The roles of interferon regulatory factors in the murine models of colitis and sepsis

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Abstract

Interferon regulatory factors (IRFs) have not only functions to induce expression of type I IFN, but play also diverse roles in innate and adaptive immune responses by regulating the expression of chemokines or cytokines, proliferation and differentiation of the lymphoid and myeloid cells. The present study was to investigate the roles of IRF4, IRF3 and IRF7 in mouse models of colitis and sepsis.

Inflammatory bowel diseases are multi etiological diseases, whose mechanisms are not completely clear. Many evidences show that imbalances between the effector T cells and regulatory T cells play an important role in the pathogenesis of IBD. Using human colon biopsies, we found out an increased IRF4 expression associated with increased IL-6 and IL-17 expression in T cells in the inflamed colon. Both expression of IL-6 and expression of IL-17 were highly correlated with expression of IRF4. Our previous study showed that IRF-4-/- mice endured mild TNBS-induced colitis and were protected completely from oxazolone-induced colitis compared with wild type (WT) mice. In addition, a protective effect was also found in T cell transfer colitis model by adoptive transferring IRF4-/- or IRF4+/+ CD4+CD45Rb^{high} T cells into RAG2-/- mice. All of these three colitis models showed an increased T cell apoptosis in absence of IRF4. Furthermore, less production of IL-6 and IL-17 was observed in RAG2-/- mice reconstituted with IRF-4-/- T cells than in RAG2-/- mice reconstituted with IRF4+/+ T cells. The former indicated also decreased expression of RORγT and RORα compared with the latter. Overexpression of IRF4 in vitro increased markedly the production of IL-17. After analyzing the sequences of mouse IL-6, IL-17 and RORα promoters, we found out possible IRF-4 binding sites within these promoters. Chromatin immunoprecipitation and electrophoretic mobility shift assay confirmed a direct IRF-4 binding site on IL-17 promoter, but not on IL-6 and on RORα promoters. The findings above suggest that IRF-4 acts as an additional factor to regulate directly IL-17 transcription besides RORα and RORγT. It plays crucial roles in chronic intestinal inflammation by direct regulation of Th17 differentiation, subsequently IL-6 production and influences the T cell apoptosis.

The other two members of IRFs, IRF 3 and IRF7 are reported to have redundant functions to increase IFN-α/β after various virus infections. In the second part of the study, the involvement of IRF3 and IRF7 in experimental colitis and abdominal sepsis was analyzed.

IRF3 or IRF7 deficient mice did not show significant differences in intestinal inflammation upon treatment with TNBS compared with TNBS treated wild type mice. Transfer of IRF-3-/- or IRF-7-/- naïve T cells into RAG1-/- mice showed mild to severe inflammation, similar to RAG1-/- mice reconstituted with wild type T cells. No alteration was found among these three groups when treated with DSS. However, IRF3 deficiency protected mice against polymicrobial sepsis whereas IRF7 deficiency and wild type mice did not by using cecal ligation and puncture (CLP) model. This protective effect of IRF-3 deficiency was associated with high bacteria clearance in peritoneum and peripheral organs. IRF3-/- septic mice indicated markedly higher migration of leukocytes in peritoneal fluid, but less in peripheral organs than WT and IRF7-/- septic mice. Less organ damage was also found in the former group than the latter. IL-6 and TNF-α production were dramatically increased after operation in all of these three groups. However, IRF-3 deficient mice produced significantly lower IL-6 and TNF-α than WT mice and IRF-7 deficient mice. These findings suggest that IRF3 and IRF7 may not involve in T cell-induced chronic colitis. However, IRF3 is more important in the acute bacterial inflammation than IRF7.

1 Introduction

1.1 The intestinal immune system

Gastrointestinal immune system is a large and complex apparatus that plays a crucial role in health. Not only because it encounters more antigens than other parts of the body, but also because it must distinguish between invasive organisms and harmless antigens such as food proteins and commensal bacteria. Generating protective immune response against these harmless agents would be inappropriate and wasteful. Aberrant immune responses of this kind are now believed to be the cause of some relatively common diseases, including celiac disease and inflammatory bowel diseases such as Crohn's disease[1]. So the gastrointestinal mucosal immune system must maintain a balance between protective immunity and homeostasis to a large number of different foreign antigens.

1.1.1 Gut-associated lymphoid tissues (GALT)

Lymphocytes and other immune system cells such macrophages and dendritic cells are found throughout the intestinal tract. Both reside in organized tissues and are scattered throughout the surface epithelium of the mucosa and an underlying layer of connective tissue called the lamina propria. The organized lymphoid tissues in the gut are known as the gut-associated lymphoid tissues (GALT). GALT include the Peyer's patches and solitary lymphoid follicles of the intestine, the appendix, the tonsils and adenoids in the throat and the mesenteric lymph nodes[1].

The Peyer's patches are macroscopic lymphoid aggregates that are found in the submucosa along the length of the small intestine. Peyer's patches are extremely important sites for the initiation of immune responses in the gut and have a distinctive appearance, forming dome-like aggregates of lymphoid cells that project into the intestinal lumen[1]. Mature Peyer's patches consist of collections of large B-cell follicles and intervening T-cell areas. The lymphoid areas are separated from the intestinal lumen by a single layer of columnar epithelial cells, known as the follicle-associated

epithelium (FAE) and a more diffuse area immediately below the epithelium, known as the subepithelial dome (SED). The FAE differs from the epithelium that covers the villus mucosa, as it has lower levels of digestive enzymes and a less pronounced brush border and it is infiltrated by large numbers of B cells, T cells, macrophages and dendritic cells (DCs)[2]. The most notable feature of the FAE is the presence of microfold (M) cells, which have a folded luminal surface instead of the microvilli present on enterocytes. Unlike enterocytes, M cells do not secrete digestive enzymes or mucus and lack a thick surface glycocalyx. They are therefore readily accessible to organisms and particles within the gut lumen and are the route by which antigen enters the Peyer's patches from the lumen[1].

Isolated lymphoid follicles can be identified microscopically both in the small and in the large intestines. These are composed of an epithelium containing M cells overlying organized lymphoid tissue, but they contain mainly B cells and develop only after birth, whereas Peyer's patches are present in the fetal gut. Peyer's patches and isolated lymphoid follicles are connected by lymphatics to the draining mesenteric lymph nodes[1].

The mesenteric lymph nodes (MLNs) are the largest lymph nodes in the body. Their development is relatively unaffected by the absence of most of the factors that are involved in the ontogeny of Peyer's patches and peripheral lymph nodes, including tumor necrosis factor (TNF), TNF receptor (TNFR), lymphotoxin-$\alpha_1\beta_2$ (LT-$\alpha_1\beta_2$) and lymphotoxin-β receptor (LTβR). Instead, these factors might have complementary roles in MLN development. Accumulation of lymphocytes in the MLNs also requires both L-selectin and $\alpha_4\beta_7$ integrin adhesion molecules, which normally direct lymphocytes to enter peripheral and mucosal tissues[2].

1.1.2 The lamina propria and epithelium of the intestinal mucosa

In addition to the organized lymphoid organs, a mucosal surface contains enormous numbers of lymphocytes and other leukocytes scattered throughout the tissue. The majority of the scattered lymphocytes have been activated by antigens and comprise effector T cells and plasma cells of the mucosal immune system. In the intestine, the effector cells are found in two main compartments: the epithelium and the lamina

propria. The lamina propria contains IgA-producing plasma cells, conventional CD4 and CD8 effector T cells, memory T cells, macrophages, dendritic cells and occasional eosinophils as well as mast cells. Neutrophils are rare in the healthy intestine. The epithelium contains mainly lymphocytes, mostly are CD8 T cells[1].

1.1.3 Induction of intestinal immune responses

Antigens present at mucosal surfaces must be transported across an epithelial barrier before they can stimulate the mucosal immune system. The M cells in the follicle-associated epithelium probably do not process antigens themselves, as they do not express MHC class II molecules. Instead they are continually taking up molecules and particles from the gut lumen by endocytosis or phagocytosis and pass on them to professional antigen presenting cells (APCs), e.g. dendritic cells, either in the epithelium or in the underlying dome region of the Peyer's patches[3]. Some of the DCs can also make their way into the epithelium or send processes through the epithelial layer without disturbing its integrity[4]. The antigen-loaded DCs then migrate to the T-cell areas and/or B-cell follicles where they can interact with naïve lymphocytes. Naïve T cells carry the chemokine receptor CCR7 and L-selectin, which direct their entry into Peyer's patches via high endothelial vunules (HEV). If they encounter antigens in the GALT, the lymphocytes become activated and lose expression of CCR7 and L-selectin. This means that they lose their homing preference for peripheral lymphoid organs and once they have exited from them they are unable to re-enter via HEV. Lymphocytes activated in Peyer's patches leave via the lymphatics, pass through mesenteric lymph nodes and eventually circulate into bloodstream through the thoracic duct[5]. The exit of the lymphocytes into the mucosa is partly because of the gut-specific homing factor - $\alpha_4\beta_7$ integrin on the lymphocytes, which can interact with mucosal addressin cell-adhesion molecule 1 (MADCAM1), expressed at high levels by the vasculature of mucosal surfaces[6]. In parallel expression of the chemokine receptor CCR9 of the gut-derived T and B cells allows them to respond to the chemokine CCL25, which is expressed selectively by small intestinal epithelial cells[7].

The lymphocytes that enter the mucosa redistribute into distinct compartments. Antigen-specific B cells are primed as IgM-producing B cells in the Peyer's patch, undergo switching to IgA production there and enter the lamina propria as IgA-producing plasma cells. CD4+ T cells also remain in the lamina propria but are distributed more evenly throughout the villus–crypt unit. CD8+ T cells migrate preferentially to the epithelium, although ~40% of T cells in the lamina propria are also CD8+. CD4+ T cells in the lamina propria produce large amounts of cytokines, particularly interferon-γ (IFN-γ), but also IL-4 and IL-10 [8, 9]. Lamina propria CD8+ T cells can also have potent cytotoxic T-lymphocyte (CTL) activity[10]. Some of these antigen-experienced lamina propria T cells might be true effector cells and might help local B cells to produce IgA. Alternatively, they might be effector memory cells, accumulated in the intestinal mucosa[11, 12]. Finally, lamina propria CD4+ T cells might be regulatory T cells and therefore responsible for maintaining local tolerance to environmental antigens[13].

1.1.4 A balance between protective immunity and homeostasis to a large number of different foreign antigens

The majority of antigens encountered by the normal intestinal immune system are not derived from pathogens but come from food and commensal bacteria. These are not only harmless but are in fact beneficial to the host. Antigens of this kind normally do not induce an immune response, despite the fact that, like any other foreign antigens, there will be no central tolerance to them because they were not present in the thymus during lymphocyte development.

Commensal bacteria have an essential role in maintaining health. They assist in the metabolism of dietary constituents as well as degrading toxins and producing essential cofactors such as Vitamin K1 and short-chain fatty acids[14]. They are in constant communication with epithelium and are also essential for maintaining the normal barrier function of the epithelium. In addition, they interfere with the ability of pathogenic bacteria to colonize and invade the gut by competing for space and nutrients and direct inhibition of the pro-inflammatory signalling pathways induced by pathogens on epithelial cells[14].

1.1.4.1 Tolerance to food proteins and commensals

Similar to the food proteins to induce oral tolerance, commensal bacteria can induce a state of systemic immune unresponsiveness. Although commensal bacteria can be recognized by the adaptive immune system, they do not possess the virulence factors necessary for penetrating the epithelium and cannot disseminate through the body. Therefore, the systemic immune system seems to remain ignorant of their presence. However, the mechanism responsible for establishing and maintaining oral tolerance to the microbiota and food-derived antigens are incompletely understood.
Dendritic cells are the key cells in controlling immunity against pathogens and tolerance towards commensals. Dendritic cells express entire spectrum of Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain proteins (NODs), enabling them to distinguish between commensals and pathogens and to either activate or silence T-cell responses[15]. Its function is regulated by their location, number and maturational state. Most antigens presented by mucosal DCs favour under physiological conditions the generation of regulatory or IgA-promoting T cells [16-18]. The activity of DCs is probably modified by TGF-ß and IL-10 in the intestinal mucosa. TGF-ß is produced constitutively by many cells in the intestine, including epithelial cells and mesenchymal cells. In addition, stromal cells constitutively produce cyclo-oxygenase 2 (COX_2)-dependent prostaglandin E_2 (PGE_2) under the influence of the physiological levels of LPS that is absorbed from intestinal flora[19]. DCs might also express COX_2 and produce PGE_2 in response to LPS[20]. All of them tend to maintain local dendritic cells in a quiescent state with low levels of co-stimulatory molecules. When such cells present antigens to naïve T cells in the mesenteric lymph node, this results in the differentiation of the naïve t cells into anti-inflammatory or regulatory T cells, rather than into the effector T cells[16].

1.1.4.2 Protective immunity caused by pathogens

When pathogens are encountered, local inflammation is induced by the effects of pathogen products mediated through TLRs and NODs. Host inflammatory response is an additional and often essential part of their invasive strategy. The pathogens can entry into the mucosa through different ways. They can be taken up by M cells or

dendrites of dendritic cells. They can also directly attach and invade epithelial cells[21]. Once delivered into subepithelial space, pathogens are able to cause more widespread infection in a variety of ways. They interact with TLRs on inflammatory cells, or induce caspase-dependent apoptosis of the phagocyte, or activate DCs to maturate. The maturated DCs, not like the quiescent DCs, produce chemokines and cytokines (including IL-7 and IL-15) to recruit and potentially maintain innate and adaptive lymphocytes. They produce the polarizing cytokines such as IL-12, IL-6 that promote Th1 and Th17 differentiation and suppress regulatory T cells[22].

1.2 Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) are a group of chronic inflammatory conditions of colon and small intestine. The two major types of IBD are ulcerative colitis and Crohn's disease. Although there are some similarities between these two forms, there are still several clinical and pathological differences.

Areas of involvement:

Crohn's disease favors the terminal ileum, but can occur anywhere along the intestinal tract from the mouth to the anus. It is characterized by the presence of segments of normal bowel between affected regions, known as skip lesions. Ulcerative colitis is limited to the colon in a continuous fashion.

Histology:

Ulcerative colitis is restricted to the mucosa with infiltration of lymphocytes and granulocytes and loss of goblet cells, whereas Crohn's disease affects the whole bowel wall.

Symptoms and complications:

The symptoms of inflammatory bowel disease include abdominal pain and diarrhea. Intestinal ulcers and bleeding are common in both Crohn disease and ulcerative colitis. But complications such as intestinal strictures, fistulas are more common in Crohn disease than in ulcerative colitis. Toxic megacolon is a life-threatening complication of ulcerative colitis and requires urgent surgical intervention. Both diseases share many extra-intestinal manifestations (autoimmunhepatitis or

cholangitis), although some of these tend to occur more commonly with one disease or the other.

Malignancy is the most dreaded long-term intestinal complication of ulcerative colitis. The risk is associated with an increased duration and extent of the disease[23]. Bernstein et al described an increased incidence rate for developing colon rectal cancer (CRC) of 2.75 [95% confidence interval (95% CI), 1.91-3.97] compared to the general population[24]. The risk of cancer in persons with Crohn disease most likely increases if the colon is involved[25].

1.2.1 Genetics

Persons with IBD have a genetic predisposition for the disease. A positive family history is the largest independent risk factor for the disease. People with Crohn's disease have a first-degree relative with Crohn's disease in 2.2-16.2% of cases and with inflammatory bowel disease in 5.2-22.5% of cases. People with ulcerative colitis have a first-degree relative with ulcerative colitis in 5.7-15.5% of cases and with inflammatory bowel disease in 6.6-15.8%[26].

The genome-wide searchings for IBD susceptibility loci have been successful in identifying genes that contribute to disease susceptibility. It has been so far indentified susceptibility regions on 12 chromosomes. Nucleotide-binding-oligomerisation-domain 2 (NOD2, CARD15), underlying gene on chromosome 16, is identified as a susceptibility gene in Crohn's disease, using positional cloning and candidate gene approaches[27]. Several additional susceptibility loci, which have linkage to inflammatory bowel disease, e.g. IBD5, IL23R and ATG16L1 have been validated in multiple studies[28-30].

1.2.2 Environmental factors

Epidemiological studies have shown a higher incidence rates for IBD in North America and northern Europe than in South America, Southeast Asia, Africa (with the exception of South Africa) and Australia[31, 32]. An accumulation of cases of IBD in

urban compared with rural communities has been also shown in North America and Europe[33-36]. Although different incident rates could result from different genetic backgrounds of the residents of these parts of the would, the other environmental factors such as quality of health care, different extents of industrialization, sanitation and hygiene could also involved in[37].

Smoking cigarettes is associated with less frequent exacerbations of ulcerative colitis. In the contrast, smoking aggravates the course of the Crohn's disease. Experimental studies suggest that the beneficial effects of nicotine in ulcerative colitis is due to increased mucus production, decreased production of proinflammtory cytokines and nitric oxide and improvement of the intestinal barrier function. Whereas nicotine seems to be related to an increased influx of neutrophils in the intestinal mucosa in Crohn's disease[38].

There are studies shown that inflammatory bowel disease is more common after gastrointestinal infections and people with the disease generally have higher concentrations of mucosal bacteria than healthy people[39, 40].

1.2.3 Immunobiology

In inflammatory bowel disease, the well controlled balance of the intestinal immune system is disturbed.

1.2.3.1 Epithelial barrier

The first line of defence of the mucosal immune system is epithelial barrier. Several studies have shown a lowered epithelial resistance with increased permeability of the inflamed and non-inflamed mucosa in inflammatory bowel disease[41, 42]. Several studies suggest T cell mediated disruption of tight junction proteins may be responsible for the increased permeability in IBD[43, 44]. An abnormal expression of TLRs on epithelial cells in IBD patients has also been reported. TLR3 is significantly downregulated in active Crohn's disease and TLR4 is strongly upregulated in both Crohn's disease and ulcerative colitis[45]. Furthermore, an upregulation of NOD2 in

epithelial cells has been reported[46]. Intestinal epithelial cell-specific inhibition of NF-κB through conditional ablation of NEMO spontaneously develops severe chronic intestinal inflammation in mice[47]. All of these suggest that regulation of NF-κB activity in epithelial cells seems to be an important point of immune response in IBD.

In addition to epithelial cells, Paneth cells and Goblet cells are also important components of the epithelium. Paneth cells reside in the base of the crypt where they secrete antimicrobial peptides, including the α-defensins. A reduction in Paneth cell α-defensin is observed in Crohn's disease with mutant NOD2[48]. Goblet cells are responsible for production of trefoil peptides, which protect epithelium and promote healing after injury. MUC2, a major goblet-cell-derived mucin, is differentially expressed in human IBD[49]. All of these data suggest that besides epithelial cells, Paneth cell and goblet cells also contribute to the maintenance of the integrity of epithelial barrier.

1.2.3.2 Dendritic cells

Antigen recognition and processing by professional antigen-presenting cells is disturbed in people with IBD. Dendritic cells incorrectly recognize commensals and induce a Th1 or Th17 proinflammatory immune response, which is normally directed by pathogens[37]. It has been reported that expressions of TLR2, TLR4 as well as the maturation/activation marker CD40 are significantly enhanced in intestinal DCs derived from patients with IBD. In addition, more colonic DCs produced IL-12 and IL-6[50], which prime T cells to differentiate into effector T cells, not regulatory T cells. Mice studies have shown that immature DCs produce IL-23 in response to TLR ligands[51]. IL-23 is believed to contribute to development of intestinal inflammation[52]. IL-23 is also essential for expansion and maintenance of Th17 cells[53]. Several studies have shown activated dendritic cells prolong their survival thereby maintaining inflammation[54, 55].

1.2.3.3 Innate immune cells

Migration of innate immune cells such as neutrophils, macrophages and dendritic cells into target mucosal tissues depends on the expression of cytokines, chemokines and adhesion molecules. These cells generate reactive oxygen species that are key effectors of inflammation and tissue injury and also increase epithelial permeability. Recruitment of activated neutrophils, dendritic cells and macrophages into the lamina propria amplifies the local immune response[56].

1.2.3.4 Effector T cells

An over-production of effector T cells, abnormal induction and/or survival of effector T cells and their production of factors such as cytokines result in tissue destruction.
Effector T cells predominate over regulatory T cells in active IBD. It is a consequence of naïve T cells preferably differentiating into Th1 or Th2, which usually resemble Crohn's disease and ulcerative colitis, respectively[57]. Th17 is a recently identified T helper subpopulation that produces interleukin 17 and can promote immune-mediated inflammation in various tissues, including gut.
Upon the various cytokines or surface proteins generated by APCs in response to pathogens, CD4 T cells are developed into distinct types of effector cells. Th1 differentiation needs the cytokines IFN-γ and IL-12. Both of them can stimulate the JAK-STAT intracellular signaling pathway, resulting in activation of specific genes. Differentiation into Th1 subset is promoted by activation of STAT1 in activated T cells by IFN-γ. STAT1 in turn induces the expression of another transcription factor, T-Bet. T-Bet switches on IFN-γ gene in the T cells and induces expression of the signaling subunit of IL-12 receptor. These T cells are now committed to become Th1 cells. Activation of STAT4 by IL-12 promotes further expansion and differentiation of the committed Th1 cells. These effector Th1 cells generate IFN-γ when they recognized antigen on a target cell[58].
IL-4 is the most powerful trigger for inducing Th1 development from naïve T cells. IL-4 activates STAT6 to promote expression of GATA-3 in the T cells. GATA-3 then activates genes for several cytokines characteristically produced by Th2 cells,

including IL-4, IL-5. In this way, GATA-3 both induces and maintains Th2 differentiation[59].

Th17 subset is distinguished by their ability to produce cytokines IL-17, but not IFN-γ or IL-4. Naïve T cells commit to the Th17 lineage when both IL-6 and TGF-ß are present, but IL-4 and IL-12 are absent. They produce besides IL-17, also IL-22, TNF and IL-6 and express IL-23R rather than IL-12R. The conversion to Th17 lineage is believed to be controlled by the transcription factors RORγT and RORα[60, 61]. Th17 is reported to be crucially involved in the pathogenesis of certain autoimmune disease such as rheumatoid arthritis, EAE (encephalomyelitis), psoriasis as well as inflammatory bowel disease.

The data supporting that Crohn's disease is a Th1-mediated inflammatory disease is quite substantial. IL-12 is overproduced by macrophages in Crohn's disease but not in ulcerative colitis[62]. T cells from the affected tissues of patients with Crohn's disease show increased amounts of activated STAT4 and T-bet and produce markedly increased amounts of IFN-γ compared with control patients[63, 64].

No evidence shows that T cells from ulcerative colitis produce increased amount of IL-4, the characterized Th2 cytokine. However, there are indirect evidences shown that Th2 contributes to ulcerative colitis. Ulcerative colitis is more associated with the production of various autoantibodies. As Th2 cells promote more efficiently the activation of B cells and induction of humoral immune responses than Th1 cells do, the presence of autoantibodies might indicate a Th2-mediated immune response than Th1[65]. Although ulcerative colitis is not associated with increased production of IL-4, the increased secretion of IL-5 has been observed[66].

Other cytokines that do not necessarily belong to either Th1 or Th2 subsets such as IL-15, IL16 and IL18 are also produced at higher levels in Crohn's disease and ulcerative colitis[67-69].

1.2.3.5 Apoptosis of T cells

The ability of T cells undergoing apoptosis has an important physiologic function in controlling of immune cell expansion in the gut. Apoptosis is a regulated process that is induced by specific extracellular signals or by the lack of signals required for

survival. Two general pathways are involved in signaling cell death, extrinsic pathway of apoptosis and intrinsic or mitochondrial pathway of apoptosis. The former is mediated by the activation of death receptors by extracellular ligands. The latter is in response to noxious stimuli including ultraviolet irradiation, chemotherapeutic drugs, or lack of the growth factors required for survival. Both pathways activate caspases, the critical step in apoptosis pathway[1].

An enhanced T cell resistance against apoptosis in IBD has been reported[70, 71]. Crohn's disease lamina propria lymphocytes from inflamed tissues express the same amount of cell surface Fas (death receptor) but are less sensitive to Fas-mediated apoptosis than control cells[70]. In addition, lamina propria lymphocytes from inflamed Crohn's disease tissues manifest increased expression of Bcl-2 (anti-apoptotic protein) after CD2 pathway stimulation and elevated Bcl-2 levels in cultures of unstimulated T cells[70]. Furthermore, cytokines such as IL-6, IL-12 and TNF-α have been shown to contribute to apoptosis resistance. The treatment with antibodies to IL-6, IL-12 and TNF-α suppresses established colitis with signs of enhanced T cell apoptosis[72-75].

1.2.3.6 Balance of regulatory T cells and effector T cells

A balance of regulatory T cells and effector T cells is disturbed in inflammatory bowel disease. Experimental inflammation occurs as a result of either excessive effector T cell function or deficient regulatory T cell function.

Mice in which essential pro-inflammatory cytokines are overproduced have been shown to develop experimental colitis. Mice deficient in AU-rich elements (ARE), are addressed to have enhanced TNF production and develop two specific pathologies, i.e., chronic inflammatory arthritis and Crohn's-like inflammatory bowel disease[76]. Transgenic mice for STAT4, which have exaggerated IL-12 signaling that leads to increased production of TNF and IFN-γ, develop also chronic intestinal inflammation[77].

On the other hand, defective regulatory T cell function causes development of IBD. A notable example is the induction of intestinal inflammation in a severe combined immunodificient (SCID) mouse by the adoptive transfer of naïve T cells ($CD45RB^{hi}$)

lacking regulatory cells. Co-transfer of mature $CD45RB^{lo}$ cells that contain a regulatory T cell subpopulation can prevent the disease[78-80]. In addition, intestinal inflammation occurs in models in which there is clear deficiency in a known regulatory cytokine such as IL-10[81] knockout mice or mice with blocked TGF-ß signaling specifically in T cells[82].

1.3 Sepsis

Definition:

Sepsis is a systemic inflammatory response to infection. The infections can be the presence of viable bacteria (bacteremia) in the blood or other infectious organisms or their toxins in the blood (septicemia) or in other normally sterile host tissue. In association with infection, manifestations of sepsis are the same as those defined for systemic inflammatory response syndrome (SIRS). SIRS is characterized as an abnormal generalized inflammatory reaction in organs remote from the initial injury. When the process is due to an infection, the terms sepsis and SIRS are synonymous. The systemic inflammatory response to a wide variety of severe clinical injuries, manifested by two or more of the following conditions: 1) temperature > 38°C or <36°C; 2) heart rate >90 beats/min; 3) respiratory rate >20 breaths/min or P_{CO2} <32mmHg; 4) WBC count >12000/mm^3 or <4000/mm^3 or >10% immature forms.

When the sepsis is associated with organ dysfunction, hypoperfusion or hypotension, it is called severe sepsis. A subset of severe sepsis is septic shock. It is defined as sepsis-induced hypotension despite adequate fluid resuscitation along with the presence of perfusion abnormalities that may include, but are not limited to, lactic acidosis, oliguria or an acute alteration in mental status[83]. Despite more than 20 years of extensive research, continued efforts and significant advances in clinical care, sepsis and SIRS remain the chief causes of death in intensive care units, with the mortality rate ranging from 30% to 70%[84].

Epidemiology and diagnosis:

Common places of infection include: abdomen, lungs, kidney, skin, generally accompanied with diseases such as peritonitis, bacterial pneumonia, urinary tract infection and cellulitis. Epidemiology shows that Gram-positive sepsis is at least as

common as Gram-negative, so that it can be misleading to focus only on endotoxin. The most common organisms are Staphylococcus aureus, Pseudomonas species and Escherichia coli. Many cases of sepsis are due to mixed infection[85].

The infection is often confirmed by a blood test. However, approximately 50% of the patients show no evidence of bacteria in the blood, which can only be diagnosed by the symptoms of SIRS. The high rate of culture-negative is probably because of the usage of antibiotics[84].

Animal models of sepis:

To understand the pathophysiology of sepsis, it is necessary to develop suitable and standardized animal models. Many models mimicking sepsis and septic shock have been developed. Some of them are based on the injection of toxins, or components of bacteria that activate TLRs or administration of viable bacteria. Other types of model involve surgical procedures to generate a septic focus leading to the immediate onset of peritonitis. Surgical models allow continuous influx of different enteric bacteria into the peritoneal cavity thereby mimicking human postoperative sepsis. Currently, the surgical sepsis models used are cecal ligation and puncture (CLP) and colon ascendens stent peritonitis (CASP). Although sepsis models may be designed to reproduced certain aspects of the human disease, it is impossible to combine all of the aspects of clinical sepsis in one single animal model, because of the high heterogeneity and the complex pathomechanisms in human sepsis[86].

1.4 Immunopathogenesis of sepsis

The pathogenesis of sepsis has often been viewed as an uncontrolled hyper-inflammatory, predominantly cytokine-mediated, response of the host. Studies have been shown that circulating TNF-α level is high and correlates with mortality[87, 88]. Therefore, many therapeutic strategies have been developed to target pro-inflammatory mediators[84] and suggested that down-regulation of immunity could be beneficial.

Corticosteroid treatment decreases TNF-α and IL-6 concentrations. Both cytokines have been shown to enhance bacterial growth and to blunt the ability of monocytes to clear bacteria in vitro[89, 90]. Several corticosteroid trials give the evidence that high-

dose steroid treatment is harmful, whereas low dose steroid given to patients with poor endogenous response to steroid-inducing hormones is clinically useful[91, 92].

The most famous target for sepsis treatment is lipopolysaccharide (LPS), endotoxin from Gram-negative bacteria. Treatment with antiserum to endotoxin is shown to be beneficial in mice challenged with LPS[93]. However, numerous clinical trials featuring blockade of LPS by antibody during sepsis show no substantial benefits[94]. The apparent inability of these antibodies to block LPS-induced cytokine production in human monocytes in vitro may explain the failure in large clinical trials.

The second most widely known target in sepsis is TNF-α. When given systemically in human being, TNF-α mimics several pathophysiological changes similar to those observed in human sepsis[95]. Studies in animal models have shown that blockage of TNF-α protects mice from lethal endotoxic shock[96, 97]. However, clinical trials haven't shown a significant benefit in blockage of TNF-α. An increase mortality is even observed[98, 99].

IL-1, another pro-inflammatory cytokine, can evoke similar pathophysiological responses as TNF-α[100]. Although the blockage of IL-1 by using recombinant IL-1 receptor antagonist results in reduced mortality in an animal model of endotoxin shock[101], no protective effects have been shown in two large phase 3 clinical trials[102, 103].

From the data above, it seems that the strategies of blocking endotoxins in order to prevent septic complications maybe simplistic. The best example is the study in C3H/HeJ mice, which have defective LPS signaling and homozygous for a TLR4 mutation. C3H/HeJ mice, which are resistant to high dose of LPS[104], have increased susceptibility to i.p. or subcutaneous Salmonella typhimurium infection[105]. Therefore, although endotoxins have deleterious effects, total blockage of endotoxins may be detrimental.

1.4.1 Microbial pathogenesis

1.4.1.1 Causative microorgarnisms

A large survey analyzing epidemiology of sepsis from 1979 to 2000 in USA has shown that although gram-negative bacteria were the predominant organisms causing sepsis from 1979 through 1987, gram-positive bacteria were reported most commonly in each subsequent year. Among the organisms reported to have caused sepsis in 2000, gram-positive bacteria accounted for 52.1% of cases, with gram-negative bacteria accounting for 37.6%, polymicrobial infections for 4.7%, anaerobes for 1.0% and fungi for 4.6%. The greatest relative changes were observed in the incidence of gram-positive infections, which was increased by an average of 26.3% per year. The number of cases of sepsis caused by fungal organisms increased by 207%[106]. The increasing frequency of fungal sepsis is a worrisome trend because this form of sepsis has a particularly poor prognosis[107]. The most commonly isolated Gram-positive bacterial pathogens are *Staphylococcus aureus* and *Streptococcus pneumonia* and the most common Gram-negative pathogens are *Escherichia coli*, *Klebsiella* spp and *Pseudomonas aeruginosa*[108].

