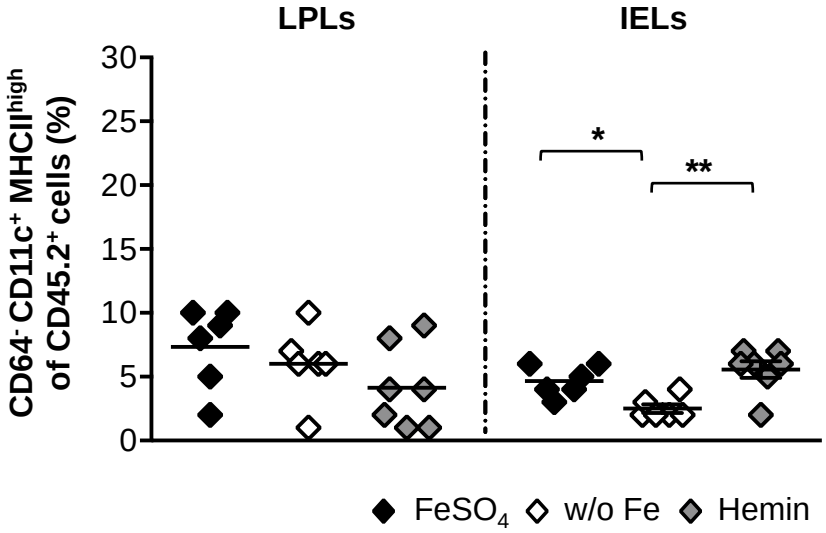

Figure 19 Effect of luminal iron depletion on colonic DCs after T cell transfer induced colitis development
T cell mediated colitis was induced in *Rag1*$^{-/-}$ 6 weeks after treatment with indicated experimental diets. Graph showing frequencies of CD64^{-}CD11c^{+}MHCII^{high} DCs within colon live-gated CD45.2^{+} LPL and IEL isolated on day 17 after T cell transfer. Dots represent data obtained from individual mice and crossbars show mean values for each diet group. One-way ANOVA followed by Tukey's post-hoc test was used to determine statistical difference ($P^{*} < 0.05$, $P^{**} < 0.01$).

According to the results obtained from steady state feeding experiments (see section **4.1.**), development of intestinal inflammation did not alter the percentage of DCs in colon LP of *Rag1*$^{-/-}$ treated with experimental diets. However, a striking change in DC subset composition was observed in colon LP in all groups during colitis characterized by a decrease of the CD103^{+}CD11b^{-} DC percentage compensated by increased frequency of CD11b single positive DCs (Figure 20).

An increased percentage of CD103 single positive DCs was found within colon IELs of colitic *Rag1*$^{-/-}$ mice fed with iron depleted diet compared to hemin treated group (w/o Fe: 17.8 ± 7.6 vs. hemin: 8.3 ± 4.0) (Figure 20**A**). Apart from this, luminal iron depletion had no significant influence on DC composition during colitis and variability was quite high especially in the hemin group. Nevertheless, it is worth mentioning that the proportion of CD103 single positive DCs in colon LP of hemin fed *Rag1*$^{-/-}$ mice was higher compared to $FeSO_4$ diet ($FeSO_4$: 8.7 ± 5.3 vs. hemin: 19.3 ± 3.5 (Figure 20**A**) while on the other hand the percentage of the CD11b^{+} single positive subset was lower in comparison to both iron depleted and $FeSO_4$ containing diets ($FeSO_4$: 66.5 ± 21.0, w/o Fe: 17.4 ± 7.1, hemin: 8.7 ± 3.3) (Figure 20**C**) suggesting a shift to the more tolerogenic DC subpopulation in the hemin group.

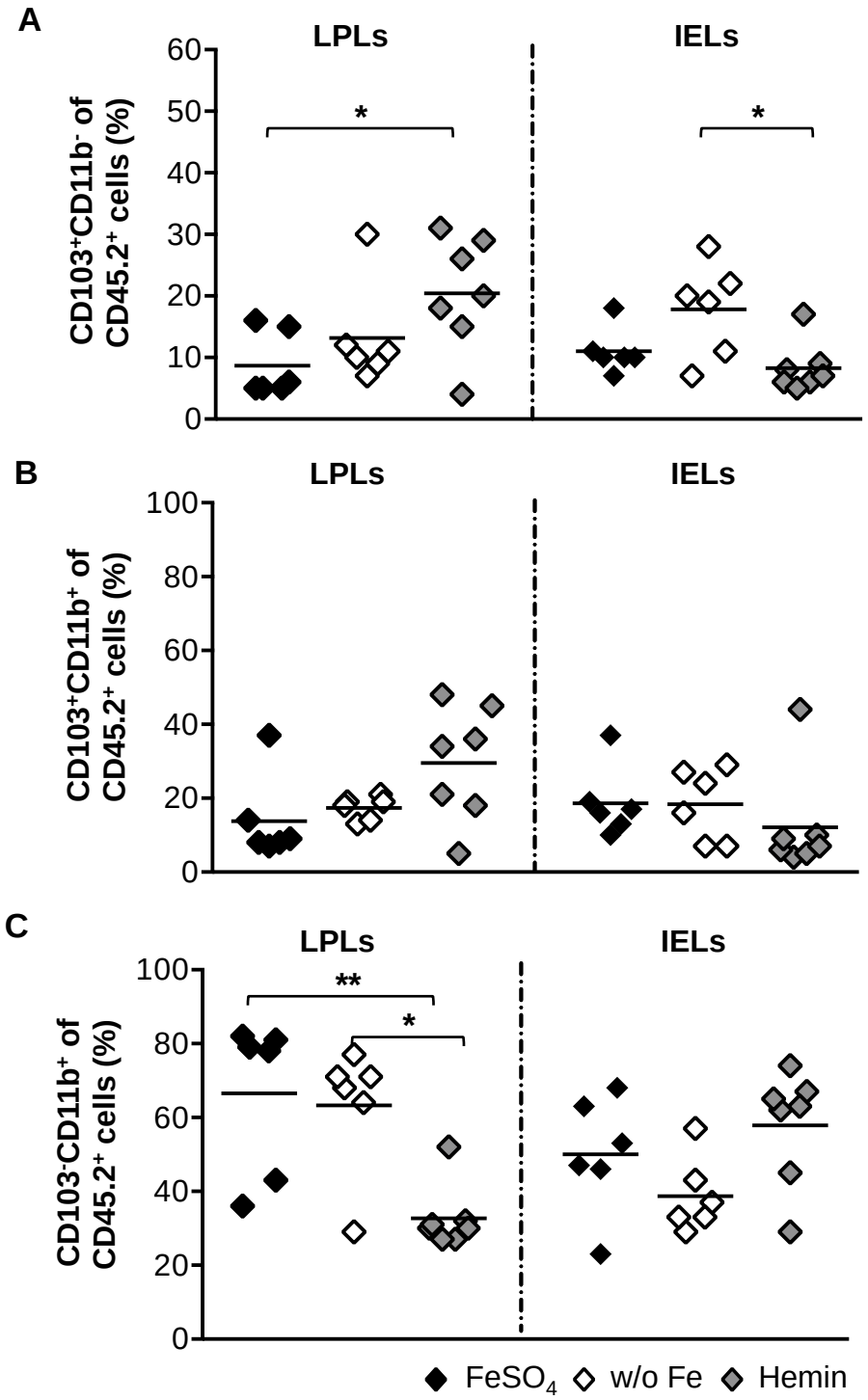

Figure 20 Effect of luminal iron depletion on colonic DC composition after colitis development
T cell transfer mediated colitis was induced in *Rag1*$^{-/-}$ mice treated with indicated experimental diets for 6 weeks. DC composition in colon LPL and IEL fraction based on CD103 and CD11b expression was analysed at day 17 after T cell transfer. Graphs showing frequencies of: **(A)** $CD103^{+}CD11b^{-}$, **(B)** $CD103^{+}CD11b^{+}$ and **(C)** $CD103^{-}CD11b^{+}$ DC subsets within live-gated $CD45.2^{+}CD64^{-}CD11c^{+}MHCII^{high}$ colon LPL and IEL. Dots represent data obtained from individual mice and crossbars show mean values for each diet group. One-way ANOVA followed by Tukey's post-hoc test was used to determine statistical difference ($P^{*} < 0.05$, $P^{**} < 0.01$).

Taken together, our results show that modulation of dietary iron contents had no striking impact on the colon leukocyte infiltration and composition during T cell transfer mediated

colitis. Still the composition of colonic LP and IEL macrophages was altered in iron deficient diet but this had no consequence for the colitis activity as shown in section **4.3.** In contrast, the effect of the inflammation itself on the colon leukocyte infiltration including blood recruited monocytes, increased frequency of proinflammatory myeloid cells and increased frequency of $CD11b^{+}$ DCs was obvious.

4.4. Effect of extended luminal iron depletion on T cell mediated intestinal inflammation development

Werner et al. demonstrated that luminal iron depletion prevented development of experimental ileitis in genetically susceptible *Tnf*$^{\Delta ARE}$ mice (Werner, Wagner et al. 2011). In my study, the protective impact of dietary iron depletion on T cell mediated colitis development was not observed. To elucidate if prolonged iron depletion can indeed suppress colonic inflammation in our experimental model, *Rag1*$^{-/-}$ mice were treated with iron-depleted (w/o Fe) and adequate ($FeSO_4$ and hemin) experimental diets for 9 weeks prior to T cell transfer.

Colitis development was confirmed by the bodyweight measurements and clinical scoring in both iron depleted and iron adequate dietary groups after adoptive transfer of T cells in *Rag1*$^{-/-}$ mice. As seen in previous colitis experiment with shorter iron starvation period, *Rag1*$^{-/-}$ mice in all three dietary groups lost weight progressively from day 10 after T cell transfer and by the day 17 more than 50% of the mice lost 15-20% of their starting bodyweight ($FeSO_4$: 91.4 ± 9.9, w/o Fe: 85.3 ± 8.0, hemin: 91.2 ± 7.4 (Figure 21**A**). In parallel the clinical score indicated increasing disease activity from day 10 after transfer (Figure 21**B**).

Additionally, histological analysis of H&E stained colon sections showed that the same degree of crypt disruption and inflammatory cell infiltration in all dietary groups, which was also confirmed by an overall histoscore as shown in Figure 21**C** and **D**.

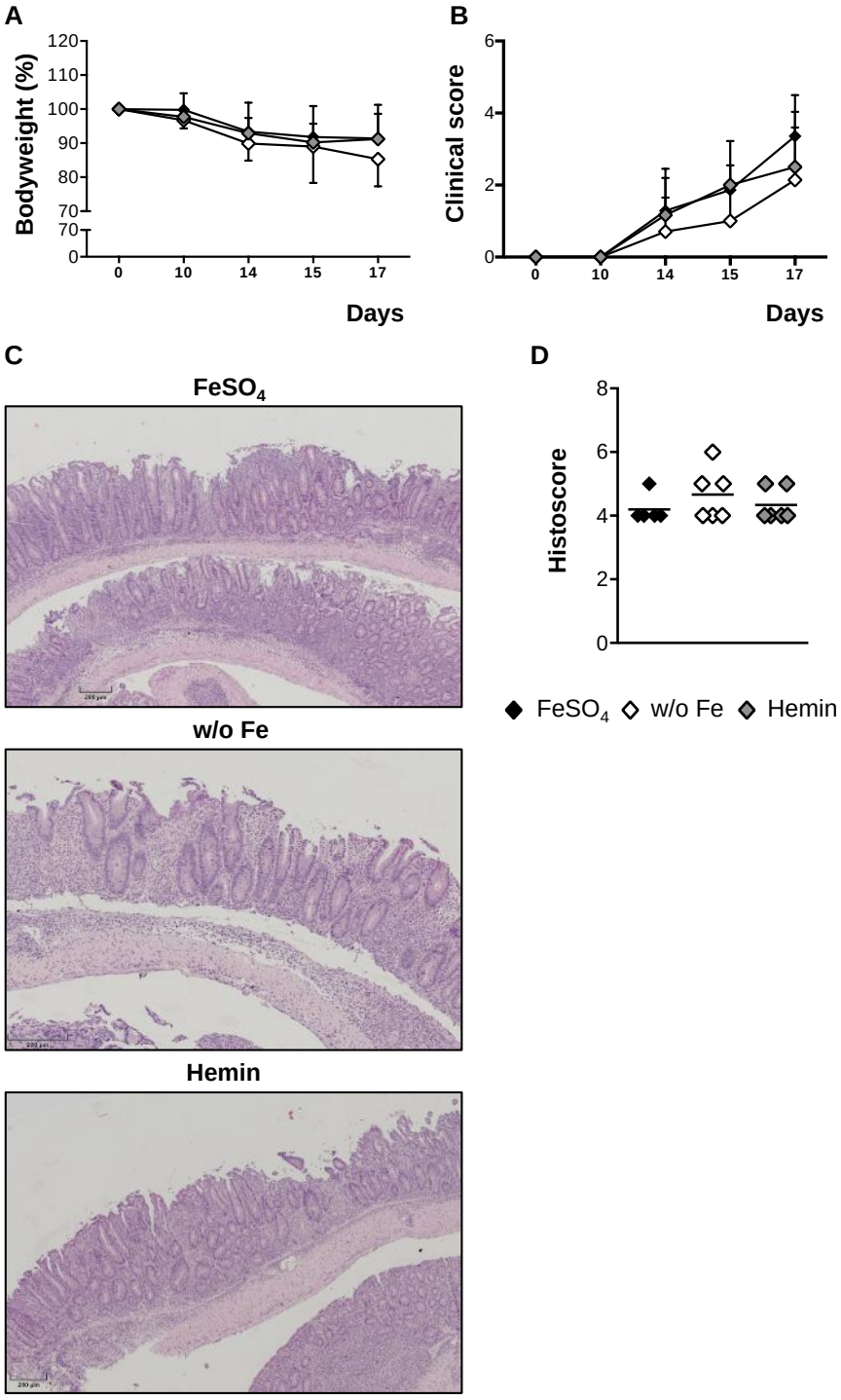

Figure 21 Development of T cell transfer induced colitis after extended dietary iron depletion
Rag1-/- mice were treated with iron-depleted or iron adequate ($FeSO_4$ or hemin) experimental diets After 6 weeks colitis was induced transferring $3*10^5$ $CD4^+CD62L^+$ splenic T cells. At day 17 after T cell transfer mice were sacrificed as the majority of them had lost 15-20% of their starting weight. **(A)** Bodyweight was measured at the indicated time points during T cell colitis development. **(B)** Colitis activity was evaluated (maximal score 15). Results shown as means ± SD (n=5-7 mice per group). **(C)** At day 17 after T cell transfer colon tissues were fixed in 4% formalin, embedded in paraffin and stained with H&E for histological analysis (final magnification 40x). **(D)** The histological score was determined 17 days after T cell transfer. Dots represent values obtained from individual mice and crossbars indicate mean values for each dietary group (n=5-7) (one-way ANOVA with Tukey's post-hoc test or Kruskal-Wallis non-parametric test).

