Functional Analysis of the Interleukin-10 (IL-10) Network
by Induction of Colitis in
Conditional IL-10 and IL-10 Receptor Knock-Out Mice

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“Characterization of pathophysiological animal models – functional and genetic analyses –“
“The only true wisdom is in knowing that you know nothing”

Socrates

- To my parents -
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Abbreviations

% per cent
°C degree Celcius
APC allophycocyanin
ASF Altered Schaedler Flora
bp base pairs
BSA bovine serum albumin
CD cluster of differentiation
CO₂ carbon dioxide
CRASF® Charles River Altered Schaedler Flora
Cre causes recombination of the bacteriophage P1
CV conventional
Cy5 cytochrom 5
d day
Da Dalton
DC dendritic cell
DNA deoxyribonuclease
dNTP 2’-deoxynuceloside-5’triphosphate
DSS dextran sulfate sodium
*e.g.* *exempli gratia*
EDTA edetic acid
*et al.* *et alterae*
FACS fluorescence activated cell sorting
FELASA Federation of European Laboratory Animal Science Associations
FGF fibroblast growth factor
Fig. figure
FITC fluorescein-isothiocyanate
flox flanked by two loxP sites
FoxP3 *fork head box p3 gene*
ABBREVIATIONS

$g$ gram
GALT gut associated lymphoid tissue
GF germfree
GM-CSF granulocyte-macrophage colony-stimulating factor
GV-SOLAS Society for Laboratory Animal Science
$h$ hour
HE hematoxylin-eosin
HZI Helmholtz Centre for Infection Research
*i.e.* it est
IBD inflammatory bowel disease
IFN interferon
IFN$\gamma$ interferon gamma
IL interleukin
IP-10 interferon inducible protein 10
JAK Janus kinase
$kb$ kilo base pairs
KC keratinocyte chemoattractant
$loxP$ genome locus crossing over (= x) in P1
LPS lipopolysaccharide
$M$ molar; mol/l
MCP-1 monocyte chemoattractant protein 1
MIG monokine induced by gamma interferon
$min$ minute
MIP-1$\alpha$ macrophage inflammatory protein-1-alpha
$ml$ milli litre
$neo$ neomyci-phosphotransferase
$ng$ nano gram
NSAID non-steroidal anti-inflammatory drug
PBS phosphate buffered saline
PCR polymerase chain reaction
PE phycoerythrin
ABBREVIATIONS

pg  pico gram
pH  potentia Hydrogenii
SCID severe combined immuno-deficent
SDS sodium dodecyl sulfate
sec  second
SPF specific pathogen free
STAT signal transducer and activator of transcription
Tab.  table
Th1/2 T helper 1/2 cell
TLR  Toll-like receptor
TNFα tumour necrosis factor alpha
U  unit
VEGF vascular endothelial growth factor
xg  fold gravitation acceleration
µg  micro gram
µl  micro litre
1 Introduction

The aim of this project was the dissection of the Interleukin-10 (IL-10) network in vivo with regard to the development of colitis. IL-10 is an anti-inflammatory cytokine produced by various cell types. The understanding of the regulation of immune responses is necessary to determine how a chronic inflammation such as Inflammatory Bowel Disease (IBD) can develop and how it can be limited. A functional IL-10 receptor (IL-10R) can be expressed on almost every cell type. To understand the influence of IL-10 on a certain cell type in the context of an entire organism, the cell type specific inactivation of IL-10R in the mouse is necessary. The analysis of a cytokine network is complicated by several features: Multiple cytokines might have a similar function; redundancy can mask the phenotype of a knock-out mouse. When inactivating cytokine receptors, the fact that cytokines often share a common signal transducing receptor needs to be considered, e.g. the common gamma chain is used for the signalling of IL-2, IL-4, IL-7, IL-9, IL-13 and IL-15 [1]. Compensatory mechanisms can replace a lacking cytokine or even produce a phenotype for example the increased production of IL-4 in IL-2 knock-out mice [2, 3]. Furthermore, a cytokine can have cell type specific effects that can further be dependant on the context of the immune response, which can only be revealed in vivo by cell type specific inactivation of a gene, e.g. mice carrying the specific deletion of IL-10 in macrophages are more susceptible to lipopolysaccharide (LPS) than mice lacking IL-10 in T-cells [4], while T-cell derived IL-10 is necessary to prevent the development of a colitis [5]. The anti-inflammatory properties of IL-10R signalling have been shown to suppress IL-6 secretion in macrophages, but not in synovial fibroblasts [6]. When working with genetically modified mice, effects of the genetic background as well as the microflora and infectious agents have to be taken into account. A review of the insights and difficulties when dealing with cytokine networks is given in [7].

In this project, analysis of the cellular IL-10 network was performed by analysing conditional IL-10R and IL-10 knock-out mice in a colitis model.
1.1 Generation of conditional knock-out mice using Cre-loxP

One possibility to dissect the cytokine network is to knock-out genes in a cell-specific or inducible way using conditional mutagenesis. Using this technique, the function of a gene for a special cell type or during a specific stage of development of the mouse can be analysed. The Cre-loxP system is a frequently used system for conditional mutagenesis.

*Cre*-recombinase (causes recombination of the bacteriophage P1 genome) was originally isolated from the bacteriophage P1. *Cre* induces recombination between two loxP sequences (genome locus of crossing-over (=x) P1) [8]. The loxP sequence is an imperfect palindrome with a 13bp direct repeat of the sequence (inverted repeats), separated by 8bp non-palindromic sequence (spacer) [9]. The non-palindromic sequence defines the two possible orientations of the loxP sequence. The original function of this system is the division of the P1 genome into 2 identical parts during replication, but it was proven to be efficient in eukaryotic cells as well [10, 11]. It can either be used to delete a gene flanked by two loxP sites that have the same orientation, or to invert a gene if the loxP sites are in opposite orientation [12]. The deletion of a gene using the *Cre*-loxP system is depicted in Figure (Fig.) 1.

![Fig. 1 The *Cre*-loxP system used for the deletion of a gene](Jaisser J. Am. Soc. Nephrol 2000 [13]. *Cre* exerts a gene flanked by 2 loxP sites of the same orientation.)

Vectors used for conditional gene targeting usually contain a part of the targeted gene flanked by two loxP-sites (floxed) and a positive selection marker, *e.g.* the *neomycin-phosphotransferase* gene (*neo*). To exert the *neo* gene after the selection for homologous recombination, a third loxP site can be used. Some vectors additionally contain a marker for...
negative selection against random integration, *e.g.* thymidine kinase of herpes simplex virus type 1. Thymidine kinase is placed outside of the region of homologous recombination.

In order to obtain mice carrying a cell type specific deletion of the gene of interest, mice carrying the floxed gene of interest are bred with mice expressing Cre under a cell type specific promoter. The offspring of these mice show a deletion of the floxed gene in the cells expressing Cre. Cre-negative (Cre−) littermates can be used as negative controls. Only mice heterozygous for the Cre allele should be used for the breeding of conditional knock-out mice, as the insertion of Cre into a gene mostly leads to non-functionality of this gene, as shown for the lyM-Cre mouse strain for example [14].

![Fig. 2 The use of the Cre-loxP system to obtain conditional, mosaic, inducible and ubiquitous deletion of a floxed gene](Rajewsky et al. J. Clin. Invest. 1996 [15].)

The efficiency and the specificity of the deletion is variable depending on the Cre and the flox mouse strains used and have to be approved for each newly bred mouse strain. Cre expression level and the location of the loxP sites to each other and on the chromosome influence the efficiency of Cre mediated recombination [16]. Cell type specificity of Cre expression is dependant on the specificity of the promoter.
1.2 Cytokines and chemokines in inflammation

Inflammation is a crucial aspect of the host defence against injury and infection. A prolonged inflammatory response though, can be detrimental for the host as seen in chronic auto-inflammatory conditions, for example in IBD. In order to give a brief overview of the cytokines and chemokines dealt with in this thesis, the immune reaction against a pathogen in the intestine is described in this chapter. Due to the various possibilities of an immune reaction depending on the pathogen, the constitution of the immune system and the multiple positive and negative feedback mechanisms, this part is roughly simplified and does not claim to explain all the possible immune reactions and interactions in the intestine.

The first line of defence against an infection or injury is the epithelial barrier. In the intestine, gut epithelial cells, mucus producing goblet cells and defensin producing Paneth cells are building this barrier. Non-immunological barrier functions of the intestinal epithelium include intestinal motility, mucus secretion and cell turn-over. Gut epithelial cells are able to recognise intestinal bacteria through Toll-like receptors (TLR) [17] and to present antigens through non-classical major-histo-compatibility (MHC) class I molecules [18]. Upon contact with a pathogen for instance, gut epithelial cells are able to produce proinflammatory cytokines [19]. Some of these proinflammatory cytokines are IL-1α/β, IL-6, TNFα and IL-17, which can be produced by macrophages upon TLR stimulation as well. An innate immune response is triggered. The vascular epithelium is activated, its permeability and the blood flow is enhanced in order to recruit more inflammatory cells. Proinflammatory cytokines also induce local tissue destruction [20]. Additionally, chemokines such as GM-CSF, MCP-1, MIG, MIP-1α, KC and IP-10 are produced by tissue cells and infiltrating leukocytes that attract and activate further immunological cells, especially macrophages, neutrophils and T-cells [21]. This innate immune response can subsequently induce an adaptive immune response, e.g. Th1 or Th2. The Th1 immune response is also designated as the cellular immune response. It is induced upon infection with intracellular pathogens and activates macrophages to destroy intracellular pathogens. The Th2 immune response is referred to as the humoral immune response. It is induced by extracellular pathogens and characterised by the differentiation of antibody producing B-cells [20]. In this context, IL-12 produced by macrophages and dendritic cells induces a Th1 polarised immune response [22].
Characteristic cytokines of a Th1 immune response are IL-12 and INFγ. Some key cytokines of a Th2 immune response are IL-4, IL-5 and IL-13. After the clearance of the infection, the immune response has to be down-regulated again by anti-inflammatory cytokines such as IL-10. Table 1 (Tab. 1) gives a brief overview of the complete designations, functions and synonyms of the cytokines and chemokines that were important for this thesis.

<table>
<thead>
<tr>
<th>Complete designation</th>
<th>Function</th>
<th>Abbreviation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin 1 alpha</td>
<td>proinflammatory</td>
<td>IL-1α</td>
<td>[23]</td>
</tr>
<tr>
<td>Interleukin 1 beta</td>
<td>proinflammatory</td>
<td>IL-1β</td>
<td>[24]</td>
</tr>
<tr>
<td>Interleukin 6</td>
<td>proinflammatory</td>
<td>IL-6</td>
<td>[25]</td>
</tr>
<tr>
<td>Interleukin 17</td>
<td>proinflammatory, Th17</td>
<td>IL-17</td>
<td>[26, 27]</td>
</tr>
<tr>
<td>Tumour necrosis factor alpha</td>
<td>proinflammatory</td>
<td>TNFα</td>
<td>[20]</td>
</tr>
<tr>
<td>Interleukin 10</td>
<td>anti-inflammatory</td>
<td>IL-10</td>
<td>[28]</td>
</tr>
<tr>
<td>Interleukin 2</td>
<td>T-cell proliferation</td>
<td>IL-2</td>
<td>[3, 29]</td>
</tr>
<tr>
<td>Interferon gamma</td>
<td>Th1</td>
<td>IFNγ</td>
<td>[30]</td>
</tr>
<tr>
<td>Interleukin 12</td>
<td>Th1</td>
<td>IL-12</td>
<td>[22, 31]</td>
</tr>
<tr>
<td>Interleukin 4</td>
<td>Th2</td>
<td>IL-4</td>
<td>[32]</td>
</tr>
<tr>
<td>Interleukin 5</td>
<td>Th2</td>
<td>IL-5</td>
<td>[33]</td>
</tr>
<tr>
<td>Interleukin 13</td>
<td>Th2, enhances epithelial cell turnover</td>
<td>IL-13</td>
<td>[34]</td>
</tr>
<tr>
<td>Monocyte chemoattractant protein 1</td>
<td>chemotaxis of monocytes and T-cells</td>
<td>MCP-1/ CCL2</td>
<td>[21]</td>
</tr>
<tr>
<td>Keratinocyte chemoattractant</td>
<td>chemotaxis and activation of neutrophils</td>
<td>KC/ CXCL1</td>
<td>[35]</td>
</tr>
<tr>
<td>Monokine induced by gamma interferon</td>
<td>chemotaxis of stimulated T-cells</td>
<td>MIG/ CXCL9</td>
<td>[21]</td>
</tr>
<tr>
<td>Macrophage inflammatory protein-1-alpha</td>
<td>recruitment of neutrophils, macrophages and T-cells</td>
<td>MIP-1α/ CCL3</td>
<td>[21]</td>
</tr>
<tr>
<td>Granulocyte-macrophage colony-stimulating factor</td>
<td>proliferation, differentiation and activation of neutrophils and macrophages</td>
<td>GM-CSF</td>
<td>[36]</td>
</tr>
<tr>
<td>Interferon inducible protein 10</td>
<td>recruitment of lymphocytes, macrophages and neutrophils, inhibits epithelial cell turnover</td>
<td>IP-10/ CXCL10</td>
<td>[21, 34]</td>
</tr>
</tbody>
</table>

Tab. 1 Brief summary of the function of some key cytokines in inflammation
1.3 **Interleukin-10**

IL-10 is an immuno-regulatory cytokine produced by various cell types including B- and T-cells, macrophages and keratinocytes. IL-10 was originally described as a product of Th2 lymphocytes, inhibiting the Th1 immune response [37]. Later on, its regulatory function for the Th2 immune response was proven [38]. Nowadays, it is widely accepted that IL-10 limits and down-regulates inflammatory responses in general [28]. IL-10 is predominantly produced by T-cells, macrophages and dendritic cells. Most types of T-cells produce IL-10 including Th1- or Th2-polarised T-cells and regulatory T-cells [39]. A self-controlling regulatory feedback loop has been described recently in Th1-cells: Th1 cells control themselves by production of IL-10 [40-42]. Innate immune responses, such as the reaction to lipopolysaccharide (LPS), have been shown to be controlled by IL-10 as well [43]. To down-regulate the immune response to LPS, macrophages and neutrophils are the major IL-10 producing cells. The innate response to CpG is another example of a reaction to a bacterial motive regulated by IL-10 [4].

1.3.1 **Interleukin-10 receptor**

IL-10R consists of two subunits, designated in literature as IL-10R\(\alpha\) and IL-10R\(\beta\) or IL-10R1 and IL-10R2, respectively. The terms IL-10R\(\alpha\) and IL-10R\(\beta\) will be used in this thesis. The *IL-10R\(\alpha\)* gene is located on mouse chromosome 9, while *IL-10R\(\beta\)* is located on mouse chromosome 16.

IL-10 selectively binds IL-10R\(\alpha\). IL-10R\(\beta\) is required to assemble the active IL-10R complex. IL-10R\(\beta\) is shared as a second subunit for signalling of at least 3 further class II cytokines: IL-22, IL-26 and IFN\(\lambda\) [44]. IL-10R activates Janus kinase (JAK)1 leading to the phosphorylation of the IL-10R\(\alpha\) chain, thereby recruiting signal transducer and activator of transcription (STAT)3 [39].

Using this pathway, IL-10 inhibits the production of proinflammatory cytokines such as IL-1\(\alpha\), IL-1\(\beta\), IL-6, IL-10 itself, IL-12, GM-CSF and TNF\(\alpha\) by macrophages [45-47] after Toll-like receptor (TLR) activation. IL-10 also inhibits the production of chemokines, *e.g.* MCP-1, MIP-1\(\alpha\) and IP-10 [48, 49], as well as the expression of MHC II. Similar effects were
described for IL-10 on neutrophils: LPS induces the production of TNF, IL-1α/β, IL-12p40, MIP-1α, MIG and IP-10 by neutrophils, which is inhibited by IL-10 with a delay of 2h post-stimulation [28, 50-52]. A simplified scheme of the overall mechanism of inhibition of the production of proinflammatory cytokines after TLR stimulation by IL-10 is shown in Fig. 3. Recently, Williams et al. confirmed that for the anti-inflammatory effects of IL-10, IL-10Rα and STAT3 are absolutely required. STAT3 activity can suppress both IL-6 and TNFα production in LPS stimulated macrophages, whereas the same signalling in synovial fibroblasts was not able to attenuate IL-6 production [6], indicating that the cellular environment plays a crucial role dictating whether STAT3 drives a pro- or anti-inflammatory response.

Fig. 3 Overall mechanism of IL-10 signalling
The highest levels of IL-10R expression are found in dendritic cells and macrophages [39]. However, IL-10Ra is constitutively expressed on haematopoietic cells and inducible on several nonhaematopoietic cells, while IL-10Rβ expression can be found on most cells and tissues [28], implicating that a functional IL-10R can be expressed on most cell types. This leads to the necessity of cell type specific deletion of IL-10R in the mouse in order to allow the determination of the function of IL-10 for single cell types "in vivo". So far, the data describing the reaction of certain cell types to IL-10 were obtained "in vitro".

1.3.2 Interleukin-10 and Inflammatory Bowel Disease

IL-10 knock-out (IL-10^{-/-}) mice develop a chronic enterocolitis similar to human IBD. Clinical symptoms are loss of bodyweight, hunchback and piloerection, diarrhoea, blood stools, and consequently anaemia. Histologically, the colonic mucosa is characterised by ulceration and invasion of inflammatory cells accompanied by hyperplasia of the epithelium. Lesions can be found at various sites throughout the entire length of the intestine [53]. The severity of the disease is dependant on the genetic background, the enteric flora and other environmental factors [54, 55]. Germfree IL-10^{-/-} mice do not develop any signs of IBD [56]. In specific pathogen free (SPF) IL-10^{-/-} mice, the severity is reduced and the onset of the inflammation is delayed compared to conventionally housed IL-10^{-/-} mice [53]. Colonisation of the intestine with certain bacteria, e.g with Helicobacter hepaticus, can accelerate the inflammation [57]. The reason for the inflammation is thought to be a dys-regulated Th1 immune response to intestinal bacteria. Analysis of conditional IL-10 knock-out mice revealed that T-cells are the most important source of IL-10 for the prevention of spontaneous IBD [5].