1.4.1.2 Microbial components

Bacterial components, which are recognized by the innate immune system, have been called pathogen-associated molecular patterns (PAMPs). In Gram-negative bacteria, LPS (known also as endotoxin) has a dominant role[109]. The outer membrane of Gram-negative bacteria is constructed of a lipid bilayer, separated from the inner cytoplasmic membrane by peptidoglycan. The LPS molecule is embedded in the outer membrane of Gram-negative bacteria. The changes of LPS conformation seem to correlate with the ability to activate host cell membranes[110]. Despite the well-known injurious host response to even minute amounts of endotoxin, LPS has no intrinsic toxic properties by itself. The toxicity of LPS is due to the host response to this microbial mediators[111].

There is no endotoxin in Gram-positive bacteria, but their cell walls do contain peptidoglycan (PGN) and lipoteichoic acid (LTA), which can bind to cell surface receptors and are pro-inflammatory[112]. An important feature of Gram-positive cells is the production of superantigens, some of which are implicated in septic shock. These superantigens (SAg) are able to bind directly to major histocompatibility complex class II (MHCII) expressed on antigen-presenting cells and cross link with a large number of T cells that bear common Vß chains and their T cell receptor. The large number of activated T cells generates a massive immune response which is not specific to any particular epitope on the SAg. Since one of the fundamental strengths of the adaptive immune system is the ability to target antigens with high specificity, SAgs produce an immune response that is not helpful. More importantly, the large number of activated T-cells secretes massive amounts of proinflammatory cytokines, which cause severe and life-threatening symptoms, including shock and multiple organ failure[113].

Several other bacterial components have been shown to have proinflammatory activity and to be able to induce shock in experimental systems. These include cell-wall structures such as flagellin[114] and unmethylated CpG sequences in naked bacterial DNA[115].

1.4.2 Host recognition of microbial component

The assumption that sepsis is the result of a hyper-inflammatory reaction of the patient to infection has been widely accepted for many years. Animals infused with large doses of bacteria or bacterial products result in a massive systemic release of an array of inflammatory mediators, many of which have been found to be directly responsible for the death of the host, including the pro-inflammatory cytokines TNF-α and IL-1[96, 97, 101]. However, most clinical sepsis trials with anti-inflammatory therapies failed to alter the outcome of patients with sepsis. There is evidence of immunosuppression in sepsis from studies showing that LPS-stimulated whole blood from patients with sepsis releases markedly smaller amounts of the inflammatory cytokines TNF-α and interleukin-1ß than that of control patients[116]. The adverse consequences of sepsis induced immunosuppression are reversed with the

administration of IFN-γ in patients with sepsis. This immune stimulant restores TNF-α production in macrophages and improves survival[117].

The states of hyper-inflammation or immunosuppression in septic patients may be changed over time of the disease development. Sepsis may be initially characterized by increased inflammatory mediators, which can propel the patient into a systemic inflammatory response syndrome (SIRS) or even multiple organ failure and shock. on the other side, the initial activation of innate immune response can also leads to the development of a compensatory anti-inflammatory response syndrome (CARS) and its associated mortality[118, 119]. The hypothesis that excessive inflammation is the main underlying cause of an adverse outcome in a septic patient requires reconsideration: the host response to sepsis induces many subsequent and concurrent processes that involve both exaggerated inflammation and immune suppression[107].

1.4.2.1 Pathogen recognition by innate immunity

Innate immune system is able to detect pathogen via a limited number of pattern-recognition receptors (PRRs). PRRs recognize conserved motifs-PAMPs that are expressed by pathogens but absent in higher eukaryotes. Additionally, endogenous danger signals released during injurious process such as trauma, ischemia or necrosis, can also activate PRRs[107].

Mannose-binding lectin:

One of PRRs is the mannose-binding lectin (MBL), which is present as a free protein in blood plasma. Besides its initiation of lectin pathway of complement activation, it can form MBL-pathogen complex, which is bound by phagocytes. The outcome is phagocytosis and killing of the pathogen and induction of other cellular response[1].

Toll-like receptor family:

The family of Toll-like receptors has a central role as PRRs in the initiation of innate immune responses. There are 10 expresssed TLR genes in mice and humans. Each of the 10 proteins is devoted to recognizing a distinct set of molecular patterns of microorganisms. TLR4 with the help of CD14 and MD-2 recognizes LPS; TLR2 is predominantly responsible for recognizing Gram-positive cell-wall structures; TLR5 is the receptor for flagellin and TLR9 recognizes CpG elements in bacterial DNA[94].

Some mammalian TLRs (TLR1,2,4,5,6) act as cell-surface receptor, whereas others act intracellularly and are located in the membranes of endosomes (TLR3,7,9).

Structurally, the TLRs are single-pass transmembrane proteins characterized by multiple copies of a leucine-rich motif in their extracellular domain and a shared motif called TIR in their cytoplasmic domain [1]. There are four adaptor proteins involved in TLR signaling: myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor-protein-inducing interferon ß (TRIF) and TRIF-related adaptor molecule (TRAM)[107].

TLR signaling induces a diverse range of responses that regulate the production of cytokines, chemokines, antimicrobial products or co-stimulatory factors. Many different signaling proteins are induced by TLR activation, including MAP kinases and PI3-kinase. The most important signaling pathway leading from TLRs is the activation of NF-κB.

Role of TLRs in septic shock models:

Mice with mutation of TLR4 show resistance to endotoxin challgenge but highly susceptible to Gram-negative infection[120]. TLR2 is shown to be responsible for septic shock induced by bacterial lipopeptides[121] and the toxic effects of CpG-DNA are mediated by TLR9[122].

The unresponsiveness to LPS and resistance to septic shock in mice deficient for TIRAP, MyD88, or TRIF indicate that these adapter proteins are essential for LPS-induced inflammatory responses[123-125]. Mice with IRAK-1 or IRAK-4 deficiency also exhibit increased resistance to endotoxin challenge[126, 127], confirming the important role of these kinases in vivo.

It should be noted, however, that both TLR2- and MyD88-null mice are highly susceptible to infection with Staphylococcus aureus[128]. These findings together with the findings on TLR4 mutant mice indicate that TLR signaling may mediate the toxic effects of high doses of microbial components, but TLRs may also be required for generation of protective immune responses upon infection with individual bacterial pathogens[86].

Role of TLRs in polymicrobial infection models:

In other studies, the contribution of TLRs to immune responses in sepsis caused by mixed-bacterial infections has been examined. Using the CASP model of septic peritonitis, no increased survival rates has been observed in mice with single or

combined deficiency for TLR2 and TLR4 compared with the wild type mice, whereas MyD88-/- mice exhibit improved survival. However, the bacterial clearance and recruitment of effector neutrophils to the infected peritoneal cavity in MyD88-/- mice are normal. The systemic hyper-inflammatory reaction is strongly attenuated, but not absent, in MyD88-deficient animals. In the absence of MyD88, production of monocyte chemoattractant protein-1 and macrophage-inflammatory protein-1alpha (responsible for neutrophil recruitment) is entirely independent of MyD88[129]. It therefore appears that during polymicrobial sepsis multiple TLRs are triggered and thereby strongly increase the complexity and intensity of the inflammatory response. Genetic ablation of MyD88 may protect mice from the detrimental outcome of polymicrobial sepsis by preventing inflammatory injury. Notably, even in the absence of a major TLR signaling pathway there appears to be sufficient residual gene expression to ensure protective neutrophil responses and anti-bacterial defense[86].

NODs:

The TLRs are located in cellular membranes, either on the cell surface or in intracellular vesicles. NODs are present in the cytosol of the cell and are able to bind microbial products there and activate NF-κB[1]. A TLR4- and MyD88-independent NF-κB activation is induced by NODs[130], suggesting that this might be yet another way for cell response to the presence of bacteria.

1.4.2.2 Proinflammatory cytokines

Activation of NF-κB by the Toll and NOD pathways leads to the production of important mediators such as cytokines and chemokines and also leads to the cell-surface expression of co-stimulatory molecules such as CD80 and CD86, that are essential for the induction of adaptive immune responses[1].

Cytokines regulate many of the pathways involved in the host inflammatory responses to sepsis. They influence cell differentiation, proliferation and activation and modulate pro-inflammatory and anti-inflammatory responses to allow the host to react appropriately to pathogens. Cytokines cause liver injury and impaired liver function, resulting in a failure to maintain normal blood glucose levels due to a lack of gluconeogenesis from stored glycogen. Overproduction of NO by cytokine-activated

cardiac myocytes and vascular smooth muscle cells leads to heart failure and loss of perfusion pressure, respectively, resulting in hemodynamic shock. The clinical trial of disseminated intravascular coagulation, hypoglycemia and cardiovascular failure classically associate in septic shock. Multiple organs show inflammation and intravascular thrombosis, which can produce organ failure.

Four cytokines, TNF-α, IL-1ß, IL-6 and IL-8 have been most strongly associated with sepsis. The binding of a cytokine to a corresponding receptor initiates intracellular signal cascades, from which the Janus-kinas/signal transducers and activators of transcription (JAK/STAT) pathway is the most involved pathway in the signaling of cytokines.

In human and experimental animal models of sepsis, cytokines are released in a sequential manner. TNF-α and IL-1ß are released during the first 30-90 minutes after exposure to LPS. Infusion of either TNF-α or IL-1ß results in a sepsis-like state and specific blockage of either of them abrogates some of the manifestations. Both of these cytokines can in turn activate a second level of inflammatory cytokines such as IL-6 and IL-8. Although the infusion of IL-6 into experimental animals does not result in a spies-like state[131] and blockage of IL-6 has not resulted in consistent benefit in experimental animal models[132], IL-6 concentration correlates more closely than other cytokines with severity and outcome of human sepsis[133]. IL-8 is likely to recruit and activate neutrophils in specific sites which can lead to tissue injury[133].

IL-12 family is also involved in the septic process. A decreased IL-12 concentration combined with a reduced IFN-γ concentration has been observed in sepsis and IL-12 therapy improves the survival in peritonitis[134]. However, other study shows a controversial result, with the protective effect of anti-IL-12 therapy in peritoneal sepsis[135]. Another study shows that EBI3 deficiency (subunit of IL-27) helps the mice to resist to CLP-induced peritonitis and blockage of IL-27 by using soluble IL-27 receptor fusion protein leads to significantly increase of survival after CLP[136].

High mobility group B1(HMGB1) has been identified as a cytokine-like product of macrophages that appears much later after LPS stimulation and may represents a more tractable target for intervention. It participates in stabilizing nucleosomes, facilitates gene transcription and modulates the activity of steroid hormone receptors. Mice injected with LPS have shown a delay of an increased HMGB1 serum

concentration about 24 hours. Administration of antibody to HMGB1 rescues the mice from the LPS-induced shock[137].

Another macrophage-derived cytokine that has been identified as a potential therapeutic target in sepsis is macrophage migration inhibitory factor (MIF). Mice with a targeted disruption of MIF gene are resistant to LPS-induce shock[138] and antibody treatment to MIF induces fully protectiion in CLP model[139].

1.4.3 Coagulation and anticoagulation

Although the coagulation system is not conventionally considered as a component of the immune reaction to infection, it is increasingly clear that the immune and coagulant responses to sepsis are intricately linked.

Disorders of coagulation are common in sepsis. Not only LPS and other microbial components, but also proinflammatory cytokines, in particular TNF, IL-1 and IL-6 are powerful inducers of coagulation. The increased expression of tissue factor (TF), the major trigger to coagulation, in turn activates a series of proteolytic cascades and promotes thrombus and clot formation[140]. An additional cause of the procoagulant state in sepsis is the downregulation of three naturally occurring anticoagulant proteins — antithrombin, protein C and tissue factor pathway inhibitor[109].

The limited intravascular coagulation is of some benefit in localized sepsis, waling-off a spreading infection and restricting bacteraemia. However, there is no doubt that the disseminated intravascular coagulation leads to microvasular thromboses throughout the body, inadequate tissue perfusion and organ failure.

1.4.4 Immune suppression and apoptosis

Animal models of sepsis as well as studies of patients with sepsis show that the initial hyper-inflammatory response in sepsis is quickly followed by the development of a sustained anti-inflammatory or immunosuppressive state[141]. Whether the occurrence of immunesuppression in sepsis is the subsequent initiation of a hyper-inflammatory

and anti-inflammatory response or it is rather a primary response than a compensatory response is still in debate.

A major mechanism of the anti-inflammatory host response during sepsis is the induction of apoptosis of the immune cells[141]. Most cells that undergo enhanced apoptosis in sepsis are lymphocytes (B cells, CD4 T cell and follicular dendritic cells) and gastrointestinal epithelial cells[141]. There are two main mechanisms, by which apoptosis contributes to immunosuppression. The first mechanism is through the deletion of critical effector cells. The second one is through the induction of anti-inflammatory cytokines, including Il-10 and TGF-ß[141].

1.5 Interferon-regulatory factors

The interferon-regulatory factor (IRF) family of transcription factors has been initially found to be involved in induction of genes that encode type I interferons. In recent years, IRF have also been shown to have essential roles in the regulation of the immune system, both in innate and in adaptive immune responses. Up to date, the mammalian IRF family comprises of nine members: IRF1, IRF2, IRF3, IRF4, IRF5, IRF6, IRF7, IRF8 and IRF9.

IRFs contain several domains that are present in all family members as well as some domains that are specific to particular family members. All IRFs are composed of a DNA-binding domain (DBD) and a regulatory domain. The DBD contains a motif of 5 tryptophan repeats. This motif mediates IRF binding to the core DNA sequence GAAA or AANNGAAA in the IFN-stimulated response elements (ISRE). All IRFs except IRF6 also contain a carboxy (C)-terminal IRF-association domain (IAD), which facilitates heterodimerization between family members. IRF2, -3, -4, -5 and -7 also contain repression domains which suppress the transcriptional activity of these factors. IRF1, -3, -5 and -7 can be activated by phosphyrilation. These modifying events, together with interactions between different IRF-family members, allow for a complex system of IRF regulation that is not restricted to alterations in IRF levels[142].

IRF1 and IRF2:

IRF1 and IRF2 mRNAs are both expressed in a variety of cell types and their expression levels, particularly IRF-1 mRNA, are dramatically upregulated upon viral

infection or IFN stimulation[143]. Besides activation of IFN gene, IRF1 is important in promotion of Th1 cell response. Mice deficient with IRF1 show defective IFN-γ production and increased IL-4 secretion[144]. IRF1 increased also the production of caspase 1, which is responsible for processing the precursor of IL-18. Processed IL-18 synergizes with IL-12 to induce expression of IFN-γ[145]. IRF2 was originally thought to function as an IRF1 antagonist, because of negative regulation of type I IFN production[146]. However, other studies show that IRF2 promotes, rather than suppresses Th1 response[142].

IRF1 has been reported to be associated with inflammatory bowel disease. Increased expression of IRF-1 in lamina propria mononuclear cells from patients with Crohn's disease has been detected[147]. In another study, mice with IRF1 deficiency showed a dramatic increase of lethality and colitis severity compared with WT mice after DSS or TNBS treatment[148].

IRF3 and IRF7:

IRF3 and IRF7, which are highly homologous, have gained much attention as the key regulators of type I IFN gene expression elicited by viruses. IRF3 is constitutively expressed in most tissue. After viral infection, IRF3 is phosphorylated by IKK-ε (IκB kinase-ε) or TBK1 or possibly by DNA-PK (DNA-dependent protein kinase). Phosphorylated IRF3 forms a dimer (either a homodimer or a heterodimer with IRF7), enabling the IRF3 to interact with the co-activators CBP (cyclic-AMP-responsive-element-binding protein (CREB)-binding protein) and translocates to the nucleus to bind DNA sequences in ISRE-containing promoters[142].

Unlike IRF3, IRF7 is expressed in small amounts in most cells and is strongly induced by viral infection or by type I IFN. Similar to IRF3, IRF7 contains an inhibitory domain and its activity is regulated by phosphorylation. The binding of type I IFNs to the type I IFN receptor results in the activation of a heterotrimeric transcriptional activator known as IFN-stimulated gene factor 3 (ISGF3), which consists of IRF9 and signal transducer and activator of transcription 1 (STAT1) and STAT2, is responsible for the induction of the IRF7 gene. IRF7 can form a homodimer or a heterodimer with IRF3 and have effects on the expression of the type I IFN gene family members. IRF3 is a potent activator of the IFN-ß gene but not the IFN-α genes, whereas IRF7 efficiently activates both IFN-α and IFN-ß genes[149]. These findings lead to the idea of positive feed back loop that IRF3 is mainly responsible for the initial induction of the

IFN-ß gene, whereas IRF7, the upregulation of which is mediated by type I IFNs themselves, is involved in the late phase induction and amplification of type I IFN gene[143].

IRF4:

IRF4 is expressed restrictively in cells of immune system, particularly in B cells, T cells and macrophages. Expression of IRF4 in splenic T cells can be triggered by CD3-specific antibodies, whereas IL-4 or antibodies specific for IgM or CD40 induce IRF4 expression in splenic B cells. The activation of macrophages by LPS or IFN-γ does not alter IRF4 level but leads to activation and nuclear transport of IRF4. The interactions of IRF4 with PU.1 or with IRF8 have been indicated[150, 151].

Besides the evidences showing that IRF4 is responsible for the differentiation of B cell into plasma cells, many studies indicate that IRF4 is associated with Th cell development. T helper cells with IRF4 deficiency show markedly decreased capacity to differentiate into Th2 cells in vitro[152, 153]. Infection with L. major in IRF4 deficient mice shows not only a complete lack of early Th2 cell development but also reduced production of IFN-γ[152]. Stimulation with TCR-specific antibodies leads to small or large reductions in IFN-γ production compared with wild-type cells[152, 153]. It seems that IRF4 has an important role in differentiation into Th2 cells but has a smaller, variable influence on development into Th1 cells. The mechanism of action of IRF4 during differentiation into Th2 cells is still unclear. Some suggest a physical interaction between nuclear factor of activated T cells 1 (NFAT2) and IRF4 shown in Th2 cells activates the IL-4 promoter[153]. The defective differentiation into Th2 cells of IRF4-/- Th cells also correlates with reduced expression of GATA3 and retroviral overexpression of GATA3 completely rescues this defect[152].

The effect in apoptosis of IRF4 is contradictory. A study shows that a high percentage of cell death is correlated with high efficiency of IRF4 knockdonwn[154]. IRF4 overexpression in a human tumor cell line increases the apoptosis of these cells and IRF4 deficient mice display defect in activation-induced cell death[155]. Another study shows a high sensitivity to apoptosis in Th cells lacking IRF4 after infection with L. major[156].

IRF5:

IRF5 acts not only as repressor or activator of type I IFN genes, but also as inducer of pro-inflammatory cytokines such as IL-6, Il-12 and TNF-α. The association of IRF5 with IBD has also been reported[157].

1.6 Aims of the study

Inflammatory bowel diseases are characterized as chronic inflammatory conditions of colon and intestine with two major types: ulcerative colitis and Crohn's disease. Although intensive studies, the etiology of IBD still remains largely uncertain. The pathogenesis of IBD seems to involve a complex interplay among certain genetic factors, environmental factors and immunological factors. In particular, a combination of genetic susceptibility factors and altered immune response driven by gut flora contributes to the initiation and development of these diseases.

Sepsis is a systemic inflammatory response to infection. Unlike IBD, sepsis is an acute inflammatory process and results in much quick mortality in clinic. Innate immune response, not adaptive immune response, is more involved in the pathogenesis of the disease.

The family of IRFs has been shown multiple roles in immune systems. IRF1 and IRF5 have been reported to be involved in IBD. IRF3 and IRF7 have been shown to be crucial factors for type I IFN production after viral infection. The roles of IRF4 in immune responses are also multifarious. It is not only responsible for the maturation of B cells, but also important in differentiation of T helper cells, especially Th 1 cells. In addition, the involvement of IRF4 in dendritc cells and macrophages has also been reported.

A previous study in our laboratory showed that IRF4 KO mice were completely protected from oxazolone-induced colitis and partially protected from TNBS-induced colitis. In the first part of the present study, the role of IRF4 in chronic colon inflammation was further investigated.

- Analyzing the influence of IRF4 in intestinal inflammation by using DSS-induced colitis model and T cell adoptive transfer colitis
- studying the role of IRF4 in regulation of T helper cell differentiation

- Identification of the influence of IRF4 in T cell apoptosis
- Characterization the mechanism of IRF4 in modulating expression of cytokines

In the second part of the study, two other members of IRFs, IRF3 and IRF7 are further investigated.

- Studying the involvement of IRF3 and IRF7 in chronic colon inflammation by using DSS-induced colitis, TNBS-induced colitis and T cell adoptive transfer colitis
- Investigating the roles of IRF3 and IRF7 in polymicrobial sepsis by using CLP model

2 Materials and methods

2.1 Materials

If it was not specialized, all the plastic materials were purchased from Greiner (Frickenhausen), Eppendorf (Hamburg), Nunc (Langenselbold), BD, BBraun (Melsungen).

All the reagents, chemicals were purchased from: Sigma (Deisenhofen), Roth (Karlsruhe), Boehringer (Mannheim), Qiagen (Hilden), Serva (Heidelberg), PAA (Austria), Santa Cruz (Heidelberg), Promega (Mannheim), Pharmingen (Hamburg), Miltenyi Biotec (Bergisch Gladbach).

2.1.1 Chemicals

Chemicals, reagents and cell culture media and other compounds were described in the methods.

2.1.2 Equipments

BD FACS CantoII	BD Biosciences, USA
Bacteria incubator	Heraeus-Holding GmbH
Balance (Adventurer Pro)	Ohaus Corporation, USA
Cellometer Auto T4 Cell counter	Nexcelom bioscience LLC. USA
Cell culture incubator	Heraeus-Holding GmbH
Centrifuge Megafuge 1.0	Thermo Electron Corporation
centrifuge 5417R	Eppendorf
Electrophoresis power supply	BioRad
Fluorescence microscope IX70	Olympus, Japan

-20°C Freezer	Liebherr, Germany
-80°C Freezer	Thermo Electron Corporation
Gel Chamber	BioRad
Gel Chamber for EMSA (SE600 Series)	Hoefer Pharmacia Biotech Inc. USA
Light Cycler 480II	Roche Diagnostics, Germany
Light Microscope	Nikon, Japan
Miniature Endoscope	Karl Storz, Tuttlingen, Germany
PCR Cycler	Thermo Electron Corporation
Retsch MM300 shaker	Retsch GMBH, Germany
Sterile work bench	Thermo Electron Corporation
Tecan infinite 200 ELISA reader	Tecan SLT-Labinstruments

2.2 Software

MS-Office-Packet (Word, Excel, PowerPoint)	Microsoft GmbH
Endnote 9.0	Thomson Reuters
Adobe Photoshop	Adobe systems Incorporated
Adobe Illustrator	Adobe systems Incorporated
GraphPad Prism	GraphPad Software Inc.
BD FACSDiva	BD Biosciences
Imaging Cell	Olympus Soft Imaging Solutions GmbH
ScenalyzerLiver Capture Program 2.1	Andreas Winter Info@
Light Cycler 480 software release 1.5	Roche Diagnostics

2.3 Methods

2.3.1 Human colon biopsy samples

Colon endoscopic biopsy specimen were obtained from patients with Crohn`s disease (CD), ulcerative colitis (UC) and patients coming to preventive medical checkup (control). The biopsy specimen were stored in RNAlater (Qiagen, Hamburg, Germany) for a long term RNA stabilization. To perform real-time PCR, we used mRNA from biopsy specimen from 10 control patients and 22 IBD patients. Groups were age-matched. Among the IBD patients, 12 suffered from Crohn`s disease, 10 from ulcerative colitis. Treatment consisted in systemic steroids, salicylates, budesonide or azathioprine. The CD group consisted of 6 male and 6 female patients between 20–60 years. The UC group consisted of 4 male and 6 female patients, 25–60 years old. The control group consisted of 10 patients (5 male, 5 female, age: 30-60 years) not receiving corticosteroids or immunosuppressant. The collection of biopsies was approved by the ethical committee and the institutional review board of the University of Mainz.

2.3.2 Mice

C57BL/6J mice, IRF4-/- mice, IRF3-/- mice, IRF7-/- were used in this study. All of the knockout mice are backcrossed with strain C57BL/6J. C57BL/6J mice were obtained from our animal facility.

IRF4-/- mice:

IRF4-/- mice used in this study were at the seventh backcross generation and were obtained from the University of Marburg (Prof. M. Lohoff), Germany [158]. Mice deficient in IRF4 were generated by replacing exons 2 and 3 of the IRF4 gene with a neomycin resistance gene. Mice were screened by PCR of tail DNA with the primers 5'-GCA ATG GGA AAC TCC GAC AGT-3' and 5'-CAG CGT CCT CCT CAC GAT TGT-3', specific for exon 2; and primers 5'-CCG GTG CCC TGA ATG AAC TGC-3' and 5'-CAA TAT CAC GGG TAG CCA ACG-3', specific for neo.

IRF3-/- mice and IRF7-/- mice:

IRF3-/- mice and IRF7-/- mice were obtained from RIKEN BioResource Center in Japan, unter the agreement of professor Taniguchi. IRF3-/- mice were generated by targeted mutation of the interferon regulatory factor 3 gene on Chr 7. A putative trascription initiation site and amino acids 1 to 35 of the mouse IRF-3 gene encompassing 2.2 Kb region was disrupted by homologous recombination. Significantly reduced production of interferon alpha/beta against virus infection. Mice were screened by PCR using primers 5'-GAA CCT CGG AGT TAT CCC GAA GG-3', 5'-GTT TGA GTT ATC CCT GCA CTT GGG-3', 5'-TCG TGC TTT ACG CTA TCG CCG CTC CCG ATT-3'. IRF7-/- mice were generated by replacing exons 2 and 3 (corresponding to amino acids 7–109; 383 base pairs) with an IRF7 gene-targeting construct - a phosphoglycerate kinase promoter-driven beta-geo-positive selection cassette (pgk-beta-geo). IRF7-/- mice were screened by PCR using primers 5'-GTG GTA CCC AGT CCT GCC CTC TTT ATA ATC T-3', 5'-TCG TGC TTT ACG GTA TCG CCG CTC CCG ATT C-3', 5'-AGT AGA TCC AAG CTC CCG GCT AAG TTC GTA C-3'. Both of them are backcrossed with CD57BL/6J mice more than seven generations. Animals were bred and maintained under specific pathogen-free conditions in our animal facility. All experiments involving animals were performed under protocols approved by the Animal Ethics and Experimentation Committee of The Johannes Gutenberg University of Mainz. 8-10 weeks mice were used for experiments.

2.3.3 Induction of colitis

2.3.3.1 TNBS-induced colitis

Colitis was induced by intrarectal application of haptenating substance TNBS (2,4,6-trinitro benzene sulfonic acid) in ethanol. Ethanol is required to break the mucosal barrier, whereas TNBS is believed to haptenize colonic autologous or microbiota proteins and render them immunogenic to the host immune system. This model is to study T helper cell-dependent mucosal immune responses[159].

Mice were sensitized on the skin with 150µl 1% TNBS (Sigma Chemical Co.) in 50% ethanol. Intrarectal injection of 150µl 2.5% TNBS in 50% ethanol was made 7 days after presensitization with the hapten reagents.

2.3.3.2 DSS-induced colitis

Application of dextran sodium sulfate (DSS) in drinking water for several days can induce acute colitis in mice. It is generally believed that DSS is directly toxic to gut epithelial cells of the basal crypts and affects the integrity of the mucosal barrier. Since T and B-cell-deficient SCID (severe combined immunedeficient) or RAG-/- mice also develop severe intestinal inflammation after DSS administration[160], the adaptive immune response does not seem to play a major role in this model.
For the induction of DSS colitis experiments, IRF4-/- mice and C57BL/6J were given 3.5% DSS (MW 36,000–50,000; MP Biomedicals) in drinking water for 5 days and followed with normal drinking water for 5 days. This procedure was repeated two times.
The induction of DSS colitis on IRF3-/-, IRF7-/- mice were performed by applying 2,5% DSS in drinking water for 7 days and followed with normal drinking water for 7 days.

2.3.3.3 Naive T cell-transfer colitis

In mice, transfer of primary naive (CD45RBhigh) CD4+ T cells to T and B cell–deficient recipients leads to wasting disease and colitis [78, 80]. Intestinal inflammation resembles that was seen in human IBD in many aspects such as marked disorder of crypt structures, depletion of goblet cells, inflammatory infiltrations of polymorphonuclear granulocytes, mononuclear leukocytes and CD4+ T cells in lamina propria. A significant population of LP CD4+ T cells express Th1 phenotype with a high production of IFN-γ, TNF-α.
Transfer colitis was performed as described by Morrissey and Powrie [78, 80]. In brief, CD4+ CD45RBhigh T cells were obtained from IRF4-/- or C57BL/6 donors, or CD4+CD25- naive T cells were obtained from IRF3-/- or IRF7-/- donors. $5x10^5$ cells

were intraperitoneally transferred into Rag2-/- mice. For the experiment combined with rmIL-17A treatment, 0.5µg rmIL-17A per mouse were intraperitoneally injected each other day.

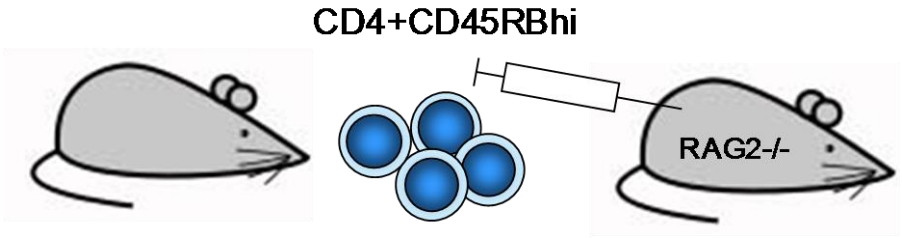

Figure 2.1 transfer colitis model. CD4+CD45RBhi T cells isolated from spleen of either IRF4-/- mice or IRF4+/+ mice. Rag2-/- mice were intraperitoneally transferred with 5x10^5 CD4+CD45RBhi T cells and the changes of weight and colon inflammation were followed.

2.3.4 Histopathology and mouse endoscopy

Histopathology:

Histological examination of colon in TNBS-induced and DSS-induced colitis model was performed in a blinded fashion by experienced pathologists (Prof. Hans A. Lehr, Lausanne) using formalin-fixed tissue sections stained with haematoxylin-eosin. Inflammation was graded on a scale from 0-7.

Table 2.1 histologic colitis score

Histologic colitis grading		
Infiltration of mononuclear cells in LP	0	normal
	1	moderate with focal infiltration
	2	severe with diffuse infiltration
Epithelial damage	0	normal
	1	focal damage
	2	extensive damage
Architectural changes	0	normal
	1	moderate disturbed
	2	extensive scarring

Presence of crypt abscesses	0	no
	1	yes

The histopathological assess of the colon in naïve T cell transfer model was performed in a blind manner by pathologist (Prof. C. Mueller, Bern). A scoring system ranging from 0-14 was used. The following parameters were considered: a) mucin-depletion/loss of goblet cells (scores from 0 to 3); b) crypt abscesses (scores from 0 to 3); c) epithelial erosions (scores from 0 to 1); d) hyperemia (scores from 0 to 2); e) cellular infiltration (scores from 0 to 3) and f) thickness of colonic mucosa (scores from 0 to 2).

