The role of CD69 molecule in the mucosal immune system of the intestine

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Abbreviations

Apc – allophycocyanin
APC – antigen presenting cells
BSA – bowine serum albumin
CD – Crohn’s disease
cDNA – complementary deoxyribonucleic acid
CFSE – carboxyfluorescin succinimidyl ester
CSF-1R – colony stimulating factor type 1 receptor
cLP – colonic lamina propria
DC – dendritic cells
DNA – deoxyribonucleic acid
dNTPs – deoxynucleoside triphosphate
dsRNA – double stranded ribonucleic acid
DSS – dextran sodium sulphate
DTH – delayed type hypersensitivity
EDTA – ethylenediaminetetraacetic acid
ELISA – enzyme-linked immunosorbent assay
FBS – fetal bowine serum
FCM – flow cytometry
FDR – false discovery rate
FITC – fluorescein isothiocyanate
GF – germ free
GIT – gastrointestinal tract
H&E – hematoxylin and eosin
HI – homing index
i.p. – intraperitoneal
i.v. – intravenously
IBD – inflammatory bowel disease
IEL – intraepithelial lymphocytes
IFA – incomplete Freund’s adjuvant
IFN – interferon
IFN-I – type I interferons
Ig – immunoglobulin
IL – interleukin
IR – input ratio
LAP – latency associated peptide
LP – lamina propria
LPL – lamina propria lymphocytes
mAb – monoclonal antibody
MACS – magnetic-activated cell sorting
MadCAM – mucosal addressin cell adhesion molecule – 1
M-cells – micro-fold cells
MHC – major histocompatibility complex
MLN – mesenteric lymph nodes
NK – natural killer
ODN – oligodeoxynucleotide
OVA – chicken ovalbumin protein
PAMP – pathogen associated molecular patterns
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PE – phycoerythrin
PerCP – streptavidin-peridinin chlorophyll protein
Poly (I:C) – polyinosinic-polycytidylic acid
PP – Peyer’s patches
qRT-PCR – quantitative real time polymerase chain reaction
RA – retinoic acid
RNA – ribonucleic acid
RPMI – Roswell Park Memorial Institute medium
RT – room temperature
s.c. – subcutaneous
S1P – sphingosine-1 phosphate
S1P₁ – sphingosine-1 phosphate receptor 1
SDS – sodium dodecyl sulphate
siLP – small intestine lamina propria
SLO – secondary lymphoid organs
SPF – standard pathogen free
ssDNA – single stranded deoxyribonucleic acid
TCR – T cell receptor
TGF – transforming growth factor
Th – T helper cells
TLR – Toll like receptor
TNBS – 2, 4, 6-trinitro benzene sulfonic acid
TNF – tumor necrosis factor
Tr1 – T regulatory type 1 cells
T_{reg} – regulatory T cells
UC – ulcerative colitis
WGA – Wheat Germ Agglutinin
1. Introduction

1.1. Mucosal immune system of the gut

1.1.1. General characteristics

Immunity represents the protection from disease and, more specifically, infectious disease [4]. The cells and molecules that are responsible for immunity constitute the immune system and their coordinated response to the introduction of danger antigen is called the immune response [4]. Various anatomically distinct compartments can be distinguished within the immune system and each of these compartments is specially adapted for generating the response against antigens encountered in the particular tissue [124]. The compartment comprising the highest number of the immune cells in the body is the mucosal immune system of the gastrointestinal tract (GIT) [108]. The lamina propria (LP) of the small and large intestine is home to approximately 80% of the all body’s immune cells [162].

The primary function of the intestine is to absorb nutrient compounds from its lumen but during the course of absorption activity the intestine is exposed to a wide variety of antigens derived from food, commensal bacteria and invading microorganisms [69]. The roughly estimated quantity of food proteins encountered in the lumen of human gut is 10-15 kg per year per person [124]. The adult human intestine is also home to a large population of resident microbes that constitute the intestinal commensal microflora. The size of this population is estimated on up to 100 trillion cells which is tenfold the total number of the cells in human body [9, 141]. The gut microbiome, which contains $\geq 100$ times the number of genes in the human genome, endows us with functional features that we have not had to evolve ourselves [9]. The commensal microbiota assists in the digestion of energy substrates (e.g. plant-derived pectin, cellulose), but also produces vitamins and hormones for the host [53]. Therefore, the mucosal immune system of the gut is facing a specific problem: it has to tolerate the beneficial food proteins and commensal microbiota constantly present in the intestine, but also has to efficiently fight pathogenic agents that try to invade the body through the intestinal route. In order to accomplish this dual role the immune system of the intestine had to adapt to this specific environment.
1.1.2. Mucosal barrier

The mucosal barrier represents a dynamic system composed of mucus and the epithelial layer that provides the first line of defence against intestinal pathogens. Consisting of only a single layer of cells (Fig.1) the intestinal epithelium must prevent the access of potentially harmful antigens and, at the same time, it has to absorb the dietary nutrients [125]. There are five different cell types in the intestinal epithelium (Goblet cells, Paneth cells, enterocytes, enteroendocrine cells and micro-fold cells (M-cells)) and all of them contribute to the barrier function [50, 53] (Fig.1).

Goblet cells produce and secrete large heavily glycosylated mucin proteins that are assembled into thick, complex bilayer of mucus that covers the GIT [111] (Fig.1). The highly hydrated, viscous mucus gel is necessarily porous to allow the diffusion of secreted and absorbed macromolecules, but provides an effective biophysical barrier for microorganisms [111]. Goblet cells also secrete other proteins (trefoil factors and antimicrobials) that contribute to the barrier function of the mucus [53, 111].

Paneth cells secrete a wide range of antimicrobial molecules into the mucus to ensure reduced bacterial load of the mucus inner layer [111] (Fig.1). These antimicrobial molecules manifest their function by interactions with and disruption of the microbial membrane [111]. Secreted antimicrobials can not discriminate between commensal and pathogen bacteria hence they are probably important in controlling the growth of the commensal microflora [53].

Enterocytes are the most numerous cells in the intestinal epithelium (Fig.1). These cells are connected with intercellular tight junctions that restrict the paracellular passage of even very small molecules [101]. The apical membrane of enterocytes forms a dense microvillous structures to increase the absorption surface and is covered with glycocalix layer [111]. Glycocalix is protecting the host from the pathogens that penetrate the mucus layer [111].

Enteroendocrine cells of the gut secrete various bioactive products (Fig.1) in the response to the luminal signals (e.g. products of microbial metabolism) [50]. Products of enteroendocrine cells include serotonin, somatostatin, gastrin, motilin, neurotensin, cholestokinin and other hormons [146]. These hormons can be secreted into the bloodstream to act on the distant tissues, but they also regulate
Figure 1. Mucosal barrier in the intestine. The intestinal epithelium is consisting of a single cell layer made of five cell types. All these cell types originate from stem cells located in the intestinal crypts. The most numerous cells in the intestinal epithelium are enterocytes that are connected with the tight junctions. Intestinal epithelial cells are covered with mucus produced by Goblet cells. Paneth cells secrete antimicrobial factors. Mucus layer above micro-fold cells (M-cells) is disrupted allowing controlled luminal antigen uptake by these cells. Enteroendocrine cells are secreting the hormones that regulate the function of the intestinal epithelium.
locally the functions of the intestinal epithelial cells (absorption, secretion of mucus and antimicrobial molecules, intestinal motility, cell proliferation and differentiation) [50, 146].

M-cells are present in the small intestine overlying Peyer’s patches (PP) and lymphoid follicles [50]. Unlike enterocytes, apical surface of M-cells lacks villus structures and glycocalix [125], which together with expression on various receptors facilitate M-cell sensing of microbes and their metabolites [50] (Fig.1). M-cells use transepithelial vesicular transport to carry the luminal antigens to the antigen presenting cells (APC) of the PP and lymphoid follicles [125]. On this way the interaction between the intestinal immune system and luminal antigens is localized and regulated.

The production and secretion of all components of mucosal barrier is modulated by the commensal microbiota. Mice kept under germ-free (GF) conditions are devoid of intestinal microflora and they have reduced Goblet cell numbers, intracellular storage of mucin proteins [73], as well as reduced expression of Paneth cell molecules [21].

1.1.3. Organization of the intestinal immune system

Enteric pathogens have developed a wide range of strategies allowing them to penetrate the mucosal barrier [111]. Protection from these pathogens is ensured by specific immune cells and tissues within the mucosal intestinal immune system. The intestinal immune system is divided into inductive and effector sites [102, 125] (Fig.2). The organized lymphoid tissues consist of mesenteric lymph nodes (MLN), PP, isolated lymphoid follicles in the small intestine and colonic patches that are responsible for the induction phase of the mucosal immune responses [50, 102, 119, 125] (Fig.2). It is thought that antigen presentation to the lymphocytes occurs at these induction sites [102, 119]. Naive T lymphocytes home to the inductive sites where they can recognize antigens presented in the major histocompatibility complex (MHC)-dependent manner by professional APC and undergo antigen-driven priming/activation, polarization and expansion to yield effector cells [84]. These effector cells then home to the effector sites of the intestinal immune system, that are the epithelium and the underlying LP, where they help to destroy invading pathogens [50, 84] (Fig.2).
Figure 2. The organization of immune tissues in the intestine. Immune tissues in the intestine can be divided into the induction and effector sites. Organized lymphoid tissues, such as Peyer’s patches and mesenteric lymph nodes, represent inductive sites where naive lymphocytes first encounter antigen presented by antigen presenting cells. Primed effector cells of both the innate (macrophages and natural killer (NK) cells) and adaptive (lymphocytes) immune systems home to the lamina propria or the epithelium that represent the effector sites.
1.2. Cells of the mucosal immune system in intestine

1.2.1. Antigen presenting cells

It is generally considered that after entering the gut through M-cells, antigens are transferred to the local APC and can be presented to the T cells in the inductive sites of the gut [119]. Both dendritic cells (DC) and macrophages play an important role in sampling pathogens and commensal bacteria in the gut [120, 135] and presenting antigens to lymphocytes, but the most important APC are DC. Murine DC can be divided into plasmacytoid CD11c⁺ B220⁺ and conventional CD11c⁺ B220⁻ that can be either CD4⁺ CD8⁻, CD4⁻ CD8⁺ or CD4⁻ CD8⁻ [134]. CD11c expression is often used as the specific marker for murine DC, but well defined intestinal macrophages can also express CD11c [120]. In the intestinal mucosa DC can be divided into a CD103 expressing and a CX₃CR1 (fractalkine receptor) expressing populations [120, 134]. CD103⁺ cells possess the features of classical DC: they migrate to MLN to prime naïve lymphocytes, imprint gut homing markers and induce the differentiation of regulatory T cells in a retinoic acid (RA)- and transforming growth factor (TGF)-β- dependent manner [37, 120, 134, 139]. CX₃CR1⁺ DC can extend their dendritic processes between the intestinal epithelial cells in a CX₃CR1-dependent manner and sample the luminal antigens directly [132]. The fact that some of the CX₃CR1⁺ cells in mucosa also express markers typical for macrophages (F4/80, CD68) [120, 133] points to the close relationship and possible plasticity of the myeloid derived cells in intestine. Furthermore, enterocytes can serve as APC by processing apically absorbed antigens and presenting them to the T cells on their basal surface [119].

In the normal non-inflamed gut all of the APC types express high levels of MHC molecules, but low levels of co-stimulatory molecules required for full T cell activation [37, 119, 139]. It is considered that these specific properties of intestinal DC contribute to the tolerance of the luminal antigens [37, 139].

1.2.2. Effector cells of the innate immune system

Innate immunity is activated rapidly by microbes [3]. It serves to prevent, control or eliminate infection of the host, but also to stimulate and direct adaptive immunity [3]. Cells of the innate immune system recognize the structures common
to the group of related microbes (pathogen associated molecular patterns, PAMP) and use phagocytosis or cytotoxic molecules as effector mechanisms [3].

Macrophages are the most abundant population among the phagocyte cells in intestine [35]. They promote tolerance in the healthy intestine, but during inflammation a new population of pro-inflammatory macrophages become dominant over tolerogenic resident ones [120]. Murine macrophages normally express F4/80, CD68, CD11b, colony stimulating factor-1 receptor (CSF-1R) and in inflamed intestine they also express high levels of Toll-like receptors (TLR), CD14, co-stimulatory molecules and produce large quantities of pro-inflammatory mediators like tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, nitric-oxide, chemokines [120].

Natural killer (NK) cells are required for the removal of transformed or infected cells and also link innate and adaptive immunity by secretion of interferon (IFN)-γ [184]. NK cells kill their target cells in a cell-contact dependent manner either by exocytosis of cytotoxic granulas containing perforines and granzymes or by engagement of death receptors on the target cell [3, 184]. CD3− NKp46+ NK cells producing high levels of IL-22, but not pro-inflammatory IFN-γ or IL-17 and also lacking cytolytic functions were found in gut LP [96] suggesting that NK cells in normal conditions can be involved in the prevention of intestinal inflammation.

1.2.3. Effector lymphocytes

Depending on their location in the mucosal immune system of the gut, effector lymphocytes can be divided into intraepithelial (IEL) and lamina propria lymphocytes (LPL) (Fig.2). Lymphocytes with a memory phenotype predominate among both IEL and LPL populations [26, 117, 119, 175] which is very important for the intestinal homeostasis because these cells can respond quickly and efficiently to the repeated assault by the same pathogen and prevent the systemic spread of it [175].

B cells are abundant among LPL [125]. After priming in the inductive sites they differentiate into the immunoglobulin (Ig) A producing plasma cells [45]. IgA is a `non-inflammatory` form of the immunoglobulin [125] meaning that plasma cells also contribute to tolerance in intestine. IgA is transcytosed across enterocytes into the intestinal lumen where it can bind pathogens and prevent their penetration
into the gut [53]. Several milligrams of IgA is produced by plasma cells and secreted to the gut lumen every day [53].

All types of T lymphocytes can be found in the gut. CD8$^+$ cytotoxic T cells are dominant among IEL and CD4$^+$ T helper (Th) cells among LPL [162]. CD8$^+$ T cells kill the infected or transformed cells when recognize the antigen presented by MHC-I molecule on the target cell surface [2]. CD4$^+$ T cells recognize the antigen presented by MHC-II molecule on APC and can differentiate into the diverse subtypes with specific cytokine and chemokine profiles [162]. These cells help the activation of the immune cells and mechanisms that will most efficiently fight the specific pathogen. In general, Th1 CD4$^+$ T cells help eliminating intracellular pathogens and produce IFN-γ, TNF-α and IL-12, while Th2 CD4$^+$ T cells produce IL-10, IL-13, IL-15 and IL-4 which contribute in defence to helmints [162] (Fig.3). The newest type of CD4$^+$ Th cells, Th17 cells are considered to help immunity against extracellular bacteria and parasites with the production of IL-17, IL-21 and IL-22 [162] (Fig.3).

1.2.4. Regulatory lymphocytes

Active suppression of immune responses by regulatory T cells is particularly important in the intestine and serves for preventing the immune reactions against self antigens (autoimmunity) and harmless antigens (e.g. commensal microflora or food antigens). The most studied type of regulatory T cells are Foxp3$^+$ CD4 T$_{reg}$ cells. These cells are characterized by constant expression of high levels of the transcription factor Foxp3 [71] which is considered to be necessary for their suppressor effects [47, 67]. Foxp3$^+$ CD4 T$_{reg}$ cells can suppress the proliferation of CD4 T cells in vitro [22, 43] and the differentiation of Th1 and Th2 effector lymphocytes [37, 54]. They also showed in vivo protective effects in mouse models of colitis [62, 122], arthritis [79], autoimmune diseases [68, 186] as well as in tumor treatment in humans [29, 83]. The importance of Foxp3$^+$ T$_{reg}$ cells is best seen in Foxp3$^{-/-}$ mice which develop lethal lymphoproliferative autoimmune syndrome [18]. TGF-β which is abundant in the normal healthy intestine [54, 71, 125] is considered to be absolutely required for the induction of Foxp3 expression and generation of T$_{reg}$ cells [22, 31, 54, 157, 183] (Fig.3). The mechanisms of suppression by Foxp3$^+$ T$_{reg}$ cells are largely
unknown, but some studies suggested a role of TGF-β in this process [126]. Other types of regulatory T cells are also described, as Th3 cells that are induced by oral tolerance and showed suppressive properties depended on their TGF-β secretion [37, 44, 91, 119] (Fig. 3). T regulatory type 1 (Tr1) cells are described as IL-10 producing cells (Fig. 3) that can be differentiated in vitro in the presence of antigen, IL-10 or interferons type I (IFN-I) [119]. Tr1 cells inhibited a number of inflammatory diseases in mice, including T cell transfer colitis via an IL-10 dependent mechanism [71], but they could not be isolated from the orally tolerized mice in vivo [119]. IL-10 is recognized as an anti-inflammatory cytokine that inhibits the release of pro-inflammatory cytokines, such as IFN-γ and IL-12, and therefore attenuates mucosal inflammation [51, 151]. Absence of IL-10 in IL-10−/− mice lead to the development of spontaneous colitis [88].

Figure 3. Types of CD4+ effector cells in intestine. After priming by antigen presenting cells naïve CD4+ T cell can differentiate into several effector cell types. Depending on the type of antigen presented, antigen presenting cells are able to give rise to the effector cell type that will ensure the best defense against a given pathogen. In general, intracellular pathogens induce the differentiation of Th1 cells, parasites induce Th2 cell differentiation, while extracellular pathogens give rise to Th17 cells. In healthy intestine luminal antigens normally promote the induction of one of the regulatory cell types (Foxp3+ Treg, Th3 or Tr1 cells).
1.3. Lymphocyte traffic in the gut

Naive T and B cells migrate from their sites of lymphopoiesis (thymus and bone marrow) through the bloodstream and enter the secondary lymphoid organs (SLO). SLO are the inductive sites in the intestine where lymphocytes can either recognize the antigen or continue to re-circulate through the body [103]. Therefore naive lymphocytes express high levels of L-selectin (CD62L) and chemokine receptor CCR7 that recognize SLO-expressed addressin and the chemokines CCL19 and CCL21, respectively [40, 60, 118]. If no antigenic stimuli have been encountered in the SLO, naive lymphocytes leave the SLO through efferent lymphatics after average residence time of 6-10 h for T cells and 12-24 h for B cells [57]. T cell egress depends on the expression of sphingosine 1-phosphate receptor type 1 (S1P₁) on the lymphocyte surface and its interaction with the ligand sphingosine 1-phosphate (S1P) that is abundant in the lymph [17, 56, 98, 109, 158]. After recognizing antigen activated lymphocytes can be retained for longer time inside the SLO, but must also exit through efferent lymphatics to reach effector sites [56]. Specialized combinations of receptors target activated lymphocytes to particular effector site, such as gut, skin or lungs [161]. Gut homing lymphocytes express the chemokine receptor CCR9 and the integrin α4β7 that bind CCL25 and mucosal addressin cell adhesion molecule-1 (MadCAM-1), respectively [40, 60]. The expression of these receptors is imprinted to the lymphocytes during an activation process in the SLO by antigen presenting DC [40, 60, 118, 161]. DC of different SLO are adapted to process local environmental factors that help them in the imprinting of lymphocyte homing receptors. DC of the intestinal inductive sites process food-derived vitamin A to the metabolic active form of RA which is necessary for the imprinting of gut homing receptors on the lymphocytes [60, 118, 161, 175]. Various chemokines secreted by intestinal epithelial cells and activated immune cells also contribute to the recruitment of specific immune cells to the inflamed gut [135]. T cell homing to the gut help the intestinal immune system in clearance of invading pathogens.
1.4. Oral tolerance

In healthy individuals inflammatory immune responses to the food- or commensal microflora-derived antigens are rare although T cell reactivity against these antigens has been described [38]. These antigens normally induce an antigen-specific state of systemic immunologic unresponsiveness, a phenomenon called oral tolerance [156]. CD4+ T cells can transfer oral tolerance \textit{in vivo} and therefore are considered the main carriers of this phenomenon [25, 63]. MLN are considered as the location where oral tolerance is induced because mice lacking MLN cannot be orally tolerized [139, 164, 182].