1.3.3 Induction of IBD in IL-10^{-/-} mice with Piroxicam

In order to overcome the variability in severity and onset of IBD in IL-10^{-/-} mice, IBD can be induced in IL-10^{-/-} mice by treatment with the non-steroidal anti-inflammatory drug (NASID) Piroxicam: Berg et al. [58] showed that treatment of 4 week old IL-10^{-/-} mice with 200ppm Piroxicam for 14 days resulted in rapid development of IBD. Colitis was
characterised by IFN\(\gamma\) producing CD4\(^+\) T-cells and macrophages. Piroxicam non-selectively inhibits cyclooxygenase (COX)-1 and COX-2, thereby inhibiting the production of prostaglandins. Prostaglandins seem to play a crucial role in the regulation of the inflammation in IL-10\(^{-}\) mice. IL-10 inhibits COX-2 expression and prostaglandin production [59]. Induction of colitis with NSAID is an interesting model for human IBD, because of the evidence that NSAID can aggravate and reactivate disease in human patients [60]. Furthermore, levels of prostaglandins correlate with the disease activity in IBD patients [61].

1.4 Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is characterised by a chronic inflammation of the intestine of unknown etiology. Crohn’s disease and ulcerative colitis are the two forms distinguished. In the Western World about 1 in 1000 people is affected by this relapsing and remitting disease, which is still incurable [62, 63]. Genetic, psychosomatic and environmental factors such as gut flora and nutrition are known to crucially influence IBD.

A Th1 immune response characterised by excessive IL-12/IL23 and IFN\(\gamma\)/IL-17 production affecting the small bowel and colon with ulceration and transmural bowel inflammation is associated with Crohn’s disease [64], whereas ulcerative colitis is characterised by a Th2 immune response with excessive production of IL-13 [65] and inflammation and ulceration of the colonic and rectal mucosa. The main hypothesis currently discussed in order to explain the chronic inflammation of the intestine is a dys-regulation of the mucosal immune system causing an excessive immune response to the enteric flora that is triggered by changes in the microflora and epithelial cell abnormalities leading to leakiness of the intestinal barrier (reviewed in [64]). One of the bacterial agents that is thought to specially trigger IBD is Mycobacterium paratuberculosis the causative agent of John’s Disease in cattle. John’s Disease is morphologically very similar to Crohn’s disease and antibodies against M. paratuberculosis have been found in 73% of the IBD patients in an Italian study and in 6 out of 15 patients but none of the 12 controls in an Irish study (reviewed in [66]).
INTRODUCTION

1.4.1 Animal models of IBD

Several animal models have been developed to mimic IBD. The key characteristics for an optimal model are: morphological alterations, inflammation, symptoms, pathophysiology and course should be similar to human IBD. Treatments applied should exhibit the same effects. The genetic background of the animal should be well defined and the immune system well characterised. Environmental factors, especially nutrition and enteric flora, should be controllable. Induction of colitis should be stable, reproducible and predictable.

Mice are relatively easy and cheap to maintain compared to other mammals and have a short reproduction cycle. The genome is 95% identical to that of humans. Inbred strains provide a stable and characterised genetic background. Furthermore, mice allow genetic manipulation, including the cell type specific deletion of genes. If mice are reared in animal facilities equipped with individually ventilated cages (IVC), environmental factors and microflora can be controlled (reviewed in [67]).

Commonly used animal models for IBD include gene knock-out, chemically induced and adoptive transfer models. The IL-2 knock-out [29] and IL-10 knock-out [53] mouse model are examples for gene knock-out models of IBD, in both models colitis is most probably due to an increased Th1 immune response accompanied by a lack of regulatory T-cells. Inducible colitis models include the induction by 2,4,6-trinitrobenzene sulfonic acid (TNBS) enema [68] or dextran sulfate sodium (DSS) [69]. TNBS and DSS are thought to induce colitis by damaging the mucosal barrier [70, 71]. Frequently applied adoptive transfer models use the transfer of CD4⁺ T-cells expressing high levels of CD45RB (CD45Rb\textsuperscript{high}) [72] or the marker for naïve T-cells CD62L [73] into mice with severe combined immunodeficiency (SCID).

The common feature of all these models is a strong influence of genetic background as well as intestinal flora and other environmental factors on the severity of the inflammation [55, 64, 67, 74].
1.4.2 Induction of colitis by dextran sulfate sodium

Dextran sulfate sodium (DSS) is a heparin-like polysaccharide containing up to three sulfate groups per glucose molecule. Induction of colitis in rodents by application of DSS in the drinking water is a widely used and well characterised model of colitis in mice, first described by Okayasu et al. 1990. Morphological changes are similar to human ulcerative colitis [69]. Additionally, anti-colitis drugs applied in human ulcerative colitis such as sulfasalazine, olsalazine and mesalazine have a therapeutic effect in DSS colitis [75, 76]. The inflammation is restricted to the large intestine [69, 77]. Erosion and inflammation of the mucosa are both most frequent and severe in the distal part of the colon [69, 77, 78]. Its severity is dependant on the concentration of DSS [79], but also on the molecular weight [80]. Concentrations described in literature range between 1% and 7%. The most commonly used molecular weight is 40,000Da.

The exact mechanism of colitis induction remains unknown. A direct toxic effect on the epithelium could lead to the inflammation [81]. In the acute phase (from day 3 to 7), prior to the appearance of inflammatory processes, an increased permeability of the intestinal mucosa has been shown [71]. Due to the damage of the epithelial barrier, toxic products of luminal bacteria such as endotoxin or peptidoglycans might permeate into the mucosa causing damage to the epithelial cells of basal crypts and thereby inducing an inflammatory reaction. As DSS is taken up by macrophages, inhibition of phagocytic capacity is another possible mechanism rendering the intestinal epithelium more susceptible to bacterial infection [78].

The role of lymphocytes in the induction and maintenance of the disease is a matter of debate and published results have so far been contradictory. Acute DSS induced colitis occurs in SCID-mice, lacking B- and T-lymphocytes and NK-cells [82, 83], though in Rag-1 knock-out mice, lacking lymphocytes in general, severity of DSS induced colitis is decreased dramatically [84]. While acute DSS colitis is characterised by proinflammatory cytokines e.g. IL-1 and TNFα accompanied by a Th1 immune response through the expression of IL-12 and IFNγ [79], chronic DSS colitis shows a mixed Th1/Th2 cytokine profile [85].
Another controversy in the DSS model is the effect of enteric bacteria. DSS treatment of germfree IQI/Jic mice leads to an even more severe colitis compared to SPF mice [86]. DSS is not degraded in the intestinal lumen [80], thus gut bacteria do not degrade DSS. Antibiotic treatment improves acute DSS colitis but has no effect on established chronic colitis [87]. Different mouse-models for Toll like receptor (TLR) signalling have given an idea about the contributions of this pathway to DSS colitis. While MyD88 knock-out mice show severe colitis after DSS treatment that is refractory to antibiotic treatment [88], colitis in TLR9 knock-out mice is reduced compared to wildtype mice [89], indicating that disturbance of multiple TLR-pathways is detrimental for the homeostasis of the intestinal epithelium, while CpG-motifs of the bacterial flora might trigger DSS colitis. Recently, probiotic bacteria, such as *Propionibacterium freudenreichii*, *Bifidobacterium infantis* and *Bifidobacterium adolescentis*, have been reported to be able to attenuate DSS colitis [90-92].

Mährer et al. described the influence of inbred background on the extent and severity of colitis after exposure to DSS: significant differences for all parameters scored have been found for all strains. Susceptibility for caecum lesions was decreasing from C3H/HeBir, C3H/HeJ, C57BL/6J to 129S2/SvPas. Concerning lesions found in the colon, C3H/HeBir, C3H/HeJ, C57BL/6J and 129S2/SvPas were highly susceptible whereas, DBA/2J were less susceptible to DSS [74, 89]. Melgar et al. found that 5 days of treatment with 3% for C57BL/6 and 5% for BALB/c revealed similar symptoms in the acute phase, however, disease resolved in BALB/c mice but became chronic in C57BL/6, indicating that BALB/c are more resistant to DSS than C57BL/6 mice [93].

Several publications have shown a crucial role for IL-10 in DSS induced colitis: IL-10 mRNA is increased [79] reaching its maximal level at day 7 [94]. In the same study Tomoyose et al. [94] showed that IL-10 reduces the production of TNFα and IL-1 in colonic tissue culture as well as various inflammatory indicators *in vivo* after DSS treatment including histological scores, colon length and rectal bleeding.
1.5 The influence of bacteria on the intestine – lessons from germfree mice

Studies on germfree mice revealed that the intestinal bacterial flora is crucial for the development of the gut associated lymphoid tissue (GALT) as well as for the architecture of the epithelium. Grossly visible changes in the architecture of the intestine in germfree mice are: A dramatically enlarged caecum that is in large parts due to the accumulation of undegraded mucus, as intestinal bacteria are responsible for the degradation of the mucus. The vili of the small intestine are longer, whereas crypts of the large intestine are shorter than in mice harbouring gut flora [95].

The composition of the GALT is different in germfree mice compared to conventional mice harbouring an intact microflora. Germfree mice exhibit a delayed development of intraepithelial lymphocytes. The lamina propria of the intestine contains less CD3\(^+\) T-cells in germfree mice. T-cells stay naïve in germfree mice, displayed by the retaining of L-selectin (CD62L\(^+\)). In contrast, the number of macrophages and CD11c\(^+\) dendritic cells was comparable to the amount seen in conventional mice [96]. Furthermore, the development of the T-cells towards regulatory T-cells is impaired in germfree mice: Recently, Östman et al. found that CD4\(^+\)CD25\(^+\) T cells of germfree mice exhibit a lower expression of the regulatory T-cell marker molecule FoxP3 (fork head box p3 gene) and exhibited a reduced suppressive potency in vitro [97]. Strauch et al. observed that CD4\(^+\)CD62L\(^+\) lymphocytes from germfree mice induce more severe colitis in SCID mice than the ones from conventional mice [98] and CD4\(^+\)CD62L\(^-\) cells from germfree mice are not able to attenuate colitis, whereas the same cell population isolated from conventional mice is able to attenuate the colitis. Moreover, cells isolated from mesenteric lymph nodes as well as T-cells from germfree mice produce less IL-10.
2 Materials and Methods

2.1 Chemicals, reagents, enzymes

All chemicals and reagents were of p.A. quality and purchased from Amersham Biosciences, BioRad, Boeringer Ingelheim, Fluka, Höchst, Invitrogen, Merck, Millipore, Promega, Riedel de Haen, Roche, Roth and Sigma Aldrich. Enzymes were delivered from New England Biolabs. Water used for solutions was deionised via a Milli-Q-System (Millipore). Mixtures of buffers and solutions used can be found in the appendix (chapter 8.1).

2.2 Description of the IL-10RFl/FI mice

In order to obtain mice with cell specific deletion of the IL-10R, mice carrying a floxed IL-10Ra gene were required. The IL-10RFl/FI mice were generated by Robert S. Jack at the Ernst-Moritz-Arndt-University of Greifswald. A loxP site excised from pGEMloxp was inserted into the Apa1 site upstream of exon 1 of IL-10Ra using a genomic clone containing the promoter region and the first three exons of the murine IL-10Ra chain. The new loxP site generated an additional EcoR1 site. A neo flox cassette excised from the vector neofox-8 was inserted into the Nhe1 site in-between exon 1 and 2. To permit counter-selection against non-homologous recombinants, a copy of the herpes simplex thymidine kinase gene was inserted. Cloning steps were monitored by sequencing all newly formed ligation junctions. The vector was inserted into embryonic stem cells via electroporation and screened for homologous recombination. The first Cre mediated deletion to exert the neo cassette was conducted in vitro. Modified embryonic stem cells were subsequently injected into blastocysts. The second step of Cre mediated deletion occurred in vivo by breeding the IL-10RFl/FI mice to the different Cre+ mice. The vector and the subsequent Cre mediated deletions are shown in Fig. 4. Additionally, the Southern strategy used to distinguish between the flox and the delta allele is depicted. Digestion with Kpn1 and labelling with the specific probe led to a fragment of 4.8kb for the wildtype (wt), 4.9kb for the flox and 3.3kb for the delta allele. This Southern strategy was applied for the breeding of IL-10R−/ mice and for the detection of the deletion in sorted cells, respectively.
**MATERIALS AND METHODS**

1. **Fig. 4 Targeting construct used to obtain IL-10R\(^{Fl/Fl}\) mice and Southern strategy used to distinguish between the flox and the delta allele**

A loxP site was inserted into the ApaI site upstream of exon 1 of IL-10R\(\alpha\) and a neo flox cassette into the Nhe site downstream. Digestion with KpnI and labelling with the IL-10R\(\alpha\) probe revealed a wildtype (wt) band of 4.8kb, a flox band of 4.9kb and a delta band of 3.3kb.

2.3 **Isolation of genomic DNA from mouse tails**

The tip of a mouse tail was digested at 54°C overnight in 720µl tail-lysis buffer (appendix 8.1) and 30µl proteinase K (10mg/ml). Cell debris and fur were separated by centrifugation at 11,000xg for 10min. The supernatant was transferred into a new reaction tube containing 600µl isopropanol. To precipitate the genomic DNA, tubes were agitated and centrifugation at 11,000xg for 5min was performed. The harvested DNA was washed with 70% ethanol, dried at room temperature for about 10min and finally dissolved TE-buffer (appendix 8.1).
2.4 Genotyping using Southern blot

Southern blotting was applied for the genotyping of IL-10R\textsuperscript{+/+} and IL-10R\textsuperscript{+/−} mice as well as for the detection of the deletion in FACS sorted cell populations. DNA was digested with KpnI (50,000U/ml, New England Biolabs). DNA fragments were separated via gelelectrophoresis using a 1% agarose gel, immobilised by alkaline transfer [99] and fixed onto a nylon membrane (Macherey-Nagel; Amersham Biosciences).

The \textit{IL-10R} specific probe was cloned into the cloning vector pGEM\textregistered-TEasy, isolated using digestion with the restriction enzyme XbaI (20,000U/ml New England BioLabs) and purified by gelpurification using the kit Nucleo Spin Extract (Macherey-Nagel).

Radioactive labelling was performed applying 50-100ng of DNA. The probe was labelled with [\textit{α}\textsuperscript{32P}]dCTP (Amersham Pharmacia Biotech) using the Ladderman\textsuperscript{TM} Labelling Kit (Takara) and purified using ProbeQuant\textsuperscript{TM} G-50 Micro Columns (Amersham Pharmacia Biotech).

After DNA transfer, the membrane was incubated at 80°C for 1h to fix the DNA permanently to the membrane. The membrane was then pre-hybridised in QuickHyb\textsuperscript{®} hybridisation solution (Stratagene) at 65°C for 60min. For hybridisation of the DNA fragments the radio-labelled probe was added and incubated at 65°C overnight. After washing the blot in Church-buffer (appendix 8.1), the DNA fragments were visualised with the phosphoimager technique (BAS 2500, Fujifilm) or x-ray film (Kodak Biomax MS).

2.5 Genotyping using PCR

To genotype IL-10R\textsuperscript{Fl/Fl} mice and the different \textit{Cre} mouse strains, respectively, the three following PCR-protocols were applied. The genotype was confirmed for all mice that were used experimentally.

2.5.1 Detection of the IL-10R flox allele

IL-10R PCR-1 and the primers LoxP and LoxP-3 were used to detect loxP3 (Fig. 4).
Primers:
LoxP: 5’-GGCCGCATAACTTCGTATAGCA-3’
LoxP-3: 5’-CCCAAGGCCAGTGGAGCCAGC-3’

<table>
<thead>
<tr>
<th>1 reaction</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5µl 10X reaction buffer</td>
<td>95°C 2min initial denaturation</td>
</tr>
<tr>
<td>0.5µl 10mM dNTPs</td>
<td>95°C 45sec denaturation</td>
</tr>
<tr>
<td>0.625µl 10µM primer LoxP</td>
<td>63°C 45sec annealing</td>
</tr>
<tr>
<td>0.625µl 10µM primer LoxP-3</td>
<td>72°C 45sec extension</td>
</tr>
<tr>
<td>0.1 5U/µl Ampli Taq polymerase</td>
<td>72°C min final extension</td>
</tr>
<tr>
<td>1µl template</td>
<td>4°C hold store</td>
</tr>
<tr>
<td>fill to 25µl H₂O</td>
<td>denaturation, annealing and extension were repeated for 35 cycles</td>
</tr>
</tbody>
</table>

Subsequent to PCR, the products were subjected to gel electrophoresis on ethidium bromide stained gels. IL-10R PCR1 gave a product of 600bp for the flox product. Wildtype DNA did not give a product in this PCR.

2.5.2 Differentiation between IL-10R<sup>Fl/Fl</sup>, IL-10R<sup>Fl/wt</sup> and IL-10R<sup>wt/wt</sup> mice
To differentiate between IL-10R<sup>Fl/Fl</sup>, IL-10R<sup>Fl/wt</sup> and IL-10R<sup>wt/wt</sup> mice IL-10R PCR-2 was performed using the primers LoxP-1 and fLoxP-1 that are flanking loxP1 (Fig. 4).

Primers:
LoxP-1: 5’-CCACCAAGAGTCAGGTAGGGAC-3’
fLoxP-1: 5’-GAGCTTGGGAACCTCCGCAG-3’

<table>
<thead>
<tr>
<th>1 reaction</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5µl 10X reaction buffer</td>
<td>95°C 5min initial denaturation</td>
</tr>
<tr>
<td>0.5µl 10mM dNTPs</td>
<td>95°C 15sec denaturation</td>
</tr>
<tr>
<td>0.5µl 10µM primer LoxP-1</td>
<td>56°C 1min annealing</td>
</tr>
<tr>
<td>0.5µl 10µM primer fLoxP-1</td>
<td>72°C 1min extension</td>
</tr>
<tr>
<td>0.5 5U/µl home Taq</td>
<td>72°C 10min final extension</td>
</tr>
<tr>
<td>1µl template</td>
<td>4°C hold store</td>
</tr>
<tr>
<td>fill to 25µl H₂O</td>
<td>denaturation, annealing and extension were repeated for 35 cycles</td>
</tr>
</tbody>
</table>
Gel electrophoresis revealed a 300bp band for the wildtype and a 350bp band for the flox allele.

### 2.5.3 Detection of Cre

Cretot PCR and the primers cretot-1 and cretot-2 was applied to detect Cre in all the Cre mouse strains.