Mouse colonscopy:

To perform experimental endoscopy, mice were anaesthetised using intraperitoneal injection of mixture of ketamin (Ratiopharm GmbH, Ulm) and xylazin (Bayer Vital GmbH, Leverkusen) with dosis of 80µl/10g of body weight. 1 ml injectionsolution contains 0.24 ml ketamin (original concentration 500mg/10ml) and 0.08 ml 2% xylazin. Mouse endoscopy was performed using a miniature endoscope (scope 1.9 mm outer diameter), a xenon light source, a triple chip camera, and an air pump (all from Karl Storz, Tuttlingen, Germany) to achieve regulated inflation of the mouse colon. The endoscopic procedure was viewed on a colour monitor and digitally recorded on the computer (see figure 3.1).

Endoscopic colitis scores were based on the observed signs of inflammation. The modified murine endoscopic index of colitis severity (MEICS) is consisted of five parameters, as indicated in figure 3.2 and ranging from 0 to 15.

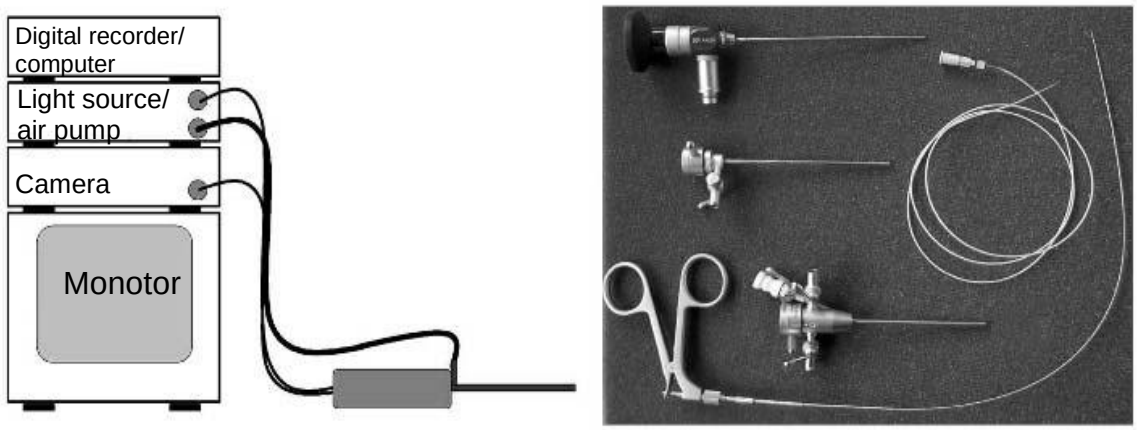

Figure 2.2 schematic diagram of endoscopic setup. Endoscopic tools used for mouse examination: straightforward telescope, examination sheath, manipulation sheath, biopsy forceps, and injection tube. [161]

	Endoscopic colitis grading				Total
Thickening of the colon	0 transparent	1 moderate	2 marked	3 intransparent	0 – 3
Changes of the vascular pattern	0 normal	1 moderate	2 marked	3 bleeding	0 – 3
Fibrin visible	0 none	1 little	2 marked	3 extreme	0 – 3
Granularity of the mucosal surface	0 none	1 moderate	2 marked	3 extreme	0 – 3
Stool consistence	0 normal+solid	1 still shaped	2 unshaped	3 spread	0 – 3

Figure 2.3 endoscopic colitis score[161]*. There are five parameters to describe the inflammation of mouse colon: thickening of the colon, changes of the vascular paltern, fibrin visible, granularity of the nucosal surface, and stool conssitence. The score was from 0 (normal) to 3 (severe) in each parameter. The final score of colon inflammation is the caculated scores including all these five parameters.*

2.3.5 Cell isolation and cultivation

2.3.5.1 Splenocytes isolation

Freshly cut spleen was mashed through 100µM cell strainer (BD Biosciences Europe, Belgium) into 50ml falcon tube. After spin down the cells, supernatant was discarded and cell pellet was resuspended in 1 ml ACK lysis buffer (1L deionized water, 8.29 g NH_4Cl, 1 g $KHCO_3$, 37.2 mg Na_2-EDTA (Carl Roth GmbH, Karlsruhe)) (pH to 7.2 - 7.4) for 2 minutes. Neutralising lysis buffer by adding 9 ml RPMI 1640 medium (Invitrogen, USA) containing 10% Fetal calf serum (FCS), 1% penicillin/Streptomycin (PAA Laboratories GmbH, Austria). Isolated splenocytes were used for further stimulation.

2.3.5.2 Naive CD4+CD25- T cell isolation

Naive CD4+CD25- T helper cells were prepared by using MACS system (Miltenyi, Germany) from spleens of mice. The sorted cells were used for transfer colitis, or stimulation. For differentiation to Th17 subset, CD4+CD25- T cells were cultured in a Th17 differentiation condition, namely with anti-CD3 (clone 145-2C11, 1 µg/ml), anti-CD28 (clone 37.51, 1 µg/ml) (both antibodies produced and purified in house), anti-IFN-γ (10µg/ml, BD Phamingen), anti-IL-4(10µg/ml, BD Phamingen), TGF-ß (5ng/ml, R&D systems), hyper-IL-6 (100ng/ml, a gift from professor Stefan Rose-John) for 4 days. Cell supernatants were collected for cytokine measurement. On day 5, cells were restimulated with phorbol 12-myristate 13-acetate (PMA) (100ng/ml, Sigma Aldrich), Ionomycin (500ng/ml, Sigma Aldrich) and GolgiPlus (10µg/ml, BD Phamingen) for further 4 hours and IL-17 intracellular staining was performed by using Fix & Perm cell permealization reagents (Caltag Laboratories).

2.3.5.3 Lamina propria mononuclear cells (LPMC) isolation

Lamina propria mononuclear cells (LPMC) were isolated as described[159]. In brief, colon was opened longitudinally and cut into small pieces. Pieces were incubated twice in 5 ml 5 mM EDTA (Carl Roth GmbH, Karlsruhe, Germany) in HBSS (Biochrom AG, Berlin, Germany) for 15 min at 37°C with slow rotation(100 rpm) to separate epithelial cells. The tissue was then digested in HBSS containing 0.5mg/ml collagenase D (Roche Diagnostics, Mannheim, Germany), DNase I (Sigma-Aldrich, Munich, Germany) and 50 U/ml dipase II (Roche Diagnostics, Germany) 3 times 20 minutes at 37°C with slow rotation. Digested tissue was passed through a 40µm cell strainer and cellular contents were separated from debris using a 40/80 Percoll (Biochrom AG, Germany) Gradient. The freshly isolated lamina propria mononuclear cells were cultured in RPMI1640 medium containing 10% FCS, 1% penicillin(100IE/ml)/streptomycin(100µg/ml), gentamycin 50µg/ml (PAA Laboratories GmbH, Austria), amphotericin B 1µg/ml (Biochrom, Berlin, Germany) and then stimulated with anti-CD3 and anti-CD28, or stimulated seperately with LPS (10ng/ml), CPG (1µmol/ml), LTA (800ng/ml) for 48 hours.

2.3.5.4 Isolation of colonic lamina propria fibroblasts (CLPFs)

Colonic lamina propria cells were isolated as described above. After 3 times digestion with collagnase D, DNase I and Dipase II, cellular contents were cultured in 25cm^2 culture flasks. Non adherent cells were removed by subsequent changes of medium. The cultured fibroblasts were stimulated with IL-6 (100ng/ml) or IL-17a (100ng/ml) for 12 hours and RNA isolated from the cell lysate was used for performance of real time PCR.

2.3.6 Cytokine measurement by using ELISA

Cytokines in cell supernatants were measured by ELISA (IL-17, R&D system; IL-6 & IFN-gamma, BD Phamingen; TNF-alpha & IL-10, eBioscience Inc., USA).

2.3.7 Immunofluorescent staining

Immunofluorescence allows the visualization of specific proteins on fixed cells or tissue sections by employing specific antibody chemically conjugated to fluorescent dye. The technique is widely used to study the cellular distribution of protein as well as the protein-protein interaction.

Human colon specimen:

Mucosal samples were snap-frozen and embedded in OCT compound to obtain cryosections. Immunofluorescence was done on gut cryosections. Tissues were fixed in 4% paraformaldehyde for 20 minutes at room temperature and washed in 1xTBST (100ml 0.05M Tris HCL pH 7.6, 8.5g NaCl, 900ml dd H_2O, 1ml Tween 20). Samples were then pretreated with 1 drop of blocking solution from Protein Blocking Kit (DAKO) for 15 minutes at room temperature and incubated overnight at 4°C with primary antibody (1:1000 dilution, polyclonal rabbit antibody against IRF4; Santa Cruz Biotechnology Inc.) in 1x TBST (2% bovine serum albumin). Samples without primary antibody served as negative control. The next day, samples were rinsed in TBST and incubated with a biotinylated secondary IgG antibody (1:1000 dilution)

followed by incubation with streptavidine-conjugated Cy2 or Cy3 (1:50 – 1:100 dilution). Samples were subjected to a second cycle of staining by using monoclonal mouse antibodies against human CD3, CD4, CD8 and CD11c as primary antibodies (all obtained from BD Pharmingen) and streptavidine-conjugated Cy3 as a chromogen. Slides were mounted with mounting medium for fluorescence (Vector Laboratories) and analyzed with an Olympus microscope. Finally, cells in 7 highpower fields (HPFs) were counted in all patients for each condition.

Murine colon samples:

To detect IRF4 and IL-6 in murine colon samples, cryosections were fixed in ice cold acetone for 10 minutes followed by sequential incubation with methanol, avidin/biotin (Vector Laboratories), and protein-blocking reagent (Dako) to eliminate unspecific background staining. Slides were then incubated overnight with primary antibodies directed against IRF4 and IL-6 (Santa Cruz Biotechnology Inc.). Subsequently, the slides were incubated for 30 minutes at room temperature with biotinylated secondary antibodies (Dianova). All samples were finally treated with streptavidine-conjugated Cy3. Before examination, the nuclei were counterstained with DAPI (Vector Laboratories).

To detect MPO or macrophages in liver or spleen in mice, cryosections were fixed in 4% paraformaldehyde for 20 minutes at room temperature. Samples were blocked sequentially by methanol, avidin/biotin and protein-blocking reagent. Rabbit anti-mouse myeloperoxidase (ABcam) with 1:100 dilution was used as primary antibody for MPO staining and rat anti-mouse F4/80 antibody with 1:1000 dilution was used for macrophage staining. After overnight incubation with primary antibody, biotinylated rabbit or rat secondary antibody was added and then followed with streptavidine conjugatged cy3 system. At last, the nuclei were counterstained with DAPI.

2.3.8 Detection of apoptosis

There are different methods to detect apoptosis. In this study, Terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) stain was used because it permits quantification of apoptotic cells in tissue sections. This assay

enzymatically labels the free 3’ OH ends of DNA independent of template. In the later stages of apoptosis, DNA cleavage occurs producing about 800 bp fragments. The free 3’ OH ends are the target trying to label to demonstrate apoptosis.
TUNEL reaction was performed using In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnositics GmbH, Germany), according to the manufacturer’s instructions. The cells were counterstained with DNA binding dye DAPI for visualisation of nuclei and mounted in VECTASHIELD (Vector Laboratories, Canada) for fluorescence microscopy.

2.3.9 RNA isolation, cDNA synthesis and Real-time PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hamburg, Germany). Reverse transcription into cDNA was performed with iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's recommendations.

Table 2.2 Reaction setup:

components	**Volume per reaction**
5x iScript reaction mix	4µl
iScript reverse transcriptase	1µl
Nuclease-free water	Xµl
RNA template (1µg)	Xµl
Total volume	20µl

Reaction protocol:
Incubate complete reaction mix:
5 minutes at 25°C
30 minutes at 42°C
5 minutes at 85°C
Cool to 4°C
5 minuntes at 85°C

Real-time PCR analysis were performed in duplicates on Light Cycler 480II (Roche).

Primer sets were listed below.

Table 2.3 Primer sequences:

Primer	Sequences	Annealing T
Mm IL-6	for 5'-CCG GAG AGG AGA CTT CAC AG-3' rev 5'-TCC ACG ATT TCC CAG AGA AC-3'	58°C
Mm IL-17	for 5'-CTC CAG AAG GCC CTC AGA CTA C-3' rev 5'- AGC TTT CCC TCC GCA TTG ACA CAG-3'	58°C
Mm IL-22	for 5'- TCC GAG GAG TCA GTG CTA AA-3' rev 5'- AGA ACG TCT TCC AGG GTG AA-3'	58°C
Mm RORγT	for 5'- CCG CTG AGA GGG CTT CAC-3' rev 5'-TGC AGG AGT AGG CCA CAT TAC A-3'	58°C
Mm ß-actin	for 5'- TGG AAT CCT GTG GCA TCC ATG AAA C-3' rev 5'- TAA AAC GCA GCT CAG TAA CAG TCC G-3'	60°C
Mm defensin	for 5'- GCT AGG GAG CAC TTG TTT GC-3' rev 5'- TTG TTT GAG GAA AGG AGG CA-3'	53°C
Hs IL-6	for 5'- AAA TTC GGT ACA TCC TCG ACG GCA-3' rev 5'- AGT GCC TCT TTG CTG CTT TCA CAC-3'	60°C
Hs GAPDH	for 5'- CCC ATC ACC ATC TTC CAG GAG CG-3' rev 5'- CAT GCC AGT GAG CTT CCC GTT CA-3'	

The other primer sets were purchased from Qiagen (Qiagen, Hamburg, Germany). Using murine ß-actin and human GAPDH as housekeeping gene, the relative quantification of cytokine mRNA was calculated with the fomular showing below:

$$\text{Relative mRNA expression} = \frac{E_T^{CpT(C) - CpT(S)}}{E_R^{CpR(C) - CpR(S)}}$$

Cp: crossing point

E: efficiency

T: target gene

R: reference gene (housekeeping gene)

C: Calibrator (Calibrators was murine spleenocytes or one human sample.)

S: sample

2.3.10 Mapping of putative IRF4 binding sites on murine promoters

Chemically, transcription factors (TF) interact with their binding sites using a combination of electrostatic (of which hydrogen bonds are a special case) and Van der Waals forces. Due to the nature of these chemical interactions, most transcription factors bind DNA in a sequence specific manner. However, not all bases in the transcription factor binding site may actually interact with the transcription factor. In addition some of these interactions may be weaker than others. Thus, transcription factors don't bind just one sequence but are capable of binding a subset of closely related sequences, each with a different strength of interaction.
To map the putative binding sites of IRF4 on murine promoters, MatInspector software was used. MatInspector is a software tool that utilizes a large library of matrix descriptions for transcription factor binding sites to locate matches in DNA sequences. It assigns a quality rating to matches and thus allows quality-based filtering and selection of matches.

2.3.11 Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) is a tool for identifying proteins interaction with specific regions of the genome by using specific antibodies that recognizes a specific protein. The initial step of ChIP is the cross-linking of protein-DNA in live cells with formaldehyde. After cross-linking, the cells were lysed and crude extracts were sonicated to shear the DNA. The cross-linked protein-DNA was immunoprecipitated. The immunoprecipitates were then reversed cross-links and the DNA fragments are purified and the detection of specific DNA sequences is performed by PCR amplification. In the present work, ChIP experiments were performed using the ChIP Assay kit (Upstate cell signaling solutions, USA) according to manufacturer's instructions.

Briefly, freshly isolated splenocytes from C57BL/6 were plated at a density of $1x10^6$/ml in a 10cm dish and stimulated with anti-CD3 and anti-CD28 as described above for 12 hours. Cross link histones to DNA by adding formaldehyde (1% final concentration) and incubate for 10 minutes at 37°C. Cells were lysed and DNA in the supernatant was sheared by Branson Digital Sonifer (Branson Ultrasonics corporation USA). The programm : 10 rounds with pulse = 10 seconds, pause= 20 seconds, power output = 40%. After removing an aliquot of sonicated samples as input material, the remainder was used for IP. 2µg anti-IRF4 (santa cruz) was added to the supernatant fraction and incubated overnight at 4°C with rotation. For a negative control, a no-antibody immunoprecipitation was performed by incubating the supernatant fraction with protein A Agarose/Salmon Sperm DNA. To reverse histone-DNA crosslinks, 20µl 5M NaCl was added to each sample and heated at 65°C for at least 4 hours. DNA was purified by using MinElute PCR Purification Kit (Qiagen) and RT-PCR was performed. Primer sequences used for PCR are listed below.

Table 2.4 Primers used for ChIP PCR:

Primer	**Sequences**	**Annealing T**
IL-6 (a) for	5'- GCG TGC CTG CGT TTA AAT A-3'	60°C
IL-6 (a) rev	5'- TAG CCC TAA GAA GCA TGA GCA -3'	60°C
IL-6 (b+c) for	5'- AAG CC ACT TTC CCC TTC CT-3'	60°C
IL-6 (b+c) rev	5'- TCA TGG GAA AAT CCC ACA TT-3'	60°C
IL-17 (a+b) for	5'- TTC CCT TCT CAT CCC TCA TC-3'	53°C
IL-17 (a+b) rev	5'- GGT TCG GTA TCA AGC CTT TC-3'	53°C
IL-17 (c) for	5'- AAC AGT TGC GGT ACT CAG-3'	53°C
IL-17 (c) rev	5'- GCC CAA AGA AAC CCA CTC AAT G-3'	53°C
RORalpha (a+b) for	5'- ATG CAA ACG ATT CAG GAA CA-3'	56°C
RORalpha (a+b) rev	5'- CCA CTG GCT AAA GGA CAA CC-3'	56°C

Table 2.5 RT-PCR setup for ChIP:

Components	**Volume (µl)**
Primer forward (10pmol/µl)	1
Primer reverse (10pmol/µl)	1

RedTag ready Mix	12.5
RNase-free water	11.5
cDNA	26.5
Total volume	26

2.3.12 Electrophoretic mobility shift assay (EMSA)

EMSA is based on the principle that the binding of protein to DNA retards the migration of free DNA in native polyacrylamide gels. When a protein binds specifically to the labeled dsDNA sequence, it migrates slower than the non-bound dsDNA resulting in discrete band corresponding to the individual protein-DNA complex. EMSA was performed using 5'-end labelled IRDye700 dsDNA and Odyssey infrared Imaging System was used for dectection of the assay.

Nuclear extract was isolated from splenocytes of IRF4-/- and IRF4+/+ mice. Freshly isolated splenocytes were stimulated with anti-CD3 and anti-CD28 for 12 hours. Cell pellets were resuspended two times in 500µl Buffer A (1mM Hepes pH7.9, 1.5mM $MgCl_2$, 10mM KCl, freshly added 1mM DTT and 0.5mM PMSF (Sigma-Aldrich, Switzerland)). After added with one drop Triton X-100, cell suspension was incubated on ice for 15 minutes and then spun down at 4000 rpm for 10 minutes. The pellet was resuspended in 50µl Buffer B (20mM Hepes pH 7.9, 25% Glycerol, 0.42M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, freshly added 1mM DTT and 0.5mM PMSF) and mixed for one hour at 4°C. After further centrifugation at 15000 rpm at 4°C, supernatant was collected as nuclear extract.

IRDye 700-labeled sense and antisense DNA oligonucleotides were synthesized by Metabion.

Table 2.6: Sequences of Oligonucleotide used for EMSA:

Oligonucleotide	**Sequences**
IRF4-1 (b) for	5'- AGT CCC TTA TTC TTT CAC TTC ATT TCC TTC C-3'
IRF4-1 (b) rev	5'- GGA AGG AAA TGA AGT GAA AGA ATA AGG GAC T-3'
IRF4-2 (a) for	5'- CCT TCA TCT TGA TTT CTA ATT CTT TCT TCG A-3'

IRF4-2 (a) rev	5'- TCG AAG AAA GAA TTA GAA ATC AAG ATG AAG G-3'
Mut IRF4-1 (b) for	5'- AGT CCC TTA TTC T***AA A***AC TTC ATT TCC TTC C-3'
Mut IRF4-1 (b) rev	5'- GGA AGG AAA TGA AGT ***TTT*** AGA ATA AGG GAC T-3'
Mut IRF4-2 (a) for	5'- CCT TCA TCT TGA TTT CTA A***AA A***TT TCT TCG A-3'
Mut IRF4-2 (a) rev	5'- TCG AAG AAA ***TTT*** TTA GAA ATC AAG ATG AAG G-3'
IRF4-3(a) for	5'-TCC ATG GAC TTT TTT TTT CCC CCA AAG TCT-3'
IRF4-3(a) rev	5'-AGA CTT TGG GGG AAA AAA AAA GTC CAT GGA-3'
IRF4-4(b) for	5'-TCC TAA CTT AAG GAA AAC ATG ACA TAA GAC-3'
IRF4-4(b) rev	5'-GTC TTA TGT CAT GTT TTC CTT AAG TTA GGA-3'
Mut IRF4-3(a) for	5'-TCC ATG GAC TTT TTT ***AAA A***CC CCA AAG TCT-3'
Mut IRF4-3(a) rev	5'-AGA CTT TGG GG***T TTT*** AAA AAA GTC CAT GGA-3'
Mut IRF4-4(b) for	5'-TCC TAA CTT AAG ***TTT T***AC ATG ACA TAA GAC-3'
Mut IRF4-4(b) rev	5'-GTC TTA TGT CAT GT***A AAA*** CTT AAG TTA GGA-3'

For competition assay, unlabeled IRF4-1, IRF4-2, IRF4-3 and IRF4-4, mutated IRF4-1, mutated IRF4-2, mutated IRF4-3 and mutated IRF4-4 were used.

For double-strand probes, sense and antisense oligonucleotides were annealed in buffer (10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 8.0) by heating to 95 °C and slowly cooled to room temperature (1°C / minute).

Electrophoretic mobility shift assay was carried out by using Odyssey Infrared EMSA Kit (Li-Cor biosciences).

Table 2.7: Binding reaction setup:

Components	**Volume (µl)**
dd H2O	X
10X Binding buffer	2
25mM DTT/2.5% Tween-20	2
1µg/µl Poly (dI.dC)	1
1%NP-40	1
100mM MgCl2	1
50% Glycerol	1
Unlabelled Oligo (200x50mM)	X
Mutated Oligo (200x50mM)	X

IRDye700-Oligo (50mM)	X
Protein extract	5µg
Anti-IRF4	4µg
Orange loading buffer	2
Total volume	22

4% native polyacrylamide gel was used to separate the binding reaction samples. The gel composition is listed below.

Table 2.8 Gel preparation for EMSA

Components	**Volume**
10xTBE	5ml
dd H2O	40ml
40% acrylamide	5ml
TEMED	32µl
10% APS	300µl
Total volume	50ml

After polymerization, the electrophoresis separation unit was setup and a pre-run was performed for 30 minutes at 140V using 0.5XTBE. The gel was allowed to run in the dark at a constant voltage of 140V for 2 hour or until orange dye migrates to the bottom of the gel. The complete gel in glass plate can be analyzed by Odyssey Infrared Imaging system.

2.3.13 Retroviral transduction

Retroviruses offer gene transfer specialists and gene therapy researchers probably the best utility for delivering genes to target cells at high efficiency in a manner that allows for long-term, stable expression of introduced genetic elements

Supernatant of Retrovirus expressing IRF4 was abtained from Professor Lohoff, Marburg. To generate the IRF4 overexpressing retrovirus, the full length IRF4 cDNA was amplified from pcDNA3.1-IRF4 with sequence-specific primers containing

additional restriction sites and cloned into pMSCV-GFP using XhoI and BglII. Virus containing supernatant was produced as discribed previously by Nolan lab.
Naïve CD4+CD25- T cells isolated from IRF4-/- or C57BL/6 mice were stimulated with anti-CD3 and ant-CD28 for 24 hours. These activated cells were then transduced with retrovirus supernatant by centrifugation for 1.5 hours at 2000 rpm in the presence of rmIL-2 (40ng/ml, R&D systems), polybrene (8µg/ml, Sigma Aldrich). After removal of the virus-containing supernatants, cells were recultured in Th17-polarizing conditions. The transduction with retrovirus was repeated on the next day and recultured as before. After 4 days of culture, cells and supernatants were harvested for intracellular staining and ELISA measurement.

2.3.14 Induction of sepsis by CLP

Several animal models to mimic sepsis have been developed. Common experimental models used in sepsis research pursue two different strategies: injection of bacteria or microbial components (such as LPS) into the peritoneal cavity or bloodstream; a septic focus originating from release of feces into the peritoneal cavity (such as CLP). CLP leads to a polymicrobial infection of the peritoneum, eventually resulting in bacteremia, SIRS, sepsis, septic shock, and usually death. CLP was used in this study since it closely resemble the course of sepsis observed in patients, characterized by an early hyperdynamic, hypermetabolic state, followed by a pronounced hypodynamic, hypometabolic state.
Mice were anesthetized by i.p injection of ketamin/xylazin. A 1-cm abdominal midline incision was made. The exposed cecum was ligated with a 5–0 silk suture (Johnson & Johnson Intl. Belgium) below the ileocecal junction without causing bowel construction, and punctured through with an 18-gauge needle. All mice received 1 ml of saline immediately after surgery. Survival was monitored for 14 days after the surgery.
In separate experiments, mice were sacrificed 24 hours after CLP. Heparinized blood was send to central laboratory for GPT, GOT, urea, creatinin analysis. EDTA blood was analyzed for heamogram. Bacterial counts were assessed (see Determination of CFU). Splenocytes from the operated mice were isolated (see method described

before) and cultured in RPMI1640 medium containing 10% heat-inactivated fetal calf serum and 1% penisilin/streptomycin on 24-well plate at a density of 10^6 cells/ ml for 48 hours.

2.3.15 Leukocyte counts

Splenocytes and peritoneal lavage cells obtained with 5 ml ice-cold PBS were counted in a Neubauer chamber. Percentage of differential cells were measured by FACS using different cell surface markers. Blood was obtained by cardiac puncture with 1mg/ml EDTA as anticoagulator. Differential cell counts of blood were performed by haemometer in central laboratory.

2.3.16 Determination of CFU

24 hours after CLP, peritoneal cavity was washed with 5 ml of sterile PBS. 0.1 g Liver, kidney and lung were collected in 1 ml PBS and homogenized by using Retsch MM300 shaker (Retsch GMBH, Haan, Germany). Heparinized peripheral blood, peritoneal lavage fluid, homogenate from liver, kidney and lung were serial diluted and plated on CASO blood agar plates (heipha Dr. Müller GmBH Germany) at 37°C. CFUs were determined after 24 hours to 48 hours.
The numbers of bacterial colonies in the colon were also determined by plating the suspension of faeces on CASO blood agar plates.

2.3.17 Fluorescence Activated Cell Sorting (FACS)

2.3.17.1 Detection of cell surface markers

Cells were incubated with different conjugated antibody for 15 minutes in dark. In this study, CD4-FITC, CD4-PE, CD25-PE, CD11c-APC, CD11c-FITC, CD11b-PE, CD11b-FITC, GR-1-PE (BD Pharmingen) were used.

2.3.17.2 Intracellular staining

Intracellular staining was performed by using Fix & Perm cell permealization reagents (Caltag Laboratories) according to the manufacture's instruction. Intracellular lL-17 was detected by using IL-17-PE (BD Pharmingen) antibody.

2.3.17.3 Phagotest

The phagocytosis process can be separated into several major stages: chemotaxis, attachment of particles to the cell surface of phagocytes, ingestion and intracellular killing by oxygen-dependent and oxygen-independent mechanisms. To analyze ingestion, Phagotest kit was used.

Phagotest kit allows the quantitative determination of leukocyte phagocytosis in heparinized whole blood. Heparinized whole blood was incubated with FITC-labelled E.coli bacteria at 37°C for 10 minutes. And then the phagocytosis was stopped by placing the samples on ice and adding quenching solution. This solution allows the discrimination between attachment and internalization of bacteria by quenching the FITC fluorescence of surface bound bacteria leaving the fluorescence of internalized particles unaltered. After two washing steps with wash solution, erythrocytes were then removed by addition of lysing solution. The DNA staining solution was added just prior flow cytometric analysis, excludes aggregation artifacts of bacteria or cells.

2.3.18 Determination of myeloperoxidase activity (MPO)

Myeloperoxidase (MPO) is a peroxidase enzyme (EC 1.11.1.7) most abundantly present in neutrophil granulocytes. It is a lysosomal protein stored in azurophilic granules of the neutrophil. When neutrophils become activated during phagocytosis, they undergo a process referred to as a respiratory burst. This respiratory burst causes production of superoxide, hydrogen peroxide, and other reactive oxygen derivatives, which are all toxic to microbes. During respiratory bursts, granule contents are released into the phagolysosomes and outside the cell, so that they can

come into contact with any presenting microbes and have important roles in microbial killing.[1] However, the infiltration of neutrophils and thereafter secreted MPO can also induce tissue damage at the same time.

In the present study, MPO activity in peritoneal fluids was determined and MPO in liver was detected by using immunfluorescent staining. Peritoneal fluids were centrifuged at 1400 rpm for 10 minutes. Serial dilution of the supernatants were added to TMB liquid substrate (BD Pharmingen) for determination of MPO activity. The reaction was stopped by addition of H_2SO_4 and the absorption determined at 450nm.

2.3.19 Depletion of neutrophils

Anti-GR1 antibody was a kindly gift from Pamela Aranda Lopez (Institute of immunology, Mainz). Anti-Gr1 (150µg, i.p.) was administered 24 hours and 4 hours prior to and 24 hours, 48 hours after performance of CLP. Survival was monitored for 14 days.

2.3.20 Statistical Analysis

Statistical significance of evaluated data was tested Student's t-Test. Results were considered as statistical significant when p values were <0.05. Results are expressed as mean values (+/- SD or +/- SEM) or as median, lower and upper quartiles, sample minimum and maximum by using GraphPad Prism (GraphPad Software, Inc. USA). The error bars in histogram figures represent standard-deviations or standard errors of the mean as indicated. Relative expression of IRF4, IL-6 mRNA, IL-17 mRNA, and IL-22mRNA from patients was correlated by using the linear correlation coefficient r to measure the strength and the direction of a linear relationship between these variables.

3 Results

3.1 IRF4 expression in human IBD patients

IRF1 and IRF5 have been reported to be associated with development of human IBD. In the present study, the involvement of IRF4 in the pathogenesis of IBD was intensively studied.

3.1.1 IRF4 protein expression in colon of IBD patients

IRF4 has been reported to be expressed in T cells, B cells, dendritic cells and macrophages. Unactivated IRF4 is in cytosol. Stimulation of the immune cells could upregulate the expression of IRF4, or increase the translocation of IRF4 into the nuclei. In the nuclei, IRF4 acts as transcription factor to regulate other genes. Immunofluorescent staining of IRF4 in human colon specimens showed an upregulation of IRF4 production in IBD patients compared with control patients. Satistical analysis of IRF4 positive cells showed a significant enhancement of IRF4 positive cells in the mucosa of UC and CU patients as compared to control patients.
Besides the increased production of IRF4, IRF4 mRNA level in the colon was also upregulated in the UC and CU patients, suggesting a transcriptional upregulation of IRF4 in inflammed tissue.

