THE ROLE OF TIM-4 IN THE INTESTINAL INFLAMMATION

By

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ABSTRACT

Inflammatory Bowel Disease (IBD) is a chronic intestinal inflammation that has caused many challenges for healthcare providers in treating the disease and also altered the quality of life of the patients. The cure for IBD is still symptomatic-based; the causes mechanism and pathogenesis of IBD are to be further investigated. Currently, IBD has been considered as an excessive immune response to commensal flora that in normal condition is tolerable to the host. Antigen presenting cells (APCs) play an important role in the pathogenesis of IBD. Macrophage is one of the professional APCs that present antigen information to T cells and induce the T cell subtype proliferation. Aside from this role, macrophages also phagocytose pathogens and clean cell debris in thebody.

T cell immunoglobulin and mucin domain (TIM)-4 is a glycoprotein expressed on the surface of macrophage, which recognizes phosphatidylserine (PS) that is expressed mainly on the surface of the early apoptotic cell phospholipid membrane; the latter is a negatively charged molecule that can bind to the TIM-4 to enhance the phagocytosing activity. In IBD, the loss of intestinal epithelial cells (IECs) due to apoptosis is prominent in the site of inflammation especially in ulcerative colitis (UC).

The aim of this study is to elucidate whether there is an increase of TIM-4 expression in colitis mice model after exposure to excessive number of apoptotic IECs and whether TIM-4 plays a role in the development of colitis in mice.

The expression of TIM-4 is measured with several tests; including flow cytometry, immunohistochemistry, western blotting and real time RT-PCR. In the first step, we tried to see if there is a difference in the TIM-4 expression in colitis mice and ethanol control mice. After the association was established, we further observed the role of TIM-4 in the pathogenesis of IBD by injecting TIM-4+ macrophages into the mice prior to inducing a mild colitis in the mice and finally injected neutralizing anti TIM-4 antibodies to block the available TIM-4 receptors.

We found that TIM-4 expression was higher in a colitis mouse model compared to the control. Also by injecting TIM-4+ macrophages into the mice, the frequency of intestinal T regulatory (Treg) cells was decreased significantly. Finally in the group treated with anti-TIM-4 neutralizing antibodies prior to colitis induction, the frequency of intestinal Treg cells increased significantly and the inflammation response was less severe than the colitis control group. This study revealed, for the first time in the world, that TIM-4 expression in the colon of colitis mice was significantly increased, which suppressed Tregs and promoted T effector cells.

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APC	Antigen Presenting Cells
CD	Crohn's Disease
FoxP3	Forkhead box P3
IBD	Inflammatory Bowel Disease
IEC	Intestinal Epithelial Cells
IFN-g	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
NFκB	Nuclear Factor kappa B
PRR	Pattern Recognition Receptor
PS	Phosphatidylserine
Th	T helper
TGF-β	Transforming Growth Factor Beta
TIM	T cell Immunoglobulin and Mucin domain
TNF-α	Tumor Necrosis Factor-alpha
Treg	T regulatory cells
UC	Ulcerative Colitis

I. INTRODUCTION

1.1 Inflammatory Bowel Disease

IBD is a chronic inflammation of the gut which relapsed and remitted overtime.[1] Patients suffer from IBD present symptoms such as rectal bleeding, severe diarrhoea, abdominal pain, fever, and weight loss. Histological features of the intestine biopsies show large number of leucocytes, particularly neutrophils, lymphocytes and monocytes in the intestinal interstitium.[2] Crohn's disease (CD) and ulcerative colitis (UC) are two major forms of IBD. CD is a multifocal, transmural inflammation process that can affect any part of the intestine while UC is a superficial, continuous inflammation, limited to the colon.[3] Despite the morphologic differences, both CD and UC may have overlapping epidemiological, clinical, radiological, endoscopic and histological characteristics.[4] It is proposed that the pathogenesis of the disease is due to a complex interaction between genetic predisposition, exogenous and endogenous factors, and modifying factors.[5-9] Despite several immunological differences in foreign antigen processing, Waterman et al found that CD and UC interestingly shared common genetic association and that both CD and UC showed impaired adaptive responses.[10] However, despite years of investigations the causative etiology of this disease is still yet to be identified.[6] Classically, IBD is viewed as an inflammation due to a diminished tolerance to the gut commensal bacteria.[1,11] The inflammation is characterized by recruitment and activation of neutrophils, lymphocytes, macrophages, and other inflammatory cells to the inflamed area. This immune hyperactivity increases chloride and water secretion leading to diarrhoea, increases permeability of the intestinal epithelial cells, and ultimately

destruction of the villi of the intestinal mucosa layer.[12,13] Moreover, adding up to the inflammatory response and the release of cytokines and chemokines by the pro-inflammatory cells, extensive intestinal tissue injury takes place resulting in edema, loss of goblet cells, decrease in mucus production, crypt hyperplasia, erosions and ulcerations.[2]