In mice oral tolerance can be established after oral administration of either a single high dose of antigen (> 20 mg) or repeated lower doses of antigen (100 ng – 1 mg) [110] followed by resting period of several days and subsequent immunization of animals with the same antigen, usually with adjuvant (Fig.4). High doses are considered to induce anergy and/or deletion of effector lymphocytes [51, 110, 139] (Fig.4). Deletion occurs by CD95 (Fas)-dependent induction of cell apoptosis, while anergy occurs through TCR engagement with inadequate co-stimulation [110]. Low dose tolerance is considered to be mediated by active suppression of effector lymphocyte immune responses by regulatory T cells [51, 110, 139] (Fig.4). Induction of Foxp3\textsuperscript{+} T\textsubscript{reg} cells [121, 172] and Th3 regulatory cells [44, 61, 119] after oral tolerance establishing is well documented while the role of Tr1 cells in this phenomenon is not yet clarified [110, 119].

The physiological role of oral tolerance is in maintaining the intestinal homeostatis and preventing immunopathologies in the gut [51], but oral tolerance can also have therapeutic implications [44]. Protective effects of oral tolerance induction are shown in murine models of autoimmune and inflammatory diseases such as experimental autoimmune encephalomyelitis [24, 25], arthritis [188], colitis [128] and diabetes [14].
Figure 4. Oral tolerance. A. Oral tolerance can be induced by small or high dose antigen (e.g. chicken ovalbumin protein (OVA)) feeding, followed with resting period and subsequent immunization with the same antigen. Control animals are usually fed with phosphate buffered saline (PBS) instead of antigen. If tolerance is established immune responses measured after the immunization should be lower compared to the control fed with placebo. Modified from Macpherson AJ and Smith K 2006 J Exp Med. Mechanisms of oral tolerance establishing are depending of the antigen dosage during feeding. B. High dose of antigen is considered to induce deletion and/or anergy of the effector cells. C. Low dose antigens are connected with the induction of regulatory cells that can actively suppress the immune responses. Regulatory cells can function through secreted immunosuppressive cytokines, cognate inhibitory receptors or surface-bound immunosuppressive cytokines. Taken from Mayer L and Shao L 2004 Nat Rev Immunol
1.5. Inflammatory bowel disease (IBD)

1.5.1. Manifestation

Breakdown of oral tolerance, disruption of the mucosal barrier and/or defects in the mucosal immune system of the gut may lead to the severe pathology as observed in patients with IBD [51, 85, 175]. IBD is an idiopathic chronic inflammatory disorder of the gut in which patients suffer from rectal bleeding, severe diarrhea, abdominal pain, fever, weight loss and fatigue [84, 138]. The onset of IBD typically occurs in the second or third decade of life and majority of the affected individuals progress toward the chronic inflammation [183].

IBD include two major types of intestinal disorders: Crohn’s disease (CD) and ulcerative colitis (UC) [1]. CD can be patchy and segmental and usually considers transmural inflammation of ileum and colon although it can affect any region of the intestine [1, 183]. UC typically represent inflammation of the mucosal layer of intestine and involves rectum, but it can spread even to the entire colon in an uninterrupted pattern [1]. CD can be associated with intestinal granulomas and fistulae which are not common findings in UC patients [1, 167].

1.5.2. Pathology

Initiation and progression of IBD involves the complex interactions between genetic, immune and environmental factors [84]. Familial clustering of cases and twin studies have established a role of genetic factors in IBD induction [1] and until now 71 risk loci were connected with CD and 47 risk loci were identified in UC [80]. Genome wide studies and animal experiments showed that disrupted mucosal barrier and deregulated immune responses contribute to the pathology of IBD.

Beside the defects in tight junctions, frequent histopathological findings in IBD are Goblet cell loss and muco-depletion [50] (Fig.5). MUC2−/− mice are deficient in Goblet cells and develop spontaneous colitis [1, 183]. The endoplasmatic reticulum stress is increased during IBD in secretory cells of the intestinal epithelium causing the defective function of especially Paneth cells and contributing to the pathology [76] (Fig.5).
The hallmark of active IBD is a pronounced infiltration of innate and adaptive immune cells into the LP (Fig. 5) which leads to the elevated levels of pro-inflammatory mediators, such as TNF-α, IL-1, IFN-γ, IL-23, IL-17 [1]. The role of the innate immune system in the pathogenesis of IBD is highlighted with the finding of the genes encoding PAMP receptors among IBD susceptibility genes. Gene encoding nucleotide oligomerization domain 2 (NOD2), a protein which serve as intracellular sensor of bacterial peptidoglycan is associated with CD [1]. Autophagy enables cells to degrade intracellular components including pathogens and it is considered to contribute to the intestinal tolerance [1]. Gene encoding autophagy related 16 like protein 1 (ATG16L1) is also associated with CD [147]. Moreover, IBD is connected to the defects of regulatory mechanism in intestine since the gene encoding inhibitory cytokine IL-10 is associated with UC [49] and defective IL-10R signalling was connected to the very early onset of IBD in children [13]. Furthermore, IL-10−/− mice develop spontaneous colitis [88]. In general, CD is associated with excess in IL-12/IL-23 and IFN-γ/IL-17 production [167] indicating deregulated Th1/Th17 responses [16, 175], while UC is considered to be Th2-dependent disease associated with increased IL-13 production [167, 175]. The genetic association between the gene encoding IL-23R and IBD is reported [39]. Since IL-23 is considered to contribute to the expansion and survival of Th17 cells [1, 183] this finding possibly implicate Th17 pathway with the pathogenesis of IBD.

The potential role of commensal bacteria in the IBD pathogenesis is suggested after the observation that mice housed in GF conditions do not develop colitis [1, 183]. Although certain alterations of intestinal microflora are observed in the IBD patients (such as depletion and reduced diversity of bacteria from phyla Firmicutes and Bacteroidetes [1]), it is not clarified weather this is the cause or the consequence of the disease.

1.5.3. Treatment

The successful treatment of IBD would represent the combination of antibiotics, anti-inflammatory agents and re-induction of oral tolerance [84, 167, 175]. Antibiotics and probiotics are useful in the treatment of some patients with UC, but have very limited effects in the patients with CD [140, 165].
Corticosteroids as non-specific anti-inflammatory agents are used for the treatment of the IBD patients with severe disease manifestations, but their efficiency is limited due to serious side effects and no proven maintenance benefit shown in either CD or UC patients [140]. Treatment with monoclonal antibodies

Figure 5. Pathogenesis of the inflammatory bowel disease. Alterations in both mucosal barrier and mucosal immune responses are seen in inflammatory bowel disease patients and animal models. Loss of Goblet cells and disruption of the mucus layer together with disrupted tight junctions and reduced secretion of antimicrobial factors by Paneth cells lead to the penetration of the luminal bacteria into the epithelial layer. Furthermore, large infiltrations of inflammatory cells (mainly macrophages and all types of effector CD4+ T cells) into the intestinal mucosa and increased production of pro-inflammatory cytokines and chemokines are observed during inflammatory bowel disease.
(mAb) that block the pro-inflammatory cytokine TNF-α is particularly effective in CD patients, but this therapy is often limited by loss of efficacy [1].

Strategies that block the recruitment of leukocytes to the sites of inflammation represent a potentially potent treatment of inflammatory diseases. The first migration-inhibitory drug approved, the anti-α4 integrin mAb, is proved to be efficient in the treatment of multiple sclerosis and CD [99]. However, block of α4 integrin increases the susceptibility to any infection, showing the need for tissue-specific migration inhibitors [98]. A possibility of cell-specific migration inhibitors is also being investigated as the mAb to chemokine CXCL-10, a ligand for the CXCR-3 receptor expressed on Th1 cells, is now in clinical trials for UC treatment [99]. The inhibitor of lymphocyte egress from SLO, FTY-720 (fingolimod), is a new promising anti-inflammatory drug [99]. FTY-720 acts as an agonist for S1P family receptors, it sequesters lymphocytes in SLO causing lymphopenia and preventing lymphocyte migration to the site of inflammation [57, 99, 149, 163]. FTY-720 is effective in the treatment of multiple sclerosis [74] and protective effects of this and other S1P receptors agonists (KRP-203 and W-061) are shown in animal models of IBD [34, 115, 149, 163]. One potential side effect of FTY-720 treatment is bradycardia [74] due to expression of S1P3 receptor on the heart [153]. Hence, development of the agonists specific for the S1P1 receptor, the dominant subtype expressed on lymphocytes [153], is needed [99].

1.6. Interferon type I system

One of the agents that were successful in the clinical trials with UC patients is IFN-β [136]. IFN-β is a member of the IFN-I cytokine family, together with numerous other members among which the most important are IFN-α subtypes [171]. IFN-I are originally described as cytokines produced after viral infection, but their role in the regulation of cell growth and differentiation as well as in the modulation of immune responses is also recognized [78, 171, 185]. IFN-I production is triggered by engagement of PAMP receptors, usually certain types of TLR [171]. Although all cells can produce IFN-I after viral infection, myeloid cells (plasmacytoid DC, conventional DC and macrophages) are considered to be
Figure 6. Pro-inflammatory effects of type I interferons. Cytokines from the type I interferon (IFN) family have effects on both innate and adaptive immune responses. They induce the maturation of dendritic cells (DC) by up-regulation of major histocompatibility complex (MHC) and co-stimulatory molecules surface expression. Type I IFN also activate cytotoxic CD8\(^+\) T cells, natural killer (NK) cells and cytolytic functions of the macrophages and promote Th1 differentiation of CD4\(^+\) T cells. Production of the pro-inflammatory cytokines, such as IL-12, IFN-\(\gamma\) and TNF-\(\alpha\) by DC, Th1 cells, NK cells and macrophages is also promoted by type I IFN.

The major IFN-I producers [78, 114, 171]. Generally, IFN-I promote immunity via stimulation of pro-inflammatory cytokines and chemokines production, induction of DC maturation by up-regulation of co-stimulatory and MHC molecules expression, promoting the activation and cytotoxic functions of CD8\(^+\) T cells, NK cells and macrophages, inhibiting Th2 and promoting Th1 responses of CD4\(^+\) T cells and extending the survival of activated lymphocytes [78, 94, 171, 185]. Therefore, IFN-I mediate the induction of both innate and adaptive immune responses [78, 94] (Fig.6) in the manner that will ensure successful elimination of viral pathogens. On the other hand, IFN-I can also suppress immune reaction and inflammation under specific conditions. It is reported that they can suppress the proliferation and promote apoptosis of activated T cells [171, 185]. Beneficial effects of the IFN-I treatment are shown in many human diseases such as multiple sclerosis, rheumatoid arthritis, myasthenia gravis, autoimmune haemolytic anemia, thyroiditis, chronic recurrent multifocal osteomyelitis, some tumors and as mentioned above IBD [171, 185]. The mechanisms of immunosuppressive effects
of IFN-I are not well understood, but it is considered that beside the suppression of cell proliferation, IFN-I can inhibit the production and/or activity of some pro-inflammatory cytokines, such as IL-12, IL-1, TNF-α and under specific conditions IFN-γ [185]. A recent study showed that IFN-β treatment of multiple sclerosis is effective if disease is induced by Th1, but not if it is induced by Th17 cells [8], although an other group showed that IFN-β inhibits Th17 cell differentiation [142]. It seems that IFN-I have many diverse effects on the immune responses and these effects depend on the various locally present factors.

1.7. CD69 molecule

1.7.1. General information

CD69 is a type II transmembrane protein of a C-type lectin domain (CTLD) family [59, 93, 190]. This disulfide-linked homodimer consists of 28 and 32 kDa subunits resulting from different glycosylation pattern [93]. The gene encoding CD69 protein in mouse genome is located on the chromosome 6 and in human genome on chromosome 12, in the NK gene cluster of both species [190]. Unlike other members of this gene cluster CD69 seems not to have role in the NK cell functions and their recognition of the target cells [169, 190]. The ligand for surface receptor CD69 is not yet known. Cristalography studies of CD69 extracellular domain suggested that the ligand can not be sugar due to the absence of the Ca²⁺-coordinating residues that mediate the classical C-type lectin sugar binding site [27, 93]. Therefore it is considered that the CD69 ligand is most probably a protein [93]. Requirement for molecular crosslinking of CD69 in order to generate signal transduction suggests that CD69 may act as a receptor for cell-associated ligands, rather than for soluble ones [169].

1.7.2. Expression pattern

CD69 is constitutively expressed on monocytes, platelets, Langerhans cells and a small percentage of resident lymphocytes in the thymus [93]. In addition CD69 expression is induced very early upon activation of T and B lymphocytes NK cells, macrophages, neutrophiles and eosinophiles [93, 123]. It is reported that
Figure 7. CD69 molecule. A. CD69 is membrane-bound protein, a homodimer of two (28 and 32 kDa) differentially glycosilated subunits. Each subunit consists of extracellular C-type lectin domain (CTLD) connected by the short neck region with the single spanning transmembrane domain and short cytoplasmatic tail. Subunits are connected with the disulfide bridge in the extracellular neck region. B. Ribbon diagram of CD69 C-type lectin domain. Secondary structure elements are shown in blue for β-strand and in red for α-helix, the disulphide bridges are represented by green ball-and-stick models. Taken from Llera AS et al 2001 J Biol Chem
surface expression of CD69 on activated T cells is detectable 2 h after stimulation, reaches peak levels by 18-24 h and declines after that [169]. Activation-induced CD69 expression precedes the expression of other cell activation markers, such as CD25 [143]. Therefore, CD69 represents the first cell surface glycoprotein detected after lymphocyte activation [170]. Only an extremely small number of circulating NK and T cells express low levels of CD69 in normal healthy individuals [169]. On the other hand, most of the gut IEL and LPL as well as T cells in lymph nodes express high levels of CD69, as expected from the lymphocytes continuously exposed to antigen challenge [33, 169].

1.7.3. Role in the immune responses

CD69 function within the immune system is not well understood, but various studies showed its involvement in the immune responses. CD69 inhibited T cell egress from the thymus as demonstrated by transgenic overexpression studies [46, 127]. Furthermore, CD69 is involved in regulation of T and B lymphocyte egress from SLO by direct interaction and blockade of the S1P₁ receptor [10, 57, 158]. IFN-I strongly induce CD69 expression which mediate their lymphopenic effect by internalization of S1P₁ receptor and subsequent retention of lymphocytes in the SLO [57, 158]. Engagement of CD69 by crosslinking antibodies led to the increased TGF-β production by the lymphocytes [41, 91, 152]. CD4⁺ CD69⁺ T cells inhibit the secretion of pro-inflammatory cytokines and proliferation of CD4⁺ CD69⁻ T cells in a process partially dependent on TGF-β1 [61]. Recent work in mice indicates a role of CD69 in the regulation of arthritis [123, 152], asthma [106, 112], myocarditis [30], pathogen clearance [176] and tumour immunity [41, 42]. Furthermore, single nucleotide polymorphisms in the CD69 gene are recently associated with the development of type I diabetes [12]. T cells in the inflamed mucosa are characterized by high expression of the activation antigen CD69 [160], but its role in the mucosal immune system is largely unknown.
1.8. Aim of the study

Wide expression of CD69 by the immune cells implicates an important role of this molecule in immune responses. Although discovered decades ago as the early activation antigen, the role of CD69 within the immune system is not yet clarified.

Since immune cells in the intestine showed high expression of CD69 [33, 169], I aimed to investigate the role of this surface receptor in the mucosal immune system of the GIT.

- To analyze the influence of intestinal microflora on the CD69 expression by CD4 T cells using SPF, GF and microflora-depleted SPF mice of different mouse strains.

- The cytokine profiles of CD4 T cells after *in vitro* CD69 activation were compared with the cytokine profiles of CD69-deficient CD4 T cells.

- CD69-dependent migration of CD4 T cells was analyzed both *in vitro* and *in vivo*.

- Finally, the role of CD69 in the intestinal immune system *in vivo* was investigated using the experimental models of oral tolerance and IBD induction.
2. Materials and methods

2.1. Mice

Inbred C57BL/6J (B6) mice, as well as RAG<sup>−/−</sup> (RAG<sup>tm1Mom</sup>) mice, CD69<sup>−/−</sup> mice, IFN-I receptor 1-deficient (IFNAR<sup>−/−</sup>) mice, transgenic OT-II, OT-II x RAG<sup>−/−</sup> and OT-II x CD69<sup>−/−</sup> mice, CD45.1 (B6.SJL-Ptprc<sup>a</sup> Pep3<sup>b</sup>/BoyJ) mice and B6.Cg-Tg (CAG-DsRed*MST) 1 Nagy/J (all on the B6 background) mice were bred and kept under specific pathogen-free (SPF) conditions in the animal facility of Ulm University (Ulm, Germany). Immunodeficient RAG<sup>−/−</sup> animals lack the cells of adaptive immunity (T and B lymphocytes) while innate immune system is intact [116]. CD69<sup>−/−</sup> mice do not express surface activation marker of leukocytes, CD69 [112, 123], while IFNAR<sup>−/−</sup> animals do not express surface receptor for IFN-I [36]. In OT-II, OT-II x RAG<sup>−/−</sup> and OT-II x CD69<sup>−/−</sup> animals CD4 T cells express a T cell receptor (TCR) specific for an epitope in chicken ovalbumin (OVA) protein [11]. Hence, these mice are used as models for investigating antigen-specific responses of CD4 T cells in the presence (OT-II [36]) or absence (OT-II x RAG<sup>−/−</sup> [133]) of CD8 T cells or in the absence of CD69 expression (OT-II x CD69<sup>−/−</sup>). CD45 or protein tyrosine phosphatase receptor type C is expressed on immune cells and it is known as leukocyte common antigen. Normal mice express the CD45.2 antigen, but CD45.1 mice strain express an unusual CD45 antigen on all the leukocytes. CD45.1 mice can hence be used in the cell transfer studies for easy differentiation between host and donor cells [148]. DsRed mice express the red fluorescent protein variant DsRed.MST under the control of the chicken beta actin promotor fused with the cytomegalovirus immediate early enhancer and all tissues of these animals express red fluorescent protein [178]. Germ free (GF) B6 mice were screened weekly for viral, bacterial and fungal contamination. Female and male mice were used at 6–12 wk of age. All animal experiments were performed according to the guidelines of the local Animal Use and Care Committee and the National Animal Welfare Law.
2.2. Cell isolation

Mice were sacrificed by inhalation of Forene (cat. no. 2594.00.00; Abbott, Wiesbaden, Germany) followed by cervical dislocation. Immune cells were isolated from the colonic and small intestine lamina propria, spleen and MLN of B6, CD69\(^{-/-}\), IFNAR\(^{-/-}\), RAG\(^{-/-}\), OT-II, OT-II x RAG\(^{-/-}\), OT-II x CD69\(^{-/-}\), CD45.1 and DsRed animals.

2.2.1. Isolation of spleen and MLN cells

Single-cell suspensions were prepared from spleen and MLN tissues by pressing them through a 40 µm cell strainer positioned on a 50 ml tube using the plunger of a 1 ml syringe and washing the strainer with phosphate buffered saline (PBS; cat. no. L182-50; Biochrom, Berlin, Germany) supplemented with 1% heat inactivated fetal bovine serum (FBS; cat. no. 10500-064; Invitrogen, Darmstadt, Germany). Red blood cells from spleen were lysed by 10 min incubation on 37ºC with 5 ml of Lysebuffer (0.16 M NH\(_4\)Cl and 0.17 M Tris, pH 7.2). Immune cells were resuspended in PBS/1%FBS.