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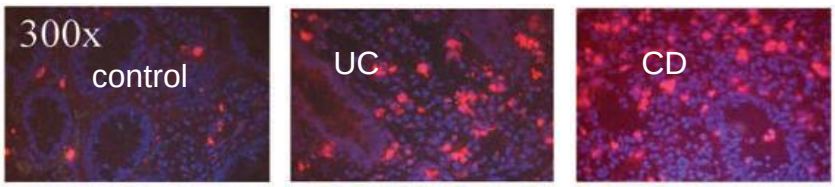


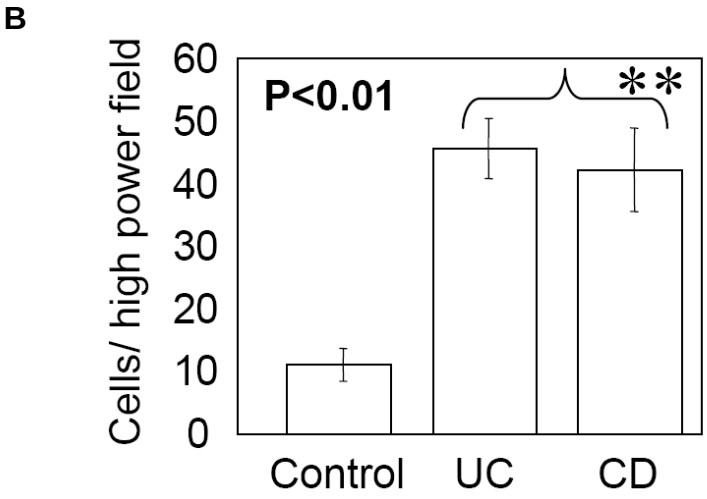


*Figure 3.1 Immunofluorescence staining of IRF4 in human colon cryosections. A) Human colon speciments (n=10 patients per group) were stained with anti-IRF4 antibody (red), and counterstained nuclei with DAPI (blue). Original magnification x300. B) Quantitative assessment of IRF4 positive cells. IRF4 positive cells were counted in 7HPFs per patient. **p<0.01. Data were published on J Clin Invest. 2008 Jul;118(7):2415-26.*

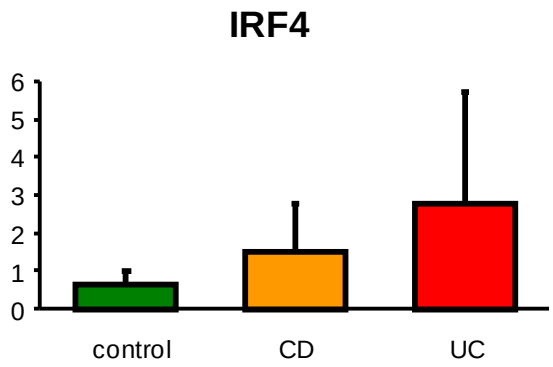


Figure 3.2 relative IRF4 expression in human colon biopsies. Colon endoscopic biopsies were obtained from patients with Crohn`s disease(n=12), ulcerative colitis (n=10) and control patients (n=10). Real-time PCR was performed to quantitatively analyze expression of IRF4.

3.1.2 IRF4 expressing cells

IRF4 has been reported to be expressed in T cells, B cells and APCs. Using immunofluorescence double staining, IRF4 expressing cells were identified in mucosa tissues of patients suffering IBD. CD4 T cells were the prominent cells in inflamed mucosa. IRF4 was expressed in all the three examined subsets: CD4, CD8

and CD11c. However, the numbers of CD4-IRF4 double positive, CD11c-IRF4 double positive cells were about two fold higher than CD8-IRF4 double positive cells. In addition, CD4 T cells were also the major population to express IRF4 in inflamed mucosa tissue.

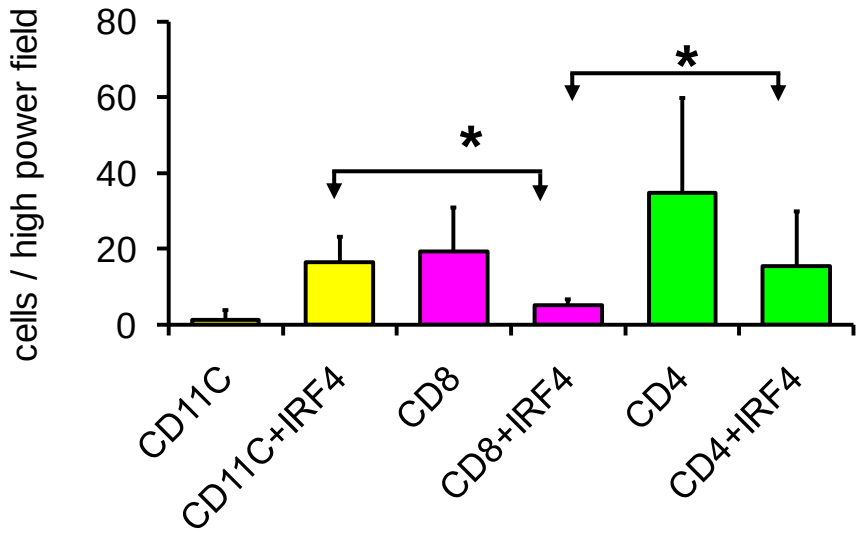


*Figure 3.3 Immunofluorescence double staining for IRF4 and CD4, CD8 and CD11c. Cells were stained with anti-IRF4 antibody. Surface staining was using either anti-CD4, or anti-CD8 or anti-CD11c. The number of positive cells was assessed in 7 HPFs per patient (n=6). *p<0.05. Data were published on J Clin Invest. 2008 Jul;118(7):2415-26.*

3.1.3 Expression of potentially IRF4 regulated cytokines in IBD patients

As shown above, expression of IRF4 was upregulated in IBD patients. And CD4 T cells were the major population to express IRF4 in inflamed mucosa tissue. It has been demonstrated that proinflammatory cytokines released from effecter T cells are responsible for the mucosal inflammation. In the next step, IL-6, IL-17 and IL-17 related cytokine mRNA in human colon mucosa were quantitative analyzed by using real time PCR.

IL-6 mRNA and IL-17 mRNA were increased in biopsies from IBD patients as compared to controls. The IL-17A level tended to be higher in UC than in CD, however the difference was not significant.

IL-22, which is produced predominantly by Th17 cells was tended to be up-regulated in the mucosa of IBD patients although without significant difference.

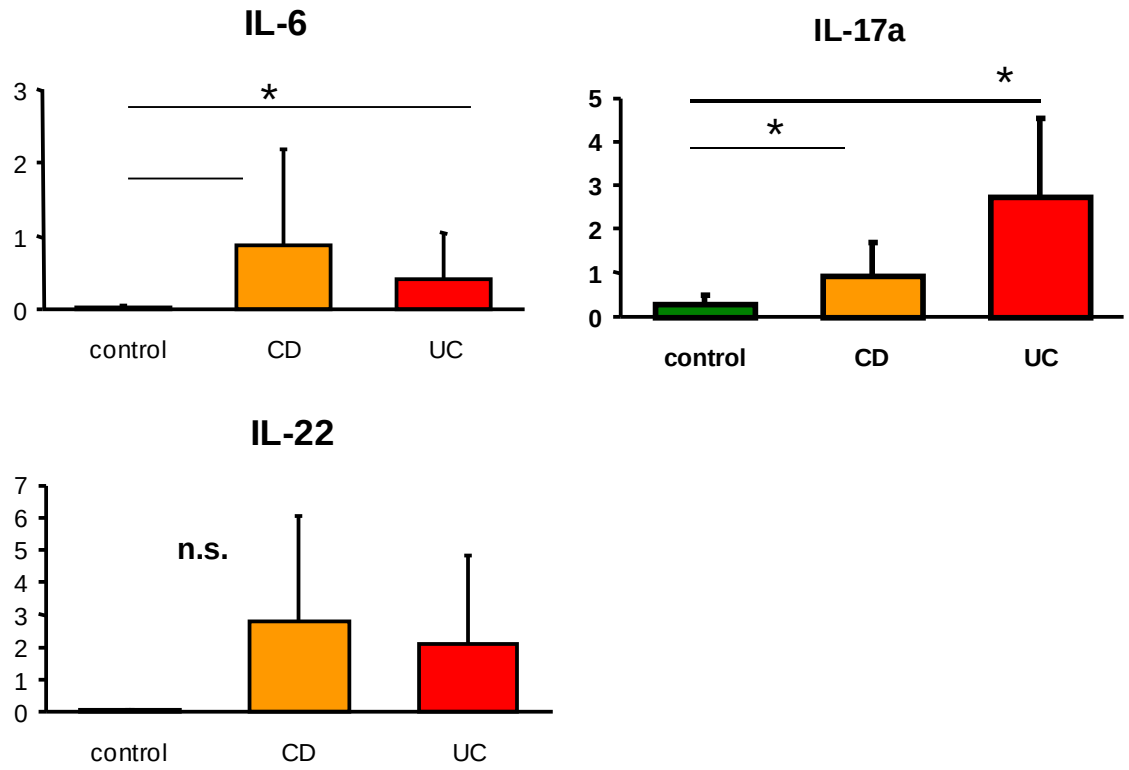


*Figure 3.4 Quantitative analysis of IL-6, IL-17 and IL-17 related cytokine mRNA in human colon mucosa. Patients with Crohn`s disease(n=12), ulcerative colitis (n=10) and control patients (n=10).*p<0.05.*

3.1.4 Correlation between IRF4 and IL-6, IRF4 and IL-17, IRF4 and IL-22

Results above showed upregulation of IRFmRNA, IL-6mRNA, IL-17mRNA. To assess how closely is the relationship between IRF4 and these cytokines, expression of these cytokines in the patients was correlated with the expression of IRF4 in the same patients. When the correlation coefficient r is higher than 0.8, it reflects a high correlation. There were high correlation between IRF4 and IL-6, and between IRF4 and IL-17, but not between IRF4 and IL-22 expression.

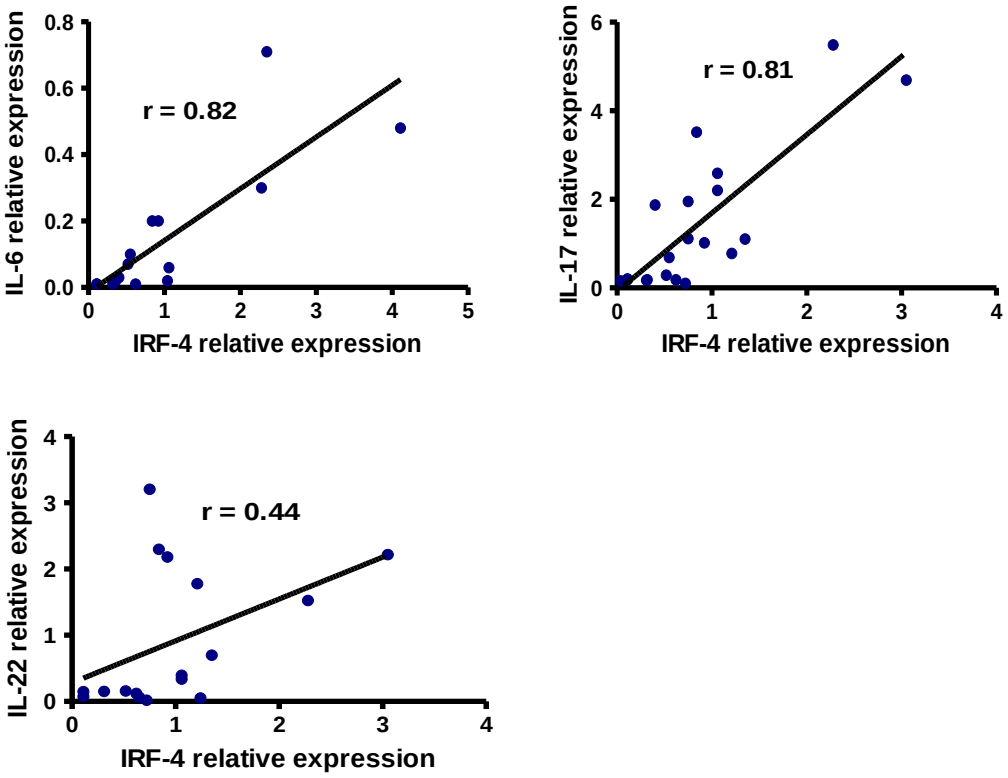


Figure 3.5 Correlation between IRF4 and IL-6, IRF4 and IL-17, IRF4 and IL-22 in human colon mucosa. Quantitative expression of IRF4, IL-6, IL-17, IL-22 were determined by using real time PCR. IRF4 relative expression was set as x axis values, relative expressions of the cytokines were set as values in y axis. Correlation was caculated by excel programm. n=18.

3.1.5 IRF4, IL-17, IL-22 expressions in inflamed mucosal tissue

Colon endoscopic biopsy specimen were obtained from patients with Crohn's disease and ulcerative colitis in the positions either with macroscopic inflammation or without macroscopic inflammation. IRF4, IL-17 and IL-22 expressions in the biopsies with macropscopic inflammations were compared with the biopsies without macroscopic inflammations. IRF4, IL-17 and IL-22 were more highly expressed in the inflammed tissue than in the macroscopicallly uninflammed tissue in the IBD patients.

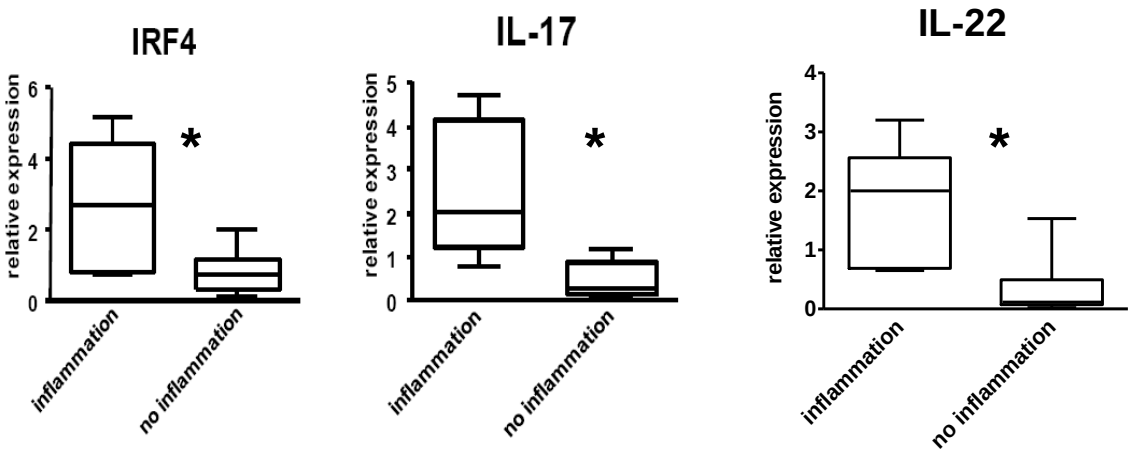


Figure 3.6 Increased expression of IRF4, IL-17 and IL-22 inflamed mucosal tissue. Colon biopsies were collected from places with or without macroscopic inflammation. Results were shown as median, lower and upper quartiles, sample minimum and maximum by using GraphPad Prism 5. N=6.

3.2 IRF 4 in T cell dependent experimental colitis models

We have shown in our lab, IRF4-/- mice were protected from oxazolone-induced colitis (data were published in JCI in 2008). After treatment with oxazolone, wild type mice lost more than 20% of the original body weight whereas IRF4-/- mice didn't.

We also indicated that IRF4-/- mice endured just mild colitis after TNBS treatment whereas wild type (C57BL/6J) mice had got severe colitis.

To study the mechanism of this protective effect of IRF4 deficient mice in colitis model, T cell transfer colitis model was further used.

$CD4^{+}CD45RB^{high}$ T cells (500,000 cells) isolated from WT or IRF4-/- mice, were adoptively transferred into immunodeficient RAG2-/- mice. Whereas RAG2-/- mice reconstituted with wild-type T cells developed severe colitis accompanied by weight loss, mice given IRF4-/- T cells were completely protected and even gained body weight, similar to un-reconstituted RAG2-/- mice.

Histological analysis of the colon showed that WT mice endured severe colon inflammation with the signs of mononuclear cells infiltration, swollen and disordered mucosa. Statistical study of histology indicated that there was significant difference between IRF4-/- and IRF4+/+ T cell transferred groups. The group transferred with

IRF4-/- T cells developed just minor inflammation in colon, whereas the group transferred with IRF4+/+ T cells developed strong colon inflammation.

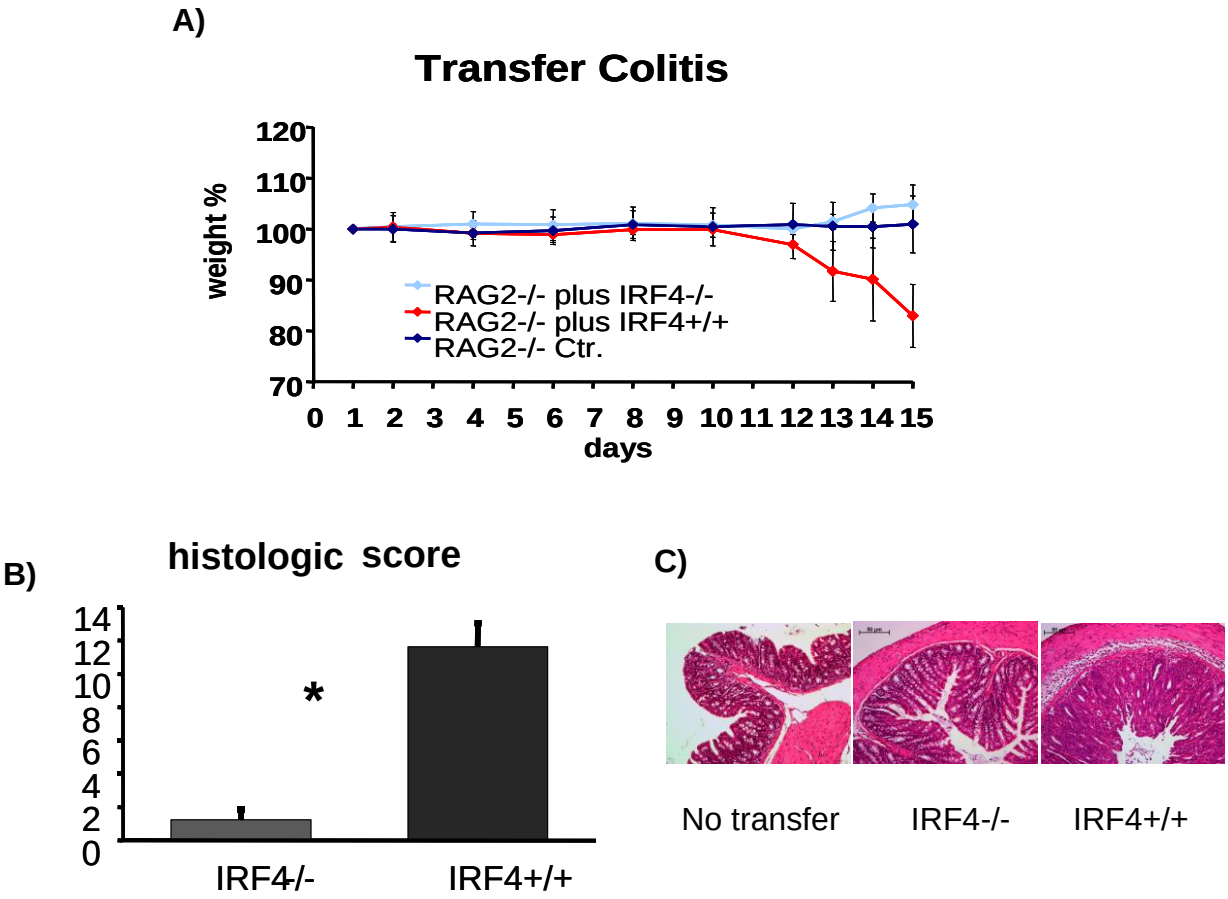


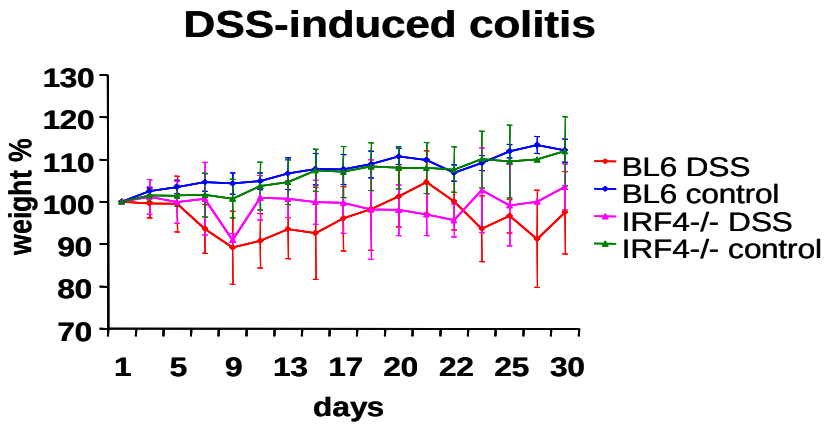
Figure 3.7 Study in transfer colitis model. CD45RBhigh CD4 positive T cells from wild-type (n=11) or IRF4-/- (n=11) mice were adoptively transferred into immunodeficient RAG2-/- mice. A) weight analysis. RAG2-/- mice reconstituted with wild-type T cells is shown in red curve, mice given IRF4-/- T cells is shown in blue curve, and unreconsituted RAG2-/- mice is in dark blue curve. B) & C) histological study. Columns in yellow or orange represent IRF4-/- T cell- or IRF4+/+ T cell-transferred RAG2-/- mice separately. One representative experiment out of three with 4 mice per group is shown in C. The differences between both groups were significant at days 14 and 15 (p<0,01).

3.3 IRF4 in DSS colitis model

Since IRF4 has been reported to be expressed also in antigen presenting cells (APC) such as dendritic cells or macrophages, we further used DSS-induced colitis model to study the role of IRF4 on innate immunity.

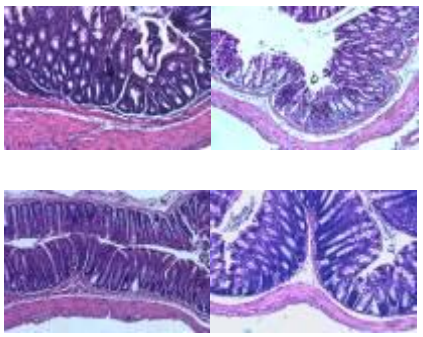
Treatment of 2.5% DSS in drinking water induced weight lose both in IRF4-/- mice and in WT mice. Both groups developed mild to severe colonic inflammation according to the histological dectection of colon. However, no significant difference was found between these two DSS treated groups.

A)



B) **C)**

BL6 **IRF4-/-**



DSS

Contr.

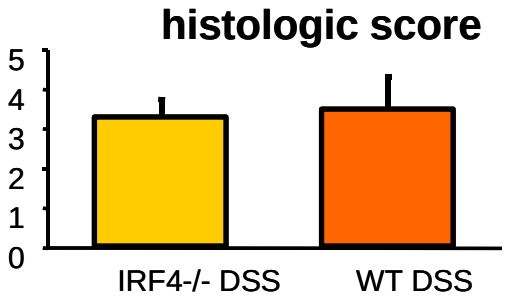


Figure 3.8 DSS-induced colitis. IRF4-/- mice or WT mice were supplied with 2.5% DSS in drinking water for 5 days, followed with 5 day- normal water. This cycle was repeated for two times. The body weights were monitored during the experiments and pathologic analysis of colon was done at the end of the experiments. A) changes of the body weights of IRF4-/- and WT mice. Data were presented for three separate experiment with n=10 of each group. B)&C) histological scoring of colon. No significant difference was shown between DSS treated IRF4-/- mice and DSS treated WT mice.

3.4 Analysis of proinflammatory cytokine pattern in murine colitis models

As transcriptional factor, IRF4 is shown to regulate B cells, T cells proliferation and differentiation. It is already known that T cells are important for the development of IBD. The proinflammatory cytokines produced by T cells such as L-6, TNF-α are responsible for the abolished T cell apoptosis, which has been confirmed as a pathological effect on colon. Data from human colon biopsies showed an upregulation of IL-6mRNA, IL-17mRNA in inflammed tissue. Early study indicated an abrogation of IL-6 expression and production in IRF4-/- mice in oxazolone colitis. Expression and production of cytokines were further analysed in other mouse colitis models.

3.4.1 Expression and production of cytokines in TNBS colitis

3.4.1.1 IL-6 and IL-17 productions in colon specimen

Colon specimens were collected in IRF4-/- and WT mice with TNBS-induced colitis. Quantitative assessment of cytokines were performed by using real time PCR. Not only the expression of IL-6, but also the expression of IL-17 was defect in TNBS-treated IRF4-/- mice.

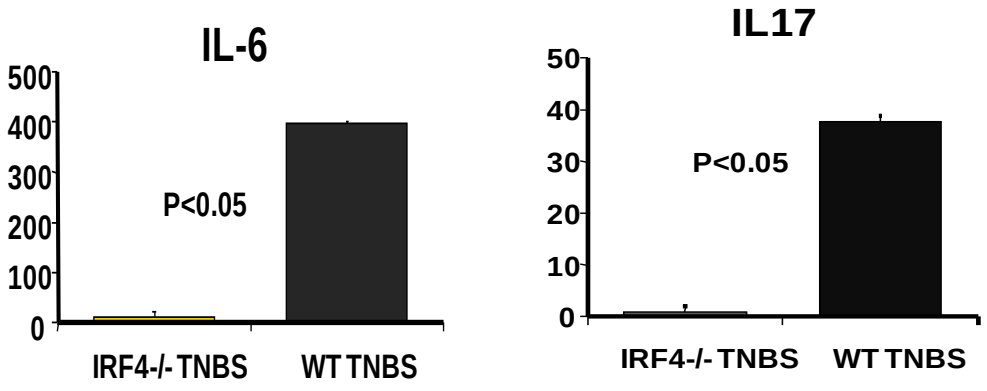


Figure 3.9 Quantitative measurement of IL-6 and IL-17 mRNA levels in TNBS colitis model. IRF4-/- mice and WT mice were sensitized with 1%TNBS on skin. Intrarectal

application of 2.5% TNBS was performed 7 days later. Relative expression of IL-6 and IL-17 in colon were measured by real time PCR. Date represent 3 separate experiments with n=6.

Immunofluorescent staining in colon showed that IL-6 expression was highly increased in inflamed mucosa of WT mice treated with TNBS compared with untreated control mice. However, IL-6 released in TNBS-treated IRF4-/- mice was significantly reduced as compare of TNBS-treated WT. After counting IL-6 positive cells in five HPF, we indicated a statistically significant enhancement of IL-6 in WT TNBS mice compared with IRF4-/- TNBS mice.

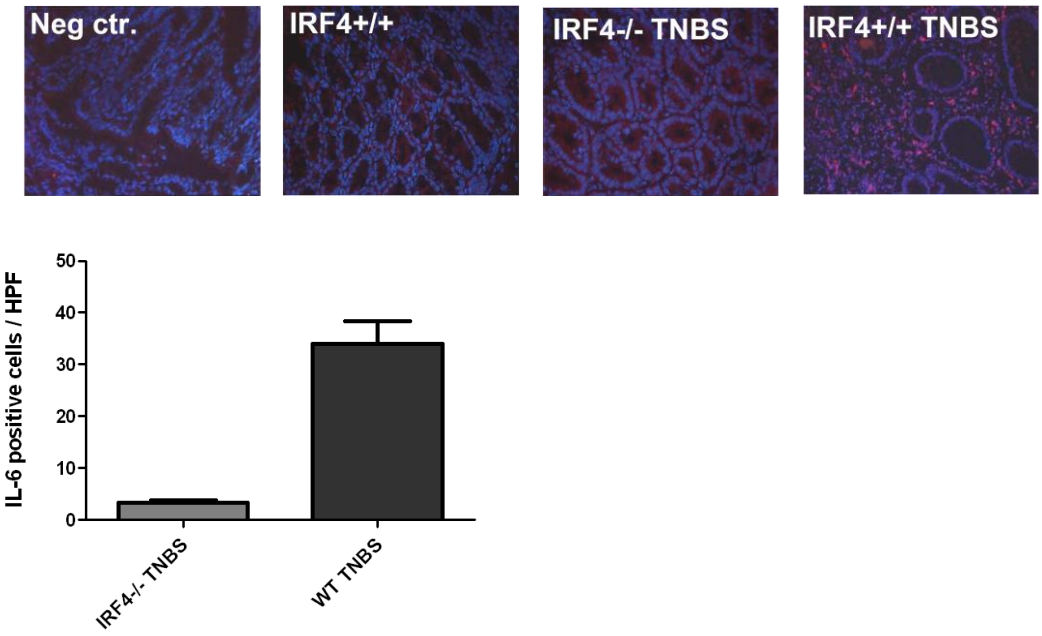


Figure 3.10 Immunofluorescence staining for IL-6 in TNBS colitis. Colon cryosections were stained with anti-IL-6 primary antibody, and biotinylated secondary antibody. IL-6 positive cells (in red) were counted for 5 HPF.original magnification x300. Significant difference was between IRF4-/- TNBS and WT TNBS with p<0.05. Data were published on J Clin Invest. 2008 Jul;118(7):2415-26,

3.4.1.2 Production of IL-6 in absence of IRF4

Decreased IL-6 production was further confirmed by measuring IL-6 concentration in supernatant of LPMC. Figure A showed IL-6 production of LPMC isolated from untreated IRF4-/- or WT mice. Figure B showed release of IL-6 of CD90+ LPMC from

TNBS-treated mice. Both figures indicated that production of IL-6 was much lower in IRF4-/- T cells than in WT mice and suggested that the defective production of IL-6 in absense of IRF4 is T cell specific.

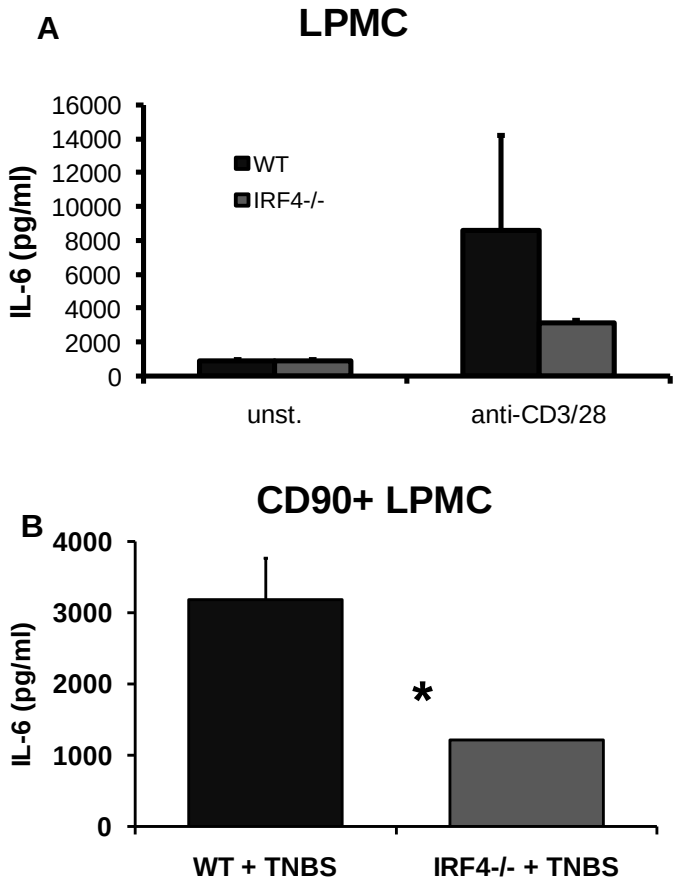


*Figure 3.11 IL-6 productions of lamina propria mononuclear cells (LPMC). A) LPMC were isolated from untreated mice and stimulated with anti-CD3/28 for 2 days. B) CD90+ T cells were selected from LPMC by using MACS selection kit and stimulated with anti-CD3/28 for 2 days. Data represented 3 separate experiments.*P<0.05.*

3.4.2 Expression and production of cytokines in transfer colitis model

Furthermore, cytokine expression and production was studied in T cell transfer colitis model. Quantitative measurement of IL-6mRNA and IL-17mRNA in the colon indicated that expressions of both cytokines were significantly diminshed in RAG2-/- mice received IRF4 deficient T cells. What's more, IL-22 expression in colon was also

decreased in the absence of IRF4. Anti-inflammatory cytokine IL-10 did not shown significant difference between these two groups.

ELISA measurement of IL-6 and IL-17 levels produced by splenocytes isolated form T cell-transferred RAG2-/- mice revealed that the Release of IL-6 and IL-17 were significantly reduced when IRF4 was absent.