1.1.1 Hygiene Hypothesis

One of the many proposed causes of IBD is an unbalanced immunological environment and immune hypersensitivity caused by the lack of exposure to pathogens. Intestinal tract has the most frequent exposure to external environment, either from the ingested food or water, in comparison to other body organs. Some non-pathogenic microorganism, also known as commensal bacteria, reside in the gut reaching more than 10^{14} bacteria with around 1000 different strain and species.[14] After birth, human gut is colonized by different variety of bacteria strain and these colonies are responsible in building the immune system of the host.[15,16] Interestingly, it is known for some time that children with atopic disease may have different strains of commensal bacteria residing in their gut compared to the non-atopic population from the same geographic area.[17-20] And children from mothers who were exposed to farming environment and cowsheds during pregnancy have lower chance of having atopic diseases. The explanation behind this finding was that the level of endotoxins is higher in the farm compared to the cities and it was suggested that long term exposure to high level of endotoxin during first year of life protect the host from asthma and atopy.[21] However, a larger scale studies is yet needed to confirm this finding.[22]

Recent findings suggested that immune disease prevalence is rising rapidly in the last three decades in countries with better hygiene and sanitary especially in children, compared to adults. In 1998, about one in five children in industrialized countries suffered from allergic disease such as asthma, allergic rhinitis or atopic dermatitis.[23] It has been demonstrated by Werner et al that at individual level, increased atopic dermatitis incidence is directly correlated with higher family income.[24] Interestingly even though innate immunity seems to play a significant role in the development of IBD, many studies have shown that IBD is directly correlated to atopic diseases and hundreds published data suggest that pulmonary hyper-responsiveness is common in patients diagnosed with IBD.[25-29]

According to the hygiene hypothesis, the balanced ratio of T helper 1 (Th1) and T helper 2 (Th2) lymphocytes is thought to be of major importance in maintaining the homeostasis. All along IBD has been categorized under Th1 response; however Heller et al initially discovered that UC has Th2 property and the high apoptotic rate in UC is caused by an increase of Interleukin (IL)-13 and IL-4, which are attributed as Th2 cytokine.[30] CD on the other hand is a Th1 response against commensal bacteria, releasing high level of interferon gamma (IFN-g), IL-1β and Tumor Necrosis Factor-alpha (TNF-α).[31] In most animal model, it is discovered that even though IBD is triggered by commensal bacteria but the development of disease is still depend on the activity of effector T cells in the gut.[5] Additionally, several reports suggested that eosinophils and eosinophil-derived mediators, which present in allergy, also contribute to the histopathology and pathophysiology of IBD.[32-36] Hence it is possible that adaptive

response similar to atopic disease takes place in the pathogenesis of IBD. Moreover, in mouse colitis model the role of transferred Treg is significant in providing negative feedbacks to suppress the activation and proliferation of Th1 and Th2 cells hence maintaining the intestinal immune homeostasis and even cure experimental colitis.[37,38]

1.1.2 Prevalence and Incidence of IBD

The incidence of IBD is rising in the last two decades mostly in the well developed countries.[39] Literally there are hundreds of articles describing the incidence of CD and UC in many regions of the world. In general, the highest incidence rate and prevalence for both CD and UC has been reported from Northern Europe, the United Kingdom, and North America, which were the geographic regions that have been historically associated with IBD. However, reports of increasing incidence and prevalence from other areas of the world such as southern or central Europe, Asia, Africa, and Latin America underscore the fact that the increase is a dynamic process.[6,40] Approximately, the prevalence of UC is about more than 0.6 million in the world, and more than 0.4 million for CD.[41-44]

1.1.3 Etiology of IBD

As mentioned above, up to now the cause of IBD still need further investigation. However, the most probable cause is believed to be an abnormal immune regulation toward commensal bacteria which induces IECs damage by the release of pro-inflammatory cytokines by innate inflammatory cells which invade the mucosal layer of the intestine.[45,46] IECs play an important protective role in the gut because the integrity of

the epithelial barrier is the first defense line against incoming pathogens. Damage to the IECs opens the access for pathogens in the lumen to invade the intestinal mucosa and eventually the blood circulation and finally to the system. The epithelial lining is a monolayer protective barrier buried deep under a thick layer of mucus. In normal condition it is extremely hard for pathogens to penetrate the mucus and damage the IECs underneath. However, if the barricade itself is flawed then the passing will be much easier. One of the several cytokines responsible for the damage and eventually apoptosis of the IECs in UC is IL-13.[47] IEC loss in UC is thought to play an important role in the pathogenesis of the disease especially in the acute inflammation sites.[48,49] Another possible cause for IBD is the increased mucosal accumulation of apoptotic-resistant-T effector cells because of an abnormal cell cycle control by p53.[50-52] Recently, a genome-wide association studies of both pediatric and adult IBD also revealed that a number of IBD susceptible genes are associated with not only innate immunity but also adaptive immunity.[53]

1.1.4 IEC's Physiology

Apart from acting as a physical barrier, IECs are capable of processing and presenting antigen with major histocompatibility complex (MHC) molecules, interacting with T cells, both in healthy gut and inflamed gut. In IBD, these IECs up-regulate the expression of both MHC and co-stimulatory molecules, which in healthy gut are anergic, and modulate the adaptive response to an unfavourable outcome.[54] IECs seem to have the ability to communicate with the immune cells lying underneath them in the gut and modulate those immune cells to maintain mucosal homeostasis.[16,55]