2.2.2. Isolation of small intestine and colon lamina propria cells

Small intestine and colon were opened longitudinally and cut into smaller segments (8-10 segments from small intestine and 3-4 from colon). Segments were than washed with PBS/1%FBS to remove debris and mucous. The epithelium was removed by incubation at 37°C for 10-15 min under gentle shaking with 1 mM dithiothreitol (and 1 mM ethylenediaminetetraacetic acid (EDTA) for colon tissue) in 25 ml PBS supplemented with 1% FBS. The remaining tissue was washed in PBS/1%FBS to remove residual epithelial cells, and the supernatants were discarded. The full length segments of small intestine and cut colon segments (2 x 2 mm pieces) were digested with 0.25 mg/ml collagenase type VIII from Clostrodium histolyticum (cat. no. C-2139; SigmaAldrich, St. Louis, MO) for 30-45 min at 37°C in 50 ml of Roswell Park Memorial Institute (RPMI) medium (RPMI powder (cat. no. A2044, 9050; AppliChem, Darmstadt, Germany), 0.01 M NaHCO\(_3\) and 0.01 M Hepes in dH\(_2\)O, filtered under pressure) under shaking. Supernatants were collected, from which LP lymphocytes were pelleted. LP lymphocytes were resuspended in RPMI medium containing 35% Percoll (density
1.124 g/ml; cat. no. L-6145; Biochrome). This cell suspension was overlaid onto 70% Percoll and centrifuged for 20 min at 750 x g. Viable cells at the 35%/70% Percoll interface were collected and washed twice with PBS/1% FBS.

2.2.3. CD4⁺ T cell enrichment

Spleen cells were enriched for CD4⁺ T cells via magnetic-activated cell sorting (MACS) isolation kit (cat. no. 130-090-860; Miltenyi Biotec, Bergisch Gladbach, Germany) using the principle of negative selection. In negative selection procedure cells that are not of interest (here non-CD4⁺ T cells, i.e. CD8⁺ T cells, B cells, DC, NK cells, monocytes, granulocytes and erythroid cells) are labelled using antibodies binding surface markers that are specifically expressed on these cells. These labelled cells will attach to the magnetic column during the separation process, while the cells of interest (here CD4⁺ T cells) cannot attach to the column and can be collected during column washing procedure as effluent that is passing through the column. Cell pellet was resuspended in 40 µl of cold (4-8°C) MACS buffer (PBS supplemented with 0.5% bovine serum albumin (BSA) and 2 nM EDTA) per 10⁷ total cells. 10 µl of biotin labelled antibody cocktail (containing anti-CD8, anti-CD11b, anti-CD45R, anti-DX5 and anti-Ter-119 binding CD8⁺ T lymphocytes, monocyte/macrophages, B lymphocytes, natural killer (NK) cells and erythroid cells respectively) per 10⁷ cells was added. After 15 min incubation on 4°C, 30 µl of cold MACS buffer and 20 µl of anti-biotin MicroBeads were added per 10⁷ total cells. After 20 min on 4°C, cells were washed twice with 10 ml and resuspended in 1 ml of cold MACS buffer. MACS LS Columns (cat. no. 130-042-401; Miltenyi Biotec) were placed in the magnetic field of MACS Separator and prepared by rinsing 3 ml of cold MACS buffer onto the column. Labelled cell suspension was added onto the column and column was washed 3 times with 3 ml of cold MACS buffer. Effluent passing through the column containing unlabelled CD4⁺ T cells was collected. Purity of isolated cells is > 95%.

2.2.4. Naive CD4⁺ CD25⁻ T cell enrichment

CD4⁺ T cells were enriched from total spleen cells via MACS isolation kit as described above. Naive CD25⁻ cells were enriched from CD4⁺ T cell population using MACS system and negative selection procedure. Cells were stained with 2
ng/10^7 cells of phycoerithrin (PE)-conjugated anti-CD25 PC61.5 mAb (cat. no. 12-0251-82; eBioscience, Frankfurt, Germany) in 100 µl of cold MACS buffer, incubated 20 min on 4°C and washed with 1 ml of cold MACS buffer. 80 µl of cold MACS buffer and 20 µl of anti-PE MicroBeads (cat. no. 130-048-801; Miltenyi Biotec) per 10^7 cells were added. After 15 min on 4°C, cells were washed twice with 10 ml and resuspended in 500 µl of cold MACS buffer. MACS MS Columns (cat. no. 130-042-201; Miltenyi Biotec) were placed in the magnetic field of MACS Separator and prepared by rinsing 500 µl of cold MACS buffer onto the column. Labelled cell suspension was added onto the column and column was washed 3 times with 500 µl of cold MACS buffer. Labelled CD25+ CD4+ T cells were attached to the column while, effluent passing through the column with the fraction of unlabelled CD4+ CD25- naïve T cells was collected.

2.2.5. CD11c+ cell enrichment

CD11c+ DC were isolated from total spleen cells via CD11c MicroBeads (cat. no. 130-052-001; Miltenyi Biotec) using MACS technology and positive selection strategy. In positive selection procedure, cells of interest are labelled using the antibody against surface marker specifically expressed on these cells (here it was anti-CD11c antibody). During separation procedure, cells of interest will attach to the magnetic column and all the other cells will be washed out from the column during washing steps. After removal of the column from the magnetic field, cells of interest can be flushed out from it. Total spleen cells were resuspended in 40 µl of cold MACS buffer and 10 µl of CD11c MicroBeads per 10^7 cells. After 15 min incubation at 4°C, cells were washed twice with 10 ml and resuspended in 1 ml of cold MACS buffer. MACS LS Columns in the magnetic field of MACS Separator were used for separation. They were prepared by rinsing 3 ml of cold MACS buffer onto the column. Labelled cell suspension was added onto the column and column was washed 3 times with 3 ml of cold MACS buffer. Column was removed from the separator and placed on a 15 ml collection tube. 1 ml of MACS buffer was added onto the column and the labelled CD11c+ cells retained in the column were immediately flushed out by firmly pushing the plunger into the column. Purity of isolated cells is > 95%.
2.3. Flow cytometry (FCM) analysis

Four-color FCM analyses were performed using a FACSCalibur (BD Biosciences, Heidelberg, Germany). The forward narrow angle light scatter was used as an additional parameter to facilitate the exclusion of dead cells and aggregated cell clumps. Non-stained CD4 T cells were used as negative control. Data were analyzed using FCS Express V3 software.

2.3.1. Extracellular staining

Cells were washed twice in PBS supplemented with 0.3% BSA and 0.1% sodium azide. Non-specific binding of antibodies to Fc receptors was blocked by preincubation of cells with mAb 2.4G2 (cat. no. 01241D; BD Biosciences) directed against the FcγRIII/II CD16/CD32 (0.5 ng mAb/10⁶ cells). Cells were washed and incubated with 1 ng/10⁶ cells of the relevant mAb for 20 min at 4°C and washed again twice. In most experiments, cells were subsequently incubated with 1 ng/10⁶ cells of the second-step reagent for 20 min at 4°C. Following reagents and mAbs from eBioscience were used: fluorescein isothiocyanate (FITC)-conjugated mAb binding CD4 GK1.5 (cat. no. 11-0041-86), allophycocyanin (Apc)-conjugated mAb binding CD4 GK1.5 (cat. no. 17-0041-83); PE-conjugated mAb binding CD69 H1.2F3 (cat. no. 12-0691-82) and IL-21R eBio4A9 (cat. no. 12-1219); biotinylated mAb binding CD25 PC61.5 (cat. no. 13-0251-81) and CD69 H1.2F3 (cat. no. 13-0691-81). From BD Biosciences, following biotinylated mAbs were used: anti–CD103 M290 (cat. no. 557493), anti–CD122 TM-β1 (cat. no. 559884), anti–IFN-γR GR20 (cat. no. 550482) and anti–ICOS 7E.17G9 (cat. no. 552145) as well as PE-conjugated anti–IL-10R1 1B1.3a (cat. no. 559914). The biotinylated mAb binding TGFβRII (cat. no. BAF532) and Apc-conjugated anti-latency associated peptide (LAP)/TGF-β1 mAb 27232 (cat. no. FAB2463A) were purchased from R&D Systems, Wiesbaden, Germany. As a second-step reagent Streptavidin-Peridinin Chlorophyll Protein (PerCP)-Cy5.5 (cat. no. 45-4317-80; eBioscience) was used.

2.3.2. Intracellular staining

Cells were isolated, washed with PBS supplemented with 0.3% BSA and 0.1% sodium azide and stained extracellularly with FITC-conjugated anti-CD4 mAb
binding CD4 GK1.5 (cat. no. 11-0041-86; eBioscience) as described above. Surface-stained cells were fixed with 4% paraformaldehyde (cat. no. P6148-500G; Sigma Aldrich, Steinheim, Germany) for 15 min in the dark, then pelleted and resuspended in 200 μl of Fixation/Permeabilization buffer (for working concentration of the buffer Fixation/Permeabilization concetrate (cat. no. 00-5123-43; eBioscience) and Fixation/Permeabilization Diluent (cat. no. 00-5223-56; eBioscience) were mixed in the ratio 1:3). After overnight incubation at 4°C in the dark, permeabilized cells were washed twice with 1 x Permeabilization buffer (10 x Permeabilization buffer (cat. no. 00-8333-56; eBioscience) was diluted with sterile dH₂O) and incubated for 15 min, 4°C with mAb 2.4G2 directed against the FcγRIII/II CD16/CD32 (0.5 ng mAb/10⁶ cells) for preventing non-specific binding of the antibodies. Cell were pelleted and after discarding the supernatant incubated for 30 min at 4°C, dark with 0.5 ng/10⁶ cells of the following PE-conjugated Abs: anti–Foxp3 FJK-16s (cat. no. 12-5773-80; eBioscience), anti–T-bet eBio4B10 (cat. no. 12-5825-80; eBioscience) anti–GATA-3 TWAJ (cat. no. 12-9966-42; eBioscience), anti–RORγt AFKJS-9 (cat. no. 12-6988-82; eBioscience). All PE-conjugated antibodies were diluted in 1 x Permeabilization buffer. Stained cells were washed once with 1 x Permeabilization buffer, once with PBS supplemented with 0.3% BSA and 0.1% sodium azide, resuspended in PBS supplemented with 0.3% BSA and 0.1% sodium azide and analyzed on FACSCalibur.

2.4. Cytokine detection by enzyme-linked immunosorbent assay (ELISA)

Cytokines in supernatants and blood serums were detected by a conventional double-sandwich ELISA. This system uses Maxisorp immuno plates (cat. no. 442404; NUNC, Roskilde, Denmark) with adsorptive polystyrene surface at the bottom of the wells. This surface captures the purified antibody (50 ng /well) directed against the certain cytokine when applied in coating buffer (0.1 M Na₂HPO₄ x 2H₂O in PBS supplied with 10% FBS, pH9) into the wells and incubated on 4°C overnight. Unspecific binding of cytokines is prevented by adding 200 μl/well of PBS/3% BSA and incubation 1 h at room temperature (RT). Cytokines from the samples (50 μl of the sample per well, 3 h incubation on RT)
added after that to the wells will bind the captured antibodies. Biotinylated antibodies directed against the specific cytokine were used for detection (50 ng Ab/well, 1 h, RT) and traced by streptavidin conjugated alkaline phosphatase (cat. no. 016-050-084; Jackson Immuno Research, Dianova, Hamburg, Germany), 1 µl of phosphatase stock diluted in 1 ml of PBS/3%BSA, 50 µl of this dilution per well, 30 min incubation on RT. This step was followed by the addition of 1 mg/ml of the alkaline phosphatase substrate 4-Nitrophenyl phosphate disodium salt hexahydrate (cat. no. 71768-5G; Sigma) diluted in substrate buffer (97 ml diethanolamin, 0.8 g MgCl x 6H2O, 0.2 g sodium azide in 1 liter dH2O, pH9.8), 10 min incubation on RT in the dark. Enzyme reaction was stop by adding 50 µl/well of 0.5 M EDTA. The intensity of the developed colour was measured at 405/490 nm on a TECAN microplate-ELISA reader using EasyWin software (both from Tecan, Wetzlar, Germany). Cytokine levels were quantified by the same software using eight standard concentrations of the purified specific cytokine and presented as pg/ml. The following mAbs (from BD Biosciences) were used for detection and capture: mAb R4-6A2 (cat. no. 551216) and biotinylated mAb XMG1.2 (cat. no. 554410) for IFN-γ, mAb TC11-18H10 (cat. no. 555068) and biotinylated mAb TC11-8H4.1 (cat. no. 555067) for IL-17A and mAb TN3-19.12 (cat. no. 557516) and biotinylated mAb C1150-14 (cat. no. 557432) for TNF-α. TGF-β1 concentration was measured by the human/mouse TGF-β1 ELISA Ready-SET-Go Kit (cat. no. 88-7344; eBioscience) using the same double-sandwich system, but a different buffers (Coating buffer provided in the kit as powder, diluted in 1 liter of dH2O, kept on the RT and filtered using a 0.22 µm filter prior every use and for blocking the unspecific binding and diluting the antibodies 5 x Assay diluent was provided in the kit and diluted with dH2O until working concentration of 1 x Assay diluent), enzyme for detection step (avidin conjugate horseradish peroxidase (HRP) that binds to biotin) and the suitable substrate (3,3´-5,5´-tetramethylbenzidine). Capturing and detection antibodies as well as the enzyme working solutions were made by the instructions of the kit manufacturer (4 µl of stock solution from the kit diluted in 1 ml of 1 x Assay diluent) and 100 µl of working solutions were added per well. In the last step 100 µl/well of substrate was added and the plate was incubated 15 min on the RT. Enzyme reaction was stoped by adding 50 µl/well of 2 N H2SO4. For activation of TGF-β1 100 µl of
each sample was treated with 20 µl 1 N HCl for 10 min and the reaction was neutralized by 20 µl of 1 N NaOH just before addition of the samples to the MaxiSorp plates. 100 µl of activated samples were used for ELISA and concentrations of TGF-β1 were calculated for the dilution factor 1.4.

2.5. qRT-PCR

2.5.1. Ribonucleic acid (RNA) isolation

RNA was prepared from approximately 30 mg of snap frozen colon, small intestinal or MLN tissue. In in vitro experiments RNA was obtained from 10^7 total or CD4^+ enriched spleen cells. In both cases RNAeasy mini kit (cat. no. 74904; Qiagen, Hilden, Germany) was used for RNA isolation. To inhibit RNase enzymes that are potentially present at the working surface, 1% sodium dodecyl sulphate (SDS; cat. no. 71725, Fluka, Sigma Aldrich) dilution was used to clean it. Frozen tissue samples were placed in 600 µl of RLT buffer (highly denaturing guanidine-thiocyanate buffer, provided in RNAeasy mini kit) containing 1% β-mercaptoethanol (cat. no. 161-0710; Bio Rad, Munich, Germany) and homogenized using rotor-stator homogenizer, while cells were disrupted only by adding 600 µl of RLT/1% β-mercaptoethanol buffer. For shearing the high-molecular-weight cellular compartments and creating a homogenous lysate, samples were than pipetted into QIAshredder columns (cat. no. 79656; Qiagen) placed in 2 ml collection tube and centrifuged in microcentrifuge 2 min at full speed. To provide appropriate binding conditions, 600 µl of 70% ethanol was added to the clear lysate in the collection tube, mixed well by pipetting and transferred to RNeasy spin columns (provided in RNAeasy mini kit). These columns contain a silica-based membrane which selectively binds total RNA and contaminants are efficiently washed away with buffers. Columns were placed in new 2 ml collection tubes and centrifuged for 15 seconds at 100 x g. Flow-through from collection tube was discarded, 350 µl of RW1 buffer (provided in RNAeasy mini kit) added to the column and centrifuged for 15 seconds at 100 x g. Flow-through was discarded and contaminating genomic deoxyribonucleic acid (DNA) was eliminated from samples by 15 min incubation on the RT with 10 µl of RNase
– free DNase I (cat.no. 1010395; Qiagen) diluted in 70 µl of RDD buffer (supplied in DNase I kit). Column was washed with 350 µl RW1 buffer and than twice with 500 µl of RPE buffer (provided in RNAeasy mini kit), centrifuging for 15 seconds and 2 min after last washing at 100 x g. For eluting RNA, columns were placed in new 1.5 ml collection tubes, 30 µl of RNase free H$_2$O was added directly to the column membrane and after 2 min incubation on RT, columns were centrifuged for 1 min at 100 x g. For increasing the RNA yield from the cells (especially from CD4 T cells which are small cells and do not have a high amount of total RNA), same 30 µl of RNase free H$_2$O used for the first elution step was added again to the column membrane, incubated 2 min on RT and centrifuged for 1 min at 100 x g. The yielded RNA concentration was measured by NanoDrop 1000 (Thermo Scientific, Schwerte, Germany).

2.5.2. Reverse transcription

A total of 2 µg of RNA isolated from tissues or 200 ng of RNA isolated from cells was reverse transcribed with SuperScript II Reverse Transcriptase (cat. no. 18064-014; Invitrogen). RNA was mixed with 100 ng of random primers (cat. no. 48190-011; Invitrogen), 0.5 mM each final dilution of deoxinucleoside triphosphate (dNTPs, cat. no. U1240; Promega, Mannheim, Germany) and dH$_2$O until 13 µl final volume. This mixture was incubated 5 min at 65°C to denature RNA secondary structure and than quickly chilled on ice to let the primers anneal to the RNA. 200 units of reverse transcriptase was added together with 4 µl buffer and 2 µl 0.1 M DTT as RNase inhibitor (both supplied in SuperScript II kit). This reaction was incubated for 50 min at 42°C, optimal temperature for reverse transcriptase activity, and than enzyme was inactivated by incubation 15 min at 70°C. For quality inspection, obtained complementary deoxyribonucleic acid (cDNA) was amplified with polymerase chain reaction (PCR) using β-actin primers (Thermo Fisher Scientific, Ulm, Germany) and analyzed by agarose gel electrophoresis. HotStar Taq Plus Master Mix Kit (ca. no. 203645; Qiagen) was used for PCR. Each reaction contained: 10 µl of HotStar Taq Plus Master Mix (containing HotStar Taq Plus DNA polymerase, unique PCR buffer and dNTPs); 3 µl of CoralLoad concentrate (provided in HotStar Taq Plus Master Mix), containing two gel tracking dyes; 0.1 µl of 100 pmol stock β-actin primer forward (5’GGG AAT
GGG TAC GAA GGA CT 3’); 0.1 µl of 100 pmol stock β-actin primer reverse (5’TGT GAT GTC ACG CAC GAT TT 3’), 5.8 µl of dH₂O and 1 µl of sample or 1 µl of dH₂O as negative control. Temperature conditions for the PCR reaction were the following: 95°C 2 min for initial denaturation; 30 cycles of 94°C 1 min, 58°C 1 min, 72°C 1.5 min; 72°C 3 min for final elongation. For successfully amplified cDNA one clear band on agarose gel was expected (Fig.8). cDNA was diluted with dH₂O in 1:8 ratio and used for quantitative real time (qRT)-PCR reactions.

![cDNA](image)

**Figure 8. cDNA.** Picture represents complementary DNA (cDNA) amplified by PCR and analyzed by agarose gel electrophoresis. Wells 1 – 13 contain cDNA obtained by reverse transcription of total tissue RNA isolated from colon. Well 14 contain dH₂O instead of cDNA (negative control). Samples were visualised by ethidium bromide and the image obtained by GeneSnap image acquisition software.