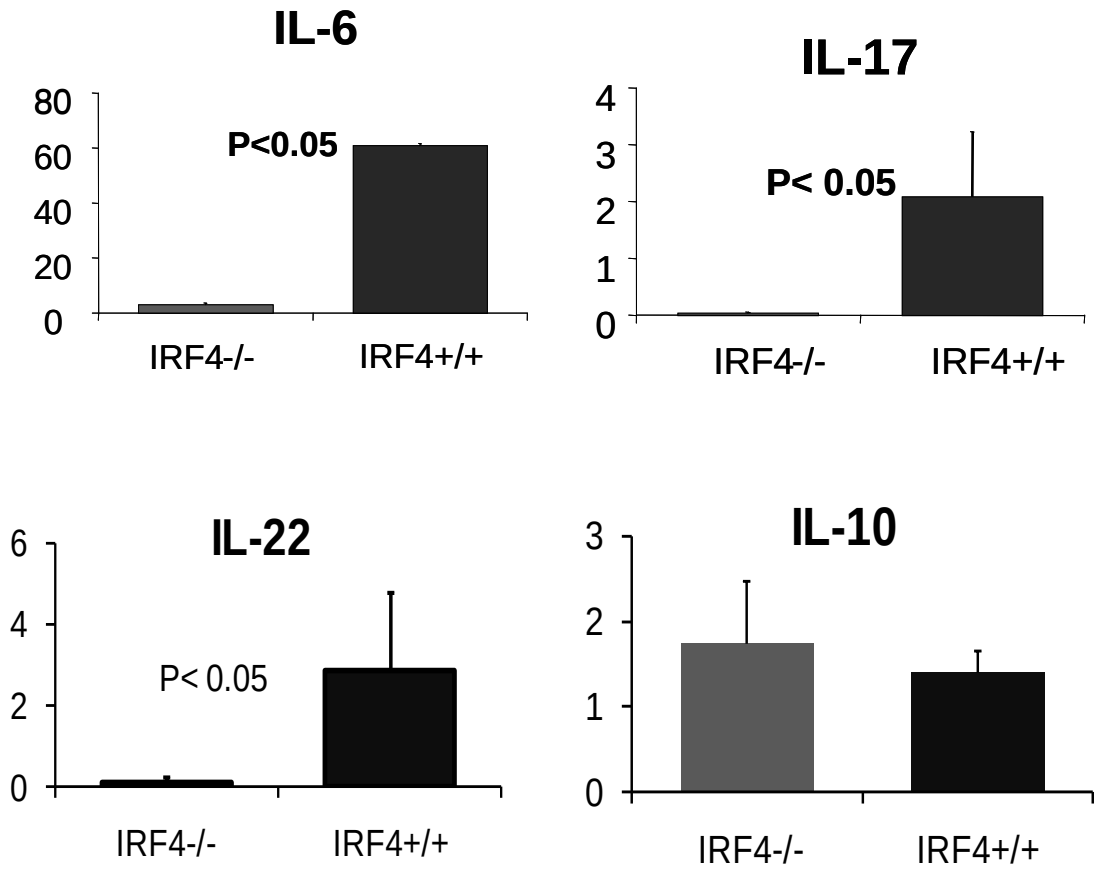


Figure 3.12 Cytokines expression in transfer colitis model. Expression levels of IL-6mRNA, IL-17mRNA, IL-22mRNA and IL-10mRNA in colon were quantitative assessed by using real time PCR. RAG2-/- mice reconstituted with IRF4 deficient T cells are shown in grey column. RAG2-/- mice received IRF4+/+ T cells transfer are shown in black column. Values represent mean ± SD from 3 separate experiments (n=6 per group).

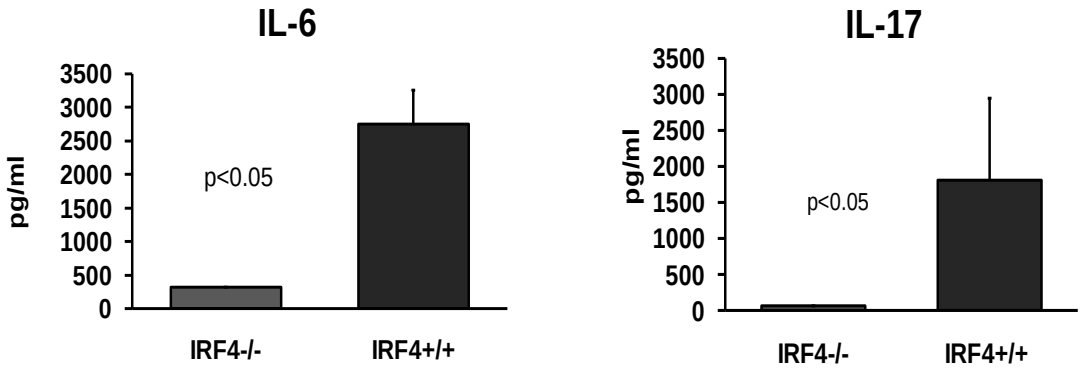
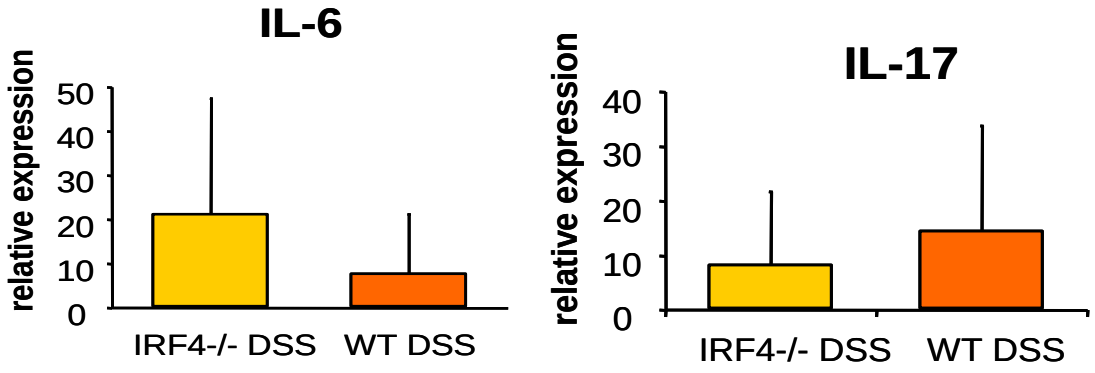


Figure 3.13 IL-6 and IL-17 productions in transfer colitis model. Transfer colitis was conducted as described above. At day 14, splenocytes from IRF4-/- and IRF4+/+ groups were isolated and stimulated with anti-CD3/28 for 2 days. Die supernatant was collected for IL-6 and IL-17 ELISA measurement. Data were from two experimetns with n=4 per group.

3.4.3 Expression of cytokines and related transcription factors in DSS colitis model

Levels of IL-6mRNA, IL-17mRNA, IL-22mRNA in colon from DSS-treated mice were assessed by real time PCR. No significant differneces in expressions of all these cytokines between DSS-treated IRF4-/- mice and DSS-treated IRF4+/+ mice were found out. Quantitative measurement of the key regulators for IL-17 revealed no alteration in ROR-γTmRNA level between DSS-treated IRF4-/- and WT mice, wehreas ROR-α mRNA was significantly diminshed when IRF4 was defective.



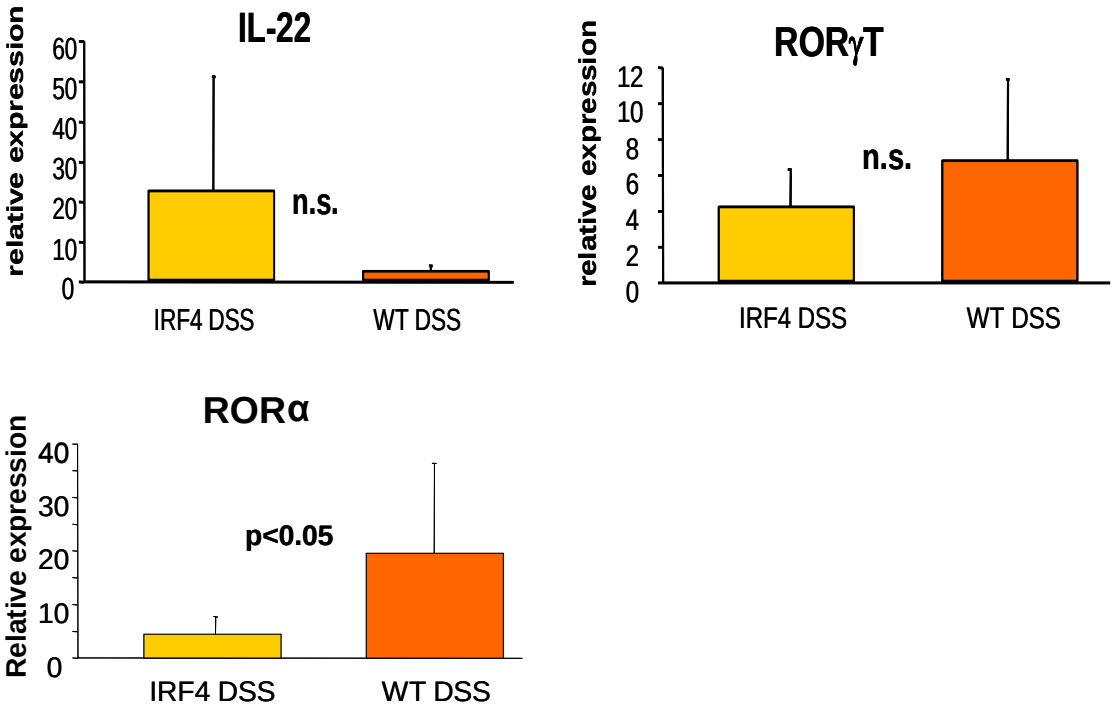


Figure 3.14 Quantitative analysis of mRNA levels of cytokines and transcriptional factors in DSS colitis colon. Relative expreesion of IL-6 mRNA, IL-17 mRNA, IL-22 mRNA, ROR-γT mRNA, and ROR-α mRNA were detected by using real time PCR. Data represented three separate experiments with n=3 per group.

3.5 Detection of apoptosis in colitis models

It has been demonstrated that proinflammatory cytokines such as IL-6, TNF-α reduce T cell apoptosis in lamina propria. Accumulated T cells in lamina propria can further aggregate intestinal inflammation.

To study apoptosis (programmed cell death) in inflammed tissue, TUNEL stain was performed. Increased apoptotic cells in colon were found in TNBS treated IRF4-/- mice compared with TNBS treated WT mice.

In mucosa of RAG2-/- mice reconstituted with IRF4 deficient T cells, much more apoptotic cells were detected than in the mucosa of RAG2-/- mice transferred with WT T cells.

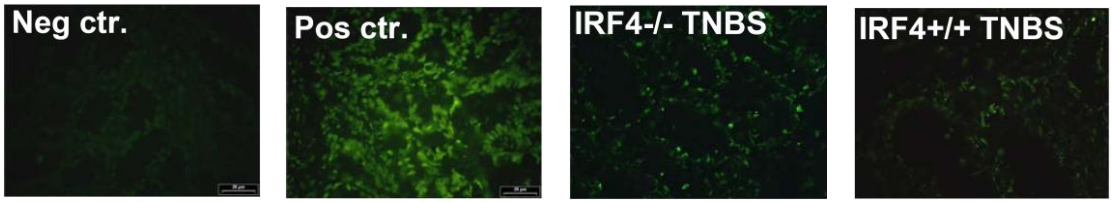


Figure 3.15 TUNEL staining of colonic cryosections form mice with TNBS-induced colitis. Cryosection of colon from TNBS-treated IRF4-/- mice and WT mice were stained for detection of apoptosis. Original magnification x300. Data were published on J Clin Invest. 2008 Jul;118(7):2415-26.

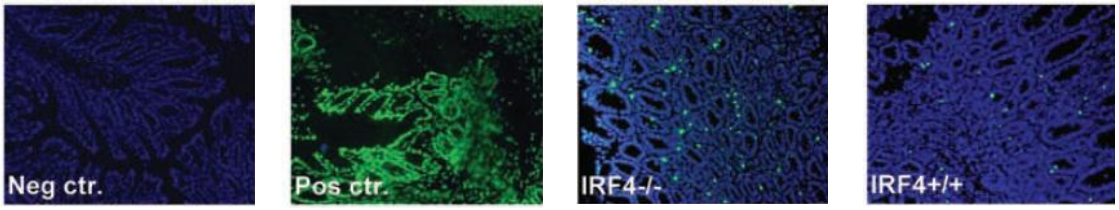


Figure 3.16 TUNEL assay on colonic cryosections from RAG2-/- mice reconstituted with either IRF4 deficient T cells or WT T cells. Original magnification x300 Data were published on J Clin Invest. 2008 Jul;118(7):2415-26.

3.6 Regulatory factors for Th17 subsets

In the last few years, a new subset of effecter T cells,Th17, was reported and investigated. Th17 cells produce IL-17, IL-22 and IL-6. Th17 cells have been reported to play an important role in autoimmune diseases. ROR-γT and ROR-α are the key transcriptional factors to modulate Th17 differentiation[61, 162].

In the present study, IRF4-/- mice were found to have a defective expression and production of IL-17. To investigate whether the abolished IL-17 expression in IRF4-/- mice was due to the decreased expression of ROR-γT and ROR-α, quantitative assessment of ROR-γT and ROR-α mRNA in IRF4-/- and IRF4+/+ transfer colitis groups were performed by using real time PCR. Both the expression of ROR-γT and expression of ROR-α in IRF4 deficient T cell transfered group were markedly downregulated compared with in IRF4+/+ T cell transfered group.

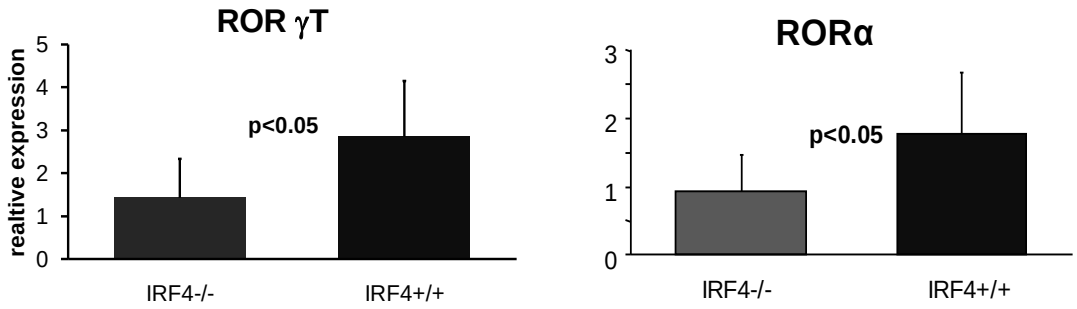


Figure 3.17 Quantitative measurement of ROR-γT and ROR-α mRNA in transfer colitis mice. RNA was isolated from colon samples of RAG-/- mice reconstituted with IRF4-/- T cells or IRF4+/+ T cells and further used for real time PCR of RORα and RORγT. N=6 per group. Values represent mean±SD.

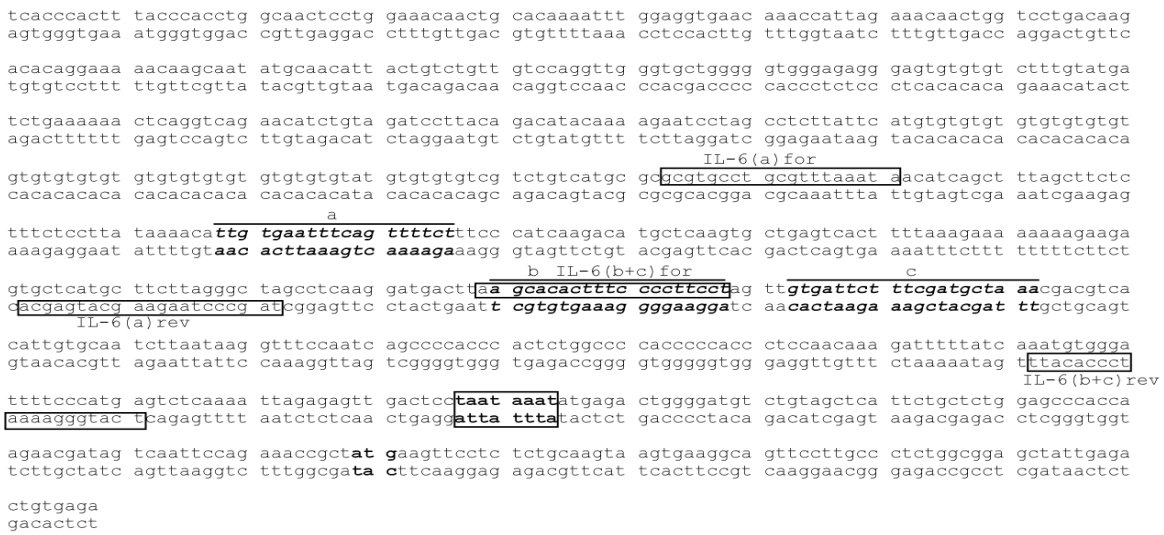
3.7 Analysis of IRF4 as transcription factor to regulate IL-6, IL-17 and ROR-α expressions

3.7.1 Promoter prediction

As it was shown above, IL-6, IL-17 and ROR-α expressions were down regulated when IRF4 was deficient. In order to differentiate to Th17 cell population from naïve T cells, IL-6 was used as stimulator. The excellular addition of IL-6 could not converse the abolishement of IL-17 on IRF4 deficient T cells, which indicated that downregulation of IL-6 in IRF4-/- cells was more like the result of decreased IL-17 than the trigger. Since it has been published that ROR-γ and ROR-α are two key factors to regulate Th17 population, we suggested whether the abolished expression of IL-17 was due to the decreased expression of ROR-α by IRF4 deficiency. Otherwise, IRF4 maybe directly modulate IL-17 production independently with ROR-α and ROR-γ.

By using software MatInspector, murine IL-6, IL-17, and ROR-α promoters were analyzed. Possible binding sites of IRF4 on these promoters were found. Three potential binding sites of IRF proteins were found within IL-6 and IL-17 promoters, and indicated as a, b and c. Two binding site of IRF were found within RORα promoter and indicated as a and b.

A) Mouse IL-6 promoter:

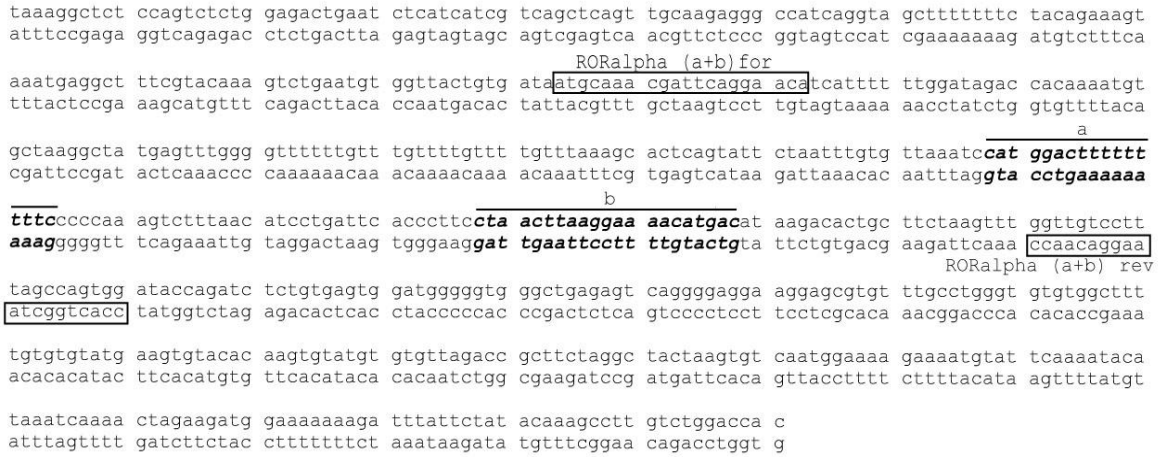


IL-6(a) forward: 5'- GCG TGC CTG CGT TTA AAT A-3'
reverse: 5'- TAG CCC TAA GAA GCA TGA GCA -3'
IL-6(b+c) forward: 5'- AAG CAC ACT TTC CCC TTC CT-3'
reverse: 5'- TCA TGG GAA AAT CCC ACA TT-3'

B) Mouse IL-17 promoter:

IL-17(a+b) forward: 5'- TTC CCT TCT CAT CCC TCA TC-3'
reverse: 5'- GGT TCG GTA TCA AGC CTT TC-3'
IL-17(c) forward: 5'- AAC AGT TGC GGT ACT CAG-3'
reverse: 5'- GCC CAA AGA AAC CCA CTC AAT G-3'

C) Mouse ROR-α promoter:



ROR alpha (a+b) for.: 5'-ATGCAAACGATTCAGGAACA-3'

ROR alpha (a+b) rev.: 5'-CCACTGGCTAAAGGACAACC-3'

Figure 3.18 localization of IRF4 binding sites on IL-6, IL-17 and ROR alpha promoters. Primers used for PCR were shown in rectangle. A) Sequence of murine IL-6 promoter. Putative cis elements were predicted by using MatInspector software. Three potential binding sites of IRF proteins within the IL-6 promoter were indicated as a, b and c. Binding sites b and c were analyzed as one by ChIP. B) Sequence of murine IL-17 promoter. Binding sites b and c of three binding elements (a, b and c) were examined together. C) Sequence of murine ROR-α promoter. Two binding sequences (a+b) of IRF4 were found on ROR-α promoter.

3.7.2 Chromatin Immunoprecipitation

Chromatin immunoprecipitation was used to verify these computer predicting binding sites of IRF4 within IL-6, IL-17 and ROR-α promoters. Wild-type (C57BL/6) splenocytes were isolated and stimulated with anti-CD3/CD28 antibodies for 12 hours. After harvesting, the cells were crosslinked with formaldehyde, and chromatin extracts were prepared as described in Material&Methods and subjected to immunoprecipitation with anti-IRF4. The purified DNA was subjected to RT-PCR with primers specific for the murine IL-6, IL-17 or ROR-α promoters. In the case of the IL-6 promoter, a primer pair was designed to analyze binding site a, while a second primer pair was designed to cover the potential IRF4 binding sites b and c. For analyzing IL-17 promoter, a primer pair including the IRF4 binding sites a and b was performed while a separate primer set was used to detect the third potential IRF4

binding site c. The binding site a and b within ROR-α promoter was analyzed together by using one primer pair.

There was no IRF4 specific signal on IL-6 promoter or on ROR-α promoter. However, a marked signal for IRF4 binding sites a and b on IL-17 promoter was detected. Thus, two potential in vivo binding sites of IRF4 within the IL-17 promoter could be discovered.

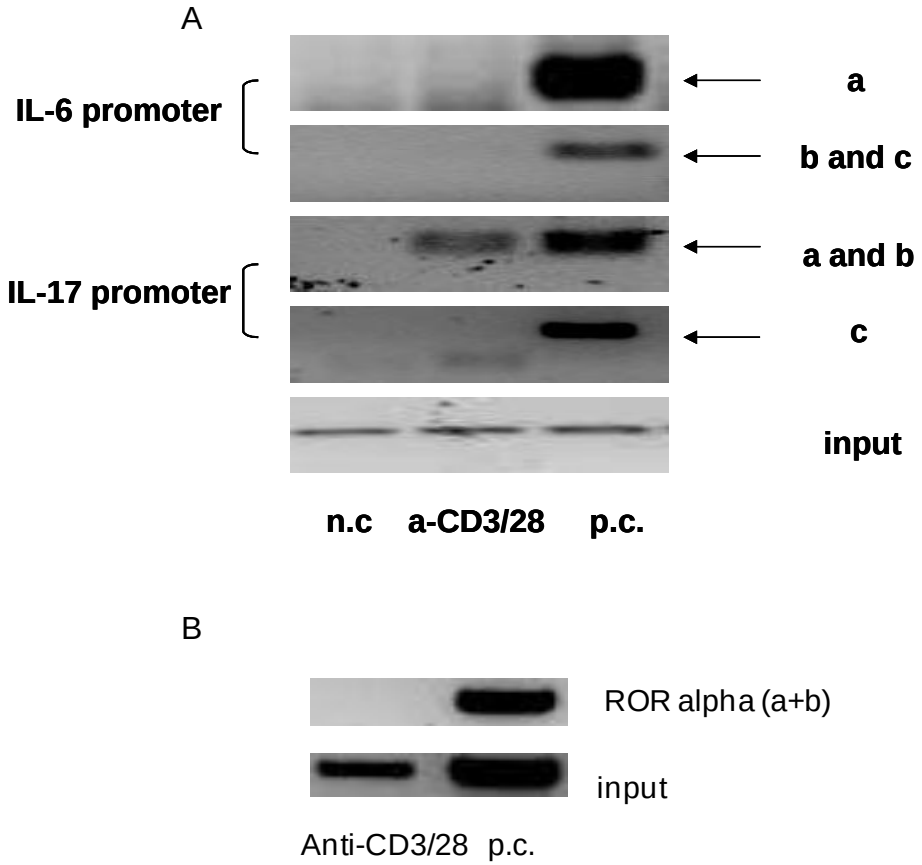


Figure 3.19 ChIP. Freshly isolated splenocytes from C57BL/6J mice were stimulated with anti-CD3/28 for 12 hours. ChIP was performed. Purified DNA samples were subjected to RT-PCR using primers listed above. N.C. (negative control) indicated non-antibody immunoprecipitations by incubating the supernatant fraction with protein Agarose/Salmon Sperm DNA. P.C (positive control) were the samples from genomic DNA. A) ChIP didn't reveal any signal of IRF4 binding on IL-6 promoter. However, lane in the mittel indicated a signal of IRF4 on IL-17 promoter by analyzing binding site a and b on IL-17 promoter. B) There was no positive signal of IRF4 binding on ROR-α promoter.

3.7.3 EMSA

To further characterize the two potential response elements of IRF4 (a and b) in the IL-17 promoter and to confirm the ChIP results of IL-17 and ROR-α promoters, we utilized oligonucleotides corresponding to the identified IRF4 binding elements within the IL-17 promoter or ROR-α promoter to carry out electrophoretic mobility shift assays (EMSA). Corresponding oligonucleotides for detection of IRF4 binding sites were designed and labeld with IRDye700. Unlabeled oligo were used to perform auto competition assay. Consensus sequences of IRF4, which were usually GAA or GAAA, were mutated to perform cross competition assay.

A)

B)

IRF4-1 (b):

Sense: 5´- AGT CCC TTA TTC TTT CAC TTC ATT TCC TTC C - 3´

Antisense: 5´- GGA AGG AAA TGA AGT GAA AGA ATA AGG GAC T - 3´

IRF4-2 (a):

Sense: 5´- CCT TCA TCT TGA TTT CTA ATT CTT TCT TCG A - 3´

Antisense: 5´- TCG AAG AAA GAA TTA GAA ATC AAG ATG AAG G - 3´

mutant IRF4-1/2: TTT

C)

D)

IRF4-3 (a):

sense: 5’-TCC ATG GAC TTT TTT TTT CCC CCA AAG TCT-3’

antisense: 5’-AGA CTT TGG GGG AAA AAA AAA GTC CAT GGA-3’

IRF4-4 (b):

sense: 5’-TCC TAA CTT AAG GAA AAC ATG ACA TAA GAC-3’

antisense: 5’-GTC TTA TGT CAT GTT TTC CTT AAG TTA GGA-3’

mut IRF4-3/4: TTTT

Figure 3.20 A) Designed oligonucleotides for EMSA contained response elements of IRF4 a and b on IL-17 promoter. B) Oligonucleotide sequences to identify IRF4 binding sites of IL-17 promoter were listed. Consensus sequence of IRF4 (GAA) were mutated to TTT. C) Designed oligonucleotides for EMSA contained response elements of IRF4 a and b on ROR-α promoter. D) Oligo sequences to study IRF4 response elements on ROR-α promoter. Unterlined consensus sequences GAAA of IRF4 were mutated to TTTT.

Band shift analysis revealed a specific signal using the IRDye700 labelled IRF4-2 oligo that covered binding site a, while the IRF4-1 showed no such signal. Specificity of this band could be shown by auto competition with unlabelled IRF4-2 oligo, while cross competition with a probe containing a mutated IRF4 core motif did not abrogate this band. Finally, this band was lacking when nuclear extracts from IRF4-/- splenic cells were used which indicated that IRF4 binds to response elements a of the IL-17 cytokine gene promoter.Another EMSA experiment to check the binding possibility of IRF4 on ROR-α promoter was performed. As the results detected by ChIP, EMSA also revealed no specific binding signal of IRF4 on ROR-α promoter.

IL-17 promoter

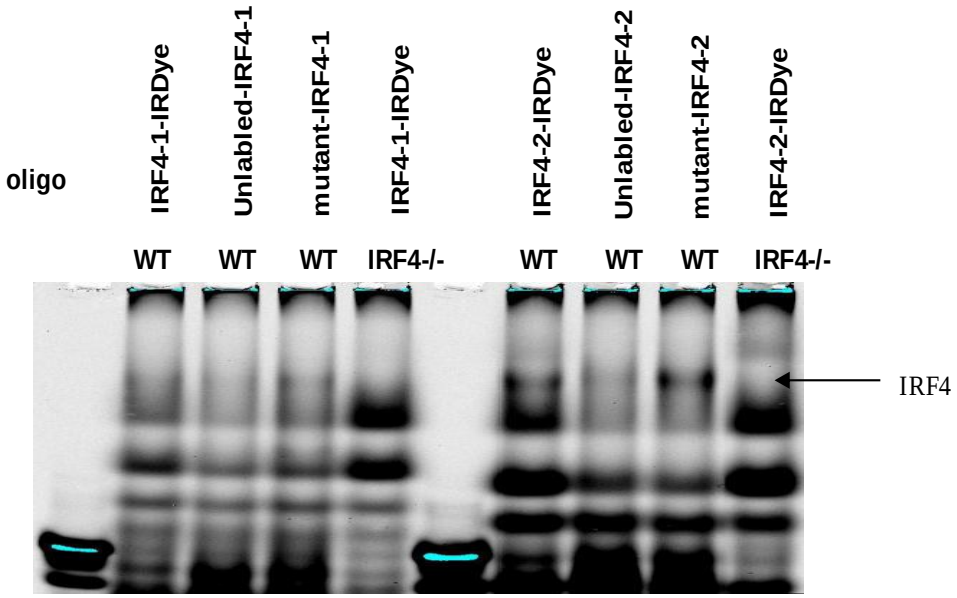


Figure 3.21 EMSA to identify IRF4 binding sites on IL-17 promoter. 5µg nuclear extracts obtained from WT or IRF4 deficient splenocytes were incubated with IRDye-700-labeled probe in the presence or absence of 200-fold excess of the indicated unlabeled competitor or IRDye-700-labeled mutant competitor. Oligo IRF4-1 was used to analyze binding site b of IRF4, whereas oligo IRF4-2 was performed to study binding site a.