IECs secretes large amounts of mucus and antibodies to maintain the homeostasis of the gut.[56] There is an inner, protected, unstirred layer of mucus that is sterile adjacent directly to the epithelial cells produced by goblet cells. In a mouse model where the MUC2 gene which produces mucin glycoprotein is mutated, this mouse group can develop spontaneous colitis. It is found by this group that there are reduced goblet cells numbers, depleted mucus layer, increased permeability, increased crypt apoptosis, and higher susceptibility to luminal inflammation-induced toxins.[12,57-60] In fact, one hallmark of IBD is the disruption of the intestinal epithelium. Normally, maintenance of the intestinal epithelium involves a dynamic process in which intestinal stem cells located at the crypt base undergo differentiation, proliferation, and migration along the crypt-villus axis and in the end undergo apoptosis.[61,62] Apoptosis is a physiologic response that eliminates unwanted, damaged or virus-infected cells. This particular process appears to be disrupted in IBD patients; their IECs exhibit hyperplasia, goblet cell depletion, and enhanced apoptosis. Anti-TNF-alpha drugs that are recently used to help reducing IBD symptoms are found to be able to inhibit epithelial cell apoptosis.[63,64]

1.1.5 Enhanced IEC Apoptosis in IBD

Several data suggest that there is a high rate of IEC apoptosis in IBD.[65-68] Data from animal and human studies show that defects of the intestinal epithelial barrier and a dysregulated gut immune system can lead to the development of IBD.[69,70] The tight junction complex of the intestinal epithelial barrier continuity is broken hence allowing macromolecules like food antigens and microbial products to penetrate to the underneath compartments.[65,71-73] Similarly, IL-13 is shown to be able to induce IEC apoptosis and

alter the epithelial barrier function via tight junction structural changes and other associated compound.[31,74,75]

Apoptosis itself is a physiological process that does not evoke an inflammatory response. However, in IBD the increased apoptotic IEC rate may somehow trigger inflammatory response due disrupted homeostasis environment and lack of continuity which is possibly related to the hyperactive immune cells underneath.[76] Apoptosis can be identified by the morphological changes in the cell membrane. Cells that undergo apoptosis show chromatin and cytoplasmic condensation, nuclear fragmentation, membrane phospholipid flipping exposing PS on the surface and formation of surface blebs. Activated cells will undergo apoptosis after some time and to be cleared from the system by phagocytes.[77-79] The reason why IEC undergo apoptosis in IBD is remain elusive, but some studies suggest that pro-inflammatory cytokines such as TNF-α, IL-6, IFN-γ and IL-13 produced by macrophages and T effector cells may be involved in the process.[63,80,81]

1.1.6 Role of T helper and Treg cells in IBD

In IBD patients, an increase in the number of activated mucosal T lymphocyte is quite commonly observed. Therapeutic intervention that target T cells and T cell derived cytokines also has shown great promise in the treatment of IBD.[12,82-86] Interestingly, it is also found that diminished forkhead box P3 (FoxP3) expression by gene targeting procedure in Treg cells produce a high level of IL-2, IL-4, IL-17 and IFN-g.[87,88] Moreover, it is suggested that natural-occurring-Treg cells may help to control the normal

immune regulation in healthy individuals.[89] Additionally in IBD patients, there is a decrease of Treg in the peripheral blood.[90-93] In mouse colitis model, activated Treg transfer not only prevent but also cure colitis.[94-98] In the acute state of intestinal inflammation, Treg population may be suppressed in the early state of inflammation hence promote the T effector cells to differentiate and invade the lumen.[99] In several studies, it is suggested that Treg cells association in IBD pathogenesis is established.[100,101]

There are two subtypes of Treg in the circulation, the natural-Tregs which are induced during thymic differentiation and the inducible-Tregs which is induced by elevated levels of transforming growth factor- beta (TGF-β) and the latter can be found mostly in the intestinal mucosa interface.[102] Moreover, TGF-β knockout mice are shown to develop multisystem inflammatory disease, including colitis. This mouse model also develops a downregulated nuclear factor kappa B (NF-κB) expression and altered pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) by IECs and APCs.[103,104] Interstingly, IECs are also able to secrete TGF-β and possess the ability to induce hypo-responsiveness to commensal bacteria.[105,106] With the enhanced loss of IECs in IBD, the role of TGF-β as a potent regulatory cytokine that inhibits T cell proliferation, differentiation and activation is also greatly diminished.[87] Additionally, TGF-β also has been shown to promote de-novo generation of Tregs, which might be able to prevent intestinal inflammation.[107-110]

1.2 TIM-4

In 2001, McIntire et al found that within a region associated with T cell and airway phenotype regulator (Tapr) gene, there is a gene family that encodes T cell membrane glycoprotein with conserved immunoglobulin variable domain and mucin domain. They called it TIM protein and found that some of the genes from this gene family were strongly associated with Th1-Th2 differentiation. This TIM family is a group of glycoprotein structure expressed mainly on the cell surface of T cell.[111]