### 2.5.3. qRT-PCR reactions

SYBR Green qPCR Master mix (cat. no. PA-012-12; SABiosciences, Qiagen) containing SYBR Green 1 Dye, AmpliTaq Gold DNA Polymerase, dNTPs with dUTP, and optimized buffer components was used for amplification and detection. qRT-PCR reactions were performed in MicroAmp Fast Optical 96-well Reaction Plate (cat. no. 4346906; Applied Biosystems, Darmstadt, Germany). All the reactions were done in duplicates and the single reaction contained: 12.5 µl of SYBR Green Master Mix; 1 µl of stock with forward and reverse primers for specific gene being amplified; 9 µl of dH₂O and 2.5 µl of template cDNA. After pipetting the reactions, plates were covered with MicroAmp Optical Adhesive Film (cat. no. 4311971; Applied Biosystems) for preventing evaporation of reaction
components. Contents of the wells were collected by centrifugation for 3 min at 3200 rounds per minute. Amplification was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems) at the following conditions: 50°C 2min, repeat 1; 95°C 10min, repeat 1; 95°C 15sec, 60°C 1min in, repeats 40; 95°C 15sec, 60°C 1min, 95°C 15sec, 60°C 15sec, repeat 1. Relative quantification of certain gene expression was performed using the housekeeping β-actin gene expression as reference. 7500 Fast System Software (Applied Biosystems) and Microsoft Excel were used for data analysis. Following primers from SABiosciences, Qiagen were used: β-actin (cat. no. PPM02945A); IFN-γ (cat. no. PPM03121A); IL-21 (cat. no. PPM03761E); IFN-β1 (cat. no. PPM03594B), TLR3 (cat. no. PPM04216B), CXCL-10 (cat. no. PPM02978D), CCL-1 (cat. no. PPM03138B), CCL-19 (cat. no. PPM03157B). TGF-β1 (forward) 5`-GTA CAG CAA GGT CCT TGC CCT-3` and TGF-β1 (reverse) 5`-TAG TAG ACG ATG GGC AGT GGC-3` primers were purchased from Thermo Scientific, Ulm, Germany and 50 mM primer stock (as mixture of both primers) was used. Expected products length was: for β-actin 154bp, IFN-γ 95bp, IL-21 189bp, IFN-β1 52bp, TLR-3 184bp, CXCL-10 106bp, CCL-1 92bp, CCL-19 100bp and TGF-β1 102bp.

2.6. Agarose gel electrophoresis

10 cm length 2% agarose (cat. no. A9539-500G; Sigma) gel was used. Electrophoresis was performed in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA. pH 8), 8 V/cm, 45 min. Gene Ruler Plus (cat. no. SM1332, Fermentas, St. Leon-Rot, Germany) was used to identify the band length. Samples were visualised by adding ethidium bromide 0.07% solution (cat. no. A2273,0015; AppliChem) in gel and using GeneGenius System (Syngene, Frankfurt, Germany) after electrophoresis. Images were obtained by GeneSnap image acquisition software.

2.7. Depletion of murine intestinal microbiota by antibiotic treatment

Mice were treated with ampicillin (cat. no. A6140-5G; Sigma) 1 g/l in water flasks and antibiotic cocktail consisting of vancomycin (cat. no. 861987-1G;
Sigma) 5 mg/ml, neomycin (cat. no. N6386-5G; Sigma) 10 mg/ml and metronidazol (cat. no. M3761-5G; Sigma) 10 mg/ml diluted in dH₂O. Gavage volume of 10 µl of antibiotic cocktail per 1 g body weight was delivered intragastrically every 12 h without prior sedation of the mice. Fresh antibiotic cocktail was mixed at every feeding point and ampicillin in fresh water was renewed every 7th day. Mice subjected to this protocol for 17 days displayed the properties of GF mice [144] and this was confirmed here by macroscopical inspection of caeca and spleen sizes after 18 days of antibiotic treatment (Fig.9).

Figure 9. Microflora-depleted mice develop the phenotype of GF animals. Photograph of A. non-treated standard pathogen free (SPF) housed B6 and antibiotic treated SPF B. OT-II and C. OT-II x RAG⁻/⁻ mice showed enlarged caeca in the antibiotic treated mice. Spleen size of the antibiotic treated SPF E. OT-II and F. OT-II x RAG⁻/⁻ mice was reduced compared to the D. non-treated SPF B6.
2.8. In vitro assays

2.8.1. CD69 activation

CD4+ T cells were enriched from total spleen cells of B6 or CD69−/− mice and 10^7 cells cultured at 37°C, 5% CO_2 in 48-well plate in 500 µl medium containing RPMI, 10% FBS, 1% Penicillin/Streptomycin (cat. no. P11-010; PAA, Pasching, Austria). Anti-CD3/anti-CD28 dynabeads (cat. no. 11452D, Invitrogen) in cell : beads ratio 10 : 1 were added to bind co-stimulatory molecules on the T cells and activate them in order to enhance surface CD69 expression. After overnight incubation PE-conjugated anti-CD69 H1.2F3 (cat.no. 13-0691-81, eBioscience) isotype Armenian hamster IgG antibody was added (1 ng/µl final concentration) to bind surface CD69. 24 h later goat anti-Armenian hamster IgG (cat. no. 127-165-160, Dianova, Hamburg, Germany) antibody was added (6 ng/µl final concentration) to cross-link the anti-CD69 antibodies and activate intracellular signals downstream of CD69. As negative controls I used: non-treated cells; cells activated with anti-CD3/anti-CD28 microbeads; PE-conjugated Armenian hamster IgG Isotype control Ab eBio299Arm (cat.no. 12-4888-81; eBioscience) and CD69−/− cells. All the assays were performed in triplets.

2.8.1. Foxp3+ T_{reg} cell induction

This experiment allows comparison of naïve CD4+CD25− T cells potential to become induced Foxp3+ T_{reg} cells under T_{reg} cell-polarizing conditions in vitro (presence of DC, IL-2 and TGF-β) [31, 58, 157]. Two different naïve T cell populations were used, from the spleens of B6 and CD69−/− mice and both were cocultured with CD11c+ DC from the spleen of B6 mice. T cells and DC were mixed in the ratio 2:1 (8 x 10^4 T cells: 4 x 10^4 DC) and cultured together with T cell activating anti-CD3/anti-CD28 dynabeads (total cell number : beads number ratio 10 : 1) in round bottom 96-well plate on 37°C, 5% CO_2 for 5 days. 200 µl final/well of medium RPMI, 10% FBS, 1% Penicillin/Streptomycin was used. To simulate conditions that favour Foxp3+ T_{reg} cells induction, 2000 U/ml of IL-2 (cat. no. 402-ML; R&D Systems) and 5 ng/ml of TGF-β1 (cat. no. 240-B; R&D Systems) were added to the cell culture. After 5 days culture, cells were washed with PBS supplemented with 0.3% BSA and 0.1% sodium azide, stained for surface CD4
with APC-conjugated mAb binding CD4 GK1.5 (cat. no. 17-0041-83; eBioscience), intracellularly for Foxp3 with FITC-conjugated anti-Foxp3 Ab FJK-16s (cat. no. 11-5773-82; eBioscience) and analyzed with FCM. As control, Foxp3 expression in both populations of naïve CD4 T cells before culture was measured by FCM.

2.9. Microarray analysis

Microarray analyses were performed using 200 ng total RNA as starting material and 5.5 µg single stranded DNA (ssDNA) per hybridization (GeneChip Fluidics Station 450; Affymetrix, Santa Clara, CA). The total RNA were amplified and labeled following the Whole Transcript (WT) Sense Target Labeling Assay (http://www.affymetrix.com). Labeled ssDNA was hybridized to Mouse Gene 1.0 ST AffymetrixGeneChip arrays (Affymetrix, Santa Clara, CA). The chips were scanned with the AffymetrixGeneChip Scanner 3000 and subsequent images analyzed using Affymetrix® Expression Console™ Software (Affymetrix).Gene expression was than analyzed using the AffymetrixGeneChip Mouse Gene 1.0 ST Array platform. Gene expression microarray files (Affymetrix .CEL files) were generated using the GCOS software (Affymetrix). Statistical analyses were carried out using R (v. 2.12.1) (R-Development-Core Team, 2010). Arrays have been normalized using robust multiple-array average (RMA) [52]. Expression data were analyzed using Bioconductoroligo package for R [20]. Differentially expressed genes were determined by the shrinkage T-statistic [137]. Functional enrichment analysis (GO category enrichment and KEGG-Pathway enrichment) was determined using the hypergeometric test statistic with all genes on the AffymetrixGeneChip Mouse Gene 1.0 ST Array as a reference set. Multiple comparison results were controlled by maintaining a false discovery rate (FDR)< 0.05 [166].
2.10. Polyinosinic-polycytidylic acid (poly (I:C)) treatment

2.10.1. In vivo

Poly (I:C) (cat. no. 27-4729-01, Amersham, Freiburg, Germany) was diluted in PBS in concentration of 1 or 0.1 µg/µl and 200 µl of this stock solution was injected intraperitoneally (i.p) to the mice.

2.10.2. In vitro

10^7 cells total (from B6 or CD69^-/- or IFNAR^-/- mice) or CD4 enriched spleen cells (form B6 mice) were cultured on 37°C, 5% CO_2 in 48-well plates for 48 h in 500 µl medium containing RPMI, 10% FBS, 1% Penicillin/Streptomycin with or without addition of 200 µg poly (I:C) per well. In one experiment, 1 x 10^6 enriched B6 CD4^+ T cells from spleen were mixed with 9 x 10^6 CD4^- CD69^-/- spleen cells or 1 x 10^6 CD4^+ T cells from spleen of CD69^-/- mixed with 9 x 10^6 CD4^- cells from spleen of B6. These mixed cells were then cultured in 48-well plate for 48 h on 37°C, 5% CO_2 in 500 µl medium containing RPMI, 10% FBS, 1% Penicillin/Streptomycin with or without addition of 200 µg poly (I:C) per well. Assays were repeated five times.

2.11. Colitis models

2.11.1. CD45RB^{high} CD4 T cell transfer colitis model

Total spleen cells of B6, CD69^-/- or IFNAR^-/- mice were isolated and stained for CD3, CD4 and CD45RB with PE-conjugated mAb binding CD3ε 145-2C11 (cat. no. 12-0031-83; eBioscience), FITC- conjugated mAb binding CD4 GK1.5 (cat. no. 11-0041-86; eBioscience) and biotinylated mAb binding CD45RB 16A (cat.no 553093; BD Biosciences), followed by the second-step reagent PerCP-Cy5.5. CD45RB^{high} CD4 T cells from the CD3^+ CD4^+ population were enriched using the FACSARia system (BD Biosciences) to a purity >95%. Purified CD45RB^{high} CD4 T cells were injected intraperitoneally (i.p.) (3 x 10^5 cells/mouse) into immunodeficient RAG^-/- mice. In some experiments hosts were i.p. treated twice a week with 20 µg of poly (I:C) per mouse. The weight of transplanted mice
and their clinical condition were monitored twice weekly. Tissue samples for histopathological examination were taken from the large intestine, fixed in neutral-buffered formalin, embedded in paraffin, sectioned on a microtome, mounted on slides, and stained with hematoxylin and eosin (H&E). Histology of the large intestine was categorized as normal (score 0); mild colitis (score 1), with few inflammatory cells in the LP, stromaedema, and a slight reduction of goblet cells; moderate colitis (score 2), with an intense inflammatory infiltration of the LP, hyperplasia of crypts, and a marked reduction of goblet cells; or severe colitis (score 3), with a spillover of leukocytes beyond the mucosa into deeper layers of the colonic wall, complete loss of goblet cells, distortion of the mucosal architecture, erosions or ulcerations, and crypt abscesses as previously published [131, 133].

2.11.2. Dextran sodium sulphate (DSS) induced colitis

B6 and CD69⁻/⁻ mice were fed 2% DSS salt (cat. no. 160110; MP Biomedicals, Illkirch, France) dissolved in sterile water ad libitum for five days. On day 5, DSS containing water was removed and replaced with normal sterile water. The weight of mice and their clinical condition were monitored daily. Mice showed the signs of colitis on day 5 and were sacrificed at the peak of disease, on day 7 after DSS administration. Histopathology of the colon and colitis scoring were performed as described above for the T cell transfer colitis.

2.12. Oral tolerance model

Mice received intragastrically 1 mg of OVA protein (cat. no. A5253; grade II, Sigma Aldrich) dissolved in 100 µl of PBS daily for five times as previously reported [113]. Control mice received 100 µl of PBS only. Seven days after the last treatment mice were immunized by subcutaneous (s.c.) injection in the base of the tail with 50 µg/mouse OVA protein and 50 µg/mouse oligodeoxynucleotide (ODN) 1826 (biomers.net, Ulm, Germany) emulsified in 50 µl/mouse incomplete Freund's adjuvant (IFA, cat. no. F5506; Sigma-Aldrich) with 50 µl/mouse PBS. One week later total spleen cells were isolated and restimulated with titrated concentrations of OVA₃₂₃-₃₃₉ peptide ISQAVHAAHAEINEAGR (cat. no.
OR266830; Thermo Fisher Scientific, Ulm, Germany) in RPMI medium containing 10% FBS and 1% Penicillin/Streptomycin. After 72 h incubation on 37°C, 5% CO₂ supernatants were collected and IFN-γ concentration was determined by ELISA. In separate experiments mice were challenged by s.c. injection of 50 µg OVA protein in 12.5 µl PBS into the right ear pinna one week after OVA immunization. As a control 12.5 µl of only PBS was injected into left ear pinna of the same mice. Ear challenge was performed after sedation of the mice with 10 mg/ml ketanest (cat. no. 0705.04; Parke-Davis, Pfizer, Karlsruhe, Germany) and 1 mg/ml Rompun (cat. no. 2607.04; Bayer, Leverkusen, Germany) diluted in PBS (150 µl of sedation dilution per mouse). Ear swelling was measured before injection and daily for 3 days after injection. Delayed-type hypersensitivity (DTH) response was measured as OVA-specific ear swelling and calculated as the following: (right ear thickness – left ear thickness)_{48h} – (right ear thickness – left ear thickness)_{0h} as 48 h after injection was the peak of swelling.

2.13. T cell migration assays

2.13.1. In vitro chemokine receptor functionality

Chemokine stocks were made in PBS supplemented with 0.1% of BSA and diluted in RPMI medium containing 10% FBS and 1% Penicillin/Streptomycin when used for experiments. Concentrations of CCL-1 (cat. no. 845-TC; R&D Systems) and CXCL-10 (cat. no. 466-CR; R&D Systems) were titrated (from 0.5 nM to 200 nM) and 600 µl of only medium or medium containing chemokine was added in the lower chamber of 24-well transwell (cat. no. 3421; Corning, New York). This transwell system includes polycarbonate membrane with 5 µm pore size that do not allow the medium to diffuse, but allows T cells to migrate from one chamber to another. After loading the lower chamber, inserts containing the membrane were carefully placed into the wells. 4 x 10^5 B6 or CD69^+ CD4 T cells in 100 µl of RPMI medium containing 10% FBS and 1% Penicillin/Streptomycin was added to the upper chambers. Plate was covered and incubated 1 hour at 37°C, 5% CO₂. Transwell inserts were carefully removed and cells in the lower chamber media counted by FACSCalibur. All the reactions were done in triplets and average cell
number per chemokine concentration per cell type was calculated. The chemotactic index was calculated as: the average number of cells migrating to the media with chemokine divided by the average number of cells migrating to the media alone as previously described [32].

2.13.2. In vivo competitive homing assay

This experiment allowed us to directly compare migratory capacity of two different T cell populations in the same mouse. Congenic markers CD45.1/CD45.2 as well as self fluorescence DsRed/labelling dye carboxyfluorescein succinimidyl ester (CFSE) were used to distinguish different cell populations.

CD4 T cells were enriched from the spleens of DsRed or CD69⁻/ mice (both on B6 CD45.2⁺ background). CD69⁻/ CD4 T cells were labelled with cell trace dye CFSE (cat. no. C34554; Invitrogen): 10⁷ cells were washed two times with PBS, resuspended in 1 ml of PBS, 0.1% BSA and than incubated 15 min on 37°C, 5% CO₂ with 5 µM final concentration of CSFE diluted in dimethylsulfoxid (DMSO; supplied in CSFE kit); labelling reaction was stopped by adding 1 ml of cold FBS and washing the cells two times with PBS.

DsRed B6 and CSFE⁺ CD69⁻/ CD4 T cells were mixed in a 1:1 ratio and resuspended in PBS. 10⁶ of each cell population in 200 µl of PBS were adoptively transferred to CD45.1⁺ B6 mice intravenously (i.v.) via tail vein injection. Small amount of cells was saved after injection for calculating input ratio (IR). These cells were extracellularly stained for CD4 with APC-conjugated mAb binding CD4 GK1.5 (cat. no. 17-0041-83; eBioscience) and for CD45.2 with biotinylated anti-CD45.2 mAb 104 (cat.no. 553771; BD Bioscience) and PerCP-Cy5.5 as a second-step reagent. Cells were analyzed by FCM and IR was calculated as:

\[
IR = \frac{\text{number of CFSE}^+ \text{ cells}}{\text{number of DsRed}^+ \text{ cells}}
\]

IR should be close to 1 as the cells are mixed in 1:1 proportion. Transferred hosts were euthanized 18 h after injection, time point suggested to showing significant T cell migration to the gut [32]. Cells were isolated from the spleen, MLN, blood, siLP and cLP of the hosts and stained same as the sample before injection (with APC-conjugated mAb binding CD4 GK1.5, biotinylated mAb binding CD45.2 104 and PerCP-Cy5.5 as a second-step reagent). Samples were analyzed by FCM and homing index (HI) for every tissue calculated as:
HI = number of CD4⁺CD45.2⁺CFSE⁺ cells / number of CD4⁺CD45.2⁺DsRed⁺ cells : IR

In a separate experiment 10⁷ of each cell population in 200 µl PBS were i.v. injected to one B6 mouse. 18 h post-injection small intestine and colon were isolated and washed from feces with PBS/1% FBS using tubing needle. Washed small intestine and colon were incubated 1 h at RT in dark with Alexa Fluor 350-conjugated Wheat Germ Agglutinin (WGA, cat. no. W11263; Invitrogen), 20 µg/ml final concentration, in 1 ml PBS/1%FBS. WGA binds mucus glycoproteins in the intestine [28] staining for the surface of intestinal epithelium. Sections of small intestine and colon were analyzed by LSM 710 Confocal Laser Scanning Microscope (Zeis, Jena, Germany) and different fluorescent colours were detected using following excitation/emission spectra: for Alexa Fluor 350 350 nm/408-464 nm, for DsRed 512 nm/591-715 nm and for CFSE 488 nm/493-525 nm. Obtained pictures were analyzed using LSM Image Browser software (Zeis, Jena).

2.14. Statistic

A one-way ANOVA test (for non-parametric) data and a t-test for two unequal variances was used. P < 0.05 was considered statistically significant.
3. Results

3.1. Expression of CD69 on the surface of CD4 T cells

CD69 is expressed on the surface of many immune system cell types upon activation [93, 95, 107, 123, 169] and also constantly expressed on the platelets [93, 123, 169]. This means that in the normal, steady-state conditions CD69 molecule is expressed in almost all the tissues in the body. This study focus is on the mucosal immune system of the GIT and CD69 expression by CD4 T cells. First step was to compare CD69 expression on the surface of CD4 T cells obtained from tissues of the different mice strains in normal conditions and after specific activation of intestinal immune system responses.