RORα promoter

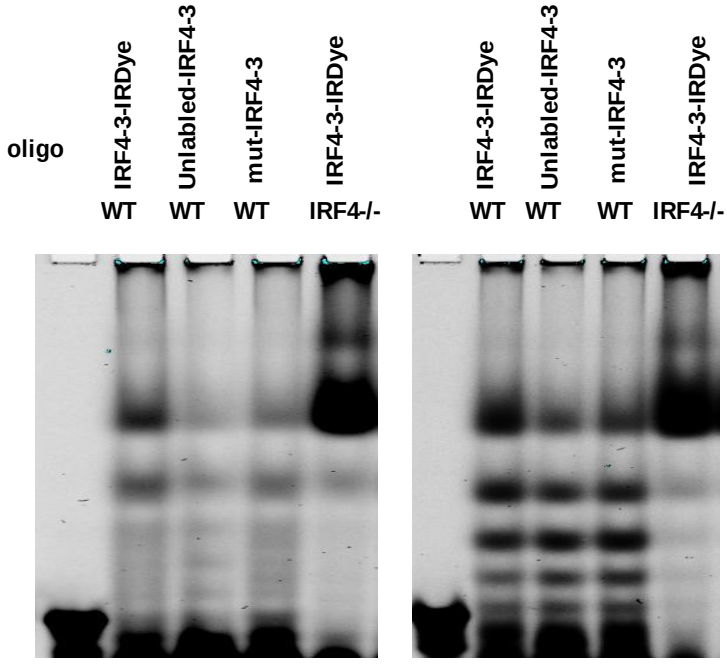
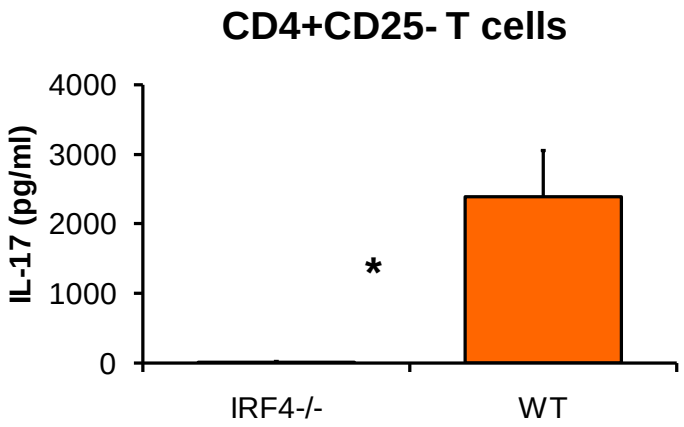


Figure 3.22 EMSA to identify IRF4 binding sites on ROR-α promoter. 5µg nuclear extracts obtained from WT or IRF4 deficient splenocytes were incubated with IRDye-700-labeled probe in the presence or absence of 200-fold excess of the indicated unlabeled competitor or IRDye-700-labeled mutant competitor. Oligo IRF4-3 was used to analyze binding site a of IRF4 on ROR-α promoter, whereas oligo IRF4-4 was used to study binding site b.

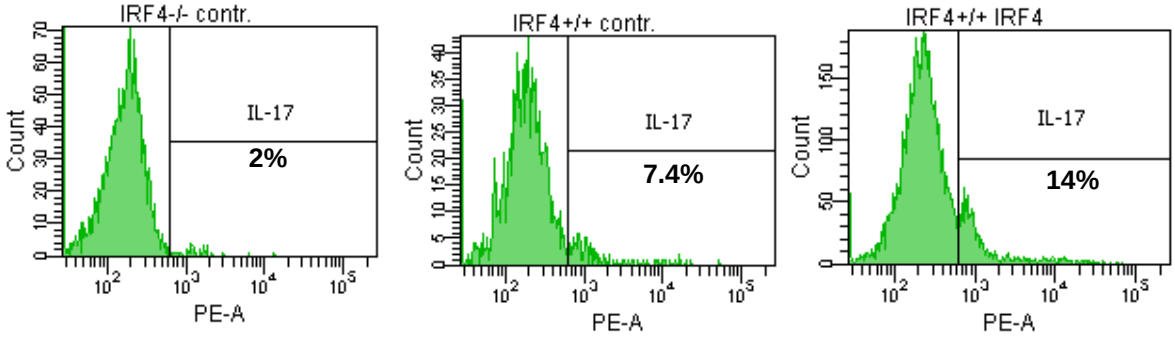
3.7.4 Overexpression of IRF4 in vitro

CD4+CD25- T cells isolated from spleen of IRF4-/- or WT mice were stimulated unter the Th17-inducing condition for 4 days. IL-17 concentration measured in the supernatant showed defective IL-17 production in IRF4-/- T cells. To analyzing whether IL-17 production will be influenced when IRF4 expression is upregulated in T cells, CD4+CD25- T cells isolated from spleen of IRF4-/- or WT mice were infected with retrovirus containing control vector or IRF4 expression vector. After 4 days cultivation unter Th17-inducing condition, supernatants were collected for IL-17 ELISA measurement and cells were further treated for intracellular IL-17 staining and then analysed by FACS. IRF4 deficient T cells infected with control vector showed only 2% intracellular produciton of IL-17, whereas IRF4+/+ T cells infected with control vector showed 7.4% production of IL-17. When the intracellular expression of IRF4 in IRF4+/+ T cells were increased by infection with retrovirus containing IRF4 expression vector, intracellular IL-17 expression was increased almost two fold compared with infection with control vector. Figure c showed statistical significance of FACS results. These results were proved by measurement of IL-17 in supernatants. IL-17 level in the supernatant collected from the same experiment indicated defective IL-17 secretion in absence of IRF4 and overexpression of IRF4 increased release of IL-17.

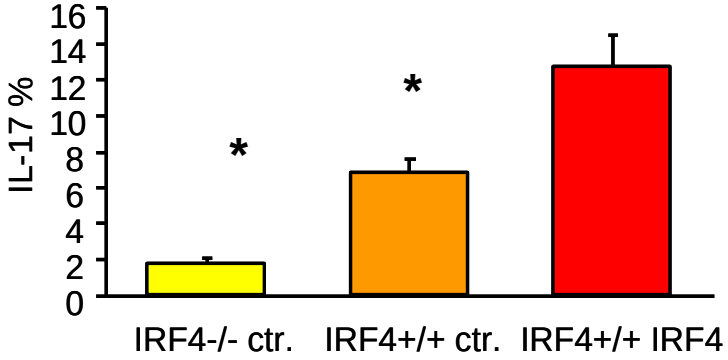
A


CD4+CD25- T cells
IL-17 (pg/ml)
4000
3000
2000
1000
0
*
IRF4-/-
WT

B)


IRF4-/- contr.
IL-17
2%
Count
PE-A
IRF4+/+ contr.
IL-17
7.4%
Count
PE-A
IRF4+/+ IRF4
IL-17
14%
Count
PE-A

C)


IL-17 %
16
14
12
10
8
6
4
2
0
*
*
IRF4-/- ctr.
IRF4+/+ ctr.
IRF4+/+ IRF4

D)

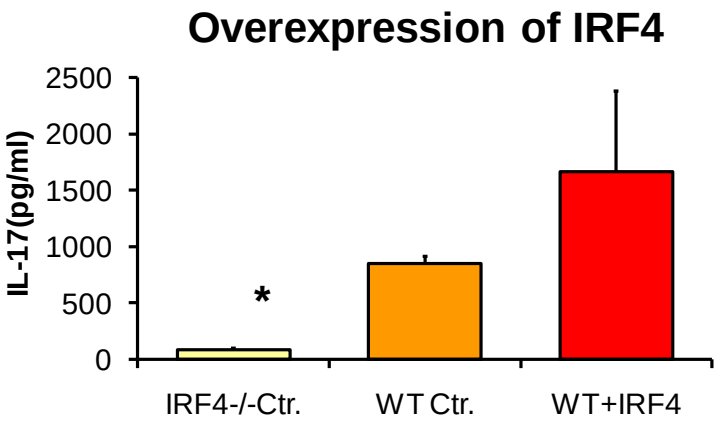


Figure 3.23 Qverexpression of IRF4 in vitro. A) CD4+CD25- splenocytes isolated from IRF4-/- or WT mice were stimulated with anti-CD3/28, anti-IFN-γ, anti-IL-4, hyper IL-6, TGF-ß for 4 days. Supernatants were collected for IL-17 measurement by ELISA. B) freshly isolated naïve T cells from IRF4-/- or WT mice were infected with retrovirus containing either control vector or IRF4 expression vector, and further stimulated unter Th17 differentiation conditions for 4 days. Intracellular IL-17 staining was performed by FACS. C) Statistic analysis of FACS results from two experiments. D) IL-17 ELISA measurement by using the supernatants from B).

3.7.5 Induction of IL-6 expression in fibroblasts

Colonic fibroblasts are not only the target of cytokines, but also a source of wide spectrum of cytokines. Colonic lamina propria fibroblasts isolated from untreated WT mice were stimulated with IL-6 or IL-17a for 12 hours. RNA was derived from cell lysate and real time PCR was performed to assess the relative expression of IL-6 mRNA. Stimulation with IL-17a induced dramatically expression of IL-6 in colonic lamina propria fibroblasts, which suggested the defective IL-17 expression could induce decreased IL-6 expression.

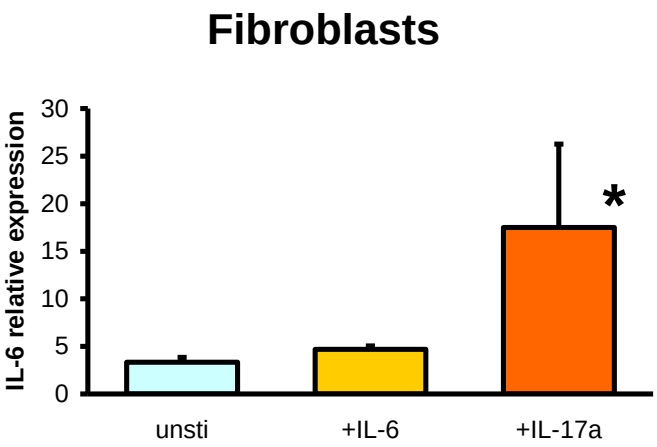


*Figure 3.24 IL-17a increased IL-6 expression in colonic lamina propria fibroblasts. Colonic lamina propria fibroblasts were isolated from untreated WT mouse and stimulated with IL-6 (100ng/ml) or IL-17a (100ng/ml) for 12 hours. RNA from the cell lysate was used for real time PCR to analyze expression of IL-6. N=2 per group.*p<0.05.*

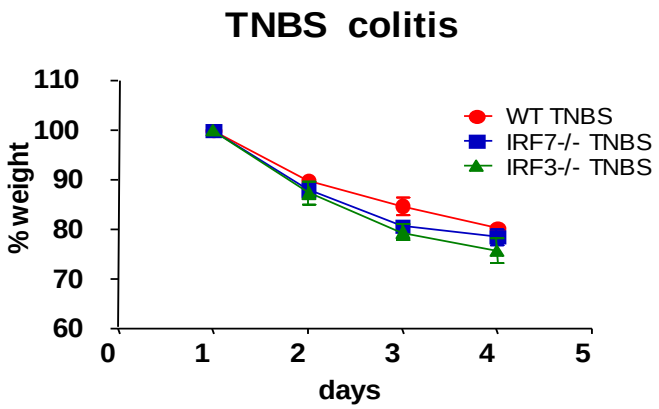
3.8 IRF3 and IRF7 in experimental colitis

IRF3 and IRF7 are two key transcriptional factors, which regulate type I IFN after virus infection. In the present study, these two factors were firstly studied in T cell-induced colitis.

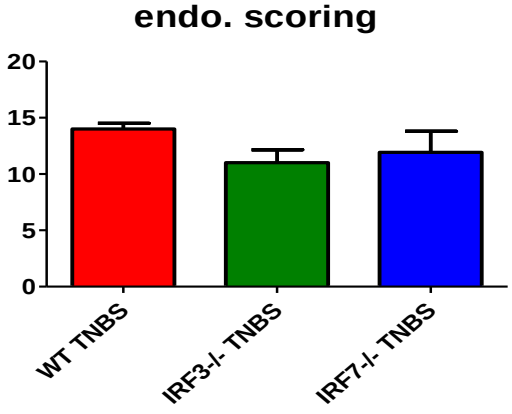
3.8.1 TNBS colitis

TNBS-induced colitis was performed as described before. IRF3-/-, IRF7-/- and WT mice were suffered from colon inflammation after treatment with TNBS. All the three groups lost body weight and showed no alteration in disease severity by endoscopic and histological analysis.

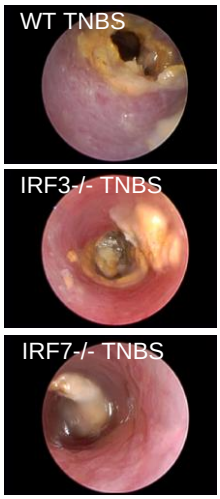
A)



B)



C)



D)

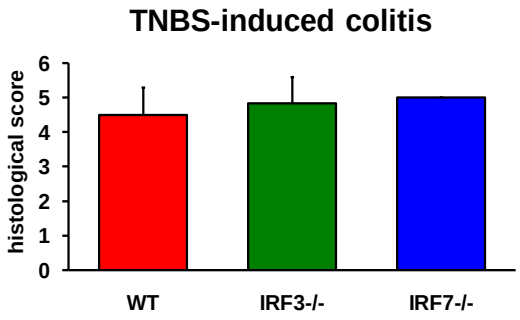


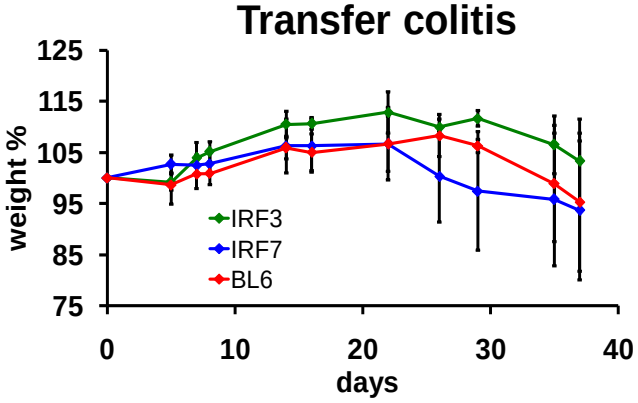
Figure 3.25 IRF3 and IRF7 in TNBS colitis. IRF3-/-, IRF7-/- or WT mice were treated with TNBS as described before. Weight was monitored (A) and at the end of experiment, colon inflammation was scored by endoscopy (B)&(C) and histology (D). Data represented three separate experiments with n=15 per group.

3.8.2 Transfer colitis

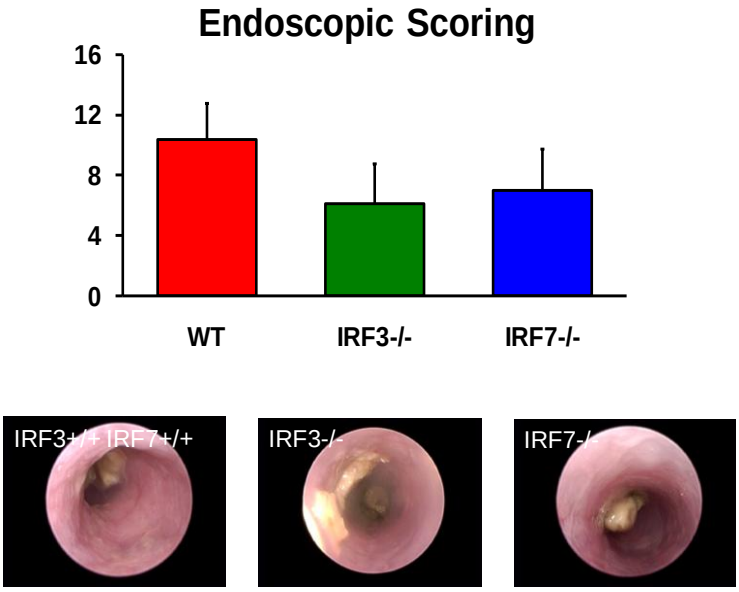
T cell transfer colitis was performed as described in materials and methods. Briefly, CD4+CD25- T cells were isolated from spleen of IRF3-/-, or IRF7-/- or WT mice and $5X10^5$ cells were i.p. injected into RAG1-/- mice. The changes of body weight were followed for one month and endoscopic and histological scoring was performed as described before.

RAG1-/- mice reconstituted either with IRF3-/- T cells, or with IRF7-/- T cells developed colitis as well as RAG1-/- reconstituted with WT T cells. No significant difference in colon inflammation among these three groups was detected by endoscopic and histological analysis.

A)



B)



C)

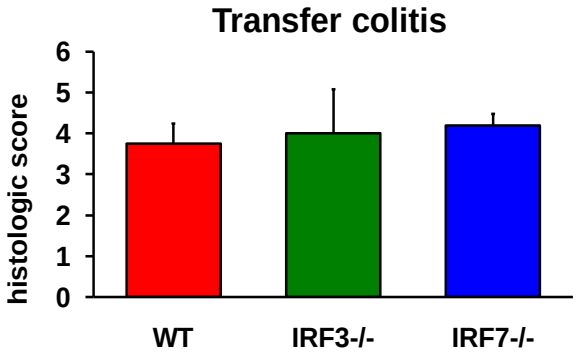
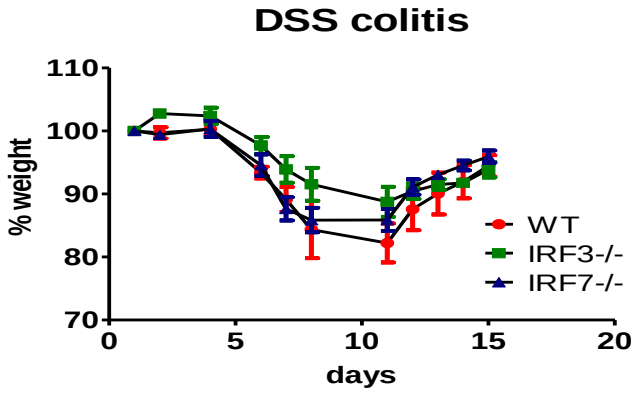


Figure 3.26 IRF3 and IRF7 in transfer colitis. CD4+CD25- T cells isolated from IRF3-/-, IRF7-/- or WT mice were i.p. injected into RAG1-/-. The changes of weight were monitored (A). Colon endoscopy (B)) and histology (C) were performed at the end of experiments. Data represented for 2 separate experiments with n=15 per group.

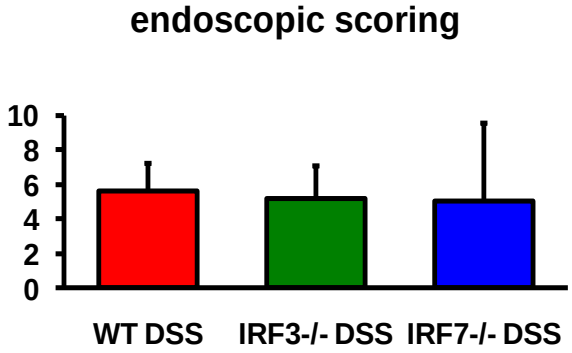
3.8.3 DSS colitis

Supplied with 2.5% DSS in drinking water for 7 days, followed with 7 days normal drink water induced colitis not only in WT mice, but also in IRF3-/- mice, and in IRF7-/- mice. There was no significant difference among these three groups in disease severity.

A)



B)



C)

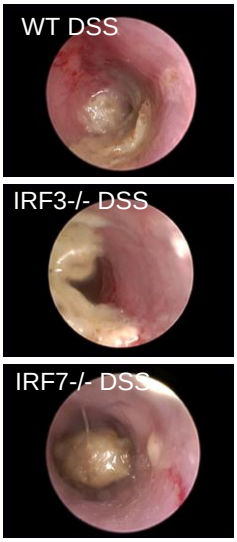


Figure 3.27 IRF3 and IRF7 in DSS colitis. IRF3-/- mice, IRF7-/- mice and WT mice were supplied with 2.5% DSS in drinking water for 7 days, followed with 7 days normal drink water. Changes of body weight were followed and colon endoscopy was conducted. Data represented from 3 separate experiments with n=15 per group.

3.9 IRF3 and IRF7 in innate immune responses

To determine the roles of IRF3 and IRF7 in innate immune responses, cecal ligation and puncture was performed to induce polymicrobial sepsisl.

In sepsis, the large amounts of microorganisms, endotoxins and exotoxins in the blood stimulate the production of enormous quantities of several cytokines, notably TNF, IL-6 and IL-1. The results are increase of circulating levels of the cytokines and changes of the form of the host response.

3.9.1 Survival analysis in polymicrobial sepsis model

IRF3-/-, IRF7-/- and WT mice were performed ceceal ligation and puncture (CLP) as described in “materials and methods” to induce polymicrobial sepsis. Survival analysis was followed for two weeks. 80% of IRF3-/- mice survived, whereas 80% of WT mice and less than 10% of IRF7-/- mice died within 2 days after operation.

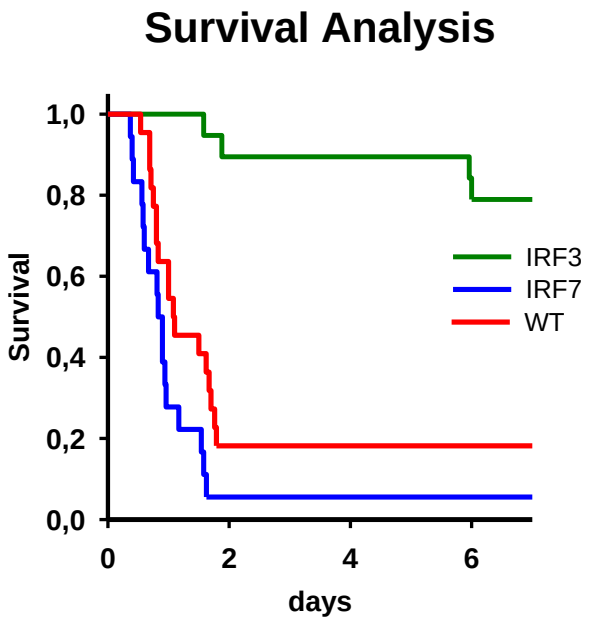
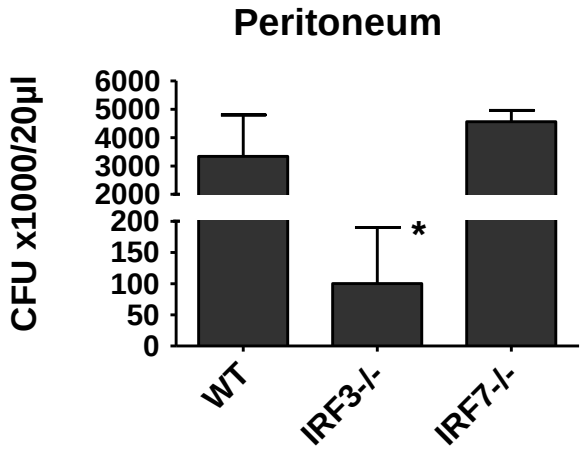


Figure 3.28 Survival analysis of polymicrobial sepsis model. IRF3-/-, IRF7-/-, and WT mice were performed CLP to induce polymicrobial sepsis. Survival rate was monitored for two weeks. Data represented three separate experiments with n=20 per groups. Significant difference was shown between IRF3-/- and WT mice, IRF3-/- and IRF7-/- mice, with p<0.01.

3.9.2 Bacterial clearance after CLP

In a separate experiment, mice were sacrificed 24 hours after CLP. Peritoneal cavity was washed with 5ml of sterile PBS. 0.1g organs (lung, liver, and kidney) were added to 1ml PBS to make homogenates. After serial dilution, 20µl peritoneal lavage fluid and homogenate from liver, lung, and kidney were plated on blood agar plates and incubated for 48 hours. IRF3-/- mice showed highest bacterial clearance both in peritoneal cavity and in organs than WT and IRF7-/- mice.

A)



B)

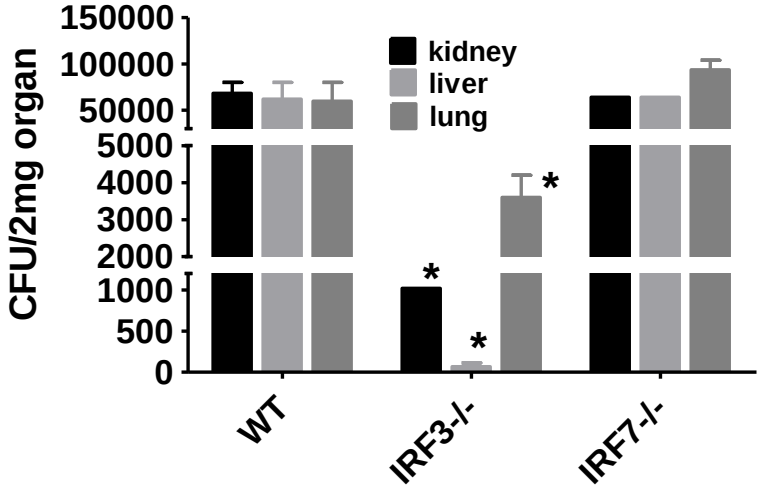


Figure 3.29 Bacterial clearance 24 hours after CLP. Dilution of peritoneal lavage fluids (A) or homogenate from organs (B) obtained from IRF3-/-, IRF7-/- and WT mice were cultured on blood agar plates for 48 hours. The numbers of bacterial colonies were counted (n=5 per group). Data shown are means of values ± SEM and were representative for two independent experiments with n=5 per group.

3.9.3 Multiple organ damage

Systemic inflammation during sepsis frequently causes multiple organ failure. The major target organs are liver, lung, kidney, spleen and gastrointestinal tract. Creatinine and urea are two markers of kidney function. GOP and GPT are two

markers for hepatic function. Mice were bled 24 hours after CLP. Separated plasma was sent to check clinical chemical values. Sepsis resulted in significant increases in the plasma level of creatinine, urea, GOT and GPT in WT mice and IRF7-/- mice compared with the levels in IRF3-/- mice.

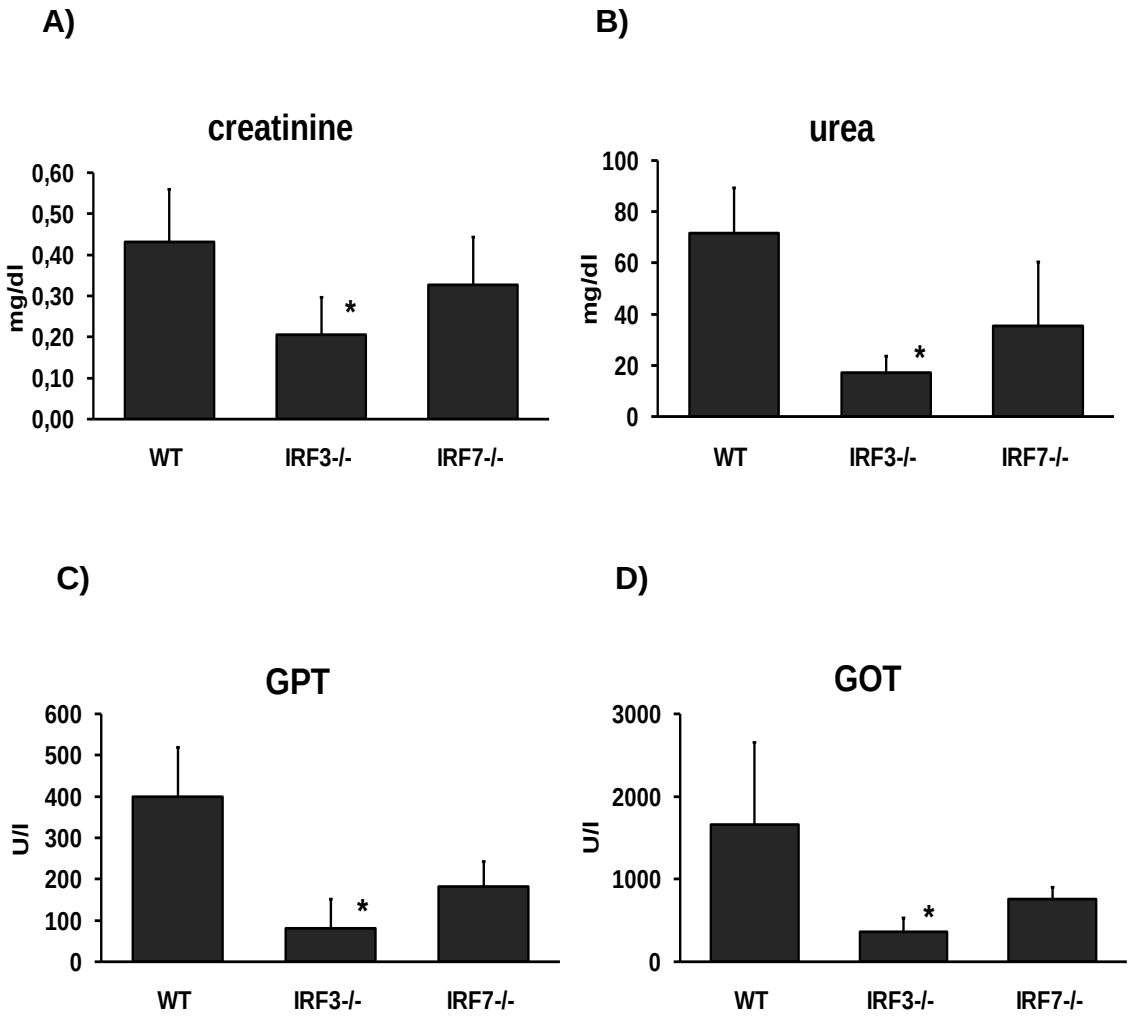
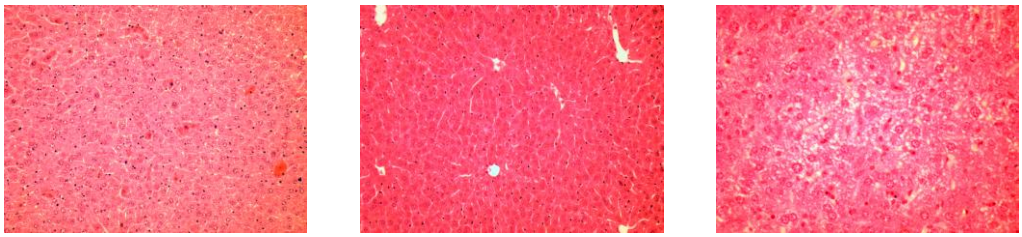


*Figure 3.30 Multiple organ damage after CLP. Mice were bled 24 hours after CLP. Liver and kidney functions were tested by checking clinical chemical values in the plasma. Creatinine (A) and urea (B) are two markers for kidney function, whereas GPT (C) and GOT (D) are two markers for liver function. Data are representative from two separate experiments with n=5 per group.*p<0.05.*

3.9.4 Histopathological analysis

There are some necrosis, increased apoptosis and lymphocyte infiltration, some ballooning and micro vascular steatosis with no specific architecture distribution pattern in the WT and IRF7-/- mice liver. The liver of IRF3-/- mice appeared normally.



WT liver IRF3-/- liver IRF7-/- liver

Figure 3.31 Histological detection of liver 24 hours after CLP. Liver from WT, IRF3-/- or IRF7-/- mice were taken out 24 hours after CLP, conserved in 4% Para formaldehyde and performed H&E stain. Histological analysis was conducted by pathologist Mr. Lehr. Original magnification x 300.

3.9.5 Cytokine production after CLP

After bacterial infection, endotoxin secreted from bacteria can induce production of chemokines and cytokines. High levels of TNF-α, IL-1 and IL-6 were major cytokines resulting further damages in the patients. Therefore, in the present study, the concentrations of these two cytokines were measured 24 hours after CLP by ELISA. The highest concentration of TNF-α is reached normally 6 to 12 hours after infection. Septic WT mice and IRF7-/- mice produced 4 times amount of TNF-α in peritoneal cavity as much as IRF3-/- mice. Splenocytes isolated from septic WT and IRF7-/- mice also produced much higher TNF- α than IRF3-/- mice. So was the production of IL-6 in peritoneal lavage and in plasma too.

