Analyses of the differential modulation of the immune response of dendritic cells and T cells by colitogenic and non-colitogenic bacteria

Untersuchungen zur differentiellen Modulation der Immunantwort von dendritischen Zellen und T Zellen durch kolitogene und nicht kolitogene Bakterien

Dissertation

der Fakultät für Biologie
der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

vorgelegt

von

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aus Bangladesh

2006

Tag der mündliche Prüfung   **27.01. 2006**

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1   Introduction

1.1   Intestinal immune system

The mucosal immune system has the complex task of responding to a vast number of ingested antigens. Under normal physiological conditions, immunological tolerance is induced to food and commensal bacteria whereas potential effector immune response occurs against pathogenic microorganisms. The immune system has a series of anatomically distinct compartments specialized to combat pathogen in a particular site of the body. For example peripheral lymph nodes and spleen respond to antigens that have entered the tissue or spreaded into the blood. Another compartment of the immune system is the mucosa associated lymphoid tissue or MALT which guards the surfaces of the body, e.g. lungs, gut, eyes, nose, mouth, throat, uterus and vagina etc. These surfaces are permeable to some extent due to their physiological functions and thus vulnerable to infection. For the gut it is more crucial as its physiological function is to absorb foreign antigens as food. It has to avoid immune reaction against food materials and commensal microorganisms but mount immune response to kill pathogenic organisms. This immune system lining the gut is known as gut associated lymphoid tissue or GALT. It is comprised of organized lymphoid tissue as well as small foci of lymphocytes and plasma cells scattered throughout the lamina propria of the gut wall.

Gut associated lymphoid tissue (GALT) comprises:

- tonsils, adenoids (Waldeyer's ring)
- Peyer's patches
- lymphoid aggregates in the appendix and large intestine
- lymphoid tissue accumulating with age in the stomach
- small lymphoid aggregates in the oesophagus
- diffusely distributed lymphoid cells and plasma cells in the lamina propria of the gut

Peyer's patches (PP) are aggregates of lymphoid follicles in the small bowel especially in the distal ileum. They form a domelike structure extending into the lumen of the intestine. The overlying layer of Peyer's patches contains specialized epithelial cells which have microfolds on their luminal surface and are called M cells. M cells are believed to be important in the transfer of antigen from the gut lumen to Peyer's patches. Peyer's patches facilitate the generation of an immune response within the mucosa. B cell precursors and memory cells are stimulated by antigen in Peyer's Patches.

GALT comprises various cell types, such as granulocytes and macrophages which form part of the innate immune system and provide non specific immunity and lymphocytes which mediate adaptive immunity. Figure 1 presents the organization of various cell types and factors involved in GALT.
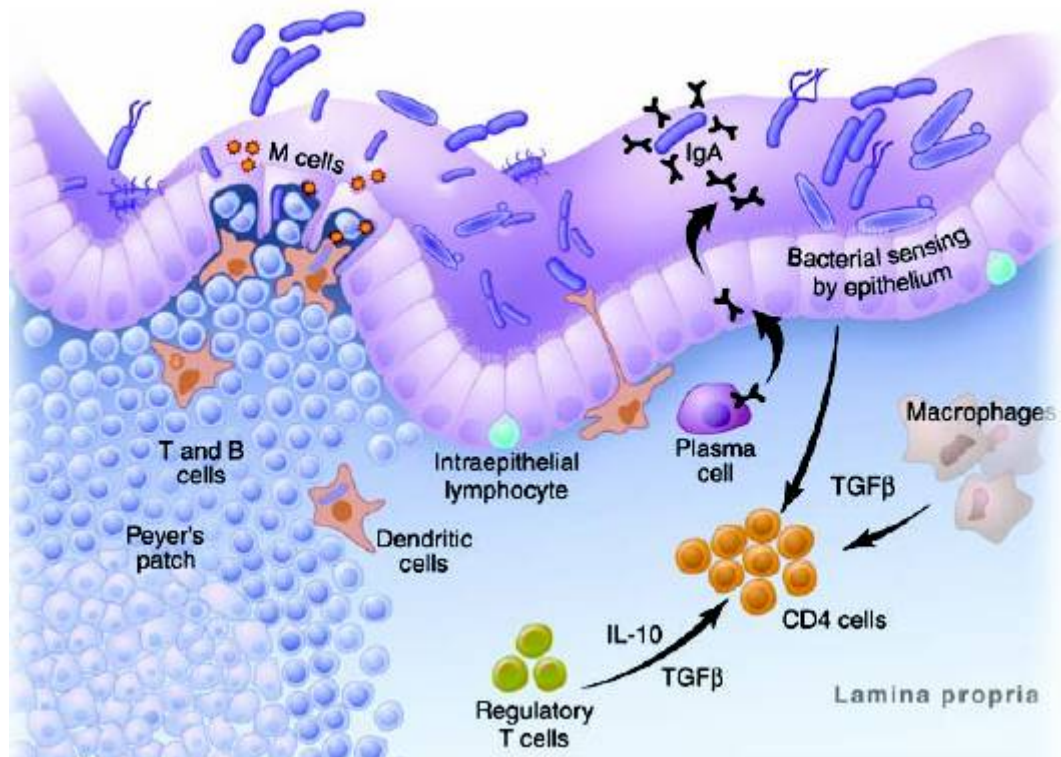
Figure 1: **Gut Associated Lymphoide tissue (GALT).** The epithelium overlying organized GALT contains specialized M cells that constantly transport gut bacteria and antigens from the gut lumen into the lymphoid tissue. DC in the lamina propria (LP) extends dendrites through epithelial cells to the lumen and also sample gut bacteria. The epithelium is filled with CD8$^+$ T cells, and the LP contains many CD4$^+$ T cells, macrophages, and IgA antibody–producing plasma cells. Potentially tissue-damaging T cell responses may be inhibited by immunosuppressive cytokines and regulatory T cells (64).

1.1.1   Physical barrier at the mucosal surface: Mucus, antimicrobial peptides.

The specialized architecture of the intestinal epithelium forms a tight barrier against the penetration of microorganisms. Tight junctions between the IECs (Intestinal epithelial cells) are involved in the barrier function of the epithelium (117). The physical barrier is supported by a thick layer of mucus containing diverse mucins. Goblet cells present both in the crypt and villus epithelium throughout the small intestine, colon and rectum produce mucus. The mucus layer forms a semipermeable protective barrier, helps to accelerate intestinal repair

especially by intestinal trefoil factor, also traps bacteria in the mucus flow and thus washes away by intestinal peristalsis.

1.1.2   Innate and adaptive immunity at the intestinal mucosa

Intestinal homeostasis is maintained by finely tuned balance between immune response and tolerance to the luminal microorganisms. Two effectors are responsible for innate immune response, antimicrobial peptides such as defensins and neutrophils. Defensins are amphipathic in character, interact with and lyse bacterial cell wall (125). In microbial colonization neutrophils are recruited to the basal side of IECs mainly in response to IL-8 produced by epithelial cells. Neutrophils then translocate through epithelial lining and in the gut lumen exert their antibacterial function (92). In addition to maintaining physical and innate immune barrier functions, the intestinal epithelium also takes part in the induction of an effective adaptive immune response at the intestinal mucosa with IEC together with antigen presenting cells and lymphoid cells. It occurs in follicle associated epithelium (FAE) which contains M cells (92).

M cells are much less prominent than absorptive gut epithelial cells, enterocytes. M cells are available on the membrane overlying Peyer's patches. M cells are adapted to interact directly with molecules and particles within the lumen of the gut and hence do not have thick mucus layer on them and also do not secrete mucus. M cells take up molecules and particles from the gut lumen by endocytosis or phagocytosis, transport them through the interior of the cell in vesicles to the basal cell membrane and release them into the extracellular space. This M cell mediated transport of antigen from lumen is called transcytosis. Lymphocytes and antigen presenting cells present at the basal surface of M cells take up this material transported by M cells and process it for antigen presentation. Some pathogens use M cells as a port of entry to subepithelial region.

Luminal microorganisms can also be captured by dendritic cells (DC) that extend pseudopods across the IECs of the epithelial lining and retract these processes before trafficking to immunocompetent sites with their bacterial cargo. Mucosal effector mechanisms include humoral factors e.g. secretory IgA as well as cellular factors of B cells, T cells and lymphocytes unique to GALT (92).

The major antibody isotype present in the lumen of gut is **secretory IgA (sIgA)**. In blood, IgA is found mainly in monomeric form but in mucosal secretions it is found as a dimer and mostly IgA2 form. IgA in gut is synthesized by plasma cells located in the lamina propria and

is transported to the lumen by immature epithelial cells present at the base of the intestinal crypts which express polymeric immunoglobulin receptor at their basolateral surface. Secreted IgA binds to the mucus layer overlying the gut epithelium where it can bind to and neutralize gut pathogens and their toxic products. IgA acts in cooperation with non-immunological defense mechanisms to mediate immune exclusion of foreign antigens by preventing epithelial adherence and penetration of invasive pathogenic microorganisms, neutralizing toxins and viral multiplication (20).

Antigen presenting cells: Dendritic cells are a widely distributed, migratory group of bone-marrow derived leukocytes that are specialized for the uptake, transport and presentation of antigens to T cells (38,70,107). At an immature stage of development DC act as sentinels in peripheral tissue continuously sampling antigens. When these DC encounter microbial products, they process them and present them on the cell surface loaded on MHC molecules. During antigen processing DC migrate to lymph nodes, become mature which is evident by up regulating costimulatory molecules, and these mature DCs interact with T cells containing specific receptor for the antigen loaded on MHC at the cell surface of DC (103).

Mucosal DC takes up antigens in several ways interacting with intestinal epithelial cells. In organized mucosal tissues, such as PP and colonic follicles, M cells directly delivers antigen from the lumen to underlying DCs. At the terminal ileum DCs extend transepithelial processes through intestinal epithelia lining the gut lumen to sample luminal antigens and microbes directly. Richard Blumberg (129) proposed another mechanism. Neonatal Fc receptors mediate the bidirectional transport of IgG, resulting in transport into the lumen and trafficking back the antigen antibody complex to the lamina propria. Due to neonatal Fc receptor, antigen antibody complex is not degraded in epithelial cells and is taken up by underlying DC. DC can also take up and process antigens from apoptotic intestinal epithelial cells (50). DC sense the microbe by recognizing evolutionarily conserved molecular patterns that are integral to microbial carbohydrates, lipids and nucleic acids through pattern recognition receptors (PRR) (47). Toll like receptors are prime members of these PRRs (85).

DCs consist of several phenotypically distinct subsets that differ in their microenvironmental localization, migration potential, PRR expression, responsiveness to microbes, and their capacity to induce and regulate distinct arms of the innate and adaptive immune systems. Major DC subsets in human blood are plasmacytoid DC, interstitial DC and Langerhans DC. In mice the major DC subsets in secondary lymphoid organs are lymphoid DC (CD8α^+), myeloid DC (CD8α^-), plasmacytoid DC and langerhans DC (83).

DC sample intestinal bacteria either directly or can engulf them after being transported by M cells. DCs present these antigenic peptides from captured microbes to B and T lymphocytes either locally at GALT or within the mescenteric lymph nodes (MLN) that drain the gut submucosa. Presentation of microbial antigens to B cells triggers production of a commensal specific IgA response that prevents the commensals from spreading beyond the gut mucosa where they could elicit a systemic inflammatory response (53).

T cells: T lymphocytes originate in the bone marrow and then develop in thymus and play a key role in adaptive immunity. T lymphocytes are subdivided into $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells. $CD4^+$ T cells are further subdivided according to their effector function in T helper 1 (Th1) and T helper 2 (Th2) and regulatory T cells. Th1 cells secrete IFN-γ and activate macrophages whereas Th2 cells secrete cytokines IL-4, IL-10 and activate B cells to produce antibodies. T cells are also classified according to their T cell receptor (TCR) type, αβ T cells and γδ T cells. In the gut there are αβ T cells bearing CD4 or CD8. They participate the conventional T helper or cytotoxic function against foreign antigens. There are some unusual T cells, e.g. CD8ααTCRαβ T cells. TCRγδ T cells are especially abundant in the gut mucosa compared to other lymphoid tissues. A subset of these T cells specifically recognizes injured epithelial cells and destroys them. γδ T cells may also have a role in oral tolerance, because tolerance appears to be reduced in mice lacking this subset of T lymphocytes.

Regulatory T cells: There are two possible and opposite outcomes of exposure to foreign antigens through the mucosa of the gut. There is often tolerance to food antigens but vigorous antibody and T cell response against pathogens. T cells specific for food antigens are either deleted by apoptosis, or become anergic that is refractory to further antigenic stimulation or suppressed by regulatory T cells. Th3 cells in Peyer's patches are involved in oral tolerance (antigen specific nonresponse to an antigen given by oral route) and secrete TGF-β (122,123). Another class of regulatory T cells named Tr1 (T regulatory cell 1) secretes IL-10 and inhibits the development of an inflammatory T cell response as well as reduces antibody response.

1.1.3   Microflora

The gastrointestinal (GI) tract of human adults contains more than 400 different species of bacteria. From birth to death we are colonized by a vast complex and dynamic array of

microorganisms. Most of these bacteria are commensals that coexist peacefully with their host, remain harmless and also do not spread beyond the gut lumen. Some commensals even are beneficial to the host (53). The commensals are not just randomly distributed throughout the GI tract of adults but are found differently distributed at different regions of the tract. The oral cavity contains microflora containing 200 species shed from oral surface such as tongue and cheeks (36). The human stomach and the upper two-thirds of the small intestine (duodenum and jejunum) contain low numbers of microbes due to low pH and relatively swift flow of stomach contents (113). The upper small intestine contains mainly acid tolerant lactobacilli and streptococci. In the distal small intestine (ileum) bacteria number increases to 10^8 bacteria /ml with decreased peristalsis and lower oxidation-reduction potentials. The large intestine (colon) is the primary site for microbial colonization (10^{10}-10^{11}bacteria/g) in humans and animals probably because of the slow intestinal motility encountered here and the very low oxidation-reduction potentials. 99.9% of the GI microflora is obligate anaerobe. There is also a difference in bacterial distribution horizontally in GI lumen, mucus layer lining the intestinal surface, deep layer of mucus at intestinal crypt and immediate surface of mucosal epithelial cell.

The microflora has some beneficial effects to the host. Microflora helps the host by interfering with further bacterial colonization, helps in the development of mucosal immune system, maintains GI tract peristalsis and intestinal mucosal integrity, detoxifies carcinogens by converting it to noncarcinogens and also produces vitamin K and B complex (9). The relationship between host and gut microbes can shift from commensalisms toward pathogenicity in certain diseases. The pathogenesis of IBD appears to involve an 'inappropriate' activation of the mucosal immune system due to a loss of tolerance to gut commensals (43).

1.1.4   Intestinal homeostasis

In order to maintain intestinal homeostasis in the gut, the immune system must tightly regulate cellular responsiveness and maintain a balance between active immunity and tolerance. Loss of tolerance to gut flora develops IBD (10). After a productive immune response to a foreign antigen, the immune system returns to a state of rest, so that the numbers and functional status of lymphocytes are reset to normal state by different mechanisms shown in figure 2. In several colitis models as well as our model of IL-2$^{-/-}$ mice one or more

mechanisms responsible to bring back the immune system to basal level after an immune response is disturbed.
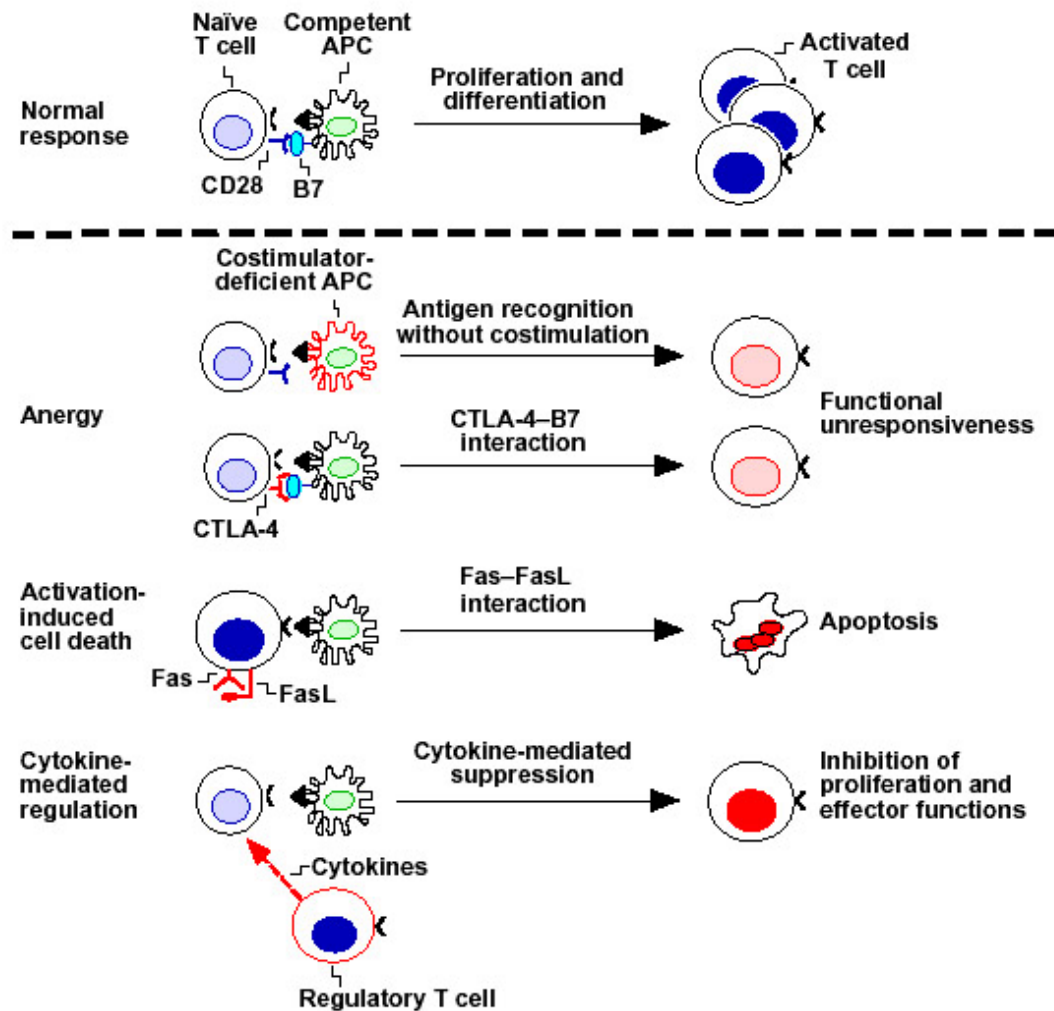
Figure 2: Mechanism of active termination of immune responses. A normal T cell response is triggered by the recognition of antigen and the co stimulatory signal. Multiple mechanisms may function to inhibit the expansion or/and effector functions of T lymphocytes. These mechanisms appear to be most important for the maintenance of self-tolerance (118).

1.2   Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) are chronic relapsing inflammatory disorders of the gastrointestinal tract. Approximately 0.1 percent of the western population suffer from Inflammatory bowel diseases (106). Disease incidence is the highest in developed, urbanized countries (12). About 20% of the individuals with Crohn's disease (CD) have a relative with some form of IBD. The age of onset is between 15 to 30 years, but both younger and older individuals may be affected (13).

IBD is a multifactorial disease, depends on genetics, environment and immune function of the individual (30). Most generally accepted pathogenesis of IBD is that it results from abnormal immune response to enteric bacteria in individuals with susceptibility due to polygenic defects (131). Microbial agents are intimately involved in four current hypotheses of IBD, first, persistent infection with a pathogen (e.g. *Mycobacterium tuberculosis*, *Listeria monocytogenes* in Crohn's disease and pathogenic *E. coli* in ulcerative colitis), second, subtle alteration in bacterial function and composition, third, altered mucosal barrier, e.g. defective mucus, tight junctions, epithelial restitution, fourth, defective regulation of mucosal immune response, e.g. less down regulation and aggressive induction of immune response (28). There are two main forms of IBD, Crohn's disease (CD) and ulcerative colitis (UC).

1.2.1   Crohn's disease

Crohn's disease is a transmural (affecting all layers of bowel), dense infiltration of lymphocytes and macrophages, with presence of granulomas in up to 60% of the patients, fissuring ulceration and submucosal fibrosis. Any part of the gastrointestinal tract can be affected, but most commonly the terminal ileum, cecum, pre-anal area and colon. It is characterized by the presence of segments of normal bowel between affected regions, known as skip lesions (12). Clinical features associated with Crohn's disease are dirrhoea, pain, narrowing of the gut lumen leading to strictures and bowel obstruction, abscess formation and fistulization to skin and internal organs (12).

1.2.2   Ulcerative colitis

The inflammatory process invariably involves the rectum and extends proximally in a continuous fashion, remaining restricted to the colon. Sometimes it is limited to the rectum as ulcerative proctitis. Inflammation affects superficial (mucosal) layers with infiltration of

lymphocytes and granulocytes and loss of goblet cells, and presence of ulceration and crypt abscesses (12). Ulcerative colitis is characterized by severe dirrhoea, blood loss and progressive loss of peristaltic function leading to a rigid colonic tube. In severe case, it leads to 'toxic megacolon' and perforation.

1.2.3   Cellular elements involved in IBD

Antigen presenting cells: APC in mucosal tissue are key cells to induce effector and regulatory cell response. A defect in T cell response which is the main cause of disease for most of the animal models of IBD may arise either from APC function or APC-T cell interactions. Macrophages, a type of APC are activated in mucosal inflammation and function mainly as effector cells.

T cells: T cells play multiple roles in mucosal inflammation both as effector cells and regulatory cells. $CD4^+$ effector T cells are one of the main cells infiltrating mucosal tissues in IBD and their deletion ameliorates inflammation. The role of $CD8^+$ T cell is not clear as its deletion has no effect on intestinal immunity though increased cytotoxic T cell function has been observed in some of the models (104). $\gamma\delta$ T cells which are confined to intra-epithelial compartment do not play an important role as effector cells in any form of colitis. There are different regulatory T cells. Th3, TGF-ß secreting T cells which are induced by antigen feeding (109) and in response to microflora, termed as oral tolerance and IL-10 secreting regulatory T cells (Tr1) which have poor proliferative capacities. Recently it has been shown that both IL-10 and IFN-γ are necessary for later ones induction (60). These T cells establish a mucosal homeostasis that ensures that most mucosal responses are self limited and do not result in inflammation. Failure in this homeostasis results in IBD which is obvious from a number of mouse models of IBD (12).

B cells: B cells play a role in either induction of mucosal inflammation or its maintenance. Autoantibodies are found in some models of colitis as well as in human IBD (109). In colitis model 'TCR-α chain deficiency', B cells have a protective role (73).

Epithelial cells: Epithelial cells form a barrier against exposure to mucosal microflora and other mucosal antigens and thus play a key role in the down regulation of mucosal immune responses. It is obvious in several colitis models that alterations in this barrier are the primary

cause of colitis (41,80) whereas in other models change in barrier is a secondary factor for mucosal inflammation (66). Epithelial cells also function as sensors of the bacterial microenvironment. They release chemokines that draw leukocytes in peri-epithelial regions which also can play a role in the initiation of mucosal inflammation (109).

1.2.4   Probiotic therapy in IBD

Probiotics are microbial species that exert beneficial effects on the host (93). Some commensal organisms from the intestinal flora have health-promoting properties and are used as probiotics to treat IBD (37). The gastrointestinal tract has developed redundant

Table 1: Mechanisms of action of probiotic agents (93).

Inhibit growth of pathogenic enteric bacteria

Decrease luminal pH
Secrete bactericidal proteins
Stimulate defensin production by epithelial and Paneth cells
Resist colonization (occupy ecologic niche)

Block epithelial attachment or invasion by pathogens

Block epithelial binding by inducing of MUC 2
Stimulate mucus production to alter biofilm
Inhibit epithelial invasion, Rho dependent and independent
pathways

Improve epithelial and mucosal barrier function

Produce short-chain fatty acids, including butyrate
Enhance mucus production
Increase barrier integrity

Alter host immune response

Induce IL-10, TGF-β and Cox2 (PGE2) expression and secretion
Stimulate secretory IgA production
Decrease TNF-α, IFN-γ expression
Activate regulatory T cells

Genetic engineering

Express and secrete IL-10 and trefoil factors

mechanisms to coexist with its resident microflora, to rapidly respond to invading pathogens and then return to homeostasis with its commensals after the pathogenic infection is cleared.

When these homeostatic mechanisms are perturbed, chronic intestinal inflammation develops (95,100). Among commensals some bacteria are beneficial and some are aggressive and detrimental. Both animal model and human studies suggest that environmental and genetic factors govern the relative balance of beneficial and detrimental bacterial species and that therapeutic manipulation of this balance can influence health and disease (95,96). One of these therapeutic approaches is administration of probiotics to alter the composition of microflora towards the more protective bacteria. *E. coli* Nissle 1917 is able to maintain remission of quiescent ulcerative colitis. Probiotics can also reverse active ulcerative colitis. Treatment of Crohn's disease with probiotics continues to be unsubstantiated, although several small trials suggest an ability to prevent relapse of quiescent disease (93). Probiotics are more efficient in preventing relapse of inflammation and infection than reversing established disease. Administration of existing probiotic preparations transiently increases luminal concentrations without permanently colonizing the intestine (93). Probiotic microbial agents and their components exert their protective effect through different mechanisms summarized in table1.

1.3   Models for colitis

The study of initiating factors in human IBD is difficult as the clinical materials are from the later stage of the disease. So, new experimental animal models of IBD are giving many clues about the early events of human IBD. Studies on animal models of mucosal inflammation started 60 years ago to understand the pathogenesis of human IBD (109). There is much development in this field and different models helped to understand some key points of IBD. These models explained key roles of microflora and epithelial barrier, over reactive lymphocytes or lack of regulatory lymphocytes in mucosal inflammation.