The cytokine expression profile in inflamed colon tissue after extended dietary iron depletion was not dramatically altered compared to 6-week dietary iron depletion. However, a striking induction in mRNA expression levels of *Ifn-γ* in colons of iron-depleted and hemin treated groups was found. In parallel, *Tnf-α* mRNA induction was reduced in the iron-depleted and hemin groups (Figure 23).

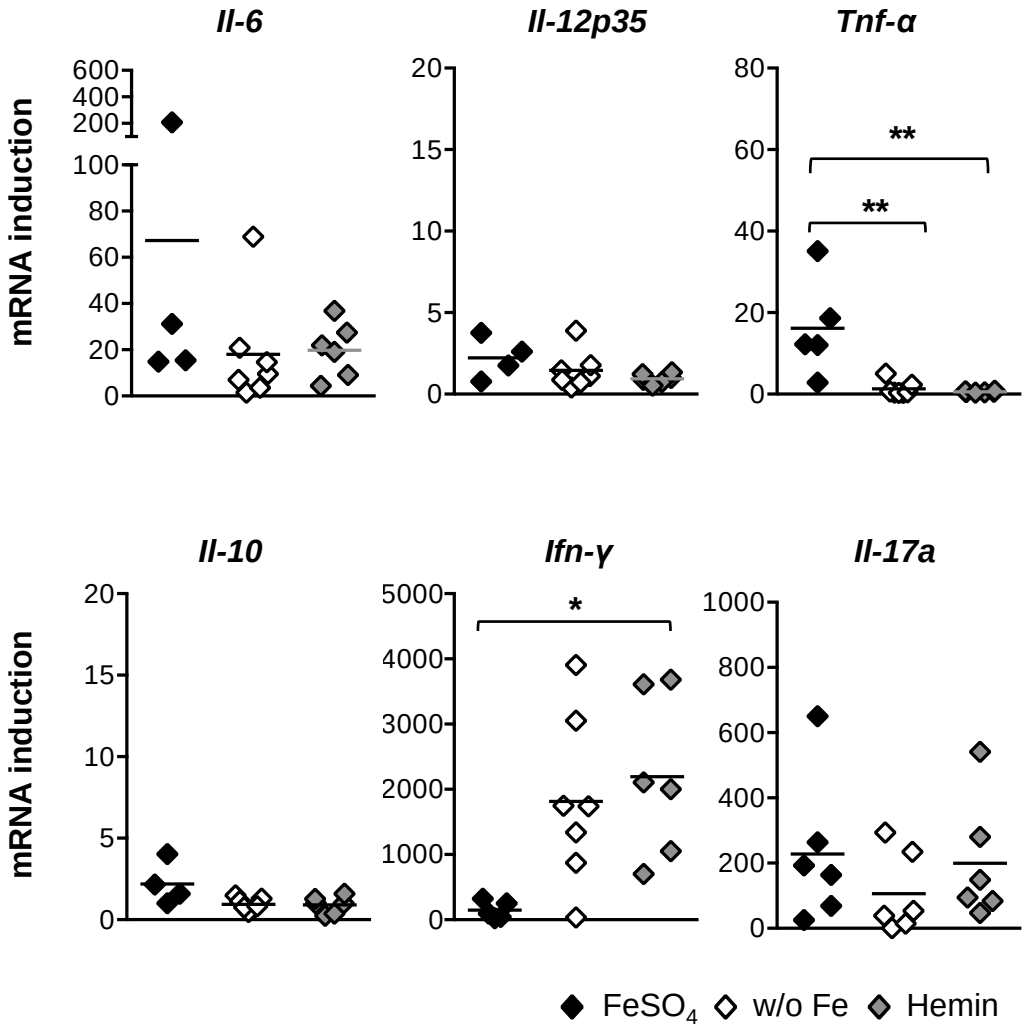

Figure 22 Effect of extended dietary treatment on mRNA cytokine expression after T cell transfer colitis development

17 days after T cell transfer RNA was isolated from proximal colon tissue samples and qRT PCR was performed. Graphs showing induction of mRNA expression for each diet group calculated relative to the expression in steady state *Rag1*$^{-/-}$ mice given the same type of diet. Dots represent the values determined from individual mice and crossbars indicate mean values (n=6-7 mice per group, one-way ANOVA followed by Tukey's post-hoc test $P^* < 0.05$, $P^{**} < 0.01$).

FACS analysis of the colon macrophage and DC populations mirrored the disease activity previously evaluated by bodyweight loss and histological colitis score. Inflammation led to approximately 8-fold higher leukocyte infiltration in both LP and IEL fractions compared to steady state mice. Yet there were no significant differences detected between iron depleted and iron adequate diet groups after extended treatment with experimental diets (Figure 23).

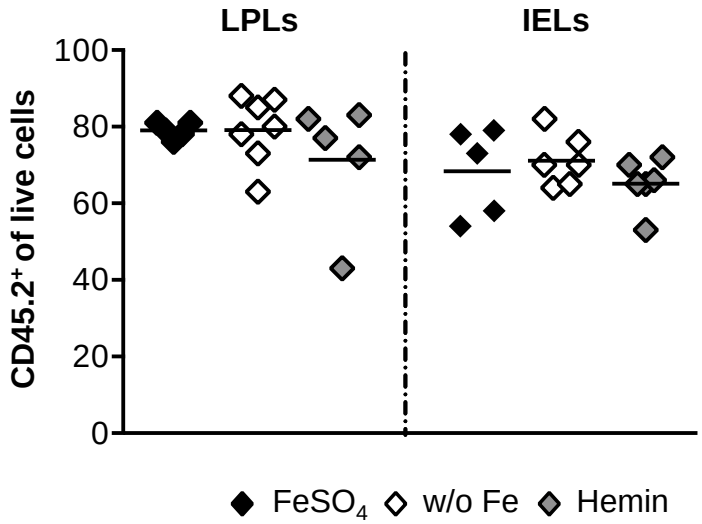

Figure 23 Expansion of leukocytes during T cell transfer colitis after extended luminal iron depletion
Colon LPL and IEL were isolated at day 17 after T cell transfer from *Rag1*$^{-/-}$ mice treated with indicated experimental diets for 9 weeks. Graph showing frequencies of CD45.2^{+} cells within live-gated population. Dots represent values obtained from individual mice and crossbars indicate mean values (n=5-7 mice per group, one-way ANOVA followed by Tukey's post-hoc test).

Colonic MΦ composition was not affected by extended luminal iron depletion in colitis *Rag1*$^{-/-}$ mice which were fed with the indicated experimental diets. Indeed, the frequencies of CD11b^{+}CD64^{+} MΦ and the distribution of MΦ subsets in colon LP and IEL fraction were even more homogeneous between dietary groups then in colitis experiment with 6-week dietary treatment. The increase in the percentage of proinflammatory MΦ, especially the Ly6C^{high}MHCII^{+} subset, during colitis was found in the iron depleted as well as in the iron adequate diets (Figure 24**A** and **B**).

Similar to the MΦ compartment, extended dietary iron depletion had no major influence on colonic DC frequencies and subset composition during T cell transfer colitis. Of note, the proportions of CD103 and CD11b single positive DCs were approximately equal in LPL and IEL fraction of all dietary groups. In contrast, in the colitis experiment with six-week feeding treatment the frequencies of CD103^{+} DCs dropped in favour of CD11b^{+} DC subset in comparison to steady state colon (Figure 25**A** and **B**).

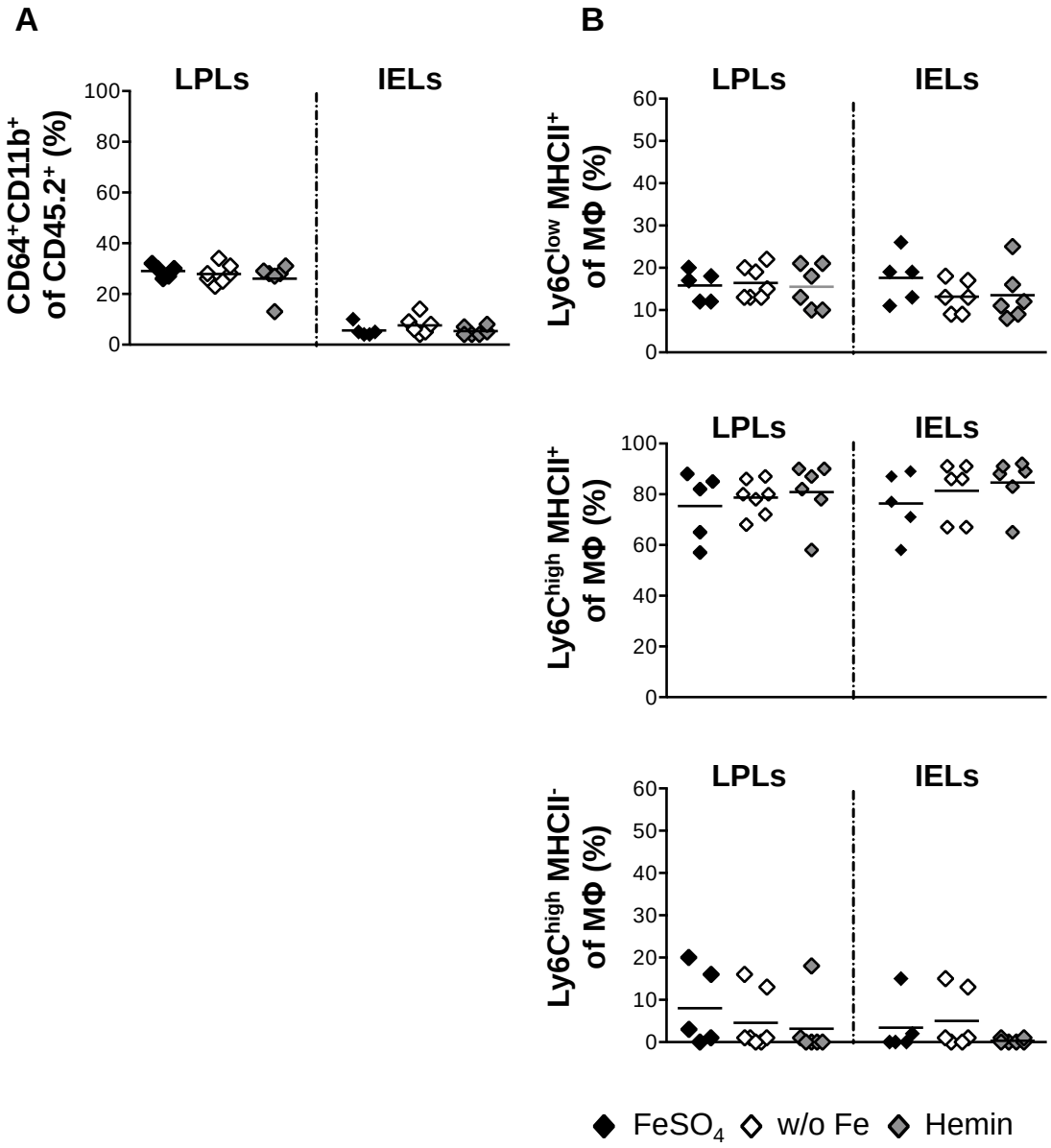

Figure 24 Effect of the extended luminal iron depletion on the colon macrophage compartment during T cell transfer colitis

Rag1$^{-/-}$ mice received the indicated experimental diets for 9 weeks after which colitis was induced by T cell transfer. Colon LPL and IEL fractions were isolated at day 17 after T cell transfer and colonic macrophages were analysed by flow cytometry. The frequencies of **(A)** CD11b^{+}CD64+ MΦ within live-gated CD45.2^{+} cells and **(B)** Mϕ subsets based on Ly6C and MHC class II expression within CD11b^{+}CD64+ MΦ in the colon LP and IEL fractions. Results are represented as values obtained from individual mice and mean values for each dietary group are indicated by crossbars (n=5-7 mice per group, one-way ANOVA with Tukey's post-hoc test).

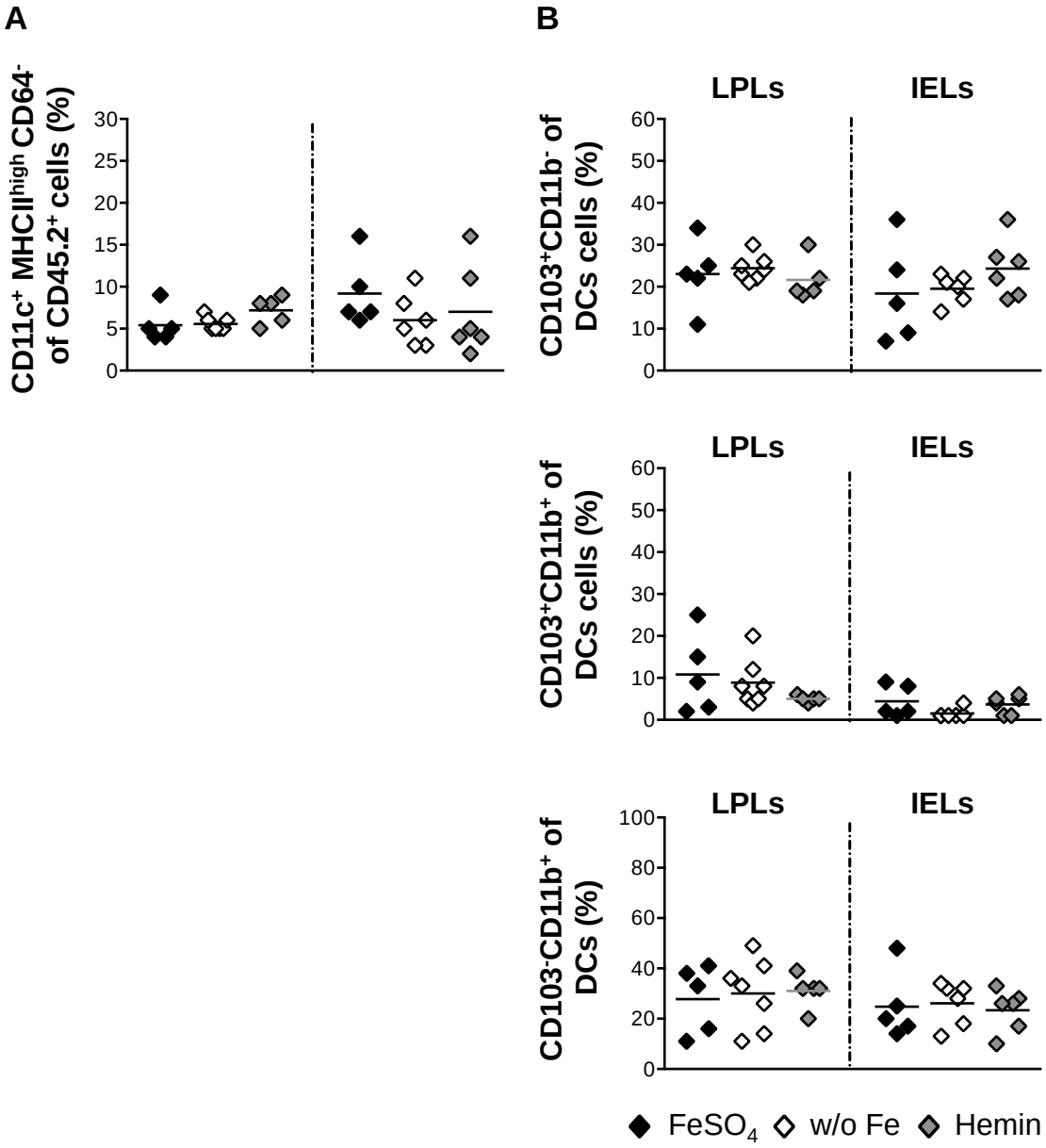

Figure 25 Effect of the extended luminal iron depletion on the colonic DC compartment after chronic experimental colitis development

Colitis was induced by T cell transfer in *Rag1*$^{-/-}$ mice after 9-week dietary treatment. Colon LPL and IEL cells were isolated at day 17 after T cell transfer and colon DCs were analysed by flow cytometry. Percentages of **(A)** CD64^{-}CD11c^{+}MHCII^{high} DCs within live-gated CD45.2^{+} cells and **(B)** DC subsets, based on CD103 and CD11b expression, within colon DCs in LPL and IEL fractions. Dots show values obtained from individual mice and crossbars indicate mean values for each dietary group (n=5-7) (One-way ANOVA with Tukey's post-hoc test.

