**Aus dem Institut für Immunologie**
**des Fachbereichs Veterinärmedizin**
**der Freien Universität Berlin**

# A transgenic probiotic bacterium as a carrier for a nematode immunomodulatory protein for the treatment of intestinal inflammation

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Rose Whelan

**Biologin aus**

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| Dekan: | Univ.-Prof. Dr. Jürgen Zentek |
| Erster Gutachter: | Univ.-Prof. Dr. Susanne Hartmann |
| Zweiter Gutachter: | Univ.-Prof. Dr. Lothar Wieler |
| Dritter Gutachter: | Prof. Dr. Britta Siegmund |

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**List of Abbreviations**

| | |
|---|---|
| ARG1 | arginase 1 |
| ATP | adenosine triphosphate |
| AvCys | *Acanthocheilonema viteae* cystatin |
| CD | Crohn's Disease |
| cDNA | complimentary deoxyribonucleic acid |
| CFU | colony forming units |
| conA | concanavalin A |
| DC | dendritic cell |
| SCID | Severe Combined Immunodeficiency |
| DAB | diaminobenzidine |
| DNA | deoxyribonucleic acid |
| DSS | dextran sodium sulfate |
| EcN | *Escherichia coli* Nissle 1917 |
| EDTA | Ethylenediaminetetraacetic acid |
| EHEC | enterohemorrhagic *Escherichia coli* |
| ELISA | enzyme linked immunosorbent assay |
| ES | excretory/secretory |
| FCS | fetal calf serum |
| Fw | forward |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| GMO | genetically modified organism |
| HBSS | Hanks balanced salt solution |
| H&E | hematoxylin and eosin |
| M-CSF | human macrophage colony stimulating factor |
| HPF | high power field |
| hrs | hours |
| IBD | Inflammatory Bowel Disease |
| IFN-γ | interferon gamma |
| IL- | interleukin- |
| IMDM | Iscove's Modified Dulbecco Medium |
| iNOS | inducible nitric oxide synthase |
| Kn | kanamycin |
| LP | lamina propria |
| LPL | lamina propria leukocytes |
| LPS | lipopolysaccharide |
| µCi | microcurie |
| MCP | monocyte chemotactic protein |
| mg | milligram |
| µg | microgram |
| min | minutes |
| MIP | macrophage inflammatory protein |
| mL | milliliter |

| | |
|---|---|
| µL | microliter |
| mLN | mesenteric lymph node |
| MLR | mixed lymphocyte reaction |
| M | molar |
| mM | millimolar |
| µM | micromolar |
| µm | micrometer |
| MRC | mannose receptor |
| ng | nanogram |
| NO | nitric oxide |
| NSAID | non-steroidal anti-inflammatory drug |
| PBS | phosphate buffered saline |
| RANTES | regulated upon activation, normal T-cell expressed, and secreted |
| rAvCys | recombinant *Acanthocheilonema viteae* cystatin |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| Rv | reverse |
| SEM | standard error of the mean |
| SCID | Severe Combined Immunodeficiency |
| SN | supernatant |
| Sm | streptomycin |
| Tc | tetracycline |
| TER | transepithelial resistance |
| TGF-β | transforming growth factor beta |
| Th- | T helper cell- |
| TLR | toll like receptor |
| TNBS | 2,4,6-trinitrobenzenesulfonic acid |
| TNF-α | tumor necrosis factor alpha |
| Treg | t regulatory cell |
| TSLP | thymic stromal lymphopoietin |
| U | units |
| UC | ulcerative colitis |

# 1. Introduction

## 1.1. Inflammatory Bowel Disease

### 1.1.1. Causes and disease pathology

Inflammatory Bowel Disease (IBD) is a chronic inflammatory disorder that manifests in a dysregulated mucosal immune response against intestinal bacteria. Human IBD can be classified into two main characteristic forms; ulcerative colitis (UC) and Crohn's Disease (CD). UC generally involves T helper cell (Th) 2 and Th17 driven inflammation, leading to superficial ulceration of the colon. CD is characterized by Th1 driven inflammation that leads to isolated, often transmural lesions and may involve the entire gastrointestinal tract (Xavier and Podolsky 2007; Khor et al. 2011). IBD patients show relapsing and remitting disease that is often lifelong.

The pathogenesis of IBD still remains incompletely understood, but the complex etiology involves multiple genetic, immunological and environmental factors. The intestinal microbiota is central for the initiation of IBD development (Jostins et al. 2012). Pathogenic or commensal bacteria trigger microbial sensing systems, which initiate pro-inflammatory responses by innate cells, such as dendritic cells (DCs) and macrophages producing interleukin (IL)-12/23, tumor necrosis factor alpha (TNF-$\alpha$), IL-6, IL-1$\beta$, reactive oxygen species and nitric oxide, which leads to disrupted epithelial barrier function in susceptible individuals. The following activation of the adaptive immune system leads to the strong production of inflammatory cytokines (interferon gamma (IFN-$\gamma$), TNF-$\alpha$ and IL-17A) by T helper cells, which constitute the dominant force driving chronic inflammation in IBD patients (Xavier and Podolsky 2007; Khor et al. 2011). Genetic inheritance is a strong component of IBD development; roughly 100 gene loci are currently linked to IBD susceptibility (Anderson et al. 2011). Pre-disposing polymorphisms linked to IBD development have been found in genes commonly associated with immune reactions to the intestinal flora and gut homeostasis. IBD associated polymorphisms affect pro-inflammatory and regulatory cytokines, their receptors as well as signaling pathways and antigen presentation molecules or epithelial innate defense factors (Anderson et al. 2011; Duerr et al. 2006; Frank et al. 2007; Khor et al. 2011). Finally, lifestyle associated factors like diets rich in fat and animal protein as well as smoking increase the risk to develop IBD (Halfvarson et al. 2006; Hou et al. 2011).

### 1.1.2. Murine models of IBD

While genetic testing, clinical trials and case studies are vital to IBD research, murine models of colitis are also necessary for the elucidation of immunological mechanisms involved in disease onset and propagation. These models are required for preliminary studies of the safety and efficacy of potential IBD therapies prior to clinical trials. Colitis can be induced through administration of inflammatory chemicals, the knock-out of regulatory cytokine expressing genes, or through the transfer of T cells lacking regulatory capacity into immune-compromised mice.

TNBS colitis is an example of a chemically induced colitis model that requires intra-rectal injection of the hapten 2,4,6-trinitrobenzenesulfonic acid (TNBS) in conjunction with ethanol to disrupt the epithelial barrier (Scheiffele and Fuss 2002). The result is the induction of an acute colitic response in approximately 3 days. Colon inflammation is induced by the production of high levels of the pro-inflammatory cytokines IL-12/23 by innate immune cells, subsequently leading to differentiation and activation of Th1/Th17 $CD4^+$ T cells (Scheiffele and Fuss 2002), similar to what is seen in human CD (Neurath et al. 2000).

Another chemical-based colitis model that resembles UC in humans (Hakansson et al. 2014) is induced by the administration of dextran sodium sulfate (DSS) in the drinking water which leads to bloody diarrhea, body weight loss, colonic inflammation and shortening of the colon that can be observed 3-7 days after initial DSS administration (Cooper et al. 1993; Hall et al. 2011; Wirtz et al. 2007; Laroui et al. 2012). While the inflammation resulting from DSS feeding was originally believed to be due to the disruption of the colon epithelial barrier and the subsequent induction of innate immune mechanisms by infiltrating lumen bacteria (Cooper et al. 1993; Hall et al. 2011; Wirtz et al. 2007), it was recently shown that the pathology is caused by the transfer of dextran moieties to the cytoplasm of colonocytes, likely directly inducing inflammatory signaling pathways (Laroui et al. 2012). Within days of the induction DCs, macrophages, and neutrophils are recruited to the gut and lead to an increase production of pro-inflammatory cytokines IL-17, IL-6, TNF-α and IFN-γ, which is consistent with findings in IBD patients (Dieleman et al. 1994; Hall et al. 2011). Early macrophage and neutrophil recruitment in response to invasion of colon bacteria is thought to be the main cause of tissue destruction in the acute DSS colitis model (Hall et al. 2011), while a strong activation of the adaptive immune system occurs in response to repetitive DSS feeding (Hall et al. 2011). Activated T cells, mostly Th17 cells, then migrate to the colon and perpetuate the inflammatory reaction.

One of the most commonly used genetically modified mouse models of colitis is the IL-10$^{-/-}$ knockout mouse model. The lack of IL-10 expression in the IL-10$^{-/-}$ knockout mouse strain leads to an inability of these mice to regulate the IL-12 induced activation of Th1 cells and the resultant expression of IFN-γ from these T cells. Both of these cytokines as well as CD4$^{+}$ T cells are required for the induction of colitis in this model. The disadvantage of the IL-10$^{-/-}$ model is that the colitis induced is spontaneous; however, the administration of the non-steroidal anti-inflammatory drug, piroxicam, to IL-10$^{-/-}$ mice allows for controlled, early onset of colitis in these mice (Berg et al. 2002; Blum et al. 2004).

Rag$^{-/-}$ mice are unable to produce mature T or B lymphocytes (Mombaerts et al. 1992) and transfer of T cells from IL-10$^{-/-}$ mice results in colitis in these mice in much the same way as in the IL-10$^{-/-}$ mice alone, with piroxicam induction allowing for the regulation of disease onset (Blum et al. 2004). This model has the added benefit that IL-10 expression is still functional in non-T cell subsets, like macrophages. Additionally, antigen specific responses can be studied if antigen specific T cells, such as OT2 ova reactive T cells, are transferred (Hang et al. 2010).

The transfer of CD4$^{+}$CD45RB$^{hi}$ T cells into mouse strains lacking functional T lymphocytes strains such as the Severe Combined Immunodeficiency (SCID) (Morrissey et al. 1993; Powrie et al. 1993), or Rag$^{-/-}$ (Ostanin et al. 2006) mice is another cell transfer murine colitis model. CD45RB$^{hi}$ cells have been elucidated to be low IL-10 and IL-4 producers, instead producing more TNF-α and IL-2 (Ten Hove et al. 2004). They are activated, differentiated and expand as Th1/Th17 cells *in vivo* leading to inflammation in the gut (Ostanin et al. 2009). The onset of severe weight loss accompanied by varying degrees of soft stools or diarrhea are observed 6-8 weeks after transfer of CD4$^{+}$CD45RB$^{hi}$ T cells and upon dissection colon dilation with infiltrating leukocytes, crypt abscesses and epithelial hyperplasia are expected. While an increase in CD4$^{+}$CD45RB$^{hi}$ T cells is observed in both UC and CD patients compared to controls, the model more closely resembles CD in that the affected tissues produce more IFN-γ and TNF-α (Ostanin et al. 2009).

### 1.1.3. Current treatment options

With numerous potential factors involved in the induction and propagation of IBD, it is not surprising that a general treatment of this condition is still lacking and that existing treatments vary in efficacy from patient to patient. The use of wide-spread immune suppressive therapies, such as corticosteroids, can be highly effective when administered long term; however, corticosteroid treatment also results in the highest adverse effect

incidences (Benchimol et al. 2008). Up to 50% of patients treated with corticosteroids develop side effects including metabolic disorders like glucose intolerance, dermatological conditions, mood/sleep disturbances, osteoporosis, cataracts and impaired growth (Mowat et al. 2011; Benchimol et al. 2008). An additional downside to corticosteroid use is a dependence on or resistance to the steroid therapies with long-term usage (Mowat et al. 2011). Corticosteroid sparing therapies are not broad spectrum and are often only effective in either UC or CD patients (Gisbert et al. 2011; Marshall et al. 2010; Prefontaine et al. 2010). Meta-analysis reviews of potentially safer alternative therapies such as dietary omega-3-fatty acids supplementation (Turner et al. 2011) or moxibustion (Lee et al. 2010), a traditional acupuncture associated therapy, show these therapies have no significant benefit when compared to controls. Clearly there is a need for the development of new therapies that are efficient in a wide range of patients, with minimal adverse secondary effects.

Nematodes infections or nematode derived therapies for IBD have been investigated in human clinical trials and showed beneficial effects (Summers et al. 2003; Summers et al. 2005). The current findings in nematode immune regulation of host responses, the effects of this nematode derived immune regulation on secondary inflammation in IBD and the prospects of nematode derived therapies for IBD treatment will be introduced.

## 1.2. Host Immune regulation by parasitic nematodes

Nematodes have evolved efficient immune evasion mechanisms enabling their prolonged survival and reproduction in the host. The modulation of the immune response is seen as an evolutionary adaptation that is also beneficial for the host, as overt immunopathology and thereby damage to the host is avoided (Maizels et al. 2004). Nematode induced immune modulation has the ability to suppress unrelated inflammatory responses as shown for airway hyper-reactivity (Hartmann et al. 2009; Kitagaki et al. 2006; Wilson et al. 2005) and inflammatory bowel disease (Summers et al. 2003; Summers et al. 2005). An understanding of how nematodes affect the different immune cells of their hosts may help to determine how these parasites can specifically interfere with inflammatory disorders such as IBD.

### 1.2.1. Evasion strategies by nematode parasites and host immune response

The term evasion may imply that parasites simply hide from the host immune system. Although this is one strategy, much more intricate mechanisms have been developed by parasitic nematodes which actively modulate the immune system from early time points of

infection in order to direct the immune responses in a way that will prevent the attack and expulsion of the parasite, while preventing extensive damage to the host as this is the nematodes primary source of protection and nutrition (Maizels et al. 2004).

One mechanism by which extensive damage is avoided in nematode infections is by the modulation of the T helper cell type 2 (Th2)-skewed host immune reaction. As strong Th2 response may lead to killing of helminth larval stages and expulsion of adult worms (Fallon et al. 2006; Filbey et al. 2014; Humphreys et al. 2008), helminths have developed strategies for modulating the immune response to suppress the activity of Th2 effector cells Nematode parasite infections induce the expression of regulatory cytokines transforming growth factor beta (TGF-β ) and IL-10 (Doetze et al. 2000; Finney et al. 2007; Rausch et al. 2008; Rausch et al. 2009; Satoguina et al. 2002; Schopf et al. 2005) that have been shown to inhibit the anti-parasite immune responses as well as immunopathology (Grainger et al. 2010; Specht et al. 2004). IL-10 and TGF-β behave in a positive feedback loop with regulatory T cell (Treg) subsets, whereby Tregs both produce and are supported by the cytokines (Murai et al. 2009; Fantini et al. 2004; Mucida et al. 2007). Predictably, nematode infections are also associated with an outgrowth of various Treg populations in both mice (D'Elia et al. 2009; Finney et al. 2007; Grainger et al. 2010; McSorley et al. 2008; Rausch et al. 2008) and humans (Babu et al. 2006; Matera et al. 2008; Metenou et al. 2010; Montes et al. 2009). This regulatory modification of a classic Th2 response by Tregs is thought to be at least partially responsible for the suppression of T effector cell responses in parasite infected hosts (D'Elia et al. 2009; Doetze et al. 2000; Fujiwara et al. 2009; Metwali et al. 2006). Furthermore, Th2 response characterized by an IL-4, IL-5, IL-10 and IL-13 cytokine milieu antagonizes inflammatory Th1 or Th17 responses. Cytokines derived from gut epithelial cells such as thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 have been shown to support the differentiation of Th2 response and antagonize Th1/Th17 driven inflammation (Saenz et al. 2008; Humphreys et al. 2008; Price et al. 2010; Taylor et al. 2009). TSLP has specifically been shown to control Th1 and Th17 responses in nematode infection (Taylor et al. 2009). Additionally, TSLP and IL-25 have been shown to decrease the expression of IL-12/23-p40, an inflammatory cytokine, by DCs (Massacand et al. 2009; Taylor et al. 2009). The suppression of DC maturation is a hallmark of nematode infection associated control of Th1 and Th17 effector cell expansion and activation, as the production of IL-12/23 by mature DCs is required for Th1/Th17 gut inflammation (Roses et al. 2008). Excretory/secretory (ES) proteins from nematodes have been shown to directly suppress DC production of IL-12 to toll like receptor (TLR) ligands such as lipopolysaccharide (LPS) as well as to suppress the expression of chemokines, co-stimulatory molecules and antigen presenting molecule MHCII (Balic et al. 2009; Li et al. 2011; Segura et al. 2007; Cruickshank et al. 2009).

### 1.2.2. Nematode suppression of colitis

The prevalence of IBD, allergies, and autoimmune disorders is increased in industrialized nations in comparison to developing countries. This correlation has been explained by the "hygiene hypothesis", which suggests that the decreased exposure to previously common infections, that have subsequently been reduced as a result of increased hygiene in the western world, may result in increased incidence of autoimmune and inflammatory disorders (Strachan 1989). Parasitic worm infections deserve special attention in this context as they are efficiently controlled by anti-helminthic drugs as well as hygiene practices in developed countries, and their eradication coincides with an increase in the development of immune disorders, including IBD (Elliott et al. 2000). As lack of exposure to helminths may be a causative factor in the development of IBD in developed nations recent studies have aimed to determine if the course of nematode infections can disrupt the development of IBD. Numerous publications have documented both the efficacy of various nematode infections (**Table 1.1**) and nematode derived immunomodulatory molecules (**Table 1.2**) in preventing the onset of murine models of IBD as well as treating established colon inflammation in these models. The evidence for helminth derived therapies for the treatment of murine models of colitis has been so compelling that several clinical trials with human IBD patients have been conducted showing variable success among the different species used (**Table 1.3**). More research is needed with human patients, including more clearly defined control groups, to determine if these therapies are both safe and effective enough to be incorporated into routine treatment strategies for IBD patients.

There are numerous cells, cytokines and pathways involved in the amelioration of murine colitis models by both nematode infections and nematode derived molecules (**Table 1.1** and **1.2**). This suggests that it is most likely not a single mechanism by which nematodes are able to suppress inflammation, but the culmination of many factors. One mechanism by which nematodes may reduce some types of inflammation is through the previously described Th2 skewed immune reaction observed in response to nematode infections that appears to be involved in the inhibition of inflammation in mouse models of IBD (**Table 1.1** and **1.2)**. The induction of Th2 counterbalances the activation of Th1 and Th17 that propagates inflammation in many of these models (Khan et al. 2002; Sutton et al. 2008; Wilson et al. 2011). For instance, *T. muris* infection was shown to increase the expression of the IL-13 receptor, which subsequently decreased the expression of pro-inflammatory Th1 and Th17 cytokines, IFN-γ and IL-17 respectively (Wilson et al. 2011). The expression of the Th2 associated cytokine, IL-4, was also shown to be important in inhibiting the expression of IL-17 during nematode infection in mice and is one proposed mechanism by which nematodes suppress inflammation in their hosts (Elliott et al. 2008).

As intestinal epithelial cells come into contact with nematodes and their molecules, it is not surprising that epithelial derived cytokines such as TSLP, IL-25 and IL-33 are also involved in the immune modulation by nematodes that suppress localized inflammation in the intestine. Various nematode infections cause an increase in the epithelial cell expression of these cytokines (Chen et al. 2012; Hepworth et al. 2012; Park et al. 2011) and these tissue derived cytokines support the Th2 response and subsequent control of pro-inflammatory Th1 and Th17 (Saenz et al. 2008; Humphreys et al. 2008; Price et al. 2010; Taylor et al. 2009). Nematode induction of these cytokines not only prevents extensive host damage within these parasitic infections, but may also be one mechanism by which intestinal inflammation in inflammatory bowel disease or murine models of colitis is ameliorated.

Tregs and the associated regulatory cytokines IL-10 and TGF-β are essential to homeostasis in the gut. Mice deficient in Tregs (Morrissey et al. 1993; Powrie et al. 1993; Ten Hove et al. 2004) or with disruptions in the IL-10 (Berg et al. 2002; Blum et al. 2004; Chaudhry et al. 2011) or TGF-β (Fahlen et al. 2005; Gorelik and Flavell 2000; Lucas et al. 2000) signaling pathways succumb to spontaneous colitis associated with dysregulated effector T cells and pro-inflammatory cytokines. It therefore, stands to reason that the induction of Tregs, IL-10 and TGF-β in both human (Babu et al. 2009; Matera et al. 2008; Metenou et al. 2010; Montes et al. 2009) and murine (Donskow-Lysoniewska et al. 2012; Ashour et al. 2013; D'Elia et al. 2009; Finney et al. 2007; Grainger et al. 2010; McSorley et al. 2008; Rausch et al. 2008) nematode infections may be one mechanism by which helminths are able to control inflammation in IBD and colitis models. Tregs not only control the pathology of helminth infections (D'Elia et al. 2009; Rausch et al. 2009), but the increase in Tregs, IL-10 and functional TGF-β signaling are found to be associated with (Adisakwattana et al. 2013; Ashour et al. 2013; Donskow-Lysoniewska et al. 2012) and sometimes necessary (Ince et al. 2009; Setiawan et al. 2007) for amelioration of colitic inflammation via helminth infections or helminth derived products. Helminth infections have also been shown to induce specific tolerogenic DCs capable of driving $FoxP3^+$ Tregs (Rimoldi et al. 2005) that inhibit effector T cell activation and proliferation (Fujiwara et al. 2009) and are necessary for the preventative effects of nematode infection in some murine colitis models (Hang et al. 2010).

Professional antigen-presenting cells, namely DCs have been shown to be a target for many helminth immune modulatory molecules. Intestinal DCs are capable of forming dendrites extending transepithelially into the gut lumen where they are able to sample antigens (Niess et al. 2005; Rescigno et al. 2001). This DC luminal sampling may be one very important way in which lamina propria immune cells can contact, and therefore be

altered by, helminth derived immunomodulatory molecules. Indeed, the phenotype of DCs varies greatly between nematode infected and naïve mice. Lower expression of co-stimulatory molecules, chemokines and suppressed reactivity to TLR induction have been described for DCs exposed to helminths (Balic et al. 2009; Fujiwara et al. 2009; Klaver et al. 2013; Li et al. 2011; Segura et al. 2007; Smith et al. 2011). This modified phenotype of DCs induced in response to helminth infection has been shown to lead to poor T effector cell activation and support for Treg induction (Fujiwara et al. 2009; Li et al. 2011; Segura et al. 2007; Smith et al. 2011).

Macrophages, like DCs, can exhibit a strong pro-inflammatory phenotype and classically activated inflammatory macrophages expressing reactive oxygen species, nitric oxide and inflammatory mediators are known to be important to the propagation of intestinal inflammation (Bar-On et al. 2011; Qualls et al. 2006). Conversely, alternatively activated macrophages (AAM) induced by Th2 cytokines are found in helminth infected hosts, where they are needed for wound healing in response to migrating parasite larvae and can also directly harm the developing parasites. (Anthony et al. 2006; Kreider et al. 2007; Loke et al. 2000; Mylonas et al. 2009; Reyes and Terrazas 2007; Weng et al. 2007). While expressing low levels of pro-inflammatory factors, they are a source of the regulatory cytokine IL-10 that can control inflammation (Reyes and Terrazas 2007; Mantovani et al. 2004). AAMs have been shown to inhibit effector T cell proliferation (Huber et al. 2010; Loke et al. 2000), regulate inflammation (Nair et al. 2009; Pesce et al. 2009), play an important role in wound healing (He and Marneros 2013; Loke et al. 2007) and ameliorate colitis when transferred to various models (Hunter et al. 2010; Rizzo et al. 2011; Weisser et al. 2011). Likewise, AAMs may play a role in amelioration of gastrointestinal inflammation in clinical IBD, as AAMs are found in higher amounts in human patients with inactive CD compared to those with active disease (Hunter et al. 2010).

While nematode infections obviously do have significant beneficial effects on the amelioration of intestinal inflammation **(Table 1.1 and 1.3)**, parasites are not commensal organisms and infections are associated with their own detriments to the host. It is thus of major interest to develop nematode derived therapies focusing on the application of specific immunomodulatory molecules produced by these parasites **(Table 1.2).**

**Table 1.1** Overview of nematode infections studied for potential benefits in murine colitis models. Changes in cytokine mileau, pathology and overall conclusions regarding cell types and pathways involved in nematode amelioration of colitic inflammation are summarized.

| Citation | Nematode Infection | IBD Model | Preventative or Curative | Changes compared to model control | Overall Outcome |
|---|---|---|---|---|---|
| (Khan et al. 2002) | *Trichinella spiralis* | DNBS | Preventative | ↑ IL-4, IL-13<br>↓ MPO activity, IL-12, IFN-ɣ | Decrease in colitis severity correlated with the induction of a Th2 response |
| (Elliott et al. 2004) | *Heligomosiodes polygyrus* | IL-10$^{-/-}$ mice with Peroxicam | Curative | ↑ IL-13 (not 4 or 5), FoxP3$^{+}$<br>↓ IL-12, IFN-ɣ | Inhibition of colitis through induction of a Treg population and an increase in Th1 antagonizing IL-13 |
| (Metwali et al. 2006) | *Heligomosiodes polygyrus* | *Rag* mice IL-10$^{-/-}$ T cell transfer, with piroxicam | Curative | ↓histological inflammation score | Described suppressive CD8$^{+}$ Tregs required for reversal of colitis, that act independently of IL-10 or TGF-B signaling. |
| (Setiawan et al. 2007) | *Heligomosiodes polygyrus* | TNBS | Preventative | ↑ IL-4, IL-5, IL-10, IL-13<br>↓ IL-12p40, IFN-ɣ | Inhibition of TNBS colitis and inflammatory cytokines in infected mice was IL-10 dependent |
| (Elliott et al. 2008) | *Heligomosiodes polygyrus* | IL-10$^{-/-}$ mice with piroxicam | Curative | ↓ IL-17 | Inhibited ongoing colitis mechanism through IL-4 dependent suppression of IL-17 |
| (Sutton et al. 2008) | *Heligomosiodes polygyrus* | TNBS | Preventative | ↑ IL-4, IL-13, mast cell infiltration, mucosal resistance<br>↓IFN-ɣ, TNF-α, secretion, neutrophils | Prevention of colitis involves antagonism of Th1 cytokine expression and may involve control of secretory function through mast cells mediated mechanisms. |
| (Hang et al. 2010) | *Heligomosiodes polygyrus* | *Rag* mice IL-10$^{-/-}$ T cell transfer | Preventative | ↑Ag-1, CD40 on pDCs<br>↓ IFN-ɣ, IL-17, CD80 and CD86 on DCs | DC phenotype alterations resulted in decreased IL-12p40 and IL-10 and antigen presentation, direct interaction with lymphocytes was unnecessary. |
| (Wilson et al. 2011) | *Trichuris muris* | IL-10$^{-/-}$ mice | *T. muris* exacerbates IL-10$^{-/-}$ colitis | ↑ IL-13Rα2 ( IL-13 receptor decoy), IFN-ɣ, IL-17A, inflammation<br>↓ IL-13 bioreactivity | Infection exacerbated IL-10$^{-/-}$ colitis due to an increase in the IL-14 receptor decoy and subsequent increase in inflammatory cytokines IFN-ɣ and IL-17A. In IL-10$^{-/-}$ IL-13Rα2$^{-/-}$ double KO mice had reduced *T. muris* associated pathology, but inflammation was restored with IL-13mAb. |
| (Donskow-Lysoniewska et al. 2012) | *Heligomosiodes polygyrus* | DSS | Curative | ↓ IL-1β, TNF-α, IL-6, MPO concentration, macrophage infiltration, MOR1, POMC and β-endorphin | The factors inhibited in the colon after L4 infection were increased in the small intestine. The increase in opoid receptors MOR1, POMC and β-endorphin in the colon after DSS and its inhibition after L4 infection suggest a role of opoids in the inhibition of colitis that was not observed in adult worm infection. |
| (Blum et al. 2012) | DC transfer from *Heligomosiodes polygyrus* infected Rag-1 mice | Rag-1 mice reconstituted with IL-10$^{-/-}$ and OVA responsive T cells, induced with piroxicam and OVA | Preventative | ↓ inflammation score, Ag-specific IFN-γ/IL-17 T cell response | DC infected Rag mice added to lamina propria mononuclear cells from colitic animals blocked OVA IFN-γ/IL-17 responses *in vitro* through direct contact. The *in vivo* amelioration did not appear to be due to regulatory T cell subsets. |
| (Leung et al. 2012) | *Heligomosiodes polygyrus* | Rag-1 mice reconstituted with IL-10$^{-/-}$ and OVA responsive T cells, induced with piroxicam and OVA | Preventative | ↑ CD4$^{+}$Foxp3$^{+}$ cells, IL-10 from non-T cells<br>↓ IL-17, IFN-γ | The decrease in IL-17 and IFN- γ was observed with or without antigen specific or polyclonal stimulation. |
| (Adisakwattana et al. 2013) | *Trichinella papuae* | DSS | Preventative | ↑IL-10<br>↓IL-4 | Infection decreased disease activity index and histopathological score, increased colon length. |
| (Ashour et al. 2013) | *Trichinella spiralis* | acetic acid-induced colitis | Preventative and curative | ↑ CD4$^{+}$ FoxP3$^{+}$<br>↓ histopathological changes and pentraxin 3 levels | The preventative model was better able to ameliorate colitis than the curative model. |

**Table 1.2** Overview of nematode derived components studied for potential benefits in murine colitis models. Resultant changes in cytokine milieu, pathology and overall conclusions regarding cell types and pathways involved in nematode amelioration of colitic inflammation are summarized.

| Citation | Nematode Component | IBD Model | Preventative or Curative | Changes compared to control | Overall outcome |
|---|---|---|---|---|---|
| (Motomura et al. 2009) | Larval antigens *Trichinella spiralis* | DNBS | Preventative | ↓ inflammatory scores, MPO activity, IL-1β, iNOS<br>↑ IL-13, TGF-β | Attenuation of colitis attributed to induction of Th2 and regulatory mechanisms via nematode antigens in the absence of live worm infection. |
| (Schnoeller et al. 2008) | Av17 Cystatin *Acanthocheilonema vitea* | DSS | Preventative | ↓ inflammatory index, cell infiltration, goblet cell hyperplasia, epithelial damage, crypt loss (histological examination) | Amelioration of innate cell driven colitis model by an immunomodulatory protein also capable of attenuating Th2 driven airway inflammation through macrophage driven mechanisms. |
| (Ruyssers et al. 2009) | *Ancylostoma caninum* | TNBS | Curative | ↓ MPO activity, clinical disease symptoms, macroscopic colonic damage and inflammatory score | Dose dependent improvements of colitis. No cytokine analysis or investigation of involved pathways were performed. |
| (Du et al. 2011) | 53kDa ES protein *Trichinella spiralis* | TNBS | Preventative | ↑ specific IgG1, IL-4, IL-13, IL-10, TGF-β , M2 (ARG1, FIZZ1)<br>↓ inflammatory scores, IFN-ɣ, TNF-α, IL-6 | Amelioration of colitis due to induction of Th2 and regulatory response, probably involving regulatory M2 macrophages. |
| (Cho et al. 2011) | *Anisakis simplex* MIF II *(*macrophage inhibitory factor homologue) | DSS | Curative | ↑ IL-10, TGF-β, Treg<br>↓ IFN-ɣ, IL-6, IL-13, weight loss, disease activity score | Increase in IL-10 in intestinal epithelial cells, DCs and fibroblasts and an increase in TGF-β in fibroblasts after exposure to recombinant *A. simplex* MIF II *in vitro*. The increase in IL-10 in IECs was shown to be a result of the activation of TLR2. |
| (Cancado et al. 2011) | *Ancylostoma ceylanicum,* crude and ES proteins | DSS | Concomitant Preventative | ↓ Th1 and Th17 cytokines, MPO and eosinophil peroxidase activity, inflammatory score | Live worms not required and both crude worm extracts and ES products are able to ameliorate disease without the application of live organisms. |
| (Ferreira et al. 2013) | *Ancylostoma caninum* ES products | DSS | No Effect | ↑ $CD4^+$ $IL\text{-}4^+$ $IL\text{-}10^+$ cells, Th2 cytokines, M2 macrophages and eosinophils<br>↓ IFN-ɣ, IL-6, iNOS, IL-17A, inflammatory score | Protease denaturation of ES products reduced induction of $CD4^+$ $IL\text{-}4^+$ $IL\text{-}10^+$ cells and ability to suppress colitis. |
| (Kron et al. 2013) | Recombinant *Brugia malayi* asparaginyl-tRNA synthase | $CD25^-$ T cell transfer into Rag-1 mice with piroxicam | Curative | ↑ $CD8^+$, IL-10, IL-4<br>↓ IFN-ɣ, IL-17A, inflammatory score | The *B. malayi* ES protein inhibited the colitis model *in vivo* and was additionally shown to increase the expression of genes involved in several signaling pathways, NK cytotoxicity as well as IL-10 and IL-22 receptors in dendritic cells *in vitro*. |

**Table 1.3** Overview of live nematode therapies studied in human clinical trials with IBD patients. Clinical outcomes of response rate, extension of remission and cytokine expression are summarized.

| Citation | Organism | Study Cohort | Treatment method | Clinical outcome |
|---|---|---|---|---|
| (Summers et al. 2003) | *Trichuris suis* | 4 active CD patients<br>3 active UC patients | 2,500 ova 1×/2week<br>12 weeks total | 6/7 patients reached remission after therapy; however, relapse was common within 12 weeks. Maintenance therapy with *T. suis* ova every 3 weeks extended remission to over a year in 3 of the 4 patients offered this option. |
| (Summers et al. 2005) | *Trichuris suis*. | 29 active CD patients | 2,500 *T. suis* ova<br>1x/3week<br>24 weeks total | Extension of previous maintenance study. At 24 weeks, 80% of the patients had responded and 73% were in remission. |
| (Croese et al. 2006) | *Necator americanus* | 5 inactive CD patients<br>4 active CD patients | Inoculation with 25-100 infective larva.<br>Three patients re-inoculated at week 27-30 | Effects on CD activity index unclear due to low number of patients and variability in *N. americanus* as well as concomitant standard therapies. |
| (Broadhurst et al. 2010) | *Trichuris trichiura* | 1 UC patient refractory to conventional treatment | Patient ingested 500 ova, 1000 more ova 3 months later and due to recurrence of symptoms 3yrs later another 2000 ova | Initial two doses resulted in chronic infection and remission for 3 years. Another dose was taken after relapse and subsequent remission was correlated with an increase in IL-22 producing T cells and a decrease in IL-17 and IL-13Rα2. |
| (Daveson et al. 2011) | *Necator americanus* | 10 celiac patients in hookworm treatment<br>10 patients in saline control group | Treatment included inoculation with 10 infective larva at week 0 and again with 5 larva at week 12 | 5 of the treatment patients experienced painful enteritis in the *N. americanus* colonization period, but were asymptomatic after 20 weeks of infection. Upon challenge with wheat, the treatment group showed no significant improvement in pathology. |

### 1.2.3. Development of nematode cystatins for IBD therapy

While infection with certain species of helminths may induce and extend remission in IBD patients, it is important to consider the consequences of administering helminths to patients as they are pathogens with the potential to induce adverse effects. High-dose infections with hookworms such as *Necator americanus* or the whipworm *Trichuris trichiura* can lead to a dysentery condition, malnutrition, and anemia as well as decreased cognitive function and retarded development in school-age children (Bager et al. 2011; Croese et al. 2006; Stephenson et al. 2000). Therefore, infections with live nematodes adapted to humans are not the ideal option for treatment of IBD. Eggs from *T. suis*, a species maladjusted to the human host, have been used in clinical trials to treat IBD **(Table 1.3)** as a way to circumvent the deleterious effects of worm infection (Summers et al. 2003; Summers et al. 2005). In a recent double-blind placebo trial, however, a significant increase in the number of patients reporting gastrointestinal symptoms, such as diarrhea and abdominal pain, was observed in *T. suis* ova-treated patients compared to the placebo administered control group (Bager et al. 2011).

The potential for nematode infection to induce pathology and increase susceptibility to secondary infections (Osborne et al. 2014; Chen et al. 2005; Chen et al. 2006; Potian et al. 2011; Tetsutani et al. 2009), as well as the lack of long-term studies regarding the safety of

these therapies, must be considered when evaluating nematode therapy. Likewise, the potential psychological effect of live worm treatment on patients accustomed to a high standard for hygiene has never been studied. For these reasons, it would be beneficial to develop treatments that utilize nematode immune modulation while avoiding the induction of live nematode infections. One potential method of bypassing live worm infection is to uncover the specific compounds produced by these parasites and reveal the manner in which they act to down-regulate inflammation in the gut. As reasoned above, ES products from nematodes and other helminths are promising candidates for the development of anti-inflammatory drugs and have as such been studied in several murine colitis models **(Table 1.2)**.

Cystatin derived from the filarial nematode *Acanthocheilonema viteae* has previously been shown to ameliorate experimental colitis when administered intra-peritoneally (Schnoeller et al. 2008). Cystatins are ubiquitous cysteine protease inhibitors involved in numerous processes from catabolism to regulation of immune activation (Klotz et al. 2011a). Parasitic nematode cystatins may have evolved a secondary role in regulating the host immune system. Since the observation that *A. viteae* cystatin is able to inhibit T cell proliferation (Hartmann et al. 1997) several other filarial cystatins both native (Pfaff et al. 2002; Schierack et al. 2003) and recombinant (Schonemeyer et al. 2001) have been reported to suppress T cell responses. Reduced antigen presentation and expression of co-stimulatory molecules by APCs has been observed when cells were exposed to filarial nematode cystatin (Manoury et al. 2001; Murray et al. 2005; Schonemeyer et al. 2001). Macrophages exposed to filarial cystatins were shown to increase the production of anti-inflammatory IL-10, as opposed to the increased IL-12 produced by macrophages exposed to *C. elegans* cystatins, supporting the theory of converging evolutionary development of cystatins in parasitic nematodes for establishment of chronic infection in the host (Schierack et al. 2003). The mechanism by which IL-10 producing macrophages are induced by *A. viteae* cystatin has been recently described (Klotz et al. 2011a). *A. viteae* cystatin is taken up by macrophages and activates the phosphorylation of mitogen-activated protein kinases (MAPK), specifically ERK1/2 and p38, resulting in the expression of IL-10 (Klotz et al. 2011a). The fine tuning of this pathway involves the regulation of MAPK phosphorylation by dual specificity phosphatases, showing that the nematode cystatin exploits activation and deactivation pathways of MAPK to induce macrophages with an IL-10 expressing phenotype (Klotz et al. 2011a).

### 1.3. Probiotics in IBD treatment

The World Health Organization defines probiotics as ‘live microorganisms which when administered in adequate amounts confer a health benefit on the host’ and further specifies

that probiotics for human use ‘must exert benefits on the host through growth and/or activity in the human body’. Meta-analyses of studies on IBD treatment with probiotics show that such treatment is beneficial in UC and inflammation of surgically produced ileal pouches (Shen et al. 2014). However, it is still unclear due to low patient numbers and a lack of well-designed clinical studies whether probiotic treatment has significant effects in CD patients (Naidoo et al. 2011).

Considering that the etiology of IBD is associated with over 100 different gene polymorphisms (Anderson et al. 2011) and there are various ways in which dysbiosis of the gastrointestinal microbiome can attribute to the onset (Hold et al. 2014), it is unsurprising that it is difficult to make vast conclusions about generalized probiotics use for IBD treatment. In addition numerous different strains of bacteria have been investigated for probiotic use. However, while many strains of bacteria show potential for probiotic effects experimentally, the strain *Escherichia coli* Nissle, and VSL#3, a combination of several *Lactobacillus, Bifidobacterium, and Streptococcus* strains, are the main probiotics with strong evidence supporting their use clinically for IBD treatment and are marketed as such (Shen et al. 2014). As reviewed recently, the specific mechanisms by which a particular probiotic strain is active, the age and development of the patient, and the time point in the disease progression or propagation at which the probiotic is administered should all be considered in the development of probiotic therapies for IBD (Sanders et al. 2013). As specific bacterial strains may show promising experimental data to support their use as a probiotic, but later fail to significantly affect IBD, more research is required before confidence in the dosage of specific bacterial species or combinations of species prescribed is achieved. Further studies will hopefully illuminate how and when probiotics, like the VSL#3 combination or *Escherichia coli* Nissle should be utilized as preventative measures, treatments or supplements to traditional pharmaceuticals in the development of IBD therapies.

### 1.3.1. *Escherichia coli* Nissle 1917

In 1917, during the First World War, the physician and researcher Dr. Alfred Nißle isolated a high grade *E. coli* strain from a stool sample of a non-commissioned officer who was resistant to the gastrointestinal ailments afflicting the rest of the troops (Nissle 1951). Shortly thereafter the live preparation of *E. coli* Nissle, patented under the name Mutaflor®, was being produced and sold as what may be the first probiotic bacteria used to treat and prevent human intestinal disorders. Being the first available probiotic treatment, the mechanisms by which *E. coli* Nissle (EcN) may protect or treat gastrointestinal disorders have been studied extensively compared to other probiotics.

Several studies have shown that when compared to placebos EcN treatment results in reduced time to remission and relapse rates **(Table 1.4)**. For instance, in maintaining remission in IBD patients EcN has comparable efficacy to the standard 5-aminosalicylic acid (mesalazine) treatment **(Table 1.4)**. Likewise, EcN was found to ameliorate colitis in experimental murine models, with marked decreases in histological inflammatory scores and expression of pro-inflammatory cytokines **(Table 1.5)**.

One mechanism by which EcN may reduce gastrointestinal inflammation is by outcompeting or inhibiting the adhesion and invasion of pathogenic bacteria in the gastrointestinal tract. EcN has been observed to inhibit the growth, adhesion or invasion of a wide range of urinary tract and gastrointestinal pathogens such as *Yersinia, Shigella, Legionella,* and *Listeria* (Storm et al. 2011); and *Pseudomona* (Storm et al. 2011)*, Escherichia, Enterococcus, Staphylococcus, Klebsiella* and *Enterobacter* (Altenhoefer et al. 2004) respectively. EcN is capable of inhibiting the invasion and translocation of *Salmonella eneterica* serovar *typhimurium* in both human (Altenhoefer et al. 2004) and swine intestinal cell lines (Schierack et al. 2011) as well as in gnotobiotic piglets (Splichalova et al. 2011). EcN was found to exclude the pathogen through modulating the virulence gene expression by *S. enterica* as well as occupying adherence sites, as the ability of EcN to inhibit *S. enterica* infection was positively correlated with the adhesion capacity of the probiotic (Schierack et al. 2011). Additionally, it has been shown that EcN outcompetes *S. enterica* for limited supplies of iron in the inflamed gut, as this nutrient competition was required for the competitive exclusion of *S. enterica* (Deriu et al. 2013).

Since competitive exclusion is normally most effective in closely related species it is unsurprising that EcN can outcompete pathogenic *Escherichia coli* species in human intestinal epithelial cell lines *in vitro* (Boudeau et al. 2003; Huebner et al. 2011) as well as in healthy piglets (Smajs et al. 2012) and murine models (Leatham et al. 2009; Maltby et al. 2013). An observed improved adherence of EcN compared to other *E. coli* species *in vitro*, likely contributes to this competitive exclusion (Rund et al. 2013). Occupation of nutritional niches is another potential mechanism by which EcN can outcompete other species, as EcN has been shown to catabolize the sugars that are necessary for the adhesion of a pathogenic strain, effectively reducing its adhesive capabilities (Maltby et al. 2013). EcN also forms strong biofilms, which is another proposed mechanism by which the probiotic may competitively exclude closely related *Escherichia* species (Hancock et al. 2010). Interestingly, EcN has not been shown to reduce the adherence of other commensal *E. coli* strains, but is most effective on pathogenic strains (Leatham et al. 2009; Maltby et al. 2013).