1.3.1   Classification of animal models

Animal models of intestinal inflammation can be divided in four categories: spontaneous models, inducible models in mice with normal immune system, adoptive transfer models in immunocompromised hosts, and genetically engineered models (transgenic mice, knockout mice).

Table 2 Characteristics of animal models of chronic intestinal inflammation [Adapted from (127)]

Model	Pathology	Affected sites
Spontaneous model		
C3H/HeJBir	Acute, chronic transmural	Cecum, right colon
Inducible model		
DSS	Acute, chronic mainly mucosal	Colon
TNBS	Acute, chronic transmural	Colon
Adoptive transfer model models		
CD4+CD45RB^{hi} transfer into SCID or RAG deficient mice	Acute, chronic transmural	Colon, duodenum
Genetically engineered or transgenic		
IL-2 knockout mice	Acute, chronic mucosal	Colon
IL-10 knockout mice	Acute, chronic transmural	Colon, sometimes jejunum/ileum
STAT4 transgenic	Acute, chronic transmural	Colon, ileum

In a spontaneous model of colitis, C3H/HeJBir mice develop colitis spontaneously with microflora reactive CD4^{+} T cells. In an inducible model for colitis, e.g. feeding mice for several days with DSS, the epithelial barrier is destroyed resulting in chronic lesions with infiltrating macrophages, CD4^{+} T lymphocytes and fissuring ulcers. In an adoptive transfer model, transfer of CD4^{+}CD45RB^{hi} T cells from wild type donor mice to immunodeficient SCID mice or RAG deficient mice causes a wasting syndrome with transmural intestinal inflammation by a proinflammatory IL-12 driven Th1 response by CD4^{+}CD45RB^{hi} cells (127). Bacterial antigens play a crucial role for the pathology because antibiotic treatment or germ free breeding of recipient SCID mice is associated with significantly less bowel inflammation (2). In genetically engineered models, IL-2$^{-/-}$ mice develop IBD associated with increased levels of IFN-γ, TNF-α and IL-1ß indicating an activation of the Th1 pathways (29,101).

1.3.2   Mechanism for colitis development

It is obvious from the above discussion that multiple factors are involved in the onset of pathogenesis in these colitis models. But they usually follow a final common pathway of

inflammation mediated by CD4$^+$ T cells, either by Th1 T cell response associated with excessive IL-12, TNF-α, IFN-γ secretion or an excessive Th2 cell response associated with increased IL-4, IL-5 secretion (109). The different colitis models that follow either of these models are summarized at table 3.

Table 3: Models for intestinal inflammation classified by nature of T cell-mediated inflammation (109).

Th1 models	Th2 models
TNBS colitis(SJL/J mice)	TCR-α chain deficiency
DSS colitis	TNBS-colitis in BALB/c mice
SCID-transfer colitis	Oxazalone colitis
TCR Tg mice with lymphopenia	WASP deficiency
IL-10 deficiency colitis	
C3H/HeJBir	
Samp1/yit mice	
T-bet Tg mice	
STAT4 Tg mice	
IL-2 deficiency colitis	
HLA-B27 Tg rats	

Mucosal immune responses are finely tuned by opposing mechanisms, effector cell and regulatory mechanism. Regulatory mechanism has two faces, T cell anergy or deletion or regulatory T cells. Models of mucosal inflammation can be classified in two broad categories, Type1 models where the cause of IBD is exacerbated effector T cell function, e.g. STAT4 transgenic mice and type 2 models where T regulatory function is impaired, e.g. IL-2 knockout mice (109).

1.3.3   Immunophysiology of normal mucosa:

The prevailing adaptive immune effector mechanism throughout the gut is the secretory IgA that performs immune exclusion at the epithelial surface. In normal conditions mucosal T cells are primed memory T cells with little CD25 on their surface which reflects no recent activation. The well balanced immunological homeostasis in the intestinal mucosa breaks down in IBD. The mucosal microvascular endothelium expresses more adhesion molecules

that favor recruitment of more B and T cells (15,16). This leads to an accumulation of large number of potentially proinflammatory IgG-producing plasma cells and recruitment of monocytes like macrophages with aggressive properties as well as T cells showing various antimicrobial specificities directed against the commensal microbiota (17-19,65). Preferential overproduction of the IgG1 subclass compared to IgG2 is seen in UC, apical deposits of IgG1 together with complement activation on the surface epithelium causes cytotoxic immune attack directed against brush border antigen(s) (13). IBD lesions contain many recently recruited and activated T cells and macrophages (CD14$^+$) and neutrophils with increased capacity for production of proinflammatory cytokines, particularly IL-1 and TNF as well as reactive metabolites of oxygen and nitrogen. Stimulation by the LPS of gram negative bacteria from invading microflora by CD14 and TLR4 probably skews the local immune response towards a Th1 profile (14). The activated Th1 cells appear to be particularly long lived because IL-12, as well as complexes of IL-6 and soluble IL-6 receptor induce antiapoptotic mechanisms (78). A strong persistent Th1 response may thus override regulatory intestinal CD4$^+$T cells that normally perform active suppression by secreting IL10 (Tr1) or TGF-ß (Th3) as part of mucosal tolerance maintenance (75). Thus perturbation of a tightly controlled cytokine network, with abnormal crosstalk between several mucosal cell types, appears to be the first step of a progressive immunopathological cascade reaction in IBD (13).

1.4   IL-2$^{-/-}$ mice as a colitis model

1.4.1   IL-2, its receptor and physiological role

IL-2 is a four bundled α-helical cytokine produced mainly by activated T lymphocytes (67). There is also evidence of IL-2 expression by naïve CD8$^+$ T cells, dendritic cells and thymic cells (6,21,34,128). In T cells IL-2 synthesis is tightly regulated at the mRNA level by signals from the TCR and CD28 (82).

IL-2 binds to and signals through a receptor complex consisting of three subunits named IL-2Rα (CD25), IL-2Rβ (CD122) and common γ chain (γ$_c$, CD132) leading to activation of signal transducer and activator of transcription 5 (STAT5) (67,77). IL-2, via interacting the

Table 4: IL-2 exerts pleiotropic effects on different cells. Effect of IL-2 on immune cells are summarized here (33).

Cell type	Primary activities of IL-2
CD4$^+$ T cells	Induces expansion of antigen specific clones via both proliferative and antiapoptotic mechanisms.
	Augments production of other cytokines
	Required for differentiation to Th1 and Th2 subsets
	Induces apoptosis of activated T cells via Fas/FasL signaling (Activation induced cell death)
	Involved in development of CD4$^+$CD25$^+$ T cells
CD8$^+$ T cells	Induces expansion of antigen specific clones
	Augments cytokine secretion
	Augments cytolytic activity
	Induces proliferation of memory CD8$^+$ T cells
B cells	Enhances antibody secretion
	Initiates immunoglobulin J chain transcription and synthesis
	Promotes proliferation
NK cells	Promotes proliferation
	Augments cytokine secretion
	Enhances cytolytic activity

IL-2R, mediates T-cell growth, and depending on the conditions, also promotes cell survival, effector function, and apoptosis (77). IL-2 sensitizes activated T cell to undergo apoptosis or activation induced cell death (AICD) by FAS and TNF-dependent pathways (59,118,119). IL-2 is required for the development, expansion, and/or function of CD4$^+$CD25$^+$ regulatory T cells (76). Different functions of IL-2 are summarized in table 4.

1.4.2   IL-2$^{-/-}$ mice develop colitis induced by microflora

IL-2$^{-/-}$ mice raised in a conventional environment develop a disorder of the hemopoietic and immune system characterized by anemia, lymphocytic hyperplasia, progressive loss of B cells, disturbances in bone marrow hemopoietic cells, and an inflammatory bowel disease (24). One prominent feature of IL-2$^{-/-}$ mice is a massive expansion of lymph nodes and spleen due to infiltration of activated T cells, which is consistent with the impaired regulation of

homeostatic proliferation (76). IL-2$^{-/-}$ mice develop normally at first 3-4 weeks of age but develop severe anemia after this time. 50% mice die from this within 4-9 weeks of age (71). The animals which survived develop a progressive colitis first detectable between 6-15 weeks of age (89). The histological appearance of this aggressive colitis resembles human ulcerative colitis. Colitis is mediated by CD4$^+$ T cells which infiltrate colon and bone marrow to cause colitis, anemia and loss of B cells respectively (54). IL-2$^{-/-}$ mice with a BALB/c background die in early age, 5 to 6 weeks, from autoimmune reaction against hemopoietic cells, anemia (29).

1.4.3   IL-2$^{-/-}$ mice have a dysregulated peripheral T cell response

At 13 weeks of age of IL-2$^{-/-}$ mice, infiltration of the superficial mucosa by lymphocytes, plasma cells, granulocytes and an increased number of mitotic epithelial cells as well as ulcerations and crypt abscesses were observed. Horak et al (63) showed that T cells are responsible for inflammatory bowel disease in IL-2$^{-/-}$ mice. IL-2$^{-/-}$ mice were bred with RAG$^{-/-}$ mice which fail to develop T cell as well as B cell and were free from IBD. In contrast IL-2$^{-/-}$ mice bred with JH$^{-/-}$ mice, which lack B cells, develop IBD. It was also shown that CD4$^+$ T cells rather than CD8$^+$ T cells which infiltrating the lamina propria are involved in bowel inflammation (105). Increased number of $\alpha\beta$TCR$^+$CD4$^+$T cells in IEL (Intra epithelial lymphocytes) and LPL (lamina propria lymphocytes) of colon from SPF (specific pathogen free) IL-2$^{-/-}$ mice were observed (120). IL-2$^{-/-}$ germ free mice showed decrease in the number of CD8$\alpha\alpha^+$, particularly $\gamma\delta^+$ T cells in IEL (24).

1.4.4   IL-2$^{-/-}$ mice fail to limit autoreactive T cells

IL-2 sensitizes activated T cells to undergo apoptosis or activation induced cell death (AICD) in vitro by upregulation of FAS (CD95) and tumor-necrosis factor receptor (TNFR) (59,118,119). It is assumed that AICD is an important mechanism that limits immune response by reducing antigen specific T cells. A failure in this mechanism is postulated to be associated with lymphoproliferation and lethal autoimmunity in IL-2$^{-/-}$ mice (67). One early hypothesis to explain the autoimmunity seen in IL-2$^{-/-}$ mice was impaired negative selection of self-reactive thymocytes. CD4$^+$CD25$^+$ T regulatory (T$_{Reg}$) cells develop in the thymus and constitute 5-10% of the circulating T cell population in healthy humans and mice. They potently inhibit T cell proliferation in vitro and suppress the autoreactive T cells in vivo (76). The evidence for this hypothesis has come from some recent findings. The number of T$_{Reg}$

cells are markedly reduced in mice that are deficient in IL-2, IL-2 receptor or a downstream signaling molecule for IL-2, STAT5 (12). Administration of IL-2 or IL-2 producing cells to IL-2$^{-/-}$ mice restores the production of T$_{Reg}$ cells and lymphoid homeostasis (1,32,51). IL-2 is required for both thymic development and peripheral expansion /maintenance of Treg cells (76). IL-2 promotes T$_{Reg}$ cell growth and suppressor function in vitro (110-112,114).

1.4.5   B cell development and survival are disturbed in IL-2$^{-/-}$ mice.

IL-2 plays an important role during an immune response by stimulating antigen-activated B lymphocytes to progress through the cell cycle and to differentiate into antibody secreting cells (115). IL-2$^{-/-}$ mice initially contain normal numbers of B and T lymphocytes. In older mice, the lamina propria of inflamed colons contains elevated levels of T cells, as well as B cells, suggesting that both T and B cells are spontaneously activated in the colonic immune response (89,98). In addition, anti-colon antibodies are regularly detected in the serum of IL-2$^{-/-}$ mice, and these antibodies have been postulated to be involved in causing bowel disease (89,108). Together, these observations suggest that in association with T cells, B cells and their autoantibody products may be important for the development of bowel disease in IL-2$^{-/-}$ mice. It was assumed that B cell response against autoantibodies is responsible for IBD development (89). To determine the role of B cells in colitis development in IL-2$^{-/-}$ mice, IL-2$^{-/-}$ mice were bred with JH$^{-/-}$ mice (these mice possess no mature B cells). There was an improvement in anemia in IL-2$^{-/-}$, JH$^{-/-}$ mice compared to IL-2$^{-/-}$ mice but there was similar onset and severity in wasting disease (63). So from above data it was clear that activated B cells against autoantibody are not crucial for IBD development. But there is a progressive loss of mature B cells in IL-2$^{-/-}$ mice with IBD progression. Schultz et al (101) showed that the level of immunoglobulins at mucosal sites and the number of peripheral B lymphocytes in mesenteric lymph nodes dramatically decrease over time in specific pathogen free (SPF) and germ free (GF) IL-2$^{-/-}$ mice. The same group later showed that B lymphocyte depletion in IL-2$^{-/-}$ mice is mainly caused by disrupted B cell development in the bone marrow (99).

1.4.6   Change in cytokine environment in intestine precedes IBD in IL-2$^{-/-}$ mice

IL-2$^{-/-}$ mice raised under conventional conditions showed higher expression of cytokines, e.g. IL-1α, IL-1β, IL-6, TNF-α, IFN-γ, and IL-10 in colon compared to wild type. Cytokine profile starts to change at the age of 10 days and this shift in cytokine pattern persists up to late age and pronounces with time. In contrast there was a weak TGF-β1 expression in

colonic tissue of WT mice compared to IL-2$^{-/-}$ mice. IL-4 was not significantly expressed in intestinal tissue of either IL-2$^{-/-}$ mice or wild type mice (3).

1.4.7   Intestinal microflora triggers IBD in IL-2$^{-/-}$ mice

Splenomegaly, lymphoadenopathy, anemia and inflammatory bowel disease are all characteristic disorders displayed by IL-2$^{-/-}$ mice. But IL-2$^{-/-}$ mice raised under germ free conditions are shown free of IBD (24,89) even though these mice showed lymphocytic hyperplasia affecting hemopoietic, lymphoid and other organs. Lymphocytes infiltration were seen in lung, pancreas and kidneys (24). Enterocolitis in IL-2$^{-/-}$ mice is due to an abnormal immune response in the mucosa to the intestinal bacteria (24). In contrast Schultz *et al* (101) showed that mild delayed focal gastrointestinal inflammation in GF IL-2$^{-/-}$ mice starts at 13 weeks of age. They hypothesized nonviable luminal bacterial fragments present in food play a role in the gastrointestinal inflammation in GF IL-2$^{-/-}$ mice. Another group also hypothesized a role of food antigen for a mild focal inflammation at 33 wk of age associated with a slight increase in Th1 cytokines in GF IL-2$^{-/-}$ mice (120).

But despite this mild focal delayed gastrointestinal inflammation in GF IL-2$^{-/-}$ mice, IL-2$^{-/-}$ mice raised under conventional conditions showed rapidly progressing intestinal inflammation characterized by mucosal hyperplasia and massive infiltration of the lamina propria with mononuclear cells (101).

1.4.8   Different strains of commensals have different capacity of colitis induction in IL-2$^{-/-}$ mice

Normal intestinal flora comprises 400 different species, some of them are beneficial to the host and others cause harm in certain clinical settings. So it is possible that not all bacterial strains present in the microflora are responsible for colitis development in IL-2$^{-/-}$ mice. Waidmann et al (121) showed this by colonizing IL-2$^{-/-}$ mice exclusively with one strain of *E. coli*, named *E. coli* mpk or *B. vulgatus*, strains isolated from the fecal flora of SPF IL-2$^{-/-}$ mice. IL-2$^{-/-}$ mice were also co colonized with both bacteria or colonized with a known probiotic bacteria, *E. coli* Nissle 1917 (56,86). Only *E. coli* mpk was able to cause colitis in IL-2$^{-/-}$ mice. In contrast *B. vulgatus* colonized mice were colitis free and it was also able to inhibit *E. coli* mpk induced colitis in IL-2$^{-/-}$ mice (121).

1.5   Objective and goals

Among different factors the microflora plays a role in IBD development as well as in colitis model IL-2$^{-/-}$ mice. Gnotobiotic IL-2$^{-/-}$ mice revealed different colitogenic potential of different species of the microflora. Therefore we investigated how different commensal bacteria modulate the immune response of DC and T cells. We wanted to generate hypothesis which might explain how *B. vulgatus* may prevent *E. coli* mpk triggered colitis development. Furthermore by Affymetrix Gene Chip oligo microarray high expression of adipsin, adiponectin etc. was found to be associated with colitis prevention (unpublished data kindly provided by a colleague from our group, Oliver Bechtold). The cellular source of this factors in the intestine was also investigated.

2   Materials and Methods

2.1   Molecular biology methods

2.1.1   RNA Isolation

All materials used in the RNA isolation process were RNase free. The working place was cleaned with 'RNA away' (Molecular bio products, CA USA). Cells were dissolved in Trizol (Invitrogen, Karlsruhe, Germany) according to manufacturers instructions. 200 µl Chloroform (Merck, Darmstadt, Germany) was added to the cell samples and were mixed well. Then the trizol-chloroform mix was transferred to a precentrifuged Phase Lock Gel (PLG) tube (eppendorf, Hamburg, Germany) and centrifuged at 14000 g, 5 minutes at room temperature. The upper aqueous layer was transferred to a 2 ml microfuge tube and 500 µl isopropanol (Merck, Darmstadt, Germany) was added (1: 0.75). The tubes were shaken and centrifuged at 12000g for 10 minutes at 4°C. Then the supernatants were discarded, carefully washed with 70% ethanol (Merck, Darmstadt, Germany) and then the pellet was dried at 37°C for 1-5 minutes until it turned to viscous. The pellet was dissolved in 10-50µl of DEPC treated water (Ambion, UK) and kept at –80°C before further use. The concentration of RNA was estimated by Spectral photometer (Ultrospec 3000, Amarsham Pharmacia biotech, Freiburg, Germany) at 260 nm wavelength.

2.1.2   Preparation of cDNA

cDNA was prepared using kit (Invitrogen, Karlsruhe, Germany) with small modifications. 1µl of oligo dT primer was added to 5-10 µg RNA, shortly spinned and incubated at 65°C heat block (Thermomixer, eppendorf, Hamburg, Germany) for 10 minutes. The samples were incubated on ice for 1 minute; 10µl 'master mix' was added to each microfuge tube and gently mixed with pipette. The samples were spinned down at 4°C and incubated at 50°C for 1 hour. The Master mix was prepared by adding 4µl 5xfirst strand buffer, 2µl 0.1M DTT, 2µl of dNTP mix (10mM each dATP, dGTP, dCTP and dTTP at neutral pH), 1µl RNase OUT^{TM} (recombinant RNase inhibitor) and 1µl of SuperScript III reverse transcriptase (200 units/µl). After one hour incubation the reaction was stopped by incubating the samples at 70°C for 15 minutes. cDNA were diluted 1:10 with DEPC treated water and stored at –20°C before further use.

2.1.3 Quantification of specific messenger RNA by Taqman PCR

Quantification of adipsin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression in adipocyte cell line was performed by Taqman real time reverse-transcription (RT)-polymerase chain reaction using Assays on demand (Applied Biosystems, Darmstadt, Germany) and the TaqMan System (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions

Real-time RT-PCR was carried out in duplicate in 96-well format on a GeneAmp 5700 Sequence detection System (Applied biosystems/Applera, Darmstadt, Germany). Each 20 µl reaction contained 10µl target gene specific Assay-on-Demand Gene Expression Assay Mix (including primers and dye-labeled hybridization probes, Applied Biosystems), 4 µl PCR grade water and 5 µl cDNA. Thermal cycling conditions for all reactions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The standard curve method was used for semiquantitative data analysis, whereas ten fold dilutions of pooled cDNA from all samples were used as standards. Data were normalized by dividing the values of target gene by the values of the housekeeping gene GAPDH.

2.2 Microbiology methods

2.2.1 Bacteria used

In this study two non-pathogenic *E. coli* strains, *E. coli* mpk and *E. coli* Nissle and an anoerobic bacteria strain, *Bacteroides vulgatus* were used. *E. coli* mpk and *B. vulgatus* were isolated from the fecal flora of specific pathogen-free (SPF) IL-2$^{-/-}$ mice (120). *E. coli* Nissle 1917 (Mutaflor,-Ardeypharm, Herdecke, Germany) is a known probiotic and effective in maintaining remission in ulcerative colitis.

2.2.2 Overnight culture of bacteria

Work with bacteria was done under sterile conditions. *E. coli* mpk was cultured in LB media overnight at 37°C with continuous shaking (200 rpm). For infection assays log phase bacteria were used. *B. vulgatus* was cultured in BHI media under anoerobic condition at 37°C for 48 hours. Bacterial suspension was centrifuged at 4000g and washed twice with PBS.

Table 5: Medium for bacteria culture

Media	LB-Medium	BHI (brain heart infusion) medium
Contents	Trypton (10 g) Yeast extract (5 g/l) NaCl 5 g/L pH 7.0 ± 0.2	BHI-Powder (37 g/L) pH 7.4

2.2.3   Preparation of cryostock

For long time storage of bacteria, bacteria cryostocks containing LB/BHI medium with 20% glycerol were prepared and stored at –80°C

2.2.4   Heat killed bacteria preparation

E. coli mpk was cultured overnight and subcultured in 100 ml LB media at 1:100 according to bacterial culture protocol described before. *B. vulgatus* was cultured in 100 ml BHI medium for 2 days according to protocol described above. Bacteria was centrifuged at 4000g, 4°C for 5 minutes, washed twice in PBS (Invitrogen, Karlsruhe, Germany) and 50µl of bacterial suspension was removed for determination of colony forming unit (CFU). The bacterial suspension was incubated at 80°C for 10 minutes and then kept on ice for 10 minutes. Heat killed bacterial suspension was centrifuged at 4000g, 4°C for 5 minutes and then resuspended in PBS (Invitrogen, Karlsruhe, Germany), and stored at –80°C until further use.

2.2.5   Counting viable bacteria by colony forming unit

Serial dilution of bacterial suspension was done; equal amount from last four dilutions was platted on agar plate and incubated at 37°C. Bacterial colonies formed on the plate were counted and the concentration of viable bacteria was calculated.

2.2.6   Preparation of bacterial culture supernatant

Bacteria from cryostock were grown overnight (*B. vulgatus* was grown two days) in cell culture media with supplements. Bacteria were centrifuged at 4000g for 10 minutes; supernatants were harvested and filter sterilized (0.20 µm, Sartorius AG, Goettingen, Germany). Supernatant was aliquoted, stored at –20°C until further use.

2.2.7   Estimation of protein content by pierce protein estimation method

Protein estimation was done according to instruction from manufacturer (BCA protein estimation kit, Pierce, USA). In 96-well plate (Greiner, Nuertingen, Germany) 10µl sample or standard (Bovine serum albumin) protein solution was incubated with Pierce reagent at 37°C for 30 minutes. Protein concentration was determined relative to standard at 562 nm.

2.3   Protein analysis

2.3.1   Analysis of protein secretion by enzyme linked immunosorbent assay (ELISA)

To determine the cytokine concentration in the cell culture supernatant, ELISA was done with standard protein, primary antibody, biotinylated secondary antibody and detection kit (OptEIA ELISA set, Becton Dickinson Heidelberg, Germany) according to their instruction. IFN-γ ELISA was done as described previously (120). Briefly 96-well Microtiter plate, round-bottom (Greiner, Nuertingen, Germany) was coated overnight using 50µl of anti-IFN-γ monoclonal antibody (10µg/ml, clone AN18), next day was washed 3 times with 150 µl PB buffer (1xPBS with 0.05% Tween 20, Sigma-Aldrich, Taufkirchen, Germany) and then blocked at 37°C with 150 µl/well same buffer. After 1 hour the plate was again washed as before, 50µl sample/ standard was pipetted and incubated at 4°C overnight. Recombinant murine IFN-γ (from Bender, Vienna, Lot: M3-RD48) was used as standard for a concentration range from 0 to 80 U/ml. At next day plate was washed and incubated with biotinylated secondary antibody (2µg/ml rat anti mouse-IFN hybridoma R46A2 in PBS) for 1 hour at 37°C. Plates were washed again, incubated with 50 µl/well of a solution containing streptavidin and biotinylated alkaline phosphatase (DAKO, Denmark) for 45 minutes at 37°C. Then, after washing 50µl/well substrate solution (1 mg/ml p-nitrophenyl-phosphate from Sigma, St Louis, Missouri, USA in Carbonate buffer) was added and optical density was measured by ELISA reader (Sunrise, Tecan, Crailsheim, Germany) at 405 nm. Concentrations of IFN-γ protein were calculated on the basis of an IFN-γ standard curve and linear regression. The lower limit of detection was 1.0 ng/ml.

2.3.2   Analysis of protein expression by Immunohistology

Colon samples were frozen in tissueTec and sectioned in 5µm width for immunohistology for adipsin. For cell culture samples, preadipocyte cells cultured and stimulated on coverslips were used for immunohistology. Before fixation samples were washed twice with PBS and fixed with 3.75% PFA for 15 minutes at RT. Cells were washed twice with PBS and

permeabilized with Triton-X (0.1% sodium citrate and 0.1% Triton X100, Sigma-Aldrich, Taufkirchen, Germany), for 2 minutes at RT, for tissue samples 5 minutes at RT. Again washed and incubated 40 minutes with H_2O_2 mix (0.2 ml 30% H_2O_2 in 11.8 ml methanol absolute) at RT (tissue sections were incubated for 10 minutes). Nonspecific binding of antibody to Fc receptor was blocked by incubating with Fc block (purified anti-mouse CD16/CD32, BD pharmingen, Becton Dickinson Heidelberg, Germany) for 40 minute at RT (tissue sections were incubated 20 minutes). Anti-adipsin antibody (P-16, Santa Kruz, USA) was added and incubated for 2 hours at RT. Coverslips were washed twice with PBS (Invitrogen, Germany) and incubated with biotin labeled secondary antibody (bovine anti goat IgG, Santa Kruz, USA) in 1:250 dilution for 1 hour at RT in a humid chamber (for tissue sections antibody was diluted 1:100). After incubation with secondary antibody coverslips / tissue sections were washed twice with PBS and incubated with streptavidin HRP detection system (Invitrogen, Karlsruhe, Germany) according to the manufacturers instruction for 5 minutes at RT, washed and incubated with freshly prepared substrate solution, DAB-solution (Di amino benzidine tetrahydro chloride, Applichem, Darmstadt, Germany), analyzed at microscope the formation of brown color and then reaction was stopped with water flow.