1.2.1 TIM-4 Structure

The nature of TIM is more likely to be associated with immune regulation but the knowledge itself is not yet considered comprehensive. This gene family shares an identical pattern one to another including an immunoglogulin (Ig) domain, a mucin domain, a transmembrane domain, and a cytoplasmic domain. TIM consists of eight members in mouse (TIM 1-8) and three in human (TIM 1, 3 and 4), in which the three in human possess a high fidelity with their counterpart in mouse.[112-114]

TIM-4, previously known as SMUCKLER, is a member that is different in its structure compared with the other TIMs for the lack of tyrosine-kinase phosphorylation tail in the cytoplasmic domain and is expressed mainly in APC, not in T cells like the other members.[115-120] In the gut, TIM-4 is highly expressed on resident peritoneal macrophages, marginal zone macrophages, and other tissue-associated macrophages.[121] TIM-4 is considered a natural ligand of TIM-1 and the interaction of these two molecules in a way induces effector T cells proliferation.[118,122] Apart from that, TIM-4 also acts as a

PS receptor and enhances phagocytosis of apoptotic cell bodies by macrophages to help maintain homeostatic state.[119,120]

1.2.2 TIM-4 role in Immunity

TIM-4 and its ligand, TIM-1, act as a co-stimulatory factor in immune response and involved in the Th1-Th2 balance.[118] The interaction between TIM-1 and TIM-4 is partly dependent on the glycosylated mucin stalk component.[123] TIM-1-TIM-4 interaction plays a role in T cell proliferation,[116] particularly Th2, because TIM-1 expression is found to be higher in activated Th2 subsets.[118,124-127] Additionally, TIM-1-TIM-4 interaction also has been reported to be responsible in the pathophysiology of renal ischemic-reperfusion injury,[128,129] and that histiocytic and dendritic cells neoplasma consistently express TIM-4.[130]

Yang et al. reported that sensitization to common commensal bacteria toxin together with ovalbumin in murine model increased the polarization of Th2 subtype which was blocked by inhibition of TIM-4 expression. They found that after exposure, TIM-4 level was slightly higher than normal and maturation of DC subsets were enhanced.[131] They also proposed that modulating TIM-4 production in APCs may probably decrease the Th2 immune response.[132] Meyers et al. reported that in the Fc fusion protein experiment they did, the binding of TIM-4 to TIM-1 was inhibited by anti-TIM-1 antibody and in vivo administration of TIM-4 fusion protein produced a higher proliferation of Th2 cells. Also they discovered that at low concentration TIM-4 inhibit T cells response, but at high concentration TIM-4 mediates positive co-stimulation.[118]

1.2.3 TIM-4 as a PS receptor

TIM-4, and several others from the same family member, specifically bound to PS on the surface of apoptotic cells via the IgV domain of the TIM molecule.[119,120,133] TIM-4 monoclonal antibodies that block the binding of PS of the apoptotic cells eventually decrease the ability of phagocyting in the mouse peritoneal macrophages.[119] TIM-4 ability to recognize apoptotic bodies is critical for an efficient clearance and prevention of autoimmunity. Rapid removal of apoptotic bodies by phagocytes is critical for the maintenance of tolerance against intracellular antigens released from the dying cells.[119,134-137] Several studies support the findings that deficiencies in PS receptor recognition molecules in mouse models lead to disrupted self-tolerance and development of autoimmunity.[138-141]

Recognition of PS is a key signal to trigger engulfment by phagocytes and via TIM-4 it will form a punctate cap when on contact with apoptotic bodies, which normally is expressed diffusely on the surface of phagocytes.[120,142,143] However, Park et al. suggested that TIM-4 probably only act as a PS tethering receptor but has no signalling pathway on its own.[144] While apoptotic cell death is a critical evolutionally conserved process for elimination of unnecessary cells, phagocytes are also capable of eliminating other dying cells such as necrotic cells and other opsonised targets, however this process is not TIM-4 dependant.[143,144]

1.2.4 Macrophage in Immunity

Macrophage plays an important role in the homeostasis by eliminating dying cells and nursing hemopoietic precursor cells.[145,146] The precursor of tissue macrophages is the blood monocytes. In the colon and small intestine mucosa, macrophage is located in the lamina propria below the epithelial layer. TNF-α is a pro-inflammatory cytokine that is mainly produced by activated macrophages and lymphocytes. Macrophages also plays a key role in the development of IBD.[147,148] Accordingly, blocking the TNF-α production pathway seems to be a good treatment option for IBD.[149-154] It is proposed that this agent helps to induce effector T cell and other mononuclear cell apoptosis not only in CD but also in UC.[155-157] Interestingly, after treatment with anti-TNF-α, the number of Treg cells population was found to be increased suggesting that this treatment is able to reverse the pathogenesis of IBD.[158]