3.1.1. The highest fraction of CD69⁺ CD4 T cells in mucosal tissues

The fraction of CD69-expressing CD4 T cells was determined in spleen, MLN, siLP and cLP of non-treated B6 and TCR-transgenic OT-II and OT-II x RAG⁻/⁻ mice. Approximately 10% of the CD4 T cells from the spleen, 17% of CD4 T cells from the MLN and 50% of CD4 T cells from the intestinal LP were expressing CD69 in B6 mice raised and kept under SPF conditions (Fig.10A). CD69⁺ CD4 T cell fraction was reduced in the tissues of SPF OT-II animals (approximately 3% in the spleen, 7% in MLN, 20% in siLP and 45% in cLP) although a substantial fraction of the intestinal LP CD4 T cells did express CD69 in these mice (Fig.10A). In both of these mice strains the fraction of CD69-expressing cells is hence increased substantially in the intestinal LP CD4 T cell population. All of the CD4 T cells obtained from OT-II x RAG⁻/⁻ animals are TCR-transgenic and hence naive when the mice are kept under SPF conditions. As expected OT-II x RAG⁻/⁻ CD4 T cells from spleen, MLN and siLP did not express the CD69 molecule on their surface, but surprisingly about 50% of OT-II x RAG⁻/⁻ CD4 T cells from cLP expressed CD69 (Fig.10A). This could be due to the especially numerous and various microflora in the colon [50], but this phenomenon needs further studies to be clarified.
Figure 10. CD69 surface expression is up-regulated by the commensal microflora. CD69 expression by CD4+ T cells from spleen, mesenteric lymph nodes (MLN), small intestinal lamina propria (siLP) and colonic lamina propria (cLP) of A. standard pathogen free (SPF) B6, OT-II and OT-II x RAG\(^{-/-}\) mice or B. germ free (GF) B6 and intestinal microflora depleted OT-II and OT-II x RAG\(^{-/-}\) animals was analyzed by multicolour flow cytometry. Filled curves represent the respective negative controls. Numbers indicate the percentage of CD4+ CD69\(^{+}\) cells. Five individual mice were analyzed per group and data from a representative mouse per each strain are shown.
These data showed the highest fraction of CD69⁺ CD4 T cells in the mucosal intestinal tissues of both B6 and TCR-transgenic animals in the normal SPF conditions.

3.1.2. Commensal microflora-dependent surface CD69 expression by CD4 T cells

CD69 expression was tested on the CD4 T cells obtained from the spleen, MLN, siLP and cLP of GF B6 and intestinal microflora-depleted SPF TCR-transgenic OT-II and OT-II x RAG⁻/⁻ mice. GF raised and kept B6 animals showed significant reduction in the fraction of CD69-expressing CD4 T cells in all the tissues tested. Approximately 4% of the CD4 T cells from the spleen, 5% of CD4 T cells from the MLN and 7% of CD4 T cells from the intestinal LP were expressing CD69 in GF B6 mice (Fig.10B). The protocol for intestinal microflora depletion by antibiotic treatment used in this study depletes cultivable intestine microbiota and reduces fecal bacterial DNA load in the mice as already reported [144]. Treatment of OT-II and OT-II x RAG⁻/⁻ mice with antibiotic cocktail for 18 days led to the establishment of GF-like phenotype in these animals as confirmed macroscopically by enlarged ceca and reduced spleen size of the mice treated with the antibiotics compared to the age and sex matched non-treated SPF B6 mice (Fig.9). This treatment also led to the reduction of CD69⁺ CD4 T cell proportion compared to the SPF OT-II and OTII-RAG⁻/⁻ animals (Fig.10B). This reduction was the most significant in the intestinal LP where only about 1% of the CD4 T cells expressed CD69 after the antibiotic treatment (Fig.10B).

These data indicate that the expression of CD69 on CD4 T cells isolated from the small intestinal and colonic LP is regulated by the intestinal microflora.

3.1.3. CD69⁺ CD4 T cells are induced by oral antigen challenge

To activate the intestinal immune responses, B6 or OTII x RAG⁻/⁻ animals were gavaged with 1 mg OVA protein dissolved in 100 µl of PBS daily for two days. Control group received PBS only. 24 h after last feeding, surface expression of the cell activation markers CD25 and CD69 was examined on CD4 T cells isolated from spleen, MLN, siLP and cLP.
Figure 11. CD69 surface expression is induced by oral antigen challenge in T cell receptor transgenic mice. A. B6 and B. OT-II x RAG−/− animals were gavaged for two days with 1 mg chicken ovalbumin protein (OVA) dissolved in 100 µl phosphate buffered saline (PBS). The control mice were fed with PBS only. The expression of the activation antigens CD69 and CD25 on the surface of CD4 T cells from spleen, mesenteric lymph nodes (MLN), small intestinal lamina propria (siLP) and colonic lamina propria (cLP) was analyzed by multicolour flow cytometry. Data from an individual, representative mouse of each strain (out of 4 mice per group analyzed) are shown. Numbers indicate the percentage of CD4 T cells that express the activation antigens CD69 or CD25.
SPF B6 mice challenged with OVA showed no change in CD69 or CD25 surface expression on CD4 T cells from the intestinal LP, the spleen and the MLN (Fig.11A). In contrast, the same treatment induced CD69 expression on CD4 T cells in spleen, MLN and siLP of OTII x RAG\(^{-/-}\) animals, but not on cLP CD4 T cells that were expressing CD69 even before OVA administration (Fig.11B). Following oral OVA challenge, 40-50% of all CD4 T cells from the intestinal LP and the spleen and approximately 20% of the CD4 T cells from MLN of OT-II x RAG\(^{-/-}\) mice were expressing CD69 on the surface (Fig.11B). Interestingly, activated OTII x RAG\(^{-/-}\) CD4 T cells did not express the activation marker CD25 that renders them responsive to IL-2 and allows their clonal expansion and survival (Fig.11B).

Hence, CD4 T cells respond to specific priming by an oral antigen challenge by expressing a CD69\(^{+}\) CD25\(^{-}\) surface phenotype in the first 24 h after treatment.

### 3.2. CD69\(^{+}\) CD4 T cells are showing the phenotype of regulatory cells

Further *in vivo* and *in vitro* studies were performed in order to investigate the function of CD69 expressed on the surface of CD4 T cells. CD69-expressing CD4 T cells were analyzed for the expression of intracellular and extracellular markers of main CD4 T cell subtypes. The responses of normal B6 cells were compared with CD69-deficient cells and with B6 cells after *in vitro* activation of CD69 molecule.

#### 3.2.1. CD69\(^{+}\) CD4 T cells induced by oral antigen challenge are expressing membrane bound TGF-\(\beta\)1/LAP

OT-II x RAG\(^{-/-}\) animals were gavaged for two days with 1 mg of OVA protein in 100 µl of PBS daily. Analyzes of the phenotype of siLP CD4 T cells from OT-II x RAG\(^{-/-}\) mice 24 h after second oral challenge with OVA, using intracellular and surface FCM analyses, revealed a significant differences between CD69\(^{-}\) and CD69\(^{+}\) population (Fig.12A). As expected, most CD69\(^{-}\) CD4 T cells were naive and did not express the master regulators T-bet, GATA-3 or ROR\(\gamma\)t (required for Th1, Th2 or Th17 CD4 T cell differentiation respective [70, 168, 189]). Almost 20%
of CD4 T cells in CD69− population were Foxp3+ Treg cells. About 20% of the intestinal LP CD69− CD4 T cells were expressing the IFN-γR (binding IFN-γ), CD122 (binding IL-2/IL-15), TGF-βRII (binding TGF-β), the TGF-β-regulated integrin CD103, IL-21R1 and a fraction of about 10% were expressing the membrane-bound LAP/TGF-β1. The cytokine receptor IL-10R and the costimulatory molecule ICOS were not expressed by CD69− CD4 T cells (Fig.12A). In contrast, none of the transcription factors T-bet, GATA-3, RORγt, the cytokine receptors IFN-γR, CD122, TGF-βRII, IL-21R1, IL-10R, the integrin CD103 or the costimulatory molecule ICOS were detected in activated intestinal LP CD69+ CD4 T cells. However, about 40% of CD69+ CD4 T cells were expressing LAP/TGF-β1 (Fig.12A).

Hence, CD69+ CD25/CD122− CD4 T cells recently activated in the intestinal LP by specific antigen oral challenge are not committed to the development of a pro-inflammatory phenotype and are largely unresponsive to key cytokines. The large fraction of activated CD69+ CD25/CD122− LP CD4 T cells expressed membrane bound LAP/TGF-β1 indicating that these cells could have regulatory properties.

**Figure 12. A.** OT-II x RAG−/− mice were fed for two days with 1 mg chicken ovalbumin protein protein dissolved in 100 µl phosphate buffered saline. Intracellular expression of T-bet, GATA-3, RORγt and Foxp3 and surface expression of IFN-γR, CD122, CD103, TGF-βRII, IL-21R, IL-10R, ICOS and LAP/TGF-β1 gated on CD69− or CD69+ CD4 T cells from small intestinal lamina propria (siLP) was analyzed by multicolour flow cytometry. Filled curves represent the respective negative controls. Four mice were analyzed and the data from a representative individual mouse are presented. Numbers represent the percentage of CD4+ cells that are positive for the indicated antigen.
3.2.2. CD69 negatively regulates the production of pro-inflammatory cytokines

Next, CD69 molecule on the surface of B6 CD4 T cells was activated \textit{in vitro} using anti-CD69 mAb and secondary cross-linking antibody. The cytokine profile of these cells was determined on both RNA and protein levels. To make sure that the response observed is specifically induced by CD69 activation,

\begin{figure}
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\includegraphics[width=\textwidth]{figure13.png}
\caption{CD69 activation increases TGF-\(\beta\)1 expression and secretion by CD4 T cells. For CD69 engagement, CD4 T cells from spleen of B6 animals were activated with anti-CD3/anti-CD28 dynabeads followed by anti-CD69 antibody (isotype Armenian Hamster IgG) and anti-Armenian Hamster IgG antibody for cross-linking. RNA from the cell lysates was reverse}
transcribed to complementary DNA and relative expression of A. TGF-β1, B. IFN-γ and C. IL-21 to β-actin was analyzed by qRT-PCR. D. TGF-β1 and E. IFN-γ concentrations after CD69 activation were measured in the supernatants by ELISA. For the control reactions without antibodies, with isotype control antibody for anti-CD69 antibody or reactions with CD69⁺ CD4 T cells were used. CD69 activation assay and all the control reactions were done in triplets. Data are representative of two individual experiments. P values were calculated with one-way ANOVA test; p < 0.05 was considered statistically significant. * p< 0.05

CD69⁻/⁻ CD4 T cells treated on the same way and B6 CD4 T cells treated with isotype control mAb instead of anti-CD69 mAb were used as control groups. Data obtained by qRT-PCR showed that CD69 engagement significantly increased TGF-β1, but decreased IFN-γ and IL-21 transcript levels (Fig.13A,B,C) in CD69-activated B6 CD4 T cells compared to the control groups. Activation of CD69 also increased the amount of TGF-β1 released into supernatants, but decreased IFN-γ secretion by B6 CD4 T cells when compared to the control groups, as measured by ELISA (Fig.13D,E).

Microarray analyzes of the CD69-dependent gene expression by comparing CD69-deficient, CD69-activated and B6 CD4 T cells confirmed the data obtained by previous experiment. When gene expression pattern in CD4 T cells from B6 animals was compared to the expression pattern in CD4 T cells from CD69⁻/⁻ animals 2472 genes were differentially regulated. The comparison of gene expression in B6 CD4 T cells with CD69-acticated CD4 T cells yielded 2641 differentially regulated genes. Comparing the gene expression pattern of CD4 T cells from CD69⁻/⁻ animals with CD69-activated CD4 T cells showed 3259 differentially regulated genes. For mapping the CD69-dependent pathways enrichment of microarray data sets in GO category and KEGG-pathways was checked. These pathways included cytokine-cytokine receptor interaction, chemokine signalling pathway, TLR signalling pathway, nod-like receptor signalling pathway and the TGF-β signalling pathway. To this study differential expression of genes encoding cytokines was relevant and it revealed that IL-21, TNF and IFN-γ genes expression were down-regulated and the TGF-β3 gene expression was up-regulated in CD69-activated CD4 T cells (Table I). The microarray data sets have been uploaded in the gene expression omnibus (GEO; accession number and can be accessed under: GSE27706).

To further investigate the influence of CD69-deficiency on cytokine production, CD69⁻/⁻ or B6 cells isolated from spleen, small intestine or colonic LP were activated in vitro using anti-CD3/anti-CD28 beads and cytokine
concentration in the supernatants produced by these activated cells was measured by ELISA. Data obtained with this experiment confirmed both microarray and CD69 activation assay results. Activated CD69−/− cells from all the tissue tested produced significantly higher amounts of IFN-γ and TNF-α, but lower amounts of TGF-β1 when compared to B6 cells (Fig.14A,B,C). Analyzes of the relative gene expression (normalized to the housekeeping β-actin gene expression) by qRT-PCR showed increased expression level of IL-21 gene in the small intestine and colon tissue of non treated CD69−/− mice compared to B6 mice (Fig.14D).

### Table I: Expression of selected genes differentially expressed in B6, CD69−/− and CD69-activated B6 CD4 T cells analyzed by microarray.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>FC</th>
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<th>FC</th>
<th>FDR</th>
<th>FDR</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>B6 vs CD69−/−</td>
<td>B6 vs CD69-A</td>
<td>CD69-A vs CD69−/−</td>
<td>B6 vs CD69−/−</td>
<td>CD69-A vs CD69−/−</td>
</tr>
<tr>
<td>Il21</td>
<td>interleukin 21</td>
<td>1.81</td>
<td>1.46</td>
<td>3.16e-14</td>
<td>1.30e-07</td>
<td>5.48e-14</td>
</tr>
<tr>
<td>Tnf</td>
<td>tumor necrosis factor</td>
<td>0.67</td>
<td>0.52</td>
<td>1.10e-12</td>
<td>2.87e-06</td>
<td>5.87e-10</td>
</tr>
<tr>
<td>Ifng</td>
<td>interferon gamma</td>
<td>0.45</td>
<td>2.57</td>
<td>1.49e-05</td>
<td>6.05e-14</td>
<td>5.48e-14</td>
</tr>
<tr>
<td>Tgfb3</td>
<td>transforming growth factor beta 3</td>
<td>0.31</td>
<td>0.46</td>
<td>0.147</td>
<td>8.89e-06</td>
<td>0.00695</td>
</tr>
<tr>
<td>Tbrg3</td>
<td>transforming growth factor beta regulated gene 3</td>
<td>0.04</td>
<td>1.07</td>
<td>0.909</td>
<td>2.72e-05</td>
<td>3.73e-05</td>
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Taken together, these data are pointing to a regulatory role of CD69 in cytokine production by CD4 T cells, making this activation marker a positive regulator of TGF-β1 and a negative regulator of pro-inflammatory cytokines (IFN-γ, TNF-α and IL-21) production. Data are suggesting that CD69+ CD4 T cells could possibly serve as regulatory cells that negatively regulate immune responses.
Figure 14. CD69 negatively regulates the production of proinflammatory cytokines. Spleen, small intestinal lamina propria (siLP) and colonic lamina propria (cLP) cells of B6 and CD69−/− mice were cultured with anti-CD3/anti-CD28 dynabeads. After 18 h on 37°C, 5% CO₂ supernatants were collected and analyzed for A. IFN-γ, B. TNF-α and C. TGF-β1 concentrations by ELISA. The mean (± SEM) of five mice per group are shown. P values were calculated with a non-parametric student’s t test; p < 0.05 was considered statistically significant. Total RNA isolated from frozen small intestine and colon tissue of B6 and CD69−/− was reverse transcribed to complementary DNA and expression of D. IL-21 was analyzed by qRT-PCR. Mean (± SEM) of five mice per group is presented. In the non-parametric student’s t test p < 0.05 was considered statistically significant. * p< 0.05
3.2.3. Proportion of peripheral Foxp3+ T_{reg} cells induced \textit{in vivo} by oral antigen administration is decreased in the absence of CD69

TGF-β is known as negative modulator of immune responses as it is needed for the induction of peripheral Foxp3+ T_{reg} cells both \textit{in vivo} [68, 86, 105] and \textit{in vitro} [22, 43, 157, 179]. As CD69 engagement induced TGF-β1 production, influence of CD69-deficiency on Foxp3+ T_{reg} cells induction was analyzed. \textit{In vivo} experiments showed that the absence of CD69 does influence the generation of Foxp3+ T_{reg} cells. In the steady state conditions, the fraction of Foxp3+ T_{reg} cells among CD4 T cells (in MLN, siLP and cLP) of CD69−/− mice was decreased compared to B6 mice (Fig.15A). In spleen there was no difference in the fraction of Foxp3+ T_{reg} cells as approximately 10% of CD4 T cells from both CD69−/− and B6 mice were expressing Foxp3 (Fig.15A). It is reported that oral feeding of the mice with specific antigen induces peripheral Foxp3+ T_{reg} cells [121, 172, 187]. However, after oral delivery of 1 mg OVA protein in 100 µl PBS daily for total of two times feeding, the fraction (Fig.15B) and the cell number (Fig.15C) of Foxp3+ CD4+ T_{reg} cells in spleen, MLN and intestinal LP of OT-II x CD69−/− mice were significantly lower than in OT-II animals (about 2-fold decrease in cell number was seen in spleen, 5-fold decrease in MLN and siLP and 3-fold decrease in cLP).

These data point to a role of CD69 in \textit{in vivo} induction of Foxp3+ CD4+ T_{reg} cells in the peripheral immune tissues and that regulatory processes involving Foxp3+ CD4 T cells in the intestinal tissues are affected in the absence of CD69.

3.2.4. CD69 increases the number of \textit{in vitro} inducted Foxp3+ T_{reg} cells

CD4+ Foxp3+ T_{reg} cells can be induced \textit{in vitro} from the naive CD4+ CD25− Foxp3− cells in the presence of APC, TGF-β and IL-2 [22, 31, 157]. To compare the potential of B6 and CD69−/− naive CD4+ CD25− T cells to become Foxp3+ T_{reg} cells \textit{in vitro}, these cells were co-cultured with B6 CD11c+ DC as APC and exogenous TGF-β1 and IL-2. Intracellular Foxp3 expression by CD4 T cells was measured by FCM after five days of culture. As the control, Foxp3 expression by the fraction of B6 and CD69−/− naive CD4+ CD25− cells was analyzed before the culture (Fig.16A). The significant fractions of both B6 and CD69−/− CD4 T cells were expressing Foxp3 after five days of culture (Fig.16A). Still, the fraction of \textit{in
vitro induced CD69−/− CD4+ Foxp3+ T_{reg} cells were significantly decreased compared to induced B6 CD4+ Foxp3+ T_{reg} cells (Fig. 16A, B).

**Figure 15.** Absence of CD69 reduces the fraction of Foxp3 regulatory T cells in vivo. A. Cells were isolated from spleen, mesenteric lymph nodes (MLN), small intestinal lamina propria (siLP) and colonic lamina propria (cLP) of non-treated B6 or CD69−/− mice and analyzed for intracellular expression of Foxp3 in CD4 T cell population by multicolour flow cytometry (FCM). Numbers indicate the percentage of CD4+ Foxp3+ cells. Four individual mice per group were analyzed and data from a representative mouse are shown. B. T cell receptor-transgenic OT-II and OT-II x CD69−/− mice were fed two days with 1 mg chicken ovalbumin protein. The intracellular expression of Foxp3 by CD4 T cells from spleen, MLN, siLP and cLP was analyzed by multicolour FCM. Numbers indicate the percentage of CD4+ Foxp3+ cells. Four individual mice per group were analyzed and data from a representative mouse are shown. C. Mean (± SEM) total number of Foxp3+ CD4 T cell number x 10^4 in spleen, MLN, siLP and cLP of two times 1 mg OVA fed OT-II and OT-II x CD69−/− mice are shown. Four mice of each strain were analyzed. In the non-parametric student's t test p< 0.05 was considered statistically significant. * p< 0.05
These data revealed that CD69 is not necessary for \textit{in vitro} Foxp3$^+$ T\textsubscript{reg} cell induction, but in the presence of CD69 on CD4 T cells proportion and cell number of induced Foxp3$^+$ T\textsubscript{reg} is increased.