Hemalaon counter stain was performed, coverslips / tissue sections were covered with Aqua Tex, analyzed by Microscope (Axiovert200) and photographed by Axiocam HR both from Carl Zeiss Vision GmbH, Munich, Germany.

2.4   Cell culture methods

2.4.1   Isolation and culture of BMDC

Bone marrow cells from C57BL/6x129Sv mouse (provided by C. Kirschning, were maintained under specific pathogen-free conditions in our breeding facility) were isolated and cultured according to Lutz *et al* (61) with small modifications. Femur and tibia were cut out from mouse, muscles were removed from bone by rubbing with isopropanol on tissue paper. Then two sides of the bone were cut down by sterile scissors and bone marrow was flushed out. Cell clumps were broken by repeated pipetting and then centrifuged at 400g for 5 minutes at room temperature, washed twice and finally the cell pellet was resuspended in complete DC media. $2x10^6$ cells were seeded in 100 mm bacteriological petri dishes (Greiner, Nuertingen, Germany) in 10 ml complete DC media. At day 3, 10 ml fresh DC media was added to each petri dish. At day 6, 10 ml of cell suspension was removed from each plate, centrifuged at 400g for 5 minutes at room temperature, cell pellet was resuspended in 10 ml fresh complete

DC media and given back to petridishes again. At day 8, dendritic cells were harvested and used for stimulation / infection.

Culture media for BMDC

RPMI 1640	Biochrom, Berlin, Germany
10% Fetal Calf Serum (inactivated at 37°C for 30 minutes)	Sigma-Aldrich, Taufkirchen, Germany
Penicillin (100 U/ml)	Biochrom, Berlin, Germany
Streptomycin (100 μg/ml)	Biochrom, Berlin, Germany
L-Glutamin (2 mM)	Gibco BRL, Eggenstein, Germany
2-Merceptoethanol (0.5 mM)	Sigma-Aldrich, Taufkirchen, Germany
Non-essential amino acids	Biochrom, Berlin, Germany
GM-CSF	200 U/ml GM-CSF (produced by mouse myeloma strain P3X63)

2.4.2   Stimulation of BMDC

At day 8 non-adherent cells were harvested, centrifuged at 400g for 5 minutes at room temperature, washed twice with PBS (Invitrogen, Germany) and then resuspended in antibiotic free complete DC media. $1x10^6$ DCs were seeded per well of 24 well plates (Beckton Dickinson Labware, France) to measure cytokine secreted by DC at different stimulations by ELISA and to collect supernatant for T cell stimulation. $1x10^6$ DCs were seeded per 100 mm bacteriological petri dish (Greiner, Nuertingen, Germany) to investigate activation of DC by flow cytometry (FACS Calibur, Becton Dickinson, Heidelberg, Germany). For infection of DC with viable bacteria antibiotic free DC media was used. DC were infected with different MOI of bacteria (0.01-1) for one hour, bacteria were killed by gentamicin (ICN, USA) and then incubated for additional 23 hours. DC were also stimulated with cytokine, cell culture supernatants etc. in 'DC complete media' with antibiotics. Cells supernatants were stored at –20°C to perform ELISA to quantify IL-12p70, TNF-α, IL-10 and IL-6. For T cell stimulation, dendritic cell supernatant were filter sterilized with 0.20 μm filter (0.20 μm, Sartorius AG, Goettingen, Germany) and stored at –20°C.

2.4.3   Analysis of activation and maturation of DC

Activation and maturation of DC was analyzed by staining surface marker of DC with PE conjugated anti-mouse CD11c, clone HL3 (Armenian Hamster IgG$_1$, λ), FITC conjugated

anti-mouse CD80, clone 16-10A1 (Armenian Hamster IgG_2, κ), FITC conjugated anti-mouse CD86, clone GL1 (Rat IgG_{2a}, κ), FITC conjugated anti-mouse I-A/I-E, clone 2G9 (Rat IgG_{2a}, κ) and FITC conjugated anti-mouse CD40, clone 3/23 (Rat IgG_{2a},κ). All antibodies used here were purchased from Becton Dickinson, Heidelberg, Germany.

2.4.4   Isolation of T cells from spleen

Spleen of stimulated or transgenic mice was removed. Splenocytes were prepared by homogenization of spleen, passing through a steel-sieve. Cells were centrifuged at 400g for 5 minutes and resuspended in 0.15 M NH_4Cl (pH 7.2) and incubated for 1 minute for erythrocyte lysis. Splenocytes were washed three times with HBSS (Biochrom, Berlin, Germany) and resuspended in cell culture media.

Culture media for T cells

RPMI 1640	Biochrom, Berlin, Germany
10% Fetal Calf Serum (inactivated t 37°C for 30 minutes)	Sigma-Aldrich, Taufkirchen, Germany
Penicillin (100 U/ml)	Biochrom, Berlin, Germany
Streptomycin (100 μg/ml)	Biochrom, Berlin, Germany
L-Glutamin (2 mM)	Gibco BRL, Eggenstein, Germany
2-Merceptoethanol (0.5 mM)	Sigma-Aldrich,Taufkirchen, Germany
Non-essential amino acids	Biochrom, Berlin, Germany
Sodium pyruvate (1mM)	Biochrom, Berlin, Germany

2.4.5   Purification of naïve $CD4^+$ T cells

DO11.10 mice transgenic for an ovalbumin$_{323-339}$ specific TCRαβ obtained from own breeding were used as a source of antigen specific T-cells (44). Splenocytes were prepared according to protocol explained before. Splenocytes were enriched for T cells by Ficoll density gradient centrifugation. Splenocytes were taken in 5 ml T cell complete culture media, 3 ml Ficoll 1:1 (1 part Ficoll 1.077kg/l, 1 part Ficoll 1.090kg/l, Sigma-Aldrich, Taufkirchen, Germany) was added carefully by Pasteur pipette (WU, Mainz, Germany) to the bottom of the tube, underneath the cell suspension and centrifuged at room temperature, 1000g for 20 minutes without break. The interphase of two layers was taken out carefully by Pasteur pipette, washed twice with HBSS (Biochrom, Berlin Germany) and cells were resuspended in 5ml complete T cell culture media.

T cells were further enriched by 'panning'. Petridishes (Greiner, Nuertingen, Germany) were coated overnight at 4°C with 15 ml antibody solution containing anti-mouse MHC-II (clone, B21-2), anti-mouse CD8 (clone, YST169.4.2.1) and anti- mouse B220 (clone, RA3) each at a concentration of 100 μg/ml. Next day, the antibody coated petridish was washed 5 times with PBS (Invitrogen, Karlsruhe, Germany), maximum $1x10^8$ cells were taken in 5 ml T cell culture medium and incubated at room temperature for 1 hour. Nonadherent cells were harvested by washing three times with fresh medium carefully.

2.4.6   Purification of CD4⁺T cells by Magnetic-activated cell sorting (MACS)

Naïve CD4⁺ T-cells were finally purified by magnetic cell sorting kit from Miltenyi Biotec, (Bergisch Gladbach, Germany) first performing negative selection of CD4⁺T cells from splenocytes and then by positive selection for CD4⁺CD62L⁺ cells according to manufacturer's instruction.

MACS isolates cells according to their surface marker profile. At first the cells are labelled with antibodies conjugated to super-paramagnetic MicroBeads. Then the cells are passed through a separation column placed in a strong permanent magnet. The magnetically labelled cells are retained in the column, while non-labelled cells pass through. After removing the column from the magnetic field, the retained cells are recovered. Two approaches for MACS are available: depletion and positive selection. Depletion means that unwanted cells are labelled and eliminated from the cell mixture. Whereas in positive selection, the retained cells labelled with antibody are isolated. MACS separation columns are used for the separation of a broad range of cell subsets. The columns used were MS and LS. The processing capacity of MS columns is 10^7 magnetically labelled cells or up to 2×10^8 total cells. LS Columns are useful for the isolation of 10^7-10^8 magnetically labelled cells from up to 2×10^9 total cells. The column matrix is composed of ferromagnetic spheres covered with a plastic coating, allowing fast and gentle selections of cells. All reagents for cell separation by MACS were also purchased from Milteny Biotec (Bergisch Gladbach, Germany).

MACS kits

CD4+ T cell isolation kit

CD62L+ Isolation kit

MS and LS columns

Depleting cell subsets by MACS

CD4$^+$ T cells were purified by depleting other cells present in cell suspension, for example B cells, Macrophages, NK cells CD8$^+$T cells, dendritic cells etc. Total 1x10^7 cells were suspended in degassed (degassed by sonification) buffer (0.1% BSA in PBS, 2 mM EDTA, pH 7.2) and were labelled with magnetic bid tagged antibodies against their surface markers according to manufacturer's instruction. Labelled cells were applied to the column placed in a strong magnetic field. Magnetic bid labelled unwanted cells retained in the column and CD4$^+$T cells passed through the column and thus purified.

Positive selection of cell subsets by MACS

To purify naïve CD4$^+$ T cells (CD4$^+$CD62L$^+$), purified CD4$^+$ T cells were resuspended in buffer (0.1% BSA in PBS, pH7.2) and were labelled with magnetic bid tagged anti-CD62L-antibody (clone, MEL-14) according to manufacturer's instruction (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were loaded onto equilibrated MS or LS columns and were allowed to pass through. Column was washed two times with buffer and was removed from the magnet, and cells were eluted from the column by flowing 3 mL of buffer (0.1% BSA in PBS, pH 7.2) through them using a plunger.

2.4.7   Stimulation of T cells with differently stimulated/ infected dendritic cell culture supernatant

2.5 x 10^5 naive T-cells, purified from spleen of DO11.10 mice (44) were cultured in complete T cell media at 5% CO_2 and 37°C in presence of 5x10^6 splenic APC (γ-irradiated, 3,000 rads) from BALB/c mice (were purchased from Harlan Winkelmann, Borchen, Germany), 0.6 µM OVA$_{323-339}$ peptide (Ovalbumin peptide AA 323-339, Th epitope; D. Palm, Wuerzburg University, Germany) and 10% supernatant from BMDC with or without stimulation/infection. BMDC supernatant were maintained for the entire period of stimulation. The cultures were splitted and the cell number was equalized in each well at day 3, day 5. Recombinant IL-2 (Biotest pharma, Dreieich, Germany), 20 U/ml was added for last 4 days of culture. As a control for T cell polarization, T helper type 1 cells were generated by adding IL-12 (10ng/ml) and anti IL-4 (10µg/ml, clone, 11B11) and T helper type 2 cells were generated by using IL-4 (10ng/ml) and anti-IL-12 (10µg/ml, clone, C 17.8) to T cell culture and incubated seven days according to the protocol explained above. The cell cultures were stimulated with PMA (50 ng/ml, Calbiochem, UK) and ionomycin (1µg/ml, Sigma-Aldrich,

Taufkirchen, Germany). After 2 hours 'Golgi stop' (Becton Dickinson, Heidelberg, Germany) solution containing Monnensin was added to half of the cell culture. The other half was cultured for further 24 hours and the supernatants were collected for assessment of IFN-γ (120), IL-4 and IL-10 (OptEIA ELISA set, Becton Dickinson Heidelberg, Germany) by ELISA. Four hours after adding golgi stop to the other half of the cell culture these cells were harvested, incubated with Fc-block (Becton Dickinson, Heidelberg, Germany), anti-FcγII/III receptors (purified 2.4G2 antibody) to block unspecific binding of fluorescent labelled antibodies to the Fc receptors of cells. Cells were stained with mouse anti-CD4–PerCP (clone RM4-5, Becton Dickinson, Heidelberg, Germany). Afterwards the cells were fixed and permeabilized with cytofix/cytoperm kit (Becton Dickinson, Heidelberg, Germany) according to manufacturer's instruction and stained with anti-IFN-γ-FITC (Clone, XMG1.2) for intracellular cytokine and analysed by flow cytometry. Antibody to IFN-γ was purchased from Becton Dickinson, Heidelberg, Germany.

2.4.8   Stimulation of T cells with stimulated/infected dendritic cells

Naïve CD4$^+$ T cells were isolated from spleen of OT-II mice according to the protocol (2.4.5-7). Bone marrow derived dendritic cells were generated according to protocol (2.4.1) and were used at day 6. BMDC were seeded $3x10^6$ in a specially treated 6-well plate (Beckton Dickinson Labware, France) and infected with bacteria at MOI 1. After 1 hour extracellular bacteria were killed with gentamicin (ICN, USA), cultured for 24 hours with Ovalbumin 100µg/ml (Sigma-Aldrich, Taufkirchen, Germany) for last 3 hours. DC were harvested, washed and counted before adding to T cell culture.

$2x10^5$ infected / control BMDCs and $8x10^5$ naïve CD4$^+$ T cells were cultured in 24 well specially treated plate (Beckton Dickinson Labware, France) for 4 days. At day 4, T cell polarization were determined by analyzing cell culture supernatants by ELISA for cytokines, IFN-γ, IL-4 and IL-10 secretion (OptEIA ELISA set, Becton Dickinson Heidelberg, Germany).

2.4.9   Proliferation assay of T cells by ^{3}[H]-thymidine uptake

T cells were cultured in round bottom 96-well (Greiner, Nuertingen, Germany) plate. 10µl of radioactive tritiated thymidine solution (0.5 ml ^{3}[H]-Thymidine, 37 MBq /ml in PBS + 9.5 ml cell culture medium) was added to each well for last 16 hours of incubation period. After this, cells were harvested on a filter, dried and analysed by scintillation counter (Top count NXT,

Microplate Scintillation and Luminescence counter, A Packard Bioscience company) according to instruction from the provider.

2.4.10 Culture method of preadipocyte cell line 3T3-L1

3T3-L1 cell line (a preadipocyte cell line) which can be differentiated to adipocyte in presence of fetal calf serum or presence of insulin, dexamethason and IBMX (iso butyl methyl xanthine, Sigma-Aldrich, Taufkirchen, Germany). This cell line was generated from BALB/c mice.

3T3-L1 cell line was cultured in 80 cm^2 cell culture flask (Nunc, Roskilde, Denmark) with medium explained above at 37°C with 5% CO_2 and 95% relative humidity. Cells were splitted 1:4 at 70% confluence.

For splitting, 3T3-L1 cells were washed with PBS and treated with Trypsin-EDTA (Gibco BRL, Eggenstein, Germany) with 3 minutes incubation at room temparature (3-5ml /80 cm^2 flask). The cell suspension was centrifuged at 400g for 5 minutes, resuspended at complete medium and taken one fourth of the cell suspension to one 80 cm^2 flask.

Culture media for 3T3-L1 cell line

D-MEM medium	Gibco BRL, Eggenstein, Germany
5-10% Neonatal Calf Serum (inactivated t 37°C for 30 minutes)	Gibco BRL, Eggenstein, Germany
Penicillin (100 U/ml)	Biochrom, Berlin, Germany
Streptomycin (100 µg/ml)	Biochrom, Berlin, Germany
L-Glutamin (2 mM)	Gibco BRL, Eggenstein, Germany

For long time storage 3T3-L1 cell line was harvested from cell culture flasks using trypsin-EDTA, cell suspension was centrifuged at 400g for 5 minutes and dissolved in 10% DMSO (Merck, Darmstadt, Germany) in FCS. Cell suspension was aliquoted in 1 ml volume in 'Nunc cryo tube' (Nunc, Roskilde, Denmark) and was stored in liquid nitrogen.

2.4.11 Stimulation of preadipocyte cell line 3T3-L1

3T3-L1 cell line was detached from the flask by treating with trypsin-EDTA (Gibco BRL, Eggenstein, Germany). $1x10^5$ cells were seeded in each well of a 24 well plate in 3T3-L1 medium (10% NCS) and incubated at 37°C, 5% CO_2 and under humid condition. Cell culture

media was changed every 2nd day until cells become confluent. After confluence cells were kept in 5% NCS containing complete media for two days more before first stimulation was done. Stimulation was done for 10 days. Every 2nd day media was changed with fresh media and stimulant.

At day 5 and day 10 of stimulation cells were washed in PBS and fixed with 10% formaldehyde at 4°C for 5 hours. Cells were washed with PBS twice and incubated for 2 hours with Oil Red O (200 µl per well in 24 well plate) at room temperature. Cells were destained by washing twice with PBS and then photographed by Microscope (Axiovert200) installed with Axiocam HR both from Carl Zeiss Vision GmbH, Munich, Germany.

2.5   Animal experiment methods

2.5.1   Adoptive transfer and stimulation of mice

DO11.10 (44) splenocytes were prepared as explained before and $2x\ 10^6$ splenocytes were transferred to BALB/c mouse by injecting through tail vein. After 3 days adoptively transferred mouse were stimulated with ovalbumin (Sigma-Aldrich, Taufkirchen, Germany) and heat killed bacteria intraperitoneally

2.5.2   Analysis of ovalbumin specific T cell proliferation and cytokine expression

$2.5x\ 10^5$ splenocytes from transferred and then stimulated mice were restimulated with different concentration of ovalbumin (20, 100 and 500µg/ml, Sigma-Aldrich, Taufkirchen, Germany) or ovapep$_{323-339}$ (Ovalbumin peptide AA 323-339, Th epitope; D. Palm, Wuerzburg University, Germany) in complete T cell culture media in 96 well round bottom plate (Nunc, Roskilde, Denmark) for 72 hours. Tritiated thymidine was added to the culture for last 16 hours. Cells were harvested and ^{3}[H]-thymidine uptake was measured according to protocol explained at 2.4.9. $2x\ 10^6$ splenocytes from transferred and then stimulated mice were also restimulated with different concentration of ovalbumin (20,100 and 500µg/ml, Sigma-Aldrich, Taufkirchen, Germany) to determine the different cytokine expression by ELISA after 72 hours of incubation.

2.6   Statistical analysis of results

Statistical analysis was performed using the paired Student's t test. P values > 0.05 were considered significant. Error bars represent ± SD

3   Results

3.1   *E. coli* mpk and *B. vulgatus* differentially modulate DC

3.1.1   Colitogenic *E. coli* mpk and noncolitogenic *B. vulgatus* bacteria differentially modulate cytokine expression in dendritic cells

Previous studies showed that colonization with different bacteria modulate colitis development in IL-2 deficient mice. Thus monocolonization of IL-2$^{-/-}$ mice with *E. coli* mpk but not *B. vulgatus* induces colitis (121). In this study we wanted to know if this difference in colitis development is related to different DC activation by these bacteria in terms of cytokine production and surface marker expression.
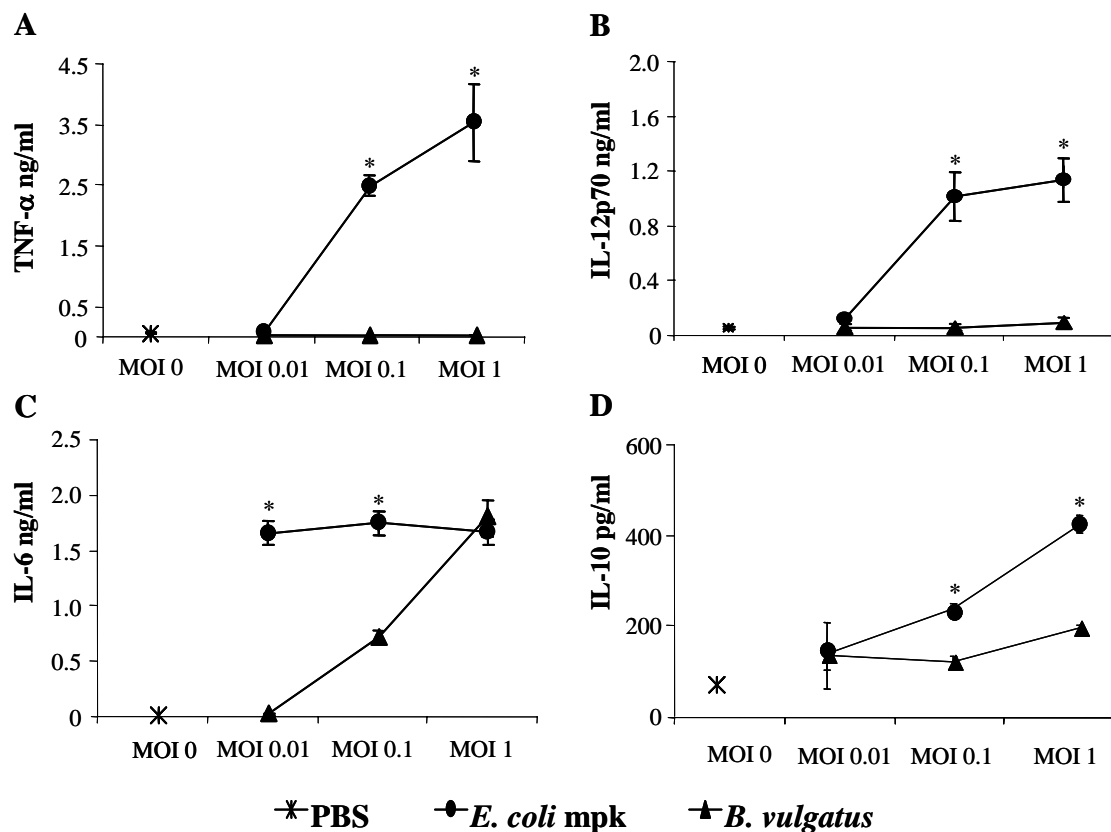
Figure 3: Different bacteria induce differential cytokine secretion by dendritic cells.
Bone marrow derived dendritic cells were infected with different MOI (0.01-1) of *E. coli* mpk and *B. vulgatus*, extracellular bacteria were killed by gentamicin after 1 hour and cells were cultured for additional 23 hours. Cell culture supernatants were analyzed by ELISA for TNF-α, IL-12p70, IL-6 and IL-10. The results are representative of three independent experiments. Each experiment was done in triplicate; error bars represent SD of duplicate. *p<0.05 compared to *E. coli* mpk.

To assess the effect of different bacteria on dendritic cells, BMDC were infected with *E. coli* mpk and *B. vulgatus* at different MOI (0.01 to 1). After one hour of infection extracellular bacteria were killed by gentamicin and cells were cultured for additional 23 hours. Cell supernatants were analyzed for cytokine secretion by ELISA. In addition DC was analyzed

for surface markers indicating activation and maturation by flow cytometry. *E. coli* mpk induced secretion of significant amounts of TNF-α, IL-12 and IL-10 upon infection with a
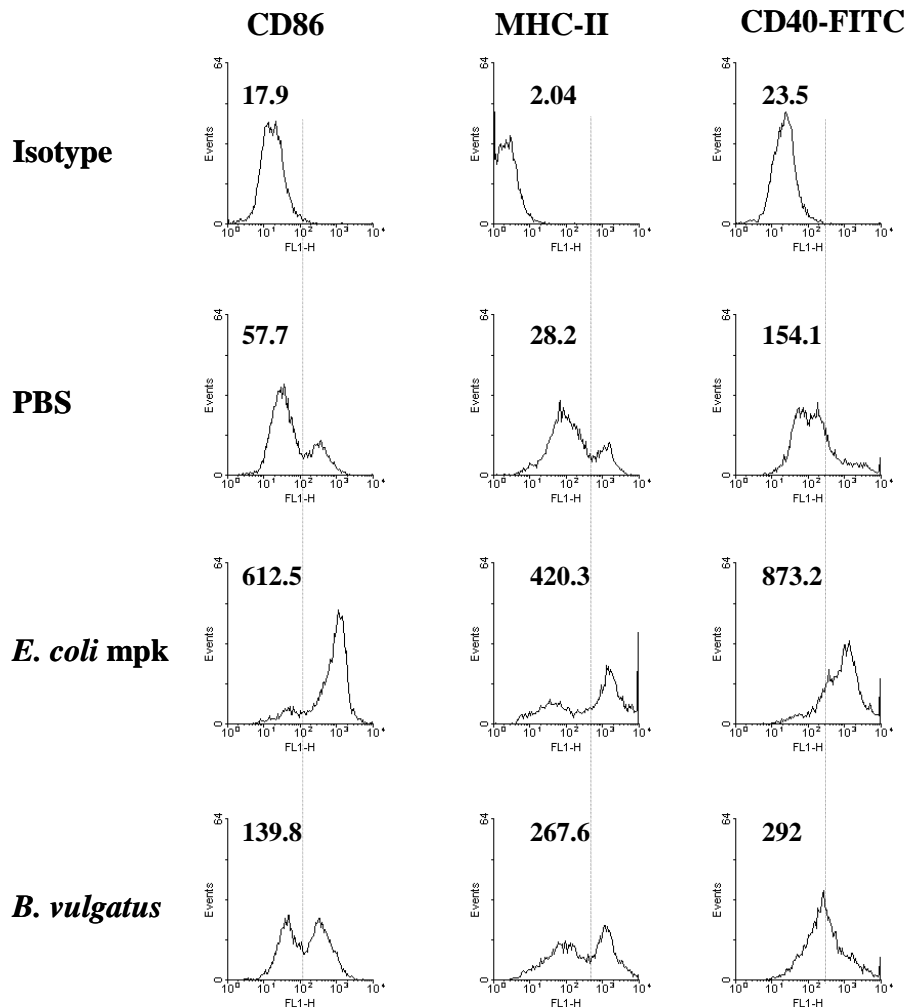
Figure 4: *B. vulgatus* keeps dendritic cells in a semimature/intermediate state. Bone marrow derived dendritic cells were cultured with different bacteria at MOI 1 for 1 hour, bacteria were killed by gentamicin and cells were cultured for additional 23 hours. After 24 hours cells were harvested, stained with anti-CD11c-PE and anti-CD86-FITC or anti-CD11c-PE and anti-MHC-II-FITC or anti-CD11c-PE and anti-CD40-FITC. Double stained cells were analyzed by flow cytometry. Histograms show expression of CD86, MHC-II and CD40 on CD11c$^+$ DC. The results are representative of three independent experiments.