1.2.5 Immunity hyper-responsiveness induced by activated macrophages

In IBD, intestinal mucosa is disrupted and invasion of the bacteria cause massive influx of leucocytes, including monocyte-derived macrophages.[159] Activated macrophage will then release cytokine and chemokine which enhances the inflammatory response that has previously taken place.[160-162] Several studies suggested that activated macrophages are the prominent constituent of the immune cells infiltration in IBD,[163-165] and depletion of macrophage population in mice model help to prevent and decrease mice susceptibility to induced-colitis.[166-168]

It was found that in both clinical and experimental colitis, colon macrophage express more CD14 and TLRs compared to the CD4+ T cells population.[169] In normal condition intestinal macrophage express lesser CD14 and TLRs due to the oral tolerance nature in the gut.[170,171] Interestingly, it was found that CD14, a lipopolysaccharide (LPS) receptor for TLR-4 recognition, is involved in the recognition of apoptotic cells but does not bind to PS.[172-174] From this finding, we can infer that when there is an increase of IECs apoptosis in IBD, at the same time there will be an increase in the intestinal macrophages expression of CD14 which makes it less tolerant to commensal flora.

1.3 Summary and Insights from this Project

TIM is a gene family that is associated with Tapr gene and TIM-4 has been discovered to have a role in the development of atopy in the intestine. It is also known that TIM-4 ligand, TIM-1, is expressed in T effector cells, suggesting that TIM-4 is associated with T cell adaptive immune regulation in the gut.[118]

In part, IBD pathogenesis also includes adaptive immune response and in mice colitis model it is known that Treg transfer can help reduce the symptom and even cure the disease. Treg proliferation and differentiation can be induced by the release of TGF-β.[94,96] Macrophages that undergo phagocytosis and IECs are known to be able to secrete TGF-β, and those groups of cells also play an important part in the pathogenesis of IBD. Moreover, there is an increase of T effector cells invading the intestine mucosa depending on the type of IBD, with a higher number of Th1 in CD and Th2 in UC. [30,103]

Taken together, IECs' enhanced apoptosis is prominent in IBD and TIM-4 expressed on the surface of macrophages may recognize any apoptotic cells.[30,119] Binding of TIM-4 to PS enhanced phagocytosis by macrophages. In addition, TIM-4 also has the ability to bind to TIM-1 on the surface of both Th1 and Th2 cells which will increase T effector cells polarization (Figure 1).[118] Moreover, Treg cells which are known to be able to inhibit the proliferation of effector T cells are significantly decreased in IBD.[158] While it is also shown that IL-13 may be the cause of the increased IEC apoptosis in IBD, the complete pathogenesis still need further investigations.[30] In conclusion, the binding of TIM-4 either on the surface of an apoptotic IEC or on the surface of T effector cells may have a role in the development of IBD.

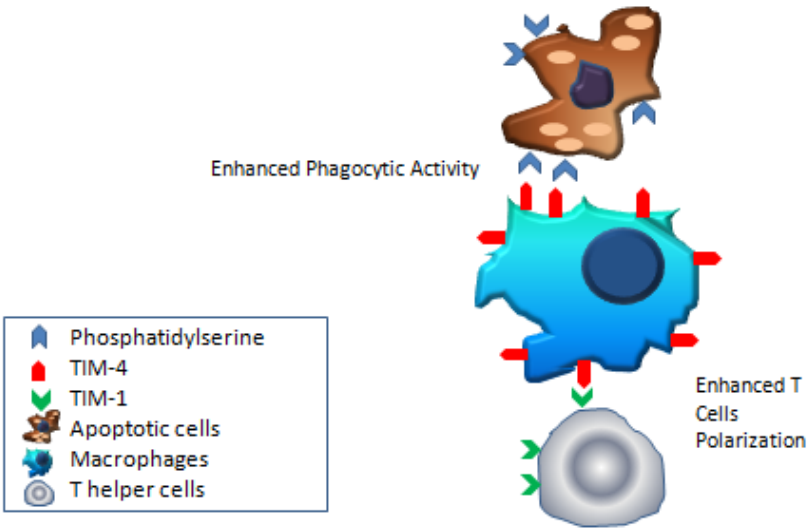

Figure 1. TIM-4 as a PS receptor and TIM-1 ligand.

II. STATEMENT OF HYPOTHESIS

2.1 Hypothesis

In previous research, our lab found that TIM-4 expression in APC is associated with intestinal immunity, particularly in Th2 food allergy murine models. Binding of TIM-4 to its ligand on Th cells under specific allergen-sensitization environment increases Th2 polarization. However, this finding is only tested in a food allergy-conditioned mouse model. Apart from that, TIM-4 was found to be a PS receptor that recognized apoptotic bodies and enhanced phagocytosis to maintain the homeostasis. Considering that IEC apoptosis in IBD holds an important role in the pathogenesis of IBD and there is a large amount of T effector cells involved in the gut inflammation, we will try to check TIM-4 expression rate under a colitis-conditioned mouse model.