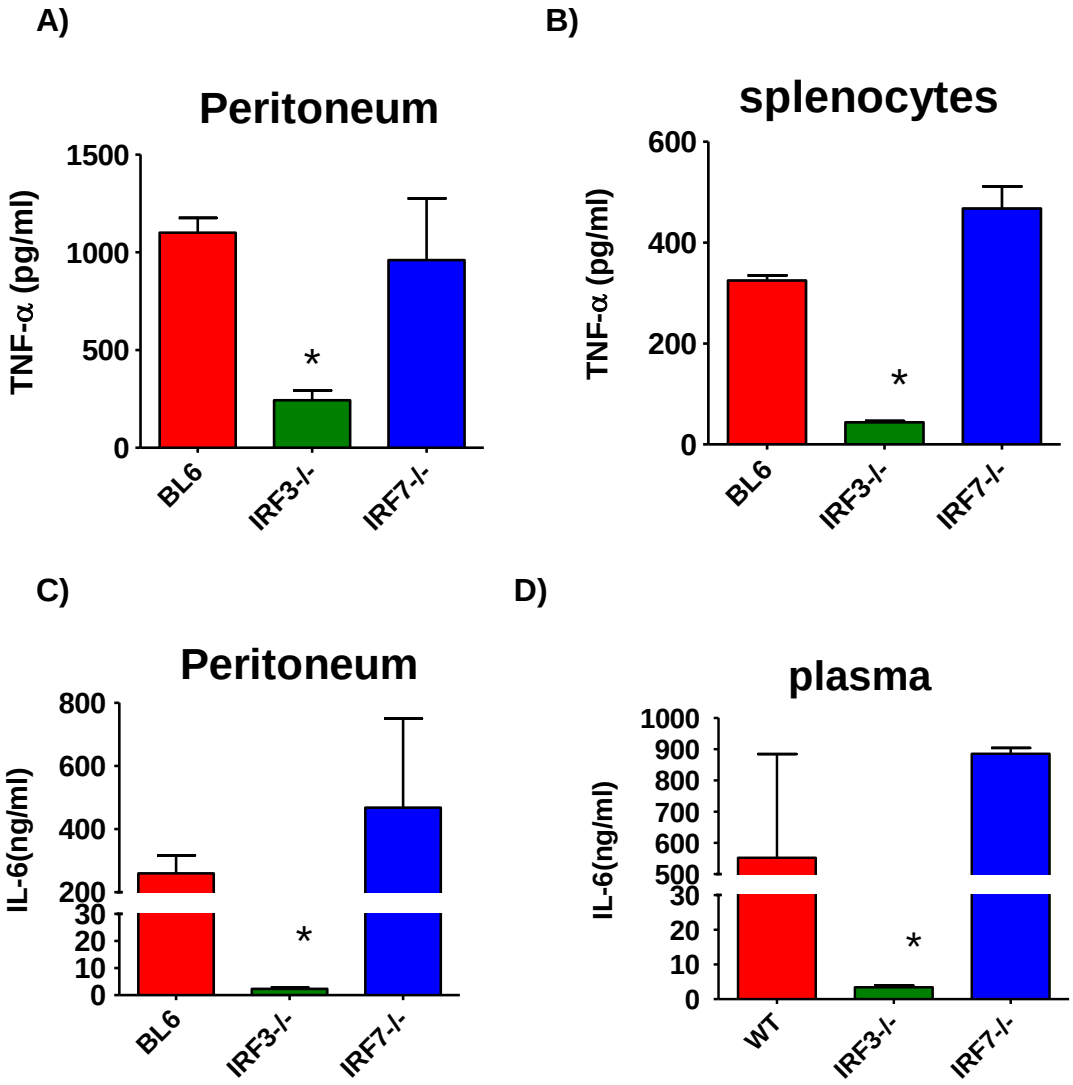


Figure 3.32 Production of cytokines in septic mice 24 hours after CLP. Mice were bled for preparation of plasma. Peritoneal fluids were obtained by washing peritoneal cavity with 5 ml PBS. Splenocytes of mice were isolated and cultivated for 48 hours and supernatant was collected. ELISA was performed to detect production of cytokines in all of these samples. TNF-α production in peritoneum (A) and from splenocytes (B). Release of IL-6 in peritoneum (C) and in plasma (D). Data were representative from two separated experiments with n=5 per group.

Several cytokine expressions in splenocytes were assessed by real time PCR. IFN-α4 mRNA and IFN-ß mRNA were obviously although not significantly less expressed in WT and IRF-7-/- mice complared with IRF3-/- mice. Proinflammatory cytokines such as IL-1ß and IL-17a were significantly higher expressed in former group than latter group. There was no significant difference in TGF-ß expression among these three groups.

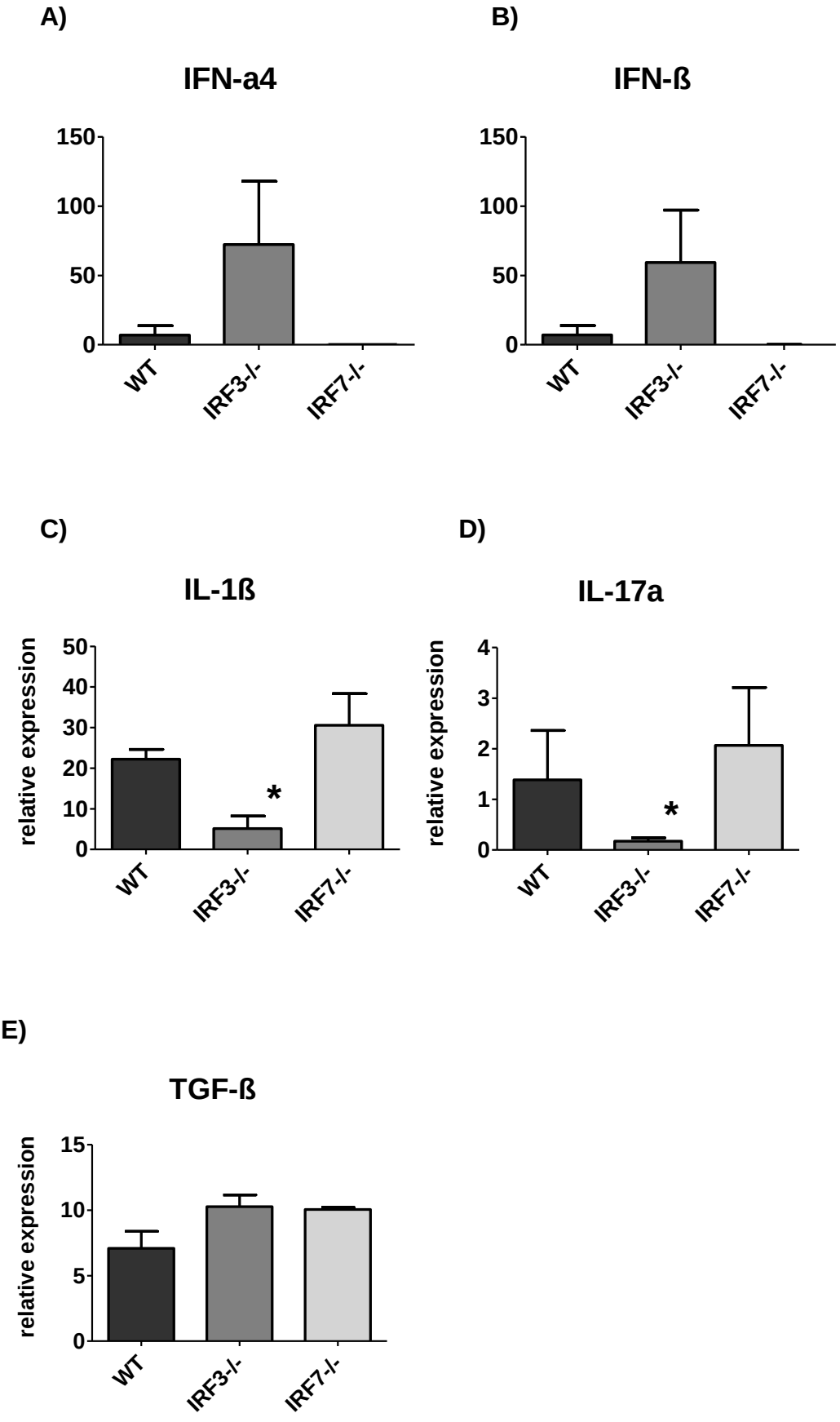

A)
IFN-a4
150
100
50
0
WT
IRF3-/-
IRF7-/-
B)
IFN-ß
150
100
50
0
WT
IRF3-/-
IRF7-/-
C)
IL-1ß
relative expression
50
40
30
20
10
0
*
WT
IRF3-/-
IRF7-/-
D)
IL-17a
relative expression
4
3
2
1
0
*
WT
IRF3-/-
IRF7-/-
E)
TGF-ß
relative expression
15
10
5
0
WT
IRF3-/-
IRF7-/-

Figure 3.33 Quantitative measurement of cytokine expression. RNA was isolated from splenocytes of septic mice. CDNA was synthesized as described in materials and methods. IFN-α4 mRNA (A) IFN-β mRNA (B), IL-1ß mRNA (c), IL-17a mRNA (D), and TGF-ß mRNA (E) were quantitative analysed by using real time PCR. N=5 per group. p<0.05.*

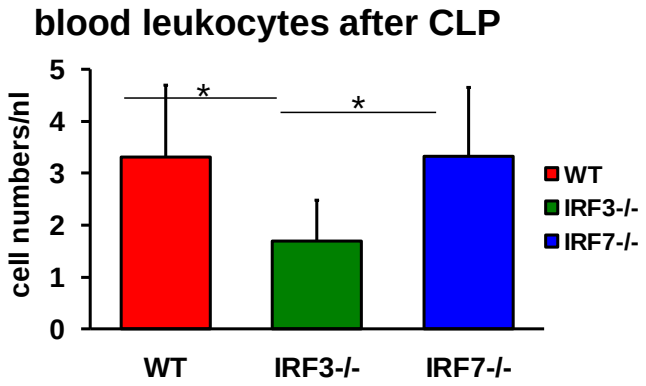
3.9.6 Leukocytes migration

Shortly after bacterial infection in peritoneum, circulating granulocytes and moncytes can migrate from plasma into local infection place. These so called phagocytes ingest pathogens and secrete reactive oxidants to kill the pathogens. They also produce chemokines and cytokines to improve further migration and immune responses.

3.9.6.1 Blood

Leukocytes in blood were measured by haemometer. There were less circulating leukocytes in IRF3-/- mice than WT and IRF7-/- mice 24 hours after CLP. However, the percentage of neutrophils and monocytes in circulation was higher in IRF3-/- mice than in WT and IRF7-/- mice, whereas the percentage of lymphocytes was less in former group than latter group.

A)



B)

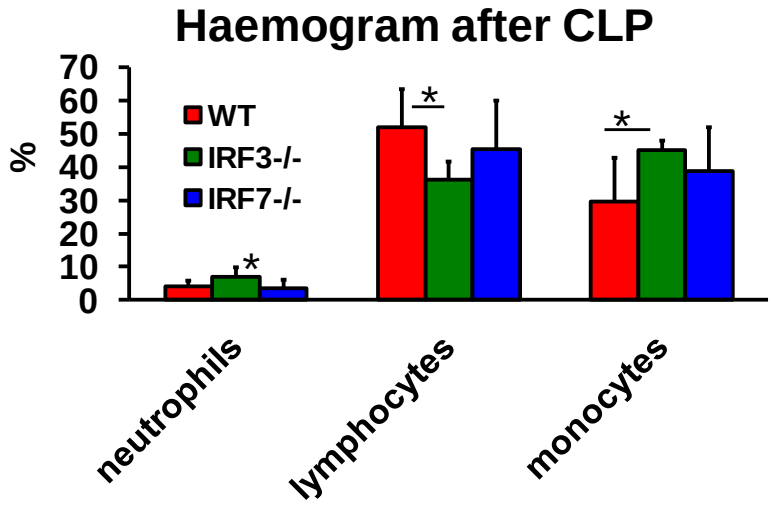


*Figure 3.34 Haemogram after CLP. Blood of mice was anticoagulated with 1mg/ml EDTA and analysed by haemometer in central laboratory. A) Total leukocyte number in blood. B) Percentage of neutrophils, lymphocytes and monocytes in blood. N=5 to 7 per group. *P<0.05.*

3.9.6.2 Peritoneum

More than two folds of cells were migrated into peritoneal cavity 24 hours after CLP in IRF3-/- mice than in WT mice. There was no significant difference in percentage of migrated neutrophils and dendritic cells.

A) **B)**

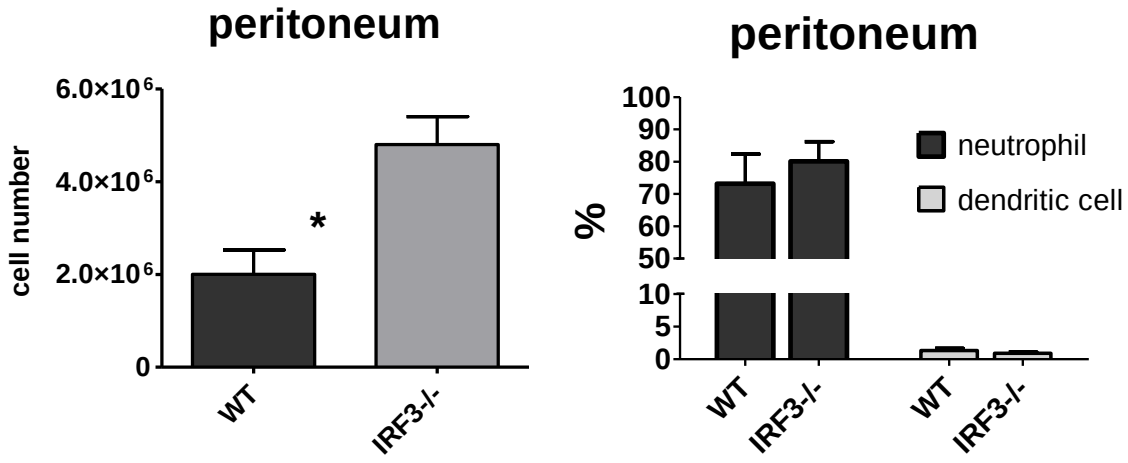


*Figure 3.35 Cell migration into peritoneum. After 24 hours of CLP, WT mice and IRF3-/- mice were sacrificed. Peritoneal cells were collected by washing with 5 ml ice cold PBS and then counted (A) in Neubauer chamber microscopically. (B) Peritoneal cells were incubated with anti-Gr1-PE or anti-CD11c-FITC and then performed FACS. Anti-Gr1 positive cells and anti-CD11c positive cells represented neutrophils and dendritic cells separately. Data shown were statistically analysed (n=3) and represented means of values ± SED.*p<0.05.*

3.9.6.3 Spleen

Spleen is an important organ to diminish died erythrocytes, store and produce haemopoitic cells. There was a slight decrease of splenocytes in IRF3-/- septic mice 24 hours after CLP compared with WT septic mice. Although the percentage of neutrophils was similar in both groups, higher percentages of macrophages and dendritc cells were observed in IRF3-/- mice than WT mice.

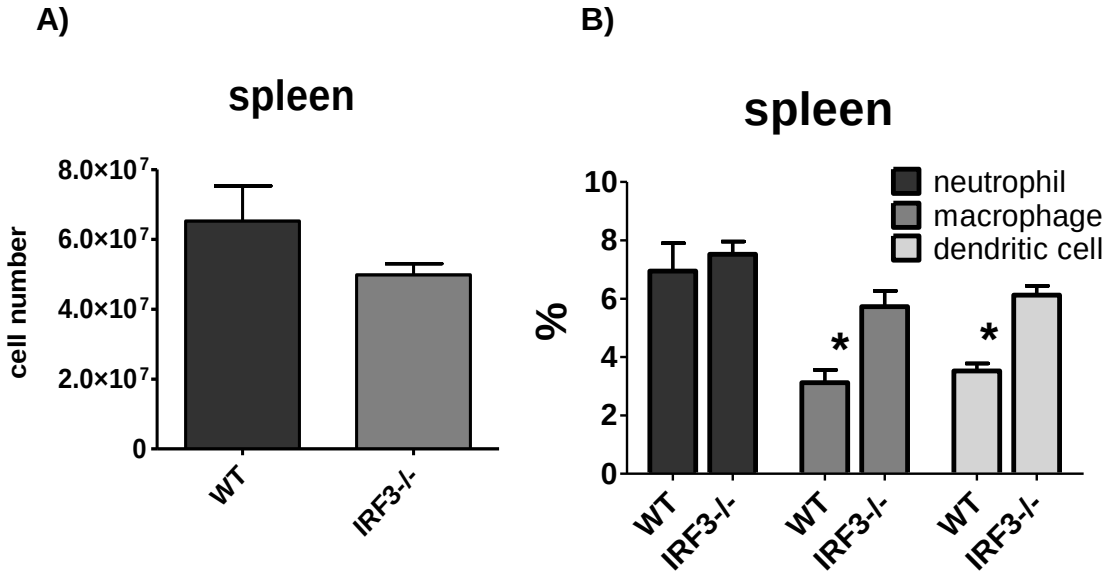


*Figure 3.36 Cell counts in spleen. After 24 hours of CLP, WT mice and IRF3-/- mice were sacrificed. splenocytes were isolated as described above and then counted (A) in Neubauer chamber microscopically. (B) splenocytes were incubated with anti-Gr1-PE, or anti-CD11b-PE, or anti-CD11c-FITC and then performed FACS. Anti-Gr1 positive cells, anti-CD11b positive cells and anti-CD11c positive cells represented neutrophils, macrophages and dendritic cells separately. Data shown were statistically analysed (n=3) and represented means of values ± SED.*p<0.05.*

3.9.7 Phagocytosis

To study the ingestion ability of the phagocytes, heparinized whole blood was collected from untreated WT mice, IRF3-/- mice and IRF7-/- mice. After the blood was incubated with FITC-labelled E.coli bacteria at 37°C for 10 minutes, the phagocytosis was stopped and performed flow cytometric analysis. Not only the percentage of phagocytozing cells were determined, but also the ingested bacteria by phagocytes were caculated.

There was no significant difference in phagocytozing abilitiy of granulocytes and monocytes in blood among WT, IRF3-/- and IRF7-/- mice. No significant difference was found in ingested baterial numbers from there cells of mice.

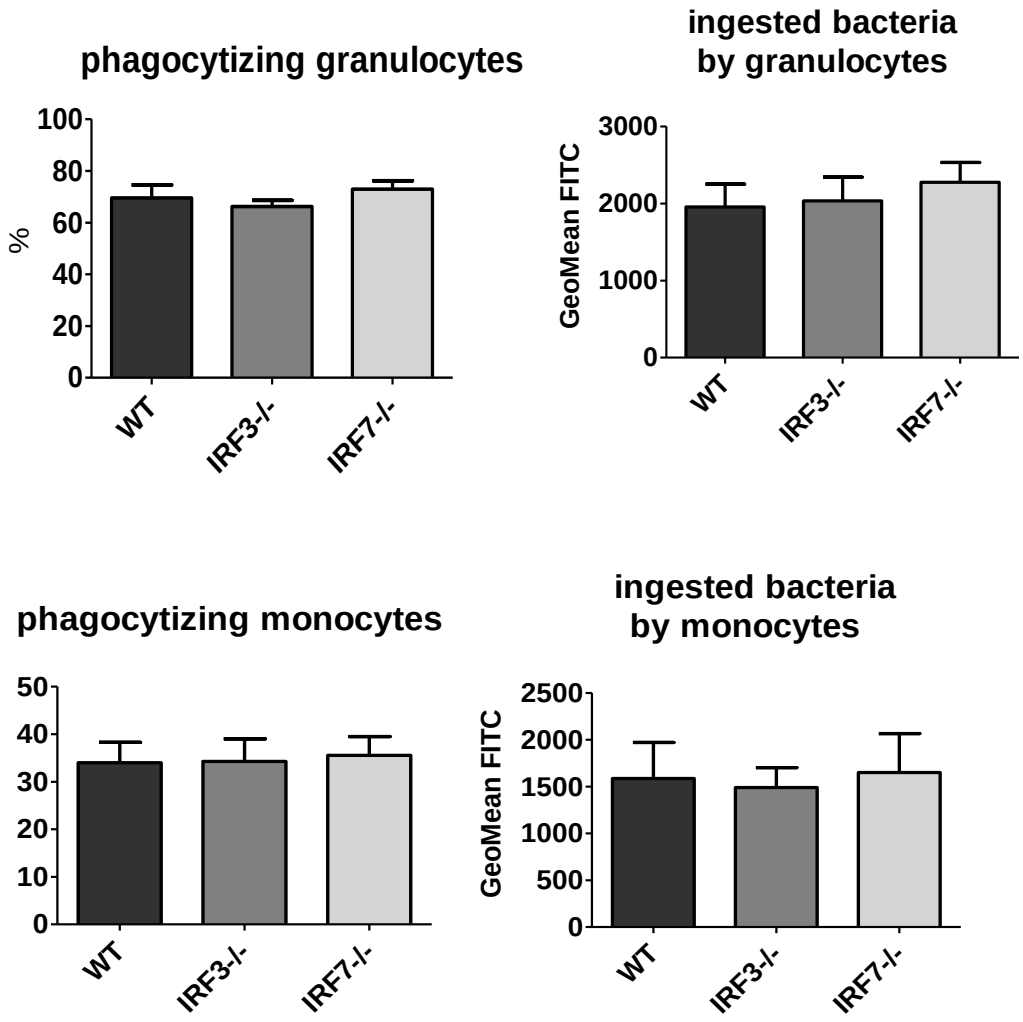


Figure 3.37 Phagocytosis. Heparinized whole blood was collected from untreated WT, IRF3-/- and IRF7-/- mice. After 10 min. on ice the blood was incubated with FITC-labelled E.coli bacteria at 37°C for 10 minutes.Then the phagocytosis was stopped by placing the samples on ice and adding quenching solution. After two washing steps with wash solution, erythrocytes were then removed by addition of lysing solution. After addition of the DNA staining solution, flow cytometric analysis was performed. Data were from two experiments with n=4 per groups.

3.9.8 MPO activity in peritoneum

Myeloperoxidase (MPO) most abundantly present in neutrophil granulocytes. It is a lysosomal protein stored in azurophilic granules of the neutrophil. When neutrophils become activated during phagocytosis, MPO is produced and released into the phagolysosomes or secreted outside the cells, where they can come into contact with microbes and kill the microbes. However, the infiltration of neutrophils and thereafter secreted MPO can also induce tissue damage at the same time.

Peritoneal fluids were centrifuged at 1400 rpm for 10 minutes. Serial dilution of the supernatants was added to TMB liquid substrate for determination of MPO activity. Confirming the results of cell migration, there was higher MPO activity in peritoneal fluids in septic IRF3-/- mice than in WT or IRF7-/- mice.

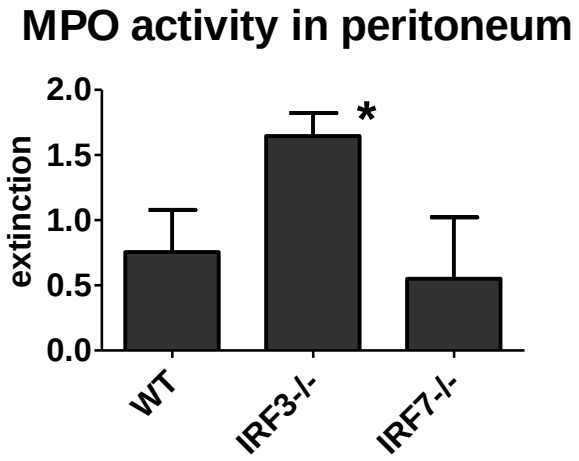


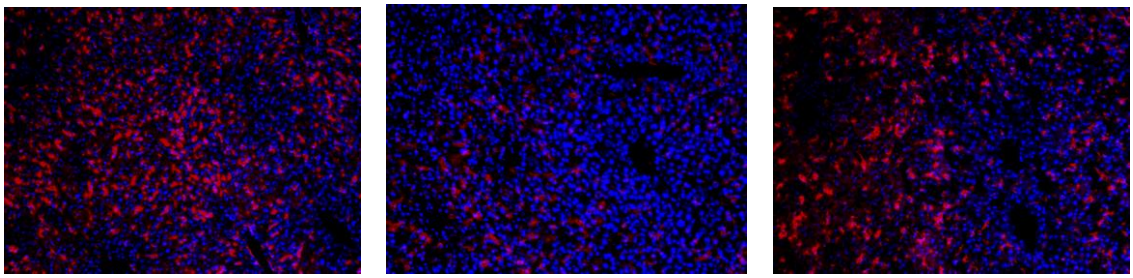
Figure 3.38 Assessment of MPO activity in peritoneal fluid. Peritoneal fluids were collected 24 hours after CLP and centrifuged at 1400 rpm for 10 minutes. Serial dilution of the supernatants was added to TMB liquid substrate and incubated for 15 minutes. The reaction was stopped by addition of H_2SO_4 and the absorption was

*determined at 450nm. Data were presented the extinction values of each samples. N= 4 per groups. *p<0.05.*

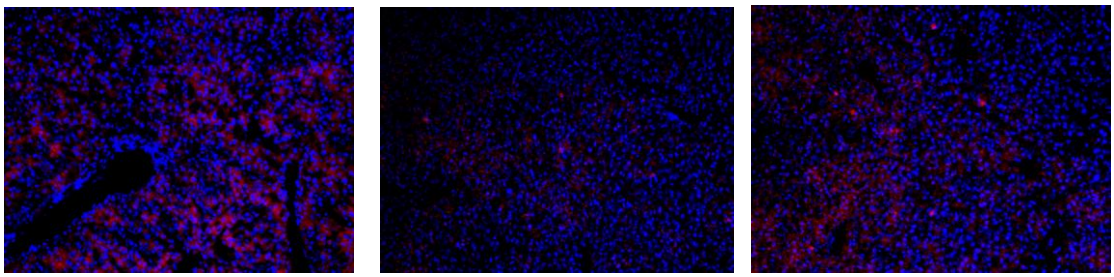
3.9.9 MPO and macrophages staining in liver

Immunfluorescent staining was performed to detect MPO and macrophages in liver as described in materials and methods.

MPO is mainly produced by neutrophils in the organs and can be referred to an indirect sign for neutrophil infiltration. There were more infiltrated neutrophils in liver of WT and IRF7-/- mice than in liver of IRF3-/- mice 24 hours after CLP. Significantly more macrophages were also detected in liver of WT mice and IRF7-/- mice than of IRF3-/- mice. Considering together with the results from the clinical chemistry analysis for liver function, it seems that increased influx of neutrophils and consequently enhanced activity of MPO in organs are at least partially responsible for the organ dysfunction.



WT Liver macrophages IRF3-/- liver macrophages IRF7-/- liver macrophages



WT liver MPO IRF3-/- liver MPO IRF7-/- liver MPO

Figure 3.39 Immunofluorescent staining for MPO and macrophages in liver. liver of the WT, IRF3-/- and IRF7-/- mice were shock freezed and prepared for the cryosections. After fixation with 4% paraformaldehyde, samples were blocked sequentially by methanol, avidin/biotin and protein-blocking reagent. Rabbit anti-

mouse myeloperoxidase with 1:100 dilution was used as primary antibody for MPO (red) staining and rat anti-mouse F4/80 antibody with 1:1000 dilution was used for macrophage (red) staining. Biotinylated rabbit or rat secondary antibody was added and then followed with streptavidine conjugatged cy3 system. The nuclei were counterstained with DAPI (blue). Original magnificantion x300.

3.9.10 Analysis of bacterial colony in faeces

Fresh faeces of untreated WT mice, IRF3-/- mice, and IRF7-/- mice were collected and resuspended in ice cold PBS. Serial dilutions of the suspension were plated on blood agar plate for 48 hours at 37°C and then bacterial colonies were counted. IRF3-/- mice had fewer amounts of bacterial colonies in colon than WT or IRF7-/- mice.

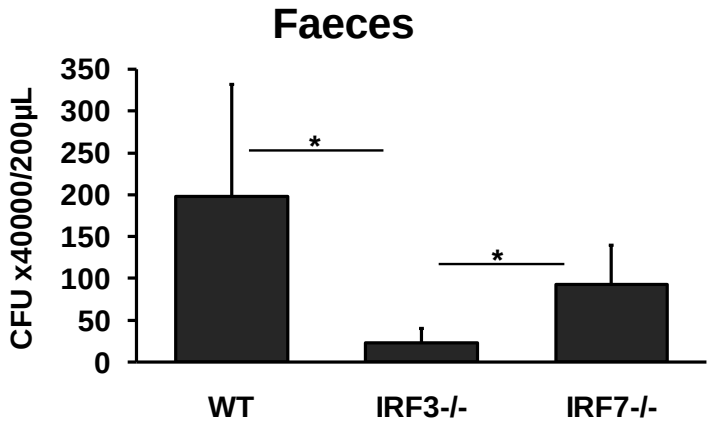


*Figure 3.40 Bacterial colony in faeces. Fresh faeces were collected from untreated WT, IRF3-/- and IRF7-/- mice, and diluted with ice cold PBS in series. 200µl suspension of the faeces in dilution 1:40000 were plated on the CASO blood agar plates at 37°C. CFU was counted 48 hours after culivation. Data were from two experiments with n=5 per group. * p<0.05.*

4 Discussion

4.1 The role of IRF4 in modulating colon inflammation

The studies to analyze the functions of IRFs in IBD indicate that IRF1 and IRF5 are involved in IBD pathogenesis. Increased expression of IRF1 has been found in lamina propria of IBD patients[147] and an insertion-deletion polymorphism in IRF5 gene has been suggested to confer risk of IBD[157]. Using mouse colitis models, Siegmund and his colleagues have reported that mice with IRF1 deficiency develop severer colitis than WT mice by using DSS and TNBS colitis models associated with reduced levels of IFN-gamma and IL-18-binding protein (IL-18BP) as well as a significant decrease of TCR gamma delta lymphocytes. They suggest a protective role for IRF-1 in intestinal inflammation with a possible anti-inflammatory role, in which IL-18BP and TCR gamma delta cells are probably the mediators[148]. Till now, the functions of other IRFs in IBD have not been intensively investigated. Our previous study showed that IRF4 KO mice were completely protected from oxazolone-induced colitis and partially protected from TNBS-induced colitis. In the first part of the present study, we further concentrated on the roles of IRF4 in colon inflammation by using two other mouse colitis models, namely DSS-induced colitis model and T cell transfer colitis model.

4.1.1 IRF4 deficiency does not influence the development of DSS-induced colitis

IRF4 has multiple functions in immune responses. It is not only involved in adaptive immunity but also in innate immune responses. IRF4 is expressed in dendritic cells and macrophages. An increased expression of IRF4mRNA during DC differentiation has been reported and a requirement of IRF4 for the generation of CD4+DCs, CD4-CD8-α DCs and for the plasmacytoid DC has also been indicated[163]. There are studies showing that TLR-dependent induction of pro-inflammatory cytokines is

markedly enhanced in macrophages from mice deficient in IRF4 gene. This enhancement is inhibited by the ectopic expression of IRF4[164, 165]. In addition, one research group suggests that IRF4 acts as an antagonist against IRF1 in immune cells[166]. Since IRF1 is thought to be a protective factor in mouse colitis models, whether IRF4 is involved in DSS-induced colitis has been further investigated.
In the present study we did not find out any difference between IRF4 KO mice and WT mice in view of colonic inflammation after DSS treatment. Both groups developed mild to severe colitis. This result was confirmed by other research group. Watanabe and his colleagues showed in the year 2008 that IRF4 deficient mice or mice treated with IRF4 siRNA were not protected from DSS-induced colitis[167]. Although IRF4 is thought to be an antagonist against IRF1 or a negative regulator of NF-κB[167], the deficiency of IRF4 cannot influence the development of DSS-induced colitis.

4.1.2 T cells are the crucial cells responsible for the experimental colitis development in IRF4-induced colitis

As mentioned above, over production of effector T cells, abnormal survival of effector T cells and their unbalanced production of pro-inflammatory cytokines, are in recent years recognized as critical factors for the intestinal inflammation. In this study, these three pathogenic factors were intensively examined.