MOI of 0.1 (Figure 3). The amounts of TNF-α, IL-12 and IL-10 secretion were further increased upon infection with a MOI of 1. Thus, upon infection with a MOI of 1 around 3.5 ng/ml TNF-α, more than 1.2 ng/ml IL-12 and approximately 500 ng/ml IL-10 were measured. In contrast infection of DC with *B. vulgatus* at a MOI of 1 did not induce significant amounts of TNF-α and IL-12. IL-10 was induced by *B. vulgatus* in DC but at a two fold lower level

than upon infection with *E. coli* mpk. After infection of DC with *E. coli* mpk at a MOI of 0.01 more than 1.5 ng/ml IL-6 were secreted. Because an increase in the infection dose did not lead to more IL-6 secretion indicating that *E. coli* mpk at MOI of 0.01 was sufficient to trigger maximal levels of IL-6 secretion by DC. In contrast infection with *B. vulgatus* at MOI of 1 was necessary to trigger similar high levels of IL-6 secretion than infection with *E. coli* mpk. Furthermore, infection with *E. coli* mpk led to high expression of CD86, MHC II, and CD40. In contrast, *B. vulgatus* triggered CD86, MHC II, and CD40 expression was much lower compared to *E. coli* mpk infected DC (Figure 4). Taken together the secretion of high levels of cytokines and expression of indicated surface markers suggest activation and maturation of DC by *E. coli* mpk. In contrast, the low expression of activation and maturation markers, CD86, MHC II and CD40 and the low amounts of cytokines indicate that *B. vulgatus* does not fully activate DC. We termed this phenotypically different DC with intermediate maturation as intermediate/semimature DC characterized by impaired cytokine secretion and low expression of surface markers.

3.1.2   *E. coli* mpk induced IL-12 secretion by DC is TLR4 and partially TLR2 dependent, but IL-6 secretion is unaffected by TLR2 and TLR4 deficiency in DC

Dendritic cells recognize pathogens or molecules from pathogens by toll like receptors (TLR). *E. coli* mpk and *B. vulgatus* are gram negative rods which contain LPS and also lipoprotein on their cell membrane. LPS in association with other molecules binds to TLR4 and lipoprotein binds to TLR2 expressed on dendritic cells surface. To assess, whether the *E. coli* mpk induced cytokine secretion by dendritic cells are TLR2 and TLR4 dependent, dendritic cells were generated from bone marrow of TLR2$^{-/-}$ mice and TLR4$^{-/-}$ mice as well as from wild type mice. These dendritic cells were infected with *E. coli* mpk and *B. vulgatus* at MOI 1. Extracellular bacteria were killed with gentamicin after one hour and cells were cultured for another 23 hours. At 24 hours of culture cell culture supernatants were analyzed for TNF-α, IL-12p70, IL-10 and IL-6 secretion by ELISA.

TNF-α secretion by TLR4$^{-/-}$ DC infected with *E. coli* mpk was 90% reduced and was 40% reduced in DC from TLR2$^{-/-}$ mice compared to DC from wild type mice infected with *E. coli* mpk (Figure 5A). *B. vulgatus* was unable to induce TNF-α secretion in TLR2$^{-/-}$, TLR4$^{-/-}$ and wild type DC. *E. coli* mpk induced IL-12 secretion was totally abolished in TLR4$^{-/-}$ DC, and reduced to about 50% in TLR2$^{-/-}$ DC compared to wild type DC (Figure 5B). DC from

TLR2$^{-/-}$, TLR4$^{-/-}$ and WT infected with *B. vulgatus* also showed similar patterns of reduction in IL-12 secretion. However, IL-12 secretion was much lower compared to *E. coli* mpk induced secretion of this cytokines by different DCs. IL-10 secretion by DC with infection of these two bacteria also depends on TLR2 and TLR4 signalling. *E. coli* mpk induced IL-10
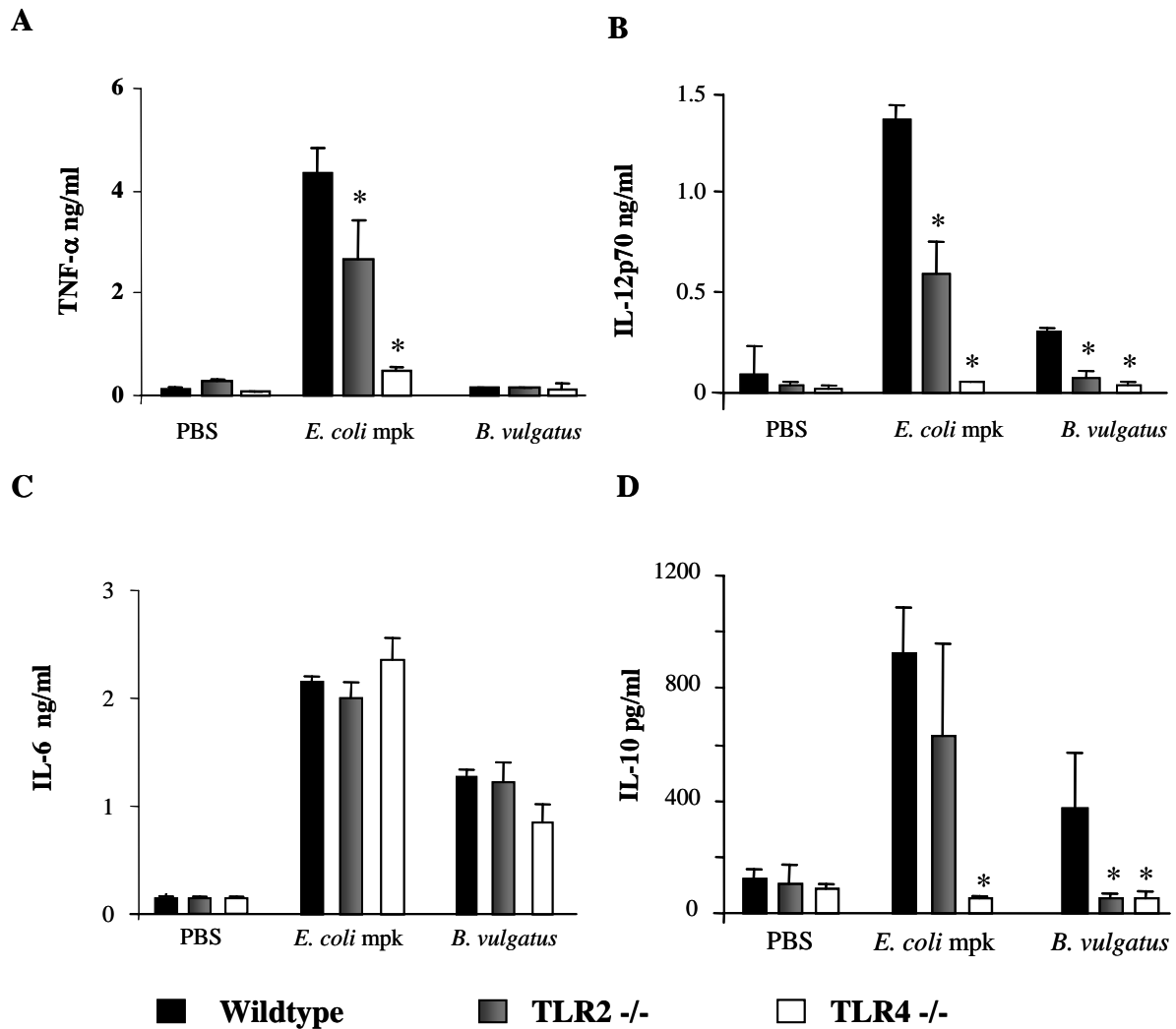
Figure 5: IL-12 induction by DC is TLR2 and TLR4 dependent. Dendritic cells derived from bone marrow of wild type, TLR2$^{-/-}$, TLR4$^{-/-}$ mice were infected with *E. coli* mpk and *B. vulgatus* at MOI 1 for 1 hour, bacteria were killed by gentamycin and cells were cultured for additional 23 hours. Cell culture supernatants were analyzed by ELISA for IL-12p70 and IL-6. The results are representative of three independent experiments. Each experiment was done in triplicate; error bars represent SD of duplicate. *p<0.05 compared to *E. coli* mpk.

secretion by DC was reduced more than 90% in TLR4$^{-/-}$ DC and about 30% in TLR2$^{-/-}$ DC compared to wild type DC (Figure 5D). But *B. vulgatus* induced IL-10 secretion was reduced in both TLR2$^{-/-}$ DC and TLR4$^{-/-}$ DC compared to wild type DC similarly (around 90% reduction). *E. coli* mpk induced similar amount of IL-6 secretion in TLR2$^{-/-}$, TLR4$^{-/-}$ and wild type dendritic cells. Similarly, *B. vulgatus* triggered IL-6 secretion was comparable in infected

DC from TLR2^{-/-}, TLR4^{-/-} and WT mice (Figure 5C). So above data suggest that IL-12, TNF-α and IL-10 secretion by DC with *E. coli* mpk infection depends on TLR4 and partially on TLR2 signalling. In contrast, IL-6 secretion by DC was not affected by TLR2 and TLR4 deficiency. So far it is unclear whether deficiency of both TLR2 and TLR4 leads to *B. vulgatus* or *E. coli* mpk triggered IL-6 secretion.

3.1.3   *B. vulgatus* can inhibit *E. coli* mpk induced TNF-α secretion by dendritic cells

Different *Lactobacillus* species modulated DC differently and among them, *Lactobacillus reuteri* was able to inhibit activation of DC with *Lactobacillus casei* infection (22).
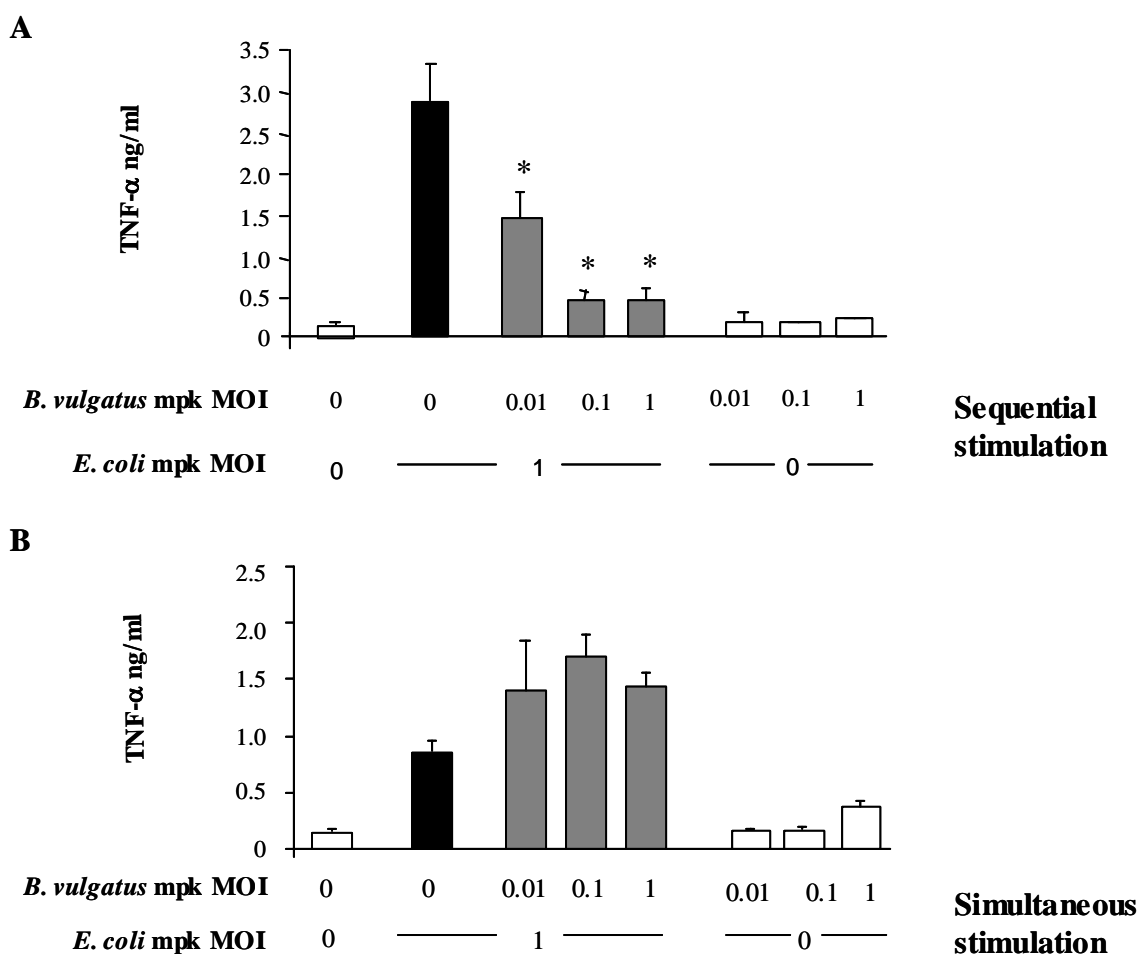
Figure 6: Modulation of *E. coli* mpk induced TNF-α expression in DC by *B. vulgatus*. Dendritic cells were (A) prestimulated with *B. vulgatus* at different MOI, after 24 hours infected with *E. coli* mpk at MOI 1 and cultured for 1 hour, extracellular bacteria were killed by gentamicin and were cultured for additional 23 hours or (B) DC were coinfected with *B. vulgatus* at different MOI and *E. coli* mpk at MOI 1, cultured for 1 hour, extracellular bacteria were killed by gentamicin and DC were cultured for additional 23 hours. Cell culture supernatants were analyzed by ELISA for TNF-α. The results are representative of three independent experiments. Each experiment was done in triplicate; error bars represent SD of duplicate. *p<0.05 compared to *E. coli* mpk.

Cocolonized IL-2$^{-/-}$ mice with *E. coli* mpk and *B. vulgatus* remain healthy whereas monocolonized IL-2$^{-/-}$ mice with *E. coli* mpk develop colitis (121). These studies pointed to a possible inhibitory role of *B. vulgatus* on *E. coli* mpk mediated immune response. Therefore we investigated whether *B. vulgatus* may suppress *E. coli* mpk triggered DC activation.

At first BMDC were infected with *E. coli* mpk and *B. vulgatus* at the same time (simultaneous stimulation). After one hour extracellular bacteria were killed by gentamicin and cells were cultured for additional 23 hours. Cell culture supernatants were analyzed for TNF-α secretion by ELISA. *B. vulgatus* did not inhibit the *E. coli* mpk induced TNF-α secretion in simultaneous infection (Figure 6B). Infection with *E. coli* mpk alone or infection with both *E. coli* mpk and *B. vulgatus* induced high TNF-α (1-1.5 ng/ml) secretion by DC. In contrast, if dendritic cells were pretreated for 24 hours with *B. vulgatus* and subsequently infected with *E. coli* mpk and cultured for additional 24 hours, TNF-α secretion by DC was lower compared to infection with *E. coli* mpk alone by DC (Figure 6A). Thus, *E. coli* mpk alone induced 3ng/ml TNF-α secretion by DC whereas DC prestimulated for 24 hours with *B. vulgatus* at MOI 1 and then stimulated with *E. coli* mpk for additional 24 hours induced only 0.5 ng/ml TNF-α. However prestimulation of DC with *B. vulgatus* does not have any effect on *E. coli* mpk induced IL-6 secretion by DC (Figure 7). The prestimulated DC, with *B. vulgatus* at MOI 1 and DC without prestimulation both secreted ~1.25 ng/ml IL-6 with further stimulation by *E. coli* mpk.
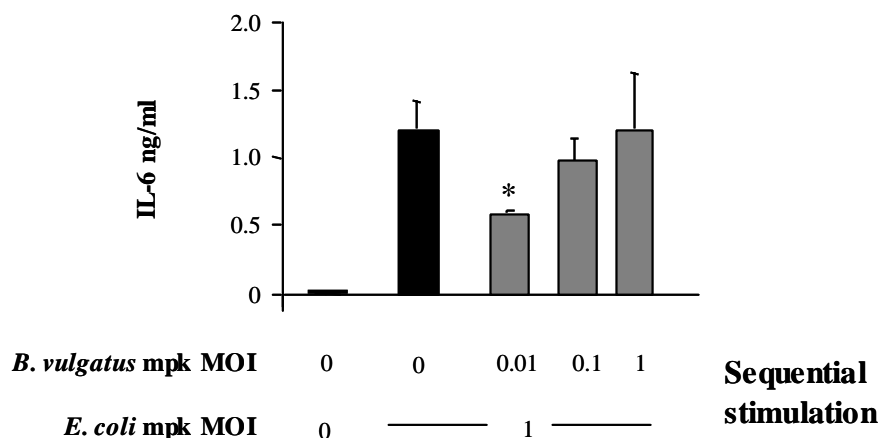
Figure 7: *B. vulgatus* does not inhibit *E. coli* mpk induced IL-6 expression in DC. Dendritic cells were prestimulated with *B. vulgatus* at different MOI (0.01-1), after 24 hours infected with *E. coli* mpk at MOI 1. After 1 hour extracellular bacteria were killed by gentamicin and cells were cultured for additional 23 hours. Cell culture supernatants were analyzed by ELISA for IL-6. The results are representative of three independent experiments. Each experiment was done in triplicate; error bars represent SD of duplicate. *p<0.05 compared to *E. coli* mpk.

These data indicate that *B. vulgatus* prestimulated DC can no longer be activated by *E. coli* mpk to induce TNF-α secretion. However, prestimulation of dendritic cells with *B. vulgatus* has no effect on *E. coli* mpk induced IL-6 secretion by DC.

3.1.4   *B. vulgatus* inhibits *E. coli* mpk induced activation and maturation of DC

Prestimulation of DC with *B. vulgatus* inhibits *E. coli* mpk induced cytokine secretion by DC (revealed from previous study). To investigate if *B. vulgatus* has the same inhibitory effect on *E. coli* mpk induced expression of surface markers for activation and maturation as well, BMDC were stimulated with *B. vulgatus* at MOI 1 for 24 hours and then were restimulated
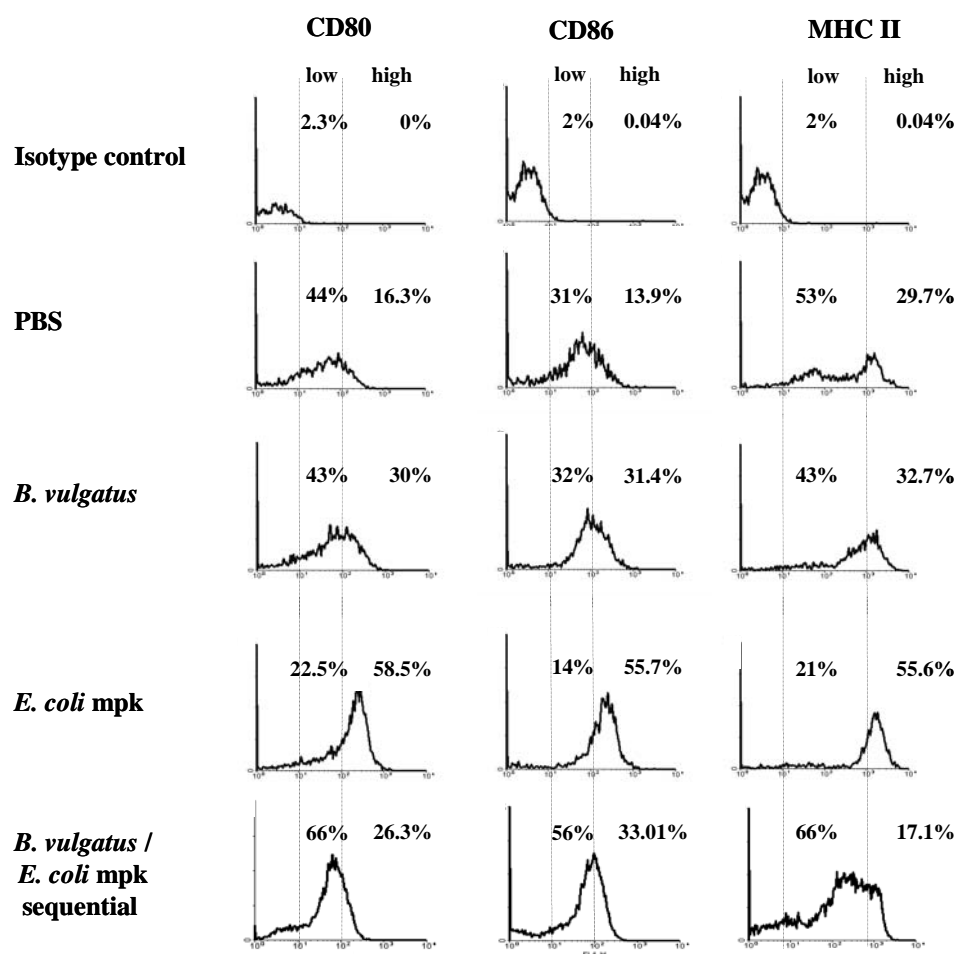
Figure 8: *B. vulgatus* can inhibit *E. coli* mpk induced activation and maturation of DC. Dendritic cells were prestimulated with *B. vulgatus* at MOI 1, after 24 hours infected with *E. coli* mpk at MOI 1 and cultured for 1 hour, extracellular bacteria were killed by gentamycin and cells were cultured for additional 23 hours. Cells were harvested, stained with anti-CD11c-PE and anti-CD80-FITC or anti-CD11c-PE and anti-CD86 or anti-CD11c-PE and anti-MHC II-FITC. Double stained cells were analyzed by flow cytometry. The results are representative of three independent experiments.

with *E. coli* mpk at MOI 1. After 1 hour, cultures were treated with gentamicin and were incubated for additional 23 hours. Cells were harvested and analyzed for expression of CD11c, CD80, CD86 and MHC-II. Diminished expression of activation and maturation markers on *B. vulgatus* prestimulated and then *E. coli* mpk induced DC was observed compared to *E. coli* mpk infected DC (Figure 8). In *E. coli* mpk infected DC, the DC population with high expression of costimulatory molecules (CD80 and CD86) and MHC II were higher compared to *B. vulgatus* prestimulated then *E. coli* mpk infected DC. In contrast to sequential stimulation of DC, with *B. vulgatus* (MOI 1) and after 24 hour with *E. coli* mpk (MOI 1), low maturation marker expressing DC population is increased compared to only *E. coli* mpk infected DC. Similar phenotype of DC, low maturation marker expression was also observed in case of *B. vulgatus* infected intermediate/semimature DC.

3.1.5   Recombinant IL-6 or supernatant of DC treated with *B. vulgatus* can inhibit *E. coli* mpk induced TNF-α secretion by dendritic cells

It was shown in previous results that *E. coli* mpk induces TNF-α, IL-12 and IL-6 secretion by DC. In contrast *B vulgatus* induces only IL-6 secretion by DC (Figure 3). Therefore it was hypothesized that IL-6 secreted by *B. vulgatus* treated DCs, in the absence of TNF-α and IL-12 primes DC to an intermediate state. This intermediate DC probably is less capable in production of proinflammatory cytokines and upregulation of activation and maturation markers with further *E. coli* mpk infection compared to activation with *E. coli* mpk alone. To investigate this hypothesis, immature dendritic cells were prestimulated with different amount of supernatant from dendritic cell culture infected with *B. vulgatus* (SN *B. vulgatus*) (Figure 9A) or different concentrations of recombinant IL-6 (Figure 9B) for 24 hours and then infected with *E. coli* mpk at MOI 1. After one hour extracellular bacteria were killed by gentamicin and cells were cultured for additional 23 hours. Cell culture supernatants were analyzed for TNF-α secretion. Activation and maturation of DCs were also analyzed by flow cytometry for the surface markers CD80, CD86 and CD40 (Figure 10). DC which were prestimulated with 10% 'SN *B. vulgatus*' and then infected with *E. coli* mpk were able to induce only 1.5 ng/ml TNF-α whereas immature DC infected with *E. coli* mpk produces around 6 ng/ml (Figure 9A). And this reduction further increases with increased dose of prestimulation (50% 'SN *B. vulgatus*). Similarly, DC primed with IL-6 (2.5 and 5 ng/ml) produces less TNF-α (<2 ng/ml) with *E. coli* mpk infection compared to TNF-α production (6 ng/ml) by immature DC infected with *E. coli* mpk alone (Figure 9B). Stimulation with IL-6 or

'SN *B. vulgatus*' prior to infection with *E. coli* mpk also led to a lower expression of the costimulatory molecules CD80, CD86 and CD40 compared to infection with *E. coli* mpk alone. From previous data (Chapter 3.1.1) it is known that culture supernatant of *B. vulgatus* infected DC ('SN *B. vulgatus*') contains IL-6. Taken together these findings suggest a crucial role of IL-6 in priming DC to an inert state and thus limiting wasting immune response.

Figure 9: IL-6 present in *B. vulgatus* infected DC supernatant inhibits *E. coli* mpk induced TNF-α production by DC. Dendritic cells were prestimulated with (A) different amount of supernatant (10% and 50%) from DC culture infected with non-colitogenic *B. vulgatus* at MOI 1 (SN *B. vulgatus*), or (B) with different concentrations of recombinant IL-6 (2.5 & 5 ng/ml). After 24 hours cultures were infected with *E. coli* mpk at MOI 1 and incubated for 1 hour, extracellular bacteria were killed by gentamicin and were cultured for additional 23 hours. Cell culture supernatants were analyzed by ELISA for TNF-α. The results are representative of three independent experiments. Each experiment was done in triplicate; error bars represent SD of duplicate. *p<0.05 compared to *E. coli* mpk.