We hypothesized that TIM-4 has a role in the pathogenesis of colitis in mice. To establish the association, we will try to examine:

A) TIM-4 expression in colitis mouse model

B) Association between TIM-4 expression rate and Treg population in colitis mouse model

III. MATERIALS AND METHODS

3.1 Reagents and Antibodies

3.1.1 Reagents

Crystal violet dimethyl sulfoxide (DMSO), ethidium bromide, EDTA, DTT, Triton X-100, Tween 20, dipotassium hydrogen orthophosphate (K_2HPO_4), sodium dihydrogen orthophosphate (NaH_2PO_4), disodium hydrogen orthophosphate (Na_2HPO_4), DNase, Blenzyme, Percoll Solution, Peptidoglycan, Hematoxylin, Eosin, protease inhibitor cocktail were purchased from Sigma, Oakville, ON.

Agarose, bovine serum albumin (BSA), Tris, glycine, sodium dodecyl sulphate (SDS), sodium citrate, sodium chloride (NaCl), O.C.T compound embedding medium, TEMED were purchased from Bioshop Burlington, ON.

Trypsin-EDTA, Trinitrobenzene Sulphonate (TNBS), Penicillin-Streptomycin, RPMI-1640 media, DEPC water, Dulbecco's Modified Eagle's Medium (DMEM), and Fetal Bovine Serum (FBS) were purchased from Invitrogen, Carlsbad, CA.

30% Acrylamide/Bis solution and 20% SDS solution were purchased from Bio-Rad, Mississauga, ON.

Glycerol, methanol, chloroform and isopropyl alcohol were purchased from Caledon Laboratories, Georgetown, ON

Reagent Alcohol, Trizol, xylene, and acetone were purchased from Fisher Scientific, Ottawa, ON.

3.1.2 Antibodies

TIM-4 recombinant mouse antibody and propidium iodide were purchased from R&D systems Inc, USA. FoxP3 mouse monoclonal antibody, TIM-4 mouse monoclonal antibody, beta actin mouse monoclonal IgG, anti-mouse HRP, anti-goat HRP were purchased from Santa Cruz Biotechnology, Inc, USA. Beside those mentioned above, other antibodies were purchased from eBioscience, Inc, USA.

3.2 Human IBD samples

All IBD patients' samples were collected from the First Hospital, Gastroenterology Department, Zhengzhou University, China. Non-IBD samples were collected from Huazhong Science and Technology University, Tongji Medical College, China.

3.3 Animal Studies

All BALB/c mice (Charles River Laboratories International, Inc.) were housed in a specific pathogen-free vivarium at Mcmaster University St'Joseph Hospital. Treatment started when the mice were around 10-12 weeks old.

3.3.1 TNBS Colitis Model

To induce colitis we used 2.5 mg TNBS in diluted 50% ethanol per mouse injected with a soft tube through the anal until approximately 4 cm deep. The injection was done under isopropyl gas anaesthesia and the mice were fasted for two days prior to

the injection.[175] Mice were sacrificed on day 7 after injection or if it reached the end-point according to our lab AUP protocol.

3.3.2 TNBS Colitis Half Dose Model

Later in our experiments, we decided to use half dose of TNBS to see whether it could develop an equal inflammation as a full dose TNBS after the mice were injected with approximately 10^8-10^9 of TIM-4+ M-HS cell lines (injected from the tail vein) 24 hours prior to the injection. We use 1.25 mg TNBS diluted in 50% ethanol per mouse, with the same procedure as described above and fasted for 48 hours prior to TNBS injection.

For the control group, we use another group of mice injected with 50% ethanol without TNBS with the same procedure and equal amount of monitoring and fasting days (Figure 2).

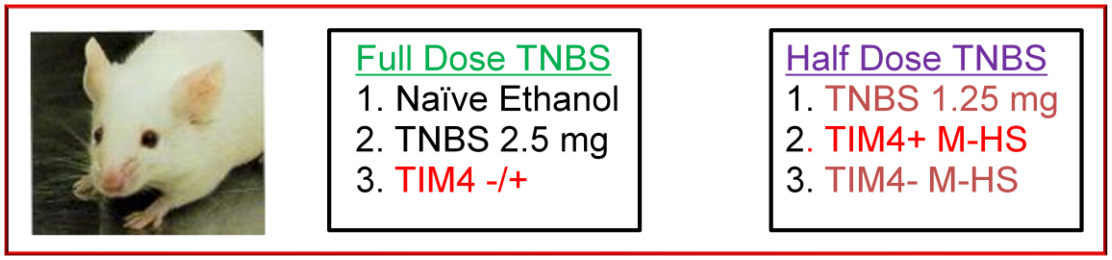

Figure 2. Experiment Design. *In vivo* test in mice conducted with 2 different dose.

3.4 Cell line and LPMC culture

3.4.1 Cell Lines

We used M-HS mouse lung macrophage cell lines, a generous gift from Dr. Zhou Xing, and cultured it according to the specified condition as advised in ATCC. For our in-vitro experiments we also used IEC mouse intestinal epithelial cell lines (a gift from Dr. Ken Cruitoru) and cultured it according to the specified condition. We used RPMI 1640 culture media with 10% FBS and 1% penicillin-streptomycin in 37^{o}C with 5% CO^2 in the tissue culture incubator.