Another probiotic mechanism by which EcN is able to ameliorate gastrointestinal inflammation is through the support of the intestinal epithelial barrier. EcN has been observed to support the integrity of tight junctions between epithelial cells in the gut in both *in vivo* murine models of colitis (Garrido-Mesa et al. 2011; Ukena et al. 2007) and in *in vitro* cell culture assays (Veltman et al. 2012; Zyrek et al. 2007).

EcN also causes immunological changes in the host. At first glance EcN appears to induce pro-inflammatory changes as EcN cultures have been shown to induce intestinal epithelial cell expression of chemokine attractants to inflammatory cells (Ukena et al. 2005; Hafez et al. 2009) and pro-inflammatory cytokines (Gad et al. 2011; Guttsches et al. 2012). However, the induction of these pro-inflammatory cytokines by immune cells in response to EcN is balanced by a concurrent and increased induction of the regulatory cytokine IL-10 (Guttsches et al. 2012). Furthermore, EcN may inhibit downstream activation of inflammatory T cells in response to pro-inflammatory cytokines. For example, despite an observed potent induction of Th17 and Th1 initiators in response to EcN, a significant increase in IL-17 production was not induced and IL-12p70 expression was actually suppressed in Th1 cells (Gad et al. 2011). EcN is also able to inhibit T cell cycling as well as the overall expression of T cell activating and pro-inflammatory cytokines, while increasing regulatory cytokine expression (Sturm et al. 2005). Interestingly, the inhibition of T cell proliferation and activation was only observed in peripheral blood T cells and not lamina propria T cells, suggesting that the probiotic does not affect the ability of resident T cells to protect the host from pathogens (Ukena et al. 2005).

In conclusion, while some pro-inflammatory effects of EcN are observed *in vitro* the overall effects of the probiotic *in vivo* are anti-inflammatory as seen by the maintenance of remission stages in human IBD clinical trials **(Table 1.4)** and the inhibition of inflammation and pro-inflammatory cytokine expression in animal models of colitis **(Table 1.5)**. Many possible mechanisms for this overall benefit *in vivo* have been elucidated and include a reduction in pathogen adhesion, support of intestinal epithelial barrier function, an induction of regulatory cytokine expression and an inhibition in pro-inflammatory T cell activation and proliferation. Experimentally it is clear that the probiotic has beneficial effects in the colon *in vivo* with the various mechanisms being summarized in **Table 1.4** and **1.5**.

**Table 1.4** Overview of the effects of *Escherichia coli* Nissle (EcN) in human clinical trials with various gastrointestinal disorders.

| Citation | Study Cohort | Treatment Method | Outcomes |
|---|---|---|---|
| (Kruis et al. 1997) | 103 patients (aged >17 years) with inactive ulcerative colitis were allocated to receive either EcN (n = 50) or 5-ASA (n = 53) | EcN group received 2.5–25×$10^9$ CFU/day orally for first 4 days and 5.0-50×$10^9$ CFU/day for the remainder of 12 weeks.<br><br>The 5-ASA group received 500 mg 3x/day with placebo. | No significant differences in relapse rates observed (EcN-16.0%, 5-ASA-11.3%)<br><br>No significant differences in relapse free days (EcN- 106 ± 5, 5-ASA- 103 ± 4 days)<br><br>No significant difference in occurrence of adverse events between the groups. |
| (Kruis et al. 2004) | 327 patients (aged 18-70 years) with ulcerative colitis in remission were allocated to receive either EcN (n=162) or 5-ASA ( n=165). | EcN group received 5-50×$10^9$ CFU/ day orally for 12 months.<br><br>The 5-ASA group received 500 mg 3x per day for 12 months. | No significant difference in relapses rates observed (EcN- 36.4%, 5-ASA- 33.9%).<br><br>No significant differences in duration or localization of the disease.<br><br>No significant difference in occurrence of adverse events between the groups. |
| (Henker et al. 2007) | 113 children (aged 2–47 months) with acute diarrhea randomized to either EcN (n=55) or placebo (n=58) | EcN group received $10^8$ CFU placebo orally 1-3x/day (depending on age) until response or ten day maximum.<br><br>Control group received the same dose of a placebo for a ten day maximum. | Response time was significantly reduced in the EcN (median 2.5 days) compared to the placebo group (median 4.8 days).<br><br>The number of responders was also higher in the EcN (94.5%) compared to the placebo group (67.2%).<br><br>No significant difference in occurrence of adverse events between the groups. |
| (Henker et al. 2008a) | 151 children (aged 1-47 months) with nonspecific diarrhea were randomized to either EcN (n = 75) or placebo (n = 76) | EcN group received $10^8$ CFU 1-3x/day (depending on age) for 21days.<br><br>Control group received same dose of placebo for 21 days. | The number of responders was significantly higher in the EcN group than the placebo group on days 14 (EcN – 93.3%, placebo - 65.8%) and 21 (EcN – 98.7%, placebo – 71.1%).<br><br>Response time was significantly reduced in the EcN (2.4 days) compared to the placebo group (5.7 days).<br><br>No significant difference in occurrence of adverse events between the groups. |
| (Henker et al. 2008b) | 34 patients (aged 11-18 years) with ulcerative colitis in remission were allocated to either EcN (n = 24) or 5-ASA (n = 10) | EcN group received 5×$10^{10}$ CFU/day orally while tapering off 5-ASA treatment over 4 weeks.<br><br>The 5-ASA group received a median dose of 1.5g/day. | Comparable relapse rates in the EcN group (25%) and 5-ASA group (30%) were observed a year after treatment (no statistics are mentioned).<br><br>After a year the occurrence of adverse effects was low and comparable between the groups. |
| (Goel et al. 2009) | 156 colon biopsies of 39 patients (aged 18-70 years) with ulcerative colitis in remission were allocated to receive either EcN (n = 25) or 5-ASA (n = 14) from the Kruis et al. 2004 study. | EcN group received 5-50×109 CFU/ day orally for 12 months.<br><br>The 5-ASA group received 500 mg 3x per day for 12 months. | Biopsies from before and after treatment were compared for microsatellite instabilities that may evolve to colorectal cancer. Microsatellite instabilities were observed in only 20% of all biopsies.<br><br>Neither treatment significantly improved (EcN – 2 patients, 5-ASA – 1 patient) or worsened (EcN –1 patient, 5-ASA – 4 patients) microsatellite stability. |
| (Matthes et al. 2010) | 90 patients (aged 18-70 years) with moderate distal activity in ulcerative colitis were randomly assigned to treatment with enemas of 40, 20 or 10mL of EcN (n = 24, 23, 23) or placebo (n = 20). | EcN enemas contained 10x$10^8$ EcN/mL. The enemas were administered once per day for at least two weeks. | A significant dose-dependent response rate was observed (EcN 40 mL group -52.9%, 20 mL group - 44.4%, 10 mL group - 27.3%, placebo group - 18.2%).<br><br>A significant dose-dependent time to remission was likewise observed (days to remission not reported).<br><br>There were no significant differences in adverse events between treatment and placebo groups. |
| (Kruis et al. 2012) | 120 patients (aged 18-65 years) with irritable bowel syndrome randomized to either EcN (n=60) or placebo (n=60) | EcN group received 2.5–25×$10^9$ CFU/day orally for first 4 days and 5.0-50×$10^9$ CFU/day for the remainder of 12 weeks.<br>Controls received the same dose of placebo for 12 weeks. | A significantly higher responder rate was observed in the EcN compared to the placebo treated group.<br><br>Most significant improvements were observed in individuals with altered enteric microflora.<br><br>No significant difference in adverse effects was observed |

**Table 1.5** Overview of *Escherichia coli* Nissle (EcN) in models of gastrointestinal inflammation. Resultant changes in cytokine milieu, pathology and overall conclusions regarding cell types and pathways involved in nematode amelioration of colitic inflammation are summarized.

| Citation | Model(s) | Treatment method | Changes from control | Overall outcomes |
|---|---|---|---|---|
| (Schultz et al. 2004) | DSS and $CD4^+CD62L^+$ T cell transfer into SCID mice | DSS model - $10^{10}$ CFU EcN administered daily from day -2 to +7<br><br>Transfer model - $10^{10}$ CFU EcN administered 3x/week from week 1 to 8 after the transfer | DSS: ↓ IFN-γ, IL-6<br>- no effect on inflammation<br><br>Transfer: ↓ IFN-γ, IL-6, IL-5, inflammatory score | Decreased pro-inflammatory cytokines in acute DSS colitis model, unaltered intestinal inflammation.<br><br>Amelioration of pathology in T cell transfer model<br><br>EcN translocation to the mesenteric lymph nodes. |
| (Schroeder et al. 2006) | Acute secretory diarrhea in piglets induced by $10^{10}$ CFU enterotoxigenic *Escherichia coli* Abbotstown (EcA) | $10^{10}$ CFU EcN daily for 10 days prior to challenge. Parameters investigated 48hrs after challenge. | ↓ forskolin-mediated stimulation of Isc<br><br>↑ paracellular permeability of tracer molecule monnitol<br><br>Model not associated with inflammation. | EcN significantly reduced the challenge associated increase in Forskolin-mediated secretory response.<br><br>Paracellular permeability was significantly decreased in challenged pigs, but not if pretreated with EcN. |
| (Ukena et al. 2007) | DSS colitis in mice | 1.5-2 x $10^8$ CFU EcN 2x/day | ↓ weight loss, colon shortening, infiltration of leukocytes in colon<br><br>↑ $Na^+$ absorptive and intestinal barrier function, expression of ZO-1 | EcN significantly inhibited colitis parameters.<br><br>The improved barrier function observed offers a possible mechanism and may be linked to the expression of the tight junction protein ZO-1. |
| (Arribas et al. 2009) | TNBS colitis and LPS induced sepsis in mice | TNBS colitis model - $10^9$ CFU EcN/day from 2 weeks before TNBS administration until 1 week after.<br><br>LPS sepsis model – $10^8$ CFU EcN in the drinking water from 2 weeks before LPS induction until 24hrs after | TNBS: ↓ in colonic MPO and TNF-α , inflammatory score<br><br>LPS: ↓ lung/plasma TNF-α; lung/colonic MPO; splenic IL-2, IL-5 IgG; plasma IgG ↑ splenic IL-10 | Anti-inflammatory effects of EcN were not restricted to the colon, but shown to have systemic effects.<br><br>EcN may have implications not only in treatment of localized colitis but in in other inflammatory disorders as well, such as systemic sepsis. |
| (Garrido-Mesa et al. 2011) | DSS colitis with two cylces (from day -4 to 0 and again from day 14 to day 18) in mice | Mice were either treated with a placebo, 50 mg/kg/day monocyclin from day 0 -7 and from day 14-18, 5x$10^8$ CFU/day EcN from day 7-26, or monocyclin and EcN | ↓ TNF-α, IL-1β, IL-2, MIP-2, MCP-1, ICAM-1, iNOS and MMP-9<br><br>↑ MUC-3 and ZO-1 | Both treatments reduced the expression of pro-inflammatory cytokines.<br><br>Combination of treatments was the only regime to significantly decrease the histological inflammation of DSS. |
| (Petersen et al. 2011) | Colonization of mice with IBD associated *E. coli* strains | Mice received 5-8 x$10^8$ CFU of two IBD associated *E. coli* strains. Six days later 8x$10^9$ CFU of EcN alone or with 0.2mg ciprofloxacin every 6 hours for 3 or 7 days either with or without a following inoculation of 9x$10^9$ CFU of EcN. | - EcN treatment resulted in co-colonization with IBD strains<br><br>- ciprofloxacin treatment eradicated both IBD strains<br><br>- ciprofloxacin + EcN eradicated one IBD strain | Ciprofloxacin treatment alone eradicate d both IBD associated *E. coli* strains.<br><br>EcN did not eradicate all the IBD associated *E. coli* and resulted in a co-colonization even with ciprofloxacin .<br><br>EcN is not an appropriate treatment for the eradication of IBD associated *E. coli*. |
| (Bures et al. 2011) | Gastrointestinal injury in piglets as a result of high doses of the NSAID indomethacin | Pigs received either a control, 3.5 x $10^{10}$ CFU/day EcN for 14 days, 15 mg/kg/day of indomethacin for 10 days, or EcN and indomethacin in combination. | **indomethacin**: ↑ height of colon crypt mucosa and colonocytes; ↓ height of stomach epithelia, jejunal crypt mucosa and villi<br>**EcN:** ↑ ileal crypt mucosa; ↓ size of stomach, jejunal, ileium and colon epithelia<br>**EcN + indomethacin**: ↓ height of jejunal/colonic crypt mucosa, jejunal crypt villi and enterocytes. | Indomethacin treatment alone caused significant changes in gastrointestinal morphology.<br><br>EcN alone resulted in significant beneficial effects on colonic morphology.<br><br>The combination of indomethacin and EcN had the most negative impact overall on gastric, jejunal, ileal and colonic morphology. |

### 1.3.2. Transgenic probiotics

The beneficial effects of nematode derived molecules as anti-inflammatory therapies for IBD are promising **(Table 1.2)**; however, the mode of administration of these molecules still requires careful consideration. Oral administration would allow for direct interaction of nematode immune modulators at the site of inflammation. Additionally, this could reduce the risk of systemic immune suppression compared to an injectable administration of nematode derived products. Likewise, compared to repeated injections, oral administration of nematode immune modulators could reduce the risk of developing an allergic responseto the nematode derived molecule, as the oral route favors tolerogenic mechanisms (Burks et al. 2008; Holmgren and Czerkinsky 2005). While encapsulation is perhaps the most likely method of ensuring passage of nematode derived proteins through the acidic environment of the stomach, probiotic bacteria have also been studied as carriers for IBD therapies. For example, the probiotic *L. lactis* has been genetically modified to produce human IL-10 and anti-TNF-α nanobodies for the treatment of colitis with apparent success (Steidler et al. 2000; Steidler et al. 2003; Vandenbroucke et al. 2010). The use of probiotic bacteria as carriers may be advantageous over encapsulation of therapeutic proteins, as colonization with these transgenic bacteria would ensure long term, continuous release of the recombinant protein. Probiotic bacteria themselves may offer benefits to the treatment of gastrointestinal disorders and have previously been reported to have beneficial effects in conjunction with some conventional treatments in IBD patients (Mallon et al. 2007).

EcN is a good candidate for a probiotic bacterium that may be genetically modified to express nematode derived proteins for IBD therapy. To test the safety of EcN as a potential carrier for a transgenic protein, a transgenic EcN expressing a model protein from the influenza virus on its surface was created and tested *in vivo* (Westendorf et al. 2005). T cells with influenza protein specific receptors were injected into both control mice and mice previously colonized with EcN, but no difference in proliferation or activation of the $CD4^+$ T populations in these two mice were observed (Westendorf et al. 2005). Transgenic EcN was also evaluated in a DSS murine colitis model and was not observed to affect the activation or expansion of specific $CD4^+$ T cells when compared to the controls even with the disruption to the epithelial barrier (Westendorf et al. 2005). Therefore, while immunological changes are observed in single *in vitro* cell culture experiments (see section 1.3.1 *Escherichia coli* Nissle) and *in vivo* animal models of gastrointestinal inflammation **(Table 1.5),** this study demonstrates that EcN does not initiate an immunogenic response in healthy animals and may in turn be a safe carrier of therapeutic molecules in healthy individuals or IBD patients in remission.

Transgenic EcN strains have been developed for the treatment of human diseases, the first published example is an EcN genetically manipulated to express anti-virals for protection against HIV transmission and was found to colonize and secrete the anti-viral peptides in the colon, rectum and vagina (Rao et al. 2005). Studies have also demonstrated the potential of EcN as a promising carrier for anti-tumor molecules. EcN was shown to migrate and colonize preferentially in tumors when administered to mice intravenously (Stritzker et al. 2007) and to reduce tumors by 50% compared to the placebo control when modified to constitutively express and secrete an anti-tumor drug (Zhang et al. 2012).

Recently, a few attempts have been made to develop genetically modified EcN for the treatment of IBD. A human IL-10 gene was introduced into EcN on a plasmid with the intent of producing the cytokine for regulation of intestinal inflammation in IBD (Pohlmann et al. 2013). However, the secreted cytokine was not bioactive and *Saccharomyces* was determined to be preferable for expression and secretion of the transgene, as the yeast is capable of the post-translational modifications necessary to produce a bioactive cytokine for human use (Pohlmann et al. 2013). In 2012, Seo et al. attempted to improve the already known effects of EcN in the treatment of IBD by creating a genetically modified variant that produces human β-defensins. The EcN produced human defensins were shown to maintain their anti-microbial effects as they were able to inhibit the growth of *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* (Seo et al. 2012).

None of the transgenic EcN strains for the treatment or detection of human disease, including IBD, are currently approved and marketed for medical use. However, as studies continue to improve the safety of transgenic probiotic administration (Stritzker et al. 2010), optimize expression and secretion of introduced genes (Pohlmann et al. 2013) and find inducible promoters for the control of transgene expression (Loessner et al. 2009) EcN may prove to be both a therapeutically and fiscally effective vehicle for diagnostic and therapeutic molecules.

## 2. Aims of the Study

The aim of this study was to develop an efficient treatment strategy for intestinal inflammation focusing on a site-directed and prolonged release of the helminth immunomodulator, AvCys, in the gut, through genetic modification of the probiotic EcN as a transgenic carrier.

In previous studies cystatin from tissue dwelling filaria *Acanthocheilonema viteae* (AvCys) was demonstrated to have strong anti-inflammatory properties when in contact with immune cells and to efficiently suppress pathology in DSS-induced colitis and ovalbumin- or birch pollen-induced allergic airway hyper-reactivity (Figueiredo et al. 2009; Schierack et al. 2003; Schnoeller et al. 2008; Danilowicz-Luebert et al. 2013). Immunomodulation by AvCys was associated with induction of IL-10 via exploiting the host cell MAPK pathway (Figueiredo et al. 2009; Klotz et al. 2011b). Together, these results provide evidence for the potential of AvCys as a candidate to ameliorate IBD.

*Escherichia coli* Nissle 1917 (EcN) was chosen as a carrier for the nematode immunomodulatory protein, as this probiotic is already successfully used to maintain remission in IBD patients (Jacobi and Malfertheiner 2011). Hence, EcN was chosen as an ideal carrier organism to test for the site-directed anti-inflammatory activities of the immunomodulatory helminth protein AvCys.

Upon development of the transgenic EcN secreting AvCys (EcN-AvCys) the aims of this study were to i) test the efficacy of EcN-AvCys murine models of colitis, ii) test the safety and efficacy of EcN-AvCys when applied in high doses to pigs as a model organism providing an intestinal tract highly similar to the human gut, and iii) address the modes of action of EcN and AvCys while elucidating any potential new mechanisms by which EcN-AvCys confers beneficial effects in the context of intestinal inflammation. The hypothesized modes of action for EcN-AvCys are outlined in **Figure 2.1.**

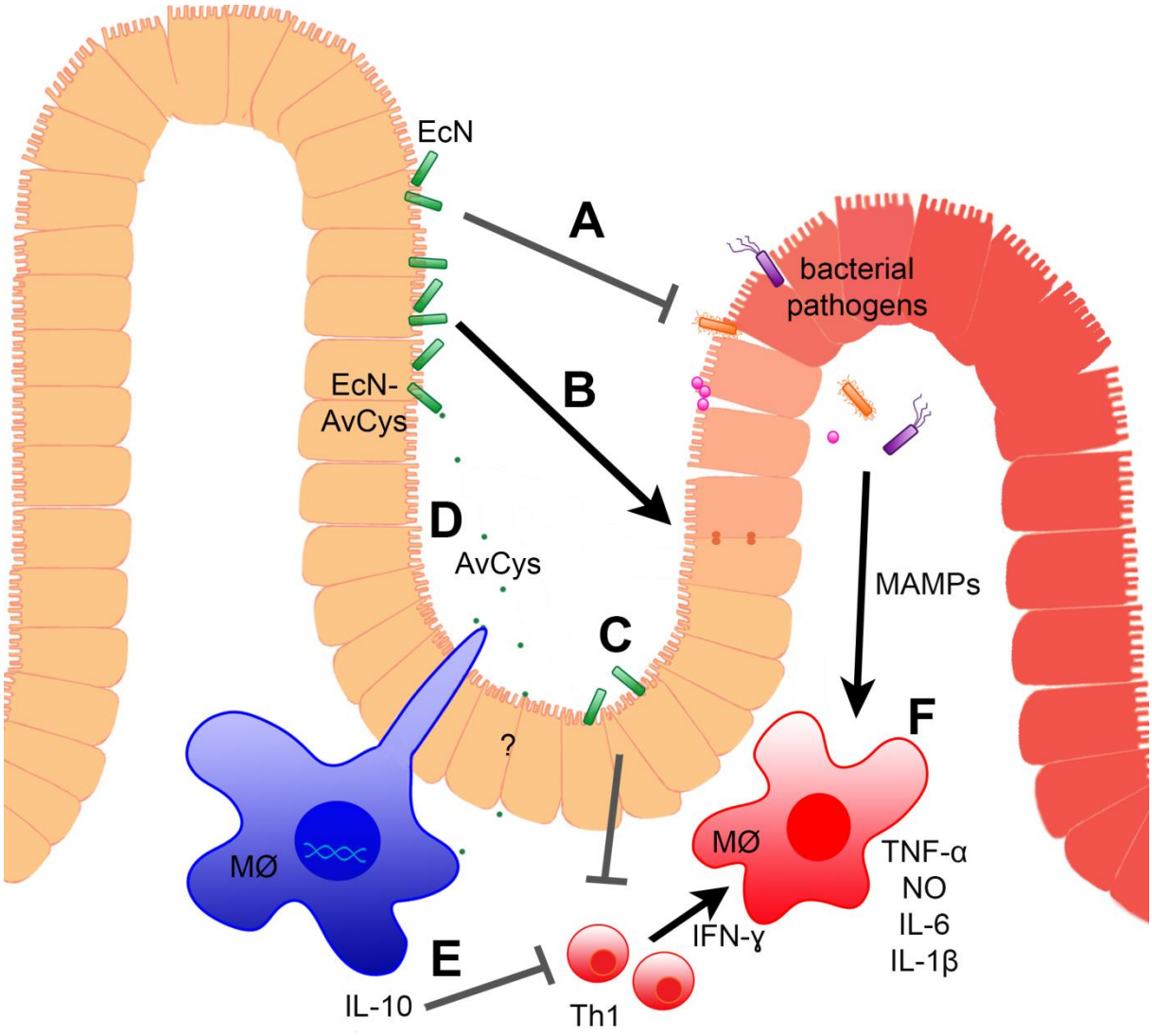


**Figure 2.1.** Hypothesized mechanisms by which EcN-AvCys may ameliorate intestinal inflammation. *Escherichia coli* Nissle (EcN) inhibits intestinal inflammation through **(A)** the inhibition of pathogen adherence, **(B)** the support of tight junction proteins and the epithelial barrier and **(C)** the inhibition of effector T cell proliferation. A nematode immune modulatory protein, AvCys, expressed and secreted from a transgenically modified EcN would have access to intestinal macrophages via **(D)** intestinal sampling or trespassing the epithelial barrier and **(E)** possibly induce or support a regulatory macrophage phenotype (blue MØ) in gut and interfere with the effector T cells proliferation, pro-inflammatory functions and/or attraction of inflammatory macrophages/monocytes (red MØ) thus **(F)** reducing the expression of inflammatory mediators such as TNF-α, nitric oxide (NO), IL-6 and IL-1β in response to microbe associated molecular patterns (MAMPs) or IFN-γ from type 1 effector T cells (Th1).

# 3. Results

## 3.1. Transgenic *E. coli* Nissle expresses and secretes *Acanthocheilonema viteae* cystatin

In order to create the transgenic EcN-AvCys the functional AvCys gene was amplified with specific primers that added an NsiI reion enzyme cut site at the 3' and 5' ends of the amplicon **(Figure 3.1A)**. A modified version (pMUT13) of the native EcN-specific plasmid (pMUT1) containing a tetracycline (Tc ) resistance cassette, a hemolysin secretion system (hly) and a multiple cloning site (MCS) within the signal sequence coding gene of a hemolysin secretion system (hlyA) was used as the cloning vector. The pMUT13 plasmid and the amplified AvCs gene were digested with the NsiI restriction enzyme **(Figure 3.1B)**. As all NsiI restriction enzyme recognition sites outside of the MCS were deleted from the pMUT13 plasmid, the ligation of digested AvCys gene into the digested pMUT13 results in the insertion of the transgene into the hlyA gene **(Figure 3.1B)**. The transgene carrying plasmid was then transformed into a chemically competent EcN strain containing a kanamycin (Kn) resistance cassette on another modified EcN specific plasmid pMUT2 and a resistance to streptomycin (Sm) of unknown origin **(Figure 3.1C)**. The transformed EcN was then cultured in liquid media to allow for repair of the competence induced cell wall damage **(Figure 3.1D)** before being cultured on LB agar plates with Tc, Kn and Sm to allow for clonal selection of EcN containing the transformed pMUT13 plasmid **(Figure 3.1E)**. PCR with AvCys-specific primers was then used to further test clones for addition of the AvCys transgene **(Figure 3.1F)**. To further select for transgenic EcN clones able to express and secrete the AvCys gene a western blot with AvCys-specific monoclonal antibodies was utilized to check for AvCys protein in the supernatant precipitate from cultures of the selected clones **(Figure 3.1G)**.

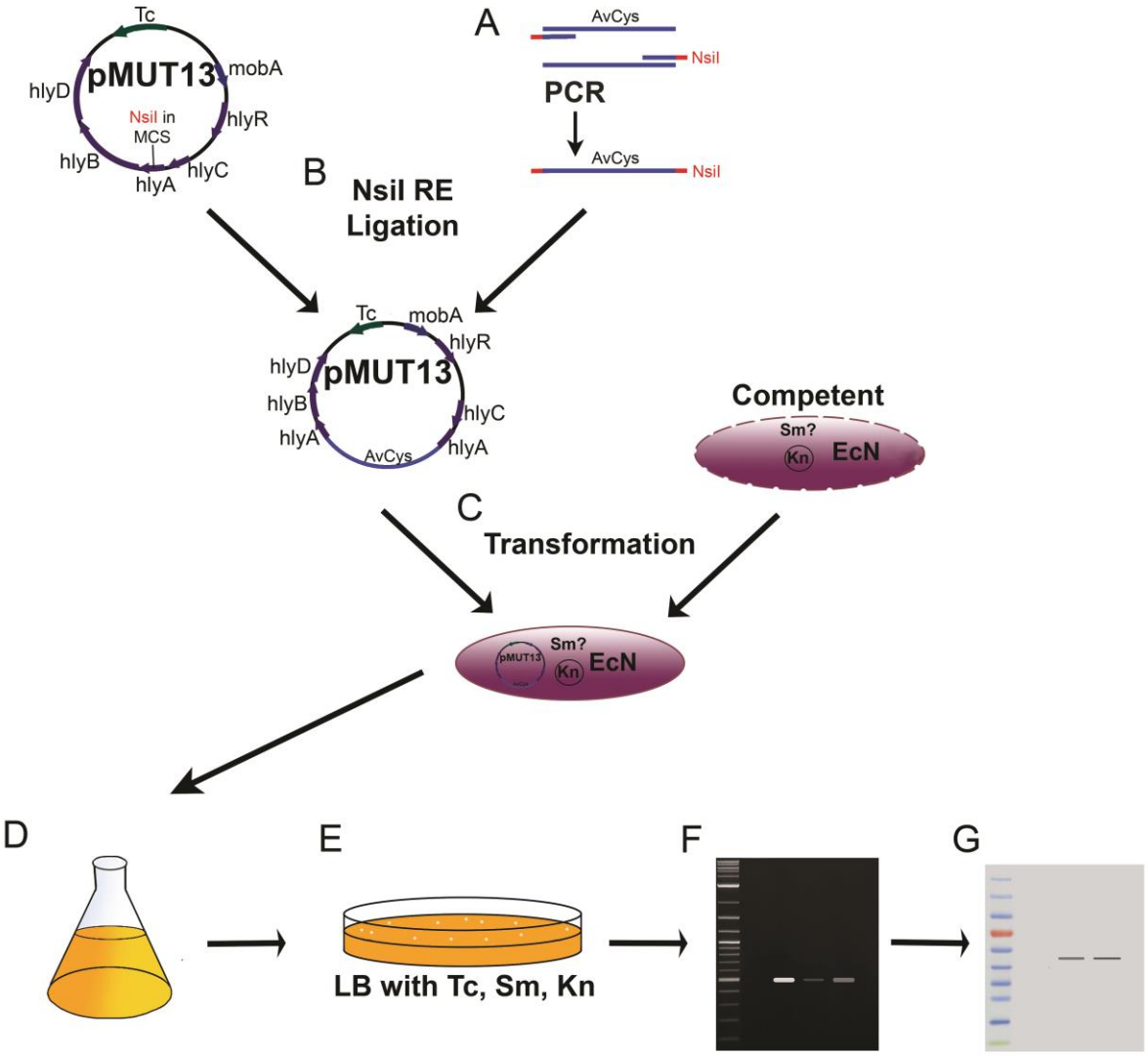


**Figure 3.1**. Construction and positive selection of transgenic AvCys expressing EcN. **(A)** The *A. viteae* cystatin (AvCys) gene was amplified with specific primers to add an NsiI restriction enzyme (RE) cut site to both the 5' and 3' tails of the gene. **(B)** NsiI RE digestion was performed on both the amplified AvCys gene and a modified EcN plasmid, pMut13, containing a hemolysin secretion system (hly), tetracycline resistance (Tc) and a multiple cloning site (MCS) with an NsiI RE recognized sequence. The sticky ends from the plasmid and the AvCys gene were then ligated with a T4 DNA ligase. **(C)** The AvCys gene containing plasmid was then transformed into a competent *E. coli* Nissle (EcN) harboring a plasmid based kanamycin resistance (Kn) and a naturally acquired streptomycin resistance (Sm) of unknown origin. **(D)** The transformed bacteria were allowed to repair cell walls and replicate in Luria broth (LB). **(E)** Clones successfully transformed with the pMut13 plasmid were then positively selected for with antibiotic supplemented LB agar plates. **(F)** Clones were then selected for positive expression of the AvCys transgene through PCR. **(G)** Clones positive for the AvCys transgene were then recultured in LB broth and precipitated supernatants were selected for the secretion of AvCys with AvCys-specific monoclonal antibodies.

The PCR analysis of plasmid DNA from EcN-AvCys with AvCys specific primers confirmed the insertion of the gene in the transgenic probiotic **(Figure 3.2A)**. Western blot analysis of EcN-AvCys culture supernatant EcN-AvCys$^{SN}$ with a monoclonal anti-AvCys antibody confirmed that EcN produced significant amounts of recombinant AvCys when cultures were grown to a concentration of 3x10$^8$ CFU EcN/mL **(Figure 3.2B)**. The slightly increased size of AvCys secreted by the transgenic EcN compared to AvCys derived from a standard expression vector is due to the presence of the hlyA secretion sequence **(Figure 3.2B)**.

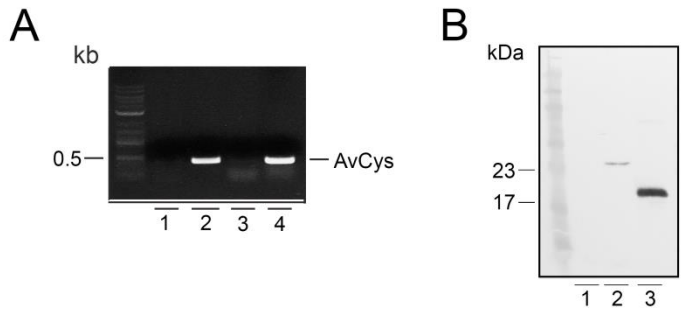


**Figure 3.2.** Generation of transgenic EcN expressing AvCys. **(A)** Polymerase Chain Reaction with AvCys specific primers amplified the AvCys gene in EcN-AvCys (2) and positive control (4), but not in EcN (1) or the negative water control (3). **(B)** Western blot analyses was performed with an AvCys-specific antibody on supernatants of EcN (1) and EcN-AvCys (2) grown in Luria broth medium as well as a recombinant AvCys positive control (3).

## 3.2. AvCys secretion by transgenic EcN-AvCys into cell culture medium

In order to further study the effects of EcN and EcN-AvCys in *in vitro* cell culture assays, supernatants that were suitable to cell culture assays were developed through modification of a method proposed originally by Yan and Polk (2002). EcN or EcN-AvCys were selectively cultured overnight in LB media containing the antibiotics Tc, Sm and Kn **(Figure 3.3A).** The overnight cultures were diluted in IMDM cell culture media and regrown to an $OD_{600}$=1, equivalent to 3x10$^8$CFU/ml **(Figure 3.3B)**. The IMDM cultures were then centrifuged and sterile filtered to remove live bacteria **(Figure 3.3C)** and a portion of the sterilized media was concentrated 100 fold **(Figure 3.3D)**. An ELISA was developed to test several dilutions of the filtered supernatants and concentrated fractions **(Figure 3.3E-J)**.

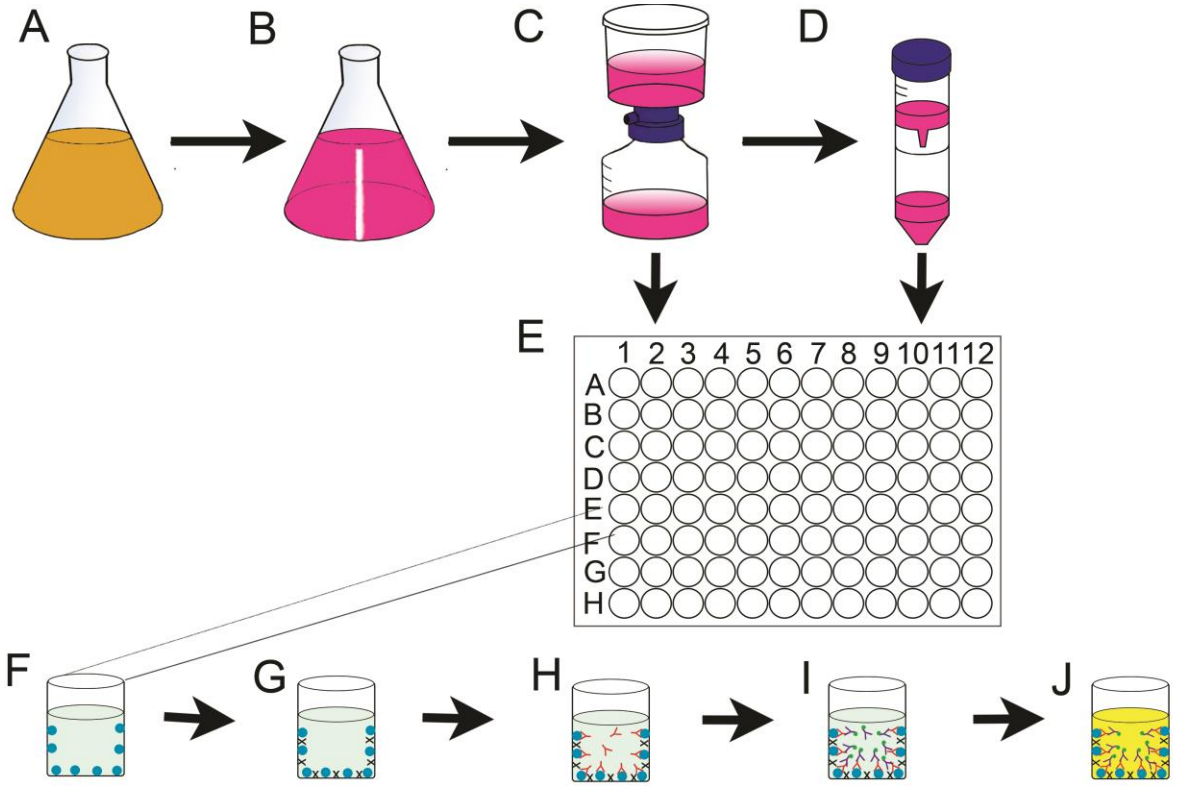


**Figure 3.3.** Production of EcN and EcN-AvCys cell culture supernatants and AvCys ELISA development. **(A)** EcN or EcN-AvCys were grown overnight in LB media. **(B)** Overnight LB cultures were diluted 1:1000 in IMDM cell culture media and cultured to an $OD_{600}$=1 before being **(C)** sterile filtered. **(D)** A fraction of the filtered supernatants was then concentrated 100x. **(E)** An AvCys-specific ELISA was developed to determine the AvCys concentration in IMDM supernatants. **(F)** Plates were coated overnight at 4°C with serial dilutions of recombinant AvCys ranging from 500 to 7.8125 ng/ml and various dilutions of the EcN-AvCys supernatants and 100 fold concentrated fractions. **(G)** Wells were washed and blocked for 2hrs at room temperature with 1% bovine serum albumin in phosphate buffered saline. **(H)** Wells were washed and incubated with a mouse anti-AvCys monoclonal antibody for 2hrs at room temperature. **(I)** After washing wells were incubated with rat anti-mouse antibody bound to horseradish peroxidase for 2hrs at room temperature. **(J)** Wells were washed and incubated for 20-30min at room temperature with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate to allow for a concentration dependent colorimetric reaction between the substrate and antibody bound enzyme before the reaction was stopped with the addition of sulfuric acid.

The AvCys concentration of the EcN ($EcN^{SN}$) or EcN-AvCys supernatants ($EcN\text{-}AvCys^{SN}$) was originally determined with a western blot using AvCys-specific antibodies and estimated to be between 10-20ng/ml based on visual comparison to band intensity of known quantities of recombinant AvCys run on the same blot **(Figure 3.4A)**. This was confirmed with the AvCys specific ELISA developed as the AvCys concentration in three separate batches of $EcN\text{-}AvCys^{SN}$ was determined to be between 12-24ng/ml **(Figure 3.4B)**.

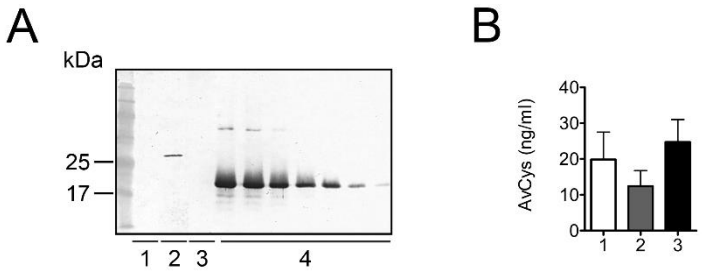


**Figure 3.4** Western Blot and ELISA analysis of AvCys concentration in supernatants from EcN-AvCys cultured in IMDM. **(A)** Western blot of 1mL of an Iscove's Modified Dulbecco Medium (IMDM) control (1), supernatant from EcN-AvCys grown in IMDM (2), supernatant from EcN-AvCys grown in IMDM (3), and 7 lanes loaded with recombinant AvCys as a standard containing from left to right 200ng, 150ng, 100ng, 50ng, 25ng, 10ng, and 5ng of the purified protein (4). **(B)** A graph representing the amounts of AvCys calculated for three different batches (1, 2, 3) of EcN-AvCys supernatant using the AvCys specific ELISA developed. Each bar represents three calculations derived from the amount of AvCys quantified in three separate dilutions of each supernatant. No significant difference in quantity of AvCys produced by these three separate batches was determined when analyzed with one-way ANOVA.

## 3.3. Transgenic EcN-AvCys is able to inhibit colitis in murine models of IBD

### 3.3.1. EcN-AvCys in the dextran sodium sulfate induced chronic colitis model

To test the potential of EcN-AvCys to inhibit colitis a chronic version of the DSS model was proposed, in which repeat cycles of DSS are administered interspaced with recovery periods with DSS free drinking water in order to allow for intestinal healing **(Figure 3.5A)**. This chronic model involves the activation of inflammatory T cells contributing to intestinal pathology (Lodinova-Zadnikova and Sonnenborn 1997; Cukrowska et al. 2002; Schulze and Sonnenborn 1995). Previous trials had demonstrated that EcN and EcN-AvCys successfully colonized the murine gut for at least four weeks after administration when supported by application of streptomycin in the drinking water (unpublished, data not shown). However, these pre-trials had shown that Sm interfered with the development of colitis in response to DSS feeding, presumably as a consequence of the reduced macrophage activation due to decreased bacteria numbers in the gut. As a compromise

permitting macrophage activation during the first DSS feeding cycles and achieving EcN-colonization by antibiotic treatment, EcN/EcN-AvCys was applied together with Sm in the drinking water during the 3rd DSS cycle (Ghia et al. 2008; Waddell et al. 2011). There did not appear to be any hindrance of the model with the addition of Sm in the third cycle as no differences in colon length, inflammatory score of the colon or production of inflammatory cytokine was observed between the DSS control groups with and without Sm administered **(Figure 3.5B, C, D)**. However, there were also no observable differences in colon length between the control groups and those treated with EcN or EcN-AvCys **(Figure 3.5B).** Analysis of histological cross section of the distal colon showed a mild trend towards a decreased inflammatory score based on cellular infiltration and alteration in the tissue of EcN and EcN-AvCys treated animals compared to both of the DSS control groups, but this was not significant **(Figure 3.5C).** Additionally, a trend towards a reduction in the expression of the pro-inflammatory cytokine IL-17A in the gut-draining mesenteric lymph nodes (mLN) was observed in EcN-AvCys treated animals specifically compared to the EcN treated controls, but again this was not significant **(Figure 3.5D)**.

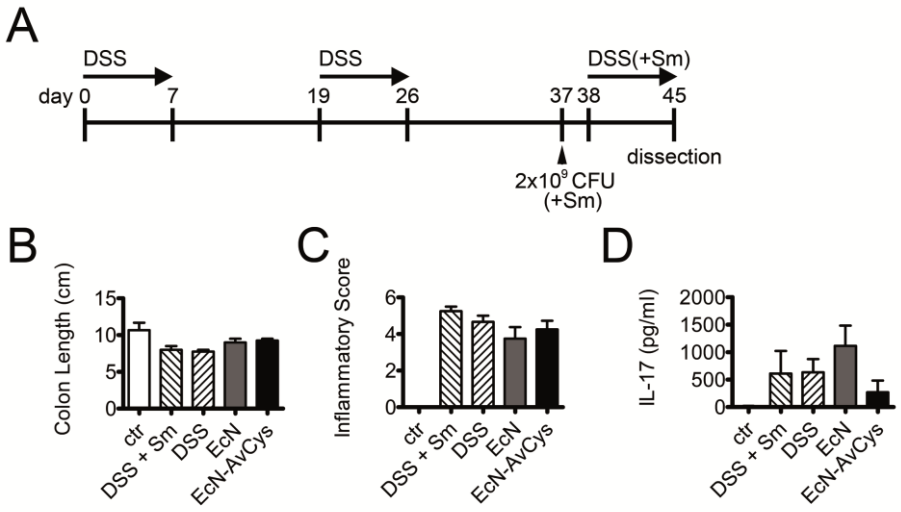


**Figure 3.5**. EcN-AvCys administration in a chronic murine DSS-colitis model. **(A)** Experimental setup. With the exception of the naïve control group (ctr, n=3), mice were administered 2.5% DSS in the drinking water for three, seven day cycles interspersed by seven days of DSS free drinking water. One day prior to the third cycle streptomycin (Sm) was added to drinking water at 5g/L and $2x10^9$ CFU of either EcN (n = 4) or EcN-AvCys (n=4) in 0.9% saline, or saline alone (DSS+Sm n=4, DSS n=3) were administered by oral gavage. After the third DSS cycle animals were euthanized. **(B)** Colon lengths of mice upon dissection are represented in cm. **(C)** Histopathological scoring of distal colon sections was compiled in inflammatory scores. **(D)** Concentration of IL-17A in supernatants of mesenteric lymph node cells stimulated with concanavalin A for 48 hrs as determined by ELISA. Mean + SEM shown. No significant differences detected between EcN-AvCys and any of the control groups observed when analyzed with Mann-Whitney U test.