Mean fluorescence	CD80	CD86	CD40
PBS	63.23	25.28	38.3
E. coli mpk	342.4	250.7	240.4
B. vulgatus	166.5	61.9	66.3
50% SN *B. vulgatus* / *E. coli* mpk sequential	282.5	174	95.7
IL-6 (5 ng/ml) / *E. coli* mpk sequential	58.6	99.5	148.4

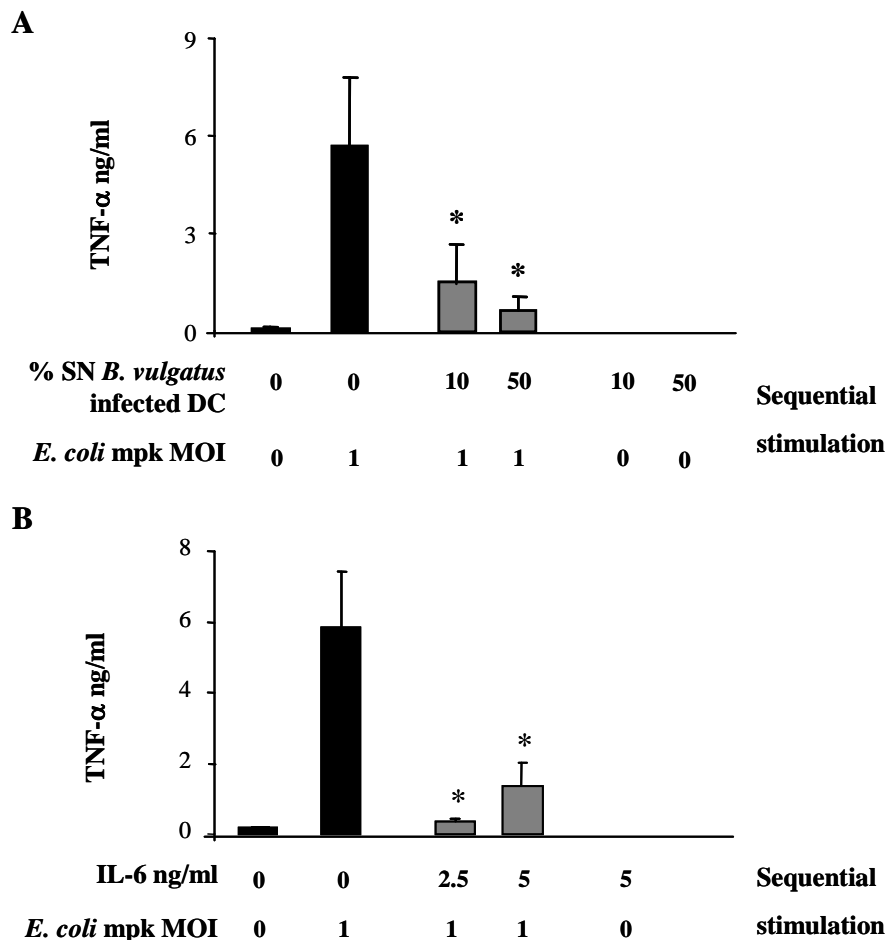**Figure 10: IL-6 present in *B. vulgatus* infected DC supernatant inhibits *E. coli* mpk induced activation of DC.** Dendritic cells were prestimulated with (A) 50% supernatant from DC culture infected with *B. vulgatus* at MOI 1(SN *B. vulgatus*), or (B) with recombinant IL-6 (5 ng/ml), after 24 hours infected with *E. coli* mpk at MOI 1, cultured for 1 hour, extracellular bacteria were killed by gentamicin and cells were cultured for additional 23 hours. Cells were harvested, stained with anti-CD11c-PE and anti-CD80-FITC or anti-CD11c-PE and anti-CD86-FITC or anti-CD11c-PE and anti-CD40-FITC. Double stained cells were analyzed by flow cytometry. The results are representative of three independent experiments.

3.1.6   Neutralization of IL-6 in *B. vulgatus* treated dendritic cell supernatant abolishes its inhibitory capacity

To further verify that pretreatment of DC with IL-6 in the supernatant of *B. vulgatus* treated DCs and not other factors are crucial to prevent the *E. coli* mpk triggered cytokine secretion and expression of surface markers, IL-6 mediated effects were neutralized by adding

antibodies against IL-6. Dendritic cells were prestimulated with this supernatant for 24 hours and then infected with *E. coli* mpk. After one hour cultures were treated with gentamycin and incubated for additional 23 hours. Cell culture supernatants were analyzed for TNF-α by ELISA (Figure 11) and cells were analyzed by flow cytometry for expression of the activation marker MHC-II (Figure 12). DC primed with 'SN *B. vulgatus*' induced less TNF-α (~50% reduction) after infection with *E. coli* mpk compared to direct treatment with *E. coli* mpk.

Figure 11: Neutralization of IL-6 present in *B. vulgatus* infected DC supernatant abolishes its ability to inhibit *E. coli* mpk induced TNF-α secretion in DC. Dendritic cells were prestimulated with different percentages of supernatant from DC cultures infected with non-colitogenic bacteria *B. vulgatus* at MOI 1 (SN *B. vulgatus*) with or without 10µg/ml anti-IL-6. After 24 hours cultures were infected with *E. coli* mpk at MOI 1, cultured for 1 hour, extracellular bacteria were killed by gentamicin and cells were cultured for additional 23 hours. Culture supernatants were harvested and analyzed for TNF-α by ELISA. The results are representative of three independent experiments. Each experiment was done in triplicate; error bars represent SD of duplicates. *p<0.05
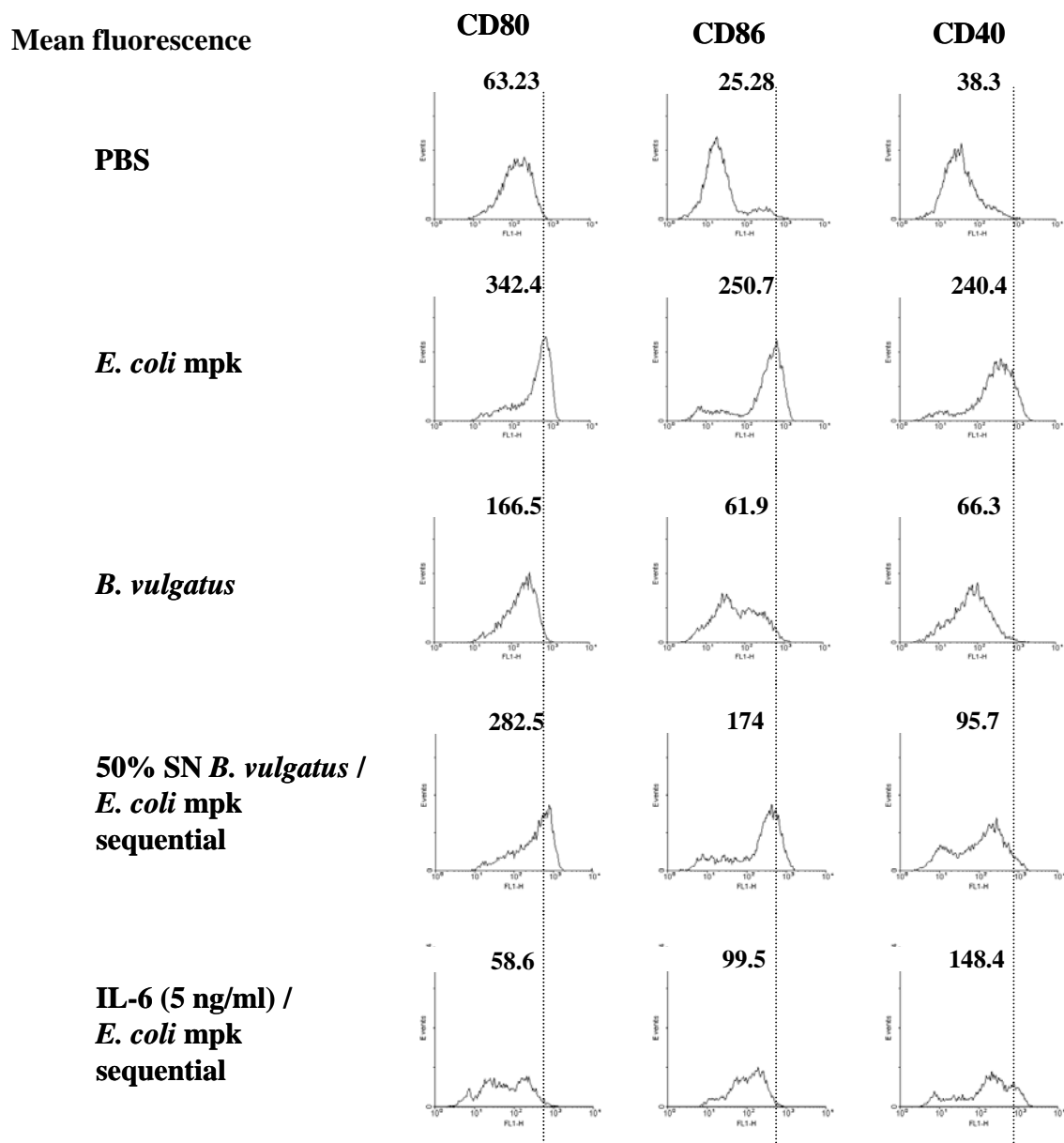
When IL-6 was neutralized in 'SN *B. vulgatus*' and DC was pretreated with it, the treatment with the supernatant could no longer prevent subsequent induction of TNF-α secretion by *E. coli* mpk. In addition the decreased expression of MHC II molecules upon *E. coli* mpk stimulation due to the pretreatment of DC with 'SN *B. vulgatus*' was restored by neutralization of IL-6 in it. These data indicate that IL-6 in the supernatant of *B. vulgatus* treated DC is crucial to prevent the stimulation by *E. coli* mpk which include TNF-α level and MHC II expression. These data further suggest the role of IL-6 in modulating the function of DC.
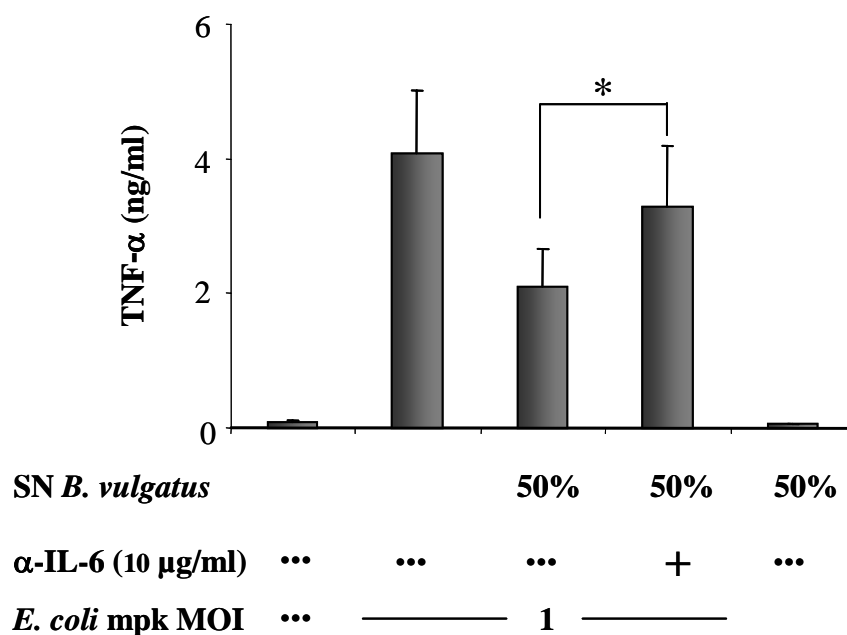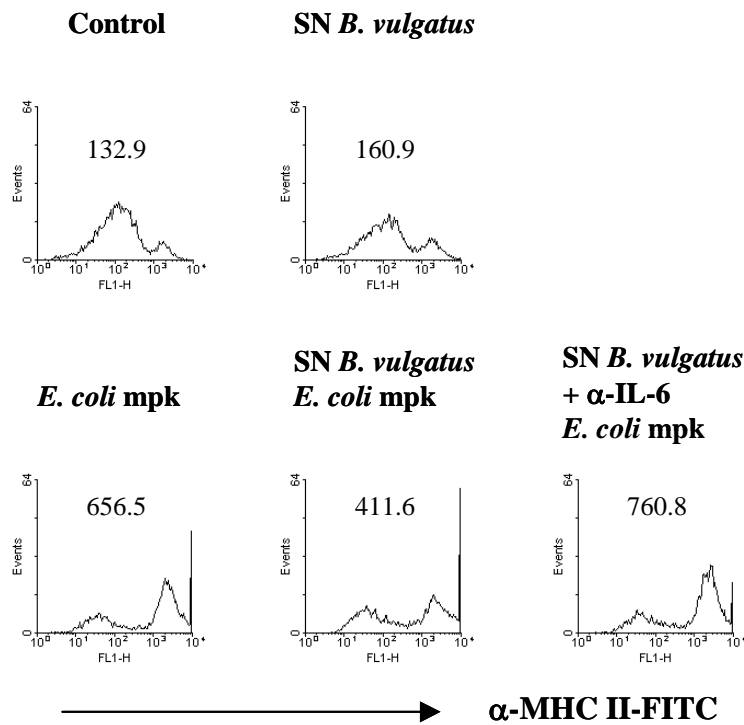
Figure 12: Neutralization of IL-6 present in *B. vulgatus* infected DC supernatant abolishes its capacity to inhibit *E. coli* mpk induced activation of DC. Dendritic cells were prestimulated for 24 hours with supernatant from DC culture infected with non-colitogenic bacteria *B. vulgatus* at MOI 1 (SN *B. vulgatus*) with or without 10µg/ml anti-IL-6. Cultures were then infected with *E. coli* mpk at MOI 1. After 1 hour, extracellular bacteria were killed by gentamicin and cells were cultured for additional 23 hours. Cells were harvested, stained with anti-CD11c-PE and anti-MHC-II-FITC. CD11c+ cells were analyzed by flow cytometry for MHC-II expression.

3.2 Colitogenic *E. coli* mpk causes Th1 polarization in vitro.

3.2.1 Supernatant of DC infected with *E. coli* mpk polarizes naïve T cells to Th1

From previous study (Chapter 3.1.1) it was clear that *E. coli* mpk and *B. vulgatus* differentially modulate DC activation. Also IL-2$^{-/-}$ mice develop Th1 driven colitis triggered by microflora (3). IL-2$^{-/-}$ mice monocolonized with *E. coli* mpk develop colitis with high expression of IFN-γ in colonic tissue whereas *B. vulgatus* monocolonized IL-2$^{-/-}$ mice do not develop colitis (121). Therefore we investigated if *E. coli* mpk stimulated DC factors are able to cause Th1 polarization and if there is any difference between *E. coli* mpk and *B. vulgatus* in this action. Naïve T cells were purified from spleen of DO11.10 mice. DO11.10 mice have transgenic CD4$^+$ T cells that recognize ovalbumin (specifically the peptide fragment from 323 to 339 amino acid of ovalbumin protein) as antigen (44). Naïve T cells from DO11.10 mice were cultured with irradiated splenocytes from BALB/c mice as antigen presenting cells (APC) and 0.6µM OVA-peptide$_{323-339}$ as antigen with 10% infected or uninfected dendritic

cell supernatant (cultured as explained at Chapter 3.1.1). The cells were splitted at day 3 and day 5 with addition of IL-2 for the last 4 days. At day 7 supernatants of cultured cells were analyzed for IFN-γ and IL-4 by ELISA and also cells were analyzed for the intracellular IFN-γ present in $CD4^+$ T cells. T cells stimulated with APC, OVA-peptide$_{323-339}$ as antigen in addition of supernatant from untreated DC produced low amounts of IFN-γ (<10 ng/ml) and 2 ng/ml IL-4. T cells stimulated with APC, OVA-peptide$_{323-339}$ in addition to *E. coli* mpk infected dendritic cell supernatant induced significantly higher IFN-γ production (~100 ng/ml)
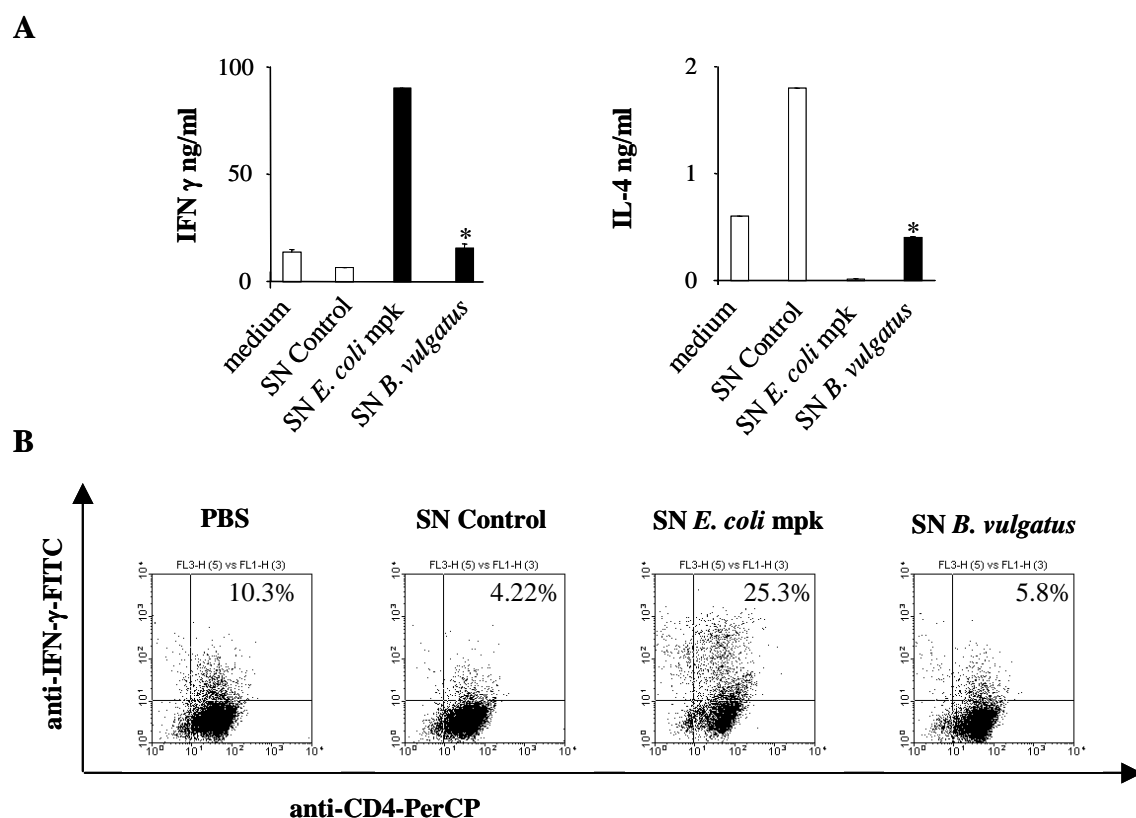
Figure 13: Supernatants from DC infected with *E. coli* mpk or *B. vulgatus* differentially polarize T-cells. Naïve $CD4^+$ T-cells from DO11.10 mice were incubated with Ova-$_{323-339}$ peptide and irradiated splenocytes from BALB/c mice in the presence of 10% supernatant from uninfected DC (SN control) and SN from DC infected with *E. coli* mpk (SN *E. coli* mpk) or *B. vulgatus* (SN *B. vulgatus*) bacteria. (A). After 7 days levels of IFN-γ and IL-4 of these culture supernatants were determined by ELISA and (B)Intracellular IFN-γ in T-cells were determined by flow cytometry. The results are representative of three independent experiments. Each experiment was done in duplicates; error bars represent SD of duplicates. *$p<0.05$ compared to *E. coli* mpk.

by antigen specific T cells compared to *B. vulgatus* infected DC supernatant (~20ng/ml) as shown by ELISA of culture supernatant (Figure 13A) and intracellular IFN-γ staining (25% vs 5.8%) and then detection by flow cytometry (Figure 13B). In contrast, 'SN *B. vulgatus*' induced some IL-4 production in T cells which was absent in supernatant *E. coli* mpk induced

T cells. So these data suggest that *E. coli* mpk induced secreted factors from DC are able to polarize naïve T cells to Th1 cells. However the supernatant of immature DC culture induced higher IL-4 secretion by T cells than *B. vulgatus* infected DC supernatant.

3.2.2   Th1 polarization needs TLR4 and TLR2 signaling in dendritic cells
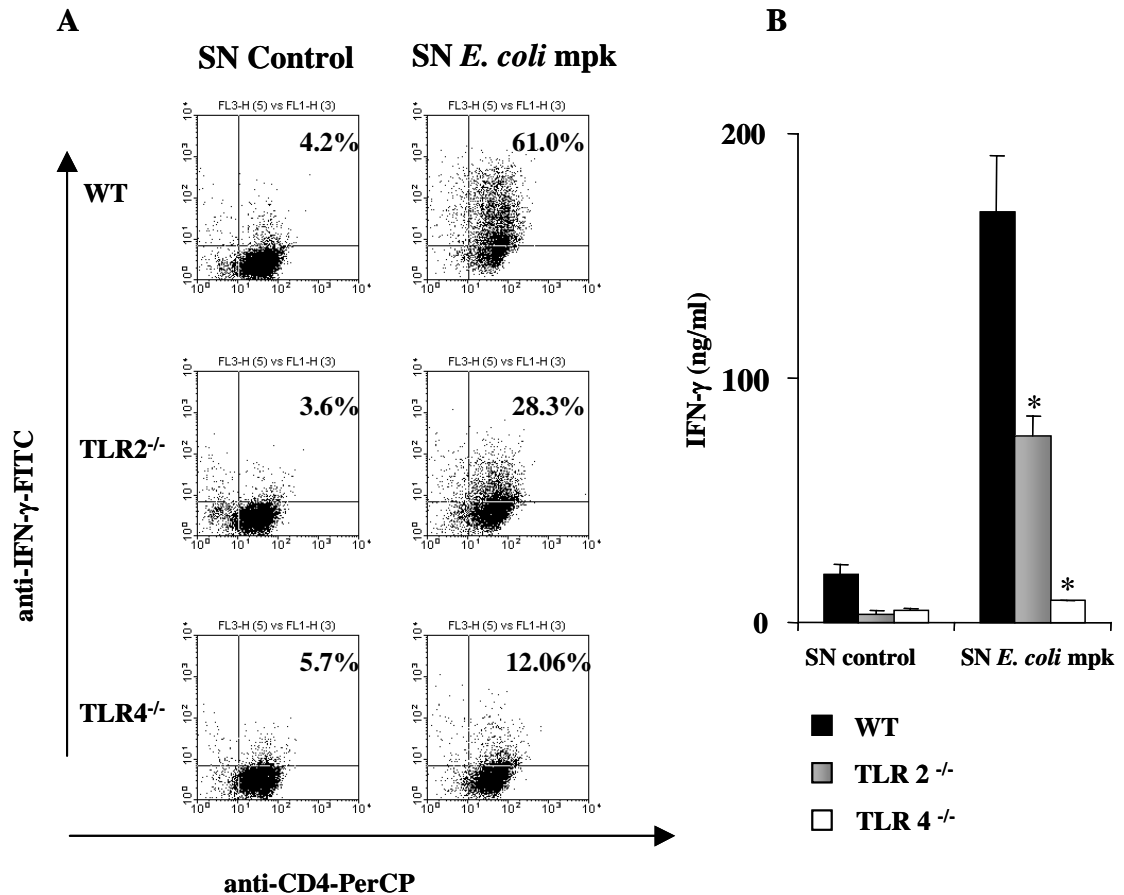
Figure 14: Th1 polarization by differently infected DC supernatant is dependent on TLR4 and TLR2. Naïve CD4+ T-cells from DO11.10 mice were incubated with Ova-$_{323\text{-}339}$ peptide and irradiated splenocytes from BALB/c mice in the presence of 10% supernatant from uninfected DC (SN PBS) and SN from DC infected with colitogenic *E. coli* mpk (SN *E. coli* mpk). DC was generated here from wild type, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice. After 7 days supernatants of these T cell cultures were harvested. Intracellular IFN-γ in T-cells was determined by flow cytometry (A), and the levels of IFN-γ in supernatant (B) were determined by ELISA. The results are representative of three independent experiments. Each experiment was done in duplicates; error bars represent SD of duplicates. *p<0.05 compared to *E. coli* mpk.

Before (Figure 5) it was shown that TNF-α and IL-12 secretion by dendritic cells depends on TLR4 and TLR2 signaling. To assess if the Th1 polarizing capacity of *E. coli* mpk is also TLR2 or TLR4 dependent, DCs were generated from bone marrow of TLR2$^{-/-}$, TLR4$^{-/-}$ and wild type mice and were infected with *E. coli* mpk. Cultures were treated with gentamicin

after one hour and incubated for additional 23 hours. Cell culture supernatants were filter sterilized and used for T cell stimulation. Naïve T cells from DO11.10 mice were cultured with irradiated splenocytes from BALB/c mice, ovalbumin$_{323-339}$ peptide in the presence of 10% infected or uninfected dendritic cell supernatant as explained before. Cultures were splitted on day 3 and day 5 and IL-2 was added for the last 4 days. On day 7 culture supernatants were analyzed for IFN-γ by ELISA (Figure 14B) and cells were also analyzed by flow cytometry for intracellular IFN-γ in CD4$^+$ T cells (Figure 14A). IFN-γ secretion by T cells was reduced to 50% in *E. coli* mpk infected culture supernatant (SN *E. coli* mpk) from TLR2$^{-/-}$ DC and reduced to basal level in case of SN *E. coli* mpk from TLR4$^{-/-}$ DC stimulation. The same was observed in IFN-γ expression analyzed by flow cytometry. Taken together these data indicate that T cell polarization by infected DC factors depends on recognition of microbial products by TLR4 and partially by TLR2 on DC.