Primers:

cretot-1: 5'- ACG ACC AAG TGA CAG CAA TG-3'
cretot-2: 5'- CTC GAC CAG TTT AGT TAC CC-3'

<table>
<thead>
<tr>
<th>1 reaction</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5µl</td>
<td>10X reaction buffer</td>
</tr>
<tr>
<td>0.5µl</td>
<td>10mM dNTPs</td>
</tr>
<tr>
<td>0.5µl</td>
<td>10µM primer LoxP</td>
</tr>
<tr>
<td>0.5µl</td>
<td>10µM primer LoxP-3</td>
</tr>
<tr>
<td>0.5</td>
<td>5U/µl home Taq</td>
</tr>
<tr>
<td>1µl</td>
<td>template</td>
</tr>
<tr>
<td>fill to 25µl</td>
<td>H₂O</td>
</tr>
</tbody>
</table>

Cretot PCR gave a product of 350bp for the Cre⁺ product. Wildtype DNA did not give a product in this PCR.

### 2.6 Cell sorting

#### 2.6.1 Isolation of cells from the spleen

Mice were euthanised with CO₂ and the spleen was excised. The spleen was homogenised using a cell strainer with pores of 100µm for homogenisation. Cells were re-suspended in 5ml PBS/0.2%BSA and stored on ice until further processing. After centrifugation at 250xg, the cell pellet was incubated 10min at room temperature with 3ml lysing buffer (BD Pharm Lyse™, BD Biosciences Pharmingen) for erythrolysis. The
suspension was filled up to 50 ml with PBS/0.2%BSA, centrifuged and the pellet re-suspended in 5ml PBS/0.2%BSA. To determine the cell number, a dilution of 1/50 was counted in a Neubauer counting chamber.

2.6.2 Isolation of cells from the peritoneal cavity

Mice were sacrificed and the fur was carefully removed. With a sterile needle, PBS/0.2%BSA was injected into the peritoneal cavity. Lavage-fluid was removed with a Pasteur-pipette. The cell suspension was further processed as the cell suspension obtained out of the homogenised spleen, but resuspended in only 1ml of PBS/0.2%BSA in the last step.

2.6.3 Antibody-stainings for FACS analyses

FACS (Fluorescence Activated Cell Sorting) was utilised to sort cells into different cell types using fluorescent labelled antibodies against surface molecules. Cells isolated by the technique described above were sorted into CD19\(^+\) B-cells, CD3\(^+\)/CD8\(^+\) or CD4\(^+\)/CD8\(^+\) T-cells and F4/80\(^+\) macrophages using specific antibodies (Tab. 2). An amount of 1x10\(^6\) to 2x 10\(^6\) cells was used for the staining.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Label</th>
<th>Specificity</th>
<th>Concentration</th>
<th>Cat-No</th>
<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td>CD19</td>
<td>APC</td>
<td>anti-mouse</td>
<td>0.2mg/ml</td>
<td>550992</td>
<td>BD Biosciences Pharmingen</td>
</tr>
<tr>
<td>CD3e</td>
<td>PE-Cy5</td>
<td>anti-mouse</td>
<td>0.2mg/ml</td>
<td>553065</td>
<td>BD Biosciences Pharmingen</td>
</tr>
<tr>
<td>CD8a</td>
<td>FITC</td>
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<td>553031</td>
<td>A Becton Dickinson</td>
</tr>
<tr>
<td>F4/80</td>
<td>RPE</td>
<td>anti-mouse</td>
<td>0.1mg/ml</td>
<td>MCA497PE</td>
<td>Serotec</td>
</tr>
<tr>
<td>CD4</td>
<td>PE</td>
<td>anti-mouse</td>
<td>0.2mg/ml</td>
<td>553730</td>
<td>A Becton Dickinson</td>
</tr>
</tbody>
</table>

Tab. 2 Antibodies used for cell sorting
Propidium iodide (2mg/ml in PBS/0.1%BSA) was applied to stain for dead cells. Cell sorting was conducted with the MoFlo cell sorter (Cytomation). The purity of the sorted cell populations ranged between 90% and 99.9%. Sorting was performed in 2 independent experiments for each mouse strain. The first experimental groups contained 3 animals, Cre− and Cre+ and CD3+/CD8+ was used to sort for T-cells. Because the amount of cells harvested was only modest and the CD4 antibody gave a better purity of the sorted T-cells, a group of 4 mice (Cre− and Cre+) was pooled for the following experiments and CD4/CD8 was used for the sorting of T-cells. All mice were at least 3 months old.

2.7 Mouse necropsy

Mice were sacrificed by CO2 asphyxiation. The peritoneal cavity was opened and the entire gastro-intestinal tract removed. 3.5% neutrally buffered formaldehyde (appendix 8.1) was injected into the intestine that was then divided into 4 parts: oral half of the small intestine, aboral half of the small intestine, caecum and colon. Small intestine and colon were rolled up to “Swiss roles” and every part was separately placed in one biopsy-cassette. Spleen, mesenteric lymph nodes and mesenteric fat including the pancreas, as well as a cross section of the liver was placed into the last biopsy-cassette. All tissues were immediately fixed in 3.5% neutral buffered formaldehyde for 24 to 48 hours.

2.8 Paraffin sections

Fixed tissue specimens were dehydrated through a graded series of ethanol, cleared in xylol and infiltrated with paraffin in a dehydration automat (Citadel 1000, Shandon) using the suggested standard protocol. Specimens were embedded in paraffin (Histoplast, Shandon) and sectioned on a microtome. Slides were dried at 37°C for 4 hours. Hematoxylin-eosin (HE) staining was performed in a staining automat (Varistain XY, Shandon). Sections were first deparaffinised in xylol (2 times, 5min), rehydrated in decreasing concentrations of ethanol (100% 4min, 90% and 70% 3min) and washed in tap water (3min). Hematoxylin staining was performed for 3min using a ready made solution from Merck. After washing in tap water and differentiation in acidified alcohol (1% HCl in 70% ethanol, 2min), sections were
counterstained with eosin (Merck) two times for 2min and 4min. The sections were washed in tap water (30sec) and dehydrated in 100% ethanol (3 times, 2min), followed by xylol (2 times, 3min).

2.9 Reticulocyte staining
Blood was taken from the heart and transferred into a tube containing 10µl 0.1M EDTA. The ACCUSTAIN® Reticulocyte Stain (Sigma Aldrich) was applied according to the user’s manual. The number of reticulocytes per 1000 erythrocytes in stained blood smears was counted using a light microscope and 630 fold magnification. Haematocrit was measured on retrobulbar blood samples using a capillary and the centrifuge “Hematocrit 210” (Hettich).

2.10 Treatment with Piroxicam
Ground feed (V 1530-030 ssniff R/M-H) irradiated with 25kGy was purchased from Ssniff. Piroxicam (P5654 Sigma Aldrich) was mixed with the ground feed by geometric dilution at 200ppm and 100ppm. Feed was moistened with drinking water taken from the water bottles of the mice and put into a clean plastic bowl. Feed was refreshed every second day. Animals of 4 weeks of age were fed with the mixture exclusively for 14 days and with the normal pellets for 14 days, subsequently. During the entire experiment bodyweight was measured every second day and clinical symptoms were assessed using the score described in chapter 2.11.1.

2.11 Induction of colitis using DSS
A solution containing 2% DSS (purchased from MP Biomedicals, Cat. No: 160110, MW: 36,000-50,000, lots: 7904H and 9244H) in autoclaved tap water was used to induce colitis in 6 week old mice. Drinking water containing 2% DSS was given for 7 days ad libitum. The DSS treatment period was followed by 7 and 14 days of normal drinking water in the preliminary experiment, and 3 days in the following experiments. During the entire period, bodyweight and clinical symptoms for each mouse were assessed daily. At day 10,
mice were sacrificed by CO₂ asphyxiation. Serum was harvested as described in chapter 2.11.4. The entire colon was excised, rolled up to a Swiss role and fixed in 4% neutrally buffered formaldehyde. Caecum and spleen, pancreas, mesenteric fat and mesenteric lymph nodes were specimens taken additionally.

2.11.1 Clinical score

The clinical score was used to monitor DSS treated mice for animal welfare reasons. It consisted of the symptoms: diarrhoea, occult blood in the faeces, blood around the anus, piloerection, hunchback and apathy. If these symptoms were observed, they were graded as 1 for each symptom. To reduce suffering and pain of the animals to a minimum, a clinical score of 4 resulted in euthanasia of the animal, as well as a loss of ≥ 20% of the initial bodyweight.

2.11.2 Disease index

The disease index summarises clinical symptoms, loss of bodyweight and macroscopical signs visible during necropsy of DSS treated animals (Tab. 3). Gain or 0% loss of bodyweight was graded as 0. Up to 5% loss of bodyweight was graded as 1, 5% to 10% resulted in a grade of 2 and more than 10% was graded as 3. Diarrhoea was graded from 1 to 3 for mild, moderate and severe. Low amounts of blood visible in faeces resulted in a grade of 1, moderate amounts were graded as 2 and if blood was visible around the anus the resulting score was 3. The behaviour of the animal was grades as 1 if piloerection was visible. Mild apathy and a hunchback position resulted in a behaviour score of 2. Moderate to severe apathy was graded as 3. Macroscopically visible inflammation of the intestine was graded as 1, if faeces were not well formed, 2 if swelling was present, 3 if swelling and hyperaemia were present.

<table>
<thead>
<tr>
<th>Loss of bodyweight</th>
<th>Diarrhoea</th>
<th>Rectal bleeding</th>
<th>Behaviour</th>
<th>Intestinal inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
</tr>
</tbody>
</table>

Disease index per mouse

0-15

Tab. 3 Composition of the disease index applied to estimate the severity of macroscopical signs of colitis
2.11.3 Histological score

The histological scoring (Tab. 4) used to evaluate the severity of colitis in DSS treated mice microscopically, was adapted from the TJL-score, developed for scoring colitis in mice by The Jackson Laboratory [54]. The colon was divided into a proximal (oral), middle and distal (aboral) section, each of about the same size. The three sections were scored for the general criteria: severity, degree of ulceration, degree of oedema, percentage of area involved. The grading was performed blinded to the genetic status of the animals.

Grades applied for severity were 0 = no alterations, 1 = mild, 2 = moderate, 3 = severe alterations. Focally small or widely separated multifocal areas of inflammation limited to the lamina propria were graded as mild lesions (1). Multifocal or locally extensive areas of inflammation extending to the submucosa were graded as moderate lesions (2). If the inflammation extended to all layers of the intestinal wall or the entire intestinal epithelium was destroyed, lesions were graded as severe (3).

Ulceration was graded as: 0 = no ulcer, 1 = 1-2 ulcers (involving up to a total of 20 crypts), 2 = 1-4 ulcers (involving a total of 20-40 crypts) and 3 = any ulcers that exceed the previous.

As hyperplasia was only occasionally found in specimens, the score was replaced by a score of oedema. Oedema was graded as 1 if only mild epithelial or submucosal oedema (less than the muscular layer in thickness) was present. Mild epithelial oedema associated with mild submucosal oedema or more moderate submucosal oedema (1 to 2 times as thick as the muscular layer) was graded as 2. Every oedema more extensive than the previous was graded as 3.

A 10% scale was used to estimate the area involved into the inflammatory process. 0 = 0%, 1 \leq 30\%, 2 = 40\% -70\%, 3 = > 70\%.

The scores were added up to a total of up to 12 per section and the scores of the three sections to a total of up to 36 per colon sample. Tab. 4 summarises the score applied for the histological examination of the colon of mice after DSS exposure.
MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Severity</th>
<th>Ulceration</th>
<th>Oedema</th>
<th>Area involved</th>
<th>Score</th>
<th>Section</th>
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<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
<td>0-12</td>
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</tr>
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<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
<td>0-12</td>
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<tr>
<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
<td>0-12</td>
<td>distal</td>
</tr>
</tbody>
</table>

**Tab. 4** Score applied for histological scoring of the colon of DSS treated mice

2.11.4 Serum analysis

Blood samples were harvested directly from the heart using a sterile 200µl syringe and a sterile needle. Via two centrifugation steps of 5,000xg for 8min serum was isolated and stored at -20°C.

For serum cytokine analysis the “Mouse Cytokine Twenty-Plex” kit, Biosource Catalogue #LM0006 was applied according to the user’s manual. The measurement was performed with the “LiquiChip” machine from Qiagen. For the measurement, an amount of 20µl of serum was diluted 1:3.

2.12 Mouse strains

An overview of the mouse strains used in this study is given in Tab. 5. All animal experiments were conducted in accordance to the German law for animal protection and permitted by the “Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit” (AZ 33.42502/07-01.05).
MATERIALS AND METHODS

Complete designation | Abbreviation | Functional aberration | Publication
---|---|---|---
C57BL/6J | B6 | wildtype | 

Complete knock-out strains:

<table>
<thead>
<tr>
<th>Complete knock-out strains:</th>
<th>Abbreviation</th>
<th>Functional aberration</th>
<th>Publication</th>
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<td>IL-4 knock-out</td>
<td>[100]</td>
</tr>
<tr>
<td>B6-Il10&lt;sup&gt;tm1Cgn&lt;/sup&gt;/J</td>
<td>IL-10&lt;sup&gt;-/-&lt;/sup&gt;</td>
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<tr>
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</table>

Floxed strains

<table>
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</tr>
<tr>
<td>C;129P2-Il10r&lt;sup&gt;tm1(flox)Grefswald&lt;/sup&gt;</td>
<td>IL-10R&lt;sup&gt;Fli/Fli&lt;/sup&gt;</td>
<td>IL-10R flox</td>
<td>unpublished</td>
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Cre strains

<table>
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<th>Cre strains</th>
<th>Abbreviation</th>
<th>Functional aberration</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6.D2-Tg(CD4-cre)1Cwi/J</td>
<td>Cd4-Cre</td>
<td>T-cells</td>
</tr>
<tr>
<td>B6-Cd19&lt;sup&gt;tm1(cre)Cgn&lt;/sup&gt;</td>
<td>Cd19-Cre</td>
<td>B-cells</td>
</tr>
<tr>
<td>B6;129P2-Lzm-s2&lt;sup&gt;tm1(cre)Cgn&lt;/sup&gt;</td>
<td>lysM-Cre</td>
<td>macrophages, neutrophils</td>
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<tr>
<td>B6.D2-Tg(KRT14-cre)1Cgn</td>
<td>K14-Cre</td>
<td>oocytes, keratinocytes</td>
</tr>
</tbody>
</table>

Tab. 5 Mouse strains used for breeding and experiments

All mouse strains were bred and maintained at the animal facility of the Helmholtz Centre for Infection Research.

The K14-Cre mouse strain expresses Cre under the human K14 promoter. Cre expression has been found in the epidermis. Additionally, in K14-Cre females, Cre induced deletion occurs in oocytes leading to a deletion of floxed genes in all offspring of K14-Cre females [105]. Thus, the breeding of K14-Cre females with IL-10R<sup>Fli/Fli</sup> males resulted in ubiquitous deletion of IL-10R.

2.13 Mouse facility

The animal housing facility at the Helmholtz Centre for Infection Research (HZI) is specific pathogen free (SPF). It is protected by barriers; personnel are required to wear sterile surgical protective clothing. All materials entering the animal rooms are autoclaved. Drinking water is acidified with sulphuric acid, then filled into bottles and autoclaved. Feed given to all mouse strains ad libitum was autoclaved Ssniff V1534-3. Mice are housed exclusively in
individually ventilated cages (IVC) (Tecniplast, Germany). All mouse strains are imported via embryo transfer using CD1 foster mice originally associated with Charles River Altered Schaedler flora (CRASF®). CRASF® consists of: *Bacteroides distasonis* (Schaedler 19X Bacteroides), *Lactobacillus acidophilus* (Schaedler L1 Lactobacillus), *Lactobacillus salivaris* (Schaedler L3 Lactobacillus), Schaedler fusiform-shaped bacterium (ASF 356), 3 strains of CRL fusiform-shaped bacterium (ASF 492, 500 and 502), CRL mouse spirochete (ASF 457), *Escherichia coli* (non-haemolytic # ICO-IM 1803) and *Streptococcus faecalis* (Enterococcus spp #ATCC 10541). To reduce the amount of LPS in the intestine, the CRASF® used for inoculation was lacking the *E. coli* strain that is contained in the original mixture. Health monitoring is performed on a quarterly basis according to the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA). Additionally, *Helicobacter species* are tested every three months. All test results were negative during the time of the breeding and the experiments for this study.

2.14 Germfree mice

Germfree IL-10⁻/⁻ mice were bred and maintained at the Hanover Medical School central animal facility (Ztm). Germfree C57BL/6 mice were purchased from Burghard Jilge, University of Ulm. DSS treatment of 8 to 9 week old IL-10⁻/⁻ and C57BL/6 mice was conducted by André Bleich according to the protocol used for SPF mice at the HZI. To reduce the risk of varying water quality between the institutes, DSS was dissolved in autoclaved tap water from the HZI. In order to confirm the germfree status of the animals, rectal smears were analysed according to the GV-SOLAS (Society for Laboratory Animal Science) recommendations after cultivation in a thioglycollate-bouillon.

2.15 Statistical analysis

Statistical analyses were performed with Graphpad Prism. For comparisons between 2 groups, Mann Whitney test was used. Kruskal-Wallis test and Dunn’s post test were applied for all analyses containing more than two groups. Differences were considered significant at p<0.05 and marked with *. ** designates p<0.005 and *** p<0.001.
3 Results

3.1 Breeding of conditional IL-10R mutant mice

In order to obtain mice with specific deletion of the IL-10R in T-cells, B-cells and macrophages, the IL-10R^Fl/Fl^ mice were mated with Cd4-Cre^+, Cd19-Cre^+ and lysM-Cre^+ mice. Mating of an IL-10R^Fl/Fl^ male with a K14-Cre^+ female lead to IL-10R^+/^ mice ubiquitously lacking the IL-10R (Fig. 5).

![Diagram of breeding scheme](image)

Fig. 5 Mouse strains used for the breeding of conditional IL-10R knock-out mice
Adapted from Rajewsky et al. [15].

The breeding scheme applied to obtain conditional knock-out mice is shown in Fig. 6. Because of the mixed genetic background, Cre negative (Cre^-) littermates were necessary as negative controls in every experiment.
RESULTS

Genotyping was performed using the PCR strategies described in chapter 2.5. An example of a typical gel picture of IL-10R PCR2 is given in Fig. 7. A homozygous wildtype (wt) animal produced a band of 300bp, PCR of a homozygous floxed animal produced a single flox band of 350bp. A heterozygous animal showed both bands.

As the delta allele, produced by mating a $K14-Cre^+$ female and a IL-10R$^{F/F}$ male, does not give a PCR product by either of the two PCR strategies used, Southern blotting as described in chapters 2.6 and 3.2 was applied for the genotyping of this mouse strain.
Backcross of the IL-10R\(^{Fl/Fl}\) mouse strain to C57BL/6 was conducted simultaneously. B6.129P2-Il10\(^{tm1(flox)Greifswald}\) are now available for the breeding of further conditional IL-10R knock-out mouse strains.