### 3.3.2. EcN-AvCys inhibits the dextran sodium sulfate induced acute colitis model

A previous study showed that recombinantly expressed AvCys (rAvCys) applied intraperitoneally reduced colon inflammation in acute DSS colitis (Schnoeller et al. 2008). Thus it was investigated whether treatment with the transgenic probiotic additionally producing the immunomodulator AvCys would further ameliorate murine colitis. Therefore, EcN-AvCys and EcN were fed to mice receiving 3.0% DSS in drinking water to induce acute inflammation of the colon. Preliminary results suggested that EcN does not naturally colonize in the gastrointestinal tracts of mice (unpublished, data not shown) as it has previously been shown to do in humans (Lodinova-Zadnikova and Sonnenborn 1997; Cukrowska et al. 2002; Schulze and Sonnenborn 1995) and swine (Kleta et al. 2006; Barth et al. 2009). Therefore, $2\times10^9$ transgenic or control bacteria were applied by oral gavage every 2nd day while control mice received saline alone **(Figure 3.6A).** When an EcN specific multiplex PCR was used to analyze feces from treated mice at day 7, EcN could be detected in the feces of EcN and EcN-AvCys treated mice but not the saline controls **(Figure 3.6B).** Additionally, specifically in DNA extracted from feces of EcN-AvCys-treated mice a PCR with AvCys specific primers could detect AvCys **(Figure 3.6C)**, indicating the presence of the transgenic probiotic in mice from these trials where the dosing regime was repeat application without streptomycin support.

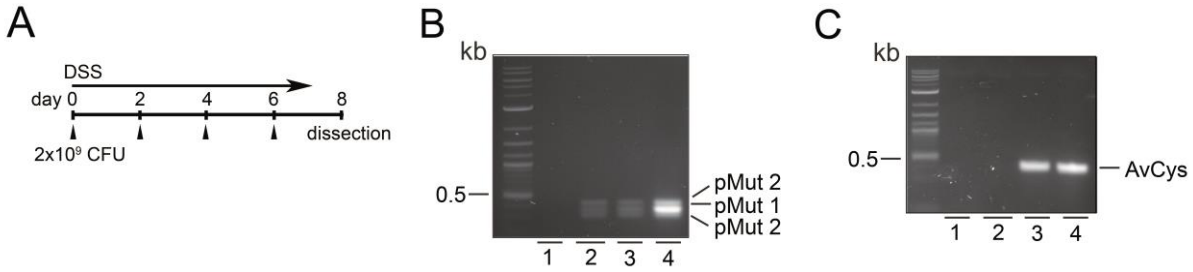


**Figure 3.6.** Acute DSS trial design and fecal detection of EcN-AvCys. **(A)** Experimental setup. EcN-AvCys and EcN treated mice were orally administered with $2x10^9$ CFU of the respective bacteria every second day while 3.0% DSS was applied via drinking water for seven days. A DSS administered control group (DSS) and healthy controls (ctr) were orally gavaged with saline every second day. **(B)** A multiplex Polymerase Chain Reaction with three primer pairs specific for EcN specific primers pMut1 and pMut2 amplified the three different amplicons from bacterial extracted from feces of mice inoculated with EcN (2) and EcN-AvCys for 7 days (3) as well as a positive control (4), but not in feces of mice inoculated with saline (1) for 7 days. **(C)** Polymerase Chain Reaction with AvCys specific primers amplified the AvCys gene from bacterial DNA extracted from feces of mice inoculated with EcN-AvCys for 7 days (3) and a positive control (4), but not in feces of mice inoculated with saline (1) or EcN (2) for 7 days. Results shown are from feces of one representative mouse per group.

While body weight development in response to treatments was relatively heterogeneous, treatment with EcN-AvCys significantly inhibited colitis-associated colon shortening compared to DSS controls **(Figure 3.7A).** Histological cross sections of the distal colon showed that the application of EcN diminished DSS-induced inflammation **(Figure 3.7B)** as described previously (Garrido-Mesa et al. 2011; Ukena et al. 2007). Compared to DSS controls this was even more apparent for EcN-AvCys which significantly reduced damage to the colon with reduced cellular infiltration, preserved tissue architecture and reduced thickening of the colon wall **(Figure 3.7B)**. As macrophages and eosinophils are essentially involved in the inflammatory process in the acute DSS colitis model (Blumberg et al. 1999; Waddell et al. 2011) both cell types were analyzed via flow cytometry and histology. Eosinophil frequencies detected in colon cross sections were similarly diminished in recipients of EcN and EcN-AvCys compared to DSS controls **(Figure 3.7C)**. Significantly lower numbers of $F4/80^{+}Ly6C^{high}$ expressing monocytes/macrophages in lamina propria (LP) leukocyte isolates from EcN-AvCys recipients were detected compared to DSS treated controls, while EcN control treatment had no such effect **(Figure 3.7D).** Similarly, only low numbers of $iNOS^{+}F4/80^{+}$ M1-type macrophages were detectable in colon cross sections of mice treated with EcN-AvCys and this was significantly reduced compared to mice treated with the control EcN **(Figure 3.7E)**.

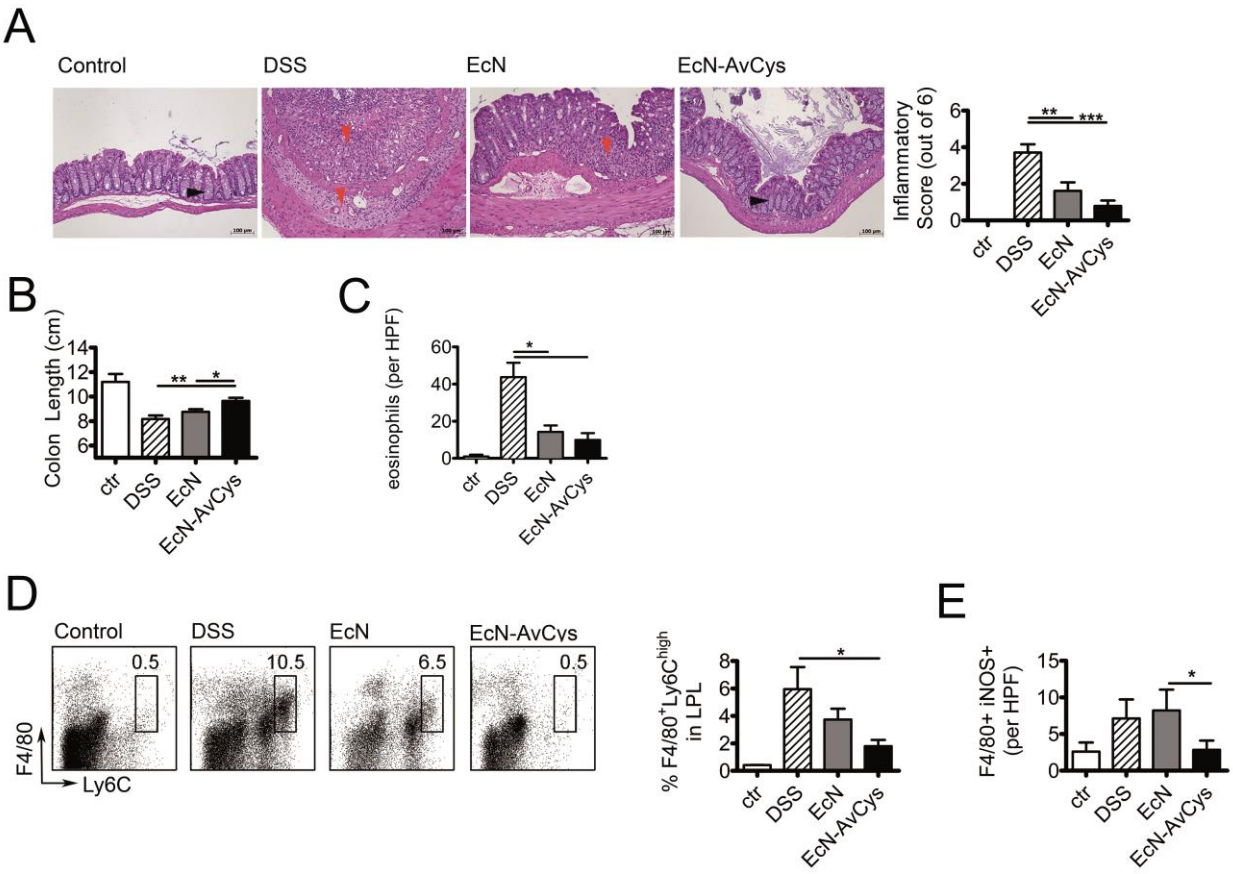


**Figure 3.7.** Effects of EcN-AvCys on acute murine DSS-colitis. **(A)** Colon lengths of mice upon dissection are represented in cm. **(B)** Representative pictures as used for histopathological scoring of distal colon tissue with red arrowheads depicting areas of strong immune cell infiltration and loss of normal tissue architecture and black arrowheads marking normal crypt architecture. A reduced colon wall thickening in response to EcN-AvCys treatment compared to DSS and EcN controls is also notable. **(C)** Numbers of eosinophils as determined by histological staining in colon cross sections. **(D)** Representative flow cytometry plots showing the detection of $F4/80^{+}$ $Ly6C^{high}$ expressing monocytes/macrophages in LPL. Bar graph shows percentages of $F4/80^{+}$ $Ly6C^{high}$ cells detected in groups of mice. **(E)** Numbers of $F4/80^{+}iNOS^{+}$ M1 macrophages detected via immunohistochemistry in colon cross sections. Mean + SEM is shown. * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$ (Mann-Whitney U test). Data are pooled from 3 individual experiments with 2-3(naïve controls) – 5 mice (DSS, DSS + probiotics) per group. LPL: lamina proria leukocytes; HPF: high power field (400× magnification). AvCys

To determine whether EcN-AvCys affected the expression of chemoattractants involved in monocyte migration to the sites of tissue damage and inflammation the expression of macrophage inflammatory protein (MIP)-1α **(Figure 3.8A)**, MIP-1β **(Figure 3.8B)**, monocyte chemoattractant protein (MCP)-1 **(Figure 3.8C)**, MCP-3 **(Figure 3.8D)** and regulated upon activation, normal T-cell expressed and secreted protein (RANTES) **(Figure 3.8E)** in colon tissue was analyzed. Compared to DSS treated controls exhibiting strong protein expression of all chemokines this was significantly suppressed in mice treated with EcN-AvCys, but not in EcN treated controls (**Figure 3.8A-E**). These effects were also apparent comparing the EcN-AvCys group to EcN controls for MIP-1β **(Figure 3.8B)**, MCP-1 **(Figure 3.8C)** and MCP-3 (**Figure 3.8D)**.

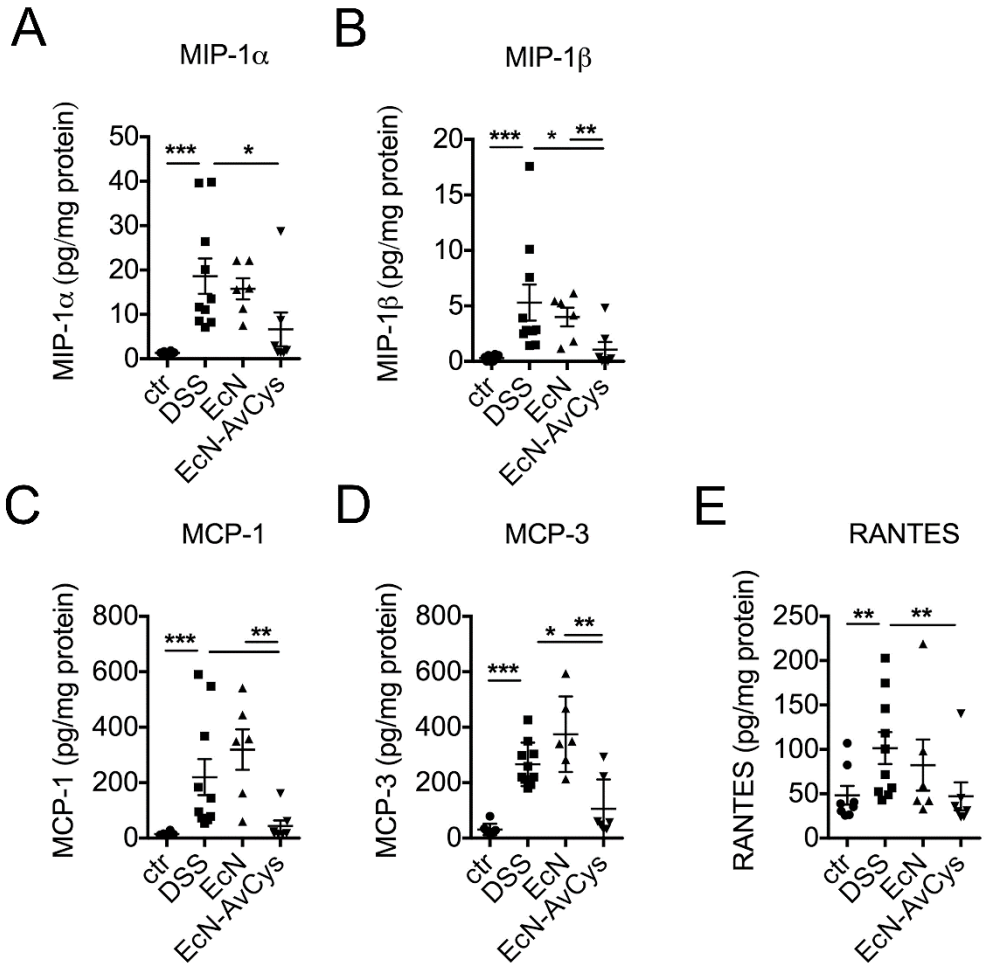


**Figure 3.8.** Effects of EcN-AvCys on colon chemokine expression in DSS-colitis. Protein levels of **(A)** MIP-1α , **(B)** MIP-1β, **(C)** MCP-1, **(D)** MCP-3 and **(E)** RANTES detected in distal colon tissue of naïve control mice (n=4), DSS controls (n=5), EcN controls (n=3) and mice fed EcN-AvCys (n=4). Chemokine levels are expressed in relation to total protein content. Mean ± SEM for 2 colon explants derived from individual mice is shown. Data derive from one of two individual experiments with similar results. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Mann-Whitney U test).

Reduced pathology was accompanied by a reduced expression of the pro-inflammatory cytokine IL-17A in the gut-draining lymph nodes of mice treated with EcN-AvCys compared to DSS controls **(Figure 3.9A)**. As Tregs were positively affected by the application of rAvCys in disease models in previous studies (Danilowicz-Luebert et al. 2013; Schnoeller et al. 2008) the frequencies of FoxP3$^+$ cells in the mLNs were analyzed. EcN-AvCys treatment led to a highly significant increase in frequencies of CD4$^+$Foxp3$^+$ Tregs compared to both healthy and DSS controls **(Figure 3.9B).** To gain insight in a possible modulation of cytokines differentially instructing/supporting Th17 cells and Tregs local levels of IL-6 and TGF-β, were assessed via culturing colon explants. Interestingly, IL-6 production was significantly suppressed comparing DSS controls and mice treated with EcN-AvCys, but not in EcN treated controls **(Figure 3.9C).** TGF-β production; however, was also significantly suppressed in mice fed the transgenic probiotic compared to DSS controls **(Figure 3.9D).**

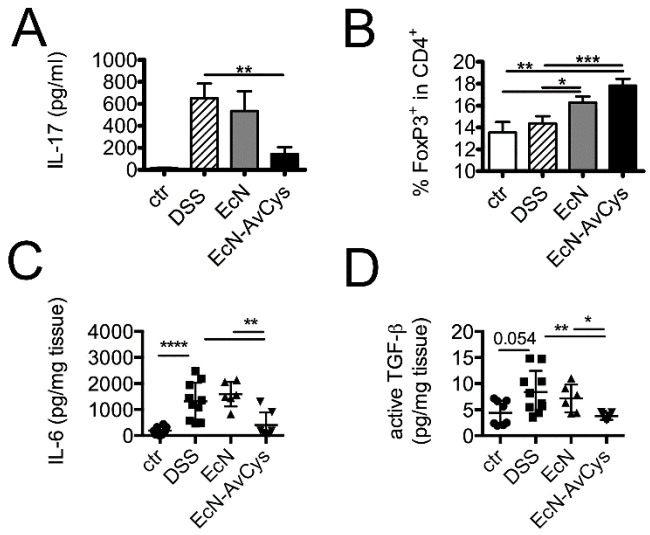


**Figure 3.9.** Effects of EcN-AvCys on local cytokine production and Tregs in DSS colitis. **(A)** Concentration of IL-17A in supernatants of mLN cells stimulated with concanavalin A for 48h as determined by ELISA. **(B)** Frequencies of Foxp3$^+$ Tregs in the CD4$^+$ population in mLN as detected by flow cytometry. **(C, D)** Levels of IL-6 **(C)** and active TGF-β **(D)** in supernatants of distal colon explants cultured for 24h expressed in relation to weight of cultured tissue. Data in A and B are pooled from 3 individual experiments with 2-3 (naïve controls) – 5 mice (DSS, DSS + probiotics) per group. Mean + SEM is shown. Data in C and D depict analyses of 2 colon explants derived from individual mice (n=3-5 per group) and mean ± SEM is shown. Data derived from one of two individual experiments with similar results. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Mann-Whitney U test).

### 3.4. EcN-AvCys is well tolerated and ameliorates post-weaning colon inflammation in piglets

The pig has a digestive tract highly similar to that of humans, (Guilloteau et al. 2010; Meurens et al. 2012; Rothkotter 2009) and post-weaning piglets develop a moderate, non-contagious inflammatory reaction in the gut as a consequence of stress and the diet change (Altenhoefer et al. 2004; Huang et al. 2012; Pedersen et al. 2012; Rothkotter 2009). To determine whether the application of EcN-AvCys is safe or might lead to adverse effects when applied repeatedly in high dosages, a feeding trial with post-weaning piglets was performed. Thirty post-weaning piglets were orally inoculated with $10^{10}$ EcN or EcN-AvCys every 48h for two weeks **(Figure 3.10A)**. During the first week of the study trial, one and two piglets succumbed to post-weaning morbidity in the saline and EcN control groups, respectively, while all recipients of EcN-AvCys survived.

In order to determine if AvCys was present in the gut of piglets after a two week feeding period fecal bacteria were grown on antibiotics to positively select for the antibiotic resistant transgenic bacteria. Feces were diluted in LB media at various concentrations and cultured on plates either without antibiotic supplementation, with Tc or a combination of Tc, Sm and Kn. In a preliminary trial with feces from naïve post-weaning piglets from the Institute of Animal Nutrition at the Freie Universität Berlin, a proportion of colonies cultured on LB agar were able to grow when stamped onto LB containing Tc; however, no multiple antibiotic-resistant clones were detected that could grow when stamped onto plates containing Tc, Sm and Kn **(Figure 3.10B)**. Therefore, the protocol was assumed to be an appropriate method for specific selection of the probiotic in the feeding trial. When the protocol was repeated with piglet feces from the feeding trial, many multiple antibiotic resistant clones could be cultured even in the control group **(Figure 3.10C)**, which then tested negative for both EcN plasmid genes and the AvCys gene by PCR (data not shown). Additionally, as Chromagar plates that have color selective properties were used in the piglet feeding trial analysis the different color and morphology of clones on the Tc, Sm and Kn containing Chromagar plates suggests that the multiple antibiotic resistant clones were comprised of diverse bacterial species **(Figure 3.10C).**

In order to ascertain that AvCys was produced by EcN-AvCys in the piglets, as selective culture protocols were unsuccessful in selectively growing the transgenic probiotic, mLN cells from the piglets were stimulated with recombinant AvCys or a control protein and analyzed for their proliferative responses. As expected only mLN cells from EcN-AvCys treated pigs proliferated specifically in response to recombinant AvCys compared to the control protein **(Figure 3.10D)**, showing that AvCys was produced *in vivo* and recognized by the immune system in piglets administered the AvCys expressing transgenic probiotic.

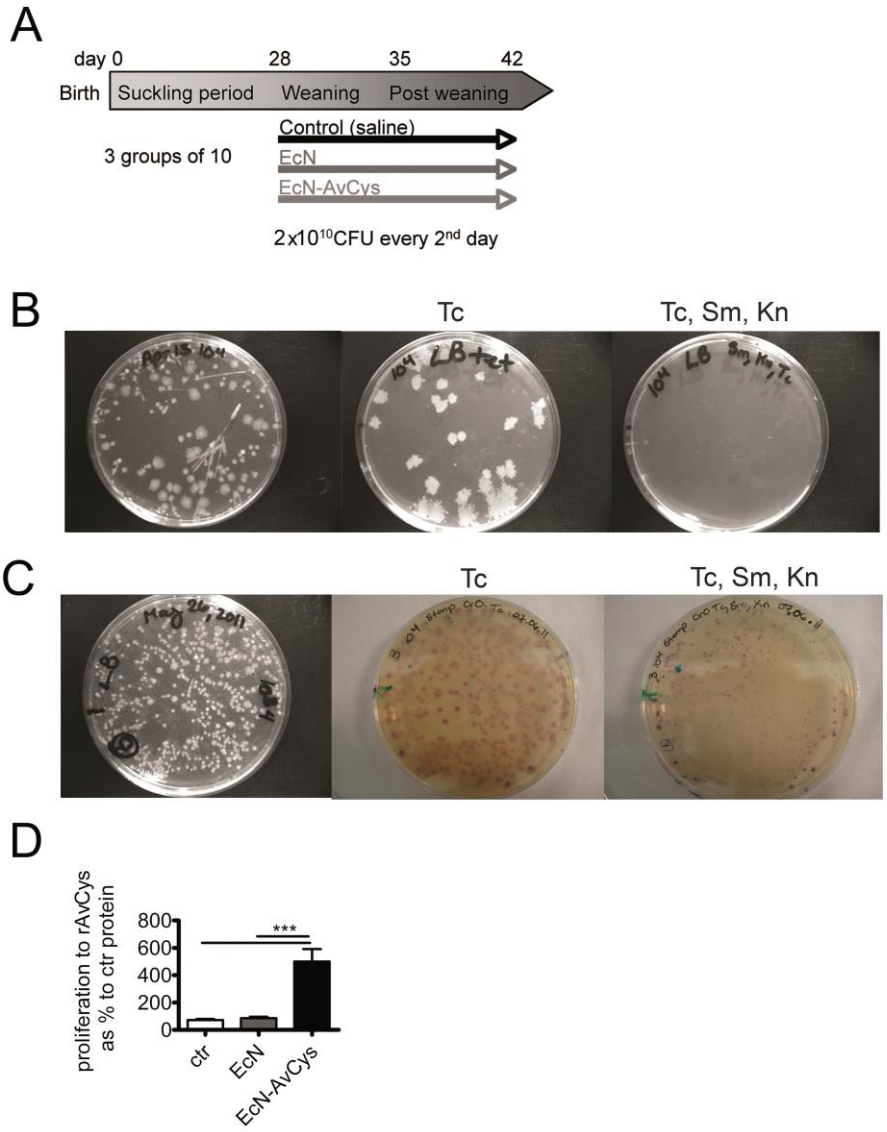


**Figure 3.10.** EcN-AvCys determination in feces from post-weaning piglets administered the transgenic probiotic and proliferative response of mLN leukocytes. **(A)** Three groups of four week old weaned piglets were inoculated with either saline (ctr, n=9), $2x10^{10}$ CFU EcN (EcN, n=8) or $2x10^{10}$ EcN-AvCys (EcN-AvCys, n=10) every 48hrs over 14 days. **(B)** A representative photo of a preliminary trial in which feces of naïve post-weaning piglets was diluted $1:10^4$ and cultured on an LB agar plate before being sequentially stamped onto LB agar containing tetracycline alone (Tc) and LB agar containing Tc, streptomycin (Sm) and kanamycin (Kn). **(C)** A representative photo of feces from a control piglet inoculated with saline in the feeding trial that was diluted $1:10^4$ and cultured on an LB agar plate before being sequentially stamped onto a Chromagar plate containing Tc alone and a Chromagar plate containing Tc, Sm and Kn. **(D)** Proliferative response of mLN leukocytes after 48h incubation with a recombinant control protein or recombinant AvCys as determined by $^3$H-thymidine incorporation. Values are expressed as proliferation indices and mean + SEM is shown. *** $p < 0.001$ (Mann-Whitney U test).

No differences in body weight were detected between the groups at day 7 or 14 of the trial **(Figure 3.11A)** and blood smears performed on the day of dissection showed that all groups had similar proportions of monocytes, granulocytes or lymphocytes in the peripheral blood **(Figure 3.11B)**. To assess whether the post-weaning associated intestinal inflammatory response was affected by probiotic treatment, the histopathology of distal colon cross sections was scored. Expectedly, pigs from the saline control group exhibited moderate signs of inflammation **(Figure 3.11C).** This inflammation was not altered by EcN feeding; however, recipients of EcN-AvCys exhibited a reduced infiltration of immune cells and significantly reduced inflammatory scores compared to the saline control group **(Figure 3.11C)**.

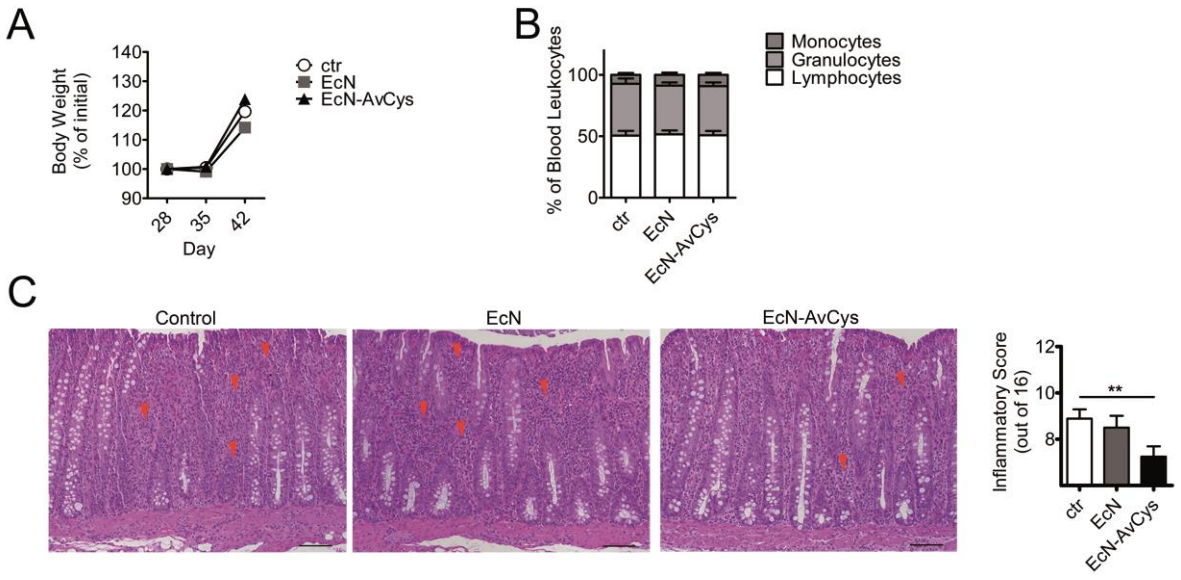


**Figure 3.11.** Physiological changes in response to EcN-AvCys administration to post-weaning piglets. **(A)** Piglets were weighed at day 28, 35 and 42 days old and body weight as a percentage of 28 day old weight is reported. **(B)** Leukocytes were microscopically counted and phenotypically characterized on venous blood smear slides and the percentage of leukocytes categorized as monocytes, granulocytes and lymphocytes was recorded. **(C)** Representative pictures used for histopatholgical scoring of distal colon sections (left) and compiled inflammatory scores for all pigs (right). Red arrowheads depict areas of extensive immune cell infiltration. **p < 0.01 (Mann-Whitney U test).

Since macrophages are targeted by AvCys (Klotz et al. 2011b; Schnoeller et al. 2008) their frequencies and SLA II expression in the colon lamina propria (LP) was assessed by flow cytometry. Neither the frequencies of $CD163^+$ macrophages nor their expression of SLA II was altered in response to EcN-AvCys application **(Figure 3.12A).** Eosinophils were also enumerated from histologically stained colon cross sections and while no significant differences were determined a trend for decreased eosinophil populations in EcN and EcN-AvCys groups compared to the saline treated controls was observed **(Figure 3.12B)**

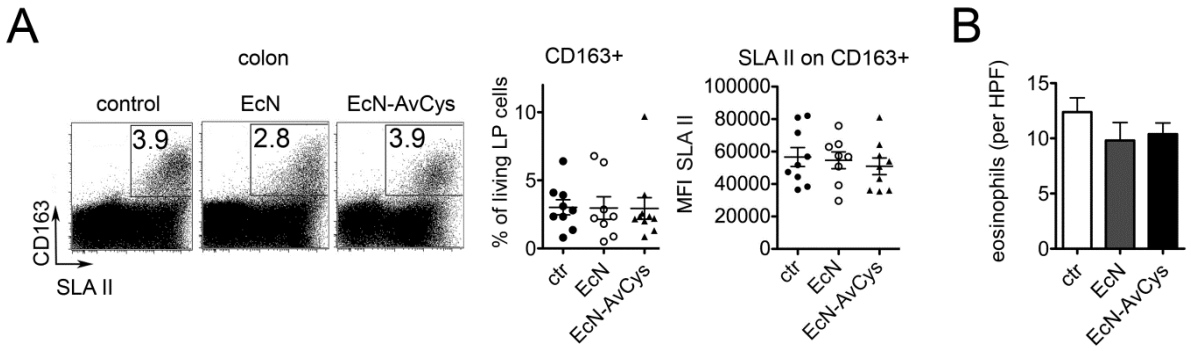


**Figure 3.12.** Innate immune parameters in response to EcN-AvCys in swine feeding trial. **(A)** Frequency of $CD163^+SLAII^+$ macrophages in live colon lamina propria leukocytes and mean fluorescence intensity (MFI) of macrophages stained for SLAII as determined by flow cytometry. **(B)** Numbers of eosinophils as determined by histological staining in colon cross sections. Individual animals and group mean + SEM is shown. No significant differences observed (Mann-Whitney U test). HPF: high power field (400× magnification)

As the numbers of Tregs and their activation status were increased after application of EcN-AvCys in the murine colitis model, mLN and colon LP $CD4^+$ cells were analyzed for frequencies of $Foxp3^+$ Tregs and expression of CD25. Frequencies of $CD4^+CD25^+Foxp3^+$ Tregs were similar in mLN **(Figure 3.13A)** and colon **(Figure 3.13B)** of all groups. In contrast, the frequencies of $CD25^+Foxp3^-$ cells were significantly increased in mLN **(Figure 3.13A)** and colon **(Figure 3.13B)** $CD4^+$ T cells of EcN-AvCys-treated pigs. To determine if the decrease in colon inflammation in the EcN-AvCys treated group was associated with local changes in cytokine production, the colonic expression of inflammatory cytokines IL-6, IL-8, IL-12, IFN-γ, and TNF-α as well as the regulatory cytokines IL-10 and TGF-β was analyzed by real time PCR; however, no significant differences were observed between groups **(Figure 3.13C).** In addition, lamina propria cells isolated from the colon were stimulated with phytohemagglutinin and cytokine levels quantified by ELISA. Trends not reaching statistical significance were observed for a decreased production of IL-6, IFN-γ and TNF-α in the EcN-AvCys compared to the saline control group **(Figure 3.13D).** Protein levels of IL-10 in EcN-AvCys treated group were similar when compared to saline controls and significantly higher when compared to EcN **(Figure 3.13D).**

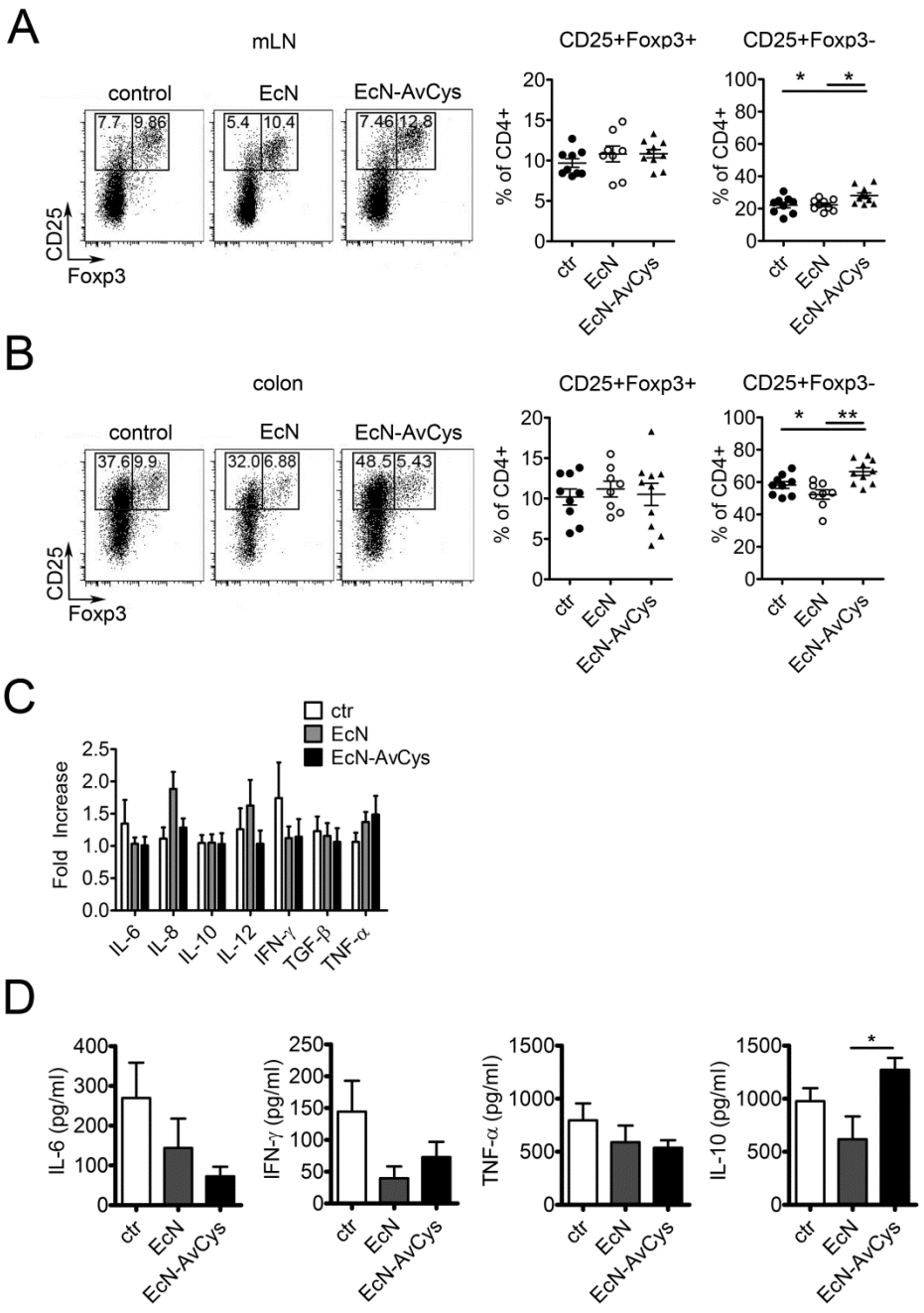


**Figure 3.13.** T cell and cytokine analyses in swine probiotic feeding trial **(A)** Representative plots from flow cytometric analysis of T cell subsets in mLN and **(B)** colon lamina propria leukocytes (LPL) based on expression of CD4, CD25 and FoxP3. Frequencies of $CD4^+CD25^+FoxP3^+$ T regulatory cells (center) and $CD4^+CD25^+FoxP3^-$ T cells (right) are shown as a percentage of total $CD4^+$ lymphocytes for all pigs. **(C)** Distal colon mRNA expression of cytokines normalized to RPL19 and expressed as fold increase above average control. **(D)** IL-6, IFN-γ, TNF-α and IL-10 protein levels detected by ELISA in supernatants of colon lamina propria cells stimulated with phytohemagglutnin for 48h. Mean + SEM is shown. * $p < 0.05$, **$p < 0.01$ (Mann-Whitney U test).

## 3.5. EcN-AvCys supports porcine gut epithelial barrier function

The following data sets for the effects of EcN-AvCys on porcine (section 3.5) and human (section 3.6) epithelial barrier function were obtained through collaborations with Dr. Dorothee Günzel, Dr. Jan Richter and Ahmed Keles at the Institute of Clinical Physiology, Charité-University Medicine Berlin; as well as Dr. Nina Hering and Prof. Dr. Jörg-Dieter Schulzke at the Department of Gastroenterology, Division of Nutritional Medicine, Charité-University Medicine Berlin.

To assess whether the secretion of AvCys affected the known support of EcN for intestinal barrier function (Altenhoefer et al. 2004; Ukena et al. 2005; Veltman et al. 2012; Zyrek et al. 2007) proximal colon tissue explants from pigs were analyzed in Ussing chambers after the feeding trial to determine the transepithelial resistance (TER) and paracellular passage of the small tracer molecule fluorescein (332 Da). While TER of explants from the EcN and especially the saline control fed group successively declined over time, colon explants from the EcN-AvCys group showed relatively stable TER values leading to a significantly higher resistance at 180 min compared to tissue from saline treated controls **(Figure 3.14A)**. Furthermore, a significant decrease in paracellular passage of the organic solute fluorescein was observed with colon tissue from EcN-AvCys treated piglets, but not in EcN inoculated controls, when compared to the saline control group **(Figure 3.14B)**. These data suggest that EcN-AvCys improved epithelial barrier function *in vivo*. To ascertain if prolonged contact of intestinal tissue with EcN-AvCys *in vivo* was necessary for the observed supportive effects on epithelial functions or whether exposure to EcN-AvCys culture supernatant (EcN-AvCys$^{SN}$) could induce similar effects, the TER of colon tissue from untreated, weaned piglets before and after exposure to either EcNcAvCys$^{SN}$ or EcN$^{SN}$ was determined. The relatively brief contact (6 hrs) of colon tissue to EcN-AvCys$^{SN}$ also significantly increased TER compared to the control group, while EcN$^{SN}$ had an intermediate effect not reaching statistical significance **(Figure 3.14C).**

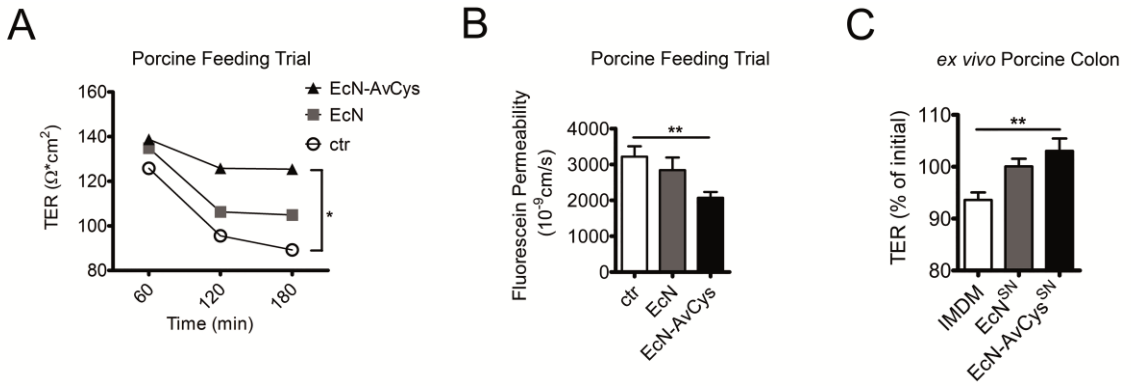


**Figure 3.14**. Effects of EcN-AvCys on porcine epithelial barrier function. **(A)** Transepithelial resistance (TER) of excised colon tissue from pigs treated with EcN (n=8), EcN-AvCys (n=10) and saline treated controls (ctr, n=9) was determined in Ussing chambers at 60, 120 and 180 min after tissue stabilization. **(B)** Colon tissue as in **(A)** was excised from euthanized piglets and permeability to the 332Da tracer molecule fluorescein was determined in Ussing chambers. **(C)** TER across colon tissues excised from untreated, healthy piglets after exposure to either IMDM (IMDM, n=7), EcN conditioned IMDM media ($EcN^{SN}$, n=7) or EcN-AvCys conditioned IMDM media ($EcN\text{-}AvCys^{SN}$, n=7) for 6hrs. Values are expressed as % of the initial value after tissue stabilization. Mean + SEM is shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way or two-way ANOVA).

## 3.6. EcN-AvCys supports barrier function of human colon epithelial cells

Whether the effects of $EcN\text{-}AvCys^{SN}$ on epithelial barrier functions were restricted to porcine gut tissue or also detectable in cultures of epithelial cells devoid of macrophages and other immune cells was then assessed. Monolayers of the human colon epithelial cell line HT-29/B6 were incubated with $EcN\text{-}AvCys^{SN}$ or $EcN^{SN}$. Compared to the control media, a significant increase in TER was observed when cells were incubated for 3h with $EcN\text{-}AvCys^{SN}$, but not with $EcN^{SN}$, while exposure for 22 hrs to either $EcN\text{-}AvCys^{SN}$ or $EcN^{SN}$ led to highly significant increases in resistance **(Figure 3.15A)**. To determine whether the increased TER of HT-29/B6 monolayers in response to $EcN\text{-}AvCys^{SN}$ and $EcN^{SN}$ could be ascribed to the differential modulation of small and large pores permitting the paracellular passage of anorganic ions, the flux of fluorescein passing through small pores (approx. 4.5Å) and labeled 4kDa dextran restricted to passage through larger pores (> 7Å) was measured. While contact to $EcN\text{-}AvCys^{SN}$ and $EcN^{SN}$ significantly decreased the passage of fluorescein, the passage of dextran was not affected significantly **(Figure 3.14B).** Hence, EcN and EcN-AvCys predominantly affect the availability of small pores for the passage of small anorganic solutes leading to an increase in TER in monocultures of human epithelial cells.