4.1.2.1 IRF4 induces resistance of T cell apoptosis by IL-6

Besides involvement in regulation of innate immune cells, there are many evidences showing that IRF4 is responsible for proliferation and differentiation of T cells.
IRF4 has been reported to regulate Th2 differentiation and to a certain extend to Th1 differentiation. Leishmania major (Lm) Ag stimulated IRF4-/- lymph node cells produce neither IL-4 nor IFN-γ. This defect to differentiation into Th1 or Th2 cells in absence of IRF4 is specific to CD4+ Th cells, as the IRF4-/- peritoneal macrophages produce normal IL-12[152].

Our previous study showed that IRF4 deficiency suppressed both oxazolone- and TNBS-induced colitis[168], which was characterized separately as Th2- or Th1-induced colitis model. In addition, we indicated that IRF4 deficient T cell failed to induce colitis in T cell adoptive transfer experiment in RAG-/- mice. All of these data suggest that T lymphocytes are a crucial factor in protective effect of IRF4 deficiency in mouse colitis models.

What's more, we found out that IRF4 was highly expressed, especially in T cells, in inflamed colon tissue, both in human and in mouse. The highly expressed IRF4 was accompanied with decreased T cell apoptosis, determined by TUNEL staining and Annexin V staining[168]. IRF4-/- mice showed markedly increased apoptosis in LPMC both in TNBS colitis model and in T cell transfer colitis model. As mentioned above, decreased apoptosis of effector T cells and subsequently increased accumulation of T cells in lamina propria result in further damage of intestine. It seems that the pro-apoptotic effect of LPMC in IRF4-/- mice contributes at least partially to the protective effect of IRF4 deficiency in colon inflammation. Confirmed with our findings, other group has also demonstrated that IRF4 protects CD4+ T cells against pro-apoptotic stimuli both in vivo and in vitro by showing that infection of IRF4-/- mice with L. major leads to the enhanced apoptosis in LNs and activation of IRF4-/- CD4+ Th cells also leads to increased apoptosis[156].

Many studies show that pro-inflammatory cytokines such as IL-6, IL-12 and TNF are responsible for the resistance of T cell apoptosis in inflammatory bowel disease. Treatment with anit-IL-6 receptor antibody suppresses established experimental colitis in various animal models, which inhibits IL-6 trans signalling by inhibition STAT3 and Bcl-2 protein and subsequently induction of T cell apoptosis[73]. As a pro-inflammatory cytokine, IL-6 was markedly increased expressed and produced in inflamed colon of WT colitis mice, as we expected. Enhanced IL-6 expression was also observed in IBD patients by analysing human biopsy samples. This upregulation of IL6 expression was highly correlated with increased expression of IRF4 in human LPMC. We further revealed a defective upregulation of IL-6 in IRF4-/- colon, in all of the three colitis models. The decreased production of IL-6 is specifically limited in LPMC, since the splenic T cells produced normal IL-6 after TNBS treatment. Isolated CD90+ T cells from LPMC produced less IL-6 when IRF4 was absent than it in WT CD90+ T cells. It seems that T cell-derived IL-6 is responsible for the IRF4-induced

anti-apoptosis in mouse colitis models. This suggestion was further verified by an experiment applying hyper IL-6 in TNBS treated IRF4-/- mice. The additional application of hyper IL-6 conversed the pro-apoptotic effect of LPMC in TNBS treated IRF4-/- mice and abolished the protective capacity of IRF4 deficiency[168].

4.1.2.2 Abnormal Th17 development in the absence of IRF4 rescues mice from T-cell induced colitis

The data from human biopsies showed an increased IL-17 expression in IBD, which was strongly correlated with upregulated IRF4 expression. In mouse colitis models, we further revealed an decreased IL-17 expression and production in the absence of IRF4. Naïve T cells isolated from spleen of untreated IRF4 KO mice under the condition favoring to Th17 polarization, failed to produce IL-17 by measuring IL-17 concentration in the supernatant and by analyzing intracellular IL-17 staining. Naïve IRF4-/- T cells adoptively transferred to RAG-/- mice showed also defective IL-17 expression and production, both in spleen and in LPMC. In addition to the decreased IL-17mRNA level, IL-22mRNA level was also downregulated in colon of RAG-/- mice reconstituted with IRF4-/- T cells. Together with the finding of defective IL-6 production in IRF4-/- T cells, we suggest that IRF4 may not only regulate Th1 and Th2 development but also the Th17 differentiation.

Th17 has been identified as a new subpopulation besides the classic subsets of T helper cells, Th1, Th2 and Treg. It is characterized by production of IL-17 (IL-17A and IL-17F). The differentiation of Th17 is related with Treg. TGF-ß is the crucial factor to induce Treg differentiation. However, in the presence of IL-6, it induces Th17 differentiation instead of Treg[169]. Two other cytokines, IL-23 and IL-21 are also suggested to be responsible for the Th17 development[170]. In addition to produce IL-17, Th17 cells also produce IL-22, IL-21, IL-6 and TNF-α[169, 170]. Th17 and their cytokines have been shown to be involved in the pathogenesis of certain autoimmune diseases such as rheumatoid arthritis, EAE (experimental autoimmune encephalomyelitis) as well as inflammatory bowel disease[171, 172]. IL-17A and IL-17F have been suggested to have redundant but highly pathogenic role in gut inflammation. Both naïve IL-17A-/- and naïve IL-17F-/- T cells induce colitis in RAG-/-

mice and inhibition any of these two factors can not abolish the inflammation, whereas inhibition of both of them at the same time reverses the disease[173]. IL-17 is also known to simulate fibroblasts, endothelial cells, macrophages and epithelial cells to secrete multiple pro-inflammatory mediators and in vivo studies have indicated that the local production of IL-17 may cause a site-specific influx and activation of inflammatory cells[172].

Although Th1 and Th2 polarized cells can produce few amount of IL-22, Th17 is the most important resource for huge amount of IL-22 production. In the present study, we showed a decreased IL-22 expression associated with protection from gut inflammation in T cell transfer colitis model. This reduction of IL-22 expression is then due to the inhibited development of Th17 cells. IL-22, a member of IL-10 family, has been reported to be anti-inflammatory or pro-inflammatory. IL-22 has been suggested to mediate IL-23 induced dermal inflammation and acanthosis[174]. Several studies propose that IL-22 induces inflammatory response and is associated with IBD development[175]. On the contrary, others suggest a protective effect of IL-22 by showing colitis in RAG-/- mice induced by IL-22-/- T cells[173], or therapeutic effect of IL-22 in experimental colitis model[176]. Although the functions of IL-22 in gut inflammation are still in debate, our data at least show that inhibited Th17 development contributes to the beneficial effect of IRF4 deficiency in experimental colitis.

4.1.2.3 Regulation of IL-17 and IL-6 by IRF4

As a unique subset of T helper cells, Th17 polarization is controlled by different cytokines as the other subsets of T helper cells. In the presence of neutralizing antibodies of IL-4 and IFN-γ, IL-6 together with TGF-ß can differentiate Th0 cells into Th17 cells. There is also an IL-6-independent way to polarizing Th17 cells. It has been reported that IL6-/- mice fail to develop Th17 and their peripheral repertoire is dominated by Foxp3+ Treg cells. However, deletion of Treg cells leads to the reappearance of Th17 cells in IL6-/- mice, suggesting an additional pathway by which Th17 cells might be generated in vivo. This alternative pathway is induced by an IL-2 cytokine family member, IL-21, cooperating with TGF-ß[170].

The key transcriptional factors responsible for Th17 differentiation are STAT3, RORγT and RORα.

Stat3 is activated downstreamly by IL-6 and IL-23. There is evidence to show that it is required for programming the Th17 cells, as well as for RORγT expression in Th17 cells. Retroviral transduction of a constitutively active Stat3 into differentiating T cell cultures enhances IL-17 production from these cells, whereas STAT3 deficiency impairs RORγT expression and subsequently Th17 differentiation[177].

The other two factors, RORγT and RORα belong to retinoic acid-related (RAR) - related orphan nuclear receptors. RORγT is highly restricted to T cells within the intestinal lamina propria. In vitro, IL-6 and TGF-ß induce transcription of RORγT in purified CD4+ T cells prior to the onset of IL-17A and IL-17F expression[162]. RORα is expressed in the central nervous system and its isoform 4 is specifically expressed in Th17 cells. Like RORγT, RORα4 is induced by TGF-ß and IL-6 in STAT3-dependent manner. RORγT and RORα work synergistically but in two parallel ways to regulate Th17 polarization because each of them alone is enough for the Th17 differentiation. Evidence of direct biochemical binding of ROR on the IL-17 promoter has been shown by Yang and his colleagues. Putative ROR response elements (ROREs) has been found in the IL17-IL17f locus at CNS2 (conserved noncoding sequence element) site. Using chromatin immunoprecipitation and EMSA they reveal the direct binding of ROR on the CNS2 containing RORE[60].

In the ways to finding the mechanisms and factors responsible for Th17 cells, IRF4, besides all the factors mentioned above, has been more concerned since the last two years. At the same time as we found the decreased Th17 population in IRF4 KO mice, Brustle and her colleagues published the involvement of IRF4 in IL-17 producing T helper cells. They showed a complete resistance to EAE in IRF4-/- mice, associated with defective Th17 development. Although the phosphorylation of STAT3 in response to IL-6 and two target genes of IL-6 (socs1 and socs3) were normal in IRF4-/- cells, IL-6 induced only small amount of RORγ-T. Downregulation of IRF4 expression by IRF4 siRNA resulted in reduced expression of RORγT. Overexpression of RORγT in IRF4-/- cells could only partially restored the Th17 differentiation[178]. All of these give us a hint that IRF4 may have a new function in Th17 differentiation.

In our study, a reduced expression of RORγT and RORα was observed in RAG-/- mice reconstituted with IRF4-/- T cells. Overexpression of IRF4 in the T cells by using retrovirus containing IRF4 expression vector could further enhanced IL-17 production in IRF4+/+ T cells. Our data confirm that IRF4 is involved in Th17 development. In the next step, we tried to find out the mechanism of IRF4 to regulating Th17 development. Although IRF4 deficiency impaired IL-17 and IL-6 productions and RORs were IL-6-dependent, destroyed Th17 polarization in IRF4 absence was probably not directly due to the decreased IL-6 generation, as the exogenous addition of IL-6 in vitro could not induce production of IL-17. To test whether IRF4 acts as transcription factor regulating expression of RORs and thereafter manipulating the Th17 development, we searched for the putative IRF4 binding sites within ROR promoters. Although we found out the putative binding possibility of IRF4 on ROR promoter, but failed to show the biochemical bindings. We further investigated whether IRF4 could directly regulate IL-17 transcription. Computer analyzing indicated several putative binding sites of IRF4 within murine IL-17 and also IL-6 promoters. The additional experiments, however, verified only a biochemical binding site of IRF4 on IL-17 promoter but not on IL-6 promoter.

This direct evidence together with the findings above leads to the following explanations about the protective effect of IRF4 in colitis models: In the absence of IRF4, IL-17 promoter can not be activated by IRF4. Th17 development is therefore inhibited, which results in decreased IL-6 production. The subsequent impairment of the IL-6-dependent RORs expression in Th17 cells can further destroy the polarization of Th17. Less IL-6 and less IL-17 contribute to the beneficial effect of IRF4 in gut inflammation with increased apoptosis of LPMC and decreased inflammation.

The function of IRF4 in Treg has been studied by some groups. Foxp3 binding within the promoter region of IRF4 in Treg cells was confirmed by ChIP. IRF4mRNA was increased in Foxp3+ Treg cells and knockdown of Foxp3 resulted in marked reduction of IRF4mRNA. However, IRF4 deficiency did not change Foxp3 expression in cells. In diseased mice, Foxp3 expression was even upregulated when IRF4-/- lacks[179]. Bruslte and her colleagues also discussed the disregulated Foxp3 expression in IRF4-/- T cells. Under the Th17 differentiation condition, Foxp3 was abnormally highly expressed in absence of IRF4. Retroviral overexpression of Foxp3

in WT Th cells reduced Th17 differentiation even in Th17-inducing condition, suggesting high expression of Foxp3 might impair Th17 differentiation. This abnormal Foxp3 expression under Th17-inducing conditions in IRF4-/- T cells is most probably indirect, because Foxp3 expression could be normally increased in IRF4-/- T cells when TGF-ß was added. The possible explanation could be the unresponse to IL-6 in IRF4-/- T cells.

4.1.3 Hypothetic model of the involvement of IRF4 in experimental colitis and IBD

Based on the results in the previous and present studies, this figure simply illustrates how the IRF4 modulates gut inflammation. IRF4 expressed T cells are important in this process. When the T cells are activated, more IRF4 can be expressed and translocated into the nuclei to regulate directly IL-17 expression and Th17 development. Production of pro-inflammatory cytokines such as IL-6 can also be strongly increased. Both of these cytokines can further recruit inflammatory cells into inflamed tissue and produce additionally pro-inflammatory mediators and induced tissue damage. What's more, IL-6 acting as an anti-apoptotic factor, enhances the resistance of T cells to apoptosis and subsequently induces accumulation of T cells in lamina propria and contributes to further damage.

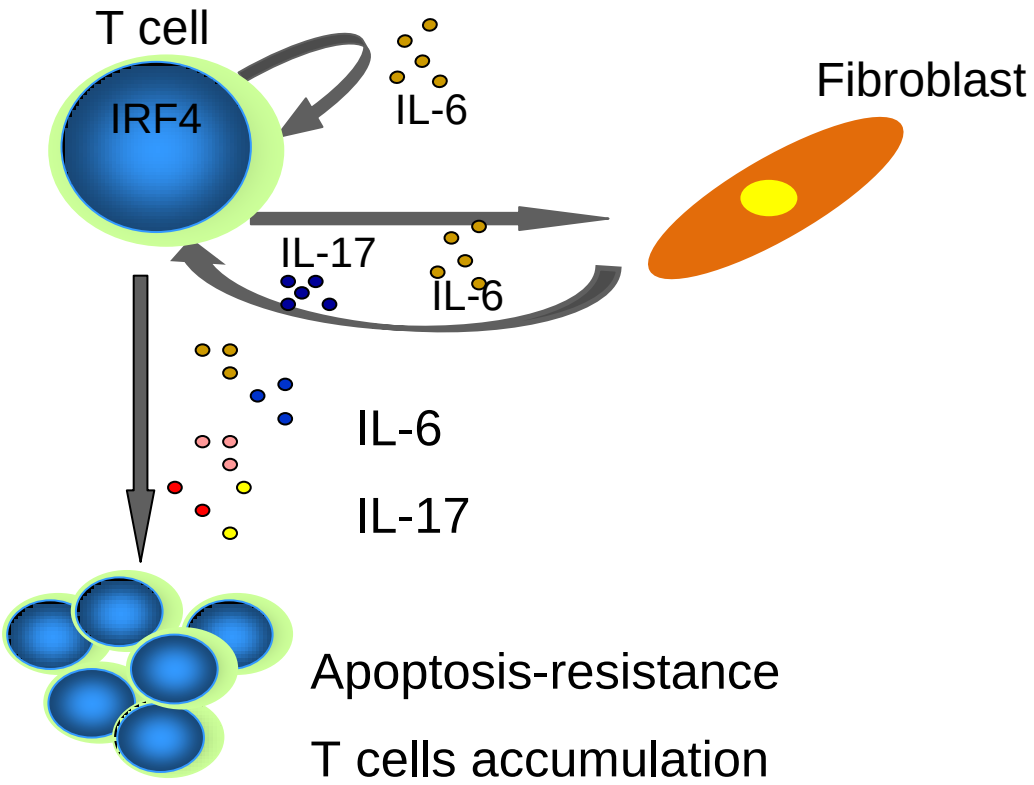


Figure 4.1 Model for IRF4 to modulate inflammation. After T cells are activated, IRF4 translocates into the nucleus in its dimeric form and binds to its specific binding site within the IL-17 promoter. Subsequently IL-17 mRNA transcription is induced. Production of IL-6 is indirectly induced by IRF4. Both of these cytokines, further recruit inflammatory cells into inflamed tissue and produce additionally proinflammatory mediators and induced tissue damage. What's more, IL-6 works as an antiapoptotic factor and enhances the resistance of T cells to apoptosis, which subsequently induces accumulation of T cells in lamina propria and contributes to further damage.

4.2 Roles of IRF3 and IRF7 in polymicrobial sepsis

IRF3 and IRF7 are highly homologous and act as the key regulators of type I IFN expression elicited by viruses. IRF3 is of the downstream of TLR3 and TLR4 signaling, whereas IRF7 is majorly activated by TLR7 and TLR9. Mice lacking IRF3 were more vulnerable to virus infection with reduced IFN-α/β gene expression. Generation of mice lacking IRF7 revealed a more important role of IRF7 in regulating type I IFN gene. Mouse embryonic fibroblasts (MRFs) from IRF7-/- mice indicated severely impaired type I IFN production when infected by ssRNA viruses. Plasmacytoid dendritic cells (pDCs) from IRF7-/- mice were completely non-

responsive to TLR7 and TLR9 ligands to induce IFN-α whereas the induction was normal in pDCs from IRF3-/- mice[143]. These two data strongly suggest that IRF7 may more essential than IRF3 in regulating type I IFN gene expression. Besides involving in regulating type I IFN, IRF3 is also reported to either directly bind to an ISRE or act as promoter-specific and signal-specific cofactor to activate transcription of a set of NF-κB-dependent genes such as chemokine CXCL(CXC-chemokine ligand) 10, CXCL9 and CLIC5 (chloride intracellular channel) etc[143]. IRF3 was also reported to contribute to secretion of IL-12 and differentiation into Th1 cells through this way[142].

To further study whether IRF3 and IRF7 were involved in mouse colitis models, TNBS colitis and T cell transfer colitis were conducted in the IRF3-/- or IRF7-/- mice. Both two groups developed similar colon inflammation as WT mice. Additional experiments of DSS colitis model did not show any alteration among these three groups. Based on these results, it seems that IRF3 and IRF7 are neither involved in chemically induced colitis nor in T cell transfer colitis.

4.2.1 High bacterial clearance is associated with high leukocyte migration into the site of infection in the absence of IRF3

To mimic sepsis, CLP was conducted. CLP is a clinically relevant model of intraabdominal sepsis developing through a polymicrobial enteric infection similar to that seen in patients with colonic perforations. IRF3-/- mice showed a distinctly higher survival rate than IRF7-/- mice and WT mice. This protective phenotype of mice lacking IRF3 was associated with increased bacterial clearance in peritoneal cavity and lower bacterial load in peripheral organs. The leukocyte migration into peritoneal cavity was much higher in IRF3-/- septic mice than in WT septic mice, whereas the number of circulating leukocytes and number of splenocytes was slightly lower in the former than in the latter group.

As mentioned above, sepsis is a very complex systemic disease, in which innate immune response plays a much more important role than adaptive immunity. Unlike LPS or other endotoxin induced sepsis, CLP induces local peritonitis and subsequently bacteriaemia with circulating microbial components. The migration of leukocytes from circulation or spleen is the crucial step for the host to protect against

infection. Reduction of leukocytes migration to infection site is associated with a poor outcome in sepsis. The migrated leukocytes, most of them are neutrophils and macrophages, can ingest and kill microorganisms and send signals to activate further immune responses. Our data showed a more than two folds of enhancement of leukocyte migration in IRF3-/- peritoneal cavity than in WT mice 24 hours after CLP. This increased large number of leukocytes, which are primarily neutrophils, evokes noticeably high ability to phagocytose microorganisms. As a result, fewer amounts of bacteria in IRF3-/- mice had the chance to enter into the blood, circulate in the body and rest in the peripheral organs. In addition to the elevated migration in the peritoneum in IRF3-/- mice, fewer amounts of bacteria in colon were also counted, showing other beneficial factor for the IRF3-/- mice. Another factor contributing to the bacterial clearance is enhanced ability of each phagocyte to ingest bacteria. In this study, individual IRF3-/- phagocyte indicated a normal capacity to ingest bacteria compared with individual WT phagocyte. Considering the results discussed above, the fewer amounts of bacteria entering into the sterile peritoneal cavity, and rashly increased large number of leukocytes migrating to the infect localization contribute to the highly bacterial clearance in IRF3-/- septic mice although the individual phagocytotic ability of IRF3-/- phagocyte is normal.

4.2.2 Decreased cell migration into peripheral organ is associated with less organ damage in IRF3-/- mice

Macrophages are resident in almost all tissues and are the mature form of monocytes, which circulating in the blood and continually migrate into tissues, where they differentiate. In addition to act as phagocytes, they help to activate and recruit other immune cells. Neutrophils the other phagocytes, which are produced in increased numbers during immune response. They migrate from the circulation into the sites of infection in response to chemokines, take up a variety of microorganism by phagocytosis and efficiently destroy them in intracellular vesicles using degradative enzymes and other antimicrobial substances stored in their cytoplasmic granules. However, neutrophils have been regarded as double-edged swords in sepsis. Opposite to their essential roles in eradication of pathogens, they are also

reported to be detrimental for injury of organs. For example, the link between overly exuberant neutrophil activation and organ injury was thought to affect the lungs in particular[141]. The pathogenesis of organ dysfunction is multifactor and incompletely understood. Tissue hypoperfusion and hypoxia are dominant factors. In addition, cellular infiltration, particularly neutrophils, damage tissue directly by release lysosomal enzymes and superoxide-derived free radicals. TNF-α and other cytokines, which increase the expression of the inducible nitric oxide synthase and increased production of nitric oxide, cause further vascular instability and may also contribute to the direct myocardial depression that occurs in sepsis[109].

In the present study, MPO activity in peritoneal fluid was much higher in IRF3-/- mice than in WT and IRF7-/- mice, whereas less MPO secretion and fewer macrophages were detected in the peripheral organs in the former than in the latter. Since MPO are secreted mainly by neutrophils, it can be accepted as a sign for migration of neutrophils to organs. Although the increased migration of neutrophils to peritoneal cavity helps the mice to improve bacterial clearance, the reduced neutrophils migration to the peripheral organs results in less organs damage, which is also beneficial for the mice to survive.

The discordant histological findings and the degree of organ dysfunction seen in patients who died of sepsis was also reported. Cell death in the heart, kidney, liver and lung was relatively minor and did not reflect the clinical evidence of more profound organ dysfunction[141]. This was confirmed by our data too. Clinical chemical analysis revealed dysfunctions of liver and kidney in WT and IRF7-/- mice. Although the histological findings showed some necrosis, increased apoptosis and lymphocyte infiltration, some ballooning and micro vascular steatosis in WT and IRF7-/ mice, these signs were far away from the lethal damages and could not echo the actual organ dysfunction.

4.2.3 Strong cytokine storm during sepsis

Following the initial host-microbial interaction there is widespread activation of the innate immune response, including secretion of cytokines. Not only the classic pro-inflammatory cytokines, IL-1, IL-6 and TNF, but also cytokines such as IL-12, IL-15,

IL-18, are released during the disease. TNF-α and IL-1 are released shortly after exposure to LPS and in turn activate a second level of inflammatory cascades including cytokines, lipid mediators and reactive oxygen species, as well as upregulating cell adhesion molecules that result in the initiation of inflammatory cell migration into tissues. Moreover, pro-inflammatory cytokines can express inducible nitric oxide synthase and increased production of nitric oxide causes vascular instability and vasodilatation, leading to tissue hypoperfusion, shock and organ failure. They are also important inducers of coagulation in sepsis. Based on the various and complex roles of cytokines in sepsis, they are considered as pivotal factors in pathogenesis of sepsis and usually as the targeted steps in the therapeutic process.

In the present study, increased cytokine secretions after CLP were indicated in WT mice, IRF3-/- mice and IRF7-/- mice. However, the classic pro-inflammatory, TNF-α, IL-6 were dramatically highly produced in WT and IRF7-/- compared with IRF3-/- mice. Real time PCR revealed an increased IL-1ß and IL-17 expression in splenocytes of WT and IRF7-/- mice, whereas expression of TGF-ß was not altered. Interestingly, the expression of IFN-α4 and IFN-ß were much higher in IRF3-/- mice than the other groups. Although expression of type I IFN is thought to be dependent on IRF3, it seems that the role of IRF7 is much more important. Honda and his colleagues showed the increased expression of IFN-α and IFN-ß could be induced in the absence of IRF3 by various stimuli, but could not be indicated in the absence of IRF7, suggesting a more pivotal role of IRF7 in regulation of type I IFN[149].

IRF3-/- mice was reported to be resistant to LPS-induced endotoxin shock. This was thought to be mediated by IFN-β, since IFN-ß KO mice were also resistant to high dose of LPS treatment. The different results in expression of type I IFN in IRF3-/- mice could be due to the different sepsis models. CLP induces polymicrobial sepsis that is much more complicated than LPS induced endotoxin shock. Our data showed an increased survival in IRF3-/- mice even with an increased expression of type I IFN, suggesting that IFN-ß should not be the critical factor to protect IRF3-/- mice from polymicrobial sepsis. The other effects such as less cytokine secretions, less neutrophil migration in the peripheral organs could play more important roles.

IRF3 has been reported to regulate several NF-κB-dependent genes such as chemokine CXCL(CXC-chemokine ligand) 10, CXCL9 and CLIC5 (chloride

intracellular channel) etc[143]. IRF3 has been also reported to contribute to secretion of IL-12. All of these factors could act as mediators or modulators in the process of sepsis. These capabilities of IRF3 may make the IRF3-/- mice behaving differently in polymicrobial sepsis to WT mice and IRF7-/- mice. However, explanation of the molecular mechanisms of IRF3 in the sepsis model needs further studies.

5 Zusammenfassung

Interferon regulatory factors (IRF1-9) steuern verschiedene Funktionen in Lymphozyten und Antigen-präsentierenden Zellen. In der vorliegenden Studie, wird der Einfluss von IRF3, IRF4 und IRF7 bei chronisch entzündlichen Darmerkrankungen (CED) und in Mausmodellen für experimentelle Colitis und Sepsis untersucht.

Es ist veröffentlicht, dass IRF4 nicht nur für die Reifung der B Zellen verantwortlich ist, sondern auch wichtig für die Proliferation und Differenzierung der T Zellen ist. Chronisch entzündliche Darmerkrankungen sind nach bisheriger Erkenntnis, durch eine gestörte Balance zwischen proinflammatorischen Effektor T Zellen und regulatorischen T Zellen vermittelt. Um die Rolle potentiell wichtiger IRF Faktoren bei CED zu analysieren, wurden humanen Darmbiopsien von Patienten mit CED untersucht. Mittels quantitativer PCR konnte eine erhöhte IRF4 Expression und gleichzeitig auch erhöhte IL-6 und IL-17 Expressionen bei CED Patienten im Vergleich zu Kontrollpatienten gemessen werden.

Im Transfer Mausmodell CD4+CD45RBhigh T Zellen werden auf RAG2-/- Mäuse transfiert. Naive T Zellen von wildtyp (WT) Mäuse induziert starke Darmentzündung und gestörte T Zellen Apoptosis in RAG2-/- Mäuse. RAG2-/- Mäuse, die mit IRF4-/- naive T Zellen transfiert waren, leiteten hingegen nur leichte Colitis. Die Expression und Produktion von IL-6 und IL-17 war signifikant niedriger in IRF4 defizienten Mäusen. Die upstream Regulatoren des IL-17 signallings RORα und RORγT waren auch runterreguliert. Durch retrovirale Expression von IRF-4 in primären murinen T-Zellen konnte die IL-17 Produktion signifikant induziert werden im Vergleich zu einem Kontrollvektor. Promotorstudien der IL-6, IL-17 und ROR Promotorregionen zeigten mögliche Bindungsstellen von IRF4. Weitere Analysen dieser möglichen Bindungsstellen durch Chromatin Immunoprecipitation (ChiP) und band shift assay (EMSA) bestätigten eine biochemische Bindung von IRF4 im IL-17 Promotor. Diese Daten weisen darauf hin, dass IRF4 als direkter regulatorischer Faktor die IL-17 Produktion induziert

Außer IRF4 wurden andere zwei IRFs- IRF3 und IRF7 in vorliegende Studie untersucht. In Vorarbeiten anderer Arbeitsgruppen wurde gezeigt, dass IRF3 und IRF7 in homo- oder heterodimerer Form die IFN-α/β Expression während viraler

Infektionen induzieren. Im Rahmen der vorliegenden Inauguraldissertation, wird ebenfalls die Bedeutung von IRF3 und IRF7 im Rahmen nicht viraler entzündlicher Prozesse bei experimenteller Colitis und "gut-derived sepsis“ analysiert.

In zwei unterschiedlichen Colitismodellen (DSS und TNBS Modell) wurden nach TNBS oder DSS Applikation keine signifikanten Unterschiede zwischen WT, IRF3-/- und IRF7-/- Mäusen gefunden. Naive T Zellen von WT Mäuse, IRF3-/- oder IRF7-/- Mäuse induzierten ähnliche starke Colitis in RAG1-/- Mäusen. Interessanterweise waren IRF3 Knockout Mäuse vor bakterieller Sepsis geschützt. Dieser Effekt korrelierte mit einer hohen Bakterien-Clearence im Peritoneum, und in den parenchymatösen Organen. Die Anzahl der eingewanderten Leukozyten im Peritoneum war bei IRF3-/- Mäusen doppelt so hoch wie bei WT Mäusen und im Leber war hingegen niedriger bei IRF3-/- Mäuse. Klinisch chemische Analysen zeigten, dass IRF-3 Knockout Mäuse geringere Organschäden aufwiesen als IRF7-/- und WT Mäuse. IRF3 Knockout Mäuse zeigten eine signifikant geringere IL-6 und TNF-α Produktion als die anderen beiden Gruppen. Die Ergebnisse zeigen, dass IRF3 und IRF7 keine wesentliche Rolle bei experimenteller Kolitis spielen. Im Rahmen eines Darm-perforierenden Geschehens mit Aktivierung der unspezifischen zellulären Immunantwort hat IRF3 Defizienz jedoch einen protektiven Effekt.

6 References

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7 Abbreviations

APC	Antigen presenting cell
CARS	Compensatory anti-inflammatory response syndrome
CASP	Colon ascendens stent peritonitis
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Crohn’s disease
cDNA	Complementary DNA
CFU	Colony-forming unit
ChIP	Chromatin immunoprecipitation
CLP	Cecal ligation and puncture
CTL	Cytotoxic T lymphocyte
CXCL	CXC-chemokine ligand
DBD	DNA-binding domain
DC	Dendritic cell
DSS	Dextran sodium sulfate
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
FACS	Fluorescent activated cell sorting
FAE	Follicle-associated epithelium
FCS	Fetal calf serum
FITC	Fluorescein-isothiocyanat
Foxp3	Forkhead box protein 3
GALT	Gut-associated lymphoid tissues
GATA-3	GATA-binding protein 3
GOT	glutamic oxaloacetic transaminase
GPT	glutamic pyruvic transaminase
HMGB	High mobility group B
HPF	High power field

IBD	Inflammatory bowel disease
IFN	interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon-regulatory factor
ISRE	IFN-stimulated response elements
I-κB	NF- κB Inhibitor
KO	knockout
LPMC	Lamina propria mononuclear cell
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M	microfold
MACS	Magnetic cell sorting
mg	Milligram
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
ml	milliliter
MLN	Mesenteric lymph node
mM	millimol
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary-response protein 88
NFAT	Nuclear factor of activated T cell
NF-κB	Nuclear factor κB
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGN	peptidoglycan
PMA	Phorbol-12-myristat-13-acetat
PRRs	Patter-recognition receptor
RAG	Recombination activating gene
ROR	RAR-related orphan receptor

rpm	Rotations per minute
SAg	Superantigen
SAg	superantigen
SCID	Severe combined immunedefficient
SD	Standard deviation
SIRS	Systemic inflammatory response syndrome
STAT	Signal transducer and activator of transcription
T-bet	T-box-containing protein expressed in T cells
TF	Tissue factor
TGF	Transforming growth factor
Th	T helper
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll like receptor
TNBS	2,4,6-trinitro benzene sulfonic acid
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor-protein-inducing interferon ß
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling a
UC	Ulcerative colitis
WT	Wild type
µg	microgram
µl	microliter

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