3.2.3   IL-12 in *E. coli* mpk infected DC supernatant plays a role in Th1 polarization

DC primed with foreign antigen or pathogen become activated, secrete cytokines such as IL-12 and IL-18 which play a role in T helper cell polarization during T cell activation. To investigate if IL-12, IL-18 plays a role in Th1 polarization by secreted factors from DC primed with *E. coli* mpk, IL-12 and IL-18 were neutralized in *E. coli* mpk infected DC

IFN-γ ng/ml
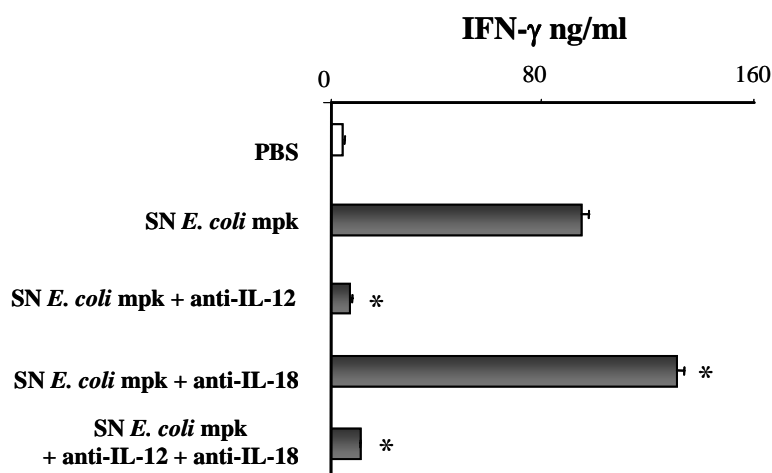
Figure 15: T cell polarization by infected DC supernatant depends on IL-12 content of the supernatant. Naïve CD4$^+$ T-cells from DO11.10 mice were incubated with Ova-$_{323-339}$ peptide and irradiated spleenocytes from BALB/c mice in presence of 10% supernatant from uninfected DC (SN PBS) and SN from DC infected with *E. coli* mpk (SN *E. coli*) with/ without α-IL-12, α-IL-18 or both. After 7 days supernatants of these cultures were harvested and the levels of IFN-γ were determined by ELISA. The results are representative of three independent experiments. Each experiment was done in duplicate; error bars represent SD of duplicate. *p<0.05 compared to *E. coli* mpk.

supernatant and were used to stimulate T cells. Immature BMDCs were infected with *E. coli* mpk for 1 hour, extracellular bacteria were killed by gentamicin and were cultured for additional 23 hours. Culture supernatants were filter sterilized. IL-12 and IL-18 in these supernatants were neutralized by adding neutralizing antibodies against IL-12 and IL-18 and were used in T cell stimulation.

Naïve T cells from DO11.10 mice were cultured with irradiated splenocytes from BALB/c mice, OVA-peptide$_{323-339}$ and in presence of 10% infected dendritic cell supernatants with/ without neutralization of IL-12 or IL-18. Cells were splitted at day 3 and day 5 with addition of IL-2 for last 4 days. At day 7 cell culture supernatants were analyzed for IFN-γ production by ELISA. There was 90% reduction of IFN-γ secretion by T cells stimulated after IL-12 neutralization in *E. coli* mpk infected DC supernatant. IL-18 neutralization had no effect on IFN-γ secretion by T cells, (Figure 15). This data suggests an important role of IL-12 which is present in DC supernatant stimulated with *E. coli* mpk for IFN-γ production by T cells and thus Th1 polarization.

3.2.4   LPS present in *E. coli* mpk infected DC supernatant also plays role in Th1 polarization

To investigate if LPS present in bacteria infected dendritic cell supernatants is responsible for IFN-γ secretion by T cells, LPS in the supernatant was neutralized by polymyxin B. Dendritic cells were infected with *E. coli* mpk or *B. vulgatus* or stimulated with LPS, cultures were treated with gentamicin after 1 hour and incubated for additional 23 hours. Culture supernatants were filter sterilized and LPS was neutralized by polymyxin B. Naïve CD4$^+$ T cells from DO11.10 mice were cultured with irradiated splenocytes from BALB/c mice, as antigen OVA-peptide$_{323-339}$ with 10% stimulated-DC supernatant with/without LPS neutralization for 7 days in presence of IL-2 for last 4 days. LPS neutralization in *E. coli* mpk infected DC supernatant reduced IFN-γ secretion (90%) by T cells upon stimulation and also induced some IL-4 secretion by these T cells. In contrast polymyxin treatment of *B. vulgatus* infected DC supernatant has no effect on IFN-γ secretion by T cells, rather the IL-4 secretion increases around 2.5 fold by LPS neutralization. LPS stimulated dendritic cell supernatant also stimulated less IFN-γ in T cell after LPS neutralization (Figure 16). Therefore, these data demonstrates that LPS together with IL-12 plays a role in Th1 polarization by *E. coli* mpk infected DC supernatant.
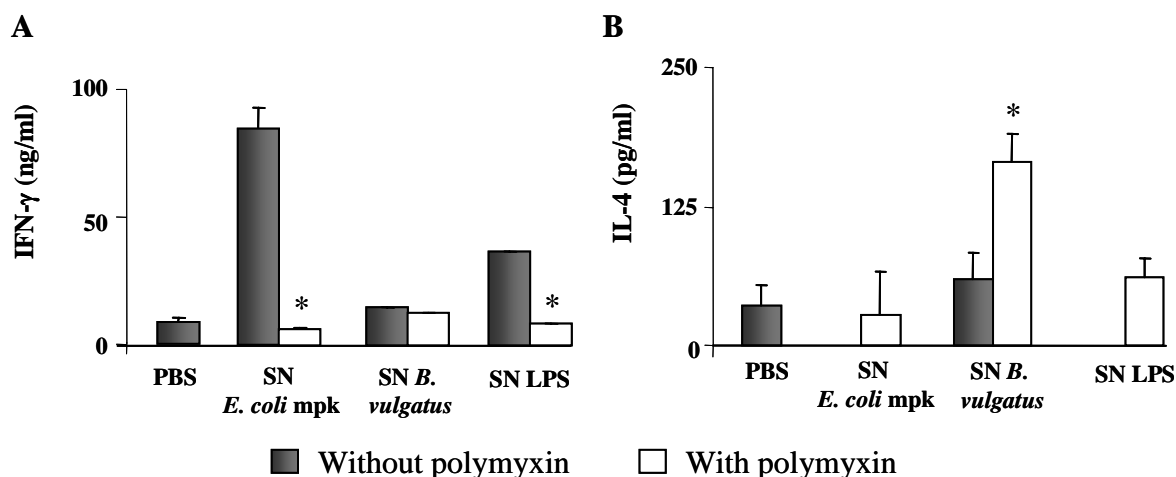
Figure 16: T cell polarization by differently infected DC supernatant is reduced by neutralization of LPS from the supernatant. Naïve CD4$^+$ T-cells from DO11.10 mice were incubated with Ova$_{323-339}$ peptide and irradiated spleenocytes from BALB/c mice in presence of 10% supernatant from uninfected DC (SN PBS) and SN from DC infected with colitogenic (SN *E. coli* mpk) or non-colitogenic (SN *B. vulgatus*) bacteria with or without polymyxin B treatment. After 7 days supernatants of these cultures were harvested and the levels of IFN-γ (A) and IL-4 (B) were determined by ELISA. The results are representative of three independent experiments. Each experiment was done in duplicates; error bars represent SD of duplicates. *p<0.05 compared to without polymyxin treated counterpart.

3.2.5   Supernatant of *B. vulgatus* infected DC can inhibit *E. coli* mpk induced Th1 polarization

It was shown earlier (Chapter 3.1.3) that *B. vulgatus* pretreatment of DC can prevent *E. coli* mpk induced cytokine secretion by DC. So far it was shown that pretreatment of DC with *B. vulgatus* prevents cytokine secretion triggered by *E. coli* mpk. In addition *E. coli* mpk treated DC supernatant but not *B. vulgatus* pretreated DC supernatant led to Th1 polarization. Therefore we hypothesized that supernatants from DCs pretreated with *B. vulgatus* and then stimulated with *E. coli* mpk should induce low IFN-γ secretion by T cells upon stimulation. To prove this T cells were stimulated with *B. vulgatus* and *E. coli* mpk infected DC supernatant. In brief, dendritic cells were prestimulated with *B. vulgatus* for 1 day and then infected with *E. coli* mpk, after one hour cultures were treated with gentamicin and incubated for another 23 hours. Culture supernatants were filter sterilized and used for T cell stimulation. Naïve CD4$^+$T cells purified from spleen of DO11.10 mouse were cultured with
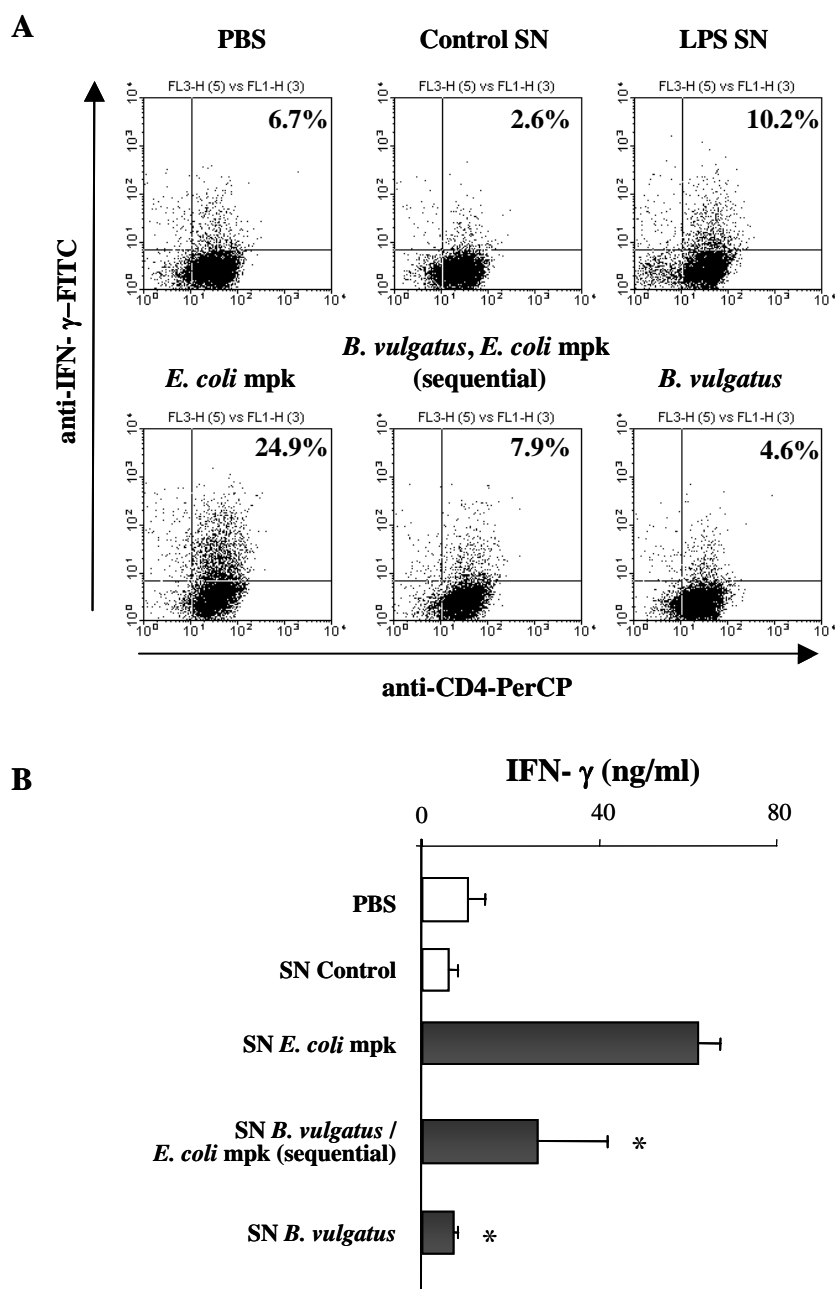
Figure 17: *B. vulgatus* **can inhibit** *E. coli* **mpk induced Th1 polarization.** Naïve CD4^{+} T-cells from DO11.10 mice were incubated with Ova-$_{323-339}$ peptide and irradiated splenocytes from BALB/c mice in presence of 10% supernatant from uninfected DC (SN Control) and SN from DC infected with *E. coli* mpk (SN *E. coli* mpk) or *B. vulgatus* (SN *B. vulgatus*) or co stimulated sequentially with *B. vulgatus* and then *E. coli* mpk (SN *B. vulgatus*, *E. coli* mpk). After 7 days cultures were harvested. (A) Intracellular IFN-γ in T-cells was determined by flow cytometry and (B) the levels of IFN-γ in supernatant were determined by ELISA. The results are representative of three independent experiments. Each experiment was done in duplicate; error bars represent SD of duplicates. *p<0.05 compared to *E. coli* mpk.

irradiated splenocytes from BALB/c mice, 0.6μM OVA peptide$_{323-339}$ in presence of 10% DC

supernatant. Cultures were splitted at day 3 and day 5 and IL-2 was added for last 4 days. At

day 7 culture supernatants were analyzed for IFN-γ by ELISA and also cells were analyzed by

flow cytometry for the intracellular IFN-γ in CD4^{+} T cells. Supernatant from *B. vulgatus* prestimulated and *E. coli* mpk infected DC induced around 50% less IFN-γ production than only *E. coli* mpk infected DC supernatant (Figure 17B). Intracellular IFN-γ was also less at costimulated DC supernatant-primed T cells (7.9%) compared to intracellular IFN-γ (24.9%) of T cells primed by DC supernatant stimulated with only *E. coli* mpk. So this data suggests an inhibitory role of *B. vulgatus* in *E. coli* mpk mediated T cell polarization.

3.2.6   *E. coli* mpk activated DC polarizes T cells

DC recognizes pathogens with its pattern recognition receptors and become activated. During activation, DC upregulate its surface markers, e. g. costimulatory molecules, MHC complex,
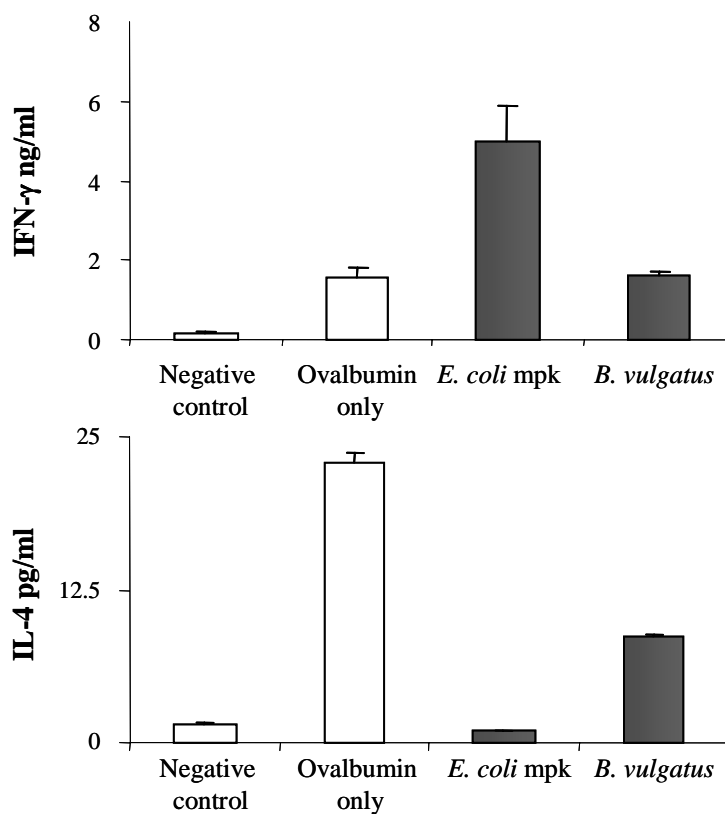
Figure 18: DC activated with colitogenic bacteria causes Th1 polarization in antigen specific T cells. Immature BMDC were stimulated with *E. coli* mpk or *B. vulgatus* or nothing for 1 hour, treated with gentamicin and incubated for additional 23 hours, last 4 hours with 100μg/ml ovalbumin was added to the cultures. Naïve CD4^{+} T cells from spleen of OT-2 mice were stimulated with these stimulated DC for 4 days and IFN-γ and IL-4 were determined in the supernatant by ELISA. The error bars represent SD of duplicate.

chemokine receptors etc. and also secretes cytokines and chemokines. In the last sections we have demonstrated the role of secreted factors from pathogen-activated DC on T cell polarization. We have shown that *E. coli* mpk and *B. vulgatus* differentially induce IL-12, TNF-α secretion by DC and also upregulates activation and maturation markers differently. The different cytokine secretion by DC with *E. coli* mpk and *B. vulgatus* has an impact on T cell polarization (Figure 13). In addition to soluble factors direct cell-to cell interaction plays an important role in T cell stimulation. Therefore to address the role of differential activation and maturation marker on bacteria-stimulated DC was investigated. Immature BMDC were infected *with E. coli* mpk or *B. vulgatus* at MOI 1, cultures were treated with gentamicin after 1 hour and cultured for additional 23 hours. DCs were pulsed with antigen, ovalbumin for last 3 hours. Naïve $CD4^+$ T cells from OT-II mice were stimulated with these stimulated DC for 4 days and IFN-γ, IL-4 levels were determined by ELISA. *E. coli* mpk infected DC polarized T cells to Th1 with high IFN-γ (6 ng/ml) but negligible IL-4 secretion. *B. vulgatus* infected DC induced less IFN-γ secretion (<2ng/ml) by T cells but more IL-4 secretion (10 pg/ml) compared to T cells primed by *E. coli* mpk infected DC (Figure 18). However DC primed with only antigen induced more IL-4 (25 pg/ml) secretion by antigen specific T cells than *B. vulgatus* stimulated antigen primed DC. It seems that in absence of active stimulus for Th1 polarization, which mediates Th1 cytokine production (IFN-γ) and inhibits Th2 cytokine (IL-4), naïve T cells produce IL-4.

3.2.7   Heat killed *E. coli* mpk and *B. vulgatus* enhance antigen specific T helper cell response in vivo

In IL-2$^{-/-}$ mice, *E. coli* mpk was found as colitogenic whereas *B. vulgatus* was non-colitogenic (121). It is known that IL-2$^{-/-}$ mice develop Th1 driven colitis (3). It was also shown in another study that *E. coli* and *P. gingivalis* LPS has different immune modulation capacity in vivo (84). Therefore, the T helper cell response against these two commensals, *E. coli* mpk and *B. vulgatus* in an antigen specific in vivo model was analyzed. For this purpose, splenocytes from DO11.10 mice (which contains 20% OVA-peptide $_{323\text{-}339}$ specific $CD4^+$ T cells) were transferred intravenously to syngenic BALB/c mice. After 3 days these BALB/c mice were immunized intraperitoneally with 2 mg ovalbumin or 2 mg ovalbumin plus 200 µg heat killed *E. coli* mpk (HK *E. coli* mpk) or plus 200µg heat killed *B. vulgatus* (HK *B. vulgatus*) respectively. After 4 days immunized mice were sacrificed, splenocytes were prepared and expansion of OVA$_{323\text{-}339}$ specific $CD4^+$ T cells was analyzed. Furthermore

splenocytes were restimulated with different concentration of ovalbumin to analyze in vitro proliferation and cytokine production. It was found that CD4$^+$ T cell population expanded in heat killed *E. coli* mpk as well as *B. vulgatus* treated reconstituted mice (Figure 19). A significant increase in OVA-peptide $_{323-339}$ specific T helper cell enhancement was only found in mice treated with ovalbumin and HK *E. coli* mpk compared to mice treated with ovalbumin alone.
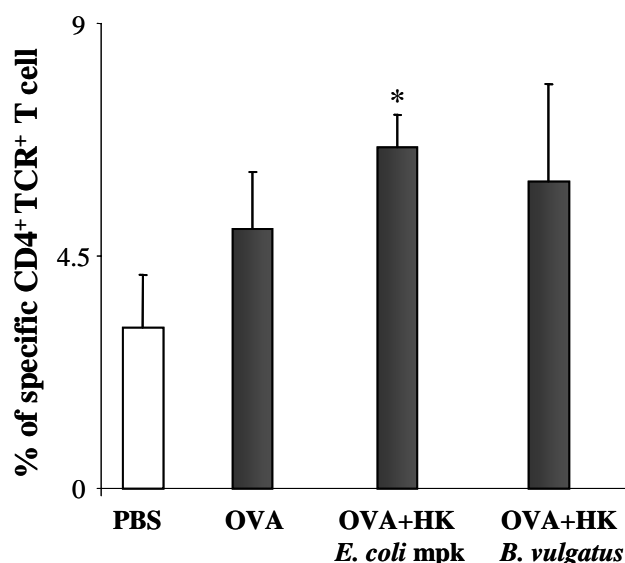
Figure 19: *E. coli* **mpk and** *B. vulgatus* **enhance T helper cell response in vivo.** BALB/c mice reconstituted with DO11.10 T cells were induced intraperitoneally with soluble ovalbumin alone, heat killed *E. coli* mpk + ovalbumin or heat killed *B. vulgatus* + ovalbumin. After 4 days splenocytes were analyzed by flow cytometry for expansion of ovalbumin specific T helper cell by staining with anti-CD4 and Kj1-26(antibody against ova specific TCR). Figure shows percentage of expanded ovalbumin specific CD4$^+$ T cells in spleen from a representative experiment. Data represents the mean of two mice in each group.

3.2.8   Heat killed *E. coli* mpk and *B. vulgatus* induce antigen specific T helper cell polarization differently

Splenocytes from mice reconstituted with OVA$_{323-339}$ specific T cells and then immunized with soluble antigen with or without heat killed bacteria (as explained before) were restimulated in vitro for 72 hours with different concentration of ovalbumin. Culture supernatants were analyzed by ELISA to measure IFN-γ production by antigen specific T cells. Cells were pulsed with ^{3}[H]-thymidine for last 16 hours to investigate T cell proliferation. .There was significant difference in IFN-γ production by cell culture from HK *E. coli* mpk and ovalbumin immunized mice compared to HK *B. vulgatus* and ovalbumin. HK *E. coli* mpk and ovalbumin immunization induced more IFN-γ secretion by T cells than HK

B. vulgatus and ovalbumin. But HK *B. vulgatus* and ovalbumin also induced some IFN-γ secretion in culture compared to only ovalbumin immunization (Figure 17). Both HK bacteria and soluble antigen stimulation induced significantly higher amounts of IL-10 secretion than only ovalbumin immunization (data not shown). T cells from mice treated only with ovalbumin proliferated nearly same like ovalbumin and heat killed bacteria immunized T cells when restimulated in vitro with ovalbumin (Figure 20). Therefore this data suggest that *E. coli* mpk, in heat-killed state is also able to enhance IFN-γ production by antigen specific T helper cell much better than heat killed *B. vulgatus*.
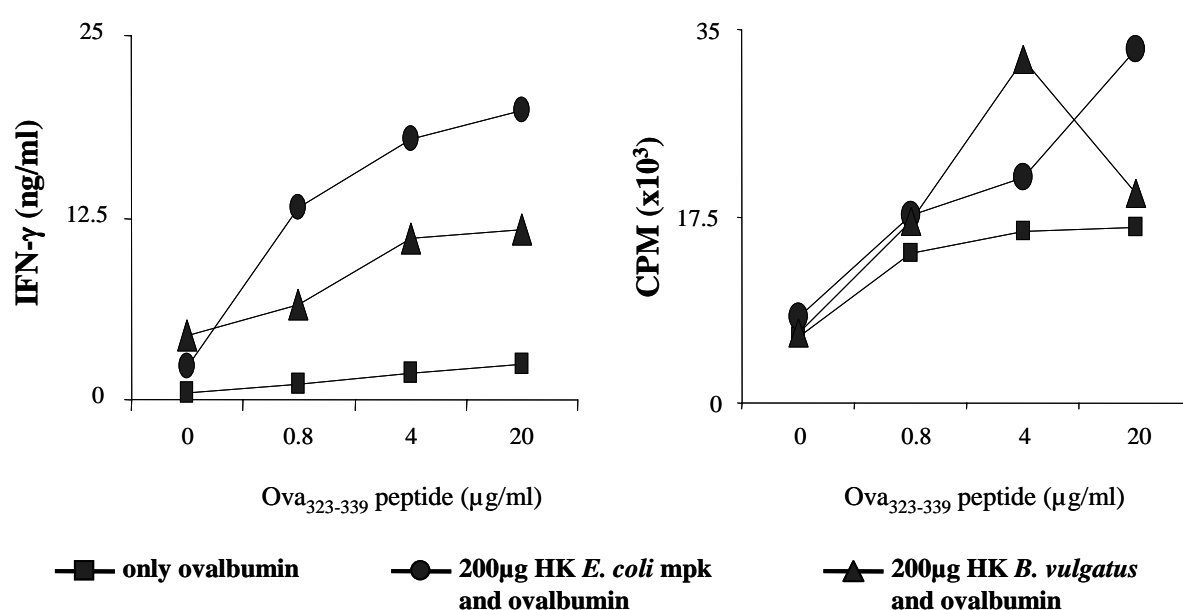
Figure 20: *E. coli* mpk enhances IFN-γ secretion by T helper cells compared to *B. vulgatus*. BALB/c mice reconstituted with DO11.10 T cells were induced intraperitoneally with soluble ovalbumin alone, heat killed *E. coli* mpk + ovalbumin or heat killed *B. vulgatus* + ovalbumin. Four days after immunization, splenocytes were restimulated with different concentration of ovalbumin and supernatants were analyzed for IFN-γ. Splenocytes were pulsed with ^{3}[H]-thymidine for last 16 hours of culture and analyzed for cell proliferation. Data is from a representative experiment.