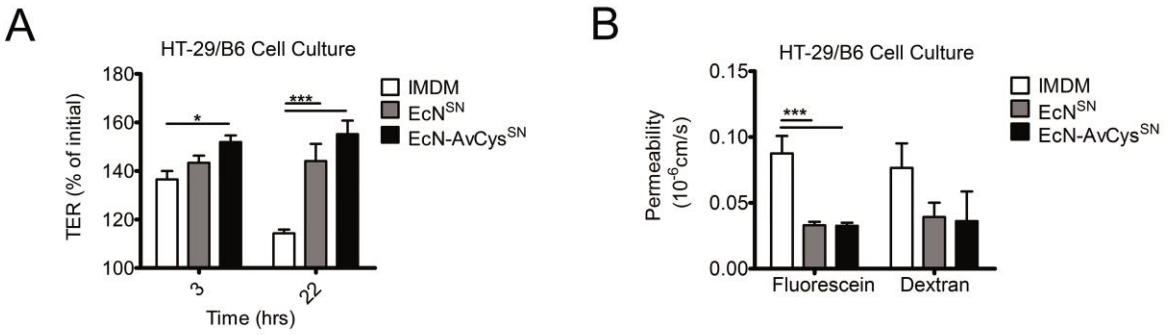


**Figure 3.15.** Effects of EcN-AvCys on human intestinal barrier function in vitro. **(A)** TER of HT-29/B6 cell monolayers exposed to either IMDM (n=6), $EcN^{SN}$( n=6) or EcN-$AvCys^{SN}$ (n=6) for 3 and 22 hrs. Values are expressed as a percentage of the initial value after tissue stabilization. **(B)** Paracellular flux of fluorescein and 4kDA dextran measured with HT-29/B6 cell monolayers in Ussing chambers after pre-incubation with $EcN^{SN}$ (n=8), EcN-$AvCys^{SN}$ (n=7) or unconditioned media as a control (n=8) for 22h. Mean + SEM is shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way or two-way ANOVA).

## 3.7. Examination of cells and mechanisms involved in EcN-AvCys immune modulation

### 3.7.1. EcN-AvCys modulation of monocyte and macrophage phenotype

It has previously been shown that contact with rAvCys causes murine macrophages to initially up-regulate pro- and anti-inflammatory marker genes such as iNOS, IL-12/23p40, TNF-α, IL-6 and IL-10 and acquire an M2a/b like phenotype marked by the expression of LIGHT, sphingosine kinase 1, arginase-1, MHCII, CD16/32, ICAM-1, PDL-1 (CD274) and PDL-2 (CD273) later on ((Klotz et al. 2011b) and unpublished). These macrophages were then shown to cause the expression of IL-10 in T cells and they efficiently suppressed inflammation when transferred to mice in the DSS colitis model (unpublished data). *Ex vivo* analyses in the pig feeding trial with EcN and the transgenic probiotic had shown no differences of $CD163^+$ monocytes/macrophages frequencies or activation as determined by SLA II expression depending on the treatment **(Figure 3.12B)**. Attempting to investigate possible phenotypic changes of porcine monocytes/macrophages in response to the transgenic probiotic, cells were investigated for the expression of markers genes after contact with cell media supernatants in which either EcN or EcN-AvCys were cultured.

As very little is known about the macrophage/monocyte phenotype in porcine compared to human or mouse cells, other than a preference for a selective induction of IDO1 in porcine cells compared to iNOS in murine cells in response to inflammatory stimuli, a preliminary phenotyping of monocytes exposed to control stimuli was conducted. Monocytes were isolated from porcine blood with anti-CD14 magnetic beads and stimulated with

recombinant IFN-γ and LPS, factors leading to the classical activation of M1 macrophages, or recombinant IL-4 and IL-13 in the presence of low amounts of activating LPS as stimuli leading to the alternative activation of M2 macrophages for 4, 16 and 48 hrs **(Figure 3.16).**

The M1 stimuli induced a trend for an increased expression of *IL12*, *IL6*, *TNFA* and *IL10* at 48 hrs, and a significant increase in the expression of *IL1B* at 48 hrs compared to 4 hrs and *IDO1* at 48 hrs compared to 4 and 16hrs **(Figure 3.16)**.

Originally IL-4 alone as well as IL-4 in conjunction with IL-13 stimuli were attempted to induce an M2 phenotype in monocytes; however, these induced no changes in marker expression (data not shown). Utilizing IL-4 and IL-13 in conjunction with low levels of TLR-activating LPS as an M2 stimulus, likewise did not induce many changes in marker expression in porcine monocytes. However, a trend towards a late increase in *TNFA* expression at 48hrs and a significant transient increase in *CD274* at 4 and 48 hrs compared to 16 hrs was observed. Unexpectedly, a trend towards a decrease in M2 associated marker *ARG1* was observed over time **(Figure 3.16)**.

Comparing marker expression between the two treatment groups, there was a trend at 48 hrs for an increased expression of *TNFA*, *IL6* and *IL12* in M1 vs M2 stimulated monocytes **(Figure 3.16)**. A significant increase in the expression of *IL10*, *IL1B* and *IDO1* in M1 compared to M2 stimulated monocytes was detected at 48 hours **(Figure 3.16).**

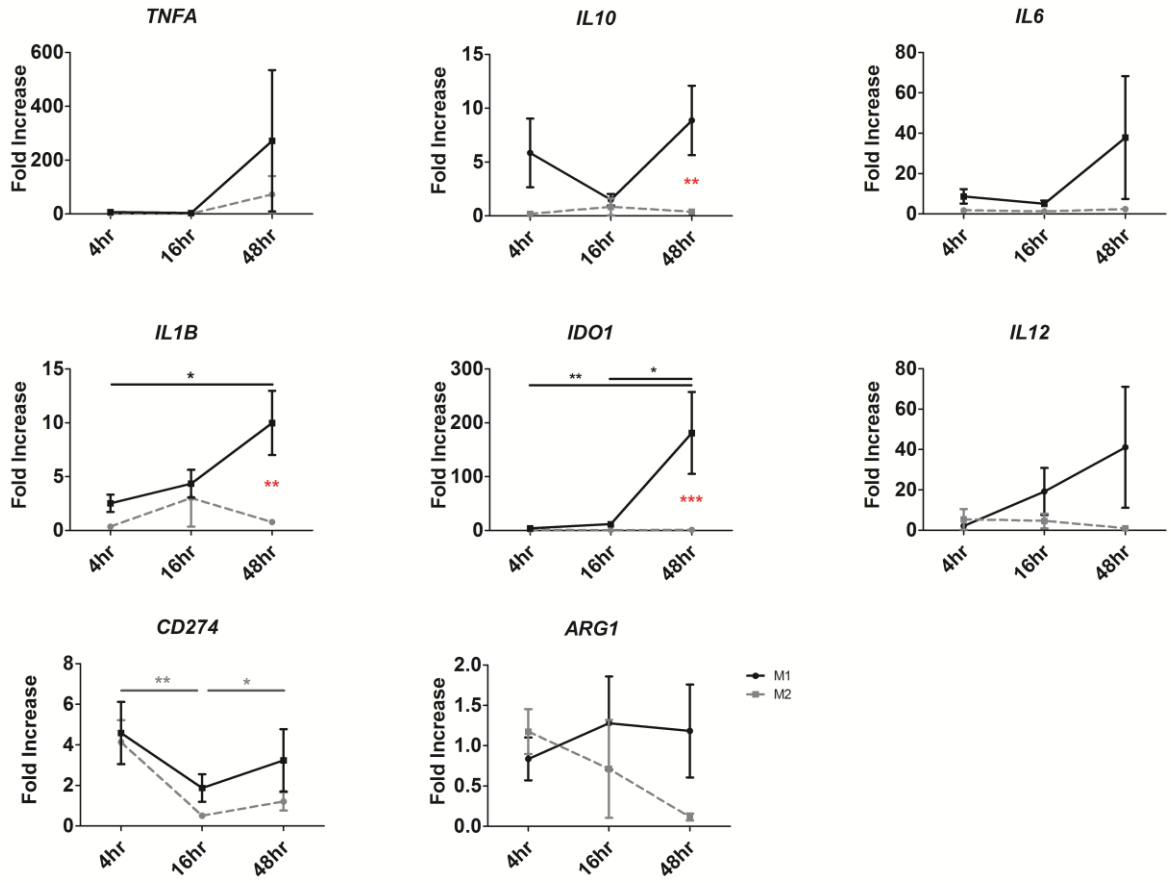


**Figure 3.16.** Effects of conventional M1 and M2 stimuli on swine monocyte phenotype. Porcine monocytes (n=5-7) were stimulated with either IMDM media, M1 classically activating stimuli LPS + IFN-γ, or M2 alternatively activating stimuli IL-4 + IL-13 in the presence of low level LPS for 4, 16 or 48 hr. The mRNA expression of macrophage phenotype marker genes *TNFA*, *IL10*, *IL6*, *IL1B*, *IDO1*, *IL12, CD274* and *ARG1*, was analyzed with RT-PCR, normalized to the housekeeping gene RPL19 and values are presented as fold increase above the media control. Mean + SEM is shown. ** $p<0.01$ *** $p<0.001$ (two-way ANOVA) represents a significant difference between treatment groups at a specific time point. * $p<0.05$ ** $p<0.01$ (one-way ANOVA) represents a significant difference in time points for M1 stimulated monocytes. * $p<0.05$ ** $p<0.01$ (one-way ANOVA) represents a significant difference in time points for M2 stimulated monocytes.

Next, the effects of $EcN^{SN}$, EcN-$AvCys^{SN}$ or purified rAvCys on the expression of monocyte phenotype markers were evaluated. Monocytes were isolated from porcine blood with anti-CD14 magnetic beads and stimulated either with an IMDM media control, $EcN^{SN}$, EcN-$AvCys^{SN}$ or rAvCys for 4, 16 or 48 hrs. Expression of macrophage phenotype markers was then analysed with RT-PCR and a fold increase compared to the media controls at each time point was calculated.

While there were no significant differences in the expression of any markers between different time points with any of the treatment conditions there were some observed trends. A trend towards early high expression of *TNFA, IL10, IDO1* and *IL12* at 4hrs in $EcN^{SN}$ or EcN-$AvCys^{SN}$ incubated monocytes was observed. Trends for an increased expression of *ARG1* were detected in response to EcN-AvCys and EcN-treatment at 48 hrs. Comparing responses to $EcN^{SN}$ and EcN-$AvCys^{SN}$ treatment revealed a significant decrease in the expression of *CD274* and a trends towards decreased expression in *ARG1* in monocytes exposed to EcN-$AvCys^{SN}$ compared to $EcN^{SN}$ at 48 hrs **(Figure 3.17)**. The exposure to purified rAvCys had no effect on the expression of the selected markers by porcine monocytes.

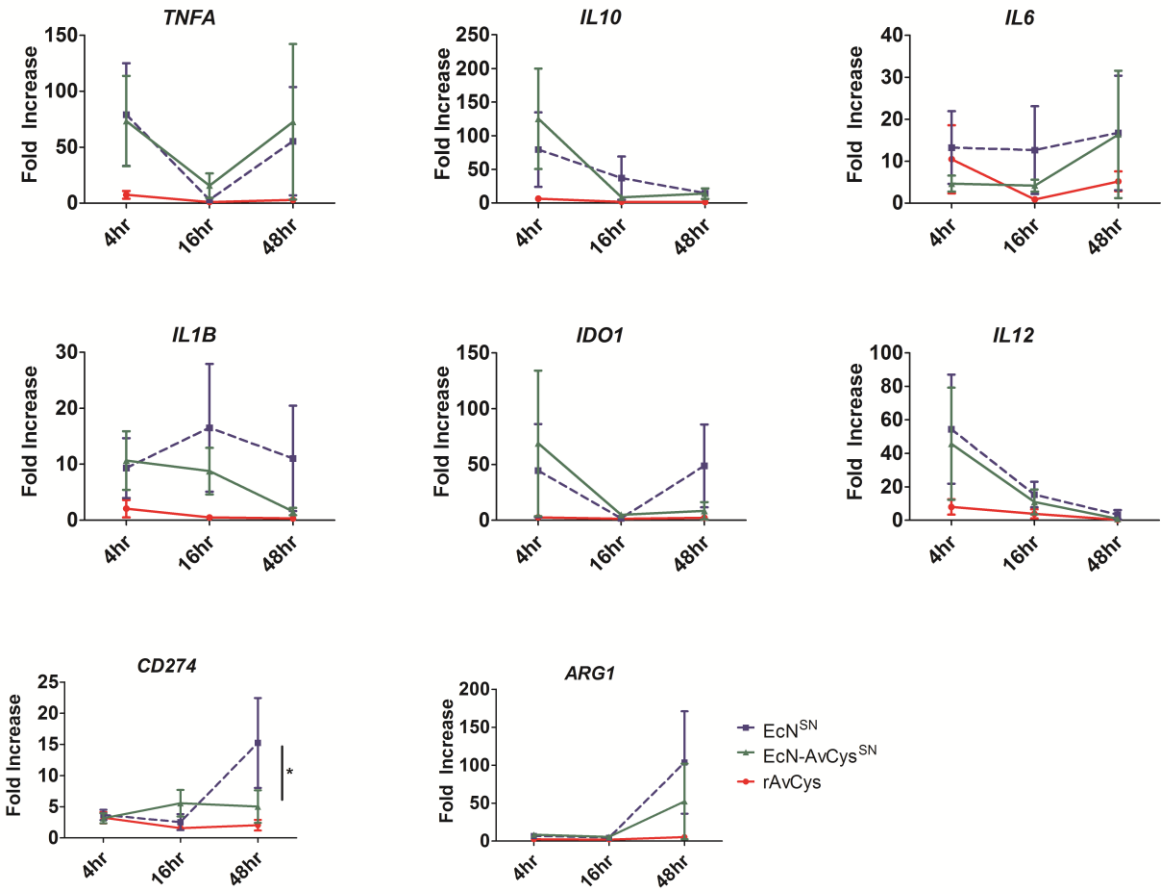


**Figure 3.17.** Effects of EcN and EcN-AvCys supernatants as well as rAvCys on swine monocyte phenotype. Porcine monocytes (n=5-7 per group) were stimulated with either IMDM media, EcN supernatant ($EcN^{SN}$), EcN-AvCys supernatant ($EcN\text{-}AvCys^{SN}$), or rAvCys for 4, 16 or 48 hrs. The mRNA expression of macrophage phenotype marker genes *TNFA*, *IL10*, *IL6*, *IL1B*, *IDO1*, *IL12*, CD274 and ARG1, was analyzed with RT-PCR, normalized to the housekeeping gene RPL19 and values are presented as fold increase above the media control. Mean + SEM is shown. * $p < 0.05$, ** $p<0.01$ (two-way ANOVA) represents a significant difference between treatment groups at a specific time point.

### 3.7.2. Effects of rAvCys on IL-1β secretion

IL-1β is a cytokine that has been shown to be vital to murine colitis (Bauer et al. 2010) and is likewise expressed in significantly higher levels in the mucosa of patients with active IBD compared to healthy individuals (Mahida et al. 1989). It is expressed as inactive pro- form that requires activation through the action of a protein complex known as the inflammasome. It was hypothesized that due to the dependence of the activation of the inflammasome on cysteine proteases (Hornung and Latz 2010), the cysteine protease inhibitor AvCys may inhibit the activation of this protein complex and therefore, inhibit the downstream activation and secretion of IL-1β.

To test the hypothesis that AvCys may inhibit the activation of the inflammasome and downstream activation and secretion of IL-1β, human blood derived monocytes were differentiated into macrophages with human rM-CSF for 7 days. Macrophages were then stimulated with LPS either in IMDM, $EcN^{SN}$ or EcN-$AvCys^{SN}$ for 4 hrs before addition of the secondary stimuli ATP or DSS. The concentration of IL-1β in the supernatants was determined by ELISA. Using ATP as a secondary signal, the presence of $EcN^{SN}$ and EcN-$AvCys^{SN}$ significantly increased the secretion of IL-1β by macrophages compared to the control stimulated only with LPS and ATP **(Figure 3.18A)**. As macrophages generally responded poorly to ATP and DSS as secondary signals to induce IL-1β secretion **(Figure 3.18A)**, undifferentiated blood-derived monocytes were also assessed for differential IL-1β responses accordingly. Monocytes reacted with a much more vigorous IL-1β secretion to both secondary stimuli, which in the case of ATP as secondary signal was not altered by the pre-incubation with LPS in $EcN^{SN}$ or EcN-$AvCys^{SN}$. In monocytes with DSS secondary stimulation EcN-$AvCys^{SN}$ significantly increased the secretion of IL-1β compared to the positive control, suggesting that the AvCys secreted in the supernatants of EcN-AvCys actually induced the activation and secretion of the cytokine **(Figure 3.18B)**. Taken together, these data do not support the hypothesis that AvCys inhibits the inflammasome activation and may actually provide evidence of a supportive role of AvCys in the activation of IL-1β.

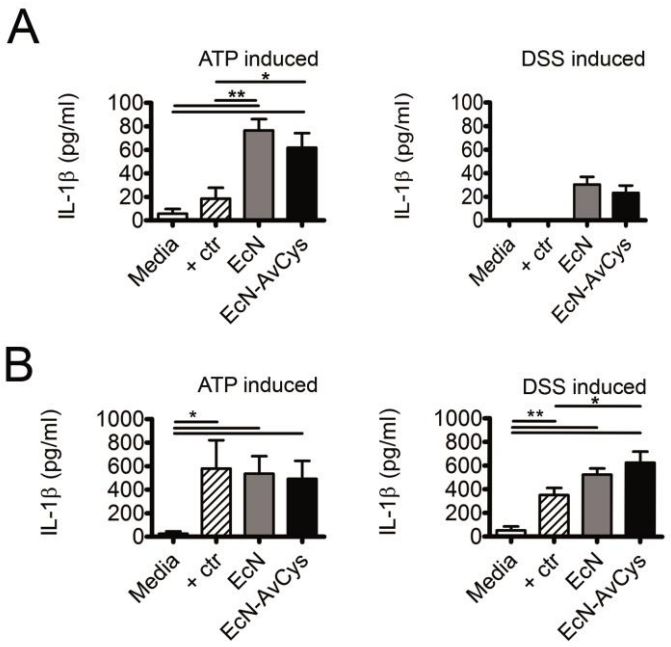


**Figure 3.18.** Effect of EcN-AvCys on the induction of IL-1β secretion by human monocyte-derived macrophages and primary human monocytes. **(A)** Human macrophages differentiated from blood-monocytes were cultured in IMDM (media) or stimulated with LPS in IMDM (+ ctr), $EcN^{SN}$ (EcN) or $EcN\text{-}AvCys^{SN}$ (EcN-AvCys) and IL-1β secretion induced with the secondary signal ATP or DSS (pooled data from 2 experiments each performed with n=3-6). Concentration of IL-1β was determined in the supernatants by ELISA. **(B)** Human blood derived monocytes were treated as in A (pooled data from 2 experiments each performed with n=3-6). Mean + SEM is shown. * $p < 0.05$, ** $p < 0.01$ (Mann-Whitney U test).

# 4. Discussion

IBD affects approximately 3.6 million North Americans and Europeans and as an incurable disease often requires lifelong treatment. There is an apparent and urgent need to develop new IBD therapies that are safe, effective, cost efficient and have the potential to treat a broad spectrum of patients suffering from IBD of varying etiologies. Cystatin from a parasitic worm (AvCys) is an interesting candidate for the treatment of IBD as the recombinant protein efficiently inhibits inflammatory responses in murine models of allergic airway hyper-reactivity and colon inflammation (Danilowicz-Luebert et al. 2013; Klotz et al. 2011b; Schnoeller et al. 2008). Mechanistically, AvCys functions through the induction of IL-10 expressing macrophages and subsequent suppression of inflammatory T cell populations (Figueiredo et al. 2009; Klotz et al. 2011b; Schnoeller et al. 2008). The probiotic *E. coli* Nissle 1917, marketed as Mutaflor®, has been shown to efficiently suppress infant diarrhea and IBD in clinical trials (Henker et al. 2008a; Kruis et al. 2004; Matthes et al. 2010) and to ameliorate colitis in animal models (Garrido-Mesa et al. 2011; Schroeder et al. 2006; Schultz et al. 2004). Additionally, EcN readily colonizes the human gut (Lodinova-Zadnikova and Sonnenborn 1997; Cukrowska et al. 2002; Schulze and Sonnenborn 1995) and is suggested as a safe carrier for therapeutic proteins (Duncker et al. 2006; Westendorf et al. 2005). Thus, the aim was to generate a transgenic probiotic EcN expressing the potent nematode immunoregulator AvCys to allow for a site-directed and prolonged release of the immunoregulator in the gut. EcN-AvCys was tested for its effects on intestinal inflammation in a mouse model of IBD and spontaneously occurring inflammation in post-weaning pigs as a genetically diverse model of the human gastrointestinal system.

## 4.1. Amelioration of murine experimental colitis by EcN-AvCys

The use of DSS to induce colitis in rodents is a commonly used model to investigate acute gut inflammation. DSS is added at concentrations of 2-5% in the drinking water which results in disruption of the intestinal epithelial barrier and activation of an inflammatory response. Physiological parameters include diarrhea often including blood, rapid weight loss, colon shortening, and an increase in both gut permeability and influx of inflammatory cell populations (Hall et al. 2011). The DSS colitis model shows some pathological and histological similarities to ulcerative colitis, one form of human IBD (Hall et al. 2011; Okayasu et al. 1990).

Preliminary trials revealed that EcN colonization in C57Bl/6 mice requires antibiotic administration. Preliminary trials conducted before the onset of this doctoral work showed that with the use of streptomycin (Sm), to which the EcN strain investigated here is

resistant, EcN was able to colonize and could be detected in the feces up to 21 days after a single inoculation (unpublished). However, when Sm administration was attempted in conjunction with the DSS model, the model failed and no intestinal inflammation was observed (unpublished). This is likely due to the requirement of intestinal bacteria to induce the inflammatory reaction in the DSS colitis model. DSS administration has been shown to alter the microbial populations in the gut to increase the numbers of inflammatory gram negative bacteria such as *Desulfovibrio* spp*., Akkermansia* spp*., Bacteriodes* spp., *Provotella* spp. and several members of Enterobacteriaceae family, as well as gram positive bacteria of the *Enterococcus* genus, that are also shown to be up-regulated in UC patients compared to healthy individuals (De Fazio et al. 2014; Hakansson et al. 2014). While cause and effect is not clearly indicated for these changes in microbial populations, TLR signaling of microbe associated molecular patterns from bacterial species in the gut are involved in the pathogenesis of DSS induced colitis. While gene knock outs that disrupt the signaling of all TLRs has been shown to exacerbate DSS colitis and induce mortality due to a complete disruption in epithelial barrier protection and repair mechanisms (Rakoff-Nahoum et al. 2004), other studies show that blocking specific TLR signaling can reduce DSS severity. For instance, TLR4 recognition of bacterial LPS is important to the DSS model as induction of TLR4 hypo-responsiveness inhibits the onset of colitis after DSS administration (Nagar et al. 2014). The hypothesis that TLR signaling in response to bacterial pathogens induces inflammation in acute DSS colitis is supported by studies showing that the presence of gram positive bacteria in the lumen is required for inflammatory pathology due to DSS administration (Petersen et al. 2011). Furthermore, germ-free rearing can reduce the severity of DSS induced intestinal inflammation compared to conventionally reared controls (Kitajima et al. 2001). It should be noted that a similar study in a different mouse strain found that DSS administration to germ free animals induced mortality within only a few days (Hudcovic et al. 2001). However, in this study the deaths were caused by gross rectal bleeding and could not be attributed directly to other colitis parameters, but were thought to be potentially due to anticoagulant properties of the DSS in this model (Hudcovic et al. 2001). This indicates that at least in some strains of mice, microbial populations in the gut lumen are important to the mechanism by which DSS causes colitis. As Sm has been shown to cause moderate (Croswell et al. 2009) to extensive (Kaiser and Hardt 2011) reduction in microbial load with broad spectrum action against both gram positive and gram negative species, it is unsurprising that Sm administration inhibited the acute DSS model that has been so well documented to require the presence of colon microbiota.

A chronic colitis model in which DSS was administered in 3 cycles interspersed with periods of healing was therefore, employed in an attempt to reduce the effects of Sm on the

model. As infiltration of T cells is observed in chronic DSS colitis models (Melgar et al. 2006), it was hypothesized that the presence of inflammatory T cell populations in the third cycle of DSS would make the system less dependent upon macrophage sensing of gut bacteria and therefore, Sm administration in this final cycle would not interfere with the induction of inflammation as is seen in the acute model. This hypothesis was supported as there were no observed differences in body weight, colon length, inflammatory score and inflammatory cytokine expression between the DSS and DSS + Sm treated groups. This data suggests that the antibiotic treatment did not interfere with development of inflammation in this modified repetitive DSS feeding approach. Unfortunately, no observed differences in the parameters measured were observed between any of the control or treatment groups in this study. This lack of differences between the DSS controls and EcN and EcN-AvCys treatment groups may be due to the overall severity of inflammation observed at this time point. However, despite the severe colitic response there were trends to suggest increased colon length as well as decreased inflammatory score and inflammatory cytokine expression in the EcN and EcN-AvCys groups, warranting further investigation in models of colitis with less extreme pathology.

The priority was thus set on the acute DSS colitis model to investigate the anti-inflammatory potential of the transgenic probiotic EcN-AvCys. In order to circumvent the inhibitory impact of antibiotics in this model and the lack of colonization of EcN and EcN-AvCys without the use of antibiotics, the probiotics were administered every 48hrs for the duration of the eight days of DSS administration. Furthermore, the probiotics were administered to the mice at $2x10^9$ CFU/48 hrs which is a relatively high dose compared to standard recommended human doses of Mutaflor® which are between $1x10^8$ – $5x10^{10}$ CFU/day **(Table 1.4)**. This regular interval dosing method used in the acute murine trial may provide a more realistic dosing regimen to the human condition as EcN, like many other probiotics, is also generally administered at regular interval doses. It has been speculated that induction of antimicrobial production in human colonic epithelial cells in response to EcN (Becker et al. 2013; Mondel et al. 2009; Schlee et al. 2007; Wehkamp et al. 2004), may be one reason for this need to repeatedly dose the probiotic as EcN is not itself resistant to these antimicrobials (Mondel et al. 2009). Therefore, while the probiotic does colonize it may require boosts to maintain colonization in the face of host expressed antimicrobial peptides.

With the acute DSS model it was confirmed that the probiotic EcN inhibits intestinal inflammation as shown in previous studies (Garrido-Mesa et al. 2011; Ukena et al. 2007). However, colon inflammation was even more efficiently controlled in mice treated with the transgenic EcN-AvCys. The beneficial effect was associated with significantly reduced

frequencies of inflammatory F4/80$^{+}$Ly6C$^{high}$ monocytes/macrophages and a decrease in iNOS$^{+}$ M1 macrophage numbers in the colon. While previous studies have shown that macrophages are the dominant target of AvCys, transiently producing high amounts of IL-10 after contact with AvCys and subsequently suppressing inflammatory T cells (Klotz et al. 2011b; Schnoeller et al. 2008), the observed reduction in infiltrating inflammatory monocytes was a new finding. The current study shows that the application of EcN-AvCys significantly suppressed the production of MIP-1α/β, MCP-1/3 and RANTES in the colon. These chemokines efficiently recruit monocytes/macrophages, T cells and granulocytes to sites of tissue damage and inflammation, activate monocytes/macrophages and lead to degranulation of attracted granulocytes (Rollins 1997). While *in vitro* co-cultures of EcN with human intestinal cell lines have reported the probiotic to increase the epithelial expression of MCP-1, RANTES and other macrophage inhibitory proteins (MIP-2α/β) (Hafez et al. 2009; Ukena et al. 2005), other studies have conversely observed a reduction in MCP-1 and MIP-2 expression when EcN was administered in a DSS model (Garrido-Mesa et al. 2011). The acute DSS trial did not support an EcN specific effect on the expression of these chemokines, but rather suggests that AvCys is responsible for the observed reduction in these chemokines in response to EcN-AvCys treatment. The increased expression of these chemokines has been associated with active disease in IBD patients (Banks et al. 2003; Mazzucchelli et al. 1996) as well as inflammation in the DSS model (Melgar et al. 2006). As activated monocytes and macrophages are important chemokine sources it is tempting to speculate that AvCys produced by the transgenic probiotic interferes with local chemokine production by tissue resident macrophages and subsequent leukocyte migration to the gut. Future studies will further address these newly detected effects of AvCys to unravel whether the drastically reduced colon inflammation in mice fed EcN-AvCys primarily reflects an effect on macrophage-mediated recruitment of inflammatory monocytes.

The reduction of eosinophils in EcN-AvCys may be a consequence of reduced expression of MCP-3 and RANTES, which are also eosinophil chemoattractants (Wedemeyer and Vosskuhl 2008). However, as eosinophils were equally decreased in EcN treated mice, to which no decrease in chemoattractant expression was observed, it is likely through another mechanism that a reduction in eosinophils was observed. The probable candidate for the common reduction in colon eosinophils in both the EcN and EcN-AvCys treated groups is a decrease in CCL11, an eosinophil specific chemokine (Wedemeyer and Vosskuhl 2008). In DSS colitis eosinophilia has been shown to be dependent upon F4/80$^{+}$Ly6C$^{+}$CD11b$^{+}$CCR2$^{+}$ inflammatory monocyte production of CCL11 (Waddell et al. 2011), so the significant decrease in overall F4/80$^{+}$Ly6C$^{high}$ expressing monocytes in response to EcN-AvCys and the observed trend toward a decrease in this population in the

EcN treated group may include an overall reduction in the CCLL expressing monocytes and provides one possible explanation for reduced eosinophilia in both the EcN control and EcN-AvCys treatment groups.

The production of IL-17A, a cytokine essentially involved in DSS-induced pathology (Ito et al. 2008) was significantly suppressed, while Treg frequencies in the gut draining lymph nodes were increased in recipients of EcN-AvCys. The contribution of Tregs to the suppressed colitic response in recipients of the transgenic probiotic was not directly tested. However, the increased frequencies of Tregs in the gut-associated lymph nodes correlating with a lower inflammatory cytokine expression and reduced gut tissue damage are in line with the established view of the central importance of Tregs in maintenance of intestinal homeostasis and control of intestinal inflammatory processes (Unutmaz and Pulendran 2009). Furthermore, in previous studies, $Foxp3^+$ Tregs were also found to be positively affected when recombinant AvCys was applied intraperitoneally in disease models of experimental airway hyper-reactivity and colitis and their depletion partially abolished the protective effects (Schnoeller et al. 2008; Danilowicz-Luebert et al. 2013). The reciprocity between Th17 and Treg development (Korn et al. 2009) allowing speculation that AvCys secreted by EcN tips the balance between Tregs and Th17 cells. In the acute DSS experiments local levels of IL-6, a cytokine driving Th17 differentiation in presence of TGF-β (Korn et al. 2009) sharply declined in response to EcN-AvCys treatment, which might be involved in the significantly lower IL-17 levels detected after feeding of the transgenic probiotic. EcN alone has been reported to reduce the expression of IL-6 in DSS colitis (Garrido-Mesa et al. 2011; Schultz et al. 2004); however, our results do not support this finding as IL-6 was only significantly reduced in the EcN-AvCys treated group. Furthermore, levels of active TGF-β were also significantly lower in mice after EcN-AvCys treatment, a factor possibly further restricting the differentiation of Th17 cells. It remains to be investigated whether IL-21 and IL-1β supporting Th17 differentiation (Fina et al. 2008; Shaw et al. 2012) are also controlled by EcN-AvCys feeding. The cytokine IL-23 known for supporting maintenance of Th17 while restraining Treg activity (Izcue et al. 2008; Stritesky et al. 2008) was actually increased by EcN-AvCys feeding this study and thus is unlikely to be responsible for the observed reduction in IL-17 or inflammatory score. While this is an unexpected observation considering the overall reduction in inflammatory cells and mediators, at least part of this induction of IL-23 could be due to EcN, as a trend for increased IL-23 was observed for EcN treated groups and EcN has been reported to increase the expression of IL-23 in human DCs (Gad et al. 2011).

Other potential mechanisms by which EcN-AvCys may increase Treg populations is through the Treg supporting mediator retinoic acid (RA). RA produced by intestinal

$CD103^+$ DC supports Treg induction and function by enhancing TGF-β and restricting IL-6 signaling (Mucida et al. 2007). Thus future trials should elucidate whether changes in intestinal DC producing RA are associated with the preferential support for Tregs and suppression of IL-17 responses by EcN-AvCys. Additionally or alternatively the effects of EcN-AvCys on MAPK pathways may be involved in the observed changes to the Treg/Th17 balance. Previous studies show that AvCys utilizes MAPK signaling resulting in IL-10 expression in macrophages (Klotz et al. 2011b). Recently it has been shown that ERK signaling is involved in the Treg/Th17 dichotomy. Blockade of ERK activation results in suppression of Th17 development by interfering with IL-6 mediated RORγt expression, while supporting TFG-β driven up-regulation of Tregs (Liu et al. 2013). Thus AvCys might interfere with the Treg/Th17 balance *in vivo* by affecting MAPK pathways in T cells.

An increased IL-10 production by T cells in response to EcN-AvCys treatment was not observed in the acute DSS trials study. It is thus unlikely that $Foxp3^+$ or $Foxp3^-$ T cells, by producing IL-10, restrained Th17 responses, a mechanism previously shown by Huber et al (Huber et al. 2011). Conversely, EcN-AvCys does appear to support Tregs, although due to the low amounts of TGF-β production observed it is unlikely to involve this Treg supporting cytokine. The support mechanism by which EcN-AvCys supports Tregs is therefore, still unidentified and future *in vitro* trials should focus on the effects of EcN-AvCys on other mediators of Treg induction in inflamed intestines such as hypoxia inducible factors (Clambey et al. 2012; Higashiyama et al. 2012), factors involved in Treg migration such as the homing integrin α4β7 (Gratz and Campbell 2014) and the expression of C-C chemokine receptors on Tregs (Huehn and Hamann 2005); as well as mediators of Treg function such as CTL-4 (Gratz and Campbell 2014) and IL-33 (Schiering et al. 2014).

The $CD4^+CD45RB^{hi}$ transfer colitis model was also assessed for EcN-AvCys effects as a model where the outgrowth of TNF-α and IL-2 expressing $CD4^+CD45RB^{hi}$ T cells introduced into the otherwise lymphocyte deficient $Rag^{-/-}$ mouse strain drives the inflammatory process (Ten Hove et al. 2004) as opposed to the macrophage dependent DSS model. This model; however, was unsuccessful as administration of Sm in these animals also prevented the onset of the disease. This finding has recently been supported by Nemoto et al, in a study where it was observed that antibiotic treatment of mice prior to the T cell transfer in this model inhibited colitis (Nemoto et al. 2013). Despite this model being driven by expanding effector memory T cells, it is hypothesized that a dependence upon microbial antigen presentation by gut APCs is still required for initial induction (Nemoto et al. 2013). As has been demonstrated with the repeated administration of EcN-

AvCys in the acute DSS model, colonization is not necessary for the amelioration of inflammation via the transgenic probiotic. Thus theoretically antibiotic supported colonization of EcN and EcN-AvCys could be replaced by continuous oral dosing of the probiotics as was performed in the acute DSS trial. However, as oral gavage induces stress in mice and experience suggests that any small inhalation of EcN or EcN-AvCys can induce mortality, continual oral gavage in the transfer colitis model (that can take more than four weeks) would likely result in mortality rates too high to be ethical or efficient. As porcine models of spontaneously occurring intestinal inflammation exist in which colonization with the probiotic can be achieved in an animal model with a greater similarity in gastrointestinal physiology, mucosal immunology and genetic variability compared to the mouse, the value of pursuing time consuming, T cell driven murine models was not prioritized.

While long term colonization in the mouse was difficult to achieve the data generated from administration of EcN-AvCys in the acute DSS model show that a reduced chemokine expression in the gut, low numbers of inflammatory monocytes/macrophages, support for Tregs by a yet unknown mechanism and suppression of IL-6 and Th17 responses correlate with a significant amelioration of intestinal inflammation.

### 4.2. EcN-AvCys ameliorates porcine post-weaning gut inflammation

To further test the transgenic probiotic in an outbred model EcN-AvCys was applied to pigs reflecting an excellent model of the human monogastric omnivorous digestive system (Guilloteau et al. 2010; Heinritz et al. 2013). Compared to mice, pigs and humans require higher quality diets as they are less dependent on nutrient sequestration by intestinal microbiota and are not nutritionally corpophagic, though some coporophagy in piglets has been observed (Guilloteau et al. 2010; Heinritz et al. 2013). In addition digestive transit times, nutrient efficiency, body composition and metabolism in humans and pigs are very similar (Guilloteau et al. 2010) making pigs an excellent model for gastrointestinal disorders in humans.

In terms of microbial populations in the gastrointestinal tracts of humans and pigs, there are strong similiarities with some notable differences. Both animals are populated mainly by bacteria of the Bacteriodes and Firmicutes phyla (Heinritz et al. 2013). However, the porcine gut microbiota is relatively low in *Bifidobacterium* and *Bacteriodes* spp. and relatively high in *Streptococci* spp. compared to that in humans. Additionally, while *Lactobaccili* spp. are fairly prominent in pig microbial populations, this genus of bacteria is highly variable among individual humans (Heinritz et al. 2013). While pigs are already a good model for intestinal physiology and microbiota in humans, recent studies have shown

that gnotobiotic reared piglets can be colonized with microbiomes from infant or adult human populations for the development of porcine models that are even more representative of gastrointestinal microbiota in humans (Zhang et al. 2013). Important to note in the context of this body of work pigs, even without humanization of the microbiome, have been reported to be susceptible to colonization with EcN (Barth et al. 2009; Kleta et al. 2006). This represents the human scenario more closely than murine models in which colonization cannot be established without antimicrobial support (unpublished).

Pigs are also closer genetically to humans than mice and additionally reflect heterogeneity in the human population, whereas murine models utilize inbred, genetically identical strains. This genetic similarity is reflected in the immune system with the porcine and human immune systems having greater similarities than mice in many respects (Fairbairn et al. 2011; Hochrein and Wagner 2004; Rothkotter 2009). The peripheral blood leukocyte proportions in healthy humans is 50-70% neutrophils and 30-50% lymphocytes; in pigs the proportions are 50-75% and 25-40% respectively, which is much more representative than mice in which 10-25% neutrophils and 75-90% lymphocytes are normal (Mestas and Hughes 2004; Nofrarias et al. 2006). The activation and cytokine expression in antigen presenting cells is also more closely related in humans and pigs than when compared to mice. For example, the expression and activation of pathogen recognition receptors such as TLR9 in swine is very comparable to that of humans; whereas these receptors are expressed on different cells and are activated by different microbe associated molecular patters, in mice (Fairbairn et al. 2011; Guilloteau et al. 2010; Kapetanovic et al. 2012). Certain aspects of the gene expression profile of LPS induced macrophages in pigs is much more similar to that of humans than of mice (Kapetanovic et al. 2012). For instance, in mice one of the main expression markers induced by LPS is a gene encoding the enzyme inducible nitric oxide synthase (iNOS), which is not induced in LPS stimulated human or pig macrophages (Fairbairn et al. 2011; Mestas and Hughes 2004). In contrast human and pig macrophages express large amounts of the *IDO1* gene that encodes a tryptophan metabolism enzyme, indoleamine 2,3-dioxygenase, in response to LPS recognition (Fairbairn et al. 2011; Kapetanovic et al. 2012). LPS also induces the up-regulation of IL-8 in human and swine macrophages, a chemokine that does not have a clearly defined ortholog in mice (Fairbairn et al. 2011; Kapetanovic et al. 2012).

Due to the similarities in physiological development of the gastrointestinal tract, the microbial populations in the gut, the immune system and genetics between pigs and humans, the pig has been used as a model for several gastrointestinal conditions in humans. Porcine models of necrotizing enterocolitis and short bowel syndrome in neonates have

been developed (Oosterloo et al. 2014; Sangild et al. 2013). Experimental radiation induced gastrointestinal syndrome in piglets has been used to mimic that observed in humans after exposure to radiation in cancer treatment (Shim et al. 2014). Models of human gastrointestinal disease caused by pathogens such as *Clostridium* (Steele et al. 2010), *Shigella* (Jeong et al. 2010), *Campylobacter* (Mansfield and Gauthier 2004), *Staphylococcus* (van Gessel et al. 2004) and *Norovirus* (Kocher et al. 2014) have also been developed in pigs. Additionally, for the study of gastrointestinal inflammation in humans, such as that observed in IBD patients, several chemically induced porcine colitis models are available including the administration of acetic acid (Wang et al. 2013), DSS (Adisakwattana et al. 2013; Bassaganya-Riera and Hontecillas 2006; Harding et al. 2010; Harding et al. 2008) or TNBS (Pouillart et al. 2010).

While chemically induced models for piglet colitis are available, for the initial trial to test EcN-AvCys in piglets, the study was designed to not only evaluate gut inflammation and functionality, but also to combine the aspect of a safety trial with prolonged feeding of the transgenic probiotic in a more naturally occurring instance of inflammation. Therefore, piglets in the post-weaning period were used as a model as they are susceptible to spontaneous gut inflammation that is not associated to any particular pathogen, but reflects a reaction to stress and the change to solid food (Boudry et al. 2004; Huang et al. 2012; Pedersen et al. 2012; Pie et al. 2004). The treatment with high doses of bacteria over two weeks was well tolerated and the piglets developed normally with respect to body weight gain and blood cell counts. One control animal and two EcN administered animals succumbed to mortality very early in the trial. This 10% loss is relatively high, but within ranges reported for commercially reared swine during the post-weaning period, so is likely independent of treatment (Dewey et al. 2006; Main et al. 2004).

In order to provide evidence that the transgenic probiotic was available in the gut, a plating scheme was proposed in which aerobically cultured fecal isolates would be stamped onto plates with both antibiotic and colorimetric selection properties. In a preliminary test with piglets reared in high hygienic conditions at the Institute for Animal Nutrition, Freie Universität zu Berlin, no natural fecal isolates were culturable on agar plates with Tc, Sm and Kn, antibiotics which the EcN control and transgenic EcN-AvCys are resistant to. However, in feces from piglets in the feeding trial many isolates were able to grow on the plates containing all three antibiotics, including feces from the control group. This suggests that there were multiple antibiotic resistant bacteria species present in the gastrointestinal microflora of the piglets in the EcN-AvCys trial aside from the transgenic probiotics administered in the treatment groups. As the piglets used for this trial were sourced from a commercial swine herd, antibiotic resistance is not unexpected due to antibiotic use in

modern livestock production. While the EU banned the use of antimicrobials for growth promotion in 2006, therapeutic doses of antibiotics for short periods as a prophylactic measure against suspected pathogen outbreak is allowable with veterinary prescription and still practiced (Barton 2014). Antibiotic resistance to tetracyclines in swine herds is widespread and multi-drug resistant *E. coli* and *Campylobacter* spp. are also common, specifically a resistance to tetracyclines, and aminocylcosides, such as streptomycin and kanamycin, in *E. coli* has been observed (Barton 2014). Our finding supports this and suggests that either the piglets sourced from the commercial barn were exposed to prophylactic antibiotics or the barn they were sourced from already harboured populations of multi-drug resistant bacteria as a result of previous antibiotic usage in that facility. Multi-antibiotic resistant bacteria were not detectable in the preliminary establishing step of the protocol as the piglets used for this trial were bred, reared and housed at the Institute for Animal Nutrition where lower housing density, higher hygiene standards, and very limited use of antibiotics is practiced.