3.2 Genetic analysis of conditional IL-10R knock-out mouse strains

To verify the efficiency and cell type specificity of the Cre mediated deletion, Southern blot analysis of DNA extracted from FACS-sorted cell populations was performed. CD8\(^+\)/CD3\(^+\) T-cells and CD8\(^+\)/CD4\(^+\) T-cells respectively, and CD19\(^+\)B-cells were harvested from the spleen. F4/80\(^+\) macrophages were isolated by peritoneal lavage as described in chapter 2.6. The purity of the sorted cell populations ranged between 90% and 99.9%.

On the Southern blot, the flox band was detectable at a size of 4.8kb, while the delta band had a size of 3.3kb (Fig. 8). For the cell populations sorted from the spleen of IL-10R\(^{Fl/Fl}\) Cd4-Cre mice, a distinct flox band, but no delta band was detected for B-cells of Cre\(^+\) and Cre\(^-\) animals, and T-cells of Cre\(^-\) animals. T-cells of Cre\(^+\) animals showed a distinct delta band, whereas no flox band was visible. Cell populations of IL-10R\(^{Fl/Fl}\) Cd19-Cre mice exhibited a distinct delta band, but no flox band in B-cells of Cre\(^+\) animals. All other cell populations exhibited the flox band only. Concerning the IL-10R\(^{Fl/Fl}\) lysM-Cre mice, solely a clear delta band was detected in macrophages out of peritoneal lavage of Cre\(^+\) animals. A low percentage of deletion, represented by a delta band of low intensity, was found in B-cells of IL-10R\(^{Fl/Fl}\) lysM-Cre\(^+\) mice. For all other cell populations a clear flox band, but no delta band was detected.
RESULTS

Hence, deletion was found to be more than 90% efficient in T-cells of IL-10R<sup>F<sub>i</sub>F<sub>i</sub></sup> Cd4-Cre<sup>+</sup> mice, in B-cells of IL-10R<sup>F<sub>i</sub>F<sub>i</sub></sup> Cd19-Cre<sup>+</sup> mice and in macrophages (macroph.) of IL-10R<sup>F<sub>i</sub>F<sub>i</sub></sup> lysM-Cre<sup>+</sup> mice. The low percentage of deletion in B-cells of IL-10R<sup>F<sub>i</sub>F<sub>i</sub></sup> lysM-Cre<sup>+</sup> mice has been shown previously for IL-10<sup>F<sub>i</sub>F<sub>i</sub></sup> lysM-Cre<sup>+</sup> mice [4]. It is unlikely to have a significant effect on the immune system of the animals as the intensity of the flox band is much greater and the population carrying the delta allele therefore, is a minority only. Thus, inactivation of the IL-10R gene in IL-10R<sup>F<sub>i</sub>F<sub>i</sub></sup> Cd4-Cre<sup>+</sup>, IL-10R<sup>F<sub>i</sub>F<sub>i</sub></sup> Cd19-Cre<sup>+</sup> and IL-10R<sup>F<sub>i</sub>F<sub>i</sub></sup> lysM-Cre<sup>+</sup> mice is efficient and cell type specific.

3.3 Histomorphological phenotyping

Since IL-10<sup>−/−</sup> mice are described as developing IBD spontaneously when they are housed in a SPF mouse facility, the first question that had to be answered was, whether IL-10<sup>−/−</sup> mice housed at the HZI mouse facility spontaneously develop IBD. Subsequently, the
conditional IL-10R knock-out mouse strains were analysed for the spontaneous development of IBD. Therefore, IL-10−/− mice of at least 6 months of age were examined for signs of IBD as well as IL-10R^{Fl/Fl}Cd4-Cre IL-10R^{Fl/Fl}Cd19-Cre and IL-10R^{Fl/Fl}lysM-Cre mice.

3.3.1 Histomorphological phenotyping of IL-10−/− mice

For the histomorphological phenotyping, mice were maintained for at least 6 months in the mouse facility at the HZI. A total of 16 IL-10−/− mice (10 males, 13 months of age, 6 females, 10 and 13 months of age) and 13 C57BL/6 mice (5 males, 6 months of age, 8 females, 9 months of age) were analysed. Neither clinical abnormalities nor macroscopical abnormalities during necropsy were found. HE stained sections of the intestine in its entire length revealed small single foci of inflammatory infiltrations in the lamina propria in 7 IL-10−/− animals. The inflammatory infiltrate consisted of granulocytes, macrophages and lymphocytes (Fig. 9).

![Fig. 9 HE-stained section of the small intestine of a 13 month old IL-10−/− mouse](image)

A: small single focus of inflammatory infiltrates into the lamina propria. B: Infiltrates consisted of lymphocytes, granulocytes and macrophages. A: Bar = 100µm, B: Bar = 25µm

The inflammation was very mild and, most likely, clinically irrelevant. Thus, IL-10−/− mice maintained at the HZI do not spontaneously develop IBD. C57BL/6 animals did not show alterations in histology.
RESULTS

HE stained sections of spleens revealed high amounts of haemosiderin in 5 of the C57BL/6, but only 2 of the IL-10-/- mice. Histological examination of the spleen was repeated in C57BL/6 (3 male, 9 female) and IL-10-/- (6 male, 5 female) mice 4 to 5 months old and gave similar results. Haemosiderin as a storage of iron resulting from the phagocytosis of aged erythrocytes is a normal finding in the mouse spleen. A low amount of haemosiderin suggests a lower renewal rate of erythrocytes in IL-10-/- mice. Reticulocytes are young erythrocytes that still contain residues of their nucleus. The number of reticulocytes correlates to the number of erythrocytes synthesised. In order to follow up the question if IL-10 might have an influence on haematopoiesis, reticulocytes in the blood of C57BL/6 and IL-10-/- mice 4 to 5 months old were counted. In contrast to histomorphology, the result in Fig. 10 shows a significant difference in the number of reticulocytes per 1000 erythrocytes in male, but not in female mice.

Male IL-10-/- mice might have a decreased number of reticulocytes in their blood. Measurement of the haematocrit did not reveal differences between C57BL/6 and IL-10-/- (data not shown).

---

Fig. 10 Comparison of histology of the spleen and reticulocytes count between C57BL/6 and IL-10-/- mice
A: Means of two groups of mice analysed by histology show about 70% female B6 mice with high amounts of haemosiderin in spleen. (Mean ± SEM) B: Significant reduction in number of reticulocytes counted per 1000 erythrocytes in IL-10-/- male, but not female mice compared to C57BL/6. Mann-Whitney test was used to compare medians.
3.3.2 Histomorphological phenotyping of conditional IL-10R knock-out mice

IL-10R\textsuperscript{Fl/Fl}Cd4-Cre and IL-10R\textsuperscript{Fl/Fl}Cd19-Cre mice did not show abnormalities during clinical and macroscopical as well as histological examination. Clinical and macroscopical examination were negative in IL-10R\textsuperscript{Fl/Fl}lysM-Cre\textsuperscript{+} also, whereas histology revealed a slightly enhanced amount of connective tissue between the crypts of the large intestine in 40% of the IL-10R\textsuperscript{Fl/Fl}lysM-Cre\textsuperscript{+} animals, but only 10% in Cre\textsuperscript{-} littermates. Special staining for connective tissue, e.g. Masson Trichrome, could be used as additional test for a mild intestinal fibrosis in IL-10R\textsuperscript{Fl/Fl}lysM-Cre\textsuperscript{+}.

3.4 Evaluation of a model of induction of colitis in IL-10\textsuperscript{−/−} mice

Since no spontaneous development of IBD could be detected in either IL-10\textsuperscript{−/−} or the conditional IL-10R knock-out mouse strains at the age of more than 6 months, a model of induction of colitis in these animals had to be developed in order to answer the question which cell type is the most important producer and target of IL-10 preventing the development of colitis.

3.4.1 Induction of colitis with Piroxicam

Piroxicam was the first agent tested to induce colitis in IL-10\textsuperscript{−/−} mice, because it was established and well described by Berg et al. in 2002. In the first trial, treatment with Piroxicam was performed according to the protocol described by this group [58]. Piroxicam was mixed into ground feed by geometric dilution. Six IL-10\textsuperscript{−/−} mice, 4 to 5 weeks of age, were fed with ground feed containing 200ppm of Piroxicam for 14 days followed by 14 days of normal feed. Surprisingly, 3 of the mice had to be euthanised during the first 4 days of the experiment. Clinical symptoms observed were apathy, hunchback, piloerection and enlargement of the abdomen. Necropsy revealed grossly enlarged small intestines while stomach, caecum and colon were empty. The small intestine was filled with ground feed. Histologically, focal erosion of the epithelium, oedema and serositis were found. In order to test whether the ground feed itself was the reason for these lesions, 6 IL-10\textsuperscript{−/−} mice of 4 to 5 weeks of age were fed with ground feed only. These animals did not show abnormalities in
RESULTS

clinical, macroscopical and histological examination. Thus, a lower dosage of 100ppm of Piroxicam was given to 6 IL-10\(^{-/-}\) and C57BL/6 mice of the same age. This time, one IL-10\(^{-/-}\) mouse was found dead in the cage at day 8. Hunchback, piloerection and loss of bodyweight were symptoms observed at day 7. Necropsy revealed cachexia, diarrhoea, enlargement of the small intestine, unformed faeces, and serositis. Rupture of the small intestine is a possible cause for these observations. All other mice did not show abnormalities in clinical examination throughout the entire experiment. Necropsy and histological examination did not reveal alterations, either. Fig. 11 compares the survival rates in the 3 experiments.

![Fig. 11 Survival rates with different doses of Piroxicam in ground feed](image)

Survival was 100% for IL-10\(^{-/-}\) fed with ground feed and C57BL/6 fed with 100ppm Piroxicam in ground feed.

Colitis could not be induced with 100ppm in IL-10\(^{-/-}\) mice, whereas 200ppm was considered a too high dose. Piroxicam most likely causes erosion of the epithelium of the small intestine resulting in rupture of the intestinal wall. Though geometric dilution was used to mix the Piroxicam powder with the ground feed, this method might not result in a equal distribution, leading to high variations in the concentration of Piroxicam ingested by individual mice. Thus, application of Piroxicam in ground feed is not an appropriate method to induce colitis in IL-10\(^{-/-}\) mice housed at the HZI mouse facility.
3.4.2 Induction of colitis with Dextran Sulfate Sodium (DSS)

As the induction of colitis with Piroxicam failed, treatment with DSS was tested for suitability to induce colitis in IL-10−/− mice. In order to answer the question whether IL-10−/− mice are more susceptible to DSS colitis than C57BL/6, 12 mice of each genotype (6 male, 6 female) and 6 weeks of age were treated with 2% DSS in their drinking water for 7 days. At day 7, 2 male and 2 female mice of each genotype were sacrificed and compared for the severity of colitis. The remaining animals were given normal drinking water until day 14 and 21. At each time point, 2 animals out of each group were sacrificed. During the entire experiment, bodyweight was measured daily and clinical symptoms were assessed using the score described in chapter 2.11.1. Because no differences were observed between male and female mice of the two genotypes, all data are depicted without differentiating between the sexes. Until day 5 no clinical symptoms of colitis were seen. From day 5 and later, both C57BL/6 and IL-10−/− mice showed blood in their faeces and diarrhoea, but symptoms were more frequent in IL-10−/− mice. IL-10−/− mice began losing weight at day 6. At day 10 and 11, three of the IL-10−/− mice showed severe symptoms of colitis including apathy and a loss of bodyweight of more than 20%. Thus, these animals were considered moribund and euthanised. C57BL/6 mice recovered starting at day 10, whereas in IL-10−/− mice diarrhoea and weight loss were present until the end of the experiment at day 21. Differences in clinical score and bodyweight between C57BL/6 and IL-10−/− were greatest at day 10 (Fig. 12).
RESULTS

A

![Graph showing bodyweight and clinical score comparison](image)

B

![Graph showing clinical score over time](image)

Fig. 12 Comparison of relative bodyweight and clinical score between IL-10\(^{-/-}\) and C57BL/6 mice upon DSS exposure

IL-10\(^{-/-}\) mice lost weight and exhibited clinical symptoms starting at day 6, whereas C57BL/6 were only marginally affected. The greatest difference in bodyweight and clinical score was around day 10. # at day 10 and 11 in total 3 animals were moribund and had to be euthanised. Graphs show mean ±SEM.

The severity of lesions seen in histology decreased in C57BL/6 mice from day 7 to day 21, whereas lesions seen in IL-10\(^{-/-}\) mice stayed at the level seen at day 7. The 3 animals euthanised at day 10 and 11 were included into the group of day 14. Histological scoring revealed the largest difference in the group of animals sacrificed at day 21 (Fig. 13).
Lesions seen in the colon at day 7 were crypt erosion, ulceration and oedema. The amount of inflammatory infiltrates was low compared to day 14 and 21, but its distribution was transmural. It consisted of granulocytes, macrophages and lymphocytes, the granulocytes were the dominating component. The lesions found in the colon displayed the same characteristics in C57BL/6 and IL-10^{-/} mice, but were more focally restricted and less extensive in C57BL/6 mice.

At day 14 the main alterations seen in C57BL/6 mice were multifocal crypt erosion and healed ulcers showing re-epithelisation. Macrophages were the predominant cell type of the inflammatory infiltrates, but also lymphocytes and occasionally granulocytes were found. Lesions seen in IL-10^{-/} mice were much more severe and characterised by large ulcerations, oedema and massive transmural acute inflammatory infiltrates dominated by granulocytes admixed with macrophages. Lymphocytes were rarely seen.

At day 21 few focally restricted sites of crypt erosion were observed in C57BL/6 mice. Inflammatory infiltrates consisted of macrophages and lymphocytes. In IL-10^{-/} mice extensive ulcerations and oedema were still observed at day 21. Inflammatory infiltrates were
RESULTS

transmural and consisted mainly of granulocytes, but also macrophages and lymphocytes were seen in large numbers.

Hence, lesions caused by 2% DSS were characterised by an acute suppurative ulcerative colitis at day 7 that was moderate in C57BL/6 and severe in IL-10\(^{-/-}\) mice. In C57BL/6 mice, lesions healed and a mild chronic fibrotic colitis was observed at day 14 and 21. In IL-10\(^{-/-}\) mice, lesions did not heal, but developed into severe chronic suppurative ulcerative colitis at day 21.

Fig. 14 Lesions seen in the colon at day 21 after exposure to 2% DSS
A: Mild chronic colitis in a C57BL/6 mouse; focal crypt distortion and inflammatory infiltrates dominated by macrophages and lymphocytes. B: Severe chronic suppurative ulcerative colitis in an IL-10\(^{-/-}\) mouse; extensive ulceration leading to destruction of the epithelium; transmural inflammatory infiltrate consisting of granulocytes, macrophages and lymphocytes. A+B: Bar = 100µm.

An additional lesion observed throughout all the histological sections of DSS treated animals was a mild squamous metaplasia of the anal-rectal transition zone.
Furthermore, light-microscopy revealed no alterations in the small intestine. Lesions seen in the caecum consisted of mixed cellular inflammatory infiltrates and ulcerations. Addition of the caecum score to the colon score did not influence the differences between C57BL/6 and IL-10<sup>−/−</sup> mice after DSS treatment. Thus, colon score was considered a valuable tool to detect strain differences.

Although the difference in histological score and characteristics of the lesions was largest at day 21, day 10 was chosen as the end of the experiment for all further experiments, because 3 IL-10<sup>−/−</sup> mice were found moribund and bodyweight curve and clinical score showed the greatest difference at this time point.

### 3.5 Validation of the DSS model – comparison of C57BL/6 and IL-10<sup>−/−</sup> mice

To further validate the model of induction of colitis using DSS, C57BL/6 and IL-10<sup>−/−</sup> mice were analysed after receiving 2% DSS for 7 days followed by a 3 day period of drinking water without additives. During these 10 days, IL-10<sup>−/−</sup> mice lost up to 20% of their
initial bodyweight, whereas C57BL/6 mice were only mildly affected by DSS (Fig. 16A). Clinically, IL-10\(^{-/-}\) mice showed a hunchback posture, piloerection and diarrhoea at day 10. Mild apathy was observed in some animals as well. Necropsy revealed swelling of the colonic wall and reduced to absent formation of faecal pellets. Sometimes hyperaemia of the colonic wall could be seen in addition. Both swelling and hyperaemia of the colonic wall were most intense and frequent in the distal to rectal part of the large intestine. Mesenteric lymph nodes were swollen. C57BL/6 mice exhibited neither clinical nor macroscopical signs of colitis at day 10. These observations were analogous to the ones made in the previous experiment (chapter 3.4.2).

The disease index summarises the clinical and macroscopical signs (chapter 2.11.2). A significant increase in disease index in IL-10\(^{-/-}\) mice is shown in Fig. 16B. Measurement of the lengths of the colon revealed significant shortening in male IL-10\(^{-/-}\), whereas females did not show significant differences (Fig. 16C) (The colon is shorter in female than in male mice, thus data can only be analysed separately). Histological colon scoring revealed a significantly higher value for both male and female IL-10\(^{-/-}\) mice compared to C57BL/6 (Fig. 16D). No sex dependant differences were found for bodyweight, disease index and histological score.
Fig. 16 Comparison between C57BL/6 and IL-10−/− mice after exposure to 2% DSS for 7 days and 3 days normal drinking water, n = 7
A: Loss of bodyweight in IL-10−/−, but not in C57BL/6. (Mean ± SEM) B: Increase in disease index in IL-10−/−. (Mean ± SEM). Kruskal-Wallis test revealed significant differences in means. C: Significant shortening of the colon in male IL-10−/−. D: Colon scores are significantly increased in IL-10−/−. C+D: Medians were compared using Mann-Whitney test. A+B+D: No significant differences between males and females.
RESULTS

Histology revealed infiltrations of inflammatory cells in varying degrees. Infiltrates consisted of granulocytes, macrophages and lymphocytes, while granulocytes were the dominating cell population. Ulcerations of differing dimensions and oedema could be seen in addition. Lesions did not differ in their characteristics dependant on the \textit{IL-10} knock-out, but only in their grade of severity: In \textit{IL-10}^{-/-} mice ulcerations and oedema were extending over larger parts of the epithelium and cell infiltrations were mostly transmural, whereas in C57BL/6 mice lesions were more focally restricted and infiltrates extended only into the lamina propria. Lesions were most severe and frequent in the distal part of the colon in both mouse strains. Examples for mild, moderate and severe lesions found in the colon of both C57BL/6 and \textit{IL-10}^{-/-} mice are depicted in Fig. 17.
Fig. 17 HE-stained sections of the colon. A-C Examples for types of lesions seen at day 10 after exposure to 2% DSS
RESULTS

Differences in disease index and histological score underline the conclusion that \( \text{IL-10}^{-/-} \) mice are more susceptible to DSS induced colitis than C57BL/6 mice, confirming the result obtained in chapter 3.4. Thus, bodyweight curve, disease index and histological colon score after 7 days of 2% DSS and a 3 days recovery period can be used to analyse conditional knock-out mouse strains. Histological score seems to be the most sensitive parameter. The experiment did not reveal sex differences.