3.3   Adipsin might have role in colitis prevention

3.3.1   *E. coli* Nissle induces adipsin expression in colon of IL-2$^{-/-}$ mice

It was found that IL-2$^{-/-}$ mice monocolonized with *E. coli* Nissle, *B. vulgatus*, *B. vulgatus* and *E. coli* mpk does not develop colitis. In contrast IL-2$^{-/-}$ mice monocolonized with *E. coli* mpk develop colitis at 22 weeks of age (121). IL-2$^{-/-}$ mice raised under specific pathogen free condition develops severe colitis. Another approach to analyze the mechanism(s) responsible

for developing colitis or prevention, the colonic total mRNA of differently colonized mice
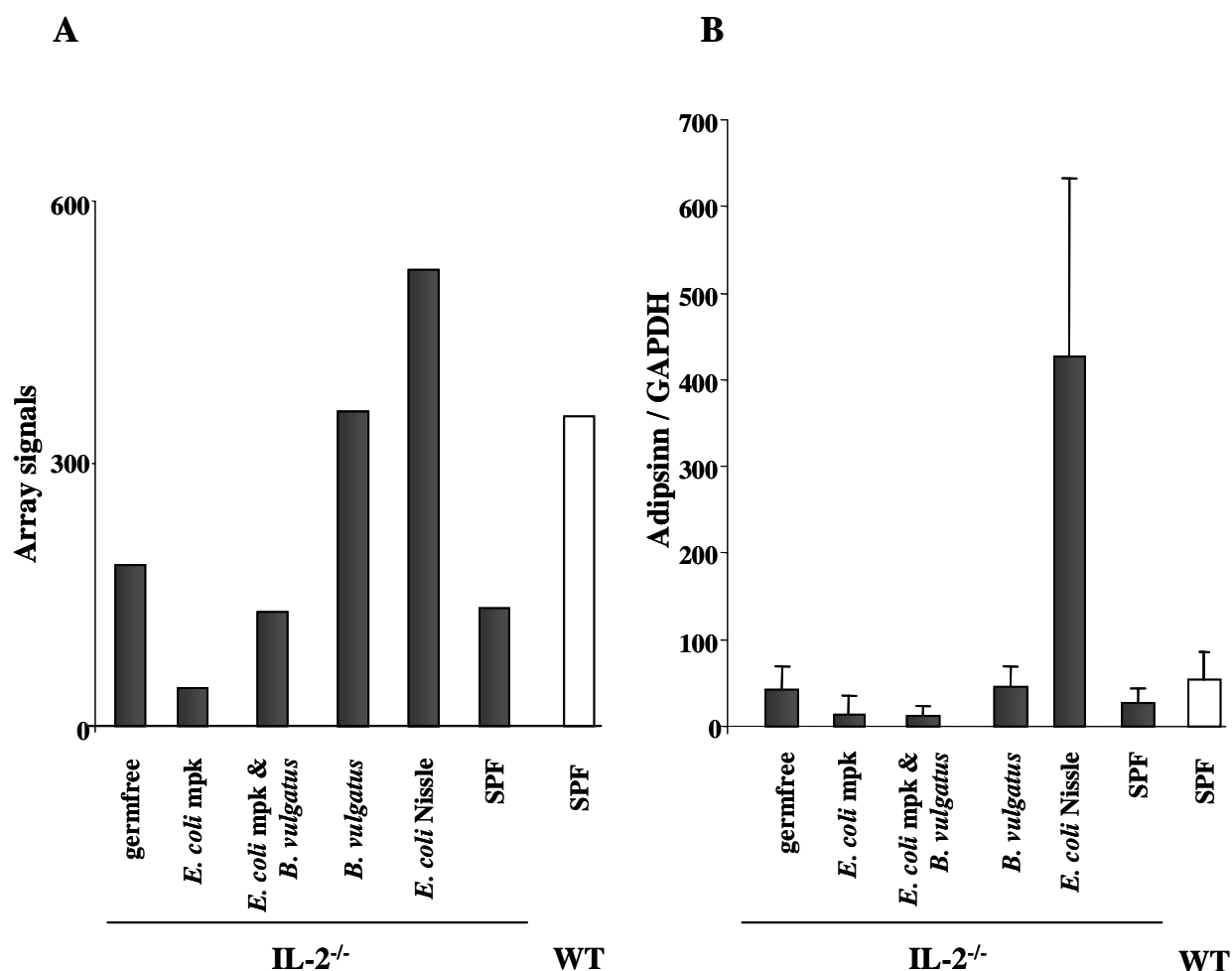
Figure 21: Higher expression of adipsin was observed at colon of *E. coli* Nissle colonized IL-2$^{-/-}$ mice. Colonic total mRNA from IL-2$^{-/-}$ mice mono colonized with *E. coli* mpk, *E. coli* Nissle, *B. vulgatus*, cocolonized with *E. coli* mpk and *B. vulgatus* or wild type and IL-2$^{-/-}$ mice kept in specific pathogen free condition or germ free condition was isolated. A, RNA was analyzed by Affymetrix Gene array signals for adipsin expression are shown. B, adipsin expression was quantified by real time RT PCR normalized with GAPDH. Error bars represent SD of 5 mice. All mice were 8 weeks old.

were analyzed by Affymetrix Gene Chip oligo microarray. One finding of this gene expression analysis was that several genes which are described to be mainly expressed by adipocytes such as adipsin, adiponectin seemed to be highly expressed in mice developing no colitis compared to mice developing colitis (Figure 21A). To confirm these findings semiquantitative RT-PCR was performed using mRNA from the colon of 5 single 8 weeks old mice per group. The highest expression for adipsin was found in IL-2$^{-/-}$ mice colonized with *E. coli* Nissle. *E. coli* mpk mice, developing colitis expressed lower amounts of adipsin

E. coli mpk   **E. coli Nissle**

B. vulgatus   **B. vulgatus / E. coli mpk**

Peptide blocker
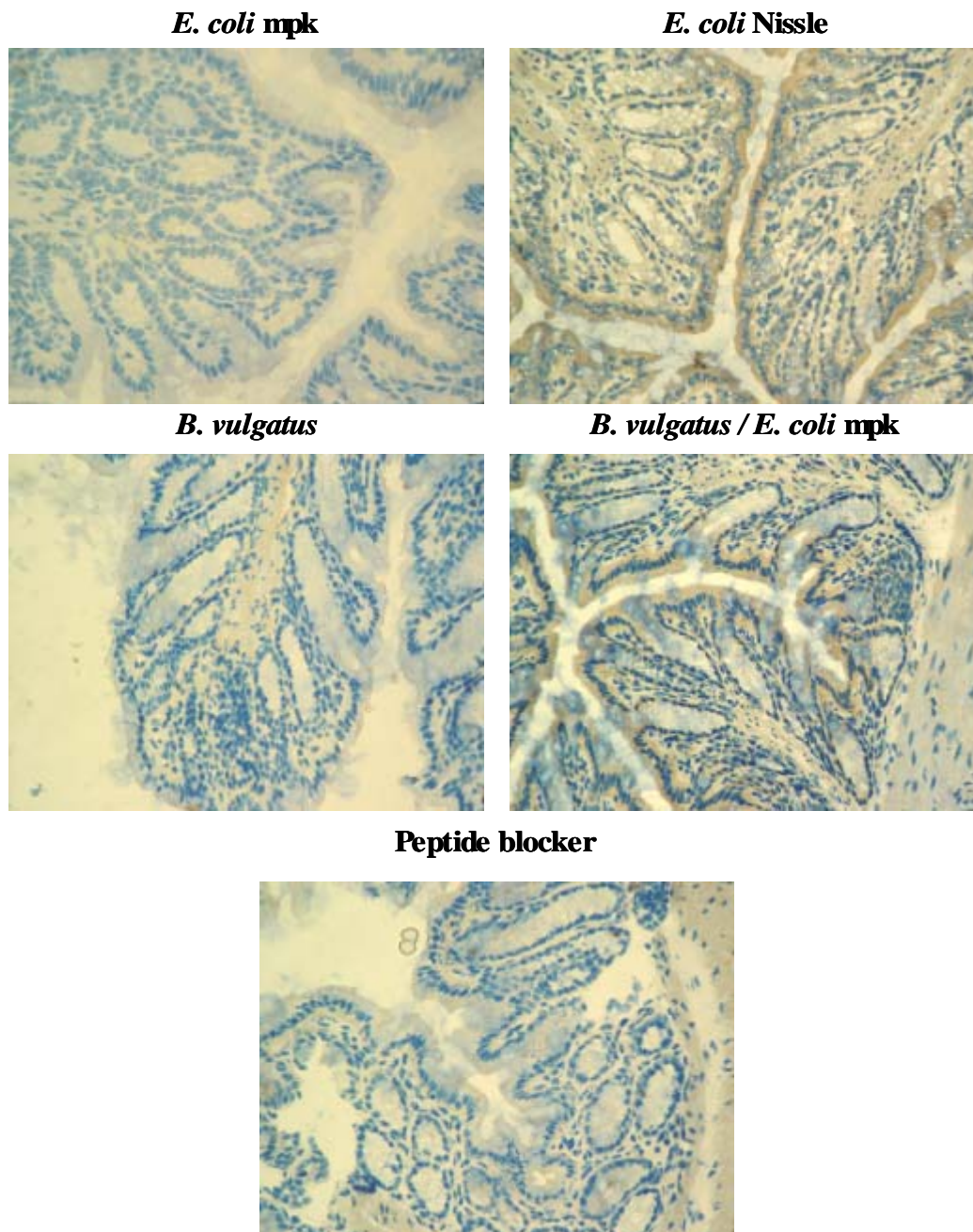
Figure 22: Immunohistochemical comparison of colonic sections from differently colonized IL-2$^{-/-}$ mice for adipsin. Colonic sections from 8 weeks old IL-2$^{-/-}$ mice mono colonized with *E. coli* mpk, *E. coli* Nissle, *B. vulgatus* or cocolonized with *E. coli* mpk and *B. vulgatus* were stained with anti- adipsin antibody and with hematoxyline/ eosin, examined at 40x magnification.

compared to *B. vulgatus* or *E. coli* Nissle monocolonized mice (Figure 21B) (unpublished data kindly provided by a colleague from our group, Oliver Bechtold). To investigate which cell types in the mucosa is the source of adipsin, colon sections from differently colonized mice were assayed by immunohistology with antibody against adipsin (Figure 22). It is known that adipocytes are the main source of adipsin. Also epithelial cells, muscle cells

express adipsin (25,49,72,124). Immunohistochemical analysis of colon section from *E. coli* Nissle colonized IL-2$^{-/-}$ mice showed higher expression of adipsin in epithelial lining of intestinal mucosa. IL-2$^{-/-}$ mice double colonized with *E. coli* mpk and *B. vulgatus* also showed specific labeling of adipsin at intestinal epithelium. In contrast *E. coli* mpk monocolonized IL-2$^{-/-}$ mice showed no specific labeling of adipsin in colonic sections.

3.3.2   *E. coli* **Nissle does not induce adipsin production by adipocytes in vitro**

To investigate if *E. coli* Nissle directly induces adipsin expression in cells, mouse intestinal epithelial cell line was (mICcl2) was infected with different bacteria and after 4 hours of infection mRNA was analyzed for adipsin expression by semiquantitative real time RT PCR. *E. coli* Nissle was unable to induce adipsin expression in epithelial cell line (data not shown). *E. coli* mpk, *B. vulgatus* was also unable to induce adipsin expression in epithelial cell line. It was found that in gastrointestinal tract adipose tissue surrounding the lymphoid cells interact with lymphoid cells. This interaction could be associated with protection against *Helicobacter felis* in a mouse infection model (74). Therefore we investigated whether *E. coli* Nissle is able to induce adipsin expression in adipose tissue and if there are differences in induction of adipsin by different bacteria. 3T3-L1, a preadipocyte cell line was used in this investigation. 3T3-L1 cells were grown to confluence. Subsequently 3T3-L1 cell line was infected with different bacteria at MOI 1, at day 0, 4 and 8 and cells were analyzed at day 10. *E. coli* Nissle as well as *E. coli* mpk and *B. vulgatus* were unable to induce adipsin expression in this cell line (Figure 23C) as analyzed by mRNA at day 10 by semiquantitative real time RT PCR. Similarly these bacteria were unable to differentiate this cell line to adipocyte (Figure 23A) as shown by 'Oil red O'staining. *E. coli* Nissle and *E. coli* mpk and also *B. vulgatus* induced cell proliferation upon infection in this cell line compared to untreated cells shown by cell count (Figure 23B). Therefore this study excludes direct differentiation of adipocytes and adipsin expression in response to *E. coli* Nissle as well as other bacteria used in this study.

A

E. coli mpk   *E. coli* Nissle   *B. vulgatus*

Negative control   Positive control

B

C

Figure 23: Infection of adipocyte cell line with different colitogenic and noncolitogenic bacteria is unable to induce adipsin expression. Preadipocyte cell line 3T3-L1 were infected with *E. coli* mpk, *E. coli* Nissle, *B. vulgatus* according to method and mRNA was analyzed at day 0, 5 and 10 for adipsin expression. As positive control cells were stimulated with insulin, dexamethason and IBMX for first two days and then insulin was maintained in culture medium for rest of the incubation. A. Cell differentiation analyzed by 'Oil red O' staining, B, cell proliferation shown by cell count at day 10 and C, Adipsin expression was quantified by Taqman RT PCR and normalized with GAPDH. Error bars represent SD of duplicates.

3.3.3   Bacteria infected dendritic cell supernatant induces proliferation in preadipocyte cell line but does not induce adipsin expression

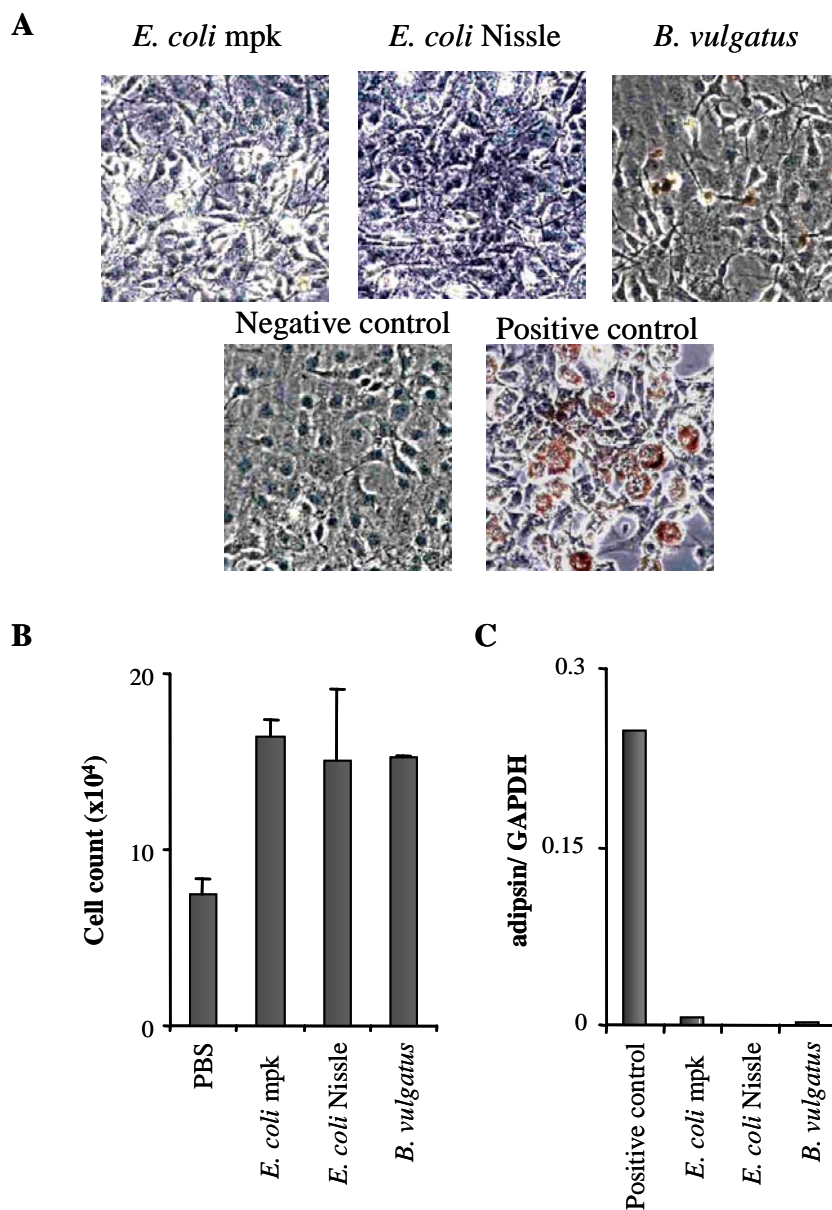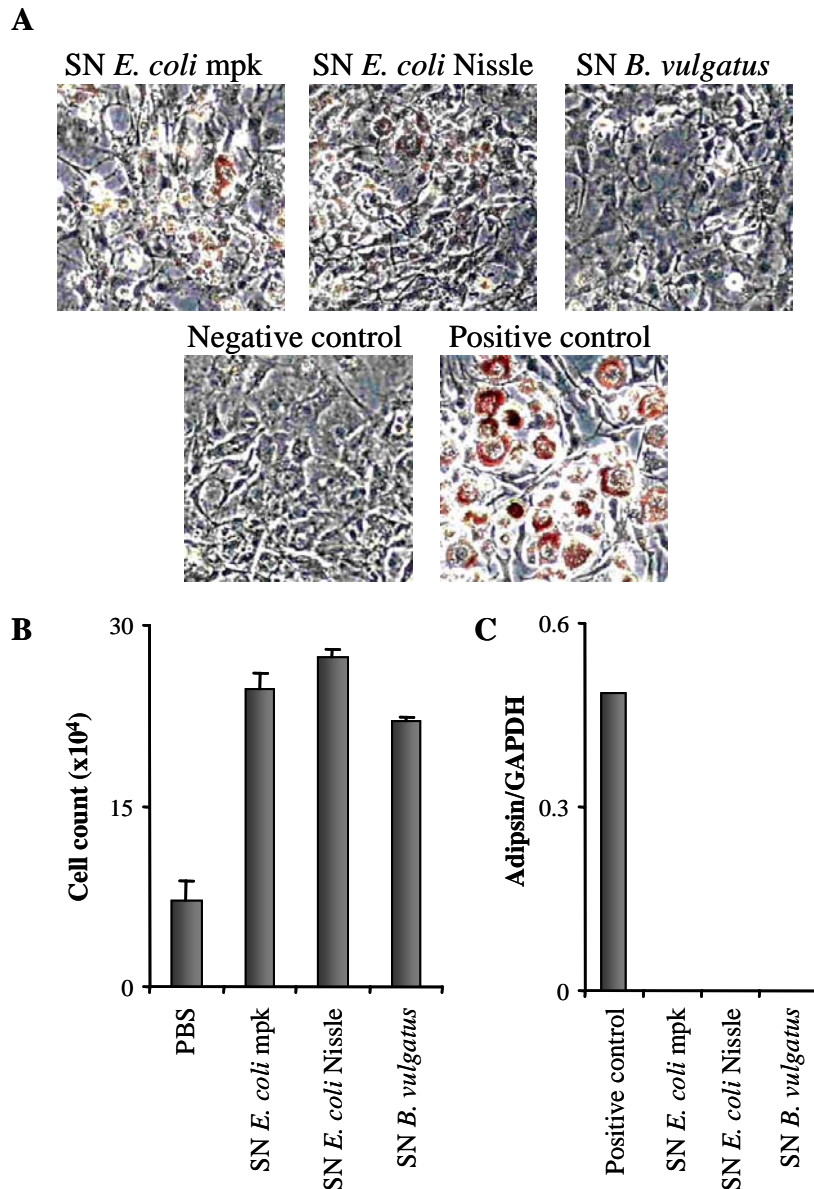**Figure 24: Infection of adipocyte cell line with supernatant of DC culture infected with different colitogenic and noncolitogenic bacteria is unable to induce adipsin expression.** Preadipocyte cell line 3T3-L1 was infected with 10% supernatant from DC cultures which were infected with different colitogenic and probiotic bacteria. As positive control cells were stimulated with insulin, dexamethason and IBMX for first two days and then insulin was maintained in culture medium for rest of the incubation. A. Cell differentiation analyzed by 'Oil red O' staining, B, Cell proliferation shown by cell count at day 10 and C, Adipsin expression was quantified by real time RT PCR and normalized with GAPDH. Error bars represent SD of duplicates.

It was assumed that *E. coli* Nissle does not directly induce adipsin in adipocyte of gut, rather it might be an indirect effect. May be *E. coli* Nissle induced gut associated lymphoid tissue in turn stimulate adipocytes to produce adipsin. To analyze this hypothesis, supernatant of DC

culture infected with *E. coli* Nissle, *E. coli* mpk or *B. vulgatus* was used to stimulate preadipocyte cell line, 3T3-L1. 3T3-L1 cells were grown to confluence and then were infected with 10% of differently infected DC supernatant at day 0, 4 and 8 and cells were analyzed at day 10. *E. coli* Nissle as well as *E. coli* mpk and *B. vulgatus* infected DC supernatant were unable to induce adipsin expression in this cell line as analyzed by Real time RT PCR. Bacteria induced DC factors were able to induce significant cell proliferation and only the DC factors induced by the both *E. coli* strains were able to differentiate this cell line to adipocyte in some extent compared to untreated cells (Figure 24). So this study also excludes a positive influence of DC mediated factors in adipsin expression by adipocyte in this experimental setup.

4   Discussion

Multiple studies have led to the view that both environmental factors and genetic susceptibility contribute to pathological immunoregulatory events causing colitis (97). Among the environmental factors the microflora is more important. Uncontrolled immune responses to nonpathogenic luminal bacteria as a cause of colitis was also supported by clinical observations such as decreasing intestinal bacterial concentrations by various ways can lead to clinical improvement and decreased intestinal inflammation (94). Furthermore an IBD model, IL-2$^{-/-}$ mice develop colitis which is triggered by microflora (89). Microflora contains plenty of different bacterial strains, part of them can cause harm in altered physiological conditions. To assess this phenomenon some research has been done on gnotobiotic mice, selectively colonizing mice with one or two bacterial strains. Waidmann *et al* (121) colonized IL-2$^{-/-}$ mice with commensal strain, *E. coli* mpk or *Bacteroides vulgatus*. *E. coli* mpk strain was colitogenic for IL-2$^{-/-}$ mice but *B. vulgatus* was not colitogenic for it. Moreover *B. vulgatus* was able to suppress *E. coli* mpk induced colitis in co colonized IL-2$^{-/-}$ mice. Mono colonization of IL-2$^{-/-}$ mice with a well known probiotic *E. coli* Nissle 1917 was also free of colitis. Similar dependence on selective bacterial colonization was observed in germ free and gnotobiotic IL-10$^{-/-}$ mice. Germ free IL-10$^{-/-}$ mice developed IBD after they were colonized with *Enterococcus feacalis*. In contrast neither germ free IL-10$^{-/-}$ mice nor IL-10$^{-/-}$ mice colonized with a pure culture of *Candida albicans*, *E. coli*, *Lactobacillus casei*, *L. ruteri*, *L. acidophilus*, a *Bifidobacterium* species, *Lactococcus lactis*, or a *Bacillus* species developed IBD (4).

In gut, commensals interact with gut associated lymphoid tissue, for example, epithelial cell, dendritic cell and then T cell. So this different colitogenic potential of different commensal strains might originate from different stimulation of gut associated lymphoid tissue and their cross talk.

4.1   Different commensals differentiate DC differently

DCs are distributed throughout the tissues, especially at the sites of interface with external environment. At mucosal interfaces, DC constantly survey and process commensal bacteria and pathogens and distinct DC subsets achieve this through different ways (45,46,48). In addition to acquiring antigens in the lamina propria, intestinal DCs located below specialized intestinal epithelial cells, M cells, also detect incoming pathogens. Recent evidence has

suggested that intestinal DC directly monitor the content of the intestinal lumen by either entering or extending dendrites into the epithelium (68,87). So DC is among the cells which encounters luminal bacteria. Therefore we investigated whether a DC in vitro model might help to explain how these bacteria may trigger or prevent colitis development in vivo. *E. coli* mpk induces higher IL-12, TNF-α, and IL-10 secretion by bone marrow derived dendritic cells than *B. vulgatus*. Also *E. coli* mpk induced higher expression of activation and maturation marker on BMDC than *B. vulgatus*. This data are in line with findings of other groups which show that different species of lactobacilli exert different DC activation patterns (22). BMDC were stimulated with six different lactobacilli strains in irradiated form. Among them *Lactobacillus casei* was a more potent inducer of cytokines (IL-12, IL-10, TNF-α and IL-6) and surface markers CD86 and MHC II by BMDC than *Lactobacillus reuteri*. The other four lactobacilli species were in between of the two strains in regards to BMDC activation and cytokine production. These data again demonstrates differential DC modulation by different bacteria which might be responsible for different colitis development in our in vivo colitis model.

4.1.1   *B. vulgatus* differentiates DC into a semimature intermediate state

It was found that stimulation of BMDC with *B. vulgatus* does not exert full activation and maturation of it. But the stimulated DC phenotype does not resemble to immature DC. Some recent data defined the semimature DCs as a DC phenotype which is phenotypically mature but functionally impaired. These DC express high levels of MHC II and co stimulatory molecules but show impaired cytokine expression (40,52,62). This phenotype is quite similar to the DC phenotype found after stimulation with *B. vulgatus*. After *B. vulgatus* treatment an impairement of IL-12 and TNF-α secretion by DC were observed. In addition the DC were unable to upregulate activation and maturation markers. Therefore, *B. vulgatus* stimulated DC were named as semimature/ intermediate DC.