While a multiplex PCR is available for the detection of EcN specific plasmids (Barth et al. 2009; Blum-Oehler et al. 2003; Duncker et al. 2006) and primers for the detection of AvCys were previously designed in house, the protocols for these PCRs involve bacterial culture prior to amplification. When selective culture was attempted prior to DNA extraction all samples were negative for both the EcN multiplex PCR and the AvCys PCR. The highly sensitive PCR primers designed for amplification of very dilute amounts of AvCys were additionally unsuccessful in amplifying the target gene in both fecal samples and colon tissue samples.

One possible explanation is that while EcN has been found to colonize piglets, that perhaps colonization at the weaning stage was unsuccessful. The weaning stage of piglets is accompanied by a reduction in feed intake and energy acquisition, physiological changes in the crypts and villi of the gut resulting in reduced epithelial surface area, as well as disturbances in barrier function that can result in secretory diarrhea (Boudry et al. 2004; Lalles et al. 2007). These factors are associated with a reduction in beneficial bacteria in the gut (Lalles et al. 2007) and could speculatively result in a reduction of EcN colonization efficiency. In a recent study in elderly human patients, EcN was unable to competitively exclude closely related multiple-antibiotic resistant *E. coli* spp (Tannock et al. 2011). So the abundance of antibiotic resistant *E. coli* spp detected in the feces of the piglets used in this trial could also inhibit the colonization of EcN.

Additionally, *in vitro* trials have revealed that EcN induces the expression of a specific antimicrobial, β-defensin 2, in human epithelial cells (Mondel et al. 2009; Schlee et al.

2007; Wehkamp et al. 2004). As EcN is not resistant to this antimicrobial peptide, it has been hypothesized that the transient colonization of EcN may be a result of this defensin expression in the host (Mondel et al. 2009). While EcN was not observed to effect the expression of several antimicrobial peptides in swine epithelial cells in a 2006 study (Duncker et al. 2006), the specific β-defensin 2 in pigs was not analyzed as the porcine ortholog has only been recently evaluated (Choi et al. 2012).

While the amounts of EcN and EcN-AvCys administered to piglets at the post-weaning phase were high compared to human doses as previously mentioned, and a tenfold increase from what was administered in the acute colitis trials in mice, it is less than has previously been administered in swine trials. In previous EcN studies in swine the probiotic was administered at a range that may have reached tenfold higher concentrations than were administered in the EcN-AvCys trial (Barth et al. 2009; Duncker et al. 2006; Kleta et al. 2006). Additionally, the other studies report a daily as compared to every second day schedule for dosing and do not mention the presence of antibiotic resistant *E. coli* populations in the gut microbiota of the experimental pigs (Barth et al. 2009; Duncker et al. 2006; Kleta et al. 2006). It can be speculated that the amounts given, dosing schedule and differences in gut microbial communities may have contributed to the inability to culture and molecularly identify EcN or EcN-AvCys in these piglets.

Taken together it can be reasoned that the difficulty in detecting EcN(-AvCys) in the treated animals may be due to any combination of a number of factors including; poor overall colonization as a result of the dysbiosis in bacterial populations and physiology at weaning, inability of EcN to compete with the high numbers of antibiotic resistant *E. coli* detected in the piglets gastrointestinal microbiome, an induction of antimicrobial expression in the host cells in response to EcN and/or an overall insufficient dosage to establish long-term colonization in the pigs.

Fortunately, while EcN-AvCys was not detectable with several culture and molecular methods attempted, leukocytes from the gut draining lymph nodes of piglets inoculated with EcN-AvCys proliferated in response to culture with rAvCys *in vitro,* while those from saline and EcN administered controls did not. This evidence strongly suggests that despite difficulty in detection, the transgenic probiotic bacteria was present in the colons of the EcN-AvCys treated piglets, was expressing and secreting AvCys and the host cells were responding to the immune modulatory protein.

No significant differences in health parameters of the piglets such as body weight and blood leukocyte proportion were observed. Additionally, no changes in macrophages and

eosinophils populations or expression of cytokines were observed in the colon. This was to be expected with EcN administration as the safety of EcN alone has already been well established in piglets. A study in 2006, in which varying doses of EcN were orally administered to healthy piglets showed no increase in specific immune cell subsets even in groups receiving high doses of EcN (Duncker et al. 2006). In general, there were also no changes observed in lymphocyte numbers in the small or large intestine. Likewise, high doses of EcN did not increase the expression of pro-inflammatory cytokines, regulatory cytokines or antimicrobial peptides (Duncker et al. 2006). However, EcN-AvCys likewise, appeared to be safe when administered to piglets despite the observation that immune cells from EcN-AvCys recognized rAvCys *in vitro*. Therefore, in this trial no evidence of detrimental effects to piglet health were observed when EcN with an additional AvCys transgene was administered.

Remarkably, EcN-AvCys fed pigs showed significant reduced signs of post-weaning inflammation in the distal colon. This reduction was not associated with changes in the mRNA expression of pro- and anti-inflammatory cytokines in the colon. It has previously been reported that initial inflammatory cytokine responses in the porcine gut decline within the second week post-weaning (Hu et al. 2013). Thus possible initial differences in gut cytokine expression patterns induced by EcN-AvCys treatment and involved in the reduction of gut inflammation may have not been detectable at the endpoint of this trial.

Leukocytes from piglets administered the transgenic probiotic recognized and specifically proliferated in response to stimulation with the transgene, AvCys. A more detailed analysis of $CD4^+$ T cells in the treatment groups showed that frequencies of $Foxp3^+$ cells were unaltered in the gut of EcN-AvCys-treated piglets; however, a significant increase in $CD4^+$ $CD25^+Foxp3^-$ T cells was observed in both mLN and distal colon from EcN-AvCys inoculated piglets. It can be postulated that these cells contain AvCys-specific T cells that proliferated antigen-specifically in response to rAvCys *in vitro;* however, no direct inference can be made. A recent study demonstrated that these $CD4^+CD25^{dim}FoxP3^-$ cells in swine produce higher amounts of cytokines, both inflammatory and regulatory, in response to stimulation than their $FoxP3^+$ or $CD25^-$ counterparts (Kaser et al. 2012). The trend towards decreased pro-inflammatory cytokines and increased IL-10 in supernatants from mitogen stimulated colon leukocytes, would suggest that these cells were potentially regulatory. However, this is merely speculative and further analysis, potentially at earlier time points, is required to determine if these AvCys induced $CD4^+CD25^{dim}FoxP3^-$ cells are involved in the observed amelioration of intestinal inflammation in EcN-AvCys treated piglets.

As previously described, macrophages have been identified as a target cell of AvCys, and these macrophages express increased levels of IL-10 upon exposure to AvCys (Klotz et al. 2011b; Schnoeller et al. 2008). Macrophages play an important role in driving inflammation in both human IBD (Heinsbroek and Gordon 2009) and murine models of the disease (Bain et al. 2013; Hall et al. 2011). The frequencies and activation status of porcine macrophages in response to feeding EcN-AvCys were therefore determined. However, no significant difference was observed in frequencies of colon tissue macrophages or expression of SLA II as a marker for activation and antigen presenting capacity. As previous murine studies indicated that it is phenotypic changes and regulation of MAPK pathways that determine the regulatory behaviour of macrophages after contact with AvCys (Figueiredo et al. 2009; Klotz et al. 2011b) future studies will focus not only on the quantity and activation, but also phenotypical changes of porcine macrophages in response to EcN-AvCys *in vivo*.

### 4.3. EcN-AvCys supports the intestinal epithelial barrier

As the gastrointestinal tract is the largest mucosal organ and regularly in contact with pathogens, the epithelial barrier is of utmost importance. The intestinal epithelial barrier must allow for passage of water or nutrients through absorptive enterocytes while blocking the entry of pathogens. A series of tight junctions, adherens junctions and desmosomes create the barrier between cells that prevents pathogen entry (Antoni 2004). Increased permeability in the intestinal epithelial barrier has been associated with IBD (Antoni et al. 2014; Merga et al. 2014). This is thought to be largely due to changes in the tight junctions between intestinal epithelial cells. For instance increased expression of tight junction proteins known as claudins, responsible for the paracellular channels, have been observed to be increased in patients with active IBD, while claudins that function to seal the gut were found to be decreased compared to healthy controls (Heller et al. 2005; Zeissig et al. 2007). This shift in claudin expression has been proposed to be at least partially responsible for the increased intestinal permeability observed in IBD patients (Antoni et al. 2014; Merga et al. 2014). Whether changes in barrier function of the gut are causative factors in the onset of the disease or a consequence of is still not well defined. However, recently several genetic polymorphisms have been reported to be more common in IBD patients that could be responsible for the changes to barrier permeability observed. For example, polymorphisms in keratin coding genes have been reported in the IBD population that have been estimated by *in situ* analysis to potentially increase permeability by 30% (Zupancic et al. 2014). Likewise, specific alleles for zonulin, one of the few well described mediators of paracellular permeability, have also been reported to be overrepresented in IBD patients compared with healthy populations (Vanuytsel et al. 2013). These IBD associated genetic polymorphisms suggest that changes in paracellular permeability are

potentially causative factors in the onset of the disease. The observation that increased intestinal permeability is also seen in first degree relatives of IBD patients, supports an underlying genetic risk factor to this physiological outcome. For example, mutations in CARD15 genes coding for a member of apoptosis regulators has not only been shown to be associated with IBD, but has been linked to higher intestinal barrier permeability in first degree relatives (Buhner et al. 2006). Additionally, increased intestinal permeability increases a patients risk of relapse (Antoni et al. 2014), further suggesting changes in epithelial barrier are involved in the induction of inflammation in the disease.

The weaning period in swine also involves changes to the intestinal epithelial barrier. Early weaning, at 21 days, commonly used in North American and European swine production has been shown to result in a reduction in transepithelial resistance (Hu et al. 2013). This is associated with an increased paracellular permeability and a decreased expression of tight junction proteins such as occludin, claudins and zona occludens (Hu et al. 2013). While these changes are transient, the increased permeability of the intestines is another parameter in which the swine post-weaning model mimics the situation in human IBD. Additionally, in piglets that suffer from extreme weaning stress, resulting in cessation of eating and rapid degradation of body composition, the changes to the intestinal epithelial barrier are even more pronounced. In these piglets that suffer from what has recently been named peri-weaning failure to thrive syndrome, the transepithelial resistance is again decreased compared to even other weaning piglets and is likely due to both paracellular and intracellular permeability as both paracellular transport of tracer molecules and intracellular ion transport induced current were increased in the peri-weaning failure to thrive piglets compared to other weaned piglets (Moeser et al. 2012). This highlights the potential importance of the barrier function in the health of weaning piglets.

Data generated in cooperation with J. Richter, N. Hering, D. Günzel, J. Schulzke and A. Kehles and their affiliations at the Charité - University Medicine Berlin showed that in the post-weaning piglet experiment EcN-AvCys positively affected epithelial barrier functions *in vivo,* as well as in *in vitro* colon tissues. An improved transepithelial barrier function in the proximal colon accompanied the decreased post-weaning inflammation after feeding of the transgenic probiotic to piglets. Transepithelial resistance (TER) was improved after feeding of high doses of EcN-AvCys for 2 weeks, but remarkably also when colon tissue from untreated, healthy pigs was subjected to culture supernatant of the transgenic probiotic for relatively short periods of time. These data clearly indicate that AvCys released by the probiotic bacteria was sufficient to support TER.

This is contradictory to most research regarding the effects of helminth infections and helminth derived molecules on the intestinal epithelial barrier. In both human intestinal nematode infections such as *Strongyolides stercoralis* (Werneck-Silva et al. 2006) and experimental intestinal nematode infections including *H. polygyrus* (Su et al. 2011; Sutton et al. 2008), *Trichuris muris* (Hasnain et al. 2011), *Strongyloides venezuelensis* (Farid et al. 2007) and *Trichenella spiralis* (Fernandez-Blanco et al. 2011) negative impacts to epithelial barrier function have been reported. While it would be easy to speculate that this is merely due to physical damage caused by the nematodes in the gut, several infection models have shown a decreased expression in tight junction proteins (Farid et al. 2007; Su et al. 2011), indicating the mode of action of these infections is more than just injury. This is supported by a recent study demonstrating an increase in intestinal permeability and decreased expression of tight junction protein in a human colon cell line after exposure to ES molecules of *T. suis* (Hiemstra et al. 2014). Several studies, indicate the induction of a strong Th2 response in intestinal dwelling nematode infections is at least partially responsible for the observed negative impacts these helminths have on intestinal epithelial barrier integrity (Shea-Donohue et al. 2001; Su et al. 2011).

The supportive role of EcN-AvCys on epithelial barrier function observed in the piglet trials and early time points of human cell lines exposed to EcN-AvCys supernatants is counter to what is observed in natural infection with intestinal nematodes. However, AvCys is not derived from an intestinal dwelling nematode, but a filarial tissue dwelling nematode. While there is little information available regarding the effects of filarial nematode ES products on intestinal epithelial barrier these nematode infections are shown to increase endothelial cell proliferation for angiogenesis around the tissue nodes in which the mature female worms live and produce microfilariae (Attout et al. 2009; Higazi et al. 2003; Mackenzie et al. 2010), likely a mechanism by which microfilariae can then have access to and circulate in blood vessels. Not only is endothelial cell proliferation induced in response to some filarial nematode infections, but selective reduction in transendothelial migration of some immune cells (Schroeder et al. 2012) suggesting that infection induces changes to the endothelial barrier. It can therefore, be speculated that AvCys may play a role in endothelial barrier support in natural infection and that similar mechanisms are involved in the EcN-AvCys induced support of the intestinal epithelial barrier function in swine. As filarial helminth infection or intestinal dwelling nematode infection could result in negative pathology, and at least in the latter case may even reduce the intestinal epithelial barrier integrity in IBD patients, EcN-AvCys is again shown to offer a superior therapy compared to live worm infection for the treatment of inflammatory disorders of the gut.

The effect of EcN-AvCys was not restricted to porcine gut tissue containing immune cells, but also detected with a human colon epithelial cell line. Of note, the fact that the TER of cultures of a human epithelial cell line also increased after contact with supernatants of EcN-AvCys indicates that the transgenic probiotic may also increase the barrier function of the human gut. While the transepithelial resistance of human epithelial cell monolayers *in vitro* was significantly improved in only the EcN-AvCys$^{SN}$ treated cultures compared to the media control at a very early time point, in a longer period of incubation both EcN$^{SN}$ and EcN-AvCys$^{SN}$ significantly increased the resistance across the monolayers. Additionally, the reduction in small pore permability across the human cell line monolayer was observed in both EcN$^{SN}$ and EcN-AvCys$^{SN}$ treated groups compared to the media control. This would suggest that the support of the epithelial barrier in this *in vitro* model is largely due to probiotic factors and not the AvCys immunomodulator. As has been mentioned several times, AvCys has previously been reported to interact with macrophages (Klotz et al. 2011b). Therefore, the absence of underlying immune cells in this cell culture experiment could be the reason why the significant EcN-AvCys specific changes observed in the porcine excised tissue experiments were not detected in the human cell line experiment. It should also be noted that both the apical and basolateral sides of the human epithelial cell monolayer were exposed to EcN$^{SN}$. This may not accurately reflect an *in vivo* situation and as EcN has been shown to be more active in inducing a chemokine response in human epithelial cell lines when applied to the basolateral side (Hafez et al. 2009), further studies should be employed to determine whether the transgenic probiotic requires access to the underlying tissues to improve barrier function in this model and whether the addition of macrophages or other immune cells to epithelial cells *in vitro* increases the positive effects of specifically the AvCys expressing transgenic probiotic on the epithelial barrier.

A beneficial effect of EcN alone was expected in barrier parameters as support of the intestinal epithelial barrier is a well-documented probiotic mechanism of EcN. EcN has been observed to up-regulate scaffolding proteins, known as zona occludens, which provide structural integrity to tight junctions between epithelial cells in the gut. This induction of zona occluden expression has been observed in both *in vivo* murine models of colitis (Garrido-Mesa et al. 2011; Ukena et al. 2007) and in *in vitro* human cell culture assays (Veltman et al. 2012; Zyrek et al. 2007). EcN was additionally shown to abrogate the reduction in zona occluden expression and transepithelial resistance, a measure of epithelial barrier function, observed in response to enteropathogenic *E. coli* (EPEC) co-incubation with intestinal epithelial cell line monolayers (Veltman et al. 2012; Zyrek et al. 2007). This inhibition of EPEC induced intestinal barrier dysfunction by EcN has been associated with the expression of microRNAs in an epithelial cell line that are responsible

for regulating the post-transcriptional expression of the scaffolding and regulatory proteins of tight junctions (Veltman et al. 2012). More recently, an EcN produced immune inhibitory component was shown to be responsible for the up-regulation of the tight junction protein claudin 14, in a human cell line through activation of protein kinase pathways (Hering et al. 2013). With such strong evidence to support the effects of EcN on intestinal barrier function it is unexpected that EcN alone had very few significant effects on the parameters of barrier function tested both *in vivo* in the post-weaning piglet trial and *in vitro* with porcine colon tissues. It is tempting to speculate that this lack of observed porcine intestinal barrier response to EcN in these trials is due to the use of supernatants rather than live bacterial cells, as the definition of probiotics outlined by the WHO in 2002 defines the need for live organisms for probiotic function (). However, the evidence of positive effects on barrier resistance in human cell culture experiments in response to EcN supernatant does not support this hypothesis. Additionally, while most of the reported positive benefits of EcN on the host epithelial barrier function were observed in either *in vivo* feeding trials (Garrido-Mesa et al. 201; Ukena et al. 2007) or *in vitro* trials with epithelial and live bacterial co-culture assays (Veltman et al. 2012; Zyrek et al. 2007) a recent study describes numerous potential functions of proteins secreted from EcN. A recent report found evidence that EcN secretes at least 192 different proteins in outer membrane vesicles (Aguilera et al. 2014). These vesicles consist of cell wall and outer membrane components and are a known strategy in which gram negative bacteria are able to communicate with other microbes or host cells from larger distances. Many of the associated proteins in EcN outer membrane vesicles were not common to gram negative pathogens and may be involved in probiotic functions such as survivability, competitive exclusion, nutrient acquisition, and modulation of host cell immune responses(Aguilera et al. 2014). Therefore it is not surprising that EcN supernatants were able to alter host epithelial barrier parameters in human cell culture experiments and is likely not the cause of a lack of response in porcine specific experiments. Rather the significant increase in transepithelial resistance and decrease small pore permeability in human cell lines exposed to EcN compared to cells exposed to control media suggests a potential host species specific difference in the effects of EcN on the intestinal epithelial barrier.

In summary EcN-AvCys was able to efficiently improve parameters of intestinal epithelial barrier function in both an *in vivo* piglet trial and *in vitro* porcine tissue explants, while both EcN and EcN-AvCys supernatants were able to improve resistance and decrease permeability at later time points of incubation in human cell culture experiments. It remains to be elucidated whether the difference between the porcine and human experiments reflects a difference of mode of action between the species or whether the

presence of underlying immune cells is required for AvCys expressed from EcN to confer additional benefits on the epithelial barrier beyond those observed for the control probiotic.

### 4.4. EcN and EcN-AvCys alter macrophage phenotype and activation

Macrophages have been divided into two major groups, classically activated M1 macrophages that are largely involved in pro-inflammatory Th1 immune responses to microbial pathogens by recruiting Th1 and NK cells **(Figure 4.1A)** and alternatively activated M2 macrophages that are involved in wound healing repair, supporting the Th2 immune response and/or function as regulators of inflammation **(Figure 4.1B, C and D).** M2 macrophages are divided further into 3 subsets. M2a macrophages are induced in response to Th2 associated cytokines and further support the Th2 response through expression of cytokines that recruit Th2 cells while antagonizing the M1 phenotype that supports Th1 recruitment **(Figure 4.1B).** M2b macrophages express some of the same pro-inflammatory cytokines observed in the M1 classically activated phenotype; however, express high amounts of the regulatory cytokine IL-10 and a decoy receptor for the inflammatory cytokine IL-1β **(Figure 4.1C)**. M2b macrophages, like M2a macrophages, recruit Th2 cells and additionally have some regulatory behaviour due to the high IL-10 expression **(Figure 4.1C).** M2c macrophages display a regulatory phenotype that is induced by and expresses high amounts of the regulatory cytokines IL-10 and TFG-β which support Tregs **(Figure 4.1D).**

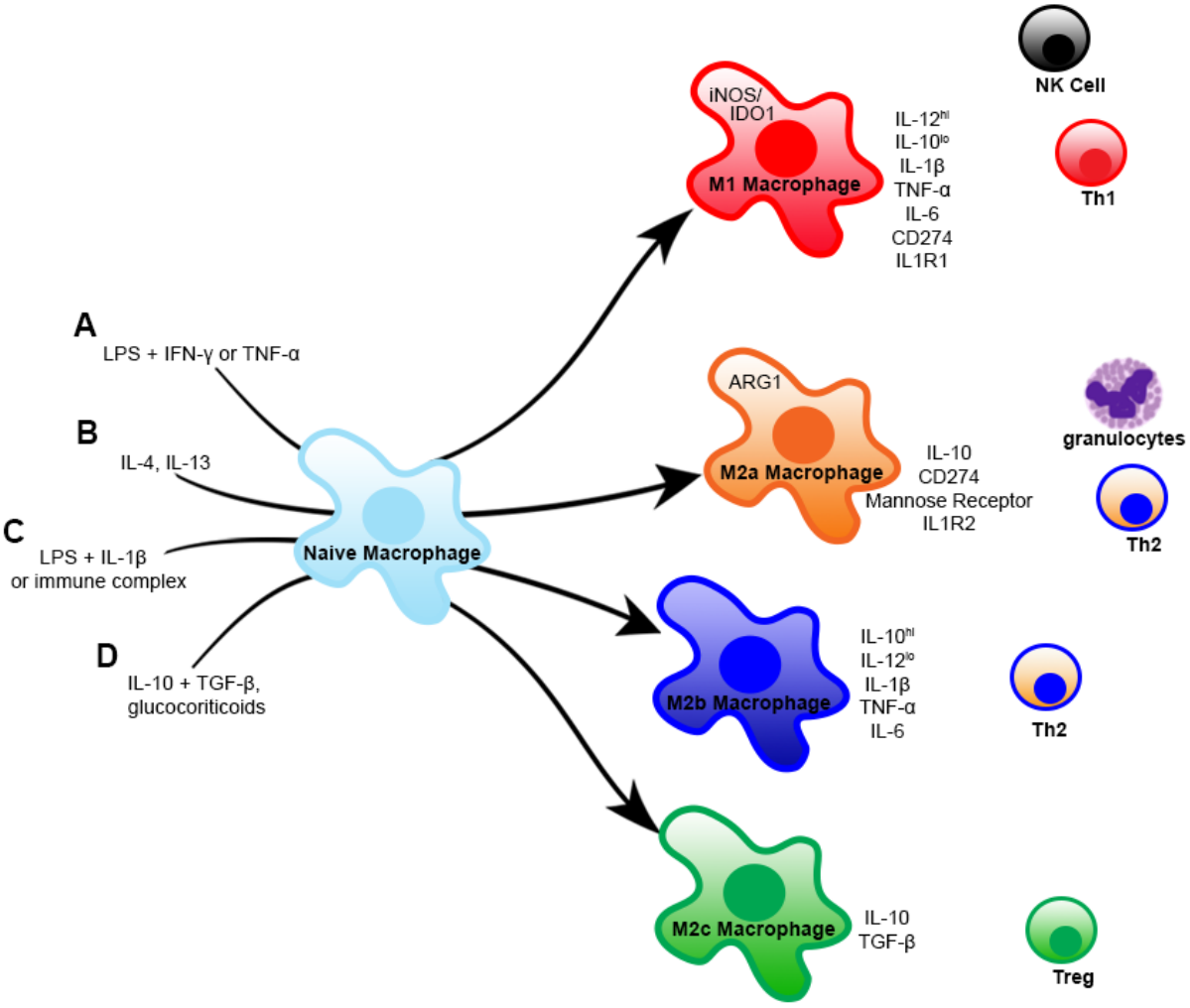


**Figure 4.1** Polarized differentiation phenotypes in human and mouse macrophages. **(A)** M1 macrophages, also known as classically activated macrophages, are induced in response to LPS with either IFN-γ, or TNF-α as can be seen in microbial infections and in turn express inflammatory markers such as iNOS (in mice) or IDO1 (in pigs and humans), proinflammatory cytokines IL-12, IL-1β, TNF-α, the surface marker CD274 and the cytokine receptor IL1R1. M1 macrophages support Th1 immune responses and natural killer (NK) cells. **(B)** M2a macrophages are activated in response to IL-4 and IL-13 and express arginase-1 (ARG1) which inhibits and competes with the M1 expression of iNOS. Additionally, M2a macrophages express CD274 as well as Mannose Receptor and support the Th2 response through recruitment of Th2 cells and granulocytes. **(C)** M2b macrophages are induced in Th2 environments in response to LPS and IL-1β stimulation or through activation with immune complexes. These macrophages, while expressing pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, and low levels of IL-12, also express high amounts of the regulatory cytokine IL-10, as well as the IL-1β decoy receptor IL1R2. M2b macrophages also support the Th2 response. **(D)** M2c macrophages are a highly regulatory phenotype of the alternatively activated macrophages induced by the regulatory cytokines IL-10 and TGF-β, or with anti-inflammatory glucocorticoids. They in turn express high amounts of regulatory cytokines IL-10 and TGF-β, which support regulatory T cell subsets (Tregs) (Mantovani et al. 2004; Martinez et al. 2008).

Macrophages have been established as a target cell of AvCys (Figueiredo et al. 2009; Klotz et al. 2011b; Schnoeller et al. 2008) and a former student, Dr. Thomas Ziegler, identified that murine macrophages exposed to AvCys acquired a mixed phenotype comprising markers of the M2a and M2b subsets of alternatively activated macrophages (unpublished). These AvCys induced M2a/b like macrophages concomitantly expressed both pro-inflammatory cytokines such as IL-12 and high amounts of the regulatory cytokine IL-10, as well as other defining phenotype markers (unpublished). These macrophages were shown to induce IL-10 production by T cells and were also able to ameliorate a murine model of colitis upon transfer into mice (unpublished). Therefore, while overall numbers of macrophages did not vary between treatment groups in the swine feeding trial it was decided that the effect of EcN, EcN-AvCys and rAvCys on porcine monocyte/macrophage phenotype should be investigated.

The phenotypical markers of porcine macrophage subsets are less well defined than that of murine or human subsets. Therefore, before a comparison of the effects of EcN-AvCys or rAvCys on porcine to murine macrophages could be made to previously generated data in mice, general phenotypic difference of macrophages to typical classically activated M1 macrophage stimuli and alternatively activated M2 macrophage stimuli needed to be investigated. While peritoneal macrophages and bone marrow cells differentiated into macrophages via M-CSF were used in the murine experiments conducted by Dr. Thomas Ziegler, it was decided that monocytes were to be used in this comparative swine study as preliminary experiments (data not shown) showed that compared to monocytes, differentiated macrophages responded very weakly to various stimuli, especially in expression of M2 markers. It is known that GM-CSF induces classically activated M1 phenotype when used to differentiate macrophages from monocytes in culture while the more commonly used M-CSF, the growth factor used to differentiate monocytes into macrophages in the murine studies conducted by Dr. Ziegler, induces an M2-biased phenotype (Fleetwood et al. 2007). At least in the case of M-CSF previous studies have shown that human macrophages differentiated with this growth factor retain the ability to polarize to either an M1 phenotype (Jaguin et al. 2013) or further polarize to a strong M2 phenotype (Mia et al. 2014) with secondary differentiation stimuli *in vitro*. This did not appear to be the case with porcine cells in initial experiments as differentiated macrophages were very resistant to stimuli, specifically in expressing M2 markers, compared to monocytes. Therefore, blood monocytes were chosen to investigate the effects of the hypothesized M1 and M2 initiating stimuli on gene marker expression as well as the effects of EcN, EcN-AvCys and rAvCys on porcine mononuclear phagocyte phenotypes in these studies.

Incubation with M1 inducing stimuli IFN-γ and LPS did induce a phenotype in porcine monocytes that was similar to what has previously been described for mouse M1 macrophages. For example, high expression levels of genes encoding the pro-inflammatory cytokines IL-12, TNF-α, IL-1β and IL-6 were observed, along with low levels of gene expression for the regulatory IL-10 which is comparable to the described murine markers of a classically activated macrophage **(Figure 4.1A)**. Furthermore, a high expression of the *IDO1* gene encoding the enzyme indoleamine 2,3-dioxygenase involved in tryptophan metabolism, was observed which is consistent with M1 phenotypes in human monocytes/macrophages (Fairbairn et al. 2011). *IDO1* expression is not however, induced in murine M1 macrophages as the up-regulation of iNOS in the murine classically activated macrophages results in the production of nitric oxide, which inhibits *IDO1* expression (Thomas et al. 1994). Therefore, while not directly comparable to the mouse model in which high iNOS production is observed by M1 macrophages, the high expression of *IDO1* in this porcine study similarly represents a marker of classically activated mononuclear phagocytes.

Inducing activation of gene expression in porcine monocytes using IL-4 and IL-13, considered traditional M2 stimuli **(Figure 4.1B),** was less successful in inducing similar marker expression to that observed in mouse and human studies. Both porcine specific IL-4 alone and IL-4 in combination with IL-13 were previously attempted as M2 stimuli with no changes in marker expression observed. It is unsurprising that IL-4 alone would be insufficient for M2 macrophage activation in porcine cells as previous studies have demonstrated that IL-4 is found in very low concentrations in circulating blood of pigs compared to other species and is replaced by IL-13 in the differentiation of DCs (Bautista et al. 2007). However, even in the case of a combination of IL-4 and IL-13 no significant changes in marker expression were observed. Therefore, a combination of IL-4 and IL-13 with low levels of LPS in order to induce gene expression in monocytes through TLR activation was attempted and finally resulted in transient changes to marker expression. These included trends towards an intermediate and late expression of the genes encoding pro-inflammatory cytokines IL-1β and TNF-α, respectively, as well as a significant early and late expression of the *CD274* gene, that encodes a ligand that when bound to its receptor on T cells inhibits activation and proliferation (Sheppard et al. 2004). While these genes are associated with M2a and M2b alternatively activated macrophage phenotypes in murine populations **(Figure 4.1B, C),** there was also an absence of expression of other gene expression markers, such as *ARG1* and *IL10* that are strongly associated with M2 macrophages in mice. As arginase (Pesce et al. 2009) and IL-10 (Klotz et al. 2011b) have regulatory functions for alternatively activated macrophages in mice, it could be conjectured that the population induced *in vitro* in porcine monocytes exposed to IL-4, IL-

13 and low level LPS are lacking regulatory properties. However, this is just mere speculation and future trials on the functional aspects (such as phagocytic activity and ability to activate or inhibit T cell responses) of porcine macrophages exposed to both these M1 and M2 stimuli should be conducted before a full comparison of these cells can be made to those in human and murine populations.

Porcine monocytes were then stimulated with $EcN^{SN}$, EcN-$AvCys^{SN}$ or rAvCys *in vitro* in order to determine the marker expression induced in the presence of the immunomodulator. Unlike the porcine monocytes incubated with the typically associated M1 and M2 stimuli, in which changes in marker expression mostly required a 48 hrs incubation period to induce, incubation with $EcN^{SN}$ or EcN-$AvCys^{SN}$ induced changes to gene expression by 4hrs in many markers tested. For example, both $EcN^{SN}$ and EcN-$AvCys^{SN}$ induced an early expression of M1 associated genes for indoleamine 2,3-dioxygenase and the pro-inflammatory cytokines TNF-α, IL-1β, and IL-12. However, a high early expression of IL-10 was also observed in the monocytes incubated with supernatants from the probiotic and transgenic probiotic cell media. Conversely a consistent and gradual increase in IL-6 encoding gene expression was observed in both groups. No significant differences in the expression of these above listed markers were observed between the $EcN^{SN}$ and EcN-$AvCys^{SN}$ treated groups. Therefore, it is likely that the expression of these markers at 4 hrs is induced by EcN specific ES products and not affected or overridden by the presence of the AvCys transgene.

Interestingly a late expression of the M2 markers *CD274* and *ARG1* was observed in $EcN^{SN}$ treated cells. This late expression of M2 markers in the probiotic treated monocytes was significantly decreased in monocytes incubated with the transgenic probiotic supernatants. In the previous murine trials rAvCys was shown to increase the late expression of M2 markers (unpublished), so this finding for a reduced expression in treatment groups where AvCys was present in porcine cells is contradictory. In porcine monocytes it would appear that AvCys inhibits the late expression of these M2 markers. Therefore, while previously unpublished data suggests that AvCys regulation of inflammation in mice is mediated through manipulation of monocyte/macrophage gene expression to induce a regulatory phenotype, this hypothesis cannot be supported as a mechanism by which inflammation is reduced in AvCys fed pigs. In fact AvCys appears to suppress the late expression of genes in porcine monocytes which may indicate that monocyte/macrophage populations in the pig reduce inflammation by being rendered anergic, and unable to activate effector T cell populations. However, as relatively few markers were analyzed in these studies that hypothesis must be further explored through

functional tests for the activity of porcine monocytes exposed to AvCys to determine if anergy or merely a phenotype incomparable to other species is induced in these cells.

The phenotype induced in porcine monocytes exposed to $EcN^{SN}$ or EcN-$AvCys^{SN}$ supports previous findings in human and murine *in vitro* cell experiments with EcN. In EcN co-cultures with murine macrophage cell lines an expression of M1 like markers such as iNOS and IL-12, some M2b markers such as IL-10 and TGF-β, as well as TNF-α, a marker found in both phenotypes has been observed (Christoffersen et al. 2014; Cross et al. 2004). Additionally, EcN molecules have been shown to cause increased expression of *IDO1*, *IL12*, *IL6*, and *IL10* in human blood derived mononuclear cells (Guttsches et al. 2012).

As many of the pro-inflammatory markers up-regulated in blood mononuclear cells in response to EcN were shown to be due to exposure to LPS (Guttsches et al. 2012), the high LPS content in $EcN^{SN}$ or EcN-$AvCys^{SN}$ could explain the apparent M1 phenotype observed in these monocytes at early time points and the lack of difference between the two treatment groups in M1 marker expression. In an *in vivo* gastrointestinal situation newly recruited monocytes would not have access to such high LPS content even in a severely compromised inflamed intestine. Likewise, $F4/80^{+}$ cells in colon lamina propria have been shown to be anergic to stimulation with LPS or NOD2 ligands, failing to produce pro-inflammatory cytokines in response (Bain and Mowat 2014). Therefore, the high expression of pro-inflammatory marker genes like *IL12* or *IDO1* by macrophages differentiated in a sterile *ex vivo* environment is likely not representative of the *in vivo* situation. In order to obtain a picture of the potential effects of AvCys on porcine macrophage phenotype that is more comparable to the previous studies conducted with murine macrophages exposed to rAvCys and more representative of an *in vivo* situation, porcine monocytes and macrophages were also treated with rAvCys in the absence of EcN.

In previous publications from members of our group it has been reported that rAvCys induces IL-10 and IL-12 early in murine macrophages and this is later regulated by dual specificity phosphatases (Klotz et al. 2011b). Furthermore, unpublished data from a former student, Dr. Thomas Ziegler, revealed the early expression of IL-12/23p40, TNF-α, iNOS, IL-6 and IL-10 in *in vitro* differentiated macrophages exposed to rAvCys which were down-regulated at later time points as the expression of other M2 markers, specifically arginase, was up-regulated (unpublished). However, when porcine monocytes were differentiated with rAvCys alone no significant changes in early marker expression were observed. This suggests that the presence of AvCys in EcN-$AvCys^{SN}$ likely had very little effect on the early expression of the markers and further supports the hypothesis that the early marker expression observed in $EcN^{SN}$ and EcN-$AvCys^{SN}$ treated monocytes is due to the probiotic products and not the presence of the transgene encoding AvCys.

In summary, while M1 stimuli in porcine monocytes appear to result in similar phenotypes to those described for other species, classic M2 stimuli for monocyte/macrophage differentiation in mouse and human do not induce strong changes in gene expression in porcine cells. Additionally, while rAvCys causes significant changes to macrophage gene expression resulting in a regulatory phenotype in mice (Klotz et al. 2011b) the protein did not induce changes in the porcine monocyte phenotype. The only potential mechanism by which AvCys may affect porcine monocyte marker expression is through an inhibition of late marker expression caused by bacterial derived products. Therefore, further experiments are required to determine if there is an alteration in other markers or macrophage function in response to AvCys in porcine cells and whether porcine monocytes/macrophages are at all involved in the improved barrier function observed in EcN-AvCys fed piglets.

### 4.5. EcN-AvCys does not inhibit macrophage or monocyte inflammasome activation

The inflammasome is a constituent of the innate immune system that can be found in macrophages, DCs and epithelial cells amongst others. The term describes protein complexes that are activated when cells are exposed to specific danger or microbe associated molecular pattern molecules (Martinon et al. 2002). The result of the inflammasome activation is the cleavage of the pro- form of caspase-1 resulting in the active protease. Functional caspase-1 is then able to cleave the pro- forms of IL-1β and IL-18 allowing for the secretion of the active forms of these pro-inflammatory cytokines from the cell (Martinon et al. 2002). While the effects of IL-18 on epithelial barrier resistance and inflammation are currently being debated, much is known about the effects of IL-1β in intestinal inflammatory response. For instance, IL-1β has been shown to increase paracellular permeability in intestinal cell lines *in vitro* (Al-Sadi et al. 2013). Dendritic cells activated with IL-1β express significantly higher amounts of other pro-inflammatory cytokines such as IL-12 and stimulate Th1 inflammation (Luft et al. 2002; Wesa and Galy 2002). Likewise, IL-1β has been reported to enhance antigen specific T cell proliferation (Khoruts et al. 2004).

IL-1β is also involved in many of the inflammatory processes observed in IBD and DSS colitis models, as well as is more highly expressed in both cells from IBD patients (Mahida et al. 1989) and those exposed to DSS (Bauer et al. 2010) than from healthy individuals or control animals respectively. Macrophages exposed to DSS secrete high amounts of IL-1β and mice with a knockout in a specific inflammasome, known as NLRP3, are resistant to the DSS-driven colitis model (Bauer et al. 2010). Genetic polymorphisms in genes relating to the NLRP3 inflammasome have also been associated with an increased risk of developing CD (Villani et al. 2009).

There are several locations in the inflammasome pathway where an active cysteine protease inhibitor such as AvCys might be able to inhibit the protein complex and the downstream activation of IL-1β **(Figure 4.2)**. Cathepsins released from lysosomes during cell damage have been shown to activate the inflammasome and downstream activation of IL-1β (Hornung and Latz 2010). Filarial cystatins have been shown previously to inhibit cathepsins (Schierack et al. 2003) and could therefore be an inhibitory target of AvCys. Likewise Caspase-1, the protease that cleaves the pro-forms of IL-1β and IL-18, is a cysteine protease and could therefore, also be a potential target by which EcN-AvCys inhibits secretion of active IL-1β. As the inflammasome is involved in both DSS models of IBD and the disease condition itself and in light of the several hypothesized ways in which AvCys may inhibit this protein complex, it was investigated as a potential mode of action by which EcN-AvCys may have ameliorated an IBD model and may eventually ameliorate the human disease.

Human blood monocytes and monocyte-derived macrophages were selected for the inflammasome experiments as induction of the inflammasome in human cells has been well established (Bauer et al. 2010; Franchi et al. 2009). Furthermore, this would offer some insight for the potential of AvCys expressing EcN in human IBD patients. The TLR ligand LPS was utilized as a primary stimulus **(Figure 4.2A)** to initiate the expression of pro-IL-1β **(Figure 4.2B).** DSS was then administered to cells as a secondary stimulus to activate the inflammasome through release of cathepsins from the phagolysosome **(Figure 4.2C)**. Additionally, extracellular ATP was used as a secondary signal, as extracellular ATP binds the PX27 receptor resulting in a $K^+$ efflux which can then in turn activate the inflammasome complex **(Figure 4.2D)**. As DSS has been shown to initiate the activation of the inflammasome independent of the PX27 transporter, this pathway offered a second model in which to test the inhibition of AvCys on the inflammasome. Furthermore, the inflammasome activation via extracellular ATP does not involve lysosomal degradation; therefore, by testing AvCys in this system it could be determined if any inhibitory behavior on the inflammasome activation was dependent upon the inhibition of lysosomal cathepsins or if the inhibition of caspase-1 directly was also involved **(Figure 4.2E).** Secreted IL-1β in the supernatants of the culture systems was used as an output as only activated IL-1β should be secreted from the cell **(Figure 4.2F).**

Monocyte derived human macrophages produced and secreted very low levels of IL-1β in the positive control group exposed to both primary LPS stimulation and secondary stimulation with ATP or DSS. A significant increase in IL-1β levels was observed when the cells were in contact with supernatants from the probiotics in the ATP stimulated system and this trend, while not significant, was also observed in the DSS stimulated

system. This may be due to the a higher expression of the pro-form of IL-1β in cells exposed to bacterial supernatants as a result of increased LPS available for TLR stimulation in these cells. This finding opposes a recent study which reports that EcN bacteria inhibit the activation of the inflammasome in a human intestinal cell line *in vitro* (Becker et al. 2014) and suggests that perhaps EcN inhibition of the inflammasome requires cell contact, and therefore $EcN^{SN}$ is not sufficient for this inhibition.

Recently, it has been reported that the inflammasome can be initiated in monocytes with only a primary LPS stimulation and in conjunction with a secondary stimulus monocytes are able to produce much more active IL-1β than macrophages (Netea et al. 2009). Therefore, undifferentiated blood derived monocytes were also utilized for the same inflammasome activation/inhibition experiments. Indeed, monocyte reacted with a far higher secretion of IL-1β in the positive controls compared to macrophages. While ATP activated cultures showed no inhibition of IL-1β production when the cells were exposed to EcN-AvCys supernatants, there was an unexpected increase in the secretion of IL-1β in EcN-AvCys treated cells in the DSS activated cells. This opposed the original hypothesis that AvCys may have inhibitory effects on the activation of the inflammasome and therefore, the secretion of active IL-1β.

The mechanism behind the increase in IL-1β secretion has not yet been elucidated. As this increase was only observed in DSS activated cells and not the ATP activated cells, the increased activation of the inflammasome in EcN-AvCys exposed cells likely involves the lysosomal cathepsins and is not a direct action on the NLRP3 inflammasome. As the connection between the release of cathepsins from the lysosome and the activation of the NLRP3 inflammasome is not well defined in the literature, it is difficult to speculate on what the mechanism of increased activation by AvCys could be. Additionally, there is the possibility that the increased secretion of IL-1β in cells that have been exposed EcN-AvCys supernatant could be due to the activation of an inflammasome other than NLRP3.