**Figure 16.** CD69-deficient CD4 T cells showed reduced potential to become Foxp3$^+$ T\textsubscript{reg} \textit{in vitro} even after addition of exogenous TGF-β1. **A.** Naive CD4$^+$ CD25$^-$ T cells were enriched from the spleen of B6 or CD69$^{-/-}$ animals and mixed with B6 CD11c$^+$ dendritic cells in the proportion 2:1. Cells were cultured for 5 days with activating anti-CD3/anti-CD28 microbeads in the presence of exogenous IL-2 and TGF-β1. Cells were pelleted, washed and intracellular Foxp3 expression was measured with multicolour flow cytometry. As the control Foxp3 expression was analyzed in the population of naive cells before the culture. Numbers indicate the percentage of Foxp3$^+$ CD4$^+$ cells. Experiment was repeated twice with ten individual samples from one mouse per strain per experiment and representative dot plots are shown. **B.** Mean (± SEM) of induced Foxp3$^+$ CD4$^+$ cell (in percentage) among B6 or CD69$^{-/-}$ naive cells for ten samples per group is presented. Experiment is repeated twice and the representative data are shown. In the non-parametric student’s t test $p<0.05$ was considered statistically significant.
3.3. The role of CD69 in IBD

Since CD69 deficiency in mice was associated with reduced numbers of Foxp3+ CD4 T_{reg} cells and an increased secretion of IFN-γ and TNF-α in the intestinal tissues, CD69^{−/−} animals were monitored for more than 6 months for the occurrence of clinical and histopathological signs of a spontaneous colitis. However, neither clinical nor histopathological signs of the development of a spontaneous colitis could be observed in animal facility of Ulm University and colon architecture in CD69^{−/−} mice stayed the same as in B6 animals (Fig.17).

![Figure 17. CD69-deficient mice do not develop spontaneous colitis.](image)

**Figure 17. CD69-deficient mice do not develop spontaneous colitis.** Large intestinal tissue samples were taken from A. B6 and B. CD69^{−/−} mice housed in the standard pathogen free conditions for more that 6 months. These sections were embedded in paraffin, sectioned on a microtome, mounted on slides, and stained with H&E. Representative images from one individual mouse per group (from two mice per group analyzed) are shown (original magnification x20).

Next, the influence of CD69-deficiency was investigated in experimentally induced models of IBD. There are several mouse models for inducing IBD. In some models the disease is chemically induced like in TNBS or oxazolone colitis where intrarectal administration of 2, 4, 6-trinitro benzene sulfonic acid (TNBS) or oxazolone respectively, induces intestinal inflammation mediated by CD4 T cells [180]. Another chemically induced IBD model is DSS colitis where mice are subjected several days to drinking water supplemented with DSS which is directly
toxic to intestinal epithelial cells [180]. Useful model to analyze the contribution of different CD4 T cell types in the pathogenesis of IBD is the cell transfer colitis model. In this model reconstitution of the immunodeficient mice (SCID or RAG$^{-/-}$) with naive CD4 T cells from immunocompetent donor leads to the intestinal inflammation [15, 138]. In this study cell transfer (CD45RB$^{\text{high}}$ CD4 T cells into RAG$^{-/-}$ hosts) and DSS induced colitis models were used to examine the role of CD69 in inflammation processes in intestine.

3.3.1. Transfer of CD69-deficient CD45RB$^{\text{high}}$ CD4 T cells into RAG$^{-/-}$ mice induce a severe colitis

B6 or CD69$^{-/-}$ CD45RB$^{\text{high}}$ CD4 T cells were sorted by FACS and transferred i.p. into RAG$^{-/-}$ hosts. This experiment should show if CD69 has an influence on the severity of CD4 T cell-induced transfer colitis. Clinical course and the severity of the colitis were monitored twice weekly and noted as body weight loss. Respecting the ethical standard, at the point when some mice showed more than 15% loss of the starting body weight (what happened twenty days after cell transfer) experiment was ended.

Data obtained in this experiment showed that transfer of CD69$^{-/-}$ CD45RB$^{\text{high}}$ CD4 T cells induced a colitis that was more severe than the colitis induced by transfer of an equal number of B6 CD45RB$^{\text{high}}$ CD4 T cells. This was evident by accelerated body weight loss (Fig.18A) and significantly higher colitis score (Fig.18B) of the RAG$^{-/-}$ hosts transferred with CD69$^{-/-}$ CD45RB$^{\text{high}}$ CD4 T cells compared to the animals transferred with B6 CD45RB$^{\text{high}}$ CD4 T cells and non-treated control RAG$^{-/-}$ animals. Analyzes of the colon histopathology revealed normal crypt and LP appearance as well as high number of mucus producing goblet cells in the colon of non-treated RAG$^{-/-}$ mice (Fig.18C) and this colon was categorized as normal (colitis score 0). Hosts transferred with B6 CD45RB$^{\text{high}}$ CD4 T cells showed some inflammatory cells in the LP and reduction of goblet cell number, slightly elongated cripts, but the basic architecture of the colon was not altered (Fig.18D) and this experimental group had the average colitis score $\sim$ 1. On the contrast, large infiltration of inflammatory cells into colonic wall, intestinal cript distortion and loss of the goblet cells was seen in the RAG$^{-/-}$ animals transplanted with CD69$^{-/-}$ CD45RB$^{\text{high}}$ CD4 T cells (Fig.18E) rendering this
experimental group under average colitis score significantly higher than other groups (~1.5).

Figure 18. CD69-deficient CD4 T cells induce a severe colitis correlating with increased IFN-γ, IL-17A and TNF-α serum levels. A. RAG<sup>−/−</sup> mice were i.p. transplanted with B6 or CD69<sup>−/−</sup> CD45RB<sup>high</sup> CD4 T cells. Mean (± SEM) of body weight loss (%) of nine mice per group is shown for control (non-treated) and cell transplanted RAG<sup>−/−</sup> animals. P values were calculated with a non-parametric student’s t test; p < 0.05 was considered statistically significant. B. Histopathological
colitis scores of colon sections taken from control or RAG⁻/⁻ mice transplanted with the indicated CD45RB<sup>high</sup> CD4 T cells. Each experimental group consisted of nine mice. In the non-parametric student’s t test p < 0.05 was considered statistically significant. Large intestinal tissue samples were taken from C. control RAG⁻/⁻, D. RAG⁻/⁻ hosts transplanted with B6 CD45RB<sup>high</sup> CD4 T cells and E. RAG⁻/⁻ hosts transplanted with CD69⁻/⁺ CD45RB<sup>high</sup> CD4 T cells, embedded in paraffin, sectioned on a microtome, mounted on slides, and stained with H&E. Representative images from one individual mouse per group (from nine mice per group analyzed) are shown (original magnification x20). F. IFN-γ, G. IL-17A and H. TNF-α concentrations were detected in blood sera of non-transplanted RAG⁻/⁻ and mice transplanted with B6 or CD69⁻/⁻ CD45RB<sup>high</sup> CD4 T cells by ELISA. Each experimental group consisted of nine mice and mean (± SEM) of indicated cytokine concentration for each group is shown. P values were calculated with the non-parametric student’s t test; p < 0.05 was considered statistically significant. N.S. – not statistically significant; * p < 0.05

### 3.3.2. Increased IFN-γ, IL-17A and TNF-α serum levels in transfer colitis induced by CD69-deficient CD4 T cells

T cell transfer colitis model is consider to be Th1/Th17 dependent model as CD4 T cells isolated from cell transplanted hosts produced large amounts of Th1- and Th17-derived cytokines [138]. Concentrations of pro-inflammatory cytokines in the blood sera of CD45RB<sup>high</sup> CD4 T cell-transferred RAG⁻/⁻ hosts were measured by ELISA in this study.

IFN-γ, IL-17A and TNF-α serum levels in RAG⁻/⁻ mice transferred with B6 CD45RB<sup>high</sup> CD4 T cells although increased, were not significantly altered compared to non-treated control animals (Fig.18F-H). Confirming the high inflammatory response and severity of the colitis, the levels of pro-inflammatory cytokines IL-17A, IFN-γ and TNF-α in blood serum of animals transferred with CD69⁻/⁻ CD45RB<sup>high</sup> CD4 T cells were increased compared to the animals transferred with B6 CD45RB<sup>high</sup> CD4 T cells and non treated RAG⁻/⁻ mice (Fig.18F-H).

These data showed that the absence of CD69 on CD4 T cells leads to the high production of pro-inflammatory cytokines <i>in vivo</i> and severe inflammation in intestine. It is possible that some regulatory mechanism that can prevent CD4 T cell-mediated inflammatory responses is missing in the absence of CD69 on CD4 T cells. Data are pointing a critical role of CD69 in regulating mucosal inflammation.
3.3.3. Increased susceptibility of CD69\(^{-/-}\) mice to DSS induced colitis

Administration of DSS in the drinking water for several days induce an acute colitis in mice, while repeated cycles of DSS administration with intervals of normal water administration in between (e.g. 5 days DSS, 14 days water) result in chronic colitis development [180]. In this study B6 and CD69\(^{-/-}\) mice were treated with 2% DSS in the drinking water for 5 days in order to induce acute colitis. Clinical condition and body weight of the mice were monitored daily and compared with the control group of non-treated B6 mice. B6 mice showed only slight body weight loss on the day 6, while CD69\(^{-/-}\) mice showed rapid and continuous body weight loss starting from day 4 (Fig.19A). All the mice were sacrificed on day 7 when CD69\(^{-/-}\) group lost about 15% of starting body weight. Histopathology of the colon revealed significantly increased average colitis score of CD69\(^{-/-}\) mice compared to the non-treated and DSS-treated B6 animals (Fig.19B). While colon tissue architecture and cell composition were normal in non-treated B6 animals and scored as 0 (Fig.19C), few inflammatory cells, slight goblet cells loss and elongation of colon crypts were observed in DSS-treated B6 mice (Fig.19D) and this group had average colitis score \(\sim 1\). On the other hand, CD69\(^{-/-}\) mice had massive infiltration of inflammatory cells in the colonic wall, complete absence of the goblet cells and the loss of normal crypt architecture (Fig.19E) with average colitis score being more than 2 in this group.

In the absence of CD69 mice are extremely susceptible to the colitis induction after DSS administration, showing more severe intestinal inflammation compared to the B6 animals. These data are once again pointing the important regulatory role of CD69 during intestinal inflammation.

3.3.4. High chemokine levels in the colon of DSS treated CD69\(^{-/-}\) animals

To investigate the reason for such a large infiltration of inflammatory cells observed in CD69\(^{-/-}\) animals after DSS-induced colitis, chemokine gene expression in the colon of non-treated B6 and DSS-treated B6 and CD69\(^{-/-}\) mice was analysed by qRT-PCR. Chemokines are low-molecular weight chemotactic factors that are secreted by many different types of cells and have the role to attract different leukocyte cell subtypes to the site of inflammation [150, 174]. Analysis revealed significant increase in the expression of CCL-1, CXCL-10 and
Figure 19. CD69-deficient mice are more susceptible to DSS induced colitis. B6 or CD69<sup>−/−</sup> are administered 2% dextrine sodium sulphate (DSS) in the drinking water for 5 days and then provided with a normal sterile water. A. Mean (± SEM) of body weight loss (%) of seven mice per DSS treated groups and five mice in control group is shown. In a non-parametric student’s t test p < 0.05 was considered statistically significant. B. Colitis scores of the histological colon sections of DSS treated B6 and CD69<sup>−/−</sup> mice and control B6 animals. Mean (± SEM) for seven mice per DSS treated groups and five mice in control group are presented. P values were calculated with a non-parametric student’s t test; p < 0.05 was considered statistically significant. Histopathological colon tissue samples taken from C. control B6 mice and DSS treated D. B6 or E. CD69<sup>−/−</sup> animals were
embedded in paraffin, sectioned on a microtome, mounted on slides, and stained with H&E. Representative images of one individual mouse per group (from seven mice per DSS treated and five in control group analyzed) are shown (original magnification x20). RNA was isolated from the frozen colon tissue samples of control B6 animals and DSS treated B6 or CD69−/− mice and reverse transcribed to complementary DNA. Relative expression of \textbf{F}, \textit{CCL-1}, \textbf{G}, \textit{CXCL-10} and \textbf{H}, \textit{CCL-19} genes compared to \( \beta \)-actin gene is measured by qRT-PCR. Mean (± SEM) for seven mice per DSS treated groups and five mice in control group are presented. In the non-parametric student’s t test \( p< 0.05 \) was considered statistically significant. N.S. – not statistically significant; * \( p< 0.05 \)

\textit{CCL-19} genes in the inflamed colon tissue of DSS-treated CD69−/− mice when compared to non-treated and DSS-treated B6 animals (\textbf{Fig.19F-H}). All of these chemokine ligands have pro-inflammatory role. \textit{CCL-1} (binding CCR-8) attracts Th2 cells, eosinophils and mast cells [81], \textit{CCL-19} (binding CCR-7) coordinates the emergence of newly generated T cells from thymus to the general circulation [48] and also attracts naive and memory T cells to the inflammation site [81] and \textit{CXCL-10} (binding CXCR-3) attracts activated T lymphocytes [19].

Severe colon inflammation with infiltration of high number of inflammatory cells in colonic wall of CD69−/− mice after DSS administration is probably due to increased expression of pro-inflammatory chemokine ligands that function to attract large number of T cells to the site of injury. Data are suggesting that CD69 could regulate intestinal inflammation through regulation of lymphocyte migration.

\textbf{3.4. The role of CD69 in the migration of CD4 T cells}

To maintain homeostasis and efficiently respond against harmful stimuli, immune cells are in the constant state of regulated migration. It is known that naive T cells continuously migrate through SLO while effector T cells migrate preferentially to extralymphoid tissues [40, 98, 158]. Chemokines are thought to provide the directional cues for movement of leukocytes in the development of both homeostasis and inflammation [97]. Having demonstrated that CD69-deficiency is associated with the increased expression of T cell-attracting chemokines during intestinal inflammation, further investigations of possible role of CD69 in lymphocyte migration were conducted. CD69 affects lymphocyte migration through direct reaction and internalization of S1P1 [10, 158]. As explained in introduction, S1P1 is necessary for lymphocyte egress from the SLO [17, 56, 98, 109, 158]. It is suggested that CD69 expression captures the
lymphocytes in the SLO and inhibits their egress to the circulation [57, 158]. In this study CD69-regulated expression of chemokine ligand and receptor genes was analysed by micro-array and qRT-PCR. Furthermore, the migration of CD69$^{-/-}$ CD4 T cells was compared both *in vitro* and *in vivo* with B6 CD4 T cells.

3.4.1. High expression of chemokine ligands and receptor genes in the CD69$^{-/-}$ CD4 T cells

Micro-array data obtained analysing CD69-dependent gene expression by comparing CD69-deficient, CD69-activated and B6 CD4 T cells were searched for differential expression of chemokine-related genes. This search showed a significant difference in the expression of chemokine ligand and their receptor genes in the CD69$^{-/-}$ compared with CD69-activated CD4 T cells (*Table II*). In CD69$^{-/-}$ CD4 T cells increased expression of the genes coding chemokine ligands that attracts T cells (XCL-1, CCL-1, CCL-3, CCL-4, CCL-19, CXCL-9 and CXCL-10 [19]) was observed (*Table II*). Some of these chemokine ligand also attracts monocytes/macrophages and DC (CCL-3, CCL-4, CXCL-9) or NK cells (XCL-1, CCL-3, CCL-4) [19]. Chemokine receptors whose gene expression was significantly up-regulated in CD69$^{-/-}$ compared to CD69-activated CD4 T cells (*Table II*) are all expressed on activated T cells [48]. Also monocytes express CCR-5 and CCR-8, NK cells express CCR-4 and CCR-8, while DC express CCR-4 and CCR-5 [48].

Micro-array data showed that activation of CD69 on CD4 T cells down-regulate the expression of chemokine-related genes with pro-inflammatory role.

3.4.2. Increased transcript levels of CCL-1, CXCL-10 and CCL-19 in the tissues of CD69-deficient mice

Expression of several genes coding T cell-attracting chemokine ligands was analysed in the MLN, small intestine and colon tissue samples from non-treated B6 and CD69$^{-/-}$ mice. In all the analysed tissue of CD69$^{-/-}$ animals the expression of CCL-1, CCL-19 and CXCL-10 was significantly increased when compared to the B6 mice (*Fig.20A-C*). Especially high increase in expression level of these chemokine ligands was observed in small intestine (approximately 4-fold increase of CCL-1 and CXCL-10 and 3-fold increase of CCL-19 gene
Table II: Expression of chemokine genes differentially expressed in CD69-activated compared to CD69−/− CD4 T cells analyzed by microarray. False discovery rate (FDR) < 0.05 was considered statistically significant

<table>
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<th>Gene</th>
<th>Description</th>
<th>Fold-change (log2)</th>
<th>FDR</th>
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expression) and colon (approximately 5-fold increase of CCL-1 and CXCL-10 and 3-fold increase in CCL-19 gene expression) of CD69−/− mice (Fig.20A-C).

CD69-deficiency leads to the increased expression of the chemokines in the intestinal tissues suggesting that CD69 could regulate lymphocyte migration by regulating the expression of chemokines and chemokine receptors.

Figure 20. Absence of CD69 leads to the increased expression of CCL-1, CXCL-10 and CCL-19 genes in the mucosal intestinal tissues. RNA was isolates from the frozen mesenteric lymph nodes (MLN), small intestine and colon tissue samples of non-treated B6 or CD69−/− animals and reverse transcribed to complementary DNA. Relative expression of A. CCL-1, B. CXCL-10 and C. CCL-19 genes compared to β-actin gene is analyzed by qRT-PCR. Mean (± SEM) for six mice per each strain is presented. In the non-parametric student’s t test p< 0.05 was considered statistically significant. * p< 0.05
3.4.3. CD69<sup>-/-</sup> CD4 T cells showed increased hemotactic index toward CCL-1 or CXCL-10 rich compartment compared to B6 CD4 T cells <em>in vitro</em>

The chemokine-dependent migration is tested <em>in vitro</em> in chemotaxis assay using transwell system. This system allows the migration of the cells toward chemokine-rich compartment using membranes to prevent medium diffusion between compartments. Chemotaxis index compares the number of the cells migrating to the chemokine-containing compartment with the number of the cells migrating to the compartment containing medium only. Higher chemotaxis index represents higher response of the cells to the indicated chemokine.

![Figure 21. CD69<sup>-/-</sup> CD4 T cells showed increased potential to migrate toward CCL-1 and CXCL-10 rich compartments <em>in vitro</em>. CD4 T cells were enriched from the spleen of B6 or CD69<sup>-/-</sup> mice and chemotactic index of these cells was analyzed <em>in vitro</em> in transwell system. Medium alone or containing indicated concentrations of CCL-1 or CXCL-10 were loaded in the lower chamber of the transwell system. Cell number migrating from the upper chamber to the chemokine containing chamber trough polycarbonate membrane after 1 h at 37°C, 5% CO<sub>2</sub> was counted by flow cytometry. All the reactions were done in triplets and average cell number per chemokine concentration per cell type was calculated. Results are presented as chemotactic index of B6 and CD69<sup>-/-</sup> CD4 T cells for A. CCL-1 and B. CXCL-10. The chemotactic index was calculated as: the average number of cells migrating to the media with chemokine divided by the average number of cells migrating to the media alone.