3.5.1 Serum cytokine levels of C57BL/6 and IL-10\(^{-/-}\) mice

In order to characterise the DSS model further, serum cytokine analysis was performed with the *Mouse Cytokine Twenty Plex kit* (chapter 2.11.4). In one experiment, 20 cytokines can be measured in 80 serum samples using this kit. Analysis revealed significant induction of the proinflammatory cytokines IL-1\(\alpha\), IL-1\(\beta\), IL-6, IL-17 and TNF\(\alpha\) in IL-10\(^{-/-}\) mice after DSS exposure (Fig. 18).
Fig. 18 Proinflammatory cytokines in serum of C57BL/6 and IL-10−/− mice after exposure to 2% DSS

0 = control, DSS = exposed to 2% DSS. Significant increases were found for all cytokines in IL-10−/− mice treated with 2% DSS. *, **, *** significant difference. Kruskal-Wallis test and Dunn’s post test were applied to compare medians.
RESULTS

IL-12 was the only Th1 immune response related cytokine significantly induced in IL-10\(^{-/-}\) mice upon DSS exposure (Fig. 19A). IFN\(\gamma\) could not be detected in any of the control and DSS treated animals (Fig. 19B). Concerning the Th2 cytokines IL-5 and IL-13, IL-13 was not significantly induced by DSS exposure (Fig. 19C). A small but significant increase was found for IL-5 in DSS treated IL-10\(^{-/-}\) mice compared to C57BL/6 (Fig. 19D). But considering that the difference between untreated and DSS treated IL-10\(^{-/-}\) mice was not significant, the increase of IL-5 can not be judged as a DSS related induction.

Fig. 19 Th1 and Th2 cytokines in serum of C57BL/6 and IL-10\(^{-/-}\) mice

Significant increase in levels of IL-12 and IL-5 in IL-10\(^{-/-}\) mice after DSS exposure. No significant differences were found for levels of IFN\(\gamma\) and IL-13. **: Significant difference. Kruskal-Wallis test and Dunn’s post test were applied to compare medians.
RESULTS

IL-12 is produced by cells of the innate immune system, i.e. macrophages and dendritic cells in response to microbial infection and induces a Th1 immune response [22]. No significant differences were found for serum levels of IL-2, a cytokine related to T-cell proliferation (appendix 8.3.1). Thus, neither a Th2 nor a sufficient Th1 immune response is induced at day 10 upon DSS exposure.

Surprisingly, high levels of IL-10 were found in sera of IL-10<sup>−/−</sup> mice after DSS exposure, though the difference between C57BL/6 and IL-10<sup>−/−</sup> mice was not significant.

![Fig. 20 Levels IL-10 in serum of C57BL/6 and IL-10<sup>−/−</sup> mice](image)

No significant differences were found. Kruskal-Wallis test and Dunn’s post test were applied to compare medians.

Cross-reactivity of the used IL-10 antibody to another IL-10-like cytokine might be the reason for this finding. Cross-reactivity of the IL-10 antibody to IL-2 and IL-4 were negatively tested according to information form the supplier of the Mouse Cytokine Twenty Plex kit (Biosource personal communication). Whether other IL-10-like cytokines cross-react with the antibody of the kit can be tested by serum proteomics of the reagent bound to the antibody. Serum levels of IL-10 were neglected for all further experiments.

Chemokines that attract and activate especially macrophages and neutrophils, but also T-cells (MCP-1, MIG, MIP-1α, KC and GM-CSF) were strongly induced in IL-10<sup>−/−</sup> mice after DSS exposure (Fig. 21).
Fig. 21 Chemokines in serum of C57BL/6 and IL-10⁻/⁻ mice

Significant increases were found for all chemokines in IL-10⁻/⁻ mice treated with 2% DSS. Kruskal-Wallis test and Dunn’s post test were applied to compare medians.
No significant differences were found in serum levels of IP-10, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (appendix 8.3.1).

Furthermore, the correlation of histological colon score to cytokine level was assessed. Significant correlation could be shown for IL-β, TNFα and MCP-1 to colon score for IL-10\(^{-/-}\), but not for C57BL/6 mice. Thus, a high colon score correlates to high levels of IL-β, TNFα and MCP-1 in IL-10\(^{-/-}\) mice treated with 2% DSS. The correlation of colon scores to serum levels of TNFα and MCP-1 is depicted in Fig. 22.

In conclusion, serum cytokine analysis revealed a strong induction of proinflammatory cytokines and chemokines in IL-10\(^{-/-}\) mice at day 10 after exposure to 2% DSS. The immune response at day 10 is an innate immune response. Th1 and Th2 cytokines are not important in this model. The severity of lesions found in histology correlates well to the induction of proinflammatory cytokines. The predomination of granulocytes seen correlates to high amounts of neutrophil and macrophage specific chemokines.
During the course of the experiments, a new lot of DSS had to be applied. No differences were found in comparison of the different values measured (bodyweight, disease index, colon score and serum cytokine levels) for the two lots.

3.6 **Induction of colitis in IL-10R<sup>−/−</sup>**

After IL-10<sup>−/−</sup> mice were proven to be more susceptible to DSS than wildtype mice, the question whether the complete knock-out of IL-10R would lead to a comparable increase in susceptibility to DSS had to be answered. IL-10R<sup>−/−</sup> mice and their IL-10R<sup>+/−</sup> littermates as controls were analysed after exposure to DSS, because of the mixed genetic background of the IL-10R<sup>−/−</sup> mouse strain. IL-10R<sup>−/−</sup> mice showed a slight loss of bodyweight compared to their IL-10R<sup>+/−</sup> littermates (Fig. 23A). Disease index was only mildly but significantly increased compared to C57BL/6 mice. The difference between IL-10R<sup>−/−</sup> and IL-10R<sup>+/−</sup> mice was not significant (Fig. 23B).

The histological score revealed a significant increase in IL-10R<sup>−/−</sup> mice compared to their IL-10R<sup>+/−</sup> littermates (Fig. 24). The characteristics of the lesions seen in colons of IL-10R<sup>−/−</sup> versus IL-10R<sup>+/−</sup> mice were the same as observed in colons of IL-10<sup>−/−</sup> versus C57BL/6.
RESULTS

Fig. 24 Comparison of histological colon scores between IL-10R\textsuperscript{+/+} and IL-10R\textsuperscript{−/−} littermates
IL-10R\textsuperscript{−/−} mice exhibited enhanced colon scores. Medians were compared using Mann-Whitney test.

Measurement of serum cytokines of IL-10R\textsuperscript{+/+} and IL-10R\textsuperscript{−/−} mice revealed a significant induction of the proinflammatory cytokines IL-1\textalpha, IL-1\textbeta, and TNF\alpha compared to C57BL/6. Levels were similar to those found in sera of IL-10\textsuperscript{−/−} mice. No significant difference was found between IL-10R\textsuperscript{+/+} and IL-10R\textsuperscript{−/−} mice. The same result was observed measuring the serum levels of the chemokines GM-CSF, KC, MCP-1, MIG and MIP-1\alpha. Serum levels of TNF\alpha and GM-CSF are shown in Fig. 25 as examples.

Fig. 25 Comparison of serum levels of TNF\alpha and GM-CSF in IL-10R\textsuperscript{+/+} and IL-10R\textsuperscript{−/−} mice
Levels of TNF\alpha and GM-CSF were significantly increased in sera of IL-10R\textsuperscript{+/+} and IL-10R\textsuperscript{−/−} compared to C57BL/6 mice, while the difference to IL-10\textsuperscript{−/−} mice was not significant. **, ***: Medians vary significantly. Kruskal-Wallis test and Dunn’s post test were applied to compare medians.
RESULTS

Differences in cytokine levels between IL-10R\(^{+-}\) and IL-10R\(^{-/-}\) mice were only found for IL-6 and IL-17, where slightly enhanced levels were measured in IL-10R\(^{-/-}\) compared to IL-10R\(^{+-}\) animals (Fig. 26):

A B

**Fig. 26 Comparison of serum levels of IL-6 and IL-17 in IL-10R\(^{+-}\) and IL-10R\(^{-/-}\) mice**

A: Levels of IL-6 were significantly enhanced in sera of IL-10R\(^{-/-}\) compared to C57BL/6 mice. B: A tendency towards higher levels of IL-17 in IL-10R\(^{-/-}\) compared to IL-10R\(^{+-}\), while there is no significant difference to C57BL/6. **, ***: Medians vary significantly. Kruskal-Wallis test and Dunn’s post test were applied to compare medians.

In summary, IL-10R\(^{-/-}\) mice exhibited increased susceptibility to DSS induced colitis compared to C57BL/6 mice as displayed by a significant increase in disease index, histological colon score and serum levels of proinflammatory cytokines and chemokines. Compared to their IL-10R\(^{+-}\) littermates, only the histological score was enhanced. No significant differences could be found in disease index and cytokine and chemokines levels.

Thus, knocking-out *IL-10 R* leads to increased susceptibility to DSS, though the difference between IL-10R\(^{-/-}\) and IL-10R\(^{+-}\) mice was less pronounced than between IL-10\(^{-/-}\) and C57BL/6 mice, respectively. IL-10R\(^{+-}\) mice already showed increased susceptibility to DSS induced colitis as indicated by cytokine and chemokine levels. This might be due to the mixed genetic background of IL-10R\(^{-/-}\) mice: IL-10R\(^{Fl/Fl}\) mice were in the 8\(^{th}\) generation of backcrossing to C57BL/6 when mated with *K14-Cre* mice. Another possible explanation would be that loss of one allele of *IL-10 R* already has an influence on the susceptibility to DSS. Again, histological
RESULTS

scoring was the most sensitive method to detect differences in susceptibility to DSS. Hence, induction of colitis by DSS can be used to detect differences in susceptibility to colitis in conditional IL-10R knock-out mouse strains, although results have to be interpreted carefully with respect to the mixed genetic background of the animals.

3.7 Effect of DSS exposure on germfree C57BL/6 and IL-10−/− mice

So far, the data of this study have shown that IL-10+/− and IL-10R−/− mice are more susceptible to DSS induced colitis than wildtype mice. However, the cause(s) of this increased susceptibility remains to be elucidated. Is a deregulation of the immune response to intestinal bacteria invading into the damaged epithelium the cause for an accelerated inflammation in IL-10−/− mice? In order to clarify the role of intestinal bacteria in induction of colitis by DSS, germfree (GF) C57BL/6 and IL-10−/− mice (8 weeks of age) were treated with 2% DSS. Unexpectedly, both germfree IL-10−/− and C57BL/6 mice were severely affected by DSS. Disease indices were even higher in germfree than in SPF IL-10−/− mice, one C57BL/6 mouse had to be euthanised at day 9, whereas histological colon scores were similar in germfree mice and SPF IL-10−/− (Fig. 27).

![Fig. 27 Comparison of disease index and colon score between germfree (GF) and SPF mice harbouring CRASF® flora (C57BL/6 and IL-10−/−)](image)

A: GF mice had significantly increased disease indices compared to IL-10−/− SPF mice. (Mean ±SEM). B: Colon scores are significantly increased compared to C57BL/6 SPF for IL-10−/− SPF, IL-10−/− GF and C57BL/6 GF mice. Mann-Whitney test was used to compare medians.
RESULTS

Though the grade of epithelial damage was similar as exhibited by histological scores, the number of inflammatory cells infiltrating the colon was strikingly lower in germfree compared to SPF IL-10\(^{-/-}\) mice. However, the distribution of inflammatory cells was transmural, as in SPF IL-10\(^{-/-}\) mice. The reduction in the amount of inflammatory cells seemed to be mainly due to a reduction in the numbers of granulocytes. Macrophages were found to be the dominating cell populations accompanied by lower amounts of lymphocytes and granulocytes. Fibrosis was observed in addition (Fig. 28). The overall severity of the lesions as well as the dimensions of ulceration and oedema in germfree mice was comparable to SPF IL-10\(^{-/-}\) mice.
Fig. 28 HE-stained sections of the colon comparing lesions seen in SPF IL-10\(^{-/}\) to GF mice after exposure to 2\% DSS
A: SPF IL-10\(^{-/}\), severe lesion, large ulceration, massive transmural mixed cellular infiltrates. B: GF IL-10\(^{-/}\), severe lesion, large ulceration, mild inflammatory response combined with fibrosis. A+B: Bar = 100\(\mu\)m. C: Mixed cellular infiltrate (granulocytes, macrophages, lymphocytes) in a SPF IL-10\(^{-/}\) mouse. D: Mixed cellular infiltrate (dominated by macrophages) in a GF IL-10\(^{-/}\) mouse. C+D: Bar = 25\(\mu\)m.
RESULTS

The cytokine profile in germfree mice exposed to 2% DSS was similar to that in SPF IL-10⁻/⁻ mice. A strong induction of the proinflammatory cytokines IL-1α, IL-1β, IL-6 and TNFα was observed. The chemokines MCP-1, MIG, MIP-1α, KC and GM-CSF were found in very high levels as well. No difference was observed between germfree C57BL/6 and germfree IL-10⁻/⁻ mice. In Fig. 29, serum levels of IL-1β and GM-CSF are displayed as examples for proinflammatory cytokines and chemokines.

![Fig. 29 IL-1β and GM-CSF in serum of germfree (GF) and SPF C57BL/6 and IL-10⁻/⁻ mice after exposure to 2% DSS](image)

Significant increase in IL-1β and GM-CSF in GF and SPF-IL-10⁻/⁻ mice. ***: Medians vary significantly.

Kruskal-Wallis test and Dunn’s post test were applied to compare medians.

The Th1 cytokines IFNγ and IL-12 were not significantly induced in germfree mice (appendix 8.3.2). Differences in cytokine levels between germfree and SPF IL-10⁻/⁻ mice were found for IL-13: germfree mice showed increased levels of IL-13, independent of their genetic status (Fig. 30A), suggesting an induction of Th2 cytokines in germfree mice. This finding was underlined by a slight, but not significant, induction of IL-5 in germfree mice (appendix 8.3.2). Another function of IL-13 is the increase the epithelial cell turnover, an effect that is antagonised by IP-10 [34]. IP-10 was found to be significantly induced in germfree mice compared to SPF as well (Fig. 30B), indicating that DSS affects epithelial cell turnover in germfree mice.
RESULTS

Fig. 30 Serum levels of IL-13 and IP-10 in germfree (GF) and SPF C57BL/6 and IL-10−/− mice
Significantly increased levels of IL-13 and IP-10 in GF compared to SPF mice. *, ***: Medians vary significantly. Kruskal-Wallis test and Dunn’s post test were applied to compare medians.

The induction of proinflammatory cytokines and chemokines correlated well to the level of epithelial damage found in histology. Whether the increased levels of IL-13 and IP-10 are induced by DSS or germfree mice have a higher level of IL-13 and IP-10 in general, should be tested by serum analysis of untreated germfree mice. Epithelial damage and cytokine levels were comparable in germfree C57BL/6 and IL-10−/− mice and IL-10−/− mice harbouring intestinal flora.

In conclusion, DSS itself induces epithelial damage that activates a strong innate immune response. Gut bacteria are not necessary to trigger the inflammatory reaction, but are even protective against epithelial damage caused by DSS in an IL-10 dependant manner.

Unfortunately, a contamination occurred during the curse of the experiment: cultivation of rectal smears revealed *Micrococcus luteus* in germfree C57BL/6 and IL-10−/− mice and *Micrococcus lylae* in addition in germfree C57BL/6 mice. Bacterial colonies were only visible after one week of incubation. Cultivation of the DSS solution and the DSS itself was negative. Mice were purchased from two different institutes. Thus, contamination is very unlikely to result from the animals themselves or from the DSS solution. Gram staining of colon sections of germfree IL-10−/− and C57BL/6 mice did not reveal microscopically visible bacterial
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colonisation. Thus, the contamination observed by cultivation was most likely not resulting from the intestines of the germfree animals. Contamination during preparation of the smears is the most probable explanation, especially as *micrococcus luteus* and *lylae* are frequent contaminants that are part of the normal flora of the human skin and the same species (*micrococcus luteus*) was found in 2 groups of animals out of 2 independent experiments.

3.8 Analysis of conditional IL-10R knock-out mice in the DSS model

In order to answer the question which cell type responding to IL-10 might be the most important one in preventing DSS induced colitis, conditional IL-10R knock-out mouse strains specific for the deletion of *IL-10R* in T-cells, B-cells and macrophages and neutrophils were analysed in the DSS model. Bodyweight curves (appendix 8.2) and disease indices did not reveal any significant differences between *Cre* and *Cre* + littermates for IL-10R<sup>F/F</sup> Cd4-Cre, IL-10R<sup>F/F</sup> Cd19-Cre and IL-10R<sup>F/F</sup> lysM-Cre mice, though a tendency towards a higher disease index was observed in IL-10R<sup>F/F</sup> lysM-Cre<sup>+</sup> mice (Fig. 31A). Likewise, histological scores did not differ significantly between *Cre* and *Cre* + littermates of these mouse strains. However, IL-10R<sup>F/F</sup> Cd19-Cre and IL-10R<sup>F/F</sup> lysM-Cre mice exhibited a huge variability in colon scores including very high values (Fig. 31B).
**RESULTS**

Fig. 31 Comparison of disease index and colon score between IL-10R\textsuperscript{Fl/Fl}Cd4-Cre, IL-10R\textsuperscript{Fl/Fl}Cd19-Cre, IL-10R\textsuperscript{Fl/Fl}lysM-Cre and IL-10R\textsuperscript{-/-} mice

No significant differences between Cre\textsuperscript{-} and Cre\textsuperscript{+} littermates for the conditional IL-10R knock-out mouse strains analysed. A: Mean ±SEM. B: Medians were compared with Mann-Whitney test.