While these inflammasome experiments in human monocytes/macrophages would suggest that AvCys may have a pro-inflammatory role this was not supported by the *in vivo* data from murine or porcine trials. Therefore, it should not be assumed from this *in vitro* experiment that AvCys would necessarily be pro-inflammatory *in vivo* in humans. It was previously mentioned that some of the pro-inflammatory effects associated with IL-1β are an increase in inflammatory cytokine expression in DCs that leads to a Th1 activated response (Luft et al. 2002; Wesa and Galy 2002), activation of Th17 cells (Acosta-Rodriguez et al. 2007), an enhanced proliferation of antigen specific T cell proliferation (Khoruts et al. 2004) and an increase in the paracellular permeability of the epithelial

barrier in the gut (Al-Sadi et al. 2013). All of these actions may be directly counteracted by AvCys, as AvCys has been shown to induce macrophages with regulatory functions that inhibit the activation and proliferation of inflammatory effector T cells at least in mice (Figueiredo et al. 2009; Hartmann et al. 1997; Klotz et al. 2011b; Schnoeller et al. 2008). Additionally, a decrease in inflammatory cytokine expression and a decrease in paracellular permeability in the gut was observed when animals were treated with EcN-AvCys in both the murine and porcine trials. Therefore, despite IL-1β secretion in response to EcN-AvCys in human cells *in vitro*, the overall effect of the treatment regulates inflammation *in vivo* in other animal models and may do likewise in an *in vivo* human scenario due to the immune regulatory properties of AvCys.

Another possible explanation for the apparent inconsistency between the presence of IL-1β in the cell culture experiments and the lack of an inflammatory response in EcN-AvCys treated animals is that the expression of IL-1 receptors in these animals may prevent the inflammatory effects of IL-1β. A recent review by Garlanda et al, describes the disparity in outcomes when IL-1β binds to various receptors (Garlanda et al. 2013). The binding of IL-1β to the IL-1R1 receptor results in activation of signaling cascades with downstream inflammatory effects. However, IL-1β is also able to bind the decoy receptor IL-1R2 with high affinity, which leads to both blocking, and in some instances active inhibition of, IL-1R1 signaling cascade activation. Therefore, future studies should be conducted to investigate the IL-1β receptor expression induced in response to EcN-AvCys and an elucidation of the role these receptors may play in the immunomodulation of macrophage phenotype induced by EcN secreted AvCys in human cell experiments.

While the *in vitro* inflammasome experiments did not show any inhibitory role of AvCys on the secretion of active IL-1β, the downstream effects of an observed increase in IL-1β secretion in human macrophages exposed to EcN-AvCys must be further investigated. Elucidating any potential pro-inflammatory or M2 macrophage polarization properties of IL-1β will further our knowledge on the potential outcomes to be expected for EcN-AvCys treatment in human patients.

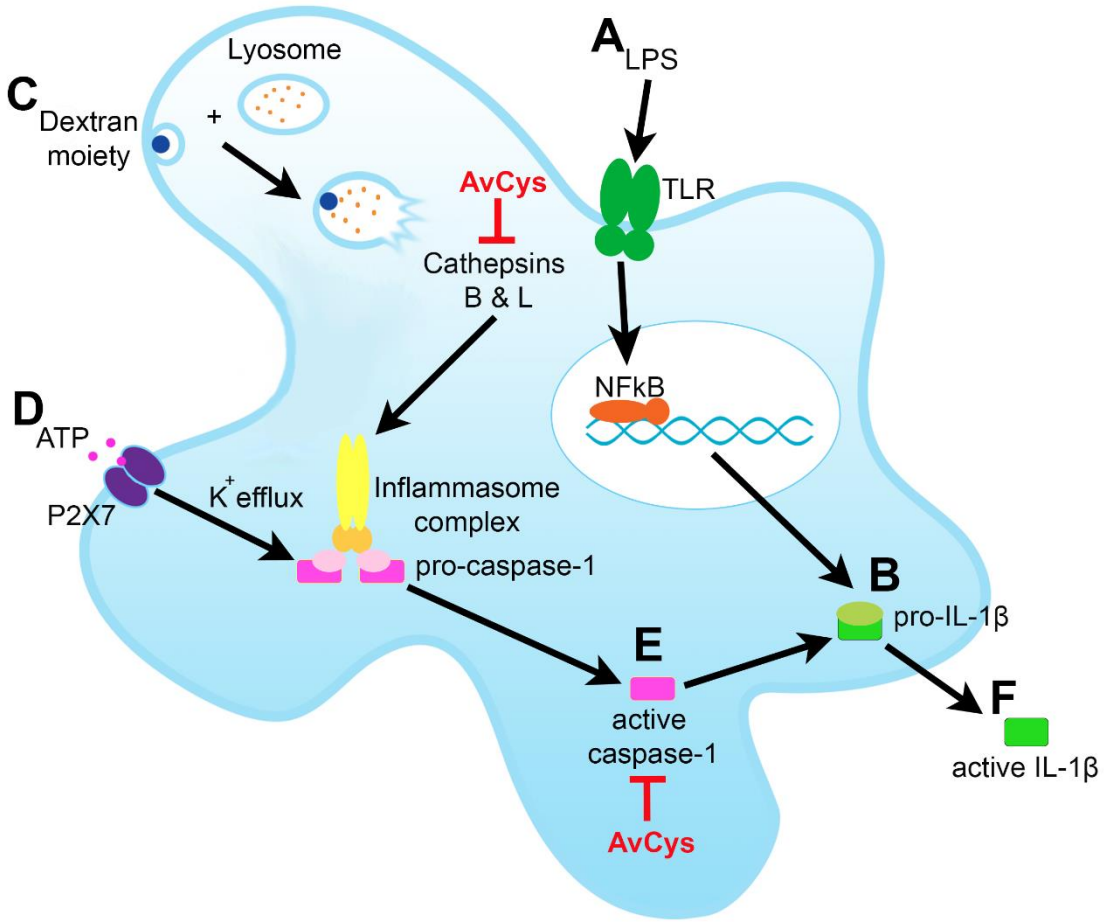


**Figure 4.2** Hypothesized modes of inflammasome inhibition by AvCys investigated in vitro. **(A)** Lipopolysaccharide (LPS) binds to toll like receptors (TLR) and causes the activation and translocation of NFkB. **(B)** NFkB initiates the expression of pro-IL-1β. **(C)** Dextran moieties from dextran sodium sulphate are phagocytosed by the macrophage and a phagolysosome is formed that degrades and releases the proteases cathepsin B and L. **(D)** Alternatively, or additionally extracellular ATP activates the P2X7 receptor causing an efflux of potassium ions ($K^+$) which in turn activates the inflammasome complex. **(E)** The functional inflammasome activates the caspase-1 enzyme. **(F)** Active caspase-1 then cleaves the pro-form of IL-1β, allowing for the activation and secretion of mature IL-1β. AvCys was hypothesized to potentially inhibit the lysosomal cathepsins and/or active caspase-1, thereby inhibiting the downstream secretion of IL-1β.

# 5. Conclusions and Future Perspectives

While current therapies for IBD exist, there is no therapy that is able to treat all UC and CD patient types and the highly efficacious treatments are often either very expensive or are associated with a high risk of the patients experiencing multiple adverse secondary effects (Benchimol et al. 2008; Mowat et al. 2011; Travassos and Cheifetz 2005; Targownik et al. 2014). This is unsurprising when one considers that over 100 different gene polymorphisms are linked to the development of IBD (Anderson et al. 2011) and there are innumerable distinct causes for microbiome disruptions which can also contribute to the onset of the disease (Hold et al. 2014). The development of EcN-AvCys, a probiotic bacterium already shown to have high success in clinical trials of IBD capable of expressing a transgenic worm gene coding for an immune regulatory molecule, presents a potential therapy for IBD that was designed to reduce the chance of side effects and treat a wide variety of patients in a safe and cost-effective manner.

While many mechanistic effects were uncovered in the experiments described in this thesis, additional experiments should be conducted to further elucidate characteristics of the modes of action currently proposed. For instance, while EcN-AvCys was able to ameliorate intestinal inflammation in post-weaning piglets through mechanisms that appear largely dependent on improving the resistance and reducing the permeability of the epithelial membrane, very few immunologic mechanisms for the transgenic probiotic were defined in this model. Potentially the lack of observed immune regulatory properties of AvCys in the pig model are simply due to the fact that the underlying cause of post-weaning inflammation in pigs is largely due to a disruption in intestinal barrier stability. Therefore, EcN-AvCys should be additionally tested in a more immune cell driven model of colitis in pigs, such as a TNBS colitis model currently being established by colleagues at the Institute of Immunology, based on TNBS colitis model for piglets previously described in the literature (Pouillart et al. 2010). The use of chemically induced, immune cell driven colitis models will allow for the further investigation of EcN-AvCys as an immune modulating therapy.

Additionally, while few changes were observed to porcine monocytes *in vitro* when exposed to rAvCys, incubation with EcN-AvCys cell media supernatants showed a trend towards reduced expression of some classically activated monocyte/macrophage markers and a significant decreased in the expression of some alternatively activated markers. This would suggest that while the effects of EcN-AvCys in pigs monocytes/macrophages are perhaps more overall immune regulatory compared to the immune modulatory effects previously observed in mice, where AvCys was shown to induce an M2 like phenotype. While the marker expression patterns allow for a predicted function of these

monocytes/macrophages based on knowledge available from murine and human studies, additional functionality experiments should be conducted *in vitro* to test the phagocytic activity of these cells as well their ability to activate or inhibit T cells. In *in vivo* settings a further investigation of the suppressive role of EcN-AvCys on monocyte/macrophage marker expression in regards to inhibition of inflammation in both post-weaning inflammation in pigs and in chemically induced TNBS porcine colitis should be also be examined.

Additional collaborative experiments with Dr. Dorothee Günzel and Ahmed Keles at the Institute of Clinical Physiology, Charité-University Medicine Berlin, are currently being conducted to investigate whether the presence of macrophages is necessary for the epithelial barrier supportive effects of EcN-AvCys in porcine cell culture models. If the supportive effects of EcN-AvCys on intestinal epithelial barrier integrity are found to be dependent upon the presence of underlying immune cells such as monocytes/macrophages further studies should be conducted to examine whether EcN-AvCys secreted proteins are necessary on the basolateral side of the epithelial layer or if apical application is sufficient. A recent study reported that *T. suis* ES products disrupt epithelial barrier function and this was proposed as a possible mechanism the nematode may have evolved in order to allow access of underlying DCs to the immunomodulatory proteins excreted/secreted by the worm (Hiemstra et al. 2014). As EcN-AvCys has been shown both *in vivo* and *in vitro* to increase the resistance and decrease permeability across the epithelial barrier, it would be interesting to define how the immunomodulator may pass the barrier and affect the underlying immune cells. It is now thought that perhaps macrophages in addition to, or instead of DCs are the resident lamina propria cells that extend dendrites between epithelial cells to sample antigens in the lumen (Mowat and Bain 2011). Therefore, this poses one possible mechanism by which AvCys secreted from transgenic EcN in the gut could access and interact with underlying immune cells despite the observed increase in epithelial barrier function.

While in some of the inflammatory parameters investigated in the animal models of colitis the addition of AvCys through genetic modification of EcN only improved the already significant effects of EcN, in a human population this combination may potentially have a more significant impact on the treatment of IBD. The human population of IBD patients is much more variable than controlled colitis models induced in genetically uniform rodents, and the combinations of underlying causes for the initiation and propagation of the disease in the human populations are highly diverse. For instance, while in mice EcN ameliorated colitis in the entire group compared to untreated controls, in clinical IBD trials only between 16-36% of IBD patients respond to EcN treatment alone (Henker et al. 2008b;

Kruis et al. 2004; Kruis et al. 1997). In patients unresponsive to the probiotic alone in the human population the addition of the potent immune modulator, AvCys, in the transgenic EcN-AvCys could improve the chances of inducing or maintaining remission. Additionally, in patients in which the etiology of the disease is caused by gene polymorphisms affecting the IL-10 signaling pathway (Anderson et al. 2011), a pathway previously shown to be important in the immune regulation initiated by AvCys (Figueiredo et al. 2009; Klotz et al. 2011b; Schnoeller et al. 2008), AvCys may have negligible effects, while the presence of EcN may nonetheless confer beneficial effects.

One proposed method of ensuring the best match of IBD treatment course to specific patients is through genetic testing for the individualization of medicine. This would allow for a determination of the IBD associated gene polymorphisms in each patient so that a treatment plan utilizing a therapy with appropriate modes of action could be devised. For instance, corticosteroid therapies are known to function through the inhibition of gene transcription necessary in IL-6, IL-1 and TNF-α production (Benchimol et al. 2008; Targownik et al. 2014). Therefore, corticosteroids have the potential to regulate these genes in a person who has a polymorphism disrupting the *IRF5* gene that encodes for a transcription factor normally regulating the expression of these cytokines in healthy individuals (Anderson et al. 2011). However, corticosteroids may be less effective, or at least have less direct effects, in IBD patients with a polymorphism in genes affecting barrier function such as the *GNA12* gene encoding a protein important to the assembly of epithelial cell tight junctions (Anderson et al. 2011). Studies have already been published in which genetic testing was used to predict the efficacy of drugs such as glucocorticoids (De Iudicibus et al. 2013) or tolerability of drugs such as azathioprine (Mascheretti and Schreiber 2005) in individual IBD patients. A recent and in depth review on genetic testing in pharmacology, proposes that the field of pharmacogenetics will require the systematic study of specific gene polymorphisms, in specific disease conditions, with specific treatments in order to slowly develop genome based individualized treatments one by one (Ma and Lu 2011). As the gene polymorphisms associated with IBD risk are fairly well defined, it is a logical step in the development of new treatments such as EcN-AvCys that *in vitro* studies in mice involving gene knockout variants, such as the IL-$10^{-/-}$ strains can be incorporated in order to make predictions as to which patient types may or may not benefit from the treatment. Additionally, in any future clinical trials with EcN-AvCys genetic testing of participant patients should be considered in order to elucidate early on in the development of the drug which patient types are good candidates for the therapy. It should not however, be overlooked that unfortunately in the early stages of genetic testing for personalized medicine very rare or undefined polymorphisms may still exist that would

still necessitate the trial and error method of prescribing to treat patients where a clear etiology for the disease is absent.

With the development of any new treatment for the human market, the cost of production and therefore, affordability for the patient as a consumer must be considered. While highly effective some IBD treatments such as monoclonal antibodies, for example Infliximab targeting the inflammatory cytokine TNF-alpha, are very cost-intensive especially when the need for long-term treatment is considered (Travassos and Cheifetz 2005). Indeed the healthcare costs for a single IBD patient for a year can be over US$40,000 with specialty drugs costing over US$20,000 (Gleason et al. 2013). In contrast the commercial brand of EcN, marketed as Mutaflor® by the company Adyepharm, costs less than €1 per capsule (www.mutaflor.com). In published clinical IBD trials patients generally receive 1-2 capsules of Mutaflor® daily **(Table 1.1)**, thus the cost of the probiotic treatment would be around €700 (US$1000) a year. There is no foreseeable reason why the addition of a transgene should largely change the production and subsequently consumer costs of EcN-AvCys compared to that of the currently marketed Mutaflor®. Therefore, EcN-AvCys is expected to be a very affordable potential therapy for IBD.

Moving towards EcN-AvCys human clinical trials the current European Union regulations need to be considered. To the authors knowledge there are no transgenic probiotics currently marketed as pharmaceuticals under medicinal legislation. However, it can be assumed that while EcN-AvCys is not a food/feed GMO that at least the same standards that the European Union Food Safety Administration upholds for GMO foods will be applied to the development of GMO medicinal therapies. Main consumer concerns regarding GMO foods surround the safety of GMO food consumption and the effects the GMO may have to the natural environment. The European Food Safety Administration GMO Panel addresses these consumer concerns and requires all GMO food applications to be analyzed by three separate standing working groups (Devos et al. 2014):

1) The molecular characterization assessment panel ensures that each applicant can provide information on the origin and sequence of the gene, methods by which the gene has been stably integrated and methods by which genetic and phenotypic changes in the GMO can be characterized.

2) The food/feed risk assessment panel requires applicants to provide detailed comparisons of the benefits of the GMO to the native organism, how the GMO may alter the health risks of the native organism and any potential toxicological or allergenic risk associated with the GMO.

3) The environmental risk assessment panel requires the applicant to provide information on how the potential changes could affect the biotic or abiotic environment and prevention methods for the spread of the GMO throughout the environment.

In regards to meeting the regulations from the molecular characterization assessment panel the origin and sequence of the AvCys gene insert can currently be provided. Additionally, the methods for the genetic (PCR) and phenotypic (western blot and ELISA) characterization of the AvCys transgene are also available. However, AvCys was not stably inserted into the EcN genome, rather the current construct was cloned with a transgene containing plasmid vector. This is not unusual as several transgenic EcN strains have been developed as vehicles for therapeutic molecules to date and all have been cloned on plasmids (Pohlmann et al. 2013; Rao et al. 2005; Seo et al. 2012; Zhang et al. 2012). The reason for plasmid cloning in the previous studies as well as EcN-AvCys is due to a previous lack of genetic information on the probiotic. However, a draft assembly of the whole EcN genome has now been published (Cress et al. 2013). Therefore, copious possibilities for developing an EcN-AvCys clone in which AvCys is stably integrated into the genome are now available. These will be further described in regards to meeting the requirements of the environmental risk panel, as there are several methods of stable integration that can decrease or eliminate the risk of environmental contamination.

To address concerns from the food/feed risk panel a detailed comparison of the native EcN to the GMO EcN-AvCys has been made utilizing both murine and porcine *in vitro* and *in vivo* models. However, no comparison between EcN and EcN-AvCys in a pathogenic disease state has been investigated. It would be naïve to ignore the possibility that a nematode derived immune regulatory protein, such as AvCys, may inhibit the host's ability to mount necessary immune responses to pathogenic organisms. Nematode infections have been shown to reduce the inflammatory Th1 and Th17 responses and enhance the susceptibility of the host to gastrointestinal bacterial pathogens (Chen et al. 2005; Chen et al. 2006), viral pathogens (Osborne et al. 2014), tuberculosis (Potian et al. 2011), and plasmodium infection (Tetsutani et al. 2009). Therefore, it is highly recommended that the potential effects of the therapy on defense mechanisms against pathogens should be tested in animal models to rule out that the transgenic probiotic interferes with immune responses necessary to control bacterial, fungal or viral pathogens in the intestine. As many of the marketed IBD therapies also involve immune suppression (Benchimol et al. 2008; Guo et al. 2013), there is the potential that the efficacy of EcN-AvCys in IBD patients may outweigh potential adverse effects that EcN-AvCys could have on host defenses. However, the severity and range of potential immunosuppression associated with EcN-AvCys should

be well defined so that the risks between EcN-AvCys and other IBD therapeutics can be compared before treatment recommendations are made.

The potential for allergenicity to the transgene must also be addressed under the food/feed risk panel for GMOs. While AvCys has been observed to be immunogenic (unpublished), this has not yet been shown to result in an allergic response. In fact airway hyper-reactivity to allergens is prevented with administration of the helminth immunomodulator (Danilowicz-Luebert et al. 2013). However, despite this observed reduction in allergic response with acute AvCys treatment in mouse models or in chronic nematode infections, it should not be overlooked that long term treatment with immunomodulator alone could induce an allergic response to the protein. In chronic nematode infections *in vivo* there may be other excretory/secretory proteins or mechanisms by which allergenicity to worm based products is prevented that would be lacking with the administration of a single protein, such as AvCys, administered with a probiotic vehicle. Therefore, further study is required to elucidate the long term effects of AvCys administration in this regard.

While many therapeutic proteins, such as AvCys, are immunogenic this may not lead to inactivation of the proteins if the antibodies produced aren't neutralizing (Schellekens 2002). Additionally, if immunogenicity of AvCys were to disrupt the immunomodulatory properties or induce allergenicity, genetic mutations can be induced that reduce the immunogenic characteristics of proteins. For instance, mutations that reduce aggregation potential can likewise reduce immunogenic potential (Ratanji et al. 2014). Alternatively, genetic manipulation of the AvCys gene in order to alter the epitopes at which antibodies may bind could also resolve any issues with immunogenicity if they occur (Schellekens 2002). In summation, while toxicity and allergenic potential of EcN-AvCys is yet to be investigated, methods are available to reduce any negative side effects if these obstacles do appear.

Finally, in regards to the environmental risk assessment panel for European Union GMO regulations, the stability of the EcN-AvCys cloning procedure and the safety or containment of the transgenic probiotic in the environment must be addressed. As was previously described the AvCys transgene is currently cloned on a plasmid and the transgenic EcN is resistant to three antibiotics. This raises legitimate concerns regarding the stability of the insert, the potential of the plasmid to be transferred to other bacteria and the risk of environmental contamination with a transgenic probiotic that is highly resistant to antibiotics. Stable integration of the AvCys gene into the genome of EcN in a manner that prevents environmental escape of bacteria and/or transgene is therefore necessary. To prevent environmental contamination of genetically modified bacteria, deletions in

sporulation genes can be used that create bacteria that are not only unable to form long lasting spores, but are additionally highly sensitive to UV damage (Kimman et al. 2008). Suicide functions can also be introduced into genetically modified bacteria in which lethal genes are triggered upon completion of the expression of the inserted transgene; however, these transgenic bacteria must then continually be administered as they cannot replicate *in vivo* (Kimman et al. 2008). Perhaps the most practical and effective containment method for transgenic probiotics is attained through gene deletion of necessary catabolic enzymes, resulting in generation of auxotrophic bacteria that are dependent upon nutrients in the gut lumen of the host that would otherwise be unavailable to them in the environment (Kimman et al. 2008). A very elegant example of the creation of a stably-cloned, auxotrophic, transgenic probiotic for IBD therapy is the *Lactococcus lactis* strain modified by Steidler et al. to express the regulatory cytokine IL-10. Through cloning of complimentary flanking regions on the IL-10 gene and double homologous crossover, a thymidylate synthase gene in *L. lactis* was replaced by the human IL-10 gene (Steidler et al. 2003). This not only allowed for stable genome integration of the gene, but the deletion of the enzyme encoding gene rendered the bacteria dependent on host intestinal sources of thymidine or thymine (Steidler et al. 2003). With the EcN genome now available (Cress et al. 2013) there are many potential methods by which stably integrating the AvCys gene can be conducted in a way that creates an auxotrophic clone for environmental containment such as the amino acid dependent transgenic *L. lactis* described.

Stable integration of AvCys in the EcN genome, as well as further testing to elucidate any risks associated with immunosuppression, toxicology and immunogenicity of the transgenic probiotic will most likely be necessary for the EU approval of EcN-AvCys in human clinical trials and its eventual approval as a medicine. Additionally, further safety testing of EcN-AvCys will be beneficial to consumer perception of the therapy. While IBD patients have been found to have a very positive perception and interest in the use of probiotic therapies for IBD (Mercer et al. 2012), consumer anxiety regarding health risks of GMO foods still exists (Nakayachi 2013). While recent studies suggest that anxiety around the safety of GMOS has increased in past years, they also report that providing consumers with information refuting the believed risks of GMOs significantly reduces the anxiety towards the perceived hazards (Nakayachi 2013). Therefore, being able to scientifically support the safety of EcN-AvCys will not only be necessary for any future approval of the novel therapy, but will additionally improve the acceptability of the therapy in the eyes of the IBD patient population. This body of work provides evidence of EcN-AvCys as a novel and effective therapy for the amelioration of intestinal inflammation in animal models and *in vitro* assays and demonstrates the promising future of the transgenic probiotic as a therapy for human IBD.

## 6. Summary

Inflammatory Bowel Disease (IBD) is an auto-inflammatory disorder characterized by unregulated intestinal inflammation. Current therapies are unable to treat all patients afflicted with the condition and are often accompanied by undesirable side effects. Therefore, an urgent demand exists for the development of novel therapies.

Parasitic nematodes actively secrete products modulating the host's immune system which enables them to persist and reproduce for long periods of time. Parasite driven immunomodulation has been shown to ameliorate murine experimental gut inflammation and clinical IBD in humans. Cystatin secreted by the filarial nematode *Acanthocheilonema viteae* (AvCys) has strong anti-inflammatory properties when in contact with immune cells, predominantly targeting macrophages. A novel treatment for IBD was developed utilizing a probiotic bacterium, *Escherichia coli* Nissle 1917 (EcN), genetically modified as a carrier for AvCys to allow for the site-directed, prolonged secretion of the immunomodulator in the gut.

The transgenic probiotic EcN-AvCys was applied in a murine model of acute colitis, where it significantly suppressed the intestinal expression of chemoattractants, infiltration of inflammatory cells and T-cell cytokine responses, leading to drastically lowered inflammatory scores. To assess the anti-inflammatory activity of EcN-AvCys in a genetically diverse, outbred model equipped with a gastrointestinal tract highly similar to that of humans, high doses were repeatedly applied to piglets suffering from post-weaning intestinal malfunction. The treatment significantly reduced post-weaning gut inflammation and, importantly, was safe in terms of body weight development and immune function. Furthermore, EcN-AvCys treatment significantly improved colon epithelial barrier functions. Similarly, short-term exposure of porcine colon tissue and human intestinal epithelial cells to EcN-AvCys supernatants improved transepithelial resistance.

To analyse the mechanistic basis for the anti-inflammatory action of EcN-AvCys, the phenotype of porcine monocytes was investigated after incubation with EcN or EcN-AvCys cell culture media supernatants, or recombinant AvCys. While recombinant AvCys had very little effect on the expression of monocyte markers related to phenotypic specialization and function, EcN and EcN-AvCys supernatants induced the indiscriminate early expression of M1 associated markers, as well as regulatory IL-10.

To test the hypothesis that AvCys may interfere with pro-inflammatory macrophage functions by interfering with the inflammasome and thus activation of the pro-inflammatory cytokine IL-1β, monocytes and macrophages where exposed to supernatants of the transgenic and control probiotic and IL-1β secretion was determined. However, EcN-AvCys supernatant supported rather than suppressed the secretion of IL-1β in response to inflammasome induction in monocytes.

Thus additional research is required to determine the mechanistic basis for the documented anti-inflammatory activity of EcN-AvCys in murine and porcine gut inflammation models and further evaluate its applicability as a treatment for human IBD.

# 7. Zusammenfassung

**Ein transgenes probiotisches Bakterium als ein Träger für ein immunmodulatorisches Nematoden-Protein für die Behandlung von intestinalen Entzündungen.**

Chronisch-entzündliche Darmerkrankungen (CED) sind Autoimmunerkrankungen, die durch eine ungeregelte Darmentzündung gekennzeichnet werden. Heutige Therapien sind nicht bei allen betroffenen Patienten wirksam und oft mit unerwünschten Nebenwirkungen verbunden. Daher besteht ein dringender Bedarf für die Entwicklung neuer Behandlungsmethoden.

Parasitische Nematoden sekretieren immunmodulatorische Moleküle, die es ihnen gestatten, längerfristig im Wirt zu überleben und sich dort fortzupflanzen. Studien mit an CED erkrankten Menschen und experimentell herbeigeführten Darmentzündungen bei Mäusen zeigten, dass die Immunmodulation der Nematoden die Symptome von CED sowohl bei Menschen als auch bei Mäusen abmilderte. Cystatin, welches von der Filarie Acanthocheilonema viteae sekretiert wird (AvCys), wirkt stark entzündungshemmend bei Kontakt mit Immunzellen, vor allem bei Makrophagen. Eine neuartige Behandlung von CED wurde unter Verwendung des probiotischen Bakteriums *Escherichia coli* Nissle 1917 (EcN) entwickelt. Hierbei werden transgene Bakterien, welche das AvCys Gen exprimieren, in den Wirtsdarm eingebracht um eine anhaltende Applikation des entzündungshemmenden Cystatins direkt vor Ort zu ermöglichen.

Der Einsatz der transgenen EcN-AvCys Probiotika bei Mäusen mit akuter Kolitis bewirkte eine deutliche Reduktion der Expression von Chemoattraktanzien, der Einwanderung von Entzündungszellen und der T-Zellantwort im Darm. Dies führte zu einer insgesamt drastisch reduzierten Entzündungsreaktion. Da der Magen-Darmtrakt von Schweinen sehr dem des Menschen ähnelt, wurde die entzündungshemmende Aktivtät von EcN-AvCys daraufhin an einer genetisch diversen Versuchstierpopulation von Hausschweinen getestet. Dabei wurden Ferkeln, die nach der Entwöhnungsphase an einer Darmfehlfunktionen litten, wiederholt hohe Dosen von EcN-AvCys verabreicht. Die Behandlung reduzierte erheblich die Darmentzündungen und stellte überdies kein Risiko für die Körpergewichtsentwicklung und Immunfunktion der Ferkel dar.

Um die mechanistische Grundlage für die antiinflammatorische Wirkung von EcN-AvCys zu analysieren, wurde der Phänotyp der Monocyten der Schweine unter verschiedenen Bedingungen untersucht: (1) nach Inkubation mit Überständen aus Zellkulturen von EcN, (2) EcN-AvCys bzw. (3) rekombinantem AvCys. Während rekombinates AvCys sehr wenig Einfluss auf die Expression von Monozyten-Markergenen hatte, induzierten die Überstände von sowohl EcN als auch EcN-AvCys Kulturen gleichsam eine frühe Expression von M1-assoziierten Markern sowie regulatorischem IL-10.

Anschließend wurde die Hypothese getestet, dass AvCys die entzündungsfördernden Makrophagen-Funktionen stören könnte indem durch Beeinträchtigung des Inflammasoms die Aktivierung des entzündungsfördernden Zytokins IL-1β gehemmt wird. Hierzu wurden Monozyten und Makrophagen mit Überständen der transgenen und Kontrollkulturen der probiotischen Bakterien inkubiert, wobei die Sekretion von IL-1β überwacht wurde. Anstatt die Sekretion von IL-1β als Reaktion auf die Induktion des Inflammasoms zu unterdrücken, förderte EcN-AvCys jedoch sogar die Sekretion des Zytokins.

Es bleiben also zusätzliche Untersuchungen erforderlich, um die mechanistische Grundlage für die dokumentierte antiinflammatorische Aktivität von EcN-AvCys im Maus- und Schweinedarmmodell zu ergründen. Diese Kenntnisse wären von Bedeutung um die Eignung von EcN-AvCys als Behandlungsmöglichkeit für die CED des Menschen besser einschätzen zu können.

# 8. Methods

## 8.1. Ethics statement

All animal experiments were approved by and conducted under licences LAGeSo Reg. Nr. G0144/10 and LAGeSo Reg. Nr. G0350/09 in accordance with guidelines of the appropriate committee (Landesamt fuer Gesundheit und Soziales, Berlin, Germany).

## 8.2. Construction of the transgenic probiotic

A spontaneously streptomycin-resistant *Escherichia coli* Nissle 1917 (EcN) strain was gifted to us graciously by Dr. Tobias Oelschläger from the University of Würzburg. The strain carries a kanamycin resistance cassette on the EcN specific plasmid pMut2 and a genetically modified version of the native EcN specific plasmid pMUT1. The modified pMUT1 plasmid, known as pMUT13, was designed for functionality as a cloning vector through the addition of a tetracycline resistance cassette, a multiple cloning site and a hemolysin A secretion system. All NsiI cut sites outside of the hlyA gene were deleted to allow for insertion of genes within the hlyA component and to ensure that protein expressed from the inserted gene are secreted from the bacteria.

For insertion of AvCys, primers were designed to add NsiI restriction enzyme cut sites to both the 3' and 5' ends of the AvCys sequence when amplified (Fw 5'-ACGTATGCATTGGTGCGCTGTGAAGA-3', Rv 5'-ACGTATGCATTCACTGATGAGAGTACT-3'). The amplified AvCys gene was then inserted into the pMU13 vector and transformed into chemically competent EcN. Successful clones were confirmed with AvCys specific primers (Fw 5'-TCGTGTCGACGGTTTTGGTGCGCTGTGAAGAAC-3', Rv 5'-ACATGCGGCCGCTCACACTGATGAGAGTA-3') and EcN plasmid specific primers (Blum-Oehler et al. 2003) via PCR. EcN-AvCys supernatants were analyzed to ensure secretion. Proteins were precipitated from 5mL of supernatant from cultures of EcN or AvCys expressing EcN (EcN-AvCys) grown to $OD_{600}$ = 1, which corresponded to a concentration of $3x10^8$ CFU EcN/mL, and analzyed via Western blot with a monoclonal AvCys-specific antibody.

## 8.3. Production of EcN and EcN-AvCys supernatant

Culture supernatants of EcN or EcN-AvCys were produced according to a modified protocol first described by Yan and Polk (Yan and Polk 2002). Cultures of EcN-AvCys and control EcN in Luria Broth media containing 20µg/mL tetracycline, 50µg/mL kanamycin and 50µg/mL streptomycin were incubated overnight at 37°C with shaking at

200rpm. Cultures were diluted 1:1000 in fresh Iscoves Modified Dulbeccos Media (IMDM) with 4 mM L-glutamine (PAA, Austria) and further incubated at 37°C with shaking at 200rpm until an $OD_{600}$ = 1.00. Cultures were then centrifuged and supernatant filtered through 0.2µm filters, before being stored at -20°C. Concentration of AvCys was determined using a direct coating ELISA using a recombinant AvCys standard curve, a murine anti-rAvCys monoclonal antibody developed in house (1µg/ml), a peroxidase bound goat anti-mouse IgG/M (0.12µg/ml, Jackson ImmunoResearc, USA) and a 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Reaction was stopped with 1M $H_2SO_4$ and colorimetric changes were read at 450nm on a Synergy HI Hybrid Reader (Biotek, USA). The concentration of LPS in each of the supernatants was determined by Limulus Amoebocyte Lysate LPS detection kit QCL 1000 (Cambrex) and ranged between 0.5-0.7µg/ml.

## 8.4. Animal experimentation

### 8.4.1. Murine dextran sodium sulfate model of chronic colitis

Male, 9-11 week old C57BL/6 mice were assigned to one of four groups: DSS controls, DSS+Sm, EcN or EcN-AvCys. All animals received 2.5% DSS (MP Biomedicals, France) in the drinking water for three, seven day cycles interspersed by twelve days of DSS free drinking water. One day prior to the third cycle streptomycin (Sm) was added to drinking water at 5g/L in the DSS+Sm, EcN and EcN-AvCys groups. On the first day of the third cycle $2x10^9$ CFU of either EcN or EcN-AvCys in 100µl 0.9% saline was administered to the EcN and EcN-AvCys groups respectively. In the DSS and DSS+Sm groups 100µl of saline alone was administered by oral gavage. After the third cycle animals were euthanized.

### 8.4.2. Murine dextran sodium sulfate model of acute colitis

Male, 9-11 week old C57BL/6 mice were assigned to one of four groups: untreated controls, DSS controls, EcN treated DSS controls and an EcN-AvCys treated DSS group. All animals except untreated controls received 3.0% DSS (MP Biomedicals, France) in the drinking water for eight days. Immediately after the introduction to DSS and every 48 hrs thereafter the EcN-AvCys group and EcN control group were treated via oral gavage with 100µl saline containing $2\times10^9$ CFU EcN-AvCys or EcN respectively. Untreated and DSS control groups received saline alone. Animals were euthanized on day eight after start of DSS feeding.

### 8.4.3. Post-weaning piglet intestinal inflammation model

Thirty male Pietran x Landrace cross piglets, weaned at age of 28 days were randomly allocated to one of three groups; a transgenic EcN-AvCys group (EcN-AvCys), an *E. coli* Nissle treated control group (EcN) and a saline treated control group (ctr). They were allocated in a level-2 biosecured, environmentally controlled experimental facility (Federal Institute for Risk Assessment, Berlin, Germany), five pigs per pen of 8.5m$^2$. Pigs in two pens formed one group. The pens were in separated rooms to prevent cross contamination. Rooms were constantly ventilated and kept at temperature of 26°C. Every 48 hrs for a total of 14 days pigs were orally inoculated with 1mL of saline containing $2\times10^{10}$ CFU/mL EcN-AvCys, 1mL saline containing $2\times10^{10}$ CFU/mL EcN respectively, or 1mL of 0.9% saline alone, respectively. Animals were weighed at day 0, 7 and 14. On day 14 animals were sedated with an intramuscular injection of azaperone (2mg/kg, Stresnil) before being deeply sedated with ketamine (25mg/kg, Ursotamin, SerumwerkeBernburg, Bernburg, Germany) and finally euthanized with a lethal intravenous dose of pentobarbital (200mg/kg, Narcoren, Merial, Hallbogmoos, Germany). Just before the euthanasia full blood was collected from heart.

## 8.5. Molecular detection of EcN-AvCys in murine feces.

Bacterial DNA was extracted from feces of mice before, during and after the administration of EcN or EcN-AvCys in the acute DSS trials with the InnuPrep stool DNA kit (Analytikjena, Germany). A PCR of the fecal extracted DNA with AvCys specific primers as described in section 7.2 was conducted, as well as a multiplex PCR with primer pairs specific to EcN plasmids previously described by Blum-Oehler *et al* (Blum-Oehler et al. 2003).

## 8.6. Culture and molecular detection attempts for EcN-AvCys in porcine feces.

In a pilot trial with feces from piglets reared at the Institut für Tierernährng, Freie Universität zu Berlin, were diluted in LB media at various concentrations, plated on LB agar and grown overnight at 37ºC, 5% $CO_2$. Colonies from these plates were then stamped onto LB agar plates containing Tc and further stamped onto plates with Tc, Sm, Kn to track which colonies had single and multiple antibiotic resistance. As no colonies were resistant to all three antibiotics in this pilot trial in the piglet feeding trial the same culture method was utilized; however, detecting CHROMagar$^{TM}$ (CHROMagar, France) was used in place of LB agar for additional colorimetric detection. Select pink colonies, indicating *E. coli* spp. growing on the CHROMagar$^{TM}$ plates supplemented with all three antibiotics were then tested for EcN plasmid genes and AvCys as previously described in section 7.2.

Molecular methods were also developed in an attempt to directly identify the AvCys gene in feces from the piglets administered EcN-AvCys in the feeding trial. Three sets of RT-PCR primers were designed for high sensitivity over specificity and optimized to be used at an annealing temperature of 60ºC **(Table 8.1).** Primer sets were then utilized in qRT-PCR assays of fecal extracted bacterial DNA from the porcine feeding trial with a standard curve developed using known amounts of AvCys DNA.

**Table 8.1** AvCys specific RT-PCR primers designed for detection of the transgene in porcine feces orally inoculated with EcN-AvCys.

| **Primer Identification** | **Forward (Fw-) and Reverse (Rv-) Primers (5'-3')** | **Product Length (base pairs)** |
|---|---|---|
| AvCys-1 | **Fw -** gCgCTgTgAAgAACCCgCAA | 81 |
| | **Rv -** TCCggATTgCgTTCCTgCCAT | |
| AvCys-2 | **Fw –** CgTTCggTgTgACgACAg | 76 |
| | **Rv -** AAATTgggCgCTTgTACC | |
| AvCys-3 | **Fw -** CTgTgAAgAACCCgCAAA | 74 |
| | **Rv -** gATTgCgTTCCTgCCATC | |

## 8.7. Histopathological analysis and immunohistochemistry

Porcine and murine distal colon sections were fixed in 3.7% formalin, dehydrated using ethanol concentrations increasing from 70-96%, cleared with xylene, embedded in paraffin, mounted and stained with hematoxilin/eosin (H&E) for histopathological scoring according to two systems. The scoring parameters for the DSS-induced colitis model were as follows: inflammation (0: no inflammation; 1: increased number of inflammatory cells in LP; 2: inflammatory cells extending into the submucosa; 3: transmural inflammatory infiltrates) and tissue damage: (0: no mucosal damage; 1: discrete epithelial lesion; 2: erosion or focal ulceration; 3: severe mucosal damage with extended ulcerations extending into bowel wall. Porcine colon cross sections were scored according to the following parameters: infiltration (1: minimal; 2: mild; 3: moderate; 4: severe), degree of infiltration (1: mucosal; 2: mucosal and focal submucosal; 3: mucosal and submucosal; 4: transmural), epithelial surface damage (1: focal denudation; 2: extensive denudation; 3: erosion; 4: ulceration), crypt epithelial damage (1: sporadic crypt abscesses; 2: multiple crypt abscesses) and hyperplasia (1: minimal; 2: mild; 3: moderate; 4: severe).

For detection of M1 macrophages, 1-2µm sections of formalin-fixed, paraffin-embedded tissue were cut, deparaffinized, and subjected to a protein induced epitope retrieval step. Slides were rinsed in Tris-buffered saline (pH 7.4) prior to incubation with monoclonal rat anti-mouse F4/80 antibody (clone BM8, Invitrogen; dilution 1:100) for 30 minutes at room temperature followed by incubation for 30 minutes with biotinylated secondary antibody donkey anti-rabbit (Invitrogen; dilution 1:200). For detection, DAKO REALTM Detection System, Alkaline Phosphatase/RED was used. The stained sections were then subjectes to a heat induced epitope retrieval step. Slides were rinsed in cool running water, washed in Tris-buffered saline (pH 7.4) and treated with Peroxidase Blocking Solution (Dako) prior to incubation with polyclonal rabbit anti mouse-iNOS antibody (Abcam; 1:100). For detection, EnVision+ System-HRP (DAB) kit (Dako) was used. Alkaline phosphatase was revealed by Fast Red as chromogen for 30 min, and peroxidase (HRP) was developed with a highly sensitive diaminobenzidine (DAB) chromogenic substrate for 5 min. Negative controls were performed by omitting the primary antibodies. Nuclei were counterstained with hematoxylin and slides mounted with gelatine (Merck). For detection of eopsinophils a modified Sirius Red stain protocol was utilized as described in Meyerholz et al. Toxicol Pathol. 2009). Images were acquired using a AxioImager Z1 microscope (Carl Zeiss MicroImaging). Cells were quantified in 10 high power fields (hpf = 0.237 mm2). All immunohistochemical evaluations were performed in a blinded manner.

## 8.8. Quantitative real time PCR

Porcine distal colon segments were snap frozen. As per manufacturers protocols, 200mg of distal colon tissue was homogenized using the FastPrep-24 Lysing Matrix Tubes D (MP Biomedical, Germany), RNA was extracted using innuPREP RNA kit (Analytikjena, Germany) and finally cDNA transcribed with the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Germany). Real-Time PCR reactions were conducted in a Mastercycler ep gradient S thermocycler with a Realplex2 detection system (Eppendorf, Germany) using FastStart Universal SYBR Green Master Mix (Roche). Specific primers for the swine housekeeping gene RPL19 as well as cytokines and macrophage phenotype markers are summarized in **Table 8.2**. The ΔΔCT method was used to determine the fold increase of these cytokines in treatment groups compared to the control group using CT values normalized to a house keeping gene (RPL19).