CD4 T cells were isolated from the spleen of B6 or CD69<sup>-/-</sup> mice and used in chemotaxis assay to compare their CCL-1 and CXCL-10 induced migration. Concentrations of chemokines were titrated from 0.5 to 200 nM, assays for every chemokine concentration were done in triplets and average chemotaxis indexes
calculated for every cell type and chemokine concentration. After 1 h at 37ºC, CD69−/− CD4 T cells migrated in higher number in response to all concentrations used of both CCL-1 (Fig.21A) and CXCL-10 (Fig.21B) when compared to B6 CD4 T cells.

Data are showing higher capacity of CD69−/− CD4 T cells to respond to CCL-1 and CXCL-10 chemoattractant signals in vitro and suggesting that CD69 affects not only the expression of chemokine genes but also the response of CD4 T cells to chemokine-derived signals.

3.4.4. Migratory capacity of CD69−/− CD4 T cells to intestinal mucosal tissues in vivo is increased compared to B6 CD4 T cells

Migration of B6 and CD69−/− CD4 T cells was also compared in vivo. CD4 T cells were isolated from the spleen of B6 and CD69-deficient mice and i.v. transferred in the same B6 host. For distinguishing donor CD4 T cells from the host cells, different isoforms of leukocyte common antigen CD45 were used: donor cells were expressing CD54.2 and host cells CD45.1 isoform. CD4 T cells of the different donors were distinguished by specific fluorescence: B6 cells were DsRed+, while CD69−/− cells were CFSE+. DsRed+ B6 and CFSE+ CD69−/− CD45.2+ CD4 T cells were mixed in the approximate ratio 1:1 (Fig.22A) and injected i.v. into CD45.1 B6 hosts. 18 h after injection donor cells were recovered from the blood, spleen, MLN, siLP and cLP of the hosts (Fig.22B). The numbers of DsRed+ and CFSE+ CD45.2+ CD4+ T cells were determined in these tissues by FCM analysis and showed significantly higher number of CD69−/− CD4 T cells migrated to the MLN, siLP and cLP (Fig.23A). Furthermore, small intestinal and colonic tissue of the transplanted host were stained with the mucus binding agglutinin and analyzed ex vivo by confocal imaging. Pictures obtained by confocal microscope showed a higher number of green CFSE+ CD69−/− CD4 T cells compared to the red DsRed+ B6 CD4 T cells in the tissues of the transplanted host (Fig.23C,D). There was no difference in the number of recovered B6 or CD69−/− CD4 T cells from the host’s spleens (Fig.23A). Homing index compared the ratio of CD69−/− to B6 CD4 T cell numbers recovered from the hosts tissues with the ratio of CD69−/− to B6 CD4 T cell numbers injected into hosts. As suggested [32] this index was normalized to the homing index in the blood to eliminate possible retention of the
one cell type in some of the secondary lymphoid tissues which could eliminate that cell type from the circulation. Homing index analyses revealed increased migration of CD69\(^{-/}\) CD4 T cells to the intestinal tissues (MLN, siLP and cLP), but not to the spleen when compared with B6 CD4 T cells (Fig.23B).

**Figure 22. In vivo competitive homing assay.** CD4 T cells were enriched from the spleen of red fluorescent DsRed or CD69\(^{+}\) animals, both on the CD45.2\(^{+}\) B6 background. CD69-deficient CD4 T cells were labelled with green fluorescent CFSE. **A.** These cells were mixed in the approximate ratio 1:1 and injected i.v. into CD45.1\(^{+}\) B6 mice. Numbers are indicating percentage of CD45.2\(^{+}\) CD4\(^{+}\) cell and percentage of DsRed\(^{+}\) or CFSE\(^{+}\) among CD45.2\(^{+}\) CD4\(^{+}\) T cell population that was injected to the hosts. **B.** 18 h later cells were isolated from spleen, mesenteric lymph nodes (MLN), small intestinal lamina propria (siLP) and colonic lamina propria (cLP) and analyzed for the surface expression of CD4, CD45.2, DsRed and CFSE by multicolour flow cytometry.

The data showed increased migratory potential of the CD4 T cells lacking CD69 expression to the intestinal tissues. Severe inflammation and high number of infiltrated inflammatory cells in the colon tissue seen after transfer of CD69-deficient CD45RB\(^{\text{high}}\) CD4 T cells into RAG\(^{-/}\) hosts or after DSS-induced colitis in CD69\(^{-/}\) mice could be due to increased migration of the CD69\(^{-/}\) CD4 T cells to intestinal tissues.
Figure 23. Increased migratory potential of CD69-deficient CD4 T cells to the mucosal intestinal tissues in vivo. 18 h after transfer of DsRed+ B6 and CFSE+ CD69−/− CD45.2 CD4 T cells into the CD45.1 host, immune cells were isolated from spleen, mesenteric lymph nodes (MLN), small intestinal lamina propria (siLP) and colonic lamina propria (cLP) and analyzed for the surface expression of CD4, CD45.2, DsRed and CFSE by multicolour flow cytometry (FCM). A. Numbers of recovered DsRed and CFSE expressing CD45.2+ CD4+ cells in the tissues was determined with FCM and presented as mean (± SEM) total number of recovered cells per tissue for four mice analyzed. P values were calculated with a non-parametric student’s t test; N.S. – not statistically significant; * p < 0.05 was considered statistically significant. B. Homing index (HI) for every tissue calculated as: HI = number of CD4+CD45.2+CFSE+ cells / number of CD4+CD45.2+DsRed+ cells : IR (where IR is input ratio calculated before the injection as: IR = number of CFSE+ cells / number of DsRed+ cells). This value was normalized by the HI in the blood, so the potential retention of the injected cells in some of the periphery organs is eliminated. Mean (± SEM) of blood-normalized HI per tissue for four mice is presented. In the non-parametric student’s t test p< 0.05 was considered statistically significant (*p< 0.05). Sections of C. small intestine and D. colon of the transferred host were stained with Alexa Fluor 350-conjugated wheat germ agglutinin (blue) analyzed for the green (CFSE) and red (DsRed) fluorescence by confocal microscope.
3.5. CD69 regulates interferon type I response

Type I IFN system (IFN-α/β) has been intensively studied for last decade. Numerous studies enlightened the importance of this complex system in shaping of both innate and adaptive immune responses [64, 66, 90]. However, the role of IFN-I in regulating mucosal immune responses has not been studied in detail. It seems that effect of IFN-I on the immune system is dependent on various factors, such as the way of delivery (systemically or locally), dosage and duration of the therapy. As it is already reported that IFN-I and their inducers are up-regulating surface CD69 expression on various cell types [72, 158], a possible connection between CD69 and IFN-I is investigated in this study.

3.5.1. Poly (I:C) is a strong CD69 inducer in a type I interferon-dependent manner

The confirmation of IFN-I-dependent induction of CD69 surface expression by CD4 T cells is obtained by an experiment in which the strong IFN-I inducer, poly (I:C), was injected i.p. to the mice. Poly (I:C) is a synthetic analog of double-stranded RNA (dsRNA), a molecular pattern associated with viral infection. Poly (I:C) is inducing strong anti-viral immune response via induction of IFN-I production [5, 6, 78, 94, 155].

Small dose (20 µg/mouse) of poly (I:C) already induced, within 24 h post-injection, CD69 surface expression on spleen, MLN, siLP and cLP CD4 T cells from B6 and OT-II x RAG⁻/⁻ mice (Fig.24). 24 h after injection of high dose (200 µg/mouse) of poly (I:C) most CD4 T cells from the spleen, MLN and intestinal LP expressed CD69 in B6 and OT-II x RAG⁻/⁻ animals (Fig.24). However, high dose of poly (I:C) did not change surface CD69 expression on spleen, MLN and intestinal LP CD4 T cells in (IFN-I unresponsive) IFNAR⁻/⁻ mice (Fig.24) confirming this effect of poly (I:C) is IFN-I dependent.

Data showed poly (I:C) as a strong and fast inducer of CD69 expression on CD4 T cells.
Figure 24. Polyinosinic-polycytidylic acid (poly (I:C)) induces CD69 surface expression in a type I interferon-dependent manner. Poly (I:C) (20 or 200 µg/mouse) was injected i.p. into B6, OT-II x RAG⁻/⁻ and IFNAR⁻/⁻ mice. 24 h later cells were isolated from spleen, mesenteric lymph nodes (MLN), small intestinal lamina propria (siLP) and colonic lamina propria (cLP) and surface expression of CD69 by CD4 T cells analyzed by multicolour flow cytometry. Three mice per group were analyzed and data from one representative individual mouse are shown. Filled curves represent the corresponding negative control. Numbers indicate the percentage of CD69⁺ CD4 T cells in indicated tissue.
3.5.2. CD69- and IFNAR-mediated signals are crucial for the development of oral tolerance

In order to maintain homeostasis, intestinal immune system has to start inflammatory responses to pathogenic antigens, but also tolerogenic responses to non-pathogenic agents. Tolerance to orally administrated antigen in mice was here induced by repeated intragastral delivery of small doses of specific antigen, followed by immunization with the same antigen injected together with adjuvant. To confirm the establishment of the oral tolerance the DTH response (as ear swelling after s.c. ear OVA challenge) and specific cytokine production by spleen cells after in vitro OVA restimulation were measured.

To examine the role of CD69 and IFN-I signals in tolerogenic processes in intestine, B6, OT-II x RAG⁻/⁻, CD69⁻/⁻ and IFNAR⁻/⁻ mice were subjected to the protocol for the induction of oral tolerance to OVA protein. Animals were fed with 1 mg OVA in 100 µl PBS or PBS only as the control, rested for seven days and immunized with OVA emulsified in IFA containing CpG-reach ODN. Splenocytes were harvested 7 days post-immunization and restimulated in vitro with titrated concentrations of the antigenic OVA₃₂₃₋₃₃₉ peptide (epitop recognized by CD4 T cells). After 72 h of restimulation, the concentration of specifically OVA-induced IFN-γ was measured in supernatants by ELISA. OVA feeding prior to immunization significantly reduced IFN-γ production by spleen cells from B6 and OT-II x RAG⁻/⁻ animals indicating the establishment of oral tolerance (Fig.25A).

Data obtained by analyzing the DTH response in B6 and OT-II x RAG⁻/⁻ mice confirmed establishment of oral tolerance in OVA-fed animals, as these animals showed significantly reduced response to ear challenge with OVA (conducted 7 days after immunization and measured as ear swelling) compared to those fed CR transgenic animals [121]. The same protocol was used to test if oral tolerance to OVA can be induced in CD69⁻/⁻ and IFNAR⁻/⁻ mice. The specific IFN-γ response of spleen cells from CD69⁻/⁻ and IFNAR⁻/⁻ mice fed with OVA, rested and immunized was not impaired comparing to the animals that were fed with PBS, rested and immunized (Fig.25A). Ear challenge with OVA 7 days after immunization revealed that DTH
Figure 25. CD69 and IFNAR-deficient animals are impaired in establishing oral tolerance. B6, OT-II x RAG\(^{-/-}\), CD69\(^{-/-}\) and IFNAR\(^{-/-}\) mice were fed for 5 days with 1 mg chicken ovalbumin protein (OVA) protein dissolved in 100 µl phosphate buffered saline (PBS) (OVA groups) or 100 µl PBS only (PBS groups). After 7 days mice were immunized s.c. with 50 µg OVA protein and 50 µg oligodeoxynucleotide emulsified in 50 µl incomplete Freud’s adjuvant with 50 µl PBS. A. Splenocytes were harvested and restimulated with ISQAV OVA peptide in indicated concentrations on day 19. After 72 h restimulation, IFN-γ was measured in the supernatants by ELISA. All the restimulation reactions were done in triplicates. Data represent mean (± SEM) of twelve samples (from four mice) per experimental group per ISQAV OVA concentration. Data are representative from two individual experiments. P values were calculated with the non-parametric student’s t test; p < 0.05 was considered statistically significant. B. Nineteen days after oral OVA feeding some of the mice were challenged by injection of 50 µg OVA protein in 12.5 µl PBS into the right ear pinna. As a control 12.5 µl of PBS was injected into left ear of the same mice. Ear swelling was measured before ear injection and daily for 3 days after injection. Peak reaction was observed after 48 hours. Delayed type hypersensitivity (DTH) response was calculated as (right ear thickness – left ear thickness)\(48h\) – (right ear thickness – left ear thickness)\(0h\). Five mice per group were used and data are representative of two individual experiments. In the non-parametric student’s t test p< 0.05 was considered statistically significant. N.S. – not statistically significant; * p< 0.05
response in OVA fed mice of these two mice strains was not altered comparing to PBS fed animals (Fig.25B).

Hence, oral tolerance cannot be induced neither in CD69<sup>−/−</sup> nor IFNAR<sup>−/−</sup> mice pointing to a critical role of CD69- and IFN-I-mediated signalling in the induction and/or maintenance of oral tolerance.

### 3.5.3. Poly (I:C) attenuates transfer colitis after transfer of B6, but not CD69- or IFNAR-deficient CD45RB<sup>high</sup> CD4 T cells

It is reported that poly (I:C) injection can diminish DSS colitis [177]. To test if poly (I:C) injections influence CD4 T cell transfer colitis, RAG<sup>−/−</sup> mice transferred with ether B6, CD69<sup>−/−</sup> or IFNAR<sup>−/−</sup> CD45RB<sup>high</sup> CD4 T cells were injected twice a week i.p. 20 µg of poly (I:C) starting from day 1 after cell transfer. The transfer of either B6, CD69<sup>−/−</sup> or IFNAR<sup>−/−</sup>CD45RB<sup>high</sup> CD4 T cells in RAG<sup>−/−</sup> hosts results in progressive body weight loss and histopathological signs of colitis (Fig 26A-D). Poly (I:C) injections attenuated the severity of transfer colitis in RAG<sup>−/−</sup> hosts transplanted with B6 CD45RB<sup>high</sup> CD4 T cells as indicated by significantly reduced body weight loss, colitis score and IFN-γ transcript levels in colon of the mice treated with poly (I:C) compared to the mice without this treatment (Fig.26A,D,E). Poly (I:C) injections did not influence the course of colitis in the RAG<sup>−/−</sup> host transplanted with CD69<sup>−/−</sup> CD45RB<sup>high</sup> CD4 T cells, as body weight loss, the severity of histopathological signs of colitis and IFN-γ transcript levels in colon were not altered in the group treated with poly (I:C) when compared to the non-treated group. (Fig.16B,D,E). Body weight loss, histopathological signs of colitis as well as IFN-γ transcript levels in colon between poly (I:C)-treated and non-treated RAG<sup>−/−</sup> hosts transplanted with IFNAR<sup>−/−</sup> CD45RB<sup>high</sup> CD4 T cells were not significantly different (Fig.26C,D,E).

Hence, poly (I:C) attenuates CD45RB<sup>high</sup> CD4 T cell transfer colitis, but this effect is dependent on the CD69 and IFNAR surface expression on CD4 T cells.
Figure 26. Polyinosinic-polycytidylic acid (poly(I:C)) attenuates transfer colitis in RAG<sup>−/−</sup> hosts transplanted with B6 but not with CD69<sup>−/−</sup> and IFNAR<sup>−/−</sup> CD4 T cells. RAG<sup>−/−</sup> mice were i.p. transplanted with A. B6, B. CD69<sup>−/−</sup> or C. IFNAR<sup>−/−</sup> CD4 T cells. Poly (I:C) (20 µg/mouse) was i.p. injected twice a week to the half of the cell transplanted mice. Mean (± SEM) loss of body weight (%) of seven mice per group is shown. P values were calculated with a non-parametric student’s t test; p < 0.05 was considered statistically significant. D. Histopathological scores of colon sections taken from RAG<sup>−/−</sup> mice transplanted with the indicated CD4 T cells and treated or not with poly (I:C) twice a week. Each experimental group consisted of seven mice. In the non-parametric student’s t test p < 0.05 was considered statistically significant. N.S. – not statistically significant; * p < 0.05 E. RNA was isolated from the frozen colon tissue samples of RAG<sup>−/−</sup> hosts transplanted with B6, CD69<sup>−/−</sup> or IFNAR<sup>−/−</sup> CD4 T cells treated or not with poly (I:C) and reverse transcribed to cDNA. Relative expression of IFN-γ gene was measured by qRT-PCR and presented as mean (± SEM) of seven mice per group. P values were calculated with a non-parametric student’s t test; p < 0.05 was considered statistically significant. N.S. – not statistically significant; * p < 0.05
3.5.4. CD69 expression on CD4 T cells affects poly (I:C)-mediated IFN-I induction

In RAG\(^{-/-}\) animals reconstituted with B6, CD69\(^{-/-}\) or IFNAR\(^{-/-}\) CD45RB\(^{\text{high}}\) CD4 T cells the observed protective effects of poly (I:C) could be a direct effect of poly (I:C) on CD4 T cells. Alternatively poly (I:C) could act on non-CD4 T cells (i.e. myeloid cells) in reconstituted RAG\(^{-/-}\) animals and indirectly elicit protective effects on CD4 T cells in animals with transfer colitis.

It is reported that poly (I:C) that is systemically administrated \textit{in vivo} or added to the culture medium \textit{in vitro} induces TLR-3 derived signals [129, 145, 185]. Investigating the difference in cell response after poly (I:C) stimuli, expression level of TLR-3 gene was analysed in the spleen cells obtained from B6, CD69\(^{-/-}\) or IFNAR\(^{-/-}\) mice. However, no significant difference in TLR-3 expression was observed among these three cell types (Fig.27A). Despite this, total spleen CD69\(^{-/-}\) cells failed to significantly increase IFN-β1 expression after poly (I:C) stimulation \textit{in vitro} in contrast to B6 and IFNAR\(^{-/-}\) spleen cells (Fig.27B). Although B6 CD4 T cells do respond directly to poly (I:C) by increasing IFN-β1 expression (Fig.27C) this 2-fold increase is low as compared to the approximately 12-fold increase in IFN-β1 expression observed after poly (I:C) treatment of total spleen cells (Fig.27B). To test if the absence of CD69 expression on CD4 T cells or myeloid cells is responsible for impaired IFN-β1 expression after poly (I:C) stimulation of CD69\(^{-/-}\) spleen cells, B6 CD4\(^{+}\) T cells were co-cultured with CD69\(^{-/-}\) CD4\(^{-}\) cells or CD69\(^{-/-}\) CD4\(^{+}\) T cells with B6 CD4\(^{-}\) cells with or without addition of poly (I:C). In the absence of CD69 on the CD4\(^{-}\) cells a strong induction of IFN-β1 expression after poly (I:C) stimulation could still be seen (Fig.27D). When CD69 was not expressed by the CD4\(^{+}\) T cells (CD69-deficient T cells), IFN-β1 up-regulation after poly (I:C) treatment could not be observed (Fig.27D).