3.4.2 LPMC Isolation and Culture

Intestines harvested from the mice were stored in a clean container filled with ice cold phosphate buffered saline (PBS, 1.36 M NaCl, 14.7 mM KH2PO4, 80 mM Na2HPO4, 26.8 mM KCl, pH 7.4) after removal from the mouse body and transferred to the nearest bio-safety cabinet hood hence the procedure was done under sterile environment. Inside the hood the intestines then were split open with scissors on a petri dish and cleaned from stools. Afterward the intestines were cut into 1cm pieces and put into a 15 ml conical tubes filled with 10 ml of EDTA and DTT solution for further washing. The tubes were then inserted into a thermo shaker for 15 minutes with 37^{o}C and shake with 60 rpm speed. After that, the intestine was cleaned from all mucous layers with tweezers then the intestines were cut into smaller pieces. These smaller pieces of intestines were then put into a 15 ml conical tubes filled with 10 ml of RPMI 1640 medium with blenzyme and dnase to digestion of the tissue. Then these tubes were put on

the thermo shaker for 10 minutes with 37^{o}C and shake with 60 rpm speed. After the shaking, the contents were then transferred to a 50 ml conical tube through a 50 um strainer, then centrifuge in room temperature for 10 minutes with 1200 rpm. The pellets were then wash once again with media and re-suspended in a 10 ml of 40% percoll solution. With a transfer pipette these suspension was carefully laid over on top of a 5 ml of 80% percoll solution in a 15 ml conical tube; without much disruption on the liquid surface. These dual-layered percoll solution tubes were then centrifuge in room temperature for 20 minutes with 2400 rpm without brake. After centrifugation, LPMC were visible as a thin layer of buffy coat in between the two layers. After retrieving this layer with a pipette carefully, these cells were re-suspended in media to remove the excess percoll twice. These cell pellets was ready to be cultured in the tissue culture incubator inside a flask with RPMI 1640 culture media with 10% FBS and 1% Penicillin-Streptomycin.[176]

3.5 Inflammatory Score and Mononuclear Cell Count

3.5.1 Hematoxylin and Eosin Staining

Paraffin sections from mice intestine were cut using a microtome and mount on a pre-baked slides. The slides were then kept to dry for 2-3 days before they were used for staining. For staining protocol, the paraffin from the sections were de-waxed and dehydrated in ethanol for 5 minutes each. The sections then were washed briefly with distilled water then stained with hemotoxylin solution for 30-60 seconds and washed in running warm tap water for 3 minutes. The sections then were re-dehydrated in 90% ethanol for 3 minutes and counterstained with eosin for 30-60 seconds. The sections then

dehydrated in ethanol and cleared in xylene for 5 minutes each. Then the sections are ready to be mounted with DPX mounting media.

3.5.2 Inflammatory Score

To determine the inflammatory score, assessment of inflammatory cells infiltration, edema and mucosa structure were used as indicators (Table 1).[177] From each mouse, 20 pictures were taken from hematoxylin and eosin stained paraffin sections and then labelled blindly with a random code by a third person. The inflammatory score results are later matched with the code from each group.

Parameter	Score	Condition
Epithelium and glands	0 1 2 3	Normal Focal destruction of epithelial surface and/or glands Zonal destruction of epithelial surface and/or zonal crypt loss Diffuse mucosal ulceration involving sub-mucosa and/or diffuse crypt loss
Inflammatory cells infiltration	0 1 2 3	Absence of infiltrate Sub-epithelial and in the lamina propria Infiltrate reaches the muscular mucosa Severe and diffuse infiltrate reaching the submucosa and/or involving the muscolaris propria
Edema	0 1 2 3	Absent Focal Zonal and/or moderately diffuse Extensive and severe

Table 1. Inflammatory Score Marking Indicator for IBD

3.5.3 Mononuclear Cell Count

From each mouse hematoxylin and eosin stained sections, 20 pictures were taken randomly and labelled blindly with codes by a third person. From these pictures, the number of mononuclear cells were counted and adjusted to the camera magnification to determine the number of cells per mm^2.

3.5.4 Myeloperoxidase Test (MPO)

Homogenized colon tissue was suspended in 50 mM potassium phosphate buffer pH 6.0 with 0.5% Hexadecyltrimethylammonium bromide (HTAB). Then the suspension went through a freeze-thaw cycle for 3 times. Then 0.1 ml of suspension was mixed with 2.9ml of 50 mM potassium phosphate buffer pH 6 with 0.167 mg/ml o-dianisidine dihydrochloride and 0.0005% H_2O_2 in a 96 wells plate. The samples were read in 450 nm within 1 minute by assuming that one unit MPO equal to degradion of 1 mM H_2O_2. [178,179]

3.6 Immunohistochemistry

3.6.1 Cryostat Section

For cryostat sections, the section was fixed and cleared from the OCT compound by using either acetone for 20-30 minutes or 4% formalin for 30-45 minutes. Then the acetone or formalin were washed with PBS for at least 3 times before starting the immunohistochemistry staining, to reduce its interference with the antibodies binding capacity and specificity.[180]