4.1.2   *B. vulgatus* inhibits *E. coli* mpk induced DC activation

Because IL-2$^{-/-}$ mice cocolonized with *E. coli* mpk and *B. vulgatus* did not develop colitis (121), we investigated whether *B. vulgatus* can protect DC activation by *E. coli* mpk in an in vitro system. A recent study showed that *L. reuteri* was able to inhibit *L. casei* induced IL-12 and TNF-α production by DC when added simultaneously (22). However, coculture of *B. vulgatus* with *E. coli* mpk did not prevent *E. coli* mpk induced TNF-α, IL-12 secretion. But

the pretreatment of DC with *B. vulgatus* converted DC in a state which made them inert against further *E. coli* mpk stimulation.

The question is how *B. vulgatus* can make DC inert to further *E. coli* mpk stimulation. We observed that *B. vulgatus* induces only IL-6 production by DC, whereas *E. coli* mpk induces IL-6 in addition to TNF-α and IL-12 production by BMDC (Figure 3). We hypothesized that IL-6 in absence of IL-12 and TNF-α primes DC to an intermediate state which stays inert to further inflammatory stimulus.

4.1.3   IL-6 plays important role in *B. vulgatus* mediated inhibition to DC

IL-6 was originally identified as an antigen nonspecific B cell differentiation factor but later on role of IL-6 on other cell type was also discovered. IL-6 now known to have pleiotropic effects on cell growth, differentiation, survival, and migration during immune response, hemopoiesis, and inflammation. IL-6 receptor consists of an α-chain and gp-130, which is shared among the receptors for the other IL-6 family cytokines. Binding of IL-6 family cytokines to their receptors activates Janus kinases (JAK1, JAK2, and TYK2), leading to the recruitment of signal-transducing molecules such as SHP2 (Src homology region 2 domain containing phosphatase 2) and STAT3 (Signal transducer and activator of transcription 3) (42).

Our data demonstrate an anti inflammatory role of IL-6. Pretreatment of DC with IL-6 as well as culture supernatant from DC infected with *B. vulgatus* (which contains IL-6 but no TNF-α or IL-12) converts immature DC to an intermediate DC, which is unable to become fully mature in response to *E. coli* mpk. Neutralization of IL-6 in culture supernatant of *B. vulgatus* primed DC was unable to induce intermediate DC and thus failed to inhibit DC activation by *E. coli* mpk. In line with our finding a recent study shows that IL-6 keeps DC in an immature/ resting state. Furthermore, pretreatment of DC with IL-6 can suppress LPS mediated activation of DC. Moreover enhanced expression of MHC II and CD86 on lymph node DC was observed in IL-6$^{-/-}$ mice compared to control mice. Similarly transgenic mice with increased signaling for IL-6 decreased the expression of these molecules on lymph node DC. In addition it was also found that STAT3 is required for the IL-6 mediated suppression of LPS-induced DC activation/ maturation. DC dependent T cell activation was enhanced in IL-6$^{-/-}$ mice but suppressed in gp130F$^{759/F759}$ mice, in which gp130-mediated STAT3 activation is enhanced (81). IL-6 was also shown to suppress CCR7 expression by DC (40)

which is necessary for migration of mature DC to lymphoid tissue. These recent findings also support this anti-inflammatory role of IL-6 in our system.

4.1.4   It is hypothesized that less migratory capacity of *B. vulgatus* induced DC might play crucial role in colitis prevention

IL-6 suppresses LPS induced NF-kB binding activity and NF-kB-dependent cytokine production and most importantly the chemokine receptor CCR7 expression (40). CCR7 is expressed on DC during maturation which is involved in homing of DC at lymph node where they encounter naïve T cells. In most nonlymphoide tissue DC are present as immature cells. These immature DC express low levels of costimulatory molecules and take up antigen very efficiently, but are poorly stimulatory for T cells. In response to maturation signals, which include microbial products and cytokines such as TNF-α, these cells change their pattern of expressed chemokine receptors and migrate to the draining lymphoid tissue (8). Immature DC express receptors for inflammatory chemokines and, upon induction of maturation, upregulate CCR7, a chemokine receptor that drives their migration to their lymphatics (27,91). CCR7 has two ligands, CCL21, which is expressed constitutively by endothelial cells of lymphatic vessels and of high endothelial venules (HEV) and by stromal cells present in the T cell zone, and CCL19, which is produced by stromal cells and mature DCs in the T cell zone (35,55,79,126). DC migration can be boosted in a paracrine fashion by TNF-α and by maturing DCs that are known to secrete TNF and other proinflammatory cytokines (69). CCR7 deficient mice has specific deficiency in T cell and DC homing into lymph nodes (31). Depending on recent data and our finding, we hypothesize that in vivo in IL-2$^{-/-}$ mice, *E. coli* mpk is able to cause maturation of intestinal DCs which migrate to lymph node and prime naïve T cells and is responsible for colitis development. In contrast *B. vulgatus* fails to induce maturation and also fails to upregulate CCR7 in intestinal DC. We speculate that these DC fail to migrate to lymph node, and do not start inflammation process. *B. vulgatus* primed DC secretes IL-6 which stimulates immature DC in a paracrine fashion to a semimature/ intermediate DC. For the situation when both bacteria are present in the gut lumen, we postulate that *E. coli* mpk activates DC and these DC migrate to lymph node to prime naïve T cells, but *B. vulgatus* converts immature DC to an intermediate phenotype with less costimulatory markers, MHC class II expression and secretes IL-6. This IL-6 converts more and more immature DC to an intermediate phenotype, which do not become activated when it face *E. coli* mpk later and thus impair colitis development. Higher expression of CCR7 on

E. coli mpk infected DCs compared to *B. vulgatus* infected DC supports this hypothesis (primary findings from a colleague of our group). More intensive study is necessary to evaluate and prove this hypothesis.

However a recent study suggests that an abnormal pattern of DC maturation, such as mature cells fail to migrate but instead remain localized in the tissue might cause chronic inflammatory process (90). But it does not contradict with our hypothesis as the intermediate DC we propose to trap in lamina propria are functionally unable to induce T cell activation.

4.2   *E. coli* mpk mediated T cells polarization towards Th1 cells correlates with the mechanism of colitis development in IL-2$^{-/-}$ mice

It was shown that T cells but not B cells are involved in colitis development in IL-2$^{-/-}$ mice (63). Later involvement of CD4$^+$ T cells rather than CD8$^+$ T cells were also reported in bowel inflammation of IL-2$^{-/-}$ mice (105). Increased expression of IFN-γ mRNA was observed in colonic tissue of these mice (3) and cytokine pattern supports involvement of Th1 cells. Microflora triggers colitis in IL-2$^{-/-}$ mice and different strain of microflora has different influence on colitis development. (121). However, this T cell priming depends on the activation of antigen presenting cell present at mucosal area. Expression of MHC molecules, B-7 molecules (CD80, CD86), CD40 etc. on APC and secretion of cytokines, chemokines are a prerequisite for T cell priming.

4.2.1   Cytokine microenvironment triggered by commensal has influence on colitis development

It was found that *E. coli* mpk and *B. vulgatus* differentially induce cytokine expression by DC in vitro. T helper cell polarization depends on different cytokine environment created by DCs. The impact of different secreted factor from DC on T cells was analyzed. In vitro *E. coli* mpk infected DC supernatant polarizes T cell to Th1 with high IFN-γ production. In contrast *B. vulgatus* infected DC supernatant induces T cells to produce slight IL-4 but no IFN-γ, with a resemblance to a Th2/Th0 phenotype. *E. coli* mpk induces IL-12 production by DC which polarizes naïve T cells to Th1 direction. IL-12 is known as a key regulator of Th1 type immune responses by binding and signaling through the high affinity IL-12 receptor (7). Neutralization of IL-12 in *E. coli* mpk infected DC supernatant abolishes its Th1 polarizing capacity.

4.2.2   Costimulation by DC is also important for *E. coli* mpk induced T cell polarization

T cell priming by activated DC requires specific binding of TCR with antigen loaded MHC molecule also called signal 1 and signal 2 via costimulatory molecule along with cytokine microenvironment (5). The influence of different cytokine environment created by different commensal infected DC is discussed in the last part. T cell stimulation in absence of costimulatory molecules results in T cell anergy while costimulation along with TCR
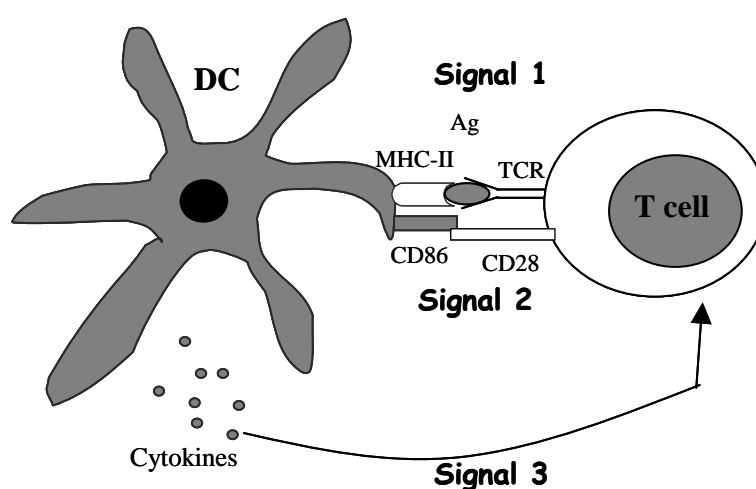

Figure 25: Schematic presentation of T cell priming by antigen loaded DC.

stimulation leads to T cell priming. Differential costimulatory molecule expression by dendritic cell was observed with different commensals used in our system. So the impact of these commensal to induce differential expression of DC surface markers on T cell priming was analyzed. *E. coli* mpk induced higher expression of CD86, MHC-II and CD40 by DC which possesses Th1 polarization capacity, whereas *B vulgatus* induces medium expression of these molecules along with some IL-4 secretion by antigen specific transgenic T cells. Taken together this findings suggest in addition to secreted cytokines direct cell-to cell contact also plays a role in *E. coli* mpk mediated T cell polarization to Th1 cells.

4.2.3   Structural difference in LPS of *E. coli* mpk and *B. vulgatus* might responsible for different immune reaction.

The surface component, LPS of gram negative bacteria plays a role in their virulence. LPS contains three distinct structural domains, O-antigen, core and lipid A. The lipid A

(endotoxin) domain of LPS is a unique, glucosamine-based phospholipid that serves as the hydrophobic anchor of LPS and is the bioactive component of the molecule that is associated with gram-negative septic shock (116). The phosphate residue at the 4´position of sugar moiety is a critical site for the bioactivity and monophosphorylated form of lipid A must be responsible for the weak bioactivity of some LPS (88). Despite the common architecture, lipid A of different bacterial origin may vary in their fine structure. Variations in structure result from the type of hexosamine present, the degree of phosphorylation, the presence of phosphate substituents and most notably, the nature, chain length, number, and location of acyl groups (88). Different structure of LPS might be responsible for different immune response by different bacteria (84). Alteration in the hydrophobic region of *E. coli* lipid A, e. g. abstraction or addition of one acyl group also causes reduction of its bioactivity (88).

It was shown that LPS from *E. coli* and *P. gingivalis* have different LPS structure which plays a role in differential cytokine production by DC and T cell polarization (84). *P. gingivalis* LPS contains multiple lipid A species, largest being pentacylated diphosphorylated species (57). Also tetra acylated monophosphorylated lipid A species present in *P. gingivalis* LPS. Interestingly it was found that the *P. gingivalis* LPS may activate host cells through either a TLR2- or a TLR4-dependent pathway (26). It was also reported that *P. gingivalis* LPS exerted antagonistic effects toward TLR4-dependent cell activation by *E. coli* LPS (23,130). The *B. vulgatus* LPS which contains lipid A carrying 4 fatty acids and one phosphate group, has similarity with *P. gingivalis* lipid A. *B. vulgatus* LPS showed significant less activity than *E. coli* LPS in inducing TNF-α production in human peripheral whole blood cells (39).We observed that heat killed *B. vulgatus* was weaker inducer for ovalbumin specific T helper cell in an adoptive transfer model than heat killed non pathogenic *E. coli* mpk and also has different T cell polarizing capacity (Figure 19). Correlating with above data from other groups we speculate that the difference in LPS structure between *B. vulgatus* and *E. coli* mpk might play a role in their different immune response but further experiments must be done with pure LPS from *E. coli* mpk and *B. vulgatus* to conclude the role of LPS in differetial DC activation and T cell polarization.

4.2.4   Possible mechanism of colitis development correlating in vitro findings

We hypothesize that *in vivo B. vulgatus* stimulates the IL-6 expression in colonic tissue but not the expression of proinflammatory cytokines like TNF-α and IL-12. This might lead to production of semimature/intermediate DC which are less responsive to *E. coli* mpk. This

semimature DC is functionally impaired, less migratory and functionally unable to activate T cell and thus does not mediate colitis. In contrast in vivo *E. coli* mpk activates DC and induces TNF-α, IL-12 and IL-6 secretion by DC. This phenotypically mature DC migrates to lymph node and there it activates T cells which cause colitis development. But when both the bacteria are present in vivo, *B. vulgatus* induces semimature DC which secretes IL-6, converts adjacent immature DCs to a semimature phenotype, and do not become activated by *E. coli* mpk later. Thus presence of semimature DC limits the *E. coli* mpk activated DC and prevents colitis in cocolonized mice.
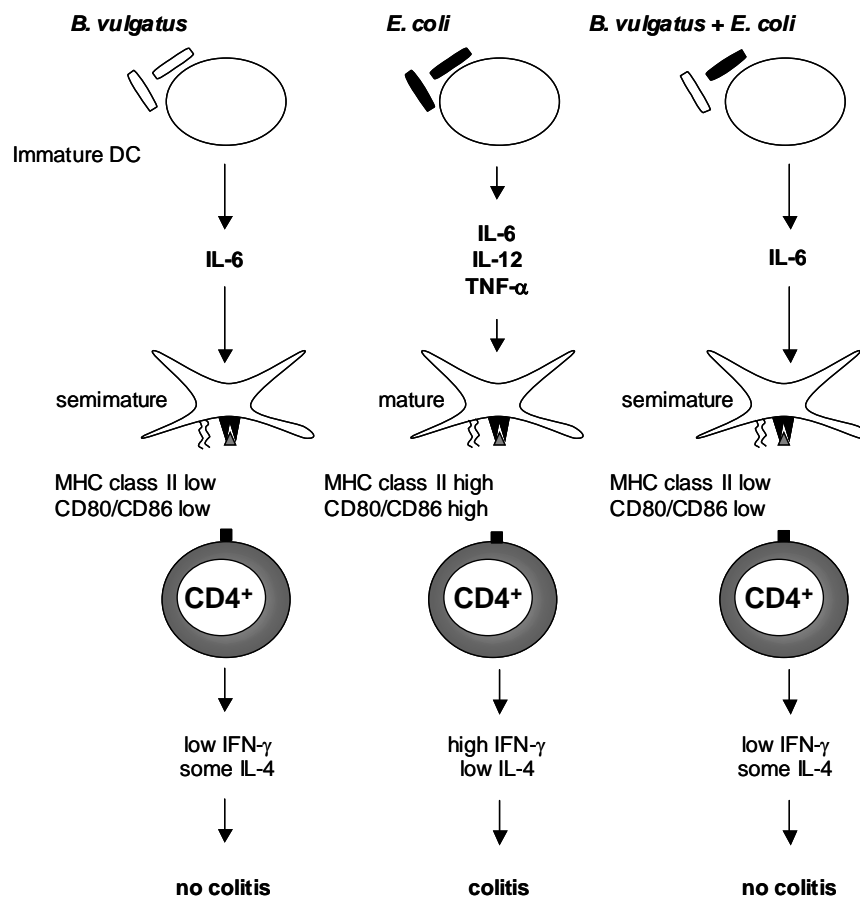

Figure 26: Schematic presentation of the mechanism involved in colitis development and prevention by the two commensals observed

Further studies are required to demonstrate whether colonic DC are actually engaged by *B. vulgatus* and/or *E. coli* mpk in vivo and whether the gene expression is affected in colonic DC in a way as observed in vitro.

4.3   Adipocyte derived factors might play a role in colitis protection

Probiotics offer physiologic, nontoxic treatment of pouchities, ulcerative colitis, and acute infectious dirrhoea. (93). *E. coli* Nissle 1917, a known probiotic is effective in maintaining remission in ulcerative colitis (56,86). In vitro studies showed that *E. coli* Nissle 1917 strain is strongly adherent to human intestinal epithelial cell line but no cytotoxicity is induced. Bordeau et al (11) hypothesized that its strong adhesion to intestinal epithelial cells could enable *E. coli* Nissle 1917 to form in vivo a biofilm of non-pathogenic bacteria that may prevent pathogenic micro-organisms from accessing the cell surface. *E. coli* Nissle also exerts a strong and specific inhibitory effect on adhesion of adherent-invasive *E. coli* strain to intestinal epithelial cells in both coinfection and preincubation experiment models (11). IL-2$^{-/-}$ mice, monocolonized with *E. coli* Nissle was colitis free with some increase in IFN-γ but no increase in TNF-α and CD14 at colon (121). Affymetrix Gene Chip oligo microarray from total mRNA of IL-2$^{-/-}$ mice with different colonization shows that *E. coli* Nissle monocolonized mice has higher expression of adipocyte derived factors, adipsin, adiponectin etc. at colonic tissue. Another colitis free group, IL-2$^{-/-}$ mice cocolonized with *E. coli* mpk and *B. vulgatus* also showed relatively higher expression of adipsin. But expression of these molecules were absent in IL-2$^{-/-}$ mice group that develop colitis.

4.3.1   Cellular source of adipsin in colitis free IL-2$^{-/-}$ mice

Adipsin is generally thought to be an adipocyte-specific gene (124) but also expressed in other tissues like monocyte/ macrophages, muscle, sciatic nerve, endometrium and intestine at low to moderate levels (25,49,72,124). Adipsin proteins are also detected at crypt region of intestinal mucosa (102). Immunohistological analysis of adipsin showed, specific staining in colonic section from *E. coli* Nissle monocolonized IL-2$^{-/-}$ mice as well as *B. vulgatus*, *E. coli* mpk cocolonized mice. The staining pattern suggests epithelial lining of colon might be the source of adipsin. Several epithelial cell lines, Mode K, mICcl2, CMT93, stimulated with *E. coli* Nissle were also unable to produce adipsin. Further investigation is necessary with different gut epithelial cell line as well as with primary epithelial cell with these commensals used in this study to conclude role of adipocyte factors in colitis prevention

4.3.2 Possible direct interaction between adipose tissue and commensals in colitis prevention were not observed

Adipocyte is no longer considered as only a storage tissue. Recent data support its role in immune function. A molecular cross talk between adjacent lymphoid and adipose cell populations in gut was observed in protected stomach from helicobacter infection model (74). *Helicobacter pylori* are associated with acute chronic gastritis, peptic ulcer disease, and two gastric malignancies etc. *H. felis* is closely related to *H. pylori*, can colonize murine stomach and induce chronic active gastritis (58). In a recent investigation (74) mice were immunized with *H. felis* sonicates and cholera toxin and after two months mice were infected intragastrically with viable *H. felis*. Among the immunized group some mice were protected from *H. felis* infection. Gene expression profiling suggested a link between adipocyte derived factors with protection. Histological comparison also showed fat tissue surrounding the protected stomach contains clusters of lymphocytes that was absent in controls. Immunohistochemical analysis further specified binding of adipsin on lymphocytes embedded in fat tissue surrounding the stomach (74).

This observation led us to analyze the influence of *E. coli* Nissle, or secreted factors from it as well as other colitogenic (*E. coli* mpk) and non-colitogenic (*B. vulgatus*) bacteria and their secreted factors on preadipocytes. No differential expression of adipsin, or preadipocyte differentiation was observed in vitro.

4.3.3 Indirect interaction between *E. coli* Nissle and adipocytes were also ruled out in this in vitro system

As dendritic cells are among the first cells that interact with luminal bacteria, we hypothesized that indirect interaction of adipocytes with commensal might be involved in colitis prevention in this IL-2$^{-/-}$ colitis model. Therefore we used different bacteria infected DC supernatant to stimulate preadipocyte cell line and analyzed adipsin expression. The indirect effect was also ruled out as bacteria infected dendritic cell supernatants did not influence adipsin expression by preadipocyte cell line, 3T3-L1. Our in vitro systems failed to explain the involvement of adipocyte derived factor in colitis protection in IL-2$^{-/-}$ mice. Further studies are necessary with different in vitro setup as well in vivo to understand the mechanism of colitis prevention by *E. coli* Nissle in IL-2$^{-/-}$ mouse model.

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5   Summary:

The cause of inflammatory bowel disease (IBD) is multifactorial and depends on genetic predisposition, environment (e.g. microflora) and the immune defense of the individual. IL-2$^{-/-}$ mice kept under germfree conditions develope no IBD. However a conventional microflora triggers colitis development. Therefore, IL-2$^{-/-}$ mice can be used as a model to study the role of the microflora for colitis development. The monocolonization of IL-2$^{-/-}$ mice with *E. coli* mpk triggers IBD while the co-colonization with *B .vulgatus* prevents IBD development. It was analyzed, how commensal bacteria might contribute to IBD development or prevention. At mucosal surfaces DC are among the first cells encountered by bacteria and play an important role in immune defense as well as maintenance of oral tolerance. The stimulation of DC with *E. coli* mpk in vitro leads to maturation of DC associated with increased IL-12, TNF-α, IL-10 und IL-6 secretion. *E. coli* mpk infected DC, pulsed with ovalbumin antigen trigger a strong T cell activation with high level of IFN-γ secretion as a marker for Th1 polarization in vitro, which is in line with the association of Th1 polarization and colitis development in vivo. *B. vulgatus* triggers only the secretion of IL-6 in DC and Th1 polarization does not occur after antigen specific T cell stimulation. Moreover the prestimulation of *E. coli* mpk treated DC with supernatants derived from *B. vulgatus* treated DC or IL-6 alone prevents the *E. coli* mpk triggered maturation of DC in line with a reduced level of cytokine secretion and Th1 polarization. Therefore we postulate that *B. vulgatus* induced IL-6 secretion transits DC in a semimature/ inert state. Such DC might no longer be activated by *E. coli* mpk in vivo which may lead to a reduced migration of mature DC into lymph nodes, less subsequent T cell activation and therefore less colitis development. Further in vitro and in vivo studies are necessary to support this hypothesis.

E. coli Nissle monocolonized mice which develop no colitis show a higher gene expression of adipocyte specific genes such as adipsin and adiponectin in the colon compared to *E. coli* mpk colonized IL-2$^{-/-}$ mice. Immunohistochemical analyses showed adipsin expression in epithelial cells. In an in vitro model system it was analysed whether adipocyte precursors can be converted in mature adipocytes by *E. coli* Nissle. This was not the case. Further studies have to be performed to investigate whether there is a functional association between *E. coli* Nissle induced adipsin expression and prevention of colitis development.

6   Abbreviations

^{3}[H]-thymidine	tritiated thymidine	Ig G	Immunoglobulin G
AA	amino acid	IgA	Immunoglobulin A
AICD	Activation induced cell death	IL	Interleukin
APC	Antigen presenting cell	LB	Luria bertani
B	Bacteroides	LPS	lipopolysaccharide
BHI	Brain heart infusion	MACS	magnetic-activated cell sorting
BMDC	Bone marrow derived dendritic cell	MALT	Mucosa associated lymphoide tissue
BSA	bovine serum albumin	MHC	Major histocompatibility complex
CCL	chemokine ligand	MLN	Mescenteric lymph node
CCR	chemokine receptor	MOI	multiplicity of infection
CD	Cluster of differentiation	mpk	Max Pettenkofer
CD	Crohn's disease	NaCl	Sodium chloride
cDNA	complimentary deoxy ribonucleic acid	NCS	neonatal calf serum
CFU	colony forming unit	NH$_4$Cl	ammonium chloride
Cox2	Cyclo oxygenase 2	NK cell	natural killer cell
dATP	deoxy adinosine triphosphate	Ova	ovalbumin
DC	Dendritic cell	PBS	phosphate buffered saline
dCTP	deoxy cytosine triphosphate	PE	phycoerythrin
DEPC	diethyl pyrocarbonate	PerCP	peridinin chlorophyll A protein
dGTP	deoxy guanidine triphosphate	PFA	paraformaldehyde
dNTP	deoxy necleoside triphosphate	PGE2	Prostaglandin E2
DSS	dextran sodium sulfate	PMA	phorbol myristate acetate
DTT	dithio threitol	PP	Peyer's Patches
dTTP	deoxy thymidine triphosphate	PRRs	Pattern recognition receptors
E. coli	*Escherichia coli*	RAG	Recombination-activating gene
e.g.	for example	RNA	Ribonucleic acid
EDTA	Ethylenediamine tetra acetic acid	RNase	Ribonuclease
ELISA	Enzyme linked immunosorbent assay	RT PCR	reverse transcript polymerase chain reaction
et al.	and others	RT	room temperature
etc.	etcetra	SCID	Severe combined immunodeficient
FACS	flourescence activated cell sorter	SD	standard deviation
FAE	Follicle associated epithelium	SPF	Specific pathogen free
FCS	fetal calf serum	STAT	Signal transducer and activator of transcription
FITC	fluoresceine isothiocyanate	TCR	T cell receptor
GALT	Gut associated lymphoid tissue	Tg	Transgenic
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	TGF	Transforming growth factor
GF	Germ free	Th	T helper cells
GI	Gastrointestinal tract	TLR	Toll like receptor
gp 130	glycoprotein 130	TNBS	Trinitrobenzene sulphonic acid
H$_2$O$_2$	hydrogen peroxide	TNF	Tumor necrosis factor
HK	heat killed	TNFR	Tumor necrosis factor receptor
IBD	Inflammatory bowel disease	Tr1	T regulatory cell 1
IEC	Intestinal epithelial cells	UC	Ulcerative colitis
IFN	Interferon		

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Acknowledgment

Curriculum Vitae

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Thema: Untersuchungen zur differentiellen Modulation der Immunantwort von dendritischen Zellen und T Zellen durch kolitogene und nicht kolitogene Bakterien