The data are showing the important role of CD69 expressed on the CD4 T cells in mediating poly (I:C) induced IFN-I expression.
Figure 27. Polyinosinic-polycytidylic acid (poly (I:C)) induction of type I interferons depend on CD69 expression by CD4 T cells A. RNA was isolated from total spleen cells of B6, CD69<sup>−/−</sup> or IFNAR<sup>−/−</sup> and reverse transcribed to complementary DNA (cDNA). Relative expression of TLR-3 was measured by qRT-PCR and the mean (± SEM) of five samples per group are shown. 8 µl of qRT-PCR products was loaded on 2% agarose gel, electrophoresis was performed and image obtained using GeneSnap acquisition software. B. Total spleen cells from B6, CD69<sup>−/−</sup> or IFNAR<sup>−/−</sup> were culture with or without addition of 200 µg/sample of poly (I:C) for 48 h. Total RNA was
isolated from cell lysates and reverse transcribed to cDNA. IFN-β1 expression was measured by qRT-PCR and mean (± SEM) of five mice per group is presented. C. Spleen cells of B6 mice were enriched for CD4 T cells and cultured with or without 200 µg poly (I:C)/well. After 48 h RNA from the cell lysates was isolated and reverse transcribed to cDNA. Relative expression of IFN-β1 was analyzed by qRT-PCR and the mean (± SEM) of five samples per group are shown. D. CD4⁺ and CD4⁻ fraction of the B6 or CD69⁻ spleen cells were separated via MACS columns. B6 CD4⁺ cells were mixed with CD69⁻ CD4⁻ cells and CD69⁻ CD4⁺ cells with B6 CD4⁻ and treated or not in vitro with 200 µg of poly (I:C). 48 h after total RNA was obtained from the cell lysates and reverse transcribed to cDNA for qRT-PCR measuring of IFN-β1 expression. Mean ± SEM of five samples per group are shown. All of these experiments were performed twice and the data from the representative one are presented. P values were calculated with a non-parametric student’s t test; p < 0.05 was considered statistically significant. N.S. – not statistically significant; * p< 0.05
4. Discussion

4.1. Surface expression of CD69 molecule

It is well documented that CD69 expression on immune cells is induced by activation [93, 123]. Induction of CD69 expression is observed after different in vitro cell activation methods [169], but it seems that at least in lymphocytes it depends from the activation of protein kinase C followed by elevation of cytoplasmatic calcium level [170]. CD69 expression is rapidly induced 2 h after cell activation, but it is transient since it declines 24 h after activation [169]. Therefore, CD69 is widely used to analyze the activation state of the leukocytes and is considered to be the marker of recently activated cells [87, 92]. As shown in this study, in mice raised under conventional SPF conditions the highest percentage of CD69-expressing CD4\(^+\) T cells is among intestinal LPL population. I showed that about half of the LP CD4\(^+\) T lymphocytes are recently activated and this observation confirms the theorem about physiological or controlled inflammation state in the healthy intestine [50]. Physiological inflammation is considered to be a specific property of intestinal mucosal compartment, allowing the immune system to efficiently eliminate pathogens and tolerate commensal at the same time point. My hypothesis was that such a high percentage of activated CD4\(^+\) T cells during homeostatic state in intestine could be derived from the constant presence of the luminal bacteria. Indeed, the fraction of CD69\(^+\) CD4 T cells significantly decreased not only in LP, but also in MLN and spleen of the mice devoid of intestinal microflora (either by housing the animals in GF conditions or by antibiotic depletion in SPF animals). The interactions between luminal microflora and mucosal immune system in intestine are already observed. It is reported that LP DC have a direct contact with luminal antigens by extending their processes into the lumen [132]. Furthermore, the introduction of luminal bacteria into the intestinal lumen of GF mice is followed by the entry of these bacteria into the PP and MLN [100]. This study confirmed the cross-talk of commensal microflora and immune system in the intestine, showing that bacteria in intestinal lumen regulate the activation state of CD4 T lymphocytes and possibly direct the further responses of these cells.
CD69 expression on CD4 T cells is also induced after oral antigen challenge. CD69 up-regulation was observed as fast as 24 h after second antigen delivery showing the fast induction of CD69 expression after cell activation in vivo, too. Strong induction of CD69 surface expression by CD4 T cells was seen even after innate stimuli (i.e. poly (I:C) injections) without engagement of TCR. Hence, the up-regulation of CD69 surface expression by the commensal microflora may require innate and TCR-specific stimuli. The observed effect of poly (I:C) was dependent of IFN-I delivered signals as IFNAR\(^{-/-}\) mice did not show this response. However, IFNAR\(^{-/-}\) mice had a high fraction of CD69-expressing CD4 T cells even before treatment (CD69 expression in intestine of IFNAR\(^{-/-}\) mice was comparable with B6 mice) suggesting that stimuli other that IFN-I are inducing CD69 expression in the steady state conditions.

4.2. CD69 as the marker of the cells with regulatory phenotype

The possible role of CD69 in the processes of the regulation of immune responses is already implied. It is reported that CD69 activation leads to the increased production of anti-inflammatory cytokine TGF-\(\beta\) [41, 91, 152] and TGF-\(\beta\) is an immunosuppressive cytokine known to suppress the cell proliferation, Th1 and Th2 differentiation, maturation of DC, activation of macrophages and NK cells [23, 54, 91]. Furthermore, CD4\(^{+}\) CD69\(^{+}\) T cells showed the regulatory properties with effector mechanisms partially dependent on TGF-\(\beta1\) [61]. In this study, I showed that CD69\(^{+}\) CD4 T cells recently activated in vivo by oral antigen feeding are characterized by LAP/TGF-\(\beta1\) surface expression. Membrane bound TGF-\(\beta\) is considered to be one of the effector mechanisms of regulatory cells by which they suppress the immune responses [55]. This study confirmed that CD69 activation is associated with an increased TGF-\(\beta1\) production, but also showed that CD69 regulates the production of pro-inflammatory cytokines. The lack of CD69 molecule was associated with increased expression and production of IFN-\(\gamma\), TNF-\(\alpha\) and IL-21 and decreased production of TGF-\(\beta1\) as shown with micro-array, RT-PCR and ELISA experiments. These data strongly suggested the role of CD69 as negative regulator of immune responses. Furthermore, CD69 affected the peripheral Foxp3\(^{+}\) T\(_{reg}\) cell pool, as demonstrated in vivo both in steady state conditions and after oral antigen challenge of OT-\(\II\) x CD69\(^{-/-}\) animals. Hence,
CD69 possibly promote the induction of peripheral Foxp3+ T\textsubscript{reg} cell population. As TGF-\(\beta\)1 is known to promote Foxp3+ T\textsubscript{reg} cell generation [68, 86, 105, 157], I speculated that reduction in Foxp3+ T\textsubscript{reg} cell fraction in CD69-deficient mice was in part due to the lack of CD69-induced TGF-\(\beta\)1 production in this animals. Still, exogenous addition of TGF-\(\beta\)1 could not restore the normal potential of naïve CD69\textsuperscript{-/-} CD4 T cells to become Foxp3+ T\textsubscript{reg} cells \textit{in vitro}. Therefore, CD69 is not necessary for the Foxp3 T\textsubscript{reg} cell induction because a population of CD69\textsuperscript{-/-} CD4 T cells was expressing Foxp3 both \textit{in vivo} and after \textit{in vitro} culturing in T\textsubscript{reg} polarizing conditions. However, CD69 contribute to the size of the Foxp3 T\textsubscript{reg} cell population, but not through TGF-\(\beta\)1-dependent pathway. It is possible that CD69 intracellular signalling interfere with Foxp3 gene expression [106] or that other cytokines, such as IL-10 are involved in these findings [82].

4.3. Regulatory role of CD69 in IBD

Although \textit{in vitro} experiments associated CD69-deficiency with the production of high amounts of pro-inflammatory cytokines and low amounts of TGF-\(\beta\)1, CD69\textsuperscript{-/-} mice did not develop spontaneous colitis when housed in animal facility of Ulm University. This shows that CD69 expression is not necessary for establishing the intestinal homeostasis. On the other hand, it is reported that TGF-\(\beta\)1\textsuperscript{-/-} mice develop strong wasting syndrome accompanied by a multifocal inflammatory cell response and infiltration of the inflammatory cells in many organs including intestine [89, 159]. TGF-\(\beta\)1\textsuperscript{-/-} mice do not survive more than 3-4 weeks after birth [89, 159]. Normal development and absence of inflammation in CD69\textsuperscript{-/-} animals shows that CD69-derived production of TGF-\(\beta\)1 contributes only partially to the total amount of this cytokine in the body.

However, using the different experimental models of colitis induction, I showed that CD69 regulates the immune responses in intestine during IBD. Reconstitution of RAG\textsuperscript{-/-} hosts with CD69\textsuperscript{-/-} CD45RB\textsuperscript{high} CD4 T cells was associated with the accelerated transfer colitis as compared to RAG\textsuperscript{-/-} hosts transplanted with CD45RB\textsuperscript{high} CD4 T cells from syngenic B6 donor mice. In this colitis model lack of CD69 on CD4 T cells led to the severe histopathology in the colon tissue and elevated levels of IFN-\(\gamma\), TNF-\(\alpha\) and IL-17 in the blood serum. This experiment connected CD69-deficiency on CD4 T cells with increased
production of the pro-inflammatory cytokines in vivo confirming my in vitro results. Hence, the increased expression of IFN-γ and TNF-α, reduced expression of TGF-β1 and reduction in the Foxp3+ Treg cell fraction in CD69−/− mice could contribute to the accelerated transfer colitis development after reconstitution of RAG−/− hosts with CD69−/− CD4 T cells. In the colitis model induced by administration of DSS in the drinking water, disease is induced by disruption of mucosal barrier followed by infiltration of inflammatory cells into the intestinal mucosa [180]. When CD69−/− mice were subjected to DSS they developed more severe disease comparing to B6 mice. In the absence of CD69 expression, the architecture and composition of intestinal epithelial layer was severely disrupted. I speculate that CD69 could also have the role in maintaining the barrier function of the intestinal epithelium by regulating the immune responses.

These experiments showed that CD69 expressed on the surface of CD4 T cells regulate in vivo immune responses and although presence of CD69 cannot prevent the colitis development it can attenuate the severity of the disease.

4.4. CD69 regulates the migration of CD4 T cells

The role of CD69 in the regulation of lymphocyte circulation through SLO has been already shown [57, 158]. CD69 directly interact with S1P1 receptor on the lymphocyte surface which leads to the internalization of S1P1 receptor [10]. On this way CD69 down-modulate the surface expression of S1P1, disables its interaction with S1P from the circulation and prevents the lymphocyte egress from the SLO [57, 158]. Trapping the lymphocytes in the SLO, CD69 causes lymphopenia and prevent recently activated lymphocytes to migrate to the site of inflammation [72, 158]. This could be one more mechanism of CD69-dependent down-regulation the immune responses. This could also mean that CD69−/− lymphocytes when activated in SLO would not down-regulate the surface expression of S1P1 receptor, possibly could continue their circulation through the body and rapidly reach the effector site where they mediate inflammation.

Indeed, in both CD45RB^{high} CD4 T cell transfer and DSS colitis models absence of CD69 lead to the increased infiltration of inflammatory cells into the intestinal mucosa. Furthermore, the expression levels of pro-inflammatory chemokines that provide the signal for the migration of various activated immune
cells were elevated in the CD69−/− mice during steady state conditions and colitis, too. Micro-array data showed significant alterations in the expression pattern of chemokine ligands and receptors by CD69-deficient CD4 T cells. In vitro chemotaxic assay showed that CD69−/− CD4 T cells have higher capacity to respond to the chemotactic stimuli. Hence, not only that CD69 regulates the expression of chemokine genes, but it also modulates the ability of CD4 T cells to answer the chemokine stimulation. The later is probably done by CD69-dependent regulation of the expression of genes for chemokine receptors. These data suggested the new role of CD69 as the regulator of chemokine-mediated migration of CD4 T cells. In in vivo competitive homing assay higher number of CD69−/− CD4 T cells was migrating to the peripheral tissues (e.g. intestine) comparing to the B6 CD4 T cells. This result is consistent with my hypothesis of increased migratory potential of CD69-deficient lymphocytes.

I speculate that the absence of CD69 leads to the increased migration of the inflammatory cells into the intestinal mucosa. The regulation of lymphocyte migration by CD69 (both S1P1- and chemokine-dependent) could contribute to the regulatory functions of CD69 observed in the IBD models. Increased number of inflammatory cells combined with the increased production of pro-inflammatory cytokines, decreased production of regulatory TGF-β1 and reduction in Foxp3 T_{reg} cells population could explain severe pathology observed in IBD models in the absence of CD69.

4.5. CD69 affects the signals of type I interferon system

Having shown that poly (I:C) strongly induce surface expression of CD69 by CD4 T cells in a IFN-I-dependent manner, I further analyzed the possible intercourse of CD69- and IFN-I- derived signals. First clue of the existence of such an intercourse was the finding that both CD69- and IFN-I-derived signals were necessary for the establishing of oral tolerance. Normal B6 and TCR-transgenic mice on the RAG−/− background were able to successfully establish oral tolerance. On the other hand CD69−/− and IFNAR−/− mice showed strong immune responses after subjected to oral tolerance induction protocol suggesting that they are impaired in fully establishing the oral tolerance.
Type I IFN have been reported to have protective effects in colitis models and patients with ulcerative colitis [65, 77, 104, 136, 181]. The ability of IFN-I to stimulate the production of IL-10 by human T cells and monocytes [7], to modulate Th1 and Th2 responses [75, 130] and inhibit the production of IL-13, a key cytokine in the development of UC [104] have been suggested to contribute to the protective effects of type I IFN in colitis models [181]. The treatment of DSS colitis with subcutaneous injections of poly (I:C) attenuates the colitis in a type I IFN-dependent manner [177]. In this study, poly (I:C) injections (20 µg/mouse) did not affect the course of transfer colitis after transfer of CD69<sup>−/−</sup> or IFNAR<sup>−/−</sup> CD45RB<sup>high</sup> CD4 T cells, while the same treatment protected from colitis the animals transplanted with B6 CD45RB<sup>high</sup> CD4 T cells. IFNAR<sup>−/−</sup> CD4 T cells are unable to respond to IFN-I showing that poly (I:C) protects from transfer colitis on IFN-I-dependent manner. On the other hand, CD69<sup>−/−</sup> cells showed an aberrant IFN-I response after poly (I:C) stimuli in vitro although they did not have the defect in the TLR-3 expression. This aberrant response was the consequence CD69-deficiency on CD4 T cells. Interactions of CD69 on the surface of CD4 T cells with a yet unknown ligand (potentially) expressed by myeloid cells may be involved in the observed effects.

It is of importance to report that injection of 200 µg of poly (I:C) per mouse to CD45RB<sup>high</sup> CD4 T cell transferred RAG<sup>−/−</sup> animals led to 50% mortality after the first injection in all the groups tested (B6, CD69<sup>−/−</sup> or IFNAR<sup>−/−</sup> CD45RB<sup>high</sup> CD4 T cell transferred groups). This could be explained by strong stimulating effects of poly (I:C) on innate immune cells. It is reported that macrophages and DC directly respond to poly (I:C). Macrophages start the production of pro-inflammatory cytokines (IFN-γ and TNF-α) and cytotoxic agents such as nitric oxide [6, 154, 171]. DC acquire mature phenotype with high expression of MHC and costimulatory molecules and also start the production of pro-inflammatory cytokines, all leading to strong stimulation of NK cells, CD8 T cell cytotoxic response and Th1 response of CD4 T helper cells [5, 114, 173]. Because of such a wide spectrum of immune stimulation effects, poly (I:C) administrated in high dose to normal immunocompetent mice can induce a lethal shock [6]. High dose of poly (I:C) injected to RAG<sup>−/−</sup> mice might start systemic pro-inflammatory response by innate cells which in these adoptive immune system-deficient
animals could not be controlled by regulatory T cells and possibly led to the death of the animals.

### 4.6. Concluding remarks

This study showed the novel role of CD69 surface receptor in the regulation of immune responses in the intestine and proposed the mechanisms for some of these CD69-mediated effects. Naive CD4 T cells do not express CD69, but they do express S1P$_1$ receptor which allows their egress from SLO and circulation through the body while they search for the specific antigen (Fig.28). Surface expression of CD69 by CD4 T cells is induced by intestinal microflora, recognition of specific antigen presented by APC or by IFN-I-derived signals (Fig.28). Activated CD4 T cells express high levels of CD69 on their surface. CD69 internalize S1P$_1$ receptor and this together with CD69-mediated down-regulation of chemokine ligands and receptors expression leads to the prevention of lymphocyte migration to the inflammation site (Fig.28). Furthermore, CD69-derived signals are inducing the production of anti-inflammatory TGF-β1 and reducing the production of pro-inflammatory IFN-γ, TNF-α and IL-21 by CD4 T cells (Fig.28). CD69 also contribute to the generation of Foxp3$^+$ T$_\text{reg}$ cell population in the intestinal tissues, but the mechanisms of this effect need to be discovered. CD69 affected IFN-I induction by the unknown mechanism. Possibly, by interaction with the potent ligand expressed on the myeloid cells (such as DC), CD69 induces the expression of IFN-I by myeloid cells (Fig.28), but further investigations need to confirm this hypothesis. All these complex processes regulated by CD69 molecule expressed by intestinal CD4 T cells are leading to the establishment of oral tolerance and attenuation of colitis (Fig.28).

Being induced by intestinal microflora activation antigen CD69 affects delicate process of cross-talk between luminal antigens and intestinal immune system. Hence, the up-regulation of CD69 by the commensal microflora is not only an indicator of lymphocyte activation, but has significant functional relevance. CD69 may help to control the potential harmful impact of the intestinal microflora to the host but further investigations are needed to address the role of CD69 in detail.
Identification of the proteins and factors involved in the intracellular signalling pathway downstream of CD69 would be very helpful for understanding how CD69 is able to mediate all these effects on the immune system. Furthermore, activation of CD69 by the specific ligand could be a novel option for the treatment of intestinal inflammation.

Figure 28. Activation antigen CD69 affects the development of oral tolerance and inflammatory bowel disease. Naive CD4 T cell do express T cell receprot, IFNAR and S1P₁ receptor on the surface, but not CD69. The intestinal microflora, specific antigen recognition or type I interferon (IFN) signals induce surface expression of CD69 by CD4 T cell. This leads to the internalization of S1P₁ receptor and prevention of CD4 T cell migration to the inflammation site. Putative, yet unknown ligand binds to the CD69 and reduces the production of pro-inflammatory cytokines (IFN-γ, TNF-α and IL-21), while increases the production of anti-inflammatory TGF-β by CD4 T cell. Possibly, the interaction between CD69 and it ligand on the myeloid cells (e.g. antigen presenting cells) might induce the IFN-β expression by myeloid cell. Through all these effects CD69 contributes to the induction of oral tolerance and attenuation of the colitis.
5. Summary

This study showed that activation antigen CD69 plays an important role in the responses of intestinal immune system:

• CD69 expression on the surface of intestinal CD4 T cells is regulated by luminal microflora; presence of intestinal microflora is inducing the expression of CD69 by CD4 T cells.

• CD69 regulates the production of cytokines by CD4 T cells; it is the negative regulator of pro-inflammatory cytokine production and induces TGF-β1 production.

• The generation of Foxp3 T<sub>reg</sub> cells on the periphery is affected by CD69; genetic deletion of CD69 in mice leads to the reduced size of Foxp3 T<sub>reg</sub> cell population in the GIT.

• The migration of CD4 T cells is regulated by CD69; beside the interaction with S1P<sub>1</sub>, CD69 is regulating chemokine gene expression.

• CD69- and IFN-I-derived signals are necessary for the establishment and/or maintenance of oral tolerance.

• CD69 expression attenuates both CD45RB<sup>high</sup> CD4 T cell transfer and DSS induced colitis; absence of CD69 expression in both colitis models leads to an accelerated disease development.

• Poly (I:C) protects from the CD45RB<sup>high</sup> CD4 T cell transfer colitis in the IFN-I- and CD69-dependent manner; CD69 affects poly (I:C)-induced IFN-I production.
6. Literature


57. Grigorova, I. L., M. Panteleev, and J. G. Cyster. 2010. Lymph node cortical sinus organization and relationship to lymphocyte egress dynamics and


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7. Appendix

7.1. List of publications


7.2. Awards

09.2010 Young investigator travel award by the European Mucosal Immunology Group (EMIG)

09.2011 Young investigator travel award by the German Gastroenterology Association (Deutsche Gesellschaft für Verdauungs- und Stoffwechselkrankheiten(DGVS))
7.3. Curriculum vitae

Not included in the online version for reasons of privacy protection.

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