Concerning the high variability, the mixed genetic background of all conditional IL-10R knock-out mouse strains has to be taken into account. IL-10R\textsuperscript{Fl/Fl} mice were originally generated in a 129 strain and then backcrossed to BALB/c, while the Cre strains they were mated to were on C57BL/6 background. Repetition of the DSS experiment with these mouse strains after backcrossing to C57BL/6 might reveal Cre dependant differences. Serum cytokine analysis was not performed for the conditional IL-10R mouse strains, because of the
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high variability in colon scores and because colon score was found to be more sensitive than serum cytokine analysis (chapter 3.5).

In conclusion, the most important target cell of IL-10 in the DSS colitis model was not found. An effect of the macrophage and neutrophil specific deletion of IL-10R might be masked by effects of the genetic background.

3.9 Analysis of conditional IL-10 knock-out mice in the DSS model

In order to find out, which cell type is the most important producer of IL-10 in this model, IL-10\textsuperscript{F\textsubscript{L}/F\textsubscript{L}}Cd4-Cre and IL-10\textsuperscript{F\textsubscript{L}/F\textsubscript{L}}lysM-Cre mice were exposed to 2% DSS. Neither significant loss of bodyweight (appendix 8.2) nor increase in disease index could be seen for both mouse strains (Fig. 32A). Light-microscopy revealed a tendency towards higher colon scores in IL-10\textsuperscript{F\textsubscript{L}/F\textsubscript{L}}Cd4-Cre\textsuperscript{+} mice compared to their \textit{Cre} littermates. In IL-10\textsuperscript{F\textsubscript{L}/F\textsubscript{L}}lysM-Cre mice colon scores were generally enhanced compared to C57BL/6, independent of \textit{Cre} (Fig. 32B).
Fig. 32 Comparison of disease index and colon score between IL-10^{Fl/Fl}Cd4-Cre, IL-10^{Fl/Fl}lysM-Cre and IL-10^{-/-} mice

Disease index and colon score reveal no significant differences between Cre^{-} and Cre^{+} littermates for the conditional IL-10 knock-out mouse strains analysed. A: Bars show mean ±SEM. B: Medians were compared with Mann-Whitney test.

Since histological scoring did not reveal significant differences between Cre^{-} and Cre^{+} littermates for IL-10^{Fl/Fl}Cd4-Cre and IL-10^{Fl/Fl}lysM-Cre mice, serum cytokine analysis was not performed for these mouse strains.

These data show that macrophages and neutrophils as well as T-cells alone are not responsible for the production of IL-10 in this model. The most important producer of IL-10 to prevent the induction of colitis by DSS was not found. However, regarding the tendency towards
higher histological scores in IL-10^{Fl/Fl}Cd4-Cre^{+} mice, IL-10 produced by T-cells might have an impact for the limitation of DSS induced inflammation. Concerning the generally enhanced colon scores of IL-10^{Fl/Fl}lysM-Cre mice, the fact that the lysM-Cre mouse strain was only in the 7^{th} generation of backcrossing to C57BL/6 when mated to the IL-10^{Fl/Fl} mouse strain has to be taken into account.

3.10 Analysis of IL-4^{-/-}IL-10^{Fl/Fl}Cd4-Cre and IL12^{-/-}IL-10^{Fl/Fl}Cd4-Cre mice in the DSS model

The most important producer and the most important target of IL-10 preventing DSS induced colitis was not found in the previous experiments. A tendency towards increased colon scores was observed in IL-10^{Fl/Fl}Cd4-Cre^{+} animals though, suggesting a strong impact of IL-10 produced by T-cells. Subsequently, the question arose whether an additional imbalance of the Th1-Th2 system induced by knocking-out IL-12 or IL-4 might reveal effects of T-cell derived IL-10 in the DSS model. IL12^{-/-} mice have an impaired Th1 immune response [31]. This imbalance towards a Th2 dominated immune response might be balanced by IL-10 from T-cells. In IL-4^{-/-} mice, Th2 responsiveness is reduced, leading to a general enhancement of Th1 immune responses in this model [38]. The additional inability of T-cells to produce IL-10 might accelerate the Th1 immune response and lead to increased susceptibility to DSS. However, bodyweight curve (appendix 8.2) and disease index did not reveal increased susceptibility to DSS in IL-4^{-/-}IL-10^{Fl/Fl}Cd4-Cre and IL12^{-/-}IL-10^{Fl/Fl}Cd4-Cre mice (Fig. 33).
RESULTS

Fig. 33 Comparison of disease index between of IL-4⁻/⁻ IL-10⁺⁻⁺Cd4-Cre, IL-12⁻/⁻ IL-10⁺⁻⁺Cd4-Cre, IL-10⁺⁻⁺Cd4-Cre and IL-10⁻/⁻ mice

No significant differences in disease index between Cre⁻ and Cre⁺ littermates of IL-4⁻/⁻ IL-10⁺⁻⁺Cd4-Cre and IL-12⁻/⁻ IL-10⁺⁻⁺Cd4-Cre. Bars show mean ±SEM.

In contrast, histological scores were enhanced in IL-12⁻/⁻ IL-10⁺⁻⁺Cd4-Cre compared to C57BL/6, independent of Cre. IL-4⁻/⁻ IL-10⁺⁻⁺Cd4-Cre⁺ mice exhibited significantly higher histological scores compared to their Cre⁻ littermates (Fig. 34).

Fig. 34 Comparison of histological colon score between of IL-12⁻/⁻ IL-10⁺⁻⁺Cd4-Cre, IL-4⁻/⁻ IL-10⁺⁻⁺Cd4-Cre, IL-10⁺⁻⁺Cd4-Cre and IL-10⁻/⁻ mice

Significant increase in colon scores in IL-12⁻/⁻ IL-10⁺⁻⁺Cd4-Cre compared to C57BL/6 and in IL-4⁻/⁻ IL-10⁺⁻⁺Cd4-Cre⁺ compared to Cre⁻ littermates. Medians were compared using Mann-Whitney test.
RESULTS

Serum cytokine analysis of proinflammatory cytokines revealed induction of IL-1β whereas IL-6 and IL-17 were not induced in IL-12−/−IL-10Fl/Fl Cd4-Cre and IL-4−/−IL-10Fl/Fl Cd4-Cre mice, independent of Cre (Fig. 35B,C,E). A tendency towards high levels of IL-1α could be seen in sera of IL-12−/−IL-10Fl/Fl Cd4-Cre and Cre+ mice without being statistically significant (Fig. 35A). TNFα levels were found enhanced in IL-12−/−IL-10Fl/Fl Cd4-Cre+ and IL-4−/−IL-10Fl/Fl Cd4-Cre+ animals, though differences were not statistically significant, either (Fig. 35D).
**RESULTS**

Fig. 35 Proinflammatory cytokines in serum of IL-4−/−IL-10<sup>Fl/Fl</sup>Cd4-Cre, IL-12−/−IL-10<sup>Fl/Fl</sup>Cd4-Cre and IL-10<sup>−/−</sup> mice

A: Increased levels of IL-1α in IL-12−/−IL-10<sup>Fl/Fl</sup>Cd4-Cre mice, independent of Cre. B: Increased levels of IL-1β in both, IL-12−/−IL-10<sup>Fl/Fl</sup>Cd4-Cre and IL-4−/−IL-10<sup>Fl/Fl</sup>Cd4-Cre mice, independent of Cre. C: No induction of IL-6 in IL-12−/−IL-10<sup>Fl/Fl</sup>Cd4-Cre and IL-4−/−IL-10<sup>Fl/Fl</sup>Cd4-Cre mice. D: No significant differences in levels of TNFα. E: No induction of IL-17 in IL-12−/−IL-10<sup>Fl/Fl</sup>Cd4-Cre and IL-4−/−IL-10<sup>Fl/Fl</sup>Cd4-Cre mice. **, ***: Medians vary significantly (Kruskal-Wallis test).
RESULTS

Sera of IL-4−/−IL-10F/F Cd4-Cre+ mice contained significantly increased amounts of IL-12 compared to their Cre− littermates and C57BL/6 mice. Serum levels of IL-13 were found enhanced in IL-12−/−IL-10F/F Cd4-Cre, both Cre− and Cre+ littermates (Fig. 36).

Fig. 36 Levels of IL-12 and IL-13 in serum of IL-4−/−IL-10F/F Cd4-Cre, IL-12−/−IL-10F/F Cd4-Cre and IL-10−/− mice
A: Increased levels of IL-12 in IL-4−/−IL-10F/F Cd4-Cre+ mice compared to Cre− littermates. B: Increased levels of IL-13 in IL-12−/−IL-10F/F Cd4-Cre, independent of Cre. ***: Medians vary significantly (Kruskal-Wallis test).

Serum levels of the cytokines GM-CSF, KC and MIG were increased in IL-12−/−IL-10F/F Cd4-Cre and IL-4−/−IL-10F/F Cd4-Cre mice, independent of Cre. As one example of this group of measured chemokines, serum levels of GM-CSF are depicted in Fig. 37.
In summary, IL-12\(^{-/-}\)IL-10\(^{Fl/Fl}\)Cd4-Cre\(^{+}\) mice exhibited increased histological scores that correlated well to the induction of the proinflammatory cytokines IL-1\(\alpha\) and IL-1\(\beta\) and the chemokines GM-CSF, KC and MIG. Thus, the knock-out of IL-12 leads to increased susceptibility to DSS. This increased susceptibility is independent of the production of IL-10 from T-cells. Confirming the specificity of the Mouse Cytokine Twenty-Plex kit, only negligible amounts of IL-12 were found in sera of IL-12\(^{-/-}\)IL-10\(^{Fl/Fl}\)Cd4-Cre\(^{+}\) mice. The lack of IL-12 leads to induction of IL-13 in the DSS model.

IL-4\(^{-/-}\)IL-10\(^{Fl/Fl}\)Cd4-Cre\(^{+}\) mice are more susceptible to DSS induced colitis than their Cre\(^{-}\) littermates, as shown by the increase in histological score and induction of IL-1\(\alpha\) and TNF\(\alpha\). Thus, in the IL-4 deficient background, where the Th1 immune response is enhanced, the lack of IL-10 from T-cells leads to an acceleration of the intestinal inflammation caused by DSS. However, the lack of IL-4 alone already leads to increased levels of the proinflammatory cytokine IL-1\(\beta\) and the chemokines GM-CSF, KC and MIG. IL-12 is induced in IL-4\(^{-/-}\)IL-10\(^{Fl/Fl}\)Cd4-Cre\(^{+}\) mice, but not in Cre\(^{-}\) littermates, to a similar extend as in IL-10\(^{-/-}\) mice. Thus, induction of IL-12 by DSS exposure is inhibited by T-cell derived IL-10.
4 Discussion

The purpose of this thesis was the elucidation of the cellular IL-10 network focussed on the impact of IL-10 on B-cells, T-cells and macrophages, and the impact of IL-10 produced by T-cells and macrophages regarding the prevention of IBD. Therefore, cell type specific IL-10 and IL-10R knock-out mice had to be analysed with respect to the development of colitis. Mouse strains carrying the conditional knock-out of IL-10 specific for various cell types were available, whereas the respective cell type specific IL-10R deficient mouse strains had to be established.

4.1 Establishment of four conditional IL-10R knock-out mouse strains

The first part of this study involved the breeding and the genetic analysis of mouse strains carrying a cell type specific deletion of the \( \text{IL-10R} \) gene in T-cells, B-cells and macrophages. Furthermore, a complete knock-out mouse strain for \( \text{IL-10R} \) had to be established. Mating of the \( \text{IL-10R}^{\text{Fl/Fl}} \) mice to \( \text{Cd4-Cre}^+ \), \( \text{Cd19-Cre}^+ \), \( \text{lysM-Cre}^+ \) and \( \text{K14-Cre}^+ \) mice, which were available at the HZI mouse facility, resulted in the required mouse strains.

Southern blot analysis of sorted cells revealed a cell type specific deletion with an efficiency of more than 90\% in T-cells of \( \text{IL-10R}^{\text{Fl/Fl}} \text{Cd4-Cre}^+ \), B-cells of \( \text{IL-10R}^{\text{Fl/Fl}} \text{Cd19-Cre}^+ \) and macrophages of \( \text{IL-10R}^{\text{Fl/Fl}} \text{lysM-Cre}^+ \) animals (Fig. 7, chapter 3.2). A low percentage of deletion was found in addition in B-cells of \( \text{IL-10R}^{\text{Fl/Fl}} \text{lysM-Cre}^+ \) mice. The same observation was made previously in B-cells of \( \text{IL-10}^{\text{Fl/Fl}} \text{lysM-Cre}^+ \) mice [4]. The intensity of the flox band in B-cells of \( \text{IL-10R}^{\text{Fl/Fl}} \text{lysM-Cre}^+ \) mice though, was much greater than the intensity of the delta band. Therefore, the population carrying the delta allele is only a minority, which is unlikely to have a significant effect on the immune system of the animals. The deletion in B-cells is most probably a result of B-cells originating from the macrophage lineage: In the adult bone marrow, lymphoid and myeloid progenitors are separately producing cells of the lymphoid lineage and the myeloid lineage [106, 107]. However, myeloid progenitor cells from the fetal liver have exhibited a limited B-lymphoid, but no T-lymphoid potential [108,
DISCUSSION

These fetal liver myeloid progenitor cells migrate into the fetal spleen [110, 111]. B-cells originating from this myeloid progenitor cell population found in the fetal spleen might result in the splenic B-cell population carrying the deletion of the \textit{IL-10R} gene seen in the \textit{IL-10R}^{Fl/Fl} lymC-Cre\(^{+}\) mice in this thesis.

Backcrossing \textit{IL-10R}^{Fl/Fl} mice to C57BL/6 was conducted simultaneously; hence, conditional \textit{IL-10R} knock-out mice on the C57BL/6 background will be available soon, allowing experimental results devoid of the variation resulting from the mixed genetic background. Breeding of a \textit{K14-Cre}\(^{+}\) female to a \textit{IL-10R}^{Fl/Fl} male resulted in mice showing solely a delta band in DNA isolated from tail tips, hence carrying the deletion of \textit{IL-10R} ubiquitously.

In summary, the establishment of mouse strains carrying a cell type specific deletion of \textit{IL-10R} in T-cells, B-cells and macrophages and the ubiquitous deletion of \textit{IL-10R} was successful.

4.2 No spontaneous IBD in \textit{IL-10}^{-/-} mice

Because the assessment of the colitis phenotype of conditional \textit{IL-10} and \textit{IL-10R} knock-out mice was the aim of this project, the phenotyping of \textit{IL-10} complete knock-out mice housed at the HZI animal facility with regard to the development of colitis had to be performed in advance.

During histomorphological phenotyping of \textit{IL-10}^{-/-} mice, decreased amounts of haemosiderin were found in spleens of \textit{IL-10}^{-/-} females. In order to test whether this decrease in iron storage could be related to an alteration of erythropoiesis, reticulocytes in blood were counted. The decreased level of reticulocytes seen in blood of male \textit{IL-10}^{-/-} mice together with the reduced iron storage, suggests a reduced erythropoiesis in \textit{IL-10}^{-/-} mice (Fig. 10, chapter 3.3). A micro- or normocytic hypochromic anaemia accompanied by reduced serum levels of iron and reduced iron stores has been reported previously in \textit{IL-10}^{-/-} mice that displayed severe IBD. Anaemia was thought to be subsequent to severe intestinal inflammation. \textit{IL-10}^{-/-} mice exhibited a decreased erythropoiesis, but increased myelopoiesis in bone marrow [53]. The results of this thesis though, suggest that \textit{IL-10} might be a crucial factor stimulating
erythropoiesis. Further investigations are needed to clarify the role of IL-10 in haematopoiesis.

No signs of spontaneous IBD were observed in IL-10\(^{-/-}\) mice housed at the HZI mouse facility, neither clinically nor histologically at the age of 13 months (chapter 3.3.1). The conditional IL-10R knock-out mice did not show signs of IBD, either (chapter 3.3.2). It is known that the severity of the IBD phenotype in IL-10\(^{-/-}\) mice is strongly dependant on the intestinal flora: germfree mice do not show intestinal inflammation [56] and several bacteria induce colitis of distinct characteristics in IL-10\(^{-/-}\) mice: e.g. *Escherichia coli* induces a mild inflammation, whereas colitis induced by *Enterococcus faecalis* is more severe and *Pseudomonas fluorescens* monoassociated IL-10\(^{-/-}\) mice do not show signs of colitis [112]. *Helicobacter hepaticus* can accelerate colitis in IL-10\(^{-/-}\) mice [57]. Moreover, antibiotics with different selective spectra have different therapeutic effects on IBD in IL-10\(^{-/-}\) mice [113], indicating that not only single bacteria but the composition of the bacterial flora influences the development of the inflammation. Conventionally housed IL-10\(^{-/-}\) mice show a more severe phenotype than mice kept under SPF conditions [53], and IL-10\(^{-/-}\) mice housed at different SPF animal facilities differ in the severity of IBD [55].

The flora of the mice housed at the HZI is based on the CRASF\(^{®}\) flora, containing the 8 ASF strains and *Enterococcus faecalis*. Recently, the integrity of this flora in the HZI mouse colony was verified, only a few contaminants *e.g.* *Rumen bacterium* and *Ruminococcus* were found [114]. The SPF mouse colony at the HZI has been free of any pathologic agent listed in the FELASA list as well as of *Helicobacter* species since it was rederived in 2001. One of the conclusions that can be drawn from this work is that neither of the ASF members nor *Enterococcus faecalis* causes colitis in IL-10\(^{-/-}\) mice on a C57BL/6 background. By infection of IL-10\(^{-/-}\) mice housed at the HZI with specific bacterial agents or mixtures of bacteria, the causative agent(s) of IBD in IL-10\(^{-/-}\) might be uncovered.

In the present study though, the IBD phenotype in conditional IL-10 and IL-10R knock-out mice should be determined, leading to the necessity of a model for the selective induction of colitis in IL-10\(^{-/-}\) mice. The use of a different genetic background would have been another
possibility: IL-10⁻/⁻ mice on C3H background for example, are much more susceptible to IBD than IL-10⁻/⁺ mice on C57BL/6 background [55]. However, this approach was considered not appropriate, because all the Cre strains available as well as the IL-10⁻/⁻ mice were on a C57BL/6 background. Thus, a model of specific induction of colitis in IL-10⁻/⁻ mice had to be established.