3.6.2 Paraffin Section

For paraffin sections the paraffin wax from the section were de-waxed with xylene and dehydrate with ethanol for 5 minutes each. Then the sections were rehydrated with distilled water and PBS for 5 minutes each. To retrieve the antigens attached to the antibodies epitopes, heat-induced method was used for 5 minutes before the immunohistochemistry staining.[180]

3.6.3 Staining Procedure

Before staining the antibodies with fluorescent-bind antibodies, the non-specific binding sites were blocked with 1% BSA for 20 minutes in a humid chamber. Then the sections were stained with primary antibodies for 1-2 hour in room temperature or 4^{o}C overnight. When the incubation time was over, the primary antibodies were washed with PBS every 5 minutes for 3 times. Then secondary antibodies staining were applied for 1-2 hour in room temperature. After the incubation time was over, the secondary antibodies were washed again with PBS every 5 minutes for 3 times,[180] and finally the sections were mounted with anti-fade mounting media. Pictures of twenty from each sample were taken with a fluorescent microscope in a room without direct light to preserve the fluorescent activity.[181]

For TIM-4 antibody staining, 1:100 dilution in 1% BSA was used for the primary antibody and 1:200 dilution in 1% BSA anti-goat FITC for the secondary antibody. For FoxP3 antibody staining, 1:100 dilution in 1% BSA was used for the primary antibody and 1:200 dilution in 1% BSA anti-mouse FITC for the secondary antibody.

3.7 Cell and Tissue Protein Analysis

3.7.1 Protein Extraction

For tissue samples, the tissues were homogenized with protein lysis buffer and protease inhibitor cocktail until there was no any visible tissue. For cells, the cells were suspended in the lysis buffer and protease inhibitor cocktail. Then the cells or homogenized tissue suspension was then kept in 4^oC for 30 minutes. Afterward, the samples were centrifuged for 20 minutes with 14,000 rpm speed in 4^oC. The pellet was discarded for when the cells lysed and the debris of the cell fragments will affect the protein purity.

3.7.2 Western Blotting

Sample protein was diluted in ddH_20 until it reached the concentration of 100 ug/ml, then sample buffer and reducing agent were added according to the company recommended ratio. The samples were then boiled at 100^oC for 5 minutes on a dry plate and then separated with a 12% SDS-polyacrylamide gel at 130 V. Proteins on the gel were then transferred to a membrane for 90 minutes at 30 V. After all the proteins were transferred to the membrane, the membrane was blocked in 5% skim milk in TBST solution for 1 hour at room temperature on a shaker with 60 rpm speed. Then the membrane was incubated in specified primary antibody overnight in 4^oC room on a shaker with 60 rpm speed or 1 hour at room temperature. After the incubation time was over, the membrane was then washed with TBST in every 15 minutes for 3 times. The membrane was then incubated in specified secondary antibody for 1 hour at room

temperature on a shaker with 60 rpm speed. When it was done, the membrane was washed again in TBST in every 15 minutes for 3 times. Afterward, the proteins on the membrane were incubated in ECL solution for about 1 minute then exposed to a film.

For TIM-4 antibodies, TIM-4 goat anti mouse was used as the primary antibody with 1:2000 dilution ratio and anti-goat HRP was used as the secondary antibody with 1:7500 dilution ratio. For FOXP3 antibodies, FOXP3 anti mouse was used as the primary antibody with 1:1000 dilution ratio and anti-mouse HRP was used as the secondary antibody with 1:16000 dilution ratio. For beta actin antibodies, anti-mouse beta actin was used as the primary antibody with 1:1000 dilution ratio and anti-mouse HRP was used as the secondary antibody with 1:16000 dilution ratio. All antibodies were diluted in 5% skim milk in TBST solution.

3.8 RNA isolation and RT-PCR

3.8.1 RNA isolation with Trizol

For tissue samples, 1 ml of TRIzol reagent was added to 100-150 mg of tissue and homogenized thoroughly until no visible tissue is seen. The suspension was then kept for 15 minutes in room temperature then centrifuge to remove the RNA from the lysed cells debris. The supernatant was then transferred to a new tube and 200 ul of chloroform was added to every 1 ml of TRIzol. The solution was mixed well and vortex vigorously for about 2 minutes and then put on ice for 15 minutes. The solution was then centrifuged in 4^{o}C for 15 minutes with no more than 12,000 g. After centrifugation 3 layers will be visible and the RNA was on the top layer with an aqueous phase. The aqueous phase was

then transferred to a new tube and re-suspended in 500 ul of isopropyl alcohol for dehydration and kept on ice for about 10 minutes. This solution was then centrifuged again for another 10 minutes with the same speed and temperature. The RNA was visible by then on the bottom of the tube. The supernatant was then removed and the RNA was then washed in 1 ml of 75% ethanol and centrifuged twice for 5 minutes in 4^{o}C with not more than 7,500 g. The supernatant was then removed and the RNA