4.5. Effect of luminal iron depletion on T cell activation in T cell transfer colitis model

As mentioned before, in our study chronic intestinal inflammation was induced by adoptive transfer of WT $CD4^+CD62L^+$ T cells into lymphopenic *Rag1*$^{-/-}$ mice lacking mature B and T lymphocytes as published by Wirtz *et al* (Mudter, Wirtz et al. 2002). Therefore, it was important to explore the effect of luminal iron depletion on T cell numbers in the colon and T cell mediated secretion of proinflamammatory cytokines IFN-γ and IL-17.

After 9-week dietary treatment, T cell mediated colitis was induced and $CD3^+CD4^+$ T cell infiltration in spleen, mLNs, colon LPL and IEL fraction was analysed at day 17 after transfer. Along the line of previous results, no major difference in the recruitment of $CD3^+CD4^+$ T cells in any of the indicated organs was detected between dietary groups Figure 26 **A** and **B**).

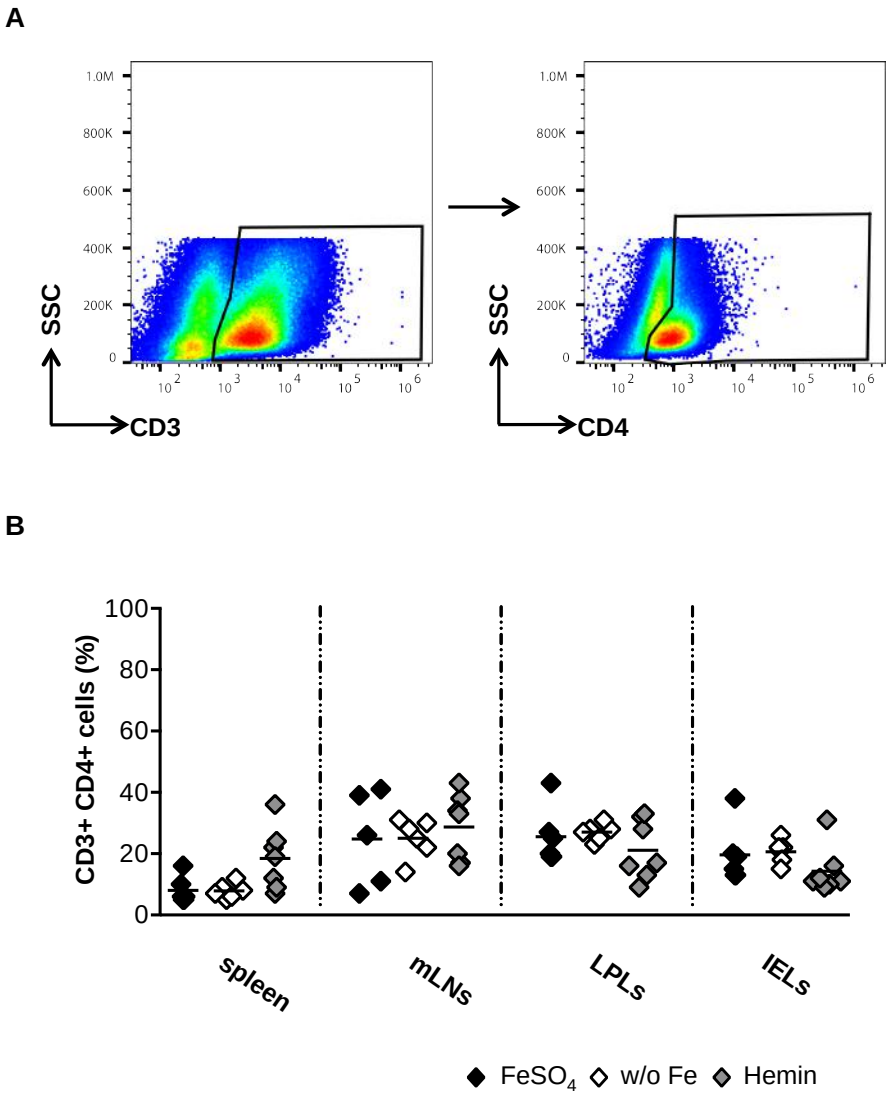

Figure 26 Effect of luminal iron depletion on the T cell infiltration after colitis development
Colitis was induced by T cell transfer in *Rag1*$^{-/-}$ mice treated with indicated experimental diets for 9 weeks. At day 17 after T cell transfer cells isolated from the spleen, mLNs, colon LPL and IEL fraction were stained for T cell membrane markers CD3e and CD4 and the T cell infiltration in indicated organs was analysed by flow cytometry. (A) Dot plots showing representative staining of colon LP T cells. (B) Graph showing frequencies of $CD3^+CD4^+$ T cells in the indicated organs. Dots represent data obtained from individual mice and crossbars indicate mean values (n=6-7, one-way ANOVA with Tukey's posthoc test).

It is well established that regulatory T cells (T_{Regs}) play an important role in the intestinal homeostasis by suppression of proinflammatory T_H1 and T_H17 responses. In addition, cotransfer of 7-10 % $Foxp3^+$ T_{Regs} ameliorated T_H1 mediated colitis development induced by adoptive transfer of $CD4^+CD62L^+$ T cells (Chen, Jin et al. 2003, Fantini, Becker et al. 2006). As shown in the Figure 4**B**, a fraction of about 7 – 10 % of $Foxp3^+$ T_{Reg} cells was detected within $CD4^+CD62L^+$ MACS sorted T cells that were transferred in *Rag1*$^{-/-}$ mice fed with the indicated experimental diets.

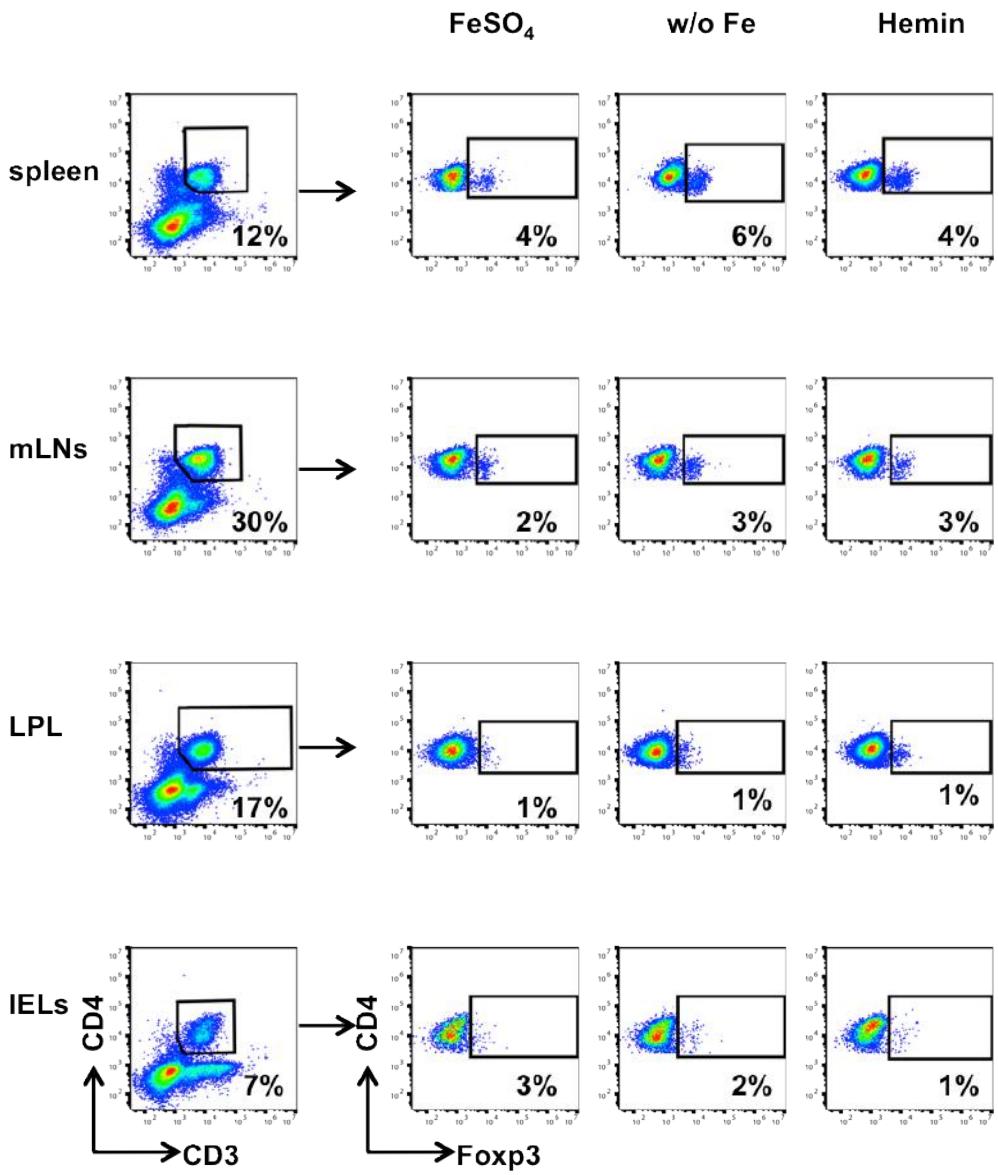

Figure 27 Effect of luminal iron depletion on T_{Reg} recruitment after T cell colitis development
After 9 weeks of treatment with indicated experimental diets, colitis was induced by transfer of $3*10^5$ $CD4^+CD62L^+$ T cells containing 7% $Foxp3^+$ Tregs. On day 17 after T cell transfer, cells from spleen, mLNs and colon LPL and IEL fractions were stained with CD3e and CD4 followed by intracellular staining against Foxp3 and the T_{reg} cell infiltration in indicated organs was analysed by FACs. Representative dot plots of intracellular Foxp3 expression in T cells in spleen, mLNs and colon LPL and IEL fraction of indicated dietary groups. Numbers indicate percentages of $CD3^+CD4^+$ T cells and $Foxp3^+$ T_{regs} determined by FACS analysis (n=3).

The frequencies of $Foxp3^+$ T_{Regs} in spleen, mLNs and colon LP and IEL fractions were analysed 17 days after transfer and no difference was found in any of indicated organs between iron depleted and iron adequate diets as shown in the Figure 27.

To investigate the role of luminal iron depletion on T cell activation, direct analysis of T cells *in vivo* was performed. At day 17 after T cell transfer, cells isolated from spleen, mLNs and the colon LP fraction were stimulated with PMA and Ionomycin and IFN-γ and IL17A

secretion by T cells was analysed. Apart from the increased IFN-γ T cell secretion seen in mLNs of the $FeSO_4$ treated group (IFN-γ^+: w/o Fe 25.6 ± 6.8 vs. $FeSO_4$ 46.5 ± 6.8 vs. hemin 24.4 ± 6.8, n=3) no differences could be detected in T_H1/T_h17 differentiation between treatment groups (Figure 28).

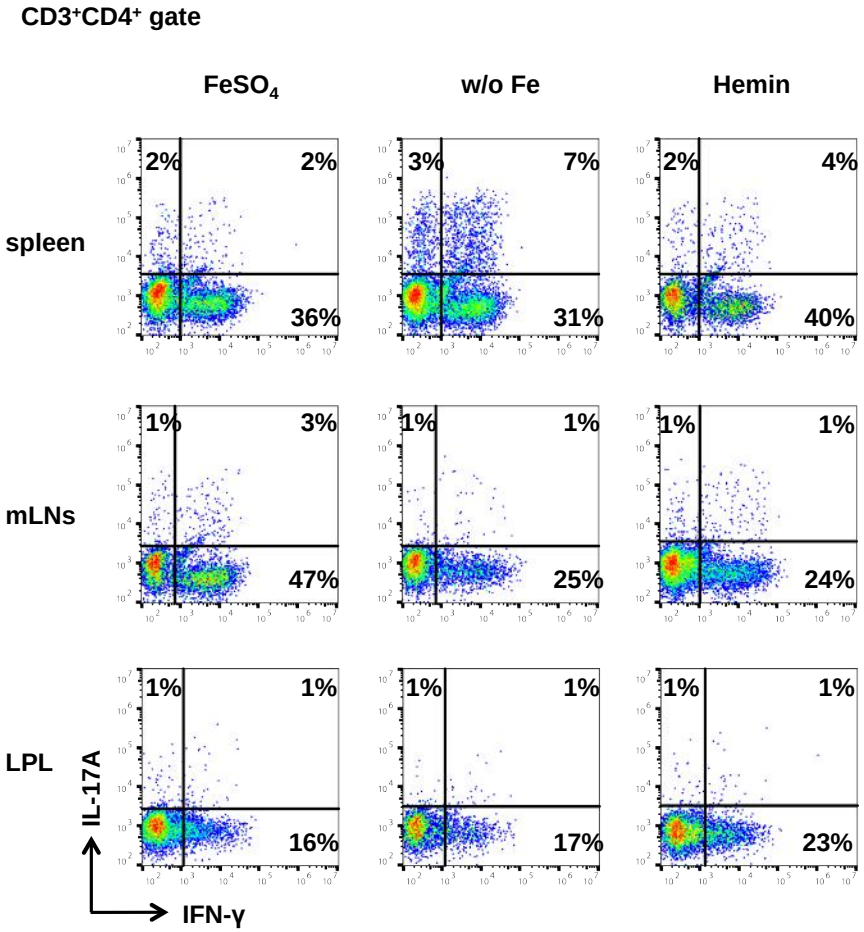

Figure 28 Effect of luminal iron depletion on IFN-γ and IL-17A secretion by T cells after colitis development
Rag1$^{-/-}$ mice were treated with indicated diets for 9 weeks followed by T cell mediated colitis induction. Cells from spleen, mLNs and colon LP were isolated on day 17 and stimulated with PMA/Ionomycin for 6 h (as described in Material & Methods section 3.2.8.). After stimulation cells were stained for membrane bound T cell markers CD3 and CD4 followed by intracellular staining for cytokines IFN-γ and IL-17A. FACS plots show expression of IFN-γ and IL-17A within CD4/CD3 double positive T cells in the indicated organs. Percentages given in quadrants are representative for the indicated groups (n=3).