4.3 Evaluation of a colitis model

4.3.1 Induction of colitis by NSAID treatment

The first model of induction of colitis in IL-10⁻/⁻ mice evaluated, was the treatment with the non-steroidal anti-inflammatory drug (NSAID) Piroxicam. This model is well established and induces colitis in IL-10⁻/⁺, but not in wildtype C57BL/6 mice [58]. Moreover, NSAID can aggravate inflammation in human IBD patients [60], rendering the animal model particularly interesting. Unexpectedly, the induction of colitis using Piroxicam could not be repeated with IL-10⁻/⁻ mice housed at the HZI: only 20% of the mice survived with the dose of 200ppm Piroxicam reported by Berg et al. [58], and the remaining two animals did not exhibit colitis in histology, whereas 100ppm did not induce colitis in IL-10⁻/⁻ mice (Fig. 11, chapter 3.4). These dramatic differences between individuals out of the same group might be due to a failure of uniform distribution of the drug in the ground feed. However, the fact that the IL-10⁻/⁻ mice used by Berg et al. spontaneously developed IBD at the age of 3 months, while IL-10⁻/⁺ mice from the HZI do not show clinically relevant signs of IBD until the age of 13 months, has to be taken into account. NSAID treatment seems to be only suitable to speed up the development of IBD that would develop spontaneously as animals grow older. Differences in the composition of the intestinal flora might be responsible for the discrepancy in the outcome of the treatment of IL-10⁻/⁺ mice with Piroxicam.

4.3.2 Induction of colitis by DSS exposure

Because the induction of colitis in IL-10⁻/⁺ mice by NSAID treatment failed, the second colitis model evaluated was the induction by dextran sulfate sodium (DSS), which was
found to be a suitable model: IL-10$^{-/-}$ mice were shown to be more susceptible to DSS than C57BL/6 control mice (Fig. 13, chapter 3.4). IL-10$^{-/-}$ mice exhibited all the symptoms of IBD over the entire course of the preliminary experiment of 21 days: they lost weight, blood was found in their faeces and diarrhoea and hunchback posture were seen as well, while C57BL/6 mice were only mildly affected. DSS exposure was chosen as a model because it is easy to conduct; DSS can be dissolved in the drinking water of the mice. Furthermore, induction of IL-10 expression by DSS exposure has been shown previously [79, 94]. The same study demonstrated an inhibiting effect of IL-10 treatment on DSS colitis and an aggravation of the colitis by treatment with an anti-IL-10 antibody [94]. A peak of IL-10 was found at day 7 of DSS exposure, correlating well with the severest symptoms seen in IL-10$^{-/-}$ mice from day 8 to 11 in this thesis.

Histologically, a severe suppurative ulcerative colitis was seen in IL-10$^{-/-}$ mice up to day 21 compared to a mild acute suppurative colitis in C57BL/6 at day 7 that developed into a mild chronic colitis. The histological picture of DSS induced colitis in IL-10$^{-/-}$ was similar to the previous observations in wildtype mice after 7 days of DSS exposure: Okayasu et al. showed a severe acute suppurative ulcerative colitis in CBA/J and BALB/c mice at day 8 [69]. A mixed cellular infiltrate consisting of neutrophiles, lymphocytes and plasma cells was seen by Cooper et al. in CBA/J and BALB/c mice at day 7 [77]. In Swiss Webster mice, Dieleman et al. found an increased number of granulocytes and macrophages, but the lesions were resolving into a chronic colitis dominated by macrophages and lymphocytes at day 21, accompanied by an increased production of IFN$\gamma$ [85].

Wildtype mice in the present study exhibited only mild colitis after exposure to 2% DSS. This could be due to different concentrations of DSS used: a concentration of 3-5% was used in the studies cited above. Furthermore, the susceptibility to DSS varies between inbred strains [74] [93] and is dependant of the composition of the intestinal flora, e.g. commensal bacteria can attenuate the colitis [90-92]. A histological phenotype of very mild colitis in C57BL/6 animals exposed to 2% DSS similar to the one observed in the present study has been described by Mannick et al. [115].
The ongoing predominantly acute, suppurative phenotype of the colitis seen in IL-10\(^{-/-}\) mice at day 21 after DSS exposure, suggests that the colitis is caused by a lack of down-regulation of an innate immune response due to the lack of IL-10.

### 4.4 Characterisation of the DSS model

Before the induction of colitis by DSS could be used as a model to analyse the conditional knock-out mouse strains, further characterisation of the model was necessary. A significant difference between IL-10\(^{-/-}\) and C57BL/6 mice was observed in bodyweight, disease index and colon score, confirming the enhanced susceptibility to DSS in IL-10\(^{-/-}\) mice. Male and female IL-10\(^{-/-}\) mice were equally susceptible to DSS induced colitis, though the difference in colon length was only significant between male IL-10\(^{-/-}\) and C57BL/6 mice (Fig. 16, chapter 3.5). Concerning the different values measured, the fact that the grading of the disease index was not conducted in a blinded fashion reduces its reliability. For the measurement of the colon length, the inflammation was most probably not severe enough to lead to a significant shortening in both males and females. Though clinical score and bodyweight measurement did reveal differences between IL-10\(^{-/-}\) and C57BL/6 mice, it was only applied to judge the clinical constitution of the mice for animal welfare reasons. The most valuable tool to grade the severity of DSS induced colitis was the histological colon score. It revealed statistically significant differences between IL-10\(^{-/-}\) and C57BL/6 mice while performed in a blinded fashion. Furthermore, it correlated well to the serum levels of proinflammatory cytokines and chemokines.

By analysis of serum cytokine levels, the reaction of IL-10\(^{-/-}\) mice to DSS exposure at day 10 could be characterised as an innate immune response dominated by proinflammatory cytokines and chemokines (chapter 3.5.1). The increased level of IL-12 observed in IL-10\(^{-/-}\) mice upon DSS exposure indicates an induction of an adaptive Th1 immune response by DSS. IL-12 is produced by innate immune cells such as macrophages and dendritic cells upon microbial infection in order to trigger a Th1 immune response [22]. An enhanced expression of the proinflammatory cytokines TNF\(\alpha\) and IL-1 accompanied by the Th1 cytokines IL-12 and IFN\(\gamma\) upon 7 days of DSS exposure has been shown previously by real-time PCR [79].
Regarding the lack of induction of IFN$\gamma$, IL-5 and IL-13 as well as the T-cell proliferation associated cytokine IL-2 though, the adaptive immune response might not be fully established in IL-10$^{-/-}$ mice at day 10 after DSS exposure. An induction of a mixed Th1/Th2 immune response in the chronic DSS model was previously reported [85]. In agreement with the data obtained in the present study, significant increase in expression of IFN$\gamma$ and IL-5 was only observed 35 days after exposure to DSS. The acute suppurative (dominated by granulocytes) character of the colitis observed in histology in IL-10$^{-/-}$ mice treated with DSS, matches well with the cytokine and chemokine profile of an innate immune response dominated by macrophages and neutrophils.

Thus, this thesis confirms the results found in earlier studies: The inhibitory effect of IL-10 on the release of these proinflammatory cytokines and chemokines by human macrophages and neutrophils that was shown in vitro (reviewed by Moore et al [28] and Murray [39]) could be shown in mice in vivo, though the target cell of IL-10 in this model was not defined, yet. The aggravation of DSS induced colitis accompanied by an enhanced expression of proinflammatory cytokines in wildtype mice by application of an anti-IL-10 antibody shown by Tomoyose et al. [94] was confirmed by using IL-10$^{-/-}$ mice in this study. Hence, the increased susceptibility to DSS induced colitis seen in IL-10$^{-/-}$ mice as well as the cytokine profile are well supported by results reported in literature. This confirms the suitability of this model for the analysis of conditional IL-10 and IL-10R knock-out mice in order to search for the most important cell producing and responding to IL-10 for the down-regulation of the innate immune response. DSS induces an innate inflammatory response that is down-regulated by IL-10 in the wildtype mouse.

The anal-rectal squamous metaplasia seen in IL-10$^{-/-}$ as well as in C57BL/6 mice (Fig. 15, chapter 3.4.2) was previously described in DSS induced colitis in mice: in C57BL/6 mice treated with 15 cycles of DSS over a total of 255 days [116] and in Swiss Webster mice treated with 4 cycles of DSS over a total of 84 days [117]. Why it was not found in studies of acute DSS induced colitis before, can only be speculated. Maybe the damage of the anal-rectal zone intermedia was higher in other studies because of higher concentrations of DSS. Squamous metaplasia might only evolve after a moderate damage to the zone intermedia,
leading to reactive hyperplasia of the epithelium. Squamous metaplasia is a pre-phase to squamous cell carcinoma and can be observed in human ulcerative colitis patients [118]. Hence, exposure of C57BL/6 mice to 2% DSS might be a good model for squamous cell carcinoma avoiding a severe colitis causing a high level of pain for the animals.

4.5 The role of intestinal bacteria in the induction of colitis by DSS

An important question remaining was what causes the induction of proinflammatory cytokines in the DSS model. Is the inflammation caused by an immune response to bacteria invading the mucosa after damage of the mucosal barrier by DSS or by a toxic effect to the epithelium by DSS itself? In order to answer this question, germfree IL-10$^{-/-}$ and C57BL/6 mice were exposed to 2% DSS. Surprisingly, germfree C57BL/6 as well as IL-10$^{-/-}$ mice were even more susceptible to DSS induced colitis than SPF IL-10$^{-/-}$ mice (Fig. 27, chapter 3.7). These results are in agreement with an increase in susceptibility for DSS colitis described previously in germfree IQI/Jic mice [86] and MyD88$^{-/-}$ mice [88]. It was recently demonstrated that the intestinal flora of the mice housed at the HZI animal facility consists of the CRASF® flora and only very few contaminants [114]. Thus, the protective effect is most probably due to one or a mixture of commensal bacteria out of the CRASF®. It was shown recently that commensal or probiotic bacteria such as *Bifidobacterium* can attenuate DSS colitis [90-92].

Bacteria are consequently not inducing the colitis seen in the DSS model, but are even protective. A degradation of DSS by gut bacteria could be the reason for this protective effect. The degradation has been postulated based on the observation of dextranase production by bacterial strains hydrolysing the α-1,6-glycosidic linkage [119-121]. DSS is a dextran additionally containing sulfate esters. Moreover, incubation of dextran with rat faecal content results in hydrolysation of dextran conjugates into small fragments. However, a fragmentation of DSS in the gut lumen of mice can be negated: Kitjiama *et al.* did not observe smaller fragments in polyacryamide gel electrophoresis (PAGE) of DSS isolated from faeces of mice [80]. Furthermore, antibiotic treatment attenuates acute DSS induced colitis [87], a result that cannot be explained if DSS was detoxified by intestinal bacteria. Thus, the protective effect of
intestinal bacteria in the DSS model is most probably not due to a loss of the toxicity of DSS induced by bacterial degradation.

Another explanation for the protective effect of gut bacteria could be an increase of the mucosal barrier due to bacteria or their products. Bacteria can alter the composition of the mucus, for example by degradation of mucus glycoproteins [95] or by adding their products into the mucus. The bacterial product butyrate has an inhibiting effect on the release of proinflammatory cytokines by intestinal epithelial cells [122] and the release of IL-12 by blood monocytes accompanied by an up-regulation of IL-10 production [123]. But again the protective effect of antibiotics leads to the exclusion of this hypothesis.

An increase of the mucosal barrier function could also be achieved by improvement of the tight junctions. Lactobacillus acidophilus has been shown to induce expression of tight junction proteins in gut epithelial cells improving the barrier function of the epithelium [124]. The induction of IL-13 seen in the sera of germfree mice treated with DSS (Fig. 30, chapter 3.7) is especially interesting as high levels of IL-13 have been found in sera of human patients suffering from UC [65]. IL-13 is a Th2 cytokine, but it also enhances the epithelial cell proliferation, e.g. rendering mice resistant for the infection with the worm Trichuris muris [125, 126]. The antagonist of IL-13, inhibiting epithelial cell proliferation, is IP-10 [34], a chemokine, which was induced in germfree mice after DSS treatment as well, indicating that DSS affects epithelial cell proliferation. Moreover, IL-13 has been shown to reduce the mucosal barrier function [127], which might accelerate the susceptibility of germfree mice to DSS induced colitis. Whether germfree mice have generally increased levels of IL-13 and IP-10 needs to be clarified.

The increased susceptibility to DSS induced colitis in SPF- and germfree housed IL-10+/− and germfree C57BL/6 mice observed in this study suggests that the protective effect of the intestinal flora is dependant on the induction of IL-10, which down-regulates the release of proinflammatory cytokines. One open question is: Which cells are the target cells of IL-10?
Cytokine analysis revealed similar levels of proinflammatory cytokines and chemokines in germfree mice compared to SPF IL-10\(^{-/-}\) mice. In contrast, histologically fewer neutrophils were observed invading the lesion site in germfree mice and macrophages were the dominating cell population. The distribution of inflammatory cells was transmural in germfree mice like in SPF IL-10\(^{-/-}\) mice though, and the inflammatory oedema had the same extent in germfree mice as in SPF IL-10\(^{-/-}\) mice. The innate immune response is therefore as strong in germfree as in SPF IL-10\(^{-/-}\) mice, only the recruitment of neutrophils is not as efficient. These results implicate that proinflammatory cytokines are produced by cells other than neutrophils. Macrophages or the damaged gut epithelial cells themselves might be inducing the innate immune response primarily by producing proinflammatory cytokines as a response to the damage induced by DSS. Expression of the proinflammatory cytokine IL-6 and the chemokine IP-10 in gut epithelial cells after infection with *Enterococcus faecalis* for example was shown to be inhibited by IL-10 [128].

Which cell type is the source of the IL-10 down-regulating proinflammatory cytokines? The fact that in germfree mice the amount of T cells is reduced, but the amount of macrophages and CD11c\(^{+}\) dendritic cells is comparable to the amount seen in conventionally housed mice [96], combined with the finding that cells isolated from spleens [97] and mesenteric lymph nodes as well as T-cells from germfree mice produce less IL-10 [98], suggests an important role of T-cells producing IL-10 in this model. A mixture of 8 different lactic acid bacteria ameliorated TNBS induced colitis by inducing IL-10 and IL-10 dependant TGF-\(\beta\) bearing regulatory T-cells [129]. Furthermore, an impaired regulatory T-cell function in germfree mice has been shown recently by two independent groups [97, 98], indicating that the cells producing IL-10 might be regulatory T-cells triggered by intestinal bacteria.

In conclusion, the data obtained by exposure of germfree mice to DSS implicate that DSS induced colitis is caused by a toxic effect to the gut epithelial cells, inducing the production of proinflammatory cytokines leading to further tissue damage. The production of proinflammatory cytokines is inhibited by IL-10. The target cells of IL-10 are most probably gut epithelial cells or macrophages. Intestinal bacteria are not causing the epithelial damage seen in the DSS model, but the damage is due to the toxic effect of DSS to epithelial cells.
DISCUSSION

only. Gut bacteria even have a protective effect against the induction of colitis by DSS. The protective effect is most probably due to the induction of IL-10 producing regulatory T-cells by intestinal bacteria.

4.6 Analysis of the IL-10 network using the DSS model

The analysis of conditional IL-10 and IL-10R knock-out mice in the DSS model was performed in order to answer the question, which cell type is the most important target and which is the most important source of IL-10 down-regulating the inflammatory response in this model.

4.6.1 The most important target cell of IL-10 in DSS induced colitis

In the conditional IL-10R knock-out mice exposed to 2% DSS, no significant difference was observed between Cre+ and Cre− littermates for the mouse strains analysed. However, the deviation of colon scores was high for IL-10R\textsuperscript{Fl/Fl}Cd19-Cre and IL-10R\textsuperscript{Fl/Fl}lysM-Cre mice and the disease index was slightly enhanced in IL-10R\textsuperscript{Fl/Fl}lysM-Cre+ comparable to the disease index in IL-10R−/− mice. Mice of the IL-10R\textsuperscript{Fl/Fl}Cd4-Cre strain though, did not exhibit this high deviation, but were resistant to DSS induced colitis, indicating that T-cells are not important as target cells of IL-10 in this model. The same data implicate that the floxed IL-10R gene itself does not affect the functionality of IL-10R. (Fig. 31, chapter 3.8). A possible effect of IL-10R\textsuperscript{Fl/Fl}lysM-Cre+ might be concealed by the effect of the mixed genetic background of the animals. Given the fact that the main cells present at the site of inflammation were macrophages and neutrophils and that the cytokines and chemokines measured in sera are mainly attracting and activating these cells, macrophages and neutrophils are still the main candidates as the most important target cells of IL-10 in this model. This hypothesis is underlined by the fact that spontaneous development of colitis has been observed in IL-10R\textsuperscript{Fl/Fl}lysM-Cre+ mice housed in a conventional mouse facility (unpublished data, Robert S. Jack). Furthermore, disruption of the STAT3 gene in macrophages and neutrophils, realised by breeding STAT3-floxed with lysM-Cre mice, led to chronic colitis in these animals [130].
Repeating the experiment after backcrossing the IL-10R^{Fl/Fl}Cd19-Cre and IL-10R^{Fl/Fl}lysM-Cre mouse strains to C57BL/6 is necessary to demonstrate the role of B-cells and macrophages and neutrophils in DSS induced colitis. In order to clarify the effect of the genetic background, analysis of single nucleotide polymorphisms (SNP) might reveal differences in susceptibility linked to certain chromosomes of the inbred strains.

However, gut epithelial cells could be the targets of IL-10 in the DSS model as well, as discussed in chapter 4.5. The analysis of mice carrying a specific deletion of IL-10R in gut epithelial cells is necessary in order to verify this hypothesis.

### 4.6.2 The most important producer of IL-10 in DSS induced colitis

The phenotype of IL-10^{-/-} mice after exposure to 2% DSS could not be obtained by the exposure of IL-10^{Fl/Fl}Cd4-Cre and IL-10^{Fl/Fl}lysM-Cre mice to DSS (Fig. 32, chapter 3.9). Thus, macrophages and neutrophils as well as T-cells alone are not the most important producers of IL-10 in this model. However, the tendency of the IL-10^{Fl/Fl}Cd4-Cre^{+} mice to show higher histological colon scores, suggests an important role of IL-10 produced by T-cells in this model. An increase in colon score compared to C57BL/6 was seen in Cre^{+} as well as Cre^{-} IL-10^{Fl/Fl}lysM-Cre mice. A similar effect was observed in IL-10R^{Fl/Fl}lysM-Cre mice (chapter 3.8). lysM-Cre mice were originally generated in 129P2 and the backcross to C57BL/6 was not finished when they were mated with the IL-10^{Fl/Fl} mice, but the lysM-Cre mouse strain was in the 7^{th} generation of backcrossing to C57BL/6. Whether residual parts of the 129P2 genome might lead to increased susceptibility can only be speculated. The knock-out of IL-10 in T-cells was previously reported to be associated with the spontaneous development of colitis. In contrast, the macrophage and neutrophil specific deletion of IL-10 did not result in spontaneous colitis [5]. A crucial impact of IL-10 produced by T-cells in the DSS colitis model might be masked by redundancy or compensatory effects of other T-cell related cytokines. Furthermore, the role of dendritic cells in DSS induced colitis needs to be clarified by cell type specific inactivation of IL-10 in these cells.
DISCUSSION

4.6.3 The role of T-cell-derived IL-10 in a Th1 or Th2 dominated immune response upon DSS exposure

The knock-out of IL-10 in T-cells did not reveal a significant increase in susceptibility to DSS. However, T-cells are still suspected to play an important role in the down-regulation of the inflammatory response in DSS induced colitis. IL-10 is regulating both the Th1 and the Th2 response [38]. The lack of IL-12 leads to an increase in Th2 immune response due a reduced Th1 induction, while the lack of IL-4 leads to an increase in Th1 immune response. To test whether IL-10 from T-cells is able to regulate the inflammatory reaction to DSS in a Th1 or Th2 dominated immune response, IL-12^−/−IL-10^Fl/FlCd4-Cre and IL-4^−/−IL-10^Fl/FlCd4-Cre double knock-out were analysed in the DSS model.