**Table 8.2** Swine specific primers for various cytokines, macrophage phenotype markers and the housekeeping gene RPL19.

| Gene Target | Forward (Fw-) and Reverse (Rv-) Primers (5'-3') | Product Length (base pairs) | Reference (NCBI Reference Sequence for primer sets designed for this study) |
|---|---|---|---|
| ARG1 | **Fw -** CCAGTCCATGGAGGTCTGTC<br>**Rv -**GTGTCTTCCCCAGAGATGGA | 334 | (Kyrova et al. 2012) |
| *IDO1* | **Fw -** GGTTTCGCTATTGGTGGAAA<br>**Rv -** CTTTTGCAAAGCATCCAGGT | 143 | (Kapetanovic et al. 2012) |
| *IL1B* | **Fw -** TGAAGTGCCGCACCCAAAACCT<br>**Rv -** CGGCTCCTCCTTTGCCACAATCA | 131 | (Pieper et al. 2012) |
| *IL6* | **Fw -** CCCACCACAAATGCCGGCCT<br>**Rv -** GAGGGAATGCCCGTGGACGG | 176 | (Pieper et al. 2012) |
| IL8 | **Fw-** GGTCTGCCTGGACCCCAAGGAA<br>**Rv-** TGGGAGCCACGGAGAATGGGTT | 124 | NM_213867.1 |
| *IL10* | **Fw -** GTCCGACTCAACGAAGAAGG<br>**Rv -** GCCAGGAAGATCAGGCAATA | 73 | (Pieper et al. 2012) |
| *IL12* | **Fw-** AGCCCGGGACCGCTACTACAG<br>**Rv-** GGGGGAGGGGTCTGCTCCATC | 144 | NM_214013.1 |
| CD274 | **Fw -** TATGGTGGTGCCGACTACAA<br>**Rv -** TGCTTGTCCAGATGACTTCG | 154 | (Lechner et al. 2010) |
| *TNFA* | **Fw-** CAAGCCACTCCAGGACCCCCT<br>**Rv-** AGAGTCGTCCGGCTGCCTGT | 72 | NM_214022.1 |
| TGFB1 | **Fw -** GAAGATGCTTGGAGCTGAGG<br>**Rv -** TGGGACTTTGTCTTGGGAAC | 121 | (Pieper et al. 2012) |
| RPL19 | **Fw -** AACTCCCGTCAGCAGATCC<br>**Rv -** AGTACCCTTCCGCTTACCG | 147 | (Badia et al. 2012) |

## 8.9. Quantification of pig blood leukocytes

Fresh venous blood was smeared onto glass slides and stained with Hemacolor Staining Kit (Merck Millipore, Germany). Proportions of monocytes, granulocytes and lymphocytes were counted per 100 leukocytes twice each by two persons and the average of the four counts reported.

## 8.10. Immune cell isolation and analysis

### 8.10.1. Cell isolation

For the isolation of leukocytes from spleen and gut draining mLNs minced tissue was passed through 70µm cell strainers. For isolation of gut LP leukocytes colon tissue was washed twice in cold Hanks` balance salt solution (HBSS) followed by two washes for 20 min at 37° and 180 rpm in HBSS/5mM EDTA. Remaining tissue was minced and stirred in

5%FCS RPMI medium containing 200 U/mL of collagenase VIII (Sigma-Aldrich) and 1 U/mL collagenase D (Roche) at 37°C for one hour. Samples were then passed over 70µm cell strainers, washed, put on a 40/70% Percoll (GE Healthcare) gradient, spun at room temperature (800×g for 20 min) and lymphocytes recovered from the interphase. Erythrocytes were lysed in 1mL erylyse buffer (0.1M $KHCO_3$, 0.155M $NH_4Cl$, 0.1mM EDTA at pH7.5) for 1-2min on ice before, spleen, mLN and LP cells were washed and resuspended in complete RPMI 1640 (PAA, Austria) containing 10% fetal calf serum, 100U/mL penicillin, 100mg/mL streptomycin and 20mM L-glutamine (PAA, Austria) (Biochrom, Germany) and counted with a CASY Cell Counter (Innovatis, Germany). Cells were plated at a concentration of $3.5\times10^5$ cells/well on 96-well plates in 200µL, stimulated for 48 hrs with media alone, concanavalin A (conA, 2µg/mL) or recombinant AvCys (0.5 µM).

### 8.10.2. Cytokine analysis

Murine specific ELISA antibody pairs for IL-17A (R&D Systems) IL-1β (ebioscience, USA) and porcine specific antibody pairs for IL-10, IFN-γ, TNF-α and IL-6 (R&D Systems) were used according to manufacturer recommendations to determine the cytokine levels from the cell culture supernatants. Cytokines in murine colon tissue were analyzed applying the ProcartaPlex system (eBioscience). In brief, 0.5 cm tissue samples were excised from the distal colon, washed in cRPMI, weighed and homogenized in 250 µl ProcartaPlex lysis buffer containing a protease inhibitor cocktail (Roche, Germany) using FastPrep-24 Lysing Matrix Tubes D (MP Biomedical, Germany). After spinning (10,000×g, 10 minutes, 4 °C), supernatants were collected and protein content measured by a bicinchoninic acid assay (Thermo Scientific Pierce, USA). MIP1-α, MIP-1β, MCP-1, MCP-3, and RANTES were quantified according to the manufacturer's instructions using the BioPlex Multiplex system (BioRad, USA) and expressed in relation to the total protein content. IL-6 and IL-23 were measured accordingly in culture supernatants of colon explants kept in RPMI 1640 (PAA, Austria) containing 1% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 20 mmol/l L-glutamine (Biochrom, Germany) at 37 °C for 24 hours and expressed in relation to mg colon tissue in cultures. Active TGF-β in colon explant supernatants was measured with the MFB-F11 reporter cell line.47 Cells were adhered for 4 hours to 96-well flat-bottom plates at 4 × 104 cells/well in DME containing 4.5% glucose 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin followed by 2 wash steps with PBS and a 2 hours starvation period in 50 µl serum-free medium. Then 50 µl of test samples was applied. Doubling dilutions of rhTGF-β1 (R&D Systems) starting with 1 ng/ml were used as a standard. After 24 hours, samples were measured on a Synergy H1 microplate reader (BioTek, USA) using a chemiluminescent SEAP Reporter Gene Assay (Roche, Germany) kit as per the manufacturer's instructions.

### 8.10.3. Flow cytometry

Murine mLN and colon LP leukocyte preparations were stained with the following antibodies purchased from eBioscience: αCD4-Percp-eFluor710 (RM4-5), αCD25-APC (PC61.5) αFoxp3-eFluor450 (FJK-16S), αF4/80-biotin (BM8), αCD11b-FITC (M1/70), αGR-1-APC (RB6-8C5), αLy6C-eFluor450 (HK1.4). αSiglec-F-PE (E50-2440) for the detection of eosinophils was purchased from (BD Biosciences). Porcine leukocytes extracted from mLN and colon LP were stained in PBS containing 0.2% bovine serum albumin with the following antibodies: unconjugated αCD25 (clone K231.3B2), unconjugated αSLA class II DR (2E9/13), αMacrophage-FITC (BA4D5), αCD163-PE (2A10/11) and biotinylated sheep αmouse IgG (all AbD Serotec, Germany). αCD4a-PE (74-12-4) was purchased from Southern Biotech (USA). αFoxP3-eFluor450 staining kit (FJK-16S), streptavidin-PE-Cy7 and eFluor 780 fixable viability dye were purchased from eBioscience (USA). Samples were acquired using a LSR II flow cytometer (BD Biosciences). Analysis was performed using FloJo software (Treestar, USA).Thymidine proliferation assay

### 8.10.4. Thymidine proliferation assay

Porcine mLN cells were incubated with rAvCys or a recombinant control protein for 72 hrs and then pulsed with 1µCi of 3H-thymidine (MP Biomedicals, Germany) for 20 hrs. The proliferative response was analyzed using a beta-counter (PerkinElmer, Germany).

## 8.11. Primary cell culture experiments

### 8.11.1. Differentiation and phenotype analysis of porcine blood monocytes

Mononuclear cells were harvested from porcine whole blood using a density gradient medium, Lymphoprep (Stemcell Technologies, France). Washed cells were then stained with human anti-$CD14^+$ Microbeads (Miltenyi Biotech, Germany), previously reported to be cross-reactive in swine (Ziegler-Heitbrock et al. 1994) and positively sorted using an autoMACSTM (Miltenyi Biotec, Germany). The $CD14^+$ cells were then plated on 48 well culture plates at a density of $3x10^5$ cells/well in IMDM supplemented with 100m L-glutamine, 10% FCS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 10µg/mL MycoKill AB (PAA, Austria). In a pilot study to define M1 vs M2 differentiated monocyte controls, $CD14^+$ cells were incubated with either 10ng/ml porcine rIFN-γ (IBI Scientific, USA) and 1mg/ml LPS (Invivogen, USA) as stimuli inducing the classical activation of M1 monocytes or 20ng/ml porcine rIL-4 (Active Bioscience, Germany), 20ng/ml porcine rIL-13 (Kingfisher Biotech, USA ) and 0.1ng/ml LPS (Invivogen, USA) to induce alternatively activated M2 monocytes for 4, 16 or 48 hrs before cell harvest. The effects of $EcN^{SN}$, EcN-$AvCys^{SN}$ or 0.5 µM rAvCys on the phenotype of $CD14^+$ monocytes was then

also determined with incubation periods of 4, 16 and 48hrs exposure to the various treatments. RNA extraction, cDNA synthesis and RT-PCR analysis was performed as described above using the primer sets summarized in **Table 8.2.** The ΔΔCT method was used to determine the fold increase of these cytokines in monocytes exposed to stimuli at the various time points compared to monocytes incubated in IMDM at the equivalent time point using CT values normalized to a house keeping gene (RPL19).

### 8.11.2. Inflammasome induction in human monocytes and macrophages

Human blood monocytes were extracted and differentiated into macrophages from buffy coats using a density gradient medium, Lymphoprep (Stemcell Technologies, France) as described in section 8.11.1. Blood derived human monocytes were differentiated into macrophages with 10ng/ml of M-CSF for 7 days. Freshly extracted blood monocytes and blood derived monocyte differentiated macrophages were incubated with 20ng/ml K12 LPS (Invivogen, USA) in complete IMDM, $EcN^{SN}$, or $EcN\text{-}AvCys^{SN}$ for 4 hrs before the addition of either 5% DSS (MP Biomedicals, France) for 24 hrs or 5mM ATP (Invivogen, USA) for 30 min. Cells were then centrifuged and supernatants collected for analysis with human IL-1β specific ELISA (ebioscience, USA).

## 8.12. Statistics

Groups were compared with one-way ANOVA, two-way ANOVA or Mann Whitney U test using GraphPad Prism software (San Diego, USA). Data are reported as means + SEM and differences were deemed significant if $p<0.05$.

## 9. References

Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F (2007) Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. Nat Immunol 8 (9):942-949

Adisakwattana P, Nuamtanong S, Kusolsuk T, Chairoj M, Yenchitsomanas PT, Chaisri U (2013) Non-encapsulated Trichinella spp., T. papuae, diminishes severity of DSS-induced colitis in mice. Asian Pac J Allergy Immunol 31 (2):106-114

Aguilera L, Toloza L, Gimenez R, Odena A, Oliveira E, Aguilar J et al. (2014) Proteomic analysis of outer membrane vesicles from the probiotic strain Escherichia coli Nissle 1917. Proteomics 14 (2-3):222-229

Al-Sadi R, Guo S, Ye D, Dokladny K, Alhmoud T, Ereifej L et al. (2013) Mechanism of IL-1beta modulation of intestinal epithelial barrier involves p38 kinase and activating transcription factor-2 activation. J Immunol 190 (12):6596-6606

Altenhoefer A, Oswald S, Sonnenborn U, Enders C, Schulze J, Hacker J et al. (2004) The probiotic Escherichia coli strain Nissle 1917 interferes with invasion of human intestinal epithelial cells by different enteroinvasive bacterial pathogens. FEMS Immunol Med Microbiol 40 (3):223-229

Anderson CA, Boucher G, Lees CW, Franke A, D'Amato M, Taylor KD et al. (2011) Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. Nat Genet 43 (3):246-252

Anthony RM, Urban JF, Jr., Alem F, Hamed HA, Rozo CT, Boucher JL et al. (2006) Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. Nat Med 12 (8):955-960

Antoni L, Nuding S, Wehkamp J, Stange EF (2014) Intestinal barrier in inflammatory bowel disease. World J Gastroenterol 20 (5):1165-1179

Arribas B, Rodriguez-Cabezas ME, Camuesco D, Comalada M, Bailon E, Utrilla P et al. (2009) A probiotic strain of Escherichia coli, Nissle 1917, given orally exerts local and systemic anti-inflammatory effects in lipopolysaccharide-induced sepsis in mice. Br J Pharmacol 157 (6):1024-1033

Ashour DS, Othman AA, Shareef MM, Gaballah HH, Mayah WW (2013) Interactions between Trichinella spiralis infection and induced colitis in mice. J Helminthol 88 (2):210-218

Attout T, Hoerauf A, Denece G, Debrah AY, Marfo-Debrekyei Y, Boussinesq M et al. (2009) Lymphatic vascularisation and involvement of Lyve-1+ macrophages in the human onchocerca nodule. PLoS One 4 (12):e8234

Babu S, Blauvelt CP, Kumaraswami V, Nutman TB (2006) Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. J Immunol 176 (5):3248-3256

Babu S, Kumaraswami V, Nutman TB (2009) Alternatively activated and immunoregulatory monocytes in human filarial infections. J Infect Dis 199 (12):1827-1837

Badia R, Zanello G, Chevaleyre C, Lizardo R, Meurens F, Martinez P et al. (2012) Effect of Saccharomyces cerevisiae var. Boulardii and beta-galactomannan oligosaccharide on porcine intestinal epithelial and dendritic cells challenged in vitro with Escherichia coli F4 (K88). Vet Res 43:4-14

Bager P, Kapel C, Roepstorff A, Thamsborg S, Arnved J, Ronborg S et al. (2011) Symptoms after Ingestion of Pig Whipworm Trichuris suis Eggs in a Randomized Placebo-Controlled Double-Blind Clinical Trial. PLoS One 6 (8):e22346

Bain CC, Mowat AM (2014) The monocyte-macrophage axis in the intestine. Cell Immunol Epub ahead of print 10.1016/j.cellimm.2014.03.012

Bain CC, Scott CL, Uronen-Hansson H, Gudjonsson S, Jansson O, Grip O et al. (2013) Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. Mucosal Immunol 6 (3):498-510

Balic A, Smith KA, Harcus Y, Maizels RM (2009) Dynamics of CD11c(+) dendritic cell subsets in lymph nodes draining the site of intestinal nematode infection. Immunol Lett 127 (1):68-75

Banks C, Bateman A, Payne R, Johnson P, Sheron N (2003) Chemokine expression in IBD. Mucosal chemokine expression is unselectively increased in both ulcerative colitis and Crohn's disease. J Pathol 199 (1):28-35

Bar-On L, Zigmond E, Jung S (2011) Management of gut inflammation through the manipulation of intestinal dendritic cells and macrophages? Semin Immunol 23 (1):58-64

Barth S, Duncker S, Hempe J, Breves G, Baljer G, Bauerfeind R (2009) Escherichia coli Nissle 1917 for probiotic use in piglets: evidence for intestinal colonization. J Appl Microbiol 107 (5):1697-1710

Barton MD (2014) Impact of antibiotic use in the swine industry. Curr Opin Microbiol 19C:9-15

Bassaganya-Riera J, Hontecillas R (2006) CLA and n-3 PUFA differentially modulate clinical activity and colonic PPAR-responsive gene expression in a pig model of experimental IBD. Clin Nutr 25 (3):454-465

Bauer C, Duewell P, Mayer C, Lehr HA, Fitzgerald KA, Dauer M et al. (2010) Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. Gut 59 (9):1192-1199

Bautista EM, Nfon C, Ferman GS, Golde WT (2007) IL-13 replaces IL-4 in development of monocyte derived dendritic cells (MoDC) of swine. Vet Immunol Immunopathol 115 (1-2):56-67

Becker HM, Apladas A, Scharl M, Fried M, Rogler G (2014) Probiotic Escherichia coli Nissle 1917 and Commensal E. coli K12 Differentially Affect the Inflammasome in Intestinal Epithelial Cells. Digestion 89 (2):110-118

Becker S, Oelschlaeger TA, Wullaert A, Vlantis K, Pasparakis M, Wehkamp J et al. (2013) Bacteria regulate intestinal epithelial cell differentiation factors both in vitro and in vivo. PLoS One 8 (2):e55620

Benchimol EI, Seow CH, Steinhart AH, Griffiths AM (2008) Traditional corticosteroids for induction of remission in Crohn's disease. Cochrane Database Syst Rev Epub ahead of print 10.1002/14651858.CD006792.pub2 (2):CD006792

Berg DJ, Zhang J, Weinstock JV, Ismail HF, Earle KA, Alila H et al. (2002) Rapid development of colitis in NSAID-treated IL-10-deficient mice. Gastroenterology 123 (5):1527-1542

Blum-Oehler G, Oswald S, Eiteljorge K, Sonnenborn U, Schulze J, Kruis W et al. (2003) Development of strain-specific PCR reactions for the detection of the probiotic Escherichia coli strain Nissle 1917 in fecal samples. Res Microbiol 154 (1):59-66

Blum AM, Hang L, Setiawan T, Urban JP, Jr., Stoyanoff KM, Leung J et al. (2012) Heligmosomoides polygyrus bakeri induces tolerogenic dendritic cells that block colitis and prevent antigen-specific gut T cell responses. J Immunol 189 (5):2512-2520

Blum AM, Metwali A, Elliott DE, Berg DJ, Weinstock JV (2004) CD4+ T cells from IL-10-deficient mice transfer susceptibility to NSAID-induced Rag colitis. Am J Physiol Gastrointest Liver Physiol 287 (2):G320-325

Blumberg RS, Saubermann LJ, Strober W (1999) Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. Curr Opin Immunol 11 (6):648-656

Boudeau J, Glasser AL, Julien S, Colombel JF, Darfeuille-Michaud A (2003) Inhibitory effect of probiotic Escherichia coli strain Nissle 1917 on adhesion to and invasion of intestinal epithelial cells by adherent-invasive E. coli strains isolated from patients with Crohn's disease. Aliment Pharmacol Ther 18 (1):45-56

Boudry G, Peron V, Le Huerou-Luron I, Lalles JP, Seve B (2004) Weaning induces both transient and long-lasting modifications of absorptive, secretory, and barrier properties of piglet intestine. J Nutr 134 (9):2256-2262

Broadhurst MJ, Leung JM, Kashyap V, McCune JM, Mahadevan U, McKerrow JH et al. (2010) IL-22+ CD4+ T cells are associated with therapeutic trichuris trichiura infection in an ulcerative colitis patient. Sci Transl Med 2 (60):60ra88

Buhner S, Buning C, Genschel J, Kling K, Herrmann D, Dignass A et al. (2006) Genetic basis for increased intestinal permeability in families with Crohn's disease: role of CARD15 3020insC mutation? Gut 55 (3):342-347

Bures J, Smajs D, Kvetina J, Forstl M, Smarda J, Kohoutova D et al. (2011) Bacteriocinogeny in experimental pigs treated with indomethacin and Escherichia coli Nissle. World J Gastroenterol 17 (5):609-617

Burks AW, Laubach S, Jones SM (2008) Oral tolerance, food allergy, and immunotherapy: implications for future treatment. J Allergy Clin Immunol 121 (6):1344-1350

Cancado GG, Fiuza JA, de Paiva NC, Lemos LD, Ricci ND, Gazzinelli-Guimaraes PH et al. (2011) Hookworm products ameliorate dextran sodium sulfate-induced colitis in BALB/c mice. Inflamm Bowel Dis 17 (2):2275-2286

Chaudhry A, Samstein RM, Treuting P, Liang Y, Pils MC, Heinrich JM et al. (2011) Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity 34 (4):566-578

Chen CC, Louie S, McCormick B, Walker WA, Shi HN (2005) Concurrent infection with an intestinal helminth parasite impairs host resistance to enteric Citrobacter rodentium and enhances Citrobacter-induced colitis in mice. Infect Immun 73 (9):5468-5481

Chen CC, Louie S, McCormick BA, Walker WA, Shi HN (2006) Helminth-primed dendritic cells alter the host response to enteric bacterial infection. J Immunol 176 (1):472-483

Chen F, Liu Z, Wu W, Rozo C, Bowdridge S, Millman A et al. (2012) An essential role for TH2-type responses in limiting acute tissue damage during experimental helminth infection. Nat Med 18 (2):260-266

Cho MK, Lee CH, Yu HS (2011) Amelioration of intestinal colitis by macrophage migration inhibitory factor isolated from intestinal parasites through toll-like receptor 2. Parasite Immunol 33 (5):265-275

Choi MK, Le MT, Nguyen DT, Choi H, Kim W, Kim JH et al. (2012) Genome-level identification, gene expression, and comparative analysis of porcine ss-defensin genes. BMC Genet 13:98

Christoffersen TE, Hult LT, Kuczkowska K, Moe KM, Skeie S, Lea T et al. (2014) In vitro comparison of the effects of probiotic, commensal and pathogenic strains on macrophage polarization. Probiotics Antimicrob Proteins 6 (1):1-10

Clambey ET, McNamee EN, Westrich JA, Glover LE, Campbell EL, Jedlicka P et al. (2012) Hypoxia-inducible factor-1 alpha-dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa. Proc Natl Acad Sci U S A 109 (41):E2784-2793

Cooper HS, Murthy SN, Shah RS, Sedergran DJ (1993) Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest 69 (2):238-249

Cress BF, Linhardt RJ, Koffas MA (2013) Draft Genome Sequence of Escherichia coli Strain Nissle 1917 (Serovar O6:K5:H1). Genome Announc 1 (2):e00047-00013

Croese J, O'Neil J, Masson J, Cooke S, Melrose W, Pritchard D et al. (2006) A proof of concept study establishing Necator americanus in Crohn's patients and reservoir donors. Gut 55 (1):136-137

Cross ML, Ganner A, Teilab D, Fray LM (2004) Patterns of cytokine induction by gram-positive and gram-negative probiotic bacteria. FEMS Immunol Med Microbiol 42 (2):173-180

Croswell A, Amir E, Teggatz P, Barman M, Salzman NH (2009) Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric Salmonella infection. Infect Immun 77 (7):2741-2753

Cruickshank SM, Deschoolmeester ML, Svensson M, Howell G, Bazakou A, Logunova L et al. (2009) Rapid dendritic cell mobilization to the large intestinal epithelium is associated with resistance to Trichuris muris infection. J Immunol 182 (5):3055-3062

Cukrowska B, LodInova-ZadnIkova R, Enders C, Sonnenborn U, Schulze J, Tlaskalova-Hogenova H (2002) Specific proliferative and antibody responses of premature infants to intestinal colonization with nonpathogenic probiotic E. coli strain Nissle 1917. Scand J Immunol 55 (2):204-209

D'Elia R, Behnke JM, Bradley JE, Else KJ (2009) Regulatory T cells: a role in the control of helminth-driven intestinal pathology and worm survival. J Immunol 182 (4):2340-2348

Danilowicz-Luebert E, Steinfelder S, Kuhl AA, Drozdenko G, Lucius R, Worm M et al. (2013) A nematode immunomodulator suppresses grass pollen-specific allergic responses by controlling excessive Th2 inflammation. Int J Parasitol 43 (3-4):201-210

Daveson AJ, Jones DM, Gaze S, McSorley H, Clouston A, Pascoe A et al. (2011) Effect of hookworm infection on wheat challenge in celiac disease--a randomised double-blinded placebo controlled trial. PLoS One 6 (3):e17366

De Fazio L, Cavazza E, Spisni E, Strillacci A, Centanni M, Candela M et al. (2014) Longitudinal analysis of inflammation and microbiota dynamics in a model of mild chronic dextran sulfate sodium-induced colitis in mice. World J Gastroenterol 20 (8):2051-2061

De Iudicibus S, Lucafo M, Martelossi S, Pierobon C, Ventura A, Decorti G (2013) MicroRNAs as tools to predict glucocorticoid response in inflammatory bowel diseases. World J Gastroenterol 19 (44):7947-7954

Deriu E, Liu JZ, Pezeshki M, Edwards RA, Ochoa RJ, Contreras H et al. (2013) Probiotic bacteria reduce salmonella typhimurium intestinal colonization by competing for iron. Cell Host Microbe 14 (1):26-37

Devos Y, Aguilera J, Diveki Z, Gomes A, Liu Y, Paoletti C et al. (2014) EFSA's scientific activities and achievements on the risk assessment of genetically modified organisms (GMOs) during its first decade of existence: looking back and ahead. Transgenic Res 23 (1):1-25

Dewey CE, Johnston WT, Gould L, Whiting TL (2006) Postweaning mortality in Manitoba swine. Can J Vet Res 70 (3):161-167

Dieleman LA, Ridwan BU, Tennyson GS, Beagley KW, Bucy RP, Elson CO (1994) Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. Gastroenterology 107 (6):1643-1652

Doetze A, Satoguina J, Burchard G, Rau T, Loliger C, Fleischer B et al. (2000) Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T(h)3/T(r)1-type cytokines IL-10 and transforming growth factor-beta but not by a T(h)1 to T(h)2 shift. Int Immunol 12 (5):623-630

Donskow-Lysoniewska K, Majewski P, Brodaczewska K, Jozwicka K, Doligalska M (2012) Heligmosmoides polygyrus fourth stages induce protection against DSS-induced colitis and change opioid expression in the intestine. Parasite Immunol 34 (11):536-546

Du L, Tang H, Ma Z, Xu J, Gao W, Chen J et al. (2011) The Protective Effect of the Recombinant 53-kDa Protein of Trichinella spiralis on Experimental Colitis in Mice. Dig Dis Sci 56 (10):2810-2817

Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ et al. (2006) A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science 314 (5804):1461-1463

Duncker SC, Lorentz A, Schroeder B, Breves G, Bischoff SC (2006) Effect of orally administered probiotic E. coli strain Nissle 1917 on intestinal mucosal immune cells of healthy young pigs. Vet Immunol Immunopathol 111 (3-4):239-250

Elliott DE, Metwali A, Leung J, Setiawan T, Blum AM, Ince MN et al. (2008) Colonization with Heligmosomoides polygyrus suppresses mucosal IL-17 production. J Immunol 181 (4):2414-2419

Elliott DE, Setiawan T, Metwali A, Blum A, Urban JF, Jr., Weinstock JV (2004) Heligmosomoides polygyrus inhibits established colitis in IL-10-deficient mice. Eur J Immunol 34 (10):2690-2698

Elliott DE, Urban JJ, Argo CK, Weinstock JV (2000) Does the failure to acquire helminthic parasites predispose to Crohn's disease? Faseb J 14 (12):1848-1855

Fahlen L, Read S, Gorelik L, Hurst SD, Coffman RL, Flavell RA et al. (2005) T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. J Exp Med 201 (5):737-746

Fairbairn L, Kapetanovic R, Sester DP, Hume DA (2011) The mononuclear phagocyte system of the pig as a model for understanding human innate immunity and disease. J Leukoc Biol 89 (6):855-871

Fallon PG, Ballantyne SJ, Mangan NE, Barlow JL, Dasvarma A, Hewett DR et al. (2006) Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. J Exp Med 203 (4):1105-1116

Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF (2004) Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. J Immunol 172 (9):5149-5153

Farid HA, Morsy ZS, Helmy H, Ramzy RM, El Setouhy M, Weil GJ (2007) A critical appraisal of molecular xenomonitoring as a tool for assessing progress toward elimination of Lymphatic Filariasis. Am J Trop Med Hyg 77 (4):593-600

Fernandez-Blanco JA, Barbosa S, Sanchez de Medina F, Martinez V, Vergara P (2011) Persistent epithelial barrier alterations in a rat model of postinfectious gut dysfunction. Neurogastroenterol Motil 23 (11):e523-533

Ferreira I, Smyth D, Gaze S, Aziz A, Giacomin P, Ruyssers N et al. (2013) Hookworm excretory/secretory products induce interleukin-4 (IL-4)+ IL-10+ CD4+ T cell responses and suppress pathology in a mouse model of colitis. Infect Immun 81 (6):2104-2111

Figueiredo AS, Hofer T, Klotz C, Sers C, Hartmann S, Lucius R et al. (2009) Modelling and simulating interleukin-10 production and regulation by macrophages after stimulation with an immunomodulator of parasitic nematodes. Febs J 276 (13):3454-3469

Filbey KJ, Grainger JR, Smith KA, Boon L, van Rooijen N, Harcus Y et al. (2014) Innate and adaptive type 2 immune cell responses in genetically controlled resistance to intestinal helminth infection. Immunol Cell Biol Epub ahead of print 10.1038/icb.2013.109

Fina D, Sarra M, Fantini MC, Rizzo A, Caruso R, Caprioli F et al. (2008) Regulation of gut inflammation and th17 cell response by interleukin-21. Gastroenterology 134 (4):1038-1048

Finney CA, Taylor MD, Wilson MS, Maizels RM (2007) Expansion and activation of CD4(+)CD25(+) regulatory T cells in Heligmosomoides polygyrus infection. Eur J Immunol 37 (7):1874-1886

Fleetwood AJ, Lawrence T, Hamilton JA, Cook AD (2007) Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. J Immunol 178 (8):5245-5252

Franchi L, Eigenbrod T, Nunez G (2009) Cutting edge: TNF-alpha mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. J Immunol 183 (2):792-796

Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR (2007) Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A 104 (34):13780-13785

Fujiwara RT, Cancado GG, Freitas PA, Santiago HC, Massara CL, Dos Santos Carvalho O et al. (2009) Necator americanus infection: a possible cause of altered dendritic cell differentiation and eosinophil profile in chronically infected individuals. PLoS Negl Trop Dis 3 (3):e399

Gad M, Ravn P, Soborg DA, Lund-Jensen K, Ouwehand AC, Jensen SS (2011) Regulation of the IL-10/IL-12 axis in human dendritic cells with probiotic bacteria. FEMS Immunol Med Microbiol 63 (1):93-107

Garlanda C, Riva F, Bonavita E, Mantovani A (2013) Negative regulatory receptors of the IL-1 family. Semin Immunol 25 (6):408-415

Garrido-Mesa N, Utrilla P, Comalada M, Zorrilla P, Garrido-Mesa J, Zarzuelo A et al. (2011) The association of minocycline and the probiotic Escherichia coli Nissle 1917 results in an additive beneficial effect in a DSS model of reactivated colitis in mice. Biochem Pharmacol 82 (12):1891-1900

Ghia JE, Galeazzi F, Ford DC, Hogaboam CM, Vallance BA, Collins S (2008) Role of M-CSF-dependent macrophages in colitis is driven by the nature of the inflammatory stimulus. Am J Physiol Gastrointest Liver Physiol 294 (3):G770-777

Gisbert JP, Chaparro M, Gomollon F (2011) Common misconceptions about 5-aminosalicylates and thiopurines in inflammatory bowel disease. World J Gastroenterol 17 (30):3467-3478

Gleason PP, Alexander GC, Starner CI, Ritter ST, Van Houten HK, Gunderson BW et al. (2013) Health plan utilization and costs of specialty drugs within 4 chronic conditions. J Manag Care Pharm 19 (7):542-548

Goel A, Mittal A, Evstatiev R, Nemeth M, Kruis W, Stolte M et al. (2009) In vivo effects of mesalazine or E. coli Nissle 1917 on microsatellite instability in ulcerative colitis. Aliment Pharmacol Ther 30 (6):634-642

Gorelik L, Flavell RA (2000) Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity 12 (2):171-181

Grainger JR, Smith KA, Hewitson JP, McSorley HJ, Harcus Y, Filbey KJ et al. (2010) Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-beta pathway. J Exp Med 207 (11):2331-2341

Gratz IK, Campbell DJ (2014) Organ-specific and memory treg cells: specificity, development, function, and maintenance. Front Immunol 5:333

Guilloteau P, Zabielski R, Hammon HM, Metges CC (2010) Nutritional programming of gastrointestinal tract development. Is the pig a good model for man? Nutr Res Rev 23 (1):4-22

Guo Y, Lu N, Bai A (2013) Clinical use and mechanisms of infliximab treatment on inflammatory bowel disease: a recent update. Biomed Res Int Epub ahead of print 10.1155/2013/581631

Guttsches AK, Loseke S, Zahringer U, Sonnenborn U, Enders C, Gatermann S et al. (2012) Anti-inflammatory modulation of immune response by probiotic Escherichia coli Nissle 1917 in human blood mononuclear cells. Innate Immun 18 (2):204-216

Hafez M, Hayes K, Goldrick M, Warhurst G, Grencis R, Roberts IS (2009) The K5 capsule of Escherichia coli strain Nissle 1917 is important in mediating interactions with intestinal epithelial cells and chemokine induction. Infect Immun 77 (7):2995-3003

Hakansson A, Tormo-Badia N, Baridi A, Xu J, Molin G, Hagslatt ML et al. (2014) Immunological alteration and changes of gut microbiota after dextran sulfate sodium (DSS) administration in mice. Clin Exp Med Epub ahead of print 10.1007/s10238-013-0270-5

Halfvarson J, Jess T, Magnuson A, Montgomery SM, Orholm M, Tysk C et al. (2006) Environmental factors in inflammatory bowel disease: a co-twin control study of a Swedish-Danish twin population. Inflamm Bowel Dis 12 (10):925-933

Hall LJ, Faivre E, Quinlan A, Shanahan F, Nally K, Melgar S (2011) Induction and activation of adaptive immune populations during acute and chronic phases of a murine model of experimental colitis. Dig Dis Sci 56 (1):79-89

Hancock V, Dahl M, Klemm P (2010) Probiotic Escherichia coli strain Nissle 1917 outcompetes intestinal pathogens during biofilm formation. J Med Microbiol 59 (4):392-399

Hang L, Setiawan T, Blum AM, Urban J, Stoyanoff K, Arihiro S et al. (2010) Heligmosomoides polygyrus infection can inhibit colitis through direct interaction with innate immunity. J Immunol 185 (6):3184-3189

Harding SV, Adegoke OA, Fraser KG, Marliss EB, Chevalier S, Kimball SR et al. (2010) Maintaining adequate nutrition, not probiotic administration, prevents growth stunting and maintains skeletal muscle protein synthesis rates in a piglet model of colitis. Pediatr Res 67 (3):268-273

Harding SV, Fraser KG, Wykes LJ (2008) Probiotics stimulate liver and plasma protein synthesis in piglets with dextran sulfate-induced colitis and macronutrient restriction. J Nutr 138 (11):2129-2135

Hartmann S, Kyewski B, Sonnenburg B, Lucius R (1997) A filarial cysteine protease inhibitor down-regulates T cell proliferation and enhances interleukin-10 production. Eur J Immunol 27 (9):2253-2260

Hartmann S, Schnoeller C, Dahten A, Avagyan A, Rausch S, Lendner M et al. (2009) Gastrointestinal nematode infection interferes with experimental allergic airway inflammation but not atopic dermatitis. Clin Exp Allergy 39 (10):1585-1596

Hasnain SZ, Thornton DJ, Grencis RK (2011) Changes in the mucosal barrier during acute and chronic Trichuris muris infection. Parasite Immunol 33 (1):45-55

He L, Marneros AG (2013) Macrophages are essential for the early wound healing response and the formation of a fibrovascular scar. Am J Pathol 182 (6):2407-2417

Heinritz SN, Mosenthin R, Weiss E (2013) Use of pigs as a potential model for research into dietary modulation of the human gut microbiota. Nutr Res Rev 26 (2):191-209

Heinsbroek SE, Gordon S (2009) The role of macrophages in inflammatory bowel diseases. Expert Rev Mol Med 11:e14

Heller F, Florian P, Bojarski C, Richter J, Christ M, Hillenbrand B et al. (2005) Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. Gastroenterology 129 (2):550-564

Henker J, Laass M, Blokhin BM, Bolbot YK, Maydannik VG, Elze M et al. (2007) The probiotic Escherichia coli strain Nissle 1917 (EcN) stops acute diarrhoea in infants and toddlers. Eur J Pediatr 166 (4):311-318

Henker J, Laass MW, Blokhin BM, Maydannik VG, Bolbot YK, Elze M et al. (2008a) Probiotic Escherichia coli Nissle 1917 versus placebo for treating diarrhea of greater than 4 days duration in infants and toddlers. Pediatr Infect Dis J 27 (6):494-499

Henker J, Muller S, Laass MW, Schreiner A, Schulze J (2008b) Probiotic Escherichia coli Nissle 1917 (EcN) for successful remission maintenance of ulcerative colitis in children and adolescents: an open-label pilot study. Z Gastroenterol 46 (9):874-875

Hepworth MR, Danilowicz-Luebert E, Rausch S, Metz M, Klotz C, Maurer M et al. (2012) Mast cells orchestrate type 2 immunity to helminths through regulation of tissue-derived cytokines. Proc Natl Acad Sci U S A 109 (17):6644-6649

Hering NA, Richter JF, Fromm A, Wieser A, Hartmann S, Gunzel D et al. (2013) TcpC protein from E. coli Nissle improves epithelial barrier function involving PKCzeta and ERK1/2 signaling in HT-29/B6 cells. Mucosal Immunol 7:369-378

Hiemstra IH, Klaver EJ, Vrijland K, Kringel H, Andreasen A, Bouma G et al. (2014) Excreted/secreted Trichuris suis products reduce barrier function and suppress inflammatory cytokine production of intestinal epithelial cells. Mol Immunol 60 (1):1-7

Higashiyama M, Hokari R, Hozumi H, Kurihara C, Ueda T, Watanabe C et al. (2012) HIF-1 in T cells ameliorated dextran sodium sulfate-induced murine colitis. J Leukoc Biol 91 (6):901-909

Higazi TB, Pearlman E, Whikehart DR, Unnasch TR (2003) Angiogenic activity of an Onchocerca volvulus Ancylostoma secreted protein homologue. Mol Biochem Parasitol 129 (1):61-68

Hochrein H, Wagner H (2004) Of men, mice and pigs: looking at their plasmacytoid dendritic cells [corrected]. Immunology 112 (1):26-27

Hold GL, Smith M, Grange C, Watt ER, El-Omar EM, Mukhopadhya I (2014) Role of the gut microbiota in inflammatory bowel disease pathogenesis: What have we learnt in the past 10 years? World J Gastroenterol 20 (5):1192-1210

Holmgren J, Czerkinsky C (2005) Mucosal immunity and vaccines. Nat Med 11 (4 Suppl):S45-53

Hornung V, Latz E (2010) Critical functions of priming and lysosomal damage for NLRP3 activation. Eur J Immunol 40 (3):620-623

Hou JK, Abraham B, El-Serag H (2011) Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature. Am J Gastroenterol 106 (4):563-573

Hu C, Song J, Li Y, Luan Z, Zhu K (2013) Diosmectite-zinc oxide composite improves intestinal barrier function, modulates expression of pro-inflammatory cytokines and tight junction protein in early weaned pigs. Br J Nutr 110 (4):681-688

Huang Y, Gauvreau H, Harding J (2012) Diagnostic investigation of porcine periweaning failure-to-thrive syndrome: lack of compelling evidence linking to common porcine pathogens. J Vet Diagn Invest 24 (1):96-106

Huber S, Gagliani N, Esplugues E, O'Connor W, Jr., Huber FJ, Chaudhry A et al. (2011) Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. Immunity 34 (4):554-565

Huber S, Hoffmann R, Muskens F, Voehringer D (2010) Alternatively activated macrophages inhibit T-cell proliferation by Stat6-dependent expression of PD-L2. Blood 116 (17):3311-3320

Hudcovic T, Stepankova R, Cebra J, Tlaskalova-Hogenova H (2001) The role of microflora in the development of intestinal inflammation: acute and chronic colitis induced by dextran sulfate in germ-free and conventionally reared immunocompetent and immunodeficient mice. Folia Microbiol (Praha) 46 (6):565-572

Huebner C, Ding Y, Petermann I, Knapp C, Ferguson LR (2011) The probiotic Escherichia coli Nissle 1917 reduces pathogen invasion and modulates cytokine expression in Caco-2 cells infected with Crohn's disease-associated E. coli LF82. Appl Environ Microbiol 77 (7):2541-2544

Huehn J, Hamann A (2005) Homing to suppress: address codes for Treg migration. Trends Immunol 26 (12):632-636

Humphreys NE, Xu D, Hepworth MR, Liew FY, Grencis RK (2008) IL-33, a potent inducer of adaptive immunity to intestinal nematodes. J Immunol 180 (4):2443-2449

Hunter MM, Wang A, Parhar KS, Johnston MJ, Van Rooijen N, Beck PL et al. (2010) In vitro-derived alternatively activated macrophages reduce colonic inflammation in mice. Gastroenterology 138 (4):1395-1405

Ince MN, Elliott DE, Setiawan T, Metwali A, Blum A, Chen HL et al. (2009) Role of T cell TGF-beta signaling in intestinal cytokine responses and helminthic immune modulation. Eur J Immunol 39 (7):1870-1878

Ito R, Kita M, Shin-Ya M, Kishida T, Urano A, Takada R et al. (2008) Involvement of IL-17A in the pathogenesis of DSS-induced colitis in mice. Biochem Biophys Res Commun 377 (1):12-16

Izcue A, Hue S, Buonocore S, Arancibia-Carcamo CV, Ahern PP, Iwakura Y et al. (2008) Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. Immunity 28 (4):559-570

Jacobi CA, Malfertheiner P (2011) Escherichia coli Nissle 1917 (Mutaflor): new insights into an old probiotic bacterium. Dig Dis 29 (6):600-607

Jaguin M, Houlbert N, Fardel O, Lecureur V (2013) Polarization profiles of human M-CSF-generated macrophages and comparison of M1-markers in classically activated macrophages from GM-CSF and M-CSF origin. Cell Immunol 281 (1):51-61

Jeong KI, Zhang Q, Nunnari J, Tzipori S (2010) A piglet model of acute gastroenteritis induced by Shigella dysenteriae Type 1. J Infect Dis 201 (6):903-911

Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY et al. (2012) Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 491 (7422):119-124

Kaiser P, Hardt WD (2011) Salmonella typhimurium diarrhea: switching the mucosal epithelium from homeostasis to defense. Curr Opin Immunol 23 (4):456-463

Kapetanovic R, Fairbairn L, Beraldi D, Sester DP, Archibald AL, Tuggle CK et al. (2012) Pig bone marrow-derived macrophages resemble human macrophages in their response to bacterial lipopolysaccharide. J Immunol 188 (7):3382-3394

Kaser T, Mullebner A, Hartl RT, Essler SE, Saalmuller A, Catharina Duvigneau J (2012) Porcine T-helper and regulatory T cells exhibit versatile mRNA expression capabilities for cytokines and co-stimulatory molecules. Cytokine 60 (2):400-409

Khan WI, Blennerhasset PA, Varghese AK, Chowdhury SK, Omsted P, Deng Y et al. (2002) Intestinal nematode infection ameliorates experimental colitis in mice. Infect Immun 70 (11):5931-5937

Khor B, Gardet A, Xavier RJ (2011) Genetics and pathogenesis of inflammatory bowel disease. Nature 474 (7351):307-317