Taken together, as seen for macrophages and DCs, luminal iron depletion had no influence on $CD3^+CD4^+$ T cell recruitment or their activation during T cell induced experimental colitis.

4.6. Luminal iron depletion fails to protect against DSS induced colitis

Several studies have demonstrated that dietary iron overload may acerbate intestinal inflammation in chemically induced colitis models (Uritski, Barshack et al. 2004, Chua, Klopcic et al. 2013). Therefore, I wanted to explore if reduction in dietary iron could have a beneficial effect on the development of acute inflammation during DSS induced colitis. To assess this, the optimal DSS dose to be used in dietary experiments was determined. According to the bodyweight development and clinical scoring (data not shown) 3% DSS was used to induce mild to moderately severe colitis, which would allow the identification of potential protective effect of luminal iron depletion. Mice were first given iron-depleted or iron adequate experimental diets ($FeSO_4$ and hemin) for 9 weeks and then exposed to 3% DSS for 5 days.

Only three days after DSS administration, a striking bodyweight loss accompanied by increased disease activity accompanied by severe diarrhea and rectal bleeding was observed in WT mice treated with the hemin diet. By day 6 after DSS administration four out of five mice died due to the severe inflammation (Figure 29). Since this phenomenon was not observed in other two groups, hemin dietary group had to be excluded from further analysis and the effect of luminal iron depletion was analysed by comparison of the iron depleted and the $FeSO_4$ supplemented group. In order to investigate a potential link between dietary hemin to increased susceptibility to colitis development, further experiments are necessary.

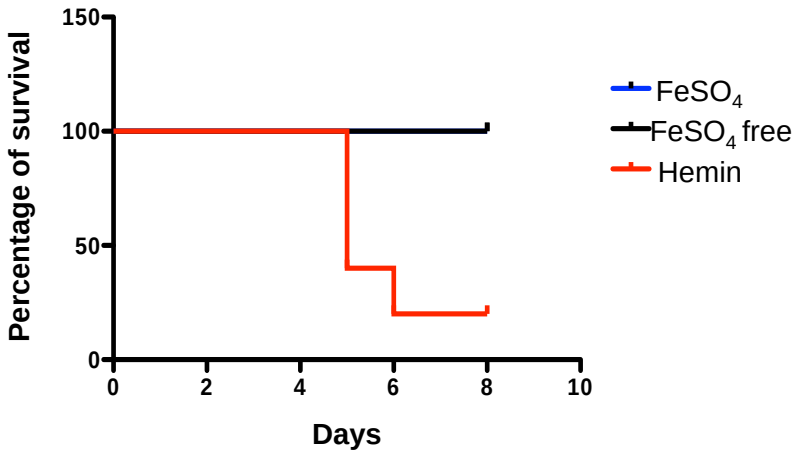

Figure 29 Survival curve during DSS induced colitis development in WT mice treated with experimental diets
WT (C57BL/6) mice were treated with 3% DSS in the drinking water for 5 days followed by 3 days of water alone. By day 8 all the mice were sacrificed. The graph showing 8-day survival curve of WT mice treated with indicated experimental diets. Survival analysis were performed by computing pair-wise comparison between the groups using the nonparametric Gehan-Breslow-Wlcoxon test and the *P* values were adjusted using Bonferroni method for multiple comparisons ($P < 0.02$)

To analyse, if luminal iron depletion has an impact on the severity of DSS induced colitis, bodyweight loss, clinical scores and colon lengths were measured in the WT mice treated with iron depleted and $FeSO_4$ containing diet. As shown in Figure 30**A** luminal iron depletion did not protect WT mice against acute colitis development since more then 50% of the mice in both groups lost between 15 – 20 % of their starting bodyweight by day 8 after start of DSS administration, which was accompanied by an increase in disease activity **(**Figure 30**B**). Luminal iron deprivation had no effect on WBC counts and colon lengths were found to be similar between two dietary groups (Figure 30**C** and **D)**. As shown in the Figure 30**E** and **F** extensive ulceration and epithelial damage together with leukocyte infiltration were observed in both dietary groups and evaluated by histoscoring.

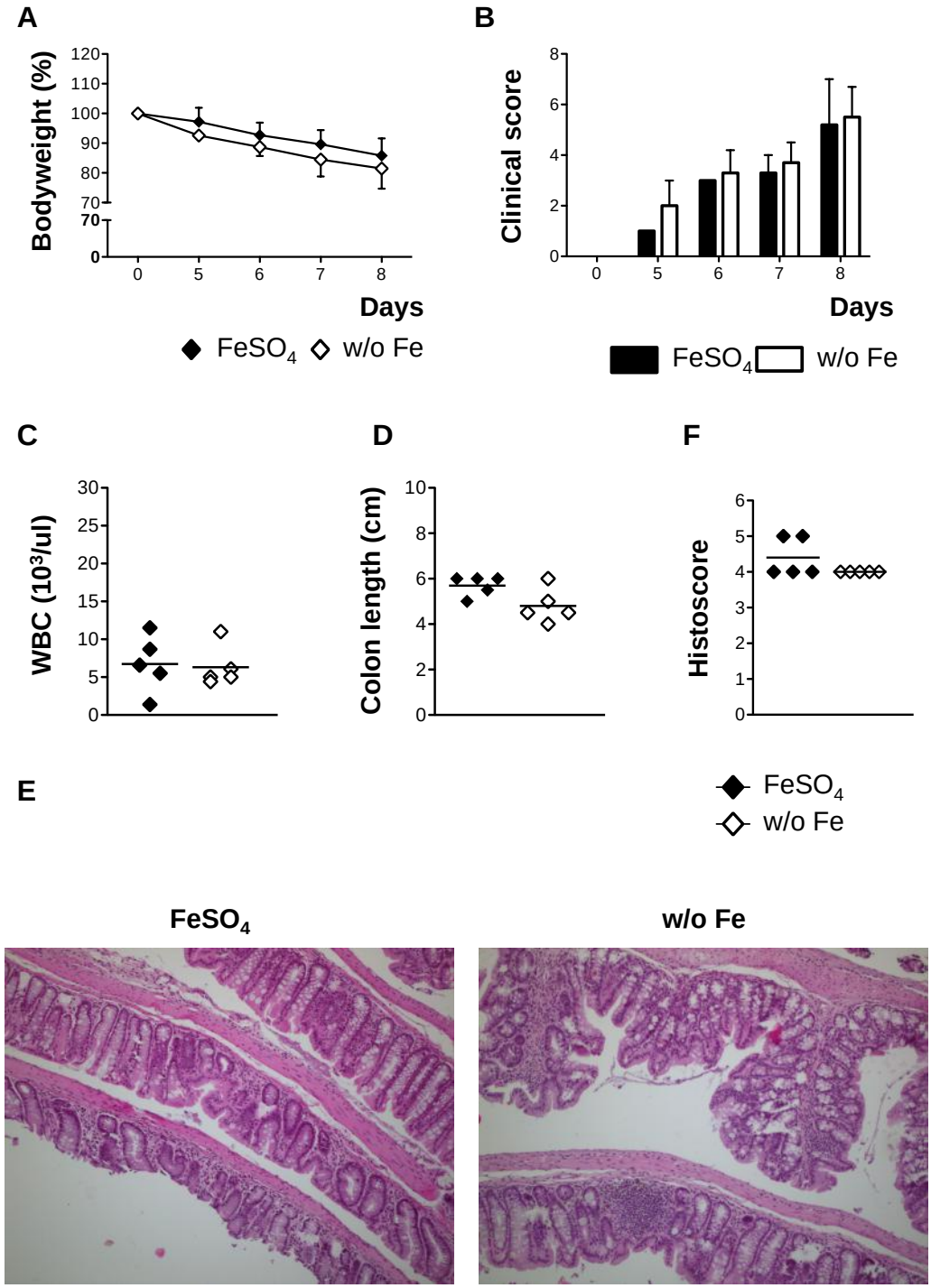

Figure 30 Effect of luminal iron depletion on DSS colitis development in WT mice
After being treated with iron adequate ($FeSO_4$) or depleted (w/o Fe) experimental diets for 9 weeks, wild type (WT) were given 3% DSS dissolved in drinking water for 5 days followed by normal water. The mice were sacrificed on day 8. (A) Bodyweights and (B) clinical score (as described in Material & Methods section 3.2.2.) records on day 0 before DSS treatment and on day 5,6,7 and 8 after starting DSS treatment. Mean values ± SD are shown for each group. (n=5) (C) White blood cell counts (WBC) from individual mice on day 8 (n=5). (D) Representative pictures of H&E stained paraffin embedded colon sections and (F) histoscore of mice treated with indicated experimental diets (n=5 mice per group, unpaired t test or Mann-Whitney non-paramteric test). Date of one of two experiments (5 mice per group) with similar results are shown.

To explore the effect of luminal iron modulation on the colon immune system, cells from mLNs and colon LPL and IEL fraction were isolated at day 8 after DSS administration and analysed by flow cytometry. As expected, luminal iron depletion had no influence on leukocyte infiltration in mLNs and colon during acute inflammation (5 mice per group, data not shown).

The same gating strategy used for characterization of colonic macrophages and DCs in T cell transfer experiments, was also used in the DSS model. Only minor changes have been implemented in order to enable precise discrimination between $CD64^{+}CD11b^{+}$ MΦ and $CD64^{-}CD11c^{high}MHCII^{high}$ DCs **(**Figure 31 and Figure 32). There was no significant difference in the percentages of $CD64^{+}CD11b^{+}$ MΦ and $CD64^{-}CD11c^{high}MHCII^{high}$ DCs in mLNs and colon LPL and IEL between iron depleted and $FeSO_4$ treatment group. Additionally, reduction in dietary iron content had no effect on subset distribution within the intestinal MΦ and DC compartment (Table 7).

Taken together, the results presented here indicate that dietary iron depletion did not suppress intestinal inflammation development in the acute DSS mediated colitis model, which was supported by comparable bodyweight loss, clinical and histoscores and colon immune cell composition.

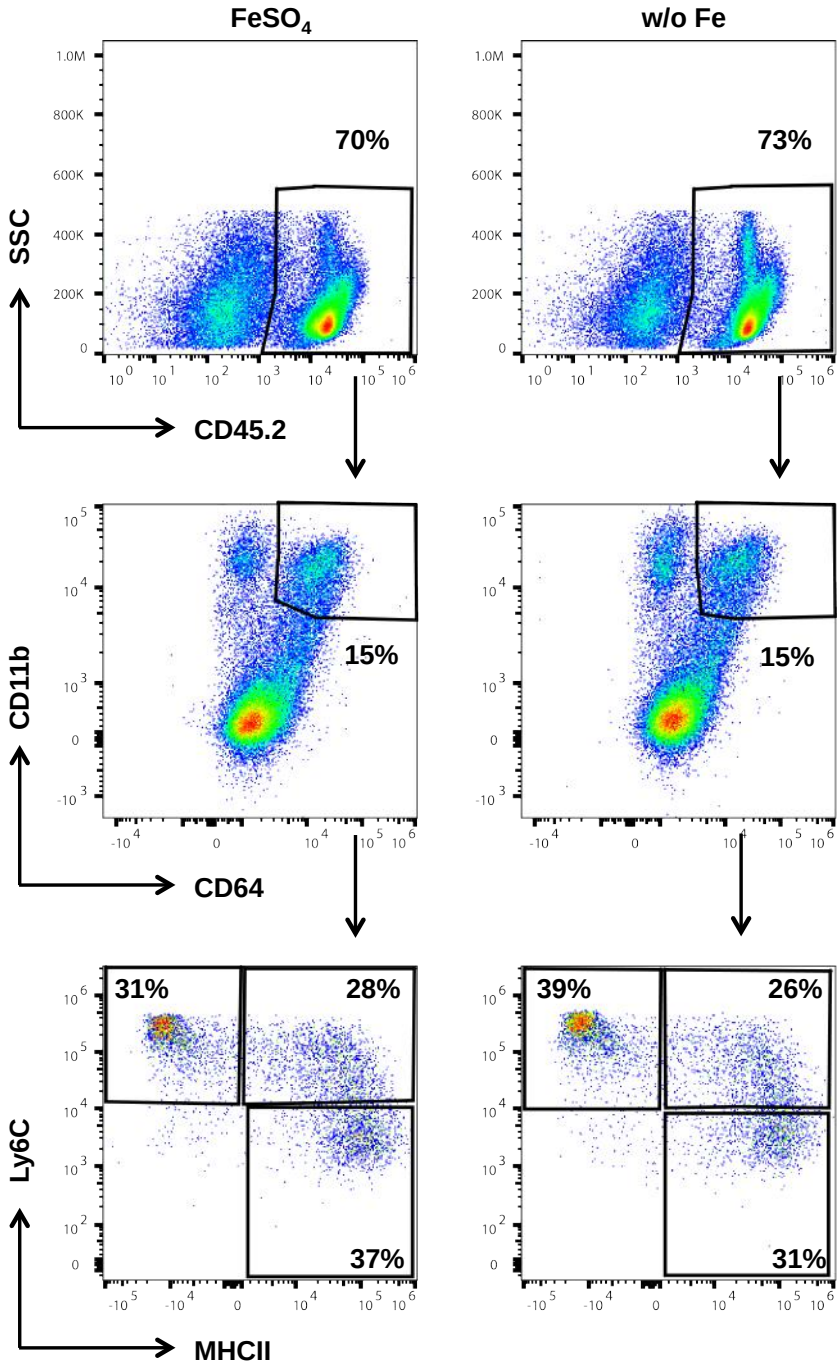

Figure 31 Characterization of colonic MΦ after luminal iron depletion and DSS colitis development
Representative FACS plots showing adjusted gating strategy for colon LP macrophages isolated from WT mice after 9-week diet treatment followed by DSS induced colitis development (n=5).

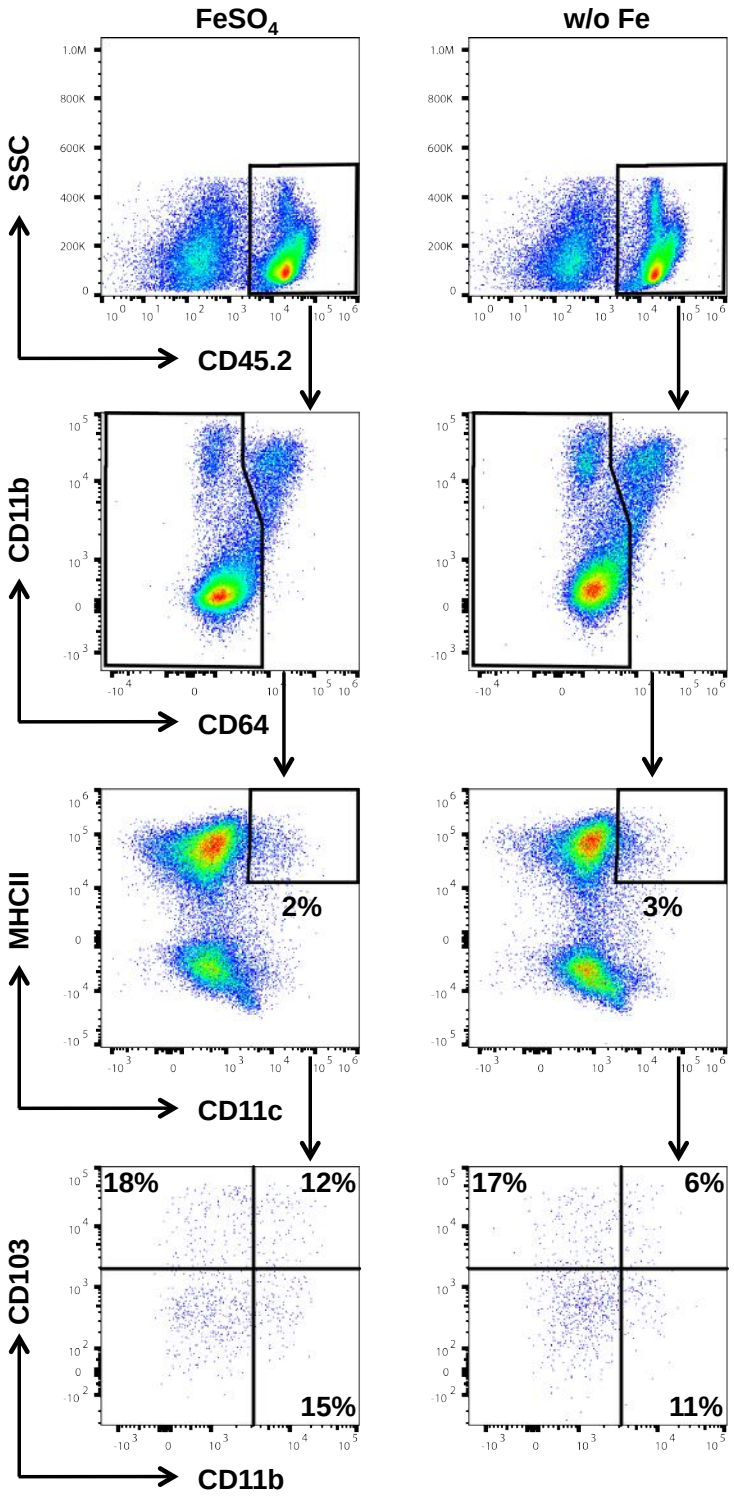

Figure 32 Characterisation of colonic DCs after luminal iron depletion and DSS mediated colitis development

Representative FACS plots showing adjusted gating strategy for colon LP DCs isolated from WT mice after 9-week diet treatment followed by DSS induced colitis (n=5 mice per group).

Diet	$CD64^+CD11b^+M\Phi$ of $CD45^+$ cells (%)	$Ly6C^{low}MHCII^+$ of MΦ (%)	$Ly6C^{high}MHCII^+$ of MΦ (%)	$Ly6C^{lhigh}MHCII^-$ of MΦ (%)	
$FeSO_4$	12.0 ± 2.6	28.0 ± 2.9	32.8 ± 3.5	32.6 ± 4.0	LP fraction
w/o Fe	13.0 ± 4.0	23.2 ± 5.5	30.4 ± 6.1	38.8 ± 6.5	
$FeSO_4$	9.6 ± 1.7	26.4 ± 3.9	34.0 ± 6.0	33.8 ± 7.0	IEL fraction
w/o Fe	9.8 ± 4.0	24.4 ± 7.4	28.6 ± 10.0	31.2 ± 7.0	
Diet	$CD64^-$ $CD11c^+MHCII^{high}$ DCs of $CD45^+$ cells (%)	$CD103^+CD11b^-$ of DCs (%)	$CD103^+CD11b^+$ of DCs (%)	$CD103^-CD11b^+$ of DCs (%)	
$FeSO_4$	2.6 ± 0.8	18.0 ± 2.2	11.2 ± 2.8	14.8 ± 1.5	LP fraction
w/o Fe	2.4 ± 1.1	14.2 ± 5.1	9.4 ± 5.1	16.6 ± 6.0	
$FeSO_4$	4.2 ± 1.6	11.8 ± 2.4	5.4 ± 2.2	10.4 ± 6.7	IEL fraction
w/o Fe	3.6 ± 1.1	17.2 ± 3.3	4.0 ± 2.0	9.4 ± 3.3	

Table 7 Effect of luminal iron depletion on mononuclear phagocytes after DSS colitis development
WT (C57BL/6) mice fed with $FeSO_4$ or iron-depleted experimental diets for 9 weeks, were treated with 3% DSS in the drinking water for 5 days followed by 3 days of water alone. By day 8 all the mice were sacrificed. Colon LPL and IEL cells were isolated and MΦ and DC compartments were analysed by flow cytometry. Data are presented as mean ± SD (unpaired t test, P=0.05) (n=5 mice per group).

5. DISCUSSION

There is a wide geographic variability in the prevalence of IBD, with highest incidence rates observed in North America and Western Europe in the last two decades (Cosnes, Gower-Rousseau et al. 2011). Over the last 20 years remarkable progress was achieved in our understanding of IBD. Together with well-defined genetic risks, environmental factors play an important role in the pathogenesis of IBD and are primarily responsible for its growing incidence around the globe (Molodecky and Kaplan 2010). The rising number of IBD cases in Western countries as well as in the developing nations of Eastern Europe and Asia is thought to be associated with "westernisation" of people`s lifestyle. Smoking, antibiotic treatments, dietary changes and hygiene are some of the risk factors linked to the pathogenesis of the IBD (Lewis 2014). A "western diet" that is rich in refined grains, processed meat and convenience foods are supposed to be associated with a higher risk for IBD development. Several studies reported a positive association of high intake of dietary ingredients such as ω-6 polyunsaturated fatty acids, refined carbohydrates and gluten with the risk of IBD (Shah, Parian et al. 2015). Micronutrients such as vitamin D, selenium, folates, zinc and iron were shown to have an immunomodulatory role during intestinal inflammation (Barnett, Bermingham et al. 2010, Kudva, Shay et al. 2015, Nielsen, Ainsworth et al. 2015, O'Sullivan 2015, Shah, Parian et al. 2015).

The role of iron in the pathogenesis of the IBD is complex. IBD patients are commonly found to develop iron deficiency anemia secondary to chronic blood loss and impaired iron absorption in the inflamed tissue. Moreover, cytokines and acute phase proteins being induced upon inflammation impair iron availability for erythropoiesis and cause a blunted biological activity of erythropoietin, and an inflammation driven impairment of erythroid progenitor cell proliferation (Nielsen, Ainsworth et al. 2015). Furthermore, inflammatory cytokines can directly inhibit iron absorption and stimulate the uptake and retention of iron in macrophages via hepcidin-dependent/independent pathways (Weiss and Schett 2013). On the other hand, oral iron administration can cause side effects and aggravate inflammatory process in active stage of the disease in humans (Rizvi and Schoen 2011). The mechanistic basis for the association between intestinal inflammation and luminal iron content are not clear. It is well established that free iron can be cytotoxic when present in high concentration due to its high potential to generate oxidative radicals that damage proteins and nucleic acids, but weather these abnormalities in iron dependent biochemical reactions might change

intestinal immune cell function and composition and contribute to the inflammation during colitis, has not been investigated in depth. Rodent studies have demonstrated that dietary iron fortification leads to exacerbation of the intestinal inflammation in chemically induced colitis models while Werner et al. showed that iron sulphate free diet prevents development of chronic ileitis in the *TNF*$^{\Delta ARE}$ mouse models (Werner, Wagner et al. 2011).

The aim of my study was to elucidate the potential protective role of luminal iron depletion in T cell dependent and independent murine IBD models focusing on the influence of luminal iron on the innate immune responses and T cell activation. To assess this, the effects of iron depleted as well as iron sulphate and hemin supplemented diets were evaluated in immunodeficient *Rag1*$^{-/-}$ mice before and after colitis induction by T cell transfer. As dietary iron depletion was not successful in preventing T cell induced colitis, the protective capacity of luminal iron depletion was explored in DSS induced acute colitis model. The bodyweight loss, tissue histopathology and mRNA cytokine profiling clearly showed that dietary iron depletion did not affect colitis development in both models. In line with this, FACS analysis confirmed that luminal iron depletion had no major effect on the colonic immune system in steady state and inflammation.

5.1. Experimental mouse colitis models

Human IBD is defined as an inflammatory state caused by many factors and mouse models have some characteristics that closely resemble human UC and CD. According to the method of the induction, these models can be broadly divided into chemically induced colitis models, genetically modified models with spontaneous colitis development, infection models and T cell transfer models (Mizoguchi 2012).

The role of luminal iron has already been investigated in several chemically induced colitis models as well is in the Crohn`s disease like murine ileitis model (Chua, Klopcic et al. 2013) (Werner, Wagner et al. 2011). In present work, I investigated the preventive effect of dietary iron depletion on development of T cell transfer and DSS induced colitis models. Both models are well established and have been used in preclinical studies for testing the efficiency of drugs, which are now approved for treating IBD patients.

5.1.1. T cell transfer colitis model

T cell transfer induced colitis is the best-characterized model of colitis induced by disruption of T cell homeostasis. The most common model developed by Powrie *et al.* consists of adoptive transfer of naive $CD4^+CD45^{high}$ T cells, isolated by FACS sorting from immunocompetent mice into immunodeficient SCID or *Rag1*$^{-/-}$ or *Rag2*$^{-/-}$ mice. This leads to wasting disease and primarily colonic inflammation that develops 5-12 weeks after transfer, depending on the microbiota (Powrie, Coffman et al. 1994, Read and Powrie 2001, Uhlig, Coombes et al. 2006). This model was modified several times in regard to T cell subsets used for transfer and genetic background of the recipient mice. In this study, the $CD4^+CD62L^+$ T cell transfer model established by Wirtz et al. was used. The major advantage of this model is that it does not require time consuming and sorting of $CD4^+CD45RB^{high}$ T cells, which also improves survival and function of the T cells. Instead the isolation of $CD4^+CD62L^+$ T cells can be performed by immunomagnetic cell separation (Mudter, Wirtz et al. 2002). In comparison to the Powrie model, adoptive transfer of $CD4^+CD62L^+$ T cells leads to more rapid bodyweight loss. In our T cell transfer experiments, *Rag1*$^{-/-}$ mice developed severe colitis within 2.5 weeks after T cell transfer. The reason for accelerated colitis development might be in the gut-homing capacity of the $CD4^+CD62L^+$ transferred T cell population. Kurmaeva et al. showed that surface T cell receptor CD62L, together with $\alpha_4\beta_7$ integrin, is essential for colitogenic function of these cells because it facilitates homing of these cells into mLNs and colon LP via interactions with MadCam and PNAd that are expressed on high endothelial venules (Kurmaeva, Boktor et al. 2013). In addition Wirtz et al. showed that CD62L expression could be used as a marker for the $CD45RB^{high}$ population. Cotransfer of $CD4^+CD62L^-$ T cells confers the same protective effect as contransfer of $CD4^+CD45RB^{low}$ T cells in $CD4^+CD45RB^{high}$ T cell induced colitis (Mudter, Wirtz et al. 2002). In both models the protective ($CD62L^-$ or $CD45RB^{low}$) T cell fraction was identified as $CD25^+Foxp3^+$ regulatory T cells population (Mudter, Wirtz et al. 2002). The cotransfer of about 7-10 % of $Foxp3^+$ T_{Regs} within the $CD4^+CD62L^+$ population had no effect on the colitis development in the T cell transfer experiments demonstrated in this study. Although the percentage of the T_{Regs} within the transferred population was much higher in comparison to Powrie`s model, the percentage of $Foxp3^+$ T_{Regs} detected in colon LP was less then 1% which was not enough to induce protection. Furthermore, the presence of *Helicobacter hepaticus* in our mouse colony might exacerbate T cell mediated colitis development supporting the induction of proinflammatory T_H1/T_H17 cells.

The $CD4^+CD62L^+$ T cell transfer into *Rag1*$^{-/-}$ mice induced colitis in predictable and reproducible manner in the experiments conducted in this study with regard to the kinetic of bodyweight loss and colitis severity. Therefore it represents a suitable model to study the effect of luminal iron on intestinal inflammation.

5.1.2. Dextran sodium sulphate (DSS) induced colitis

DSS is a water-soluble chemical colitogen, which when administered in the drinking water induces colitis resembling the acute phase of human UC. Today there are numerous studies using DSS-induced colitis model to investigate pathogenesis of colitis and different factors affecting colitis. The mechanism by which DSS induces colitis most likely involves damage of the epithelial monolayer allowing excessive stimulation of underlying innate immune cells by bacteria and food components (Chassaing, Aitken et al. 2014). The mechanisms of how DSS passes through the mucosal epithelial cells remains unclear, but it has been shown that the impairment of the epithelial barrier function is associated with loss of the tight junction proteins such as occludin, ZO-1 and claudins within first three days of DSS treatment (Poritz, Garver et al. 2007, Mennigen, Nolte et al. 2009, Ichikawa-Tomikawa, Sugimoto et al. 2011). Because of cytotoxicity of DSS, both increased apoptosis and decreased proliferation of the epithelium due to the ER stress take place during acute DSS colitis (Araki, Mukaisyo et al. 2010). Indeed, excessive ER stress in IECs was demonstrated to accelerate inflammatory pathways such as NF-κB cascade or induce IECs apoptosis (Araki, Mukaisyo et al. 2010, Choi, Koh et al. 2015). In addition, DSS-mediated colitis is associated with the upregulation of various cytokines, chemokines, nitric oxide and inducible nitric oxide synthase (iNOS). Increased expression of TNF-α, IL-1β, IFN-γ, IL-17 and Il-12 was observed already after 1 day of DSS treatment and the production of these cytokines increased progressively during DSS colitis (Yan, Kolachala et al. 2009). In addition, distinct cytokine profiles were observed between acute and chronic state of DSS colitis switching from the T_H1/T_H17 mediated acute inflammation to predominantly T_H2 inflammatory responses (increased IL-4 and IL-10) during chronic phase (Alex, Zachos et al. 2009).

Since it has already been shown by others that 10 – 100 fold dietary iron fortification enhances intestinal inflammation during DSS induced colitis in rats and mice (Reifen, Matas et al. 2000, Aghdassi, Carrier et al. 2001, Carrier, Aghdassi et al. 2001, Carrier, Aghdassi et al. 2002, Seril, Liao et al. 2002), we hypothesised that luminal iron depletion most likely will have a protective effect against colitis development in this model. In addition, unlike human

disease, T and B cells are not required for colitis development in response to DSS and therefore this model is suitable for studying the role of iron on the composition of colonic innate immune cells primarily macrophages and DCs.

The successful and reproducible induction of DSS-induced colitis depends on numerous factors, including DSS source, lot number, molecular weight, concentration, duration, mouse strains as well as environmental factors including the hygienic condition in the animal facility (Chassaing, Aitken et al. 2014). For example iron fortification increased susceptibility to DSS colitis in C57BL/6 mice after administration of 1% (w/v) DSS for 7 days in drinking water(Seril, Liao et al. 2002). To apply this model in the dietary experiments, concentration and the time of the exposure had to be established. After several trial experiments, administration of 3% (w/v) DSS for 5 days led to development of moderate to severe inflammation and by day 8 after DSS application, C57BL/6 mice fed with $FeSO_4$ supplemented experimental diet had to be sacrificed. Similar kinetics of colitis development was observed in WI mice fed with standard chow suggesting that experimental diet itself showed no effect on colitis susceptibility in WT mice.

Using the DSS colitis model, I was able to evaluate the role of dietary iron depletion in prevention of acute colitis development in comparison to normal iron content found in standard chow.

5.2. The effect of luminal iron depletion in mouse experimental colitis models

Concern has been raised that oral/luminal iron could exacerbate intestinal inflammation in IBD. Many physicians avoid the use of oral iron replacement therapy in IBD patients with active disease for this reason and because oral iron is less well tolerated and less effective than i.v. iron in active IBD with anemia. However the available literature provides no clear evidence from clinical trials to link oral iron supplementation or iron-enriched diet to the risk of IBD development or exacerbation of the disease. Interestingly, in a large trial on iron fortification in African children (without IBD diagnosis) increased number of enterobacteria and decreased number of lactobacilli correlated with higher concentration of fecal calprotectin, a marker of gut inflammation, after 6 month of iron fortified diet (Zimmermann,

Chassard et al. 2010). Findings from recent animal studies clearly demonstrated that oral iron could increase the oxidative stress leading to excessive intestinal inflammation. Furthermore oral iron supplementation induces changes in microbiota (Zimmermann, Chassard et al. 2010, Dostal, Lacroix et al. 2014). Majority of these studies were done in rats using chemically induced colitis models. For example, Carrier et al. demonstrated in their studies that increased disease activity, clinical scores and oxidative stress parameters were observed during DSS induced colitis in rats when fed with nonpurified diet containing 3000 – 30 000 mg iron/kg in form of iron sulphate. In the same study the rats fed with same type of diet containing 270 mg iron/kg developed mild colitis but with significantly lower clinical score (Carrier, Aghdassi et al. 2001, Carrier, Aghdassi et al. 2002). In DNBS induced colitis, rats fed with iron-deprived diet containing 50 mg iron/kg developed less mucosal damage while dietary iron supplementation with 1700 mg iron /kg worsened the disease activity in comparison to standard chow (200 mg iron/kg). Although it was not clear from the study weather the deprivation of dietary iron protected rats from DNBS induced colitis, the observed decrease in the level of tissue damage suggests that the reduction of oral iron might have protective effect against colitis development (Barollo, D'Inca et al. 2005). Recent clinical trials suggested that lower doses of oral iron (60 mg iron per day) reduced the side effects in IBD patients (Rizvi and Schoen 2011). In addition, Werner et al. showed that depletion of luminal iron was successful in prevention of ileitis in $TNF^{\Delta ARE}$ mice. In the $TNF^{\Delta ARE}$ model iron-depleted diet implicated changes in gut microbiota and reduced ER stress in IECs (Werner, Wagner et al. 2011).

To investigate the potential protective role of luminal iron depletion in T cell transfer and DSS induced colitis models, the dietary protocol published by Werner *et al.* was adjusted and used for dietary treatment of $Rag1^{-/-}$ and C57BL/6 mice followed by colitis induction by T cell transfer or DSS. In both colitis models standard chow was gradually replaced by experimental diets during 2 week long adaptation phase because of the high sensitivity of both mouse strains to the dietary change. The reason for poor tolerance of experimental diets is currently not clear. Other experimental diets such as high fat diet can also be poorly tolerated by some mouse strains. Dietary treatments in steady state $Rag1^{-/-}$ mice clearly showed no effect of dietary intervention on the bodyweight development and iron status when compared to standard chow.

In contrast to previous findings, we showed that *Rag1*$^{-/-}$ mice fed with iron depleted or iron adequate diets ($FeSO_4$ or hemin) develop severe colitis when transferred with $CD4^+CD62L^+$ T cell regardless of the iron content in the diet and the time period of dietary iron depletion. The colitis development in all three dietary groups was confirmed by bodyweight loss and clinical scoring and later evaluated by histoscoring and cytokine profiling at mRNA level. The disease activity, monitored by clinical assessment of the mice, was similar between iron-depleted and iron adequate groups which was opposite to the previous findings by others demonstrating no or milder disease development in mice fed with low iron diet (< 50mg Fe /kg) in both chemical (DSS, TNBS) and genetically predisposed murine IBD models (*TNF*$^{\Delta ARE}$, *Il-10*$^{-/-}$ etc.). Thus, dietary iron deprivation did not prevent T cell mediated colitis onset nor had impact on the colitis development since mice on iron-depleted diet did not recover after losing 15-20% of their starting weight.

Enhanced colonic IL-6, IL-1β and IFN-γ production was shown in DSS induced colitis model after oral iron supplementation that was correlated with the increased risk of CRC development (Seril, Liao et al. 2002). It has been showed by Oldenburg *et al.* that oral and rectal iron administration affected proinflammatory cytokine (IL-1, IL-6, TNF-α and IFN-γ) production in colon mucosa of *Il-10*$^{-/-}$ mice that are genetically predisposed to develop a chronic intestinal inflammation (Oldenburg, van Berge Henegouwen et al. 2000). My results show that the mRNA expression of the pronflammatory cytokines IL-6, IL-12p35 and TNF-α was shown to be upregulated in the colon tissue of the *Rag1*$^{-/-}$ after T cell transfer. Iron sulphate supplemented diet induced an upregulation of TNF-α during T cell transfer colitis development. On the other hand, dietary iron content did not affect mRNA levels of IL-12p35 and IL-6 cytokines. The expression level of IL-10, a cytokine that was shown to have a protective effect in T cell transfer model (Murai, Turovskaya et al. 2009), was not affected by dietary iron. High levels of IFN-γ and IL-17A found in inflamed colon tissue of the *Rag1*$^{-/-}$ were in the line with previous findings showing that T_H1/T_H17 cells drive intestinal inflammation in the T cell transfer model. Indeed, the increased frequencies of IFN-γ and IL17-A producing T cells were found in the spleen, mLNs and colon LP of *Rag1*$^{-/-}$ regardless of the iron content in the experimental diet they received.

Therefore, dietary iron deprivation did not protect *Rag1*$^{-/-}$ mice from colitis development after $CD4^+CD62L^+$ T cell transfer. The reason for this is not clear. Based on the results from the *TNF*$^{\Delta ARE}$ ileitis model, we would have expected reduced colitis activity (Werner, Wagner et

al. 2011) However, it is known that $CD4^+CD62L^+$ T cell mediated chronic inflammation results in very rapid bodyweight loss within only 2-3 weeks. It is possible that we would have seen the protective effect of luminal iron depleted diet in a colitis model which develops less rapidly (e.g. transfer of $CD4^+CD45RB^{high}$ cells (Powrie, Coffman et al. 1994)) or genetically predisposed models that are more dependent on innate immune responses or ER stress. However, the $CD4^+CD62L^+$ T cell transfer colitis model showing similar kinetics has already been used by Heiseke et al. to elucidate the role of DC specific CCL17 chemokine on colitis development, demonstrating that this is a valid model to study IBD pathophysiology (Heiseke, Faul et al. 2012). In case of genetically predisposed colitis models (e.g. *Stat3LyMCre*$^{-/-}$, TRUC etc.) with slow development of intestinal inflammation, dietary iron depletion would have to be prolonged which might contribute to development of systemic anemia and therefore systemic iron replacement would be required as shown by Werner *et al.* in *TNF*$^{\Delta ARE}$ ileitis model (Werner, Wagner et al. 2011).

Recent studies have shown that 10-100 fold fortification of dietary iron leads to enhancement of inflammation during DSS induced colitis (Seril, Liao et al. 2002). As demonstrated in present study, C57BL/6 mice developed severe colitis regardless of dietary iron content after exposure to 3% DSS. Interestingly, mice fed with hemin containing diet showed higher susceptibility to colitis induction since more then 80% of the mice developed severe diarrhoea with visible rectal bleeding that resulted in rapid bodyweight loss in the first four days of DSS treatment and therefore this group was excluded from further analysis. This was surprising since Zhong *et al.* showed that intraperitoneal injection of hemin upregulated HO-1 that in turn induced expansion of $CD4^+CD25^+Foxp3^+$ T_{reg} that was correlated with attenuation of DSS induced colitis (Zhong, Xia et al. 2010). Hemin supplementation of experimental diet represents a model of `Western diet` in the present study. Red meat is one of the main components of Western diet, which has already been linked to colorectal cancer. Heme iron in the red meat was suggested to be one of the potential triggers for cancer development since the link between CRC and white meat that is poor in heme iron is much lower (Oostindjer, Alexander et al. 2014). Indeed, when rats received diet supplemented with hemin, increased levels of ROS in fecal water were observed leading to the oxidative stress in mucosa, which was shown to correlate with epithelial proliferation and thus the risk for colon cancer development (Sesink, Termont et al. 1999). Ijssennagger et al. showed that dietary heme induced ROS production and lipid perroxidation represent a acute effects of heme that are observed already within first 2 days of heme diet treatment while epithelial hyperproliferation

and hyperplasia are delayed and occur after 4 days of the dietary treatment (Ijssennagger, Rijnierse et al. 2013). Thus, dietary hemin may have increased the ROS production and activated ER stress responses in the IECs leading to higher sensitivity of the epithelium monolayer to the cytotoxic effect of DSS in our acute model. Whether iron or other heme metabolites are responsible for the cytotoxic effect of heme in the intestinal epithelium is not clear. However, Werner *et al*. demonstrated that $FeSO_4$ containing diet activated oxidative and ER stress responses in IECs leading to increased cytotoxic T cell mediated epithelial apoptosis in $TNF^{\Delta ARE}$ CD like ileitis model (Werner, Wagner et al. 2011). Beside the toxic effect of heme on intestinal epithelium, heme-induced cytotoxicity and hyperproliferation is dependent on sulfid producing and mucus degrading members of gut microbiota that degrade protective mucus barrier and facilitate the internalization of cytotoxic heme factors (CHF) that are formed by binding dietary heme to peroxidized lipids(Ijssennagger, Belzer et al. 2015). Therefore to verify the observed proinflammatory effect of dietary hemin in colitis development further experiments are required.

To evaluate the protective effect of dietary iron depletion in the DSS colitis model the iron sulfate dietary group was compared to the iron-depleted group. The data presented in this study clearly show that dietary iron depletion failed to protect mice against DSS induced acute colitis development. Indeed, no difference could be found in bodyweight loss and clinical scoring. Dietary iron depletion had no effect on histological signs of colitis and cytokine mRNA expressionin colon tissue. There are several factors that should be considered when interpreting these findings in context of the published literature. At first, the majority of published studies suggesting a proinflammatory effect of dietary iron supplementation during DSS induced colitis were performed in rats. Only few studies used mouse models for this purpose and mice were fed with iron-supplemented diet containing 10-100 fold higher iron content compared to standard chow which may have caused the enhanced ROS generation and ER stress induction leading to the increase in disease activity after iron supplementation (Rizvi and Schoen 2011). In the experiments presented here 3% DSS administration for 5 days was used since this experimental protocol resulted in rapid and reproducible bodyweight loss under our housing conditions and experimental diet treatment. In the published studies that performed similar experiments, 1-2% DSS was used and time period of administration varied from 5-7 days (Seril, Liao et al. 2002). In contrast, 1-2% DSS given to C57BL/6 mice in our animal facility caused insignificant bodyweight loss (5% of starting bodyweight) and mice failed to develop colon inflammation. Therefore, a higher DSS dose was used to obtain

reliable colitis induction. The acute DSS model with similar kinetic as ours has been used in many studies to investigate the functions of the innate immune system, epithelial barrier function and tissue regeneration. It is therefore a valid model to study the role of luminal iron in acute colitis and we found no effect of dietary iron content on colitis development.

The dietary system used in present study was previously published by Werner et al. and only the time period of the dietary treatment was adapted to our models(Werner, Wagner et al. 2011). In their study, Werner et al. showed that 11 week long dietary iron deprivation protected $TNF^{\Delta ARE}$ mice from CD like ileitis while in our case 2 week shorter treatment with the same diet failed to prevent T cell dependent/independent colitis. Since $TNF^{\Delta ARE}$ model bears a histopathology resembling that of human CD and mice develop $CD8^+$ T cell dependent transmural inflammation in terminal ileum (Apostolaki, Manoloukos et al. 2008), we speculate that differences in the iron absorption, microbiota composition and immune cell compositon between SI and colon may be the reason why we were not able to observe the protective effect of luminal iron depletion in our colitis models. First, luminal iron absorption in colon has been estimated to only 14% of the iron absorption in SI (Blachier, Vaugelade et al. 2007), which minimizes the chance of direct effect of luminal iron on IECs and immune cells. Second, SI and colon harbour distinct niches, each containing a different microbial ecosystem that varies according to the location within the GI tract, which is already demonstrated by the fact that the microbial density is higher in colon then in SI (Booijink, Zoetendal et al. 2007). While the knowledge about colon microbiota is well established, there is only limited insight into SI microbiota composition that is shown to be highly influenced by gastric acid, bile and pancreatic secretions entering SI in the duodenum (Booijink, Zoetendal et al. 2007). Furthermore, the products of food enzymatic digestion are mainly absorbed in jejunum and ileum. The findings emerging from studies with ileal biopsies collected during surgical intervention demonstrated that the composition of the microbiota in ileostomy effluent is less diverse and less stable from that of the faecal microbiota (Booijink, El-Aidy et al. 2010). Therefore, the effect of $FeSO_4$ derived iron on the composition of the microbiota might differ along the GI, which may be one of the reasons for distinct effects of luminal iron on inflammation development between SI and colon. In the end, the differences in anatomy between small intestine and colon primarily in thickness and permeability of mucus layer, as well as compositon of IECs and immune cells underlying intestinal epithelium may be the potential factors that are responsible for distinct effects of luminal iron in the $TNF^{\Delta ARE}$ model and in T cell transfer and DSS colitis models (Mowat, Millington et al. 2004).

5.3. Effect of dietary iron on colonic macrophages and DCs composition during intestinal inflammation

Intestinal macrophages and DCs represent guardians of intestinal homeostasis and key drivers of intestinal inflammation during IBD. These cells have quite distinct functions with MΦ having a primate in maintenance of intestinal homeostasis and tissue repair while DCs play an essential role in antigen uptake and T cell priming. However, recent studies have demonstrated a tight collaboration between macrophages and DCs during luminal antigen uptake. Indeed, DCs were shown to be efficient in taking up and transporting a particular bacterial antigen (e.g. *Salmonella*) while being inefficient in the uptake of soluble food-derived components (Mazzini, Massimiliano et al. 2014). The CX_3CR1^{high} MΦ were shown to be more efficient in uptake of this sort of antigens via transepithelial dendrites. Specific dietary components and micronutrients have been associated with higher risk of IBD and colon cancer. Yet, very few studies investigated the effect of these nutrients on the composition and function of intestinal MΦ and DCs. This is not surprising since performance of these experiments is quite challenging due to the long isolation protocols and difficulties in identification of these cells. Since iron absorptive capacity in the small intestine is limited, significant amounts of non absorbed iron may reach the colon. In the colon, excess iron may be sensed by microbiota, IECs as well as the underlying myeloid/macrophage immune cells among which CX_3CR1^{int} DCs and resident CX_3CR1^{high} MΦ could directly sense luminal iron by extending TEDs into the gut lumen. Since it was shown by Werner et al. that luminal iron alters commensal microbiota and drives IEC layer destruction leading to increased inflammation in $TNF^{\Delta ARE}$ model, we used the same dietary system in order to evaluate the potential effect of dietary iron content on the composition and function of the colonic MΦ and DCs during homeostasis and T cell dependent and independent inflammation.

In order to accomplish this, the isolation protocol and multi-colour staining panel for flow cytometry were established and applied to characterize colonic MΦ and DCs first in resting mice treated with experimental diets. When I started my PhD there were various different protocols for isolation of intestinal cells using different combinations of enzymes and time periods for tissue digestion. While working on the improvement of existing isolation protocol, I realized that successful isolation of intestinal MΦ and DCs is highly dependent on the mouse strain and intestinal microflora and therefore final enzyme concentrations and duration of digestion had to be tested. On the other hand a remarkable improvement was made in

establishment of common gating strategies for identification of intestinal MΦ and DCs and by the time I started to work on my project, it had been suggested that intestinal macrophages can be characterized by the expression of CD64, (FcγRI) and the chemokine receptor CX_3CR1, as well as the F4/80 and the integrins CD11b and CD11c (Bain, Scott et al. 2013, Bain and Mowat 2014, Mazzini, Massimiliano et al. 2014). Since the $CX_3CR1^{+/gfp}$ reporter mouse was not available in our animal facility by the time started my project, I adjusted the gating strategy for WT mice previously suggested by Bain *et al.* Using combination of CD11b, CD11c, MHC class II and CD64 markers, colonic MΦ were defined as $CD45.2^{+}CD11b^{+}CD64^{+}$ and DCs as $CD45.2^{+}CD64^{-}CD11c^{+}MHCII^{high}$. Furthermore, three distinct subsets with regards to MHC class II and Ly6C expression could be found in the colonic MΦ population in the line with findings from Bain et al. (Bain, Scott et al. 2013). $Ly6C^{high}MHCII^{-}$ cells, derived from $Ly6C^{high}$ blood monocytes, have a high proinflammatory capacity. However, in the steady state these cells undergo differentiation into colon macrophages, which is accompanied upregulation of MHC class II and some other surface markers such as CX_3CR1, CD64 and F4/80 and downregulation of Ly6C. Therefore $Ly6C^{high}MHCII^{+}$ cells represent an intermediate state in the process of MΦ maturation in the gut and finally differentiate into $Ly6C^{low}MHCII^{+}$ colonic MΦ, which acquire a characteristic anti-inflammatory gene expression profile (Bain and Mowat 2011, Bain, Scott et al. 2013). It is well established that this process is disturbed in during intestinal inflammation. Indeed, after T cell transfer or DSS colitis induction in mice treated with experimental diets, an expansion of colonic leukocytes and increased number of colonic MΦ could be observed in both 6 weeks and 9 week dietary treatments. Furthermore, inflammation affected the differentiation process within myeloid cell/MΦ compartment. Indeed a 2-3 fold decrease of anti-inflammatory MΦ was observed after colitis development in comparison to the resting state while the frequencies of proinflammatory $Ly6C^{high}MHCII^{-}$ and $Ly6C^{high}MHCII^{+}$ were much higher in the inflamed colons. In contrast to the well-established impact of inflammation on the intestinal MΦ compartment, the effect of luminal iron content as one of the potential factors influencing the intestinal MΦ maturation process in resting and inflamed state was not observed. The higher frequencies of antiinflammatory $Ly6C^{low}MHCII^{+}$ and intermediate $Ly6C^{high}MHCII^{+}$ MΦ were observed in the iron deficient *Rag1*$^{-/-}$ mice after 6 weeks of the experimental diet treatment and T cell transfer colitis development. However this effect could not be seen in T cell transfer colitis experiment after extended dietary treatment in which MΦ composition was found to be quite similar between the dietary groups. Therefore our findings suggest that luminal iron plays no role in colonic MΦ maturation in

homeostasis and inflammation at the iron concentrations which were tested here (high-normal and very low). Little is known about macrophage involvement in iron uptake in both small intestine, a site of iron absorption and colon, where non-absorbed iron is found before being excreted. However, macrophages from reticuloendothelial system recycle iron from senescent erythrocytes and play an important role in iron homeostasis (Andrews and Schmidt 2007). Corna et al. showed that M1 and M2 subsets derived from primary murine monocytes have a different iron handling profile; proinflammatory M1 macrophages have relatively sealed iron content and anti-inflammatory M2 macrophages are with high ability to recycle this metal (Corna, Campana et al. 2010). Moreover, some recent data suggests that iron accumulation in macrophages can directly activate macrophages to the proinflammatory M1 phenotype, highlighting a putative role of macrophage iron retention in the pathogenesis of chronic inflammatory and autoimmune diseases (Recalcati, Locati et al. 2010). Although the results shown here suggest that luminal iron is not one of the factors driving influx of proinflammatory macrophages, the expression of iron related proteins in colonic MΦ subsets was not included in this study as no major differences in colitis development and MΦ frequencies could be found between dietary groups in both colitis models. Despite the fact that luminal iron had no effect on MΦ composition in colon, it remains to be elucidated if iron could affect composition of macrophage subsets at the site of its absorption in the small intestine. However this was not the focus of my work and remains to be investigated.

The $CD45.2^{+}CD64^{-}CD11c^{+}MHCII^{high}$ colonic DCs could be further divided into three subsets; $CD103^{+}CD11b^{-}$, $CD103^{+}CD11b^{+}$ and $CD103^{-}CD11b^{+}$ cells. In contrast to macrophages, the overall frequencies of colonic DCs were not affected significantly by the colitis development. However the percentage of CD103 single positive DCs was lower after T cell induced colitis induction. These cells migrate to mLNs during steady state and inflammation and prime T cells to become effector T cells or induce generation of T_{Regs} (Jang, Sougawa et al. 2006). In the T cell transfer colitis experiment that involved 6-week dietary intervention, higher frequencies of $CD103^{+}CD11b^{+}$ cells and a significant increase of $CD11b^{+}$ DCs was found. On the other hand, FACS analysis of the composition of colonic DCs in the T cell transfer induced colitis after extended dietary treatment indicated that only the frequencies of CD103 single positive DCs were lower after inflammation induction. $CD103^{+}CD11b^{+}$ DCs are less common in colon whereas in SI they make up the majority of the DC population. These cells, as CD103 single positive DCs, were shown to migrate to mLNs where they can induce the differentiation of IgA-producing B cells, Th17 cells, Th1

cells or IFNγ^+ effector CD8$^+$ T cells (Uematsu, Fujimoto et al. 2008, Fujimoto, Karuppuchamy et al. 2011, Cerovic, Houston et al. 2013). The CD11b$^+$ subset that also expresses CX_3CR1 is similar to MΦ in their function but they expand in response to Flt3L and do not develop from Ly6C^{high} monocytes. This subset of DCs is present in SI and colon LP as well as in the mLNs where they are able to induce the differentiation of IFN-γ^+ and IL-17$^+$ T cells (Cerovic, Bain et al. 2014). In my T cell transfer colitis experiments, increased levels of T_H1/T_h17 cells were found in spleens and mLNs independent of iron content of the experimental diet while the frequency of these cells in colon LP was lower. The CD103$^-$ CD11b$^-$ DCs could also be detected in the colon LP of both steady state and inflamed *Rag1*$^{-/-}$ mice, but the function and the ontogeny of these cells which were also found by others is not known yet and therefore was not highlighted in this study.

To my knowledge, the role of dietary iron on the composition and function of intestinal DCs has not been studied before. According to our findings, dietary iron reduction resulted in minor differences, e.g. a decrease in the percentage of DCs and CD103$^+$CD11b$^-$ DC subset but only in colon IEL fraction after 6 week dietary depletion and T cell colitis development, while no differences in the colonic DC compartment were observed in T cell transfer experiment after extended dietary treatment. The reason for such discrepancy between these two dietary treatments, which was also observed when analysing colonic MΦ, could be due to the differences in the level of the inflammation on the cellular level that however could not be detected by bodyweight loss or histoscoring. Prolonged dietary treatment could also have generated more stable microbiota. Since it is well known that microbiota composition affects the MΦ and DC compartment and luminal iron depletion was shown to alter the microbiota, it is surprising that I did not observe consistent changes in MΦ/DCs composition. Obviously the development and functional differentiation of these cells is quite robust and not much affected by dietary intervention such as shown here for iron reduction. In addition, no effect of luminal iron depletion on colonic DC and MΦ compartment could be detected in DSS induced acute colitis model.

5.4. The effect of luminal iron depletion on T cell differentiation after T ceil induced colitis development

To elucidate the effect of dietary iron depletion on immunopathology of T cell transfer colitis, the impact of luminal iron on T cell differentiation was investigated as the outcome of this process is shown to be critical for chronic inflammation development in this model. The protection of immunodeficient *Rag1*$^{-/-}$ mice against colitis development is associated with expansion of $CD25^{+}Foxp3^{+}$ T_{Regs} once cotransferred with $CD4^{+}CD45RB^{high}$ or $CD4^{+}CD62L^{+}$ T cells (Mudter, Wirtz et al. 2002, Uhlig, Coombes et al. 2006). The cotranfer of 7-10% $Foxp3^{+}$ T_{Regs} did not resulted in protection against colitis since *Rag1*$^{-/-}$ mice developed severe colitis by day 17 after transfer independently of dietary treatment as obtained before (Heiseke, Faul et al. 2012). Although $Foxp3^{+}$ T_{Regs} could be detected in spleen, mLNs and colon LP, the frequencies of this population were too low to make any impact on inflammation development and further luminal iron modulation did not affect T_{Regs} in colon. In contrast, in previous study T_{Reg} expansion was observed in mice, which were protected from colitis development due to CCL17 deficiency in the same model (Heiseke, Faul et al. 2012).

In addition, T_H1/T_H17 induction was not altered by luminal iron depletion 17 days after T cell transfer. An expansion of IFN-γ producing T cells was detected in spleen, mLNs and colon LP of all dietary groups. This was in accordance with high mRNA levels of *Il-12p35* and *ifn-γ* detected in proximal colon tissue taken from *Rag1*$^{-/-}$ mice after dietary treatment and T cell transfer. Of note, higher induction of IFN-γ producing T cells was found in mLNs of $FeSO_4$ treated mice, but this had no effect on colitis development. Surprisingly, the frequencies of IL17A or IFN-γ/IL-17A double producing cells were around 1% suggesting that on experimental diet, IL-17A production from T cells is reduced.

5.5. Conclusion

In the spirit of the predominant conviction that dietary iron supplementation can enhance intestinal inflammation in animal colitis models, in the present study the potential protective effect of luminal iron depletion was tested in T cell dependent and acute DSS murine colitis models. The presented results clearly show that dietary iron deprivation did not protect mice from colitis development in both models. Although colon inflammation itself induced upregulation of proinflammatory cytokines, leukocyte infiltration and contributed to the

expansion of $CD64^{+}CD11b^{+}Ly6C^{high}$ monocytes in colon, luminal iron content did not affect this process. In addition, T_H1/T_H17 differentiation was not affected by luminal iron depletion in the T cell transfer model. The effect of iron depleted experimental diet was compared to the same type of diets containing $FeSO_4$ or Hemin as source of iron with final concentration comparable to the standard chow, which has a "normal" iron content. Whether dietary iron fortification would show a higher proinflammatory effect in comparison to iron depletion as shown by others, was not investigated here. Thus, the results of this study do not support a reduction of iron in the diet for colitis patients, which would be a quite severe dietary restriction. Also the current notion that oral iron supplementation should be avoided in IBD patients with active disease due to the proinflammatory activity of luminal iron is not based on convincing clinical or experimental evidence. Nevertheless, the poor tolerability and slow response to oral iron supplementation are good reasons for preference of intravenous iron supplementation in patients with active IBD and iron-deficiency.

6. SUMMARY

Inflammatory bowel diseases (IBD) are spontaneously relapsing, immunological disorders of the gastrointestinal tract characterized by excessive inflammation as a result of uncontrolled and persistent activation of innate immunity, T_H17 responses and/or deficiency of regulatory T cells. Crohn's disease (CD) and ulcerative colitis (UC) are two major forms of this disorder with long-term morbidity. Although the disease activity has been assigned to complex interplay between environmental factors and commensal bacteria in genetically susceptible host, the essential triggers for the disease onset are yet to be identified. Along this line, "Westernized lifestyle" is associated with increased incidence of IBD and smoking, antibiotic treatment and diet have been considered as potentially reversible risk factors for IBD development. Western like diet mostly composed of meat and other iron rich foods were shown to correlate with inflammation in IBD patients and oral iron supplementation is poorly tolerated by IBD patients with active disease. The mechanism that explains the influence of luminal iron on intestinal inflammation is not fully understood. However, the most likely cause to this is the high prooxidative capacity of iron, which leads to ROS and iNOS production and therefore activates the intestinal immune system in the already damaged mucosa.

The aim of my study was to investigate the potential protective role of dietary iron reduction against development of acute and chronic murine colitis with main focus on the impact of iron on the intestinal DCs and macrophages and their interaction with T cells in the gut. In order to study this, *Rag1*$^{-/-}$ mice lacking B and T cells, and WT (C57BL/6) mice were treated with iron adequate experimental diets ($FeSO_4$ or hemin) or iron depleted experimental diet prior to induction of colitis by T cell transfer or dextran sodium sulfate (DSS) respectively.

In the present study, I could show that luminal iron depletion had no influence on colitis development in two murine models, T cell transfer mediated colitis and DSS induced colitis.

Bodyweight assessment, clinical scoring and cytokine profiling on the mRNA level showed that regardless of the dietary iron content, lymphopenic *Rag1*$^{-/-}$ mice were not protected against colon inflammation during T cell induced colitis. Histological analysis demonstrated same level of intestinal disruption with presence of the epithelial damage and leukocyte

infiltrations present in all dietary groups. Additionally, prolonged luminal iron depletion also showed no effect on T cell mediated colitis development.

FACS analysis of the colon immune cells showed that luminal iron depletion had no effect on the composition of the colonic macrophage and DC compartment in steady state as well as during inflammation after development of T cell transfer colitis. An increased percentage of $CD64^{+}CD11b^{+}$ both $Ly6C^{high}MHCII^{+}$ and $Ly6C^{high}MHCII^{-}$ proinflammatory macrophages was observed in all three dietary groups due to the ongoing inflammation. Furthermore, T cell infiltration, T_{Reg} abundance and T_H1/T_H17 responses were not affected by the diet during T cell transfer colitis development.

In contrast to the studies showing colitis aggravation as a result of dietary iron fortification in the DSS model, I did not observe a protective effect of lower dietary iron content compared to normal iron content in this model. After DSS administration, WT mice treated with iron adequate ($FeSO_4$) but also depleted diet developed severe colitis accompanied with significant bodyweight loss, increased disease activity and colon shortening.

In conclusion, the results of this study indicate that reduction in dietary iron content has no effect on development of intestinal inflammation in both T cell dependent and independent murine colitis models when compared to adequate iron content in the diet.

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8. APPENDIX

8.1. Appendix 1

Nutritional composition of experimental diets and rodent standard chow used in present study is indicated in following data sheets obtained from manufacturers of the diets.

8.1.1. Experimental diets

Altromin Spezialfutter GmbH & Co. KG
Im Seelenkamp 20 - D-32791 Lage
Tel.: +49 (0)5232/6088-0 - Fax: +49 (0)5232/6088-20
E-Mail: info@altromin.de - http://www.altromin.de

Nummer **Lfd. Nummer** Änderung 05.05.2009 13:07:04
10100000 100000 C 1000 Kontrolldiät Ratte/Maus | Control Diet Rats/Mice

Inhaltsstoff	Einheit	Bedarf	Gehalt	Differenz
Rohprotein / Crude Protein	mg/kg		176115,000	
Rohfett / Crude Fat	mg/kg		50830,000	
Rohfaser / Crude Fibre	mg/kg		40450,000	
Rohasche / Crude Ash	mg/kg		54943,225	
Feuchtigkeit / Moisture	mg/kg		81735,625	
Disaccharide(s)	mg/kg		110960,500	
Polysaccharide(s)	mg/kg		471700,000	
Umsetzb. Energie/Metab. Energy	kcal/kg		3518,055	
Lysin / Lysine	mg/kg		17400,970	
Methionin / Methionine	mg/kg		10688,000	
Cystin / Cystine	mg/kg		3196,180	
Threonin / Threonine	mg/kg		7154,170	
Tryptophan	mg/kg		1976,960	
Arginin / Arginine	mg/kg		9828,790	
Histidin / Histidine	mg/kg		5275,790	
Isoleucin / Isoleucine	mg/kg		7222,820	
Leucin / Leucine	mg/kg		14762,770	
Phenylalanin / Phenylalanine	mg/kg		7171,970	
Valin / Valine	mg/kg		3296,140	
Alanin / Alanine	mg/kg		2528,000	
Asparaginsäure / Aspartic acid	mg/kg		3583,140	
Glutaminsäure / Glutamic acid	mg/kg		23674,970	
Glycin / Glycine	mg/kg		3136,000	
Prolin / Proline	mg/kg		12762,980	
Serin / Serine	mg/kg		5267,800	
Tyrosin / Tyrosine	mg/kg		9285,010	
Vitamin A	I.E./kg		15000,000	
Vitamin D3	I.E./kg		500,000	
Vitamin E	mg/kg		163,900	
Vitamin K3 als/as Menadion(e)	mg/kg		10,000	
Vitamin B1	mg/kg		20,040	
Vitamin B2	mg/kg		20,322	
Vitamin B6	mg/kg		15,034	
Vitamin B12	mg/kg		0,030	
Nikotinsäure / Nicotinic acid	mg/kg		50,170	
Pantothensre./Pantothenic acid	mg/kg		50,106	
Folsäure / Folic acid	mg/kg		10,00240	
Biotin	mg/kg		0,201	
Cholinchlorid/Choline chloride	mg/kg		1011,500	
P-Aminobenzoesre./Benzoic acid	mg/kg		100,000	
Inosit / Inositol	mg/kg		111,000	
Vitamin C	mg/kg		20,000	
Calcium	mg/kg		9310,506	
Ges.Phosphor / Phosphorus	mg/kg		7522,765	
Verd.Phosphor/Digest.Phosporus	mg/kg		7199,565	
Magnesium	mg/kg		683,506	
Natrium / Sodium	mg/kg		2488,262	
Kalium / Potassium	mg/kg		7088,682	
Schwefel / Sulfur	mg/kg		2791,540	
Chlor / Chlorine	mg/kg		3630,000	

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Nummer **Lfd. Nummer** Änderung 28.01.2005 08:40:39

10103800 103800 C 1038 Eisenarme Diät | Iron deficient diet

Inhaltsstoff	Einheit	Bedarf	Gehalt	Differenz
Rohprotein / Crude Protein	mg/kg		174465,000	
Rohfett / Crude Fat	mg/kg		50500,000	
Rohfaser / Crude Fibre	mg/kg		39460,000	
Rohasche / Crude Ash	mg/kg		55959,810	
Feuchtigkeit / Moisture	mg/kg		58159,275	
Disaccharide(s)	mg/kg		435761,860	
Polysaccharide(s)	mg/kg		178000,000	
Umsetzb. Energie/Metab. Energy	kcal/kg		3557,743	
Lysin / Lysine	mg/kg		17351,800	
Methionin / Methionine	mg/kg		10655,000	
Cystin / Cystine	mg/kg		3161,200	
Threonin / Threonine	mg/kg		7091,800	
Tryptophan	mg/kg		1966,400	
Arginin / Arginine	mg/kg		9748,600	
Histidin / Histidine	mg/kg		5228,600	
Isoleucin / Isoleucine	mg/kg		7158,800	
Leucin / Leucine	mg/kg		14561,800	
Phenylalanin / Phenylalanine	mg/kg		7089,800	
Valin / Valine	mg/kg		3217,600	
Alanin / Alanine	mg/kg		2396,000	
Asparaginsäure / Aspartic acid	mg/kg		3471,600	
Glutaminsäure / Glutamic acid	mg/kg		23361,800	
Glycin / Glycine	mg/kg		3070,000	
Prolin / Proline	mg/kg		12609,200	
Serin / Serine	mg/kg		5182,000	
Tyrosin / Tyrosine	mg/kg		9213,400	
Vitamin A	I.E./kg		15000,000	
Vitamin D3	I.E./kg		500,000	
Vitamin E	mg/kg		163,900	
Vitamin K3 als/as Menadion(e)	mg/kg		10,000	
Vitamin B1	mg/kg		20,040	
Vitamin B2	mg/kg		20,322	
Vitamin B6	mg/kg		15,034	
Vitamin B12	mg/kg		0,030	
Nikotinsäure / Nicotinic acid	mg/kg		50,170	
Pantothensre./Pantothenic acid	mg/kg		50,106	
Folsäure / Folic acid	mg/kg		10,00240	
Biotin	mg/kg		0,201	
Cholinchlorid/Choline chloride	mg/kg		1011,500	
P-Aminobenzoesre./Benzoic acid	mg/kg		100,000	
Inosit / Inositol	mg/kg		111,000	
Vitamin C	mg/kg		20,000	
Calcium	mg/kg		9450,408	
Ges.Phosphor / Phosphorus	mg/kg		7606,757	
Verd.Phosphor/Digest.Phosporus	mg/kg		7586,557	
Magnesium	mg/kg		669,168	
Natrium / Sodium	mg/kg		2484,994	
Kalium / Potassium	mg/kg		7071,889	
Schwefel / Sulfur	mg/kg		2697,140	
Chlor / Chlorine	mg/kg		3684,548	

Harlan Laboratories

2018

Teklad Global 18% Protein Rodent Diet

Product Description- 2018 is a fixed formula, non-autoclavable diet manufactured with high quality ingredients and designed to support gestation, lactation, and growth of rodents. 2018 does not contain alfalfa, thus lowering the occurrence of natural phytoestrogens. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from 150 to 250 mg/kg. Exclusion of alfalfa reduces chlorophyll, improving optical imaging clarity. Absence of animal protein and fish meal minimizes the presence of nitrosamines. **Also available certified (2018C) and irradiated (2918). For autoclavable diet, refer to 2018S (Sterilizable) or 2018SX (Extruded & Sterilizable).**

Ingredients (in descending order of inclusion)- Ground wheat, ground corn, wheat middlings, dehulled soybean meal, corn gluten meal, soybean oil, calcium carbonate, dicalcium phosphate, brewers dried yeast, iodized salt, L-lysine, DL-methionine, choline chloride, kaolin, magnesium oxide, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B_{12} supplement, folic acid, biotin, vitamin D_3 supplement, cobalt carbonate.

Standard Product Form: Pellet

Macronutrients		
Crude Protein	%	18.6
Fat (ether extract) [a]	%	6.2
Carbohydrate (available) [b]	%	44.2
Crude Fiber	%	3.5
Neutral Detergent Fiber [c]	%	14.7
Ash	%	5.3
Energy Density [d]	kcal/g (kJ/g)	3.1 (13.0)
Calories from Protein	%	24
Calories from Fat	%	18
Calories from Carbohydrate	%	58
Minerals		
Calcium	%	1.0
Phosphorus	%	0.7
Non-Phytate Phosphorus	%	0.4
Sodium	%	0.2
Potassium	%	0.6
Chloride	%	0.4
Magnesium	%	0.2
Zinc	mg/kg	70
Manganese	mg/kg	100
Copper	mg/kg	15
Iodine	mg/kg	6
Iron	mg/kg	200
Selenium	mg/kg	0.23
Amino Acids		
Aspartic Acid	%	1.4
Glutamic Acid	%	3.4
Alanine	%	1.1
Glycine	%	0.8
Threonine	%	0.7
Proline	%	1.6
Serine	%	1.1
Leucine	%	1.8
Isoleucine	%	0.8
Valine	%	0.9
Phenylalanine	%	1.0
Tyrosine	%	0.6
Methionine	%	0.4
Cystine	%	0.3
Lysine	%	0.9
Histidine	%	0.4
Arginine	%	1.0
Tryptophan	%	0.2

Vitamins		
Vitamin A [e,f]	IU/g	15.0
Vitamin D_3 [e,g]	IU/g	1.5
Vitamin E	IU/kg	110
Vitamin K_3 (menadione)	mg/kg	50
Vitamin B_1 (thiamin)	mg/kg	17
Vitamin B_2 (riboflavin)	mg/kg	15
Niacin (nicotinic acid)	mg/kg	70
Vitamin B_6 (pyridoxine)	mg/kg	18
Pantothenic Acid	mg/kg	33
Vitamin B_{12} (cyanocobalamin)	mg/kg	0.08
Biotin	mg/kg	0.40
Folate	mg/kg	4
Choline	mg/kg	1200
Fatty Acids		
C16:0 Palmitic	%	0.7
C18:0 Stearic	%	0.2
C18:1ω9 Oleic	%	1.2
C18:2ω6 Linoleic	%	3.1
C18:3ω3 Linolenic	%	0.3
Total Saturated	%	0.9
Total Monounsaturated	%	1.3
Total Polyunsaturated	%	3.4
Other		
Cholesterol	mg/kg	--

[a] Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

[b] Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

[c] Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber.

[d] Energy density is a calculated estimate of *metabolizable energy* based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

[e] Indicates added amount but does not account for contribution from other ingredients.

[f] 1 IU vitamin A = 0.3 µg retinol

[g] 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.

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10. CURRICULUM VITAE

PERSONAL INFORMATION

- **Name:** Anamamarija Markota
- **Date and Place of Birth:** May, 30, 1987 in Split, Croatia

EDUCATION

11/2011 to date	**PhD thesis – "The role of luminal iron as environmental factor influencing intestinal immune responses in mouse colitis models"** Research group of Prof. Dr. Anne Krug (Institute for Immunology, Ludwing-Maximilians Universität Munich) and the research fellow of TUM Graduate School 1482
10/2010 – 07/2011	**Master in Bioindustrial Techniques** University of Orleans, France
10/2009 – 07/2011	**Master of Molecular Biotechnology** University of Zagreb, Croatia
10/2006 – 07/2009	**Bachelor of Biotechnology** University of Zagreb, Croatia
09/2002 – 07/2006	**High School Graduation** Language Gymnasium, Split, Croatia

PUBLICATIONS

Heiseke A.F., Jeuk B.H,. **Markota A**., Straub T., Lehr H.A., Reindl W., Krug A.B. IRAK1 drives intestinal inflammation by Promoting the Generation of Effector Th Cells with Optimal Gut.Homing Capacity. J Immunol, 2015.

CONFERENCES

- **International Conference on Mucosal Immunology, Berlin, Germany, Jul. 2015**

Poster "Luminal iron depletion fails to protect against T cell induced colitis

- **Munich Interact, Germany, Mar. 2015**

Poster "Dietary iron as environmental factor influencing antigen presentation and T cell differentiation in IBD"

- **13th International DC Meeting, Tours, France, Sept. 2014**

Poster "The role of DC-specific chemokine CCL17 in murine models of colitis and associated colon cancer development"

- **Autumn School of immunology, Merseburg, Germany, Oct. 2013**

Poster "Dietary iron as environmental factor influencing antigen presentation and T cell differentiation in IBD"

SCHOLARSHIPS

11/2011 – 04/2015	Deutsche Forschungsgemeinschaft (DFG) scholarship
01/2011 – 06/2011	Erasmus scholarship