Unexpectedly, the analysis of IL-12^−/−IL-10^Fl/FlCd4-Cre mice after exposure to 2% DSS revealed an increase in colon scores independent of Cre underlined by an increase in serum levels of proinflammatory cytokines and chemokines (chapter 3.10). Thus, the loss of IL-12 increases the susceptibility to DSS induced colitis and IL-10 is not able to inhibit this inflammation. This result was surprising because the inhibition of IL-12 is thought to be attenuating IBD by down-regulating the pathological Th1 immune response. Anti-IL-12 antibodies were shown to abrogate TNBS colitis in mice [68] and were successfully used in clinical trials as a treatment of Crohn’s disease in human patients [131]. The initial hypothesis was that the IL-12 knock-out would be balanced by IL-10 from T-cells leading to an increase in DSS susceptibility in the animals lacking both IL-12 and IL-10 from T-cells.

An induction of IL-13 was observed in the sera of IL-12^−/−IL-10^Fl/FlCd4-Cre animals, confirming the activation of a Th2 immune response upon DSS exposure due to the lack of IL-12. As discussed in chapter 4.5, IL-13 also attenuates the mucosal barrier function and increases epithelial cell proliferation, an effect that might aggravate DSS induced colitis.

The lack of IL-4 leads to non Th2 conditions, where Th1 can be accelerated [38]. Recently, several groups described that IL-10 produced by T-cells down-regulates the Th1 immune response by a self control mechanism of Th1 polarised T-cells [40-42]. In order to test whether an increased Th1 immune response due to the lack of IL4 is accelerated by the lack
of IL-10 from T-cells and whether this might result in increased inflammation upon DSS exposure, IL-4\(^{-/-}\)IL-10\(^{Fl/Fl}\)Cd4-Cre animals were analysed in the DSS model. In agreement with the recent literature, an increased susceptibility to DSS colitis was observed in IL-4\(^{-/-}\)IL-10\(^{Fl/Fl}\)Cd4-Cre\(^{+}\) mice, displayed by a significantly enhanced colon score accompanied by an induction of proinflammatory cytokines and chemokines (chapter 3.10). This confirmed the fact that IL-10 from T-cells can regulate the Th1 immune response even in the Th1 dominated immune response caused by the lack of IL-4. The induction of proinflammatory cytokines and chemokines, was also present in IL-4\(^{-/-}\)IL-10\(^{Fl/Fl}\)Cd4-Cre\(^{-}\) animals, indicating that IL-4 alone can inhibit the release of proinflammatory cytokines and chemokines after DSS exposure.

Increased levels of IL-12 found in the sera of IL-4\(^{-/-}\)IL-10\(^{Fl/Fl}\)Cd4-Cre\(^{+}\) mice but not in their Cre\(^{-}\) littermates demonstrate that IL-10 from T-cells inhibits the production of IL-12. Levels of IL-12 were as high in IL-4\(^{-/-}\)IL-10\(^{Fl/Fl}\)Cd4-Cre\(^{+}\) as in IL-10\(^{-/-}\) mice. Increased levels of IL-12 have been observed previously in IL-10\(^{-/-}\) mice after LPS exposure [5].

In conclusion, the analysis of conditional IL-10 and IL-10R knock-out mice in the DSS model did not clearly reveal the most important producer and target of IL-10. Gut epithelial cells and macrophages remain the strongest candidates as target cells for IL-10. T-cells have been shown to be important sources of IL-10 in the DSS colitis model, in agreement with the results obtained for the prevention of spontaneous colitis, where the lack of IL-10 in T-cells led to colitis [5]. The analysis of IL-12\(^{-/-}\)IL-10\(^{Fl/Fl}\)Cd4-Cre and IL-4\(^{-/-}\)IL-10\(^{Fl/Fl}\)Cd4-Cre mice confirmed the important role of T-cells in this model: the lack of IL-12, which is mainly produced by Th1 cells, as well as the lack of IL-4 in combination with the IL-10 deficiency of T-cells led to an increased susceptibility to DSS induced colitis. Based on the cytokine profile, DSS colitis in IL-10\(^{-/-}\) mice was characterised as inducing an innate immune response (chapter 3.5.1).

The adaptive immunity might not be necessary to induce the inflammation: It was previously shown that B- and T-cell deficient as well as SCID mice are as susceptible to DSS as wildtype mice [82, 84]. Athymic mice that lack only T-cells though, were more susceptible to DSS,
suggesting a protective role of thymus-derived T-cells [83]. The innate immune response is most probably down-regulated by an adaptive immune response, independent of the Th1 or Th2 type of response induced. An accelerated Th1 as well as an accelerated Th2 immune response might aggravate the inflammation. In order to confirm the role of innate and adaptive immunity in DSS induced colitis in IL-10 deficient mice, mice lacking lymphocytes and IL-10, e.g. Rag-1^{-/-}IL-10^{-/-} double knock-out mice, should be tested for their susceptibility to DSS induced colitis.

4.7 General conclusions

In summary, the data obtained in this project implicate that DSS exposure induces colitis by a toxic effect to the epithelium. The damage of the epithelial cells induces the release of proinflammatory cytokines and chemokines, most probably by macrophages and/or epithelial cells. This innate inflammatory reaction is subsequently inhibited by an adaptive immune response, inducing the release of IL-10. A lack of IL-10 leads to a dys-regulated immune response causing further release of proinflammatory cytokines and chemokines, thereby inducing tissue damage resulting in chronic intestinal inflammation.

![Fig. 38 Current hypothesis of the immune response to DSS](image)

**Fig. 38 Current hypothesis of the immune response to DSS**
DSS causes damage of epithelial cells, thereby inducing an innate immune response. The innate immune response triggers an adaptive immune response down-regulating the innate immune response by the production of IL-10 (grey line). The lack of IL-10 leads to a prolonged innate immune response causing further tissue damage and subsequent chronic intestinal inflammation (black dotted line).
The major candidates for the target cells are consequently macrophages and epithelial cells, whereas the cells producing IL-10 are most probably T-cells. The interaction of the immune system with enteric bacteria triggers an IL-10 rich milieu, most probably by the induction of regulatory T cells.

Fig. 39 Current hypothesis of cellular interactions upon DSS exposure
DSS causes damage of the intestinal epithelial cells. These induce an innate immune response in interaction with macrophages and neutrophils. The inflammatory response leads to further tissue damage and inflammation. T-cells produce IL-10 down-regulating the inflammatory response. The IL-10 producing T-cells are induced by gut bacteria and/or their products.
5 Summary

Elucidation of the cellular interleukin-10 (IL-10) network is an important issue for understanding how IL-10 regulates inflammatory responses. The comprehension of such regulatory mechanisms might help find a way to limit the immune response in a chronic inflammation such as Inflammatory Bowel Disease (IBD). The generation and analysis of conditional knock-out mice is a way to demonstrate the cell specific function of a gene in the context of an entire organism. The work in this thesis involves the establishment of 3 conditional IL-10 receptor (IL-10R) mouse strains and the development of a colitis model in order to analyse these strains and conditional IL-10 knock-out mouse strains in addition, for their susceptibility to colitis.

In the first part, 3 conditional IL-10R knock-out mouse strains, specific for B-cells, T-cells and macrophages and neutrophils, were bred and the efficiency and the specificity of the deletion was confirmed by Southern blot analysis of sorted cell populations. Furthermore, a mouse strain carrying the ubiquitous IL-10R knock-out (IL-10\(^{-/-}\)) was established.

The evaluation of a colitis model was the next step conducted, because IL-10 complete knock-out mice maintained at the Helmholtz Centre for Infection Research were found to be devoid of signs of colitis. Induction of colitis using dextran sulfate sodium (DSS) was found to be a suitable model and gave the following results:

IL-10\(^{-/-}\) mice developed a severe acute suppurative colitis, whereas wildtype mice exhibited only a mild colitis. The cytokine profile in IL-10\(^{+/+}\) mice was characterised by proinflammatory cytokines and chemokines. These results demonstrate the induction of an innate immune response by DSS and its inhibition by IL-10.

In order to elucidate the role of the intestinal bacteria in DSS induced colitis, germfree mice were exposed to DSS. Surprisingly, germfree wildtype as well as IL-10\(^{-/-}\) mice were even more susceptible to DSS than IL-10\(^{+/+}\) mice harbouring intestinal flora. This implies that intestinal bacteria are protective against DSS induced damage and that the protective effect is mediated by IL-10. Hence, DSS causes colitis through a toxic effect to the intestinal epithelial cells.
The analysis of the mouse strains carrying a cell type specific deletion of the IL-10R in B-cells, T-cells and macrophages and neutrophils in the DSS colitis model did not reveal significant differences to wildtype controls. The mixed genetic background of the animals might be the cause for the large deviation observed. A backcross of the three conditional IL-10R mouse strains to C57BL/6 might reveal differences that were masked by the background effect in this thesis.

The exposure of T-cell and macrophage and neutrophil specific conditional IL-10 knock-out mice to DSS did not reveal significant differences, either. Mice carrying the specific deletion of IL-10 in T-cells though, exhibited a tendency towards an enhanced susceptibility to DSS.

In order to elucidate the role of T-cell derived IL-10 in a Th1 or Th2 dominated immune response upon DSS exposure, double knock-out mice carrying the deletion of IL-10 in T-cells and the complete knock-out of IL-4 or IL-12 in addition, were analysed in the DSS colitis model. Unexpectedly, the lack of IL-12 led to an increased susceptibility to DSS induced colitis. The lack of T-cell derived IL-10 in combination with the lack of IL-4 increased the susceptibility to DSS induced colitis as well. Hence, a T helper cell immune response seems to down-regulate the innate immune response to DSS induced damage of the epithelium, independent of a Th1 or Th2 polarisation.

In conclusion the data obtained in this study indicate that DSS causes damage to the intestinal epithelium due to a toxic effect, thereby inducing an innate immune response. This innate immune response is inhibited by IL-10. The main IL-10 producing cells are most probably T-cells that are induced by intestinal bacteria. The most important target cell of IL-10 was not found in this study, the cytokine profile of an innate immune response though, suggests a crucial role of macrophages and neutrophils or intestinal epithelial cells.

The analysis of the recently available mouse strain carrying the Cre-transgene for intestinal epithelial cells would be of interest in the DSS colitis model. Furthermore, the role of dendritic cells in this model remains to be determined.
6 Zusammenfassung

Die Analyse des Interleukin-10 (IL-10) Netzwerkes ist notwendig um zu verstehen, wie IL-10 Entzündungsreaktionen reguliert. Das bessere Verständnis solcher immuno-regulatorischen Mechanismen kann helfen, Immunantworten, die chronischen Entzündungen wie z.B chronisch entzündlichen Darmerkrankungen (IBD) zugrunde liegen, einzudämmen. Ziel dieser Arbeit war es, anhand der Analyse konditionaler IL-10 Rezeptor (IL-10R) knock-out Mäuse die Funktion von IL-10 auf T-Zellen, B-Zellen und Makrophagen und Neutrophilen im Hinblick auf die Entwicklung einer IBD zu untersuchen. Des Weiteren sollte die Analyse konditionaler IL-10 knock-out Mäuse zeigen, ob von T-Zellen oder von Makrophagen und Neutrophilen produziertes IL-10 die Entwicklung von IBD verhindert. Dazu wurden zunächst drei konditionale IL-10R knock-out sowie eine IL-10R komplett knock-out Mauslinien gezüchtet und anschließend die Effizienz und Zellspezifität der Deletion des IL-10R mittels einer Southern-Blot-Analyse von FACS sortierten Zellpopulationen nachgewiesen.
Da die am Helmholtz Zentrum für Infektionsforschung gehaltenen IL-10 defizienten Mäuse keine spontane Darmentzündung zeigten, wurde durch Verabreichung von Dextran Sodium Sulphat (DSS) eine Kolitis ausgelöst. Das DSS Modell ergab folgende Ergebnisse: IL-10⁻/⁻ Mäuse entwickelten eine hochgradige akut eitrige Kolitis, während wildtyp Mäuse nur eine geringgradige Kolitis zeigten. Das Zytokinprofil in IL-10⁻/⁻ Mäusen war charakterisiert durch proinflammatorische Zytokine und Chemokine. Die Induktion einer angeborenen Immunantwort durch DSS und deren Hemmung durch IL-10 konnte damit gezeigt werden.
Um zu untersuchen ob die Darmentzündung in IL-10⁻/⁻ Mäusen durch kommensale Darmbakterien ausgelöst wird, wurden keimfreie Mäuse mit DSS behandelt. Unerwarteter Weise zeigten keimfreie Mäuse eine dramatischere Darmentzündung als IL-10⁻/⁻ Mäuse mit normaler Darmflora, unabhängig davon ob sie den IL-10 Knock-out trugen oder nicht. DSS löst demnach eine Darmentzündung durch einen direkten toxischen Effekt auf das Darmepithel aus. Darmbakterien schützen vor der Schädigung durch DSS durch einen IL-10 vermittelten Effekt.
Bei der Analyse der konditionalen IL-10R knock-out Mutanten konnte kein eindeutiger Unterschied zu den Kontrolltieren in den drei untersuchten Linien festgestellt werden. Der Grund dafür könnte in dem gemischten genetischen Hintergrund der Tiere zu finden sein. Eine Wiederholung des DSS Versuches mit den drei Linien nach Rückkreuzung auf C57BL/6 könnte Unterschiede hervorbringen.


Die Analyse von Doppel-knock-out Mutanten, die zusätzlich zu der IL-10 Defizienz in T-Zellen einen Knock-out von IL-12 oder IL-4 trugen, wurde durchgeführt um zu untersuchen, ob IL-10 produzierende T-Zellen in der Lage sind, eine vorherrschende Th1 oder Th2 Immunantwort im DSS Modell zu begrenzen. Unerwarteter Weise löste bereits das Fehlen von IL-12 eine erhöhte Empfindlichkeit gegenüber DSS aus. Das Fehlen von IL-10 in T-Zellen in Kombination mit dem Fehlen von IL-4 führte ebenfalls zu einer erhöhten Empfindlichkeit. Eine T-Helferzell-Immunantwort, unabhängig ob sie Th1 oder Th2 polarisiert ist scheint demnach die angeborene Immunantwort auf die Schädigung des Epithels durch DSS zu regulieren.

Zusammenfassend lässt sich sagen, dass DSS eine toxische Schädigung des Epithels verursacht, die eine angeborene Immunantwort auslöst, welche durch IL-10 gehemmt wird. Die IL-10 produzierenden Zellen sind wahrscheinlich T-Zellen, deren Aktivität von Darmbakterien abhängig ist. Welche die wichtigste Zielzelle von IL-10 in diesem Modell ist, konnte nicht beantwortet werden. Das Zytokinprofil der angeborenen Immunantwort weist jedoch auf Makrophagen und Neutrophile oder Epithelzellen als wichtigste Zielzellen hin.

Seit kurzem sind auch Cre-transgene Mauslinien für Darmepithelzellen verfügbar, deren Analyse in diesem Experiment von Interesse wäre. Des Weiteren bleibt die Rolle der dendritischen Zellen noch zu klären.
REFERENCES

7 References

REFERENCES


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REFERENCES


8 Appendix

8.1 Buffers and solutions

Neutrally buffered formaldehyde, 3.5%
4g $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$
8.125g $\text{Na}_2\text{HPO}_4 \times \text{H}_2\text{O}$
dissolve in 900ml $\text{H}_2\text{O}$
add 100ml Formaldehyde (35%)

TE pH 7.6
2.5ml 2M tris-HCl pH 7.6
1ml 0.5 M EDTA
496.5ml $\text{H}_2\text{O}$

Tail lysis buffer
100ml 2M tris-HCl pH 8.5
20ml 0.5 M EDTA pH 8.0
40ml 10% SDS
80ml 5M NaCl
1760ml $\text{H}_2\text{O}$

Church buffer
500ml 20% SDS
400ml 1M NaHPO$_4$ pH 6.8
9100ml $\text{H}_2\text{O}$
8.2 Additional results – bodyweight curves

A

B

C

D

E
Fig. 40 Bodyweight curves of mice during the DSS experiment
No significant differences were observed for any of the strains analysed. (Mean±SEM)
8.3 Additional results- cytokine measurement

8.3.1 Serum cytokines of IL-10⁻/⁻ and C57BL/6 mice in the DSS model

Fig. 41 Serum cytokines of IL-10⁻/⁻ and C57BL/6 mice after exposure to 2% DSS
A-D: No significant differences. Kruskal-Wallis test was used to compare medians.
8.3.2 Serum cytokines germfree and SPF mice in the DSS model

A. IL-1α

B. IL-6

C. TNFα

D. IL-17

E. IL-2

F. IFNγ
Fig. 42 Serum cytokines germfree and SPF mice after exposure to 2% DSS
8.3.3 Serum cytokines IL-4⁻/⁻IL-10⁺⁺Fl⁻⁺Cd4-Cre and IL12⁻/⁻IL-10⁺⁺Fl⁻⁺Cd4-Cre mice in the DSS model

A. IL-2

B. IL-5

C. IFNγ

D. MCP-1

E. MIG

F. MIP-1α
Fig. 43 Serum cytokines IL-4+/IL-10<sup>Fl<sup>/Fl</sup>Cd4-Cre and IL12<sup>+/</sup>IL-10<sup>Fl<sup>/Fl</sup>Cd4-Cre mice after exposure to 2% DSS

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