Khoruts A, Osness RE, Jenkins MK (2004) IL-1 acts on antigen-presenting cells to enhance the in vivo proliferation of antigen-stimulated naive CD4 T cells via a CD28-dependent mechanism that does not involve increased expression of CD28 ligands. Eur J Immunol 34 (4):1085-1090

Kimman TG, Smit E, Klein MR (2008) Evidence-based biosafety: a review of the principles and effectiveness of microbiological containment measures. Clin Microbiol Rev 21 (3):403-425

Kitagaki K, Businga TR, Racila D, Elliott DE, Weinstock JV, Kline JN (2006) Intestinal helminths protect in a murine model of asthma. J Immunol 177 (3):1628-1635

Kitajima S, Morimoto M, Sagara E, Shimizu C, Ikeda Y (2001) Dextran sodium sulfate-induced colitis in germ-free IQI/Jic mice. Exp Anim 50 (5):387-395

Klaver EJ, Kuijk LM, Laan LC, Kringel H, van Vliet SJ, Bouma G et al. (2013) Trichuris suis-induced modulation of human dendritic cell function is glycan-mediated. Int J Parasitol 43 (3-4):191-200

Kleta S, Steinruck H, Breves G, Duncker S, Laturnus C, Wieler LH et al. (2006) Detection and distribution of probiotic Escherichia coli Nissle 1917 clones in swine herds in Germany. J Appl Microbiol 101 (6):1357-1366

Klotz C, Ziegler T, Danilowicz-Luebert E, Hartmann S (2011a) Cystatins of parasitic organisms. Adv Exp Med Biol 712:208-221

Klotz C, Ziegler T, Figueiredo AS, Rausch S, Hepworth MR, Obsivac N et al. (2011b) A helminth immunomodulator exploits host signaling events to regulate cytokine production in macrophages. PLoS Pathog 7 (1):e1001248

Kocher J, Bui T, Giri-Rachman E, Wen K, Li G, Yang X et al. (2014) Intranasal P particle vaccine provided partial cross-variant protection against human GII.4 norovirus diarrhea in gnotobiotic pigs. J Virol Epub ahead of print 10.1128/JVI.01249-14

Korn T, Bettelli E, Oukka M, Kuchroo VK (2009) IL-17 and Th17 Cells. Annu Rev Immunol 27:485-517

Kreider T, Anthony RM, Urban JF, Jr., Gause WC (2007) Alternatively activated macrophages in helminth infections. Curr Opin Immunol 19 (4):448-453

Kron MA, Metwali A, Vodanovic-Jankovic S, Elliott D (2013) Nematode asparaginyl-tRNA synthetase resolves intestinal inflammation in mice with T-cell transfer colitis. Clin Vaccine Immunol 20 (2):276-281

Kruis W, Chrubasik S, Boehm S, Stange C, Schulze J (2012) A double-blind placebo-controlled trial to study therapeutic effects of probiotic Escherichia coli Nissle 1917 in subgroups of patients with irritable bowel syndrome. Int J Colorectal Dis 27 (4):467-474

Kruis W, Fric P, Pokrotnieks J, Lukas M, Fixa B, Kascak M et al. (2004) Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine. Gut 53 (11):1617-1623

Kruis W, Schutz E, Fric P, Fixa B, Judmaier G, Stolte M (1997) Double-blind comparison of an oral Escherichia coli preparation and mesalazine in maintaining remission of ulcerative colitis. Aliment Pharmacol Ther 11 (5):853-858

Kyrova K, Stepanova H, Rychlik I, Faldyna M, Volf J (2012) SPI-1 encoded genes of Salmonella Typhimurium influence differential polarization of porcine alveolar macrophages in vitro. BMC Vet Res 8:115

Lalles JP, Bosi P, Smidt H, Stokes CR (2007) Nutritional management of gut health in pigs around weaning. Proc Nutr Soc 66 (2):260-268

Laroui H, Ingersoll SA, Liu HC, Baker MT, Ayyadurai S, Charania MA et al. (2012) Dextran sodium sulfate (DSS) induces colitis in mice by forming nano-lipocomplexes with medium-chain-length fatty acids in the colon. PLoS One 7 (3):e32084

Leatham MP, Banerjee S, Autieri SM, Mercado-Lubo R, Conway T, Cohen PS (2009) Precolonized human commensal Escherichia coli strains serve as a barrier to E. coli O157:H7 growth in the streptomycin-treated mouse intestine. Infect Immun 77 (7):2876-2886

Lechner MG, Liebertz DJ, Epstein AL (2010) Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. J Immunol 185 (4):2273-2284

Lee DH, Kim JI, Lee MS, Choi TY, Choi SM, Ernst E (2010) Moxibustion for ulcerative colitis: a systematic review and meta-analysis. BMC Gastroenterol 10:36-42

Leung J, Hang L, Blum A, Setiawan T, Stoyanoff K, Weinstock J (2012) Heligmosomoides polygyrus abrogates antigen-specific gut injury in a murine model of inflammatory bowel disease. Inflamm Bowel Dis 18 (8):1447-1455

Li Z, Liu G, Chen Y, Liu Y, Liu B, Su Z (2011) The phenotype and function of naturally existing regulatory dendritic cells in nematode-infected mice. Int J Parasitol 41 (11):1129-1137

Liu H, Yao S, Dann SM, Qin H, Elson CO, Cong Y (2013) ERK differentially regulates Th17- and Treg-cell development and contributes to the pathogenesis of colitis. Eur J Immunol 43 (7):1716-1726

Lodinova-Zadnikova R, Sonnenborn U (1997) Effect of preventive administration of a nonpathogenic Escherichia coli strain on the colonization of the intestine with microbial pathogens in newborn infants. Biol Neonate 71 (4):224-232

Loessner H, Leschner S, Endmann A, Westphal K, Wolf K, Kochruebe K et al. (2009) Drug-inducible remote control of gene expression by probiotic Escherichia coli Nissle 1917 in intestine, tumor and gall bladder of mice. Microbes Infect 11 (14-15):1097-1105

Loke P, Gallagher I, Nair MG, Zang X, Brombacher F, Mohrs M et al. (2007) Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection. J Immunol 179 (6):3926-3936

Loke P, MacDonald AS, Robb A, Maizels RM, Allen JE (2000) Alternatively activated macrophages induced by nematode infection inhibit proliferation via cell-to-cell contact. Eur J Immunol 30 (9):2669-2678

Lucas PJ, Kim SJ, Melby SJ, Gress RE (2000) Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta II receptor. J Exp Med 191 (7):1187-1196

Luft T, Jefford M, Luetjens P, Hochrein H, Masterman KA, Maliszewski C et al. (2002) IL-1 beta enhances CD40 ligand-mediated cytokine secretion by human dendritic cells (DC): a mechanism for T cell-independent DC activation. J Immunol 168 (2):713-722

Ma Q, Lu AY (2011) Pharmacogenetics, pharmacogenomics, and individualized medicine. Pharmacol Rev 63 (2):437-459

Mackenzie CD, Huntington MK, Wanji S, Lovato RV, Eversole RR, Geary TG (2010) The association of adult Onchocerca volvulus with lymphatic vessels. J Parasitol 96 (1):219-221

Mahida YR, Wu K, Jewell DP (1989) Enhanced production of interleukin 1-beta by mononuclear cells isolated from mucosa with active ulcerative colitis of Crohn's disease. Gut 30 (6):835-838

Main RG, Dritz SS, Tokach MD, Goodband RD, Nelssen JL (2004) Increasing weaning age improves pig performance in a multisite production system. J Anim Sci 82 (5):1499-1507

Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD, Allen JE (2004) Helminth parasites--masters of regulation. Immunol Rev 201:89-116

Mallon P, McKay D, Kirk S, Gardiner K (2007) Probiotics for induction of remission in ulcerative colitis. Cochrane Database Syst Rev Epub ahead of print 10.1002/14651858.CD005573.pub2 (4):CD005573

Maltby R, Leatham-Jensen MP, Gibson T, Cohen PS, Conway T (2013) Nutritional basis for colonization resistance by human commensal Escherichia coli strains HS and Nissle 1917 against E. coli O157:H7 in the mouse intestine. PLoS One 8 (1):e53957

Manoury B, Gregory WF, Maizels RM, Watts C (2001) Bm-CPI-2, a cystatin homolog secreted by the filarial parasite Brugia malayi, inhibits class II MHC-restricted antigen processing. Curr Biol 11 (6):447-451

Mansfield LS, Gauthier DT (2004) Lymphoglandular complexes are important colonic sites for immunoglobulin A induction against Campylobacter jejuni in a swine disease model. Comp Med 54 (5):514-523

Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M (2004) The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 25 (12):677-686

Marshall JK, Thabane M, Steinhart AH, Newman JR, Anand A, Irvine EJ (2010) Rectal 5-aminosalicylic acid for induction of remission in ulcerative colitis. Cochrane Database Syst Rev Epub ahead of print 10.1002/14651858.CD004115.pub2 (1):CD004115

Martinez FO, Sica A, Mantovani A, Locati M (2008) Macrophage activation and polarization. Front Biosci 13:453-461

Martinon F, Burns K, Tschopp J (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell 10 (2):417-426

Mascheretti S, Schreiber S (2005) Genetic testing in Crohn disease: utility in individualizing patient management. Am J Pharmacogenomics 5 (4):213-222

Massacand JC, Stettler RC, Meier R, Humphreys NE, Grencis RK, Marsland BJ et al. (2009) Helminth products bypass the need for TSLP in Th2 immune responses by directly modulating dendritic cell function. Proc Natl Acad Sci U S A 106 (33):13968-13973

Matera G, Giancotti A, Scalise S, Pulicari MC, Maselli R, Piizzi C et al. (2008) Ascaris lumbricoides-induced suppression of total and specific IgE responses in atopic subjects is interleukin 10-independent and associated with an increase of CD25(+) cells. Diagn Microbiol Infect Dis 62 (3):280-286

Matthes H, Krummenerl T, Giensch M, Wolff C, Schulze J (2010) Clinical trial: probiotic treatment of acute distal ulcerative colitis with rectally administered Escherichia coli Nissle 1917 (EcN). BMC Complement Altern Med 10:13-20

Mazzucchelli L, Hauser C, Zgraggen K, Wagner HE, Hess MW, Laissue JA et al. (1996) Differential in situ expression of the genes encoding the chemokines MCP-1 and RANTES in human inflammatory bowel disease. J Pathol 178 (2):201-206

McSorley HJ, Harcus YM, Murray J, Taylor MD, Maizels RM (2008) Expansion of Foxp3+ regulatory T cells in mice infected with the filarial parasite Brugia malayi. J Immunol 181 (9):6456-6466

Melgar S, Drmotova M, Rehnstrom E, Jansson L, Michaelsson E (2006) Local production of chemokines and prostaglandin E2 in the acute, chronic and recovery phase of murine experimental colitis. Cytokine 35 (5-6):275-283

Mercer M, Brinich MA, Geller G, Harrison K, Highland J, James K et al. (2012) How patients view probiotics: findings from a multicenter study of patients with inflammatory bowel disease and irritable bowel syndrome. J Clin Gastroenterol 46 (2):138-144

Merga Y, Campbell BJ, Rhodes JM (2014) Mucosal barrier, bacteria and inflammatory bowel disease: possibilities for therapy. Dig Dis 32 (4):475-483

Mestas J, Hughes CC (2004) Of mice and not men: differences between mouse and human immunology. J Immunol 172 (5):2731-2738

Metenou S, Dembele B, Konate S, Dolo H, Coulibaly SY, Coulibaly YI et al. (2010) At homeostasis filarial infections have expanded adaptive T regulatory but not classical Th2 cells. J Immunol 184 (9):5375-5382

Metwali A, Setiawan T, Blum AM, Urban J, Elliott DE, Hang L et al. (2006) Induction of CD8+ regulatory T cells in the intestine by Heligmosomoides polygyrus infection. Am J Physiol Gastrointest Liver Physiol 291 (2):G253-259

Meurens F, Summerfield A, Nauwynck H, Saif L, Gerdts V (2012) The pig: a model for human infectious diseases. Trends Microbiol 20 (1):50-57

Mia S, Warnecke A, Zhang XM, Malmstrom V, Harris RA (2014) An optimized Protocol for Human M2 Macrophages using M-CSF and IL-4/IL-10/TGF-beta Yields a Dominant Immunosuppressive Phenotype. Scand J Immunol 79 (5):305-314

Moeser AJ, Borst LB, Overman BL, Pittman JS (2012) Defects in small intestinal epithelial barrier function and morphology associated with peri-weaning failure to thrive syndrome (PFTS) in swine. Res Vet Sci 93 (2):975-982

Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE (1992) RAG-1-deficient mice have no mature B and T lymphocytes. Cell 68 (5):869-877

Mondel M, Schroeder BO, Zimmermann K, Huber H, Nuding S, Beisner J et al. (2009) Probiotic E. coli treatment mediates antimicrobial human beta-defensin synthesis and fecal excretion in humans. Mucosal Immunol 2 (2):166-172

Montes M, Sanchez C, Verdonck K, Lake JE, Gonzalez E, Lopez G et al. (2009) Regulatory T cell expansion in HTLV-1 and strongyloidiasis co-infection is associated with reduced IL-5 responses to Strongyloides stercoralis antigen. PLoS Negl Trop Dis 3 (6):e456

Morrissey PJ, Charrier K, Braddy S, Liggitt D, Watson JD (1993) CD4+ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified CD4+ T cells. J Exp Med 178 (1):237-244

Motomura Y, Wang H, Deng Y, El-Sharkawy RT, Verdu EF, Khan WI (2009) Helminth antigen-based strategy to ameliorate inflammation in an experimental model of colitis. Clin Exp Immunol 155 (1):88-95

Mowat AM, Bain CC (2011) Mucosal macrophages in intestinal homeostasis and inflammation. J Innate Immun 3 (6):550-564

Mowat C, Cole A, Windsor A, Ahmad T, Arnott I, Driscoll R et al. (2011) Guidelines for the management of inflammatory bowel disease in adults. Gut 60 (5):571-607

Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M et al. (2007) Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science 317 (5835):256-260

Murai M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H et al. (2009) Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nat Immunol 10 (11):1178-1184

Murray J, Manoury B, Balic A, Watts C, Maizels RM (2005) Bm-CPI-2, a cystatin from Brugia malayi nematode parasites, differs from Caenorhabditis elegans cystatins in a specific site mediating inhibition of the antigen-processing enzyme AEP. Mol Biochem Parasitol 139 (2):197-203

Mylonas KJ, Nair MG, Prieto-Lafuente L, Paape D, Allen JE (2009) Alternatively activated macrophages elicited by helminth infection can be reprogrammed to enable microbial killing. J Immunol 182 (5):3084-3094

Nagar J, Ranade S, Kamath V, Singh S, Karunanithi P, Subramani S et al. (2014) Therapeutic potential of chloroquine in a murine model of inflammatory bowel disease. Int Immunopharmacol 21 (2):328-335

Naidoo K, Gordon M, Fagbemi AO, Thomas AG, Akobeng AK (2011) Probiotics for maintenance of remission in ulcerative colitis. Cochrane Database Syst Rev Epub ahead of print 10.1002/14651858.CD007443.pub2 (12):CD007443

Nair MG, Du Y, Perrigoue JG, Zaph C, Taylor JJ, Goldschmidt M et al. (2009) Alternatively activated macrophage-derived RELM-{alpha} is a negative regulator of type 2 inflammation in the lung. J Exp Med 206 (4):937-952

Nakayachi K (2013) The unintended effects of risk-refuting information on anxiety. Risk Anal 33 (1):80-91

Nemoto Y, Kanai T, Takahara M, Oshima S, Okamoto R, Tsuchiya K et al. (2013) Th1/Th17-mediated interstitial pneumonia in chronic colitis mice independent of intestinal microbiota. J Immunol 190 (12):6616-6625

Netea MG, Nold-Petry CA, Nold MF, Joosten LA, Opitz B, van der Meer JH et al. (2009) Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. Blood 113 (10):2324-2335

Neurath M, Fuss I, Strober W (2000) TNBS-colitis. Int Rev Immunol 19 (1):51-62

Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA et al. (2005) CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science 307 (5707):254-258

Nissle A (1951) [Mutaflor and its medical significance]. Z Klin Med 2 (3-4):68

Nofrarias M, Pujols J, Segales J, Gibert X, Majo N (2006) Changes in peripheral blood leukocyte populations in pigs with naturally occurring exudative epidermitis. Res Vet Sci 81 (2):211-214

Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R (1990) A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology 98 (3):694-702

Oosterloo BC, Premkumar M, Stoll B, Olutoye O, Thymann T, Sangild PT et al. (2014) Dual purpose use of preterm piglets as a model of pediatric GI disease. Vet Immunol Immunopathol 159 (3-4):156-165

Osborne LC, Monticelli LA, Nice TJ, Sutherland TE, Siracusa MC, Hepworth MR et al. (2014) Coinfection. Virus-helminth coinfection reveals a microbiota-independent mechanism of immunomodulation. Science 345 (6196):578-582

Ostanin DV, Bao J, Koboziev I, Gray L, Robinson-Jackson SA, Kosloski-Davidson M et al. (2009) T cell transfer model of chronic colitis: concepts, considerations, and tricks of the trade. Am J Physiol Gastrointest Liver Physiol 296 (2):G135-146

Ostanin DV, Pavlick KP, Bharwani S, D'Souza D, Furr KL, Brown CM et al. (2006) T cell-induced inflammation of the small and large intestine in immunodeficient mice. Am J Physiol Gastrointest Liver Physiol 290 (1):G109-119

Park HK, Cho MK, Choi SH, Kim YS, Yu HS (2011) Trichinella spiralis: infection reduces airway allergic inflammation in mice. Exp Parasitol 127 (2):539-544

Pedersen KS, Kristensen CS, Nielsen JP (2012) Demonstration of non-specific colitis and increased crypt depth in colon of weaned pigs with diarrhea. Vet Q 32 (1):45-49

Pesce JT, Ramalingam TR, Mentink-Kane MM, Wilson MS, El Kasmi KC, Smith AM et al. (2009) Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis. PLoS Pathog 5 (4):e1000371

Petersen AM, Schjorring S, Gerstrom SC, Krogfelt KA (2011) Treatment of inflammatory bowel disease associated E. coli with ciprofloxacin and E. coli Nissle in the streptomycin-treated mouse intestine. PLoS One 6 (8):e22823

Pfaff AW, Schulz-Key H, Soboslay PT, Taylor DW, MacLennan K, Hoffmann WH (2002) Litomosoides sigmodontis cystatin acts as an immunomodulator during experimental filariasis. Int J Parasitol 32 (2):171-178

Pie S, Lalles JP, Blazy F, Laffitte J, Seve B, Oswald IP (2004) Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. J Nutr 134 (3):641-647

Pieper R, Kroger S, Richter JF, Wang J, Martin L, Bindelle J et al. (2012) Fermentable fiber ameliorates fermentable protein-induced changes in microbial ecology, but not the mucosal response, in the colon of piglets. J Nutr 142 (4):661-667

Pohlmann C, Thomas M, Forster S, Brandt M, Hartmann M, Bleich A et al. (2013) Improving health from the inside: Use of engineered intestinal microorganisms as in situ cytokine delivery system. Bioengineered 4 (3):172-179

Potian JA, Rafi W, Bhatt K, McBride A, Gause WC, Salgame P (2011) Preexisting helminth infection induces inhibition of innate pulmonary anti-tuberculosis defense by engaging the IL-4 receptor pathway. J Exp Med 208 (9):1863-1874

Pouillart PR, Depeint F, Abdelnour A, Deremaux L, Vincent O, Maziere JC et al. (2010) Nutriose, a prebiotic low-digestible carbohydrate, stimulates gut mucosal immunity and prevents TNBS-induced colitis in piglets. Inflamm Bowel Dis 16 (5):783-794

Powrie F, Leach MW, Mauze S, Caddle LB, Coffman RL (1993) Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. Int Immunol 5 (11):1461-1471

Prefontaine E, Macdonald JK, Sutherland LR (2010) Azathioprine or 6-mercaptopurine for induction of remission in Crohn's disease. Cochrane Database Syst Rev Epub ahead of print 10.1002/14651858.CD000545.pub3 (6):CD000545

Price AE, Liang HE, Sullivan BM, Reinhardt RL, Eisley CJ, Erle DJ et al. (2010) Systemically dispersed innate IL-13-expressing cells in type 2 immunity. Proc Natl Acad Sci U S A 107 (25):11489-11494

Qualls JE, Kaplan AM, van Rooijen N, Cohen DA (2006) Suppression of experimental colitis by intestinal mononuclear phagocytes. J Leukoc Biol 80 (4):802-815

Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 118 (2):229-241

Rao S, Hu S, McHugh L, Lueders K, Henry K, Zhao Q et al. (2005) Toward a live microbial microbicide for HIV: commensal bacteria secreting an HIV fusion inhibitor peptide. Proc Natl Acad Sci U S A 102 (34):11993-11998

Ratanji KD, Derrick JP, Dearman RJ, Kimber I (2014) Immunogenicity of therapeutic proteins: influence of aggregation. J Immunotoxicol 11 (2):99-109

Rausch S, Huehn J, Kirchhoff D, Rzepecka J, Schnoeller C, Pillai S et al. (2008) Functional analysis of effector and regulatory T cells in a parasitic nematode infection. Infect Immun 76 (5):1908-1919

Rausch S, Huehn J, Loddenkemper C, Hepworth MR, Klotz C, Sparwasser T et al. (2009) Establishment of nematode infection despite increased Th2 responses and immunopathology after selective depletion of Foxp3+ cells. Eur J Immunol 39 (11):3066-3077

Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R et al. (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol 2 (4):361-367

Reyes JL, Terrazas LI (2007) The divergent roles of alternatively activated macrophages in helminthic infections. Parasite Immunol 29 (12):609-619

Rimoldi M, Chieppa M, Salucci V, Avogadri F, Sonzogni A, Sampietro GM et al. (2005) Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. Nat Immunol 6 (5):507-514

Rizzo A, Monteleone I, Fina D, Stolfi C, Caruso R, Fantini MC et al. (2011) Inhibition of colitis by IL-25 associates with induction of alternatively activated macrophages. Inflamm Bowel Dis 18 (3):449-459

Rollins BJ (1997) Chemokines. Blood 90 (3):909-928

Roses RE, Xu S, Xu M, Koldovsky U, Koski G, Czerniecki BJ (2008) Differential production of IL-23 and IL-12 by myeloid-derived dendritic cells in response to TLR agonists. J Immunol 181 (7):5120-5127

Rothkotter HJ (2009) Anatomical particularities of the porcine immune system--a physician's view. Dev Comp Immunol 33 (3):267-272

Rund SA, Rohde H, Sonnenborn U, Oelschlaeger TA (2013) Antagonistic effects of probiotic Escherichia coli Nissle 1917 on EHEC strains of serotype O104:H4 and O157:H7. Int J Med Microbiol 303 (1):1-8

Ruyssers NE, De Winter BY, De Man JG, Loukas A, Pearson MS, Weinstock JV et al. (2009) Therapeutic potential of helminth soluble proteins in TNBS-induced colitis in mice. Inflamm Bowel Dis 15 (4):491-500

Saenz SA, Taylor BC, Artis D (2008) Welcome to the neighborhood: epithelial cell-derived cytokines license innate and adaptive immune responses at mucosal sites. Immunol Rev 226:172-190

Sanders ME, Guarner F, Guerrant R, Holt PR, Quigley EM, Sartor RB et al. (2013) An update on the use and investigation of probiotics in health and disease. Gut 62 (5):787-796

Sangild PT, Thymann T, Schmidt M, Stoll B, Burrin DG, Buddington RK (2013) Invited review: the preterm pig as a model in pediatric gastroenterology. J Anim Sci 91 (10):4713-4729

Satoguina J, Mempel M, Larbi J, Badusche M, Loliger C, Adjei O et al. (2002) Antigen-specific T regulatory-1 cells are associated with immunosuppression in a chronic helminth infection (onchocerciasis). Microbes Infect 4 (13):1291-1300

Scheiffele F, Fuss IJ (2002) Induction of TNBS colitis in mice. Curr Protoc Immunol Chapter 15:Unit 15 19

Schellekens H (2002) Immunogenicity of therapeutic proteins: clinical implications and future prospects. Clin Ther 24 (11):1720-1740

Schierack P, Kleta S, Tedin K, Babila JT, Oswald S, Oelschlaeger TA et al. (2011) E. coli Nissle 1917 Affects Salmonella adhesion to porcine intestinal epithelial cells. PLoS One 6 (2):e14712

Schierack P, Lucius R, Sonnenburg B, Schilling K, Hartmann S (2003) Parasite-specific immunomodulatory functions of filarial cystatin. Infect Immun 71 (5):2422-2429

Schiering C, Krausgruber T, Chomka A, Frohlich A, Adelmann K, Wohlfert EA et al. (2014) The alarmin IL-33 promotes regulatory T-cell function in the intestine. Nature Epub ahead of print 10.1038/nature13577

Schlee M, Wehkamp J, Altenhoefer A, Oelschlaeger TA, Stange EF, Fellermann K (2007) Induction of human beta-defensin 2 by the probiotic Escherichia coli Nissle 1917 is mediated through flagellin. Infect Immun 75 (5):2399-2407

Schnoeller C, Rausch S, Pillai S, Avagyan A, Wittig BM, Loddenkemper C et al. (2008) A helminth immunomodulator reduces allergic and inflammatory responses by induction of IL-10-producing macrophages. J Immunol 180 (6):4265-4272

Schonemeyer A, Lucius R, Sonnenburg B, Brattig N, Sabat R, Schilling K et al. (2001) Modulation of human T cell responses and macrophage functions by onchocystatin, a secreted protein of the filarial nematode Onchocerca volvulus. J Immunol 167 (6):3207-3215

Schopf L, Luccioli S, Bundoc V, Justice P, Chan CC, Wetzel BJ et al. (2005) Differential modulation of allergic eye disease by chronic and acute ascaris infection. Invest Ophthalmol Vis Sci 46 (8):2772-2780

Schroeder B, Duncker S, Barth S, Bauerfeind R, Gruber AD, Deppenmeier S et al. (2006) Preventive effects of the probiotic Escherichia coli strain Nissle 1917 on acute secretory diarrhea in a pig model of intestinal infection. Dig Dis Sci 51 (4):724-731

Schroeder JH, Simbi BH, Ford L, Cole SR, Taylor MJ, Lawson C et al. (2012) Live Brugia malayi microfilariae inhibit transendothelial migration of neutrophils and monocytes. PLoS Negl Trop Dis 6 (11):e1914

Schultz M, Strauch UG, Linde HJ, Watzl S, Obermeier F, Gottl C et al. (2004) Preventive effects of Escherichia coli strain Nissle 1917 on acute and chronic intestinal inflammation in two different murine models of colitis. Clin Diagn Lab Immunol 11 (2):372-378

Schulze J, Sonnenborn U (1995) Re.: Oral administration of a certain strain of live Escherichia coli for intestinal disorders? (Infection 23 [1995] 51-54). Infection 23 (3):184-188

Segura M, Su Z, Piccirillo C, Stevenson MM (2007) Impairment of dendritic cell function by excretory-secretory products: a potential mechanism for nematode-induced immunosuppression. Eur J Immunol 37 (7):1887-1904

Seo EJ, Weibel S, Wehkamp J, Oelschlaeger TA (2012) Construction of recombinant E. coli Nissle 1917 (EcN) strains for the expression and secretion of defensins. Int J Med Microbiol 302 (6):276-287

Setiawan T, Metwali A, Blum AM, Ince MN, Urban JF, Jr., Elliott DE et al. (2007) Heligmosomoides polygyrus promotes regulatory T-cell cytokine production in the murine normal distal intestine. Infect Immun 75 (9):4655-4663

Shaw MH, Kamada N, Kim YG, Nunez G (2012) Microbiota-induced IL-1beta, but not IL-6, is critical for the development of steady-state TH17 cells in the intestine. J Exp Med 209 (2):251-258

Shea-Donohue T, Sullivan C, Finkelman FD, Madden KB, Morris SC, Goldhill J et al. (2001) The role of IL-4 in Heligmosomoides polygyrus-induced alterations in murine intestinal epithelial cell function. J Immunol 167 (4):2234-2239

Shen J, Zuo ZX, Mao AP (2014) Effect of probiotics on inducing remission and maintaining therapy in ulcerative colitis, Crohn's disease, and pouchitis: meta-analysis of randomized controlled trials. Inflamm Bowel Dis 20 (1):21-35

Sheppard KA, Fitz LJ, Lee JM, Benander C, George JA, Wooters J et al. (2004) PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. FEBS Lett 574 (1-3):37-41

Shim S, Jang WS, Lee SJ, Jin S, Kim J, Lee SS et al. (2014) Development of a new minipig model to study radiation-induced gastrointestinal syndrome and its application in clinical research. Radiat Res 181 (4):387-395

Smajs D, Bures J, Smarda J, Chaloupkova E, Kvetina J, Forstl M et al. (2012) Experimental administration of the probiotic Escherichia coli strain Nissle 1917 results in decreased diversity of E. coli strains in pigs. Curr Microbiol 64 (3):205-210

Smith KA, Hochweller K, Hammerling GJ, Boon L, MacDonald AS, Maizels RM (2011) Chronic helminth infection promotes immune regulation in vivo through dominance of CD11cloCD103- dendritic cells. J Immunol 186 (12):7098-7109

Specht S, Volkmann L, Wynn T, Hoerauf A (2004) Interleukin-10 (IL-10) counterregulates IL-4-dependent effector mechanisms in Murine Filariasis. Infect Immun 72 (11):6287-6293

Splichalova A, Trebichavsky I, Rada V, Vlkova E, Sonnenborn U, Splichal I (2011) Interference of Bifidobacterium choerinum or Escherichia coli Nissle 1917 with Salmonella Typhimurium in gnotobiotic piglets correlates with cytokine patterns in blood and intestine. Clin Exp Immunol 163 (2):242-249

Steele J, Feng H, Parry N, Tzipori S (2010) Piglet models of acute or chronic Clostridium difficile illness. J Infect Dis 201 (3):428-434

Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W et al. (2000) Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. Science 289 (5483):1352-1355

Steidler L, Neirynck S, Huyghebaert N, Snoeck V, Vermeire A, Goddeeris B et al. (2003) Biological containment of genetically modified Lactococcus lactis for intestinal delivery of human interleukin 10. Nat Biotechnol 21 (7):785-789

Stephenson LS, Latham MC, Ottesen EA (2000) Malnutrition and parasitic helminth infections. Parasitology 121 Suppl:S23-38

Storm DW, Koff SA, Horvath DJ, Jr., Li B, Justice SS (2011) In vitro analysis of the bactericidal activity of Escherichia coli Nissle 1917 against pediatric uropathogens. J Urol 186 (4 Suppl):1678-1683

Strachan DP (1989) Hay fever, hygiene, and household size. Bmj 299 (6710):1259-1260

Stritesky GL, Yeh N, Kaplan MH (2008) IL-23 promotes maintenance but not commitment to the Th17 lineage. J Immunol 181 (9):5948-5955

Stritzker J, Hill PJ, Gentschev I, Szalay AA (2010) Myristoylation negative msbB-mutants of probiotic E. coli Nissle 1917 retain tumor specific colonization properties but show less side effects in immunocompetent mice. Bioeng Bugs 1 (2):139-145

Stritzker J, Weibel S, Hill PJ, Oelschlaeger TA, Goebel W, Szalay AA (2007) Tumor-specific colonization, tissue distribution, and gene induction by probiotic Escherichia coli Nissle 1917 in live mice. Int J Med Microbiol 297 (3):151-162

Sturm A, Rilling K, Baumgart DC, Gargas K, Abou-Ghazale T, Raupach B et al. (2005) Escherichia coli Nissle 1917 distinctively modulates T-cell cycling and expansion via toll-like receptor 2 signaling. Infect Immun 73 (3):1452-1465

Su CW, Cao Y, Kaplan J, Zhang M, Li W, Conroy M et al. (2011) Duodenal helminth infection alters barrier function of the colonic epithelium via adaptive immune activation. Infect Immun 79 (6):2285-2294

Summers RW, Elliott DE, Qadir K, Urban JF, Jr., Thompson R, Weinstock JV (2003) Trichuris suis seems to be safe and possibly effective in the treatment of inflammatory bowel disease. Am J Gastroenterol 98 (9):2034-2041

Summers RW, Elliott DE, Urban JF, Jr., Thompson RA, Weinstock JV (2005) Trichuris suis therapy for active ulcerative colitis: a randomized controlled trial. Gastroenterology 128 (4):825-832

Sutton TL, Zhao A, Madden KB, Elfrey JE, Tuft BA, Sullivan CA et al. (2008) Anti-Inflammatory mechanisms of enteric Heligmosomoides polygyrus infection against trinitrobenzene sulfonic acid-induced colitis in a murine model. Infect Immun 76 (10):4772-4782

Tannock GW, Tiong IS, Priest P, Munro K, Taylor C, Richardson A et al. (2011) Testing probiotic strain Escherichia coli Nissle 1917 (Mutaflor) for its ability to reduce carriage of multidrug-resistant E. coli by elderly residents in long-term care facilities. J Med Microbiol 60 (Pt 3):366-370

Targownik LE, Nugent Z, Singh H, Bernstein CN (2014) Prevalence of and outcomes associated with corticosteroid prescription in inflammatory bowel disease. Inflamm Bowel Dis 20 (4):622-630

Taylor BC, Zaph C, Troy AE, Du Y, Guild KJ, Comeau MR et al. (2009) TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. J Exp Med 206 (3):655-667

Ten Hove T, The Olle F, Berkhout M, Bruggeman JP, Vyth-Dreese FA, Slors JF et al. (2004) Expression of CD45RB functionally distinguishes intestinal T lymphocytes in inflammatory bowel disease. J Leukoc Biol 75 (6):1010-1015

Tetsutani K, Ishiwata K, Ishida H, Tu L, Torii M, Hamano S et al. (2009) Concurrent infection with Heligmosomoides polygyrus suppresses anti-Plasmodium yoelii protection partially by induction of CD4(+)CD25(+)Foxp3(+) Treg in mice. Eur J Immunol 39 (10):2822-2830

Thomas SR, Mohr D, Stocker R (1994) Nitric oxide inhibits indoleamine 2,3-dioxygenase activity in interferon-gamma primed mononuclear phagocytes. J Biol Chem 269 (20):14457-14464

Travassos WJ, Cheifetz AS (2005) Infliximab: Use in Inflammatory Bowel Disease. Curr Treat Options Gastroenterol 8 (3):187-196

Turner D, Shah PS, Steinhart AH, Zlotkin S, Griffiths AM (2011) Maintenance of remission in inflammatory bowel disease using omega-3 fatty acids (fish oil): a systematic review and meta-analyses. Inflamm Bowel Dis 17 (1):336-345

Ukena SN, Singh A, Dringenberg U, Engelhardt R, Seidler U, Hansen W et al. (2007) Probiotic Escherichia coli Nissle 1917 inhibits leaky gut by enhancing mucosal integrity. PLoS One 2 (12):e1308

Ukena SN, Westendorf AM, Hansen W, Rohde M, Geffers R, Coldewey S et al. (2005) The host response to the probiotic Escherichia coli strain Nissle 1917: specific up-regulation of the proinflammatory chemokine MCP-1. BMC Med Genet 6:43-56

Unutmaz D, Pulendran B (2009) The gut feeling of Treg cells: IL-10 is the silver lining during colitis. Nat Immunol 10 (11):1141-1143

van Gessel YA, Mani S, Bi S, Hammamieh R, Shupp JW, Das R et al. (2004) Functional piglet model for the clinical syndrome and postmortem findings induced by staphylococcal enterotoxin B. Exp Biol Med (Maywood) 229 (10):1061-1071

Vandenbroucke K, de Haard H, Beirnaert E, Dreier T, Lauwereys M, Huyck L et al. (2010) Orally administered L. lactis secreting an anti-TNF Nanobody demonstrate efficacy in chronic colitis. Mucosal Immunol 3 (1):49-56

Vanuytsel T, Vermeire S, Cleynen I (2013) The role of Haptoglobin and its related protein, Zonulin, in inflammatory bowel disease. Tissue Barriers 1 (5):e27321

Veltman K, Hummel S, Cichon C, Sonnenborn U, Schmidt MA (2012) Identification of specific miRNAs targeting proteins of the apical junctional complex that simulate the probiotic effect of E. coli Nissle 1917 on T84 epithelial cells. Int J Biochem Cell Biol 44 (2):341-349

Villani AC, Lemire M, Fortin G, Louis E, Silverberg MS, Collette C et al. (2009) Common variants in the NLRP3 region contribute to Crohn's disease susceptibility. Nat Genet 41 (1):71-76

Waddell A, Ahrens R, Steinbrecher K, Donovan B, Rothenberg ME, Munitz A et al. (2011) Colonic eosinophilic inflammation in experimental colitis is mediated by Ly6C(high) CCR2(+) inflammatory monocyte/macrophage-derived CCL11. J Immunol 186 (10):5993-6003

Wang Q, Hou Y, Yi D, Wang L, Ding B, Chen X et al. (2013) Protective effects of N-acetylcysteine on acetic acid-induced colitis in a porcine model. BMC Gastroenterol 13:133

Wedemeyer J, Vosskuhl K (2008) Role of gastrointestinal eosinophils in inflammatory bowel disease and intestinal tumours. Best Pract Res Clin Gastroenterol 22 (3):537-549

Wehkamp J, Harder J, Wehkamp K, Wehkamp-von Meissner B, Schlee M, Enders C et al. (2004) NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium. Infect Immun 72 (10):5750-5758

Weisser SB, Brugger HK, Voglmaier NS, McLarren KW, van Rooijen N, Sly LM (2011) SHIP-deficient, alternatively activated macrophages protect mice during DSS-induced colitis. J Leukoc Biol 90 (3):483-492

Weng M, Huntley D, Huang IF, Foye-Jackson O, Wang L, Sarkissian A et al. (2007) Alternatively activated macrophages in intestinal helminth infection: effects on concurrent bacterial colitis. J Immunol 179 (7):4721-4731

Werneck-Silva AL, Alvares EP, Gama P, Damiao AO, Osaki LH, Ogias D et al. (2006) Intestinal damage in strongyloidiasis: the imbalance between cell death and proliferation. Dig Dis Sci 51 (6):1063-1069

Wesa A, Galy A (2002) Increased production of pro-inflammatory cytokines and enhanced T cell responses after activation of human dendritic cells with IL-1 and CD40 ligand. BMC Immunol 3:14-24

Westendorf AM, Gunzer F, Deppenmeier S, Tapadar D, Hunger JK, Schmidt MA et al. (2005) Intestinal immunity of Escherichia coli NISSLE 1917: a safe carrier for therapeutic molecules. FEMS Immunol Med Microbiol 43 (3):373-384

Wilson MS, Ramalingam TR, Rivollier A, Shenderov K, Mentink-Kane MM, Madala SK et al. (2011) Colitis and intestinal inflammation in IL10-/- mice results from IL-13Ralpha2-mediated attenuation of IL-13 activity. Gastroenterology 140 (1):254-264

Wilson MS, Taylor MD, Balic A, Finney CA, Lamb JR, Maizels RM (2005) Suppression of allergic airway inflammation by helminth-induced regulatory T cells. J Exp Med 202 (9):1199-1212

Wirtz S, Neufert C, Weigmann B, Neurath MF (2007) Chemically induced mouse models of intestinal inflammation. Nat Protoc 2 (3):541-546

Xavier RJ, Podolsky DK (2007) Unravelling the pathogenesis of inflammatory bowel disease. Nature 448 (7152):427-434

Yan F, Polk DB (2002) Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. J Biol Chem 277 (52):50959-50965

Zeissig S, Burgel N, Gunzel D, Richter J, Mankertz J, Wahnschaffe U et al. (2007) Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. Gut 56 (1):61-72

Zhang Q, Widmer G, Tzipori S (2013) A pig model of the human gastrointestinal tract. Gut Microbes 4 (3):193-200

Zhang Y, Zhang Y, Xia L, Zhang X, Ding X, Yan F et al. (2012) Escherichia coli Nissle 1917 targets and restrains mouse B16 melanoma and 4T1 breast tumors through expression of azurin protein. Appl Environ Microbiol 78 (21):7603-7610

Ziegler-Heitbrock HW, Appl B, Kafferlein E, Loffler T, Jahn-Henninger H, Gutensohn W et al. (1994) The antibody MY4 recognizes CD14 on porcine monocytes and macrophages. Scand J Immunol 40 (5):509-514

Zupancic T, Stojan J, Lane EB, Komel R, Bedina-Zavec A, Liovic M (2014) Intestinal cell barrier function in vitro is severely compromised by keratin 8 and 18 mutations identified in patients with inflammatory bowel disease. PLoS One 9 (6):e99398

Zyrek AA, Cichon C, Helms S, Enders C, Sonnenborn U, Schmidt MA (2007) Molecular mechanisms underlying the probiotic effects of Escherichia coli Nissle 1917 involve ZO-2 and PKCzeta redistribution resulting in tight junction and epithelial barrier repair. Cell Microbiol 9 (3):804-816

## 10. Publications, Patents and Conference Proceedings

### Publications

1. **Whelan RA** and Rausch S, Ebner F, Günzel D, Richter JF, Hering NA, Schulzke JD, Kühl AA, Keles A, Janczyk P, Nöckler K, Wieler LH, Hartmann S. 2014. A transgenic probiotic secreting a parasite immunomodulator for site-directed treatment of gut inflammation. Molecular Therapy. doi: 10.1038/mt.2014.125. [Epub ahead of print].

2. **Whelan RA**, Hartmann S, Rausch S. 2012. Nematode modulation of inflammatory bowel disease. Protoplasma. 249 (4): 871-886.

### Patents

1. Hartmann S, Rausch S, **Whelan RA**, Wieler L. Probiotic bacteria as a carrier for a helminth derived immunomodulator for the treatment of inflammatory disorders. 06.09.2012. PTC 12 183 268.7

### Conference Proceedings

1. **Whelan RA**, Rausch S, Ebner F, Richter JF, Hering NA, Günzel D, Schulzke JD, Kühl AA, Janczyk P, Nöckler K, Wieler LH, Hartmann S. A transgenic probiotic bacterium secreting a nematode immunomodulator for site-directed treatment of intestinal inflammation. Annual Meeting 'Veterinärmedizinischer Arbeitskreis' of the German Society for Immunology (DGfI), 2-3.5.2014; Jena, Germany.

2. **Whelan RA,** Rausch S, Ebner F, Richter JF, Hering NA, Günzel D, Schulzke JD, Kühl AA, Janczyk P, Nöckler K, Wieler LH, Hartmann S. A transgenic probiotic bacterium secreting a nematode immunomodulator for site-directed treatment of intestinal inflammation. Probiota 2014, 4-5.2.2014; Amsterdam, Netherlands.

3. **Whelan RA,** Rausch S, Ebner F, Kühl AA, Janczyk P, Nöckler K, Wieler LH, Hartmann S. The effect of transgenic, nematode cystatin expressing, probiotic in colitis models. The 17th Annual Woodshole Immunoparasitology Conference, 28-30.4.2013; Woods Hole, USA.

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## 12. Affidavit

I hereby declare that the thesis has been written by myself without any external unauthorized help and that I have not used other than the declared sources.

Rose Whelan
Berlin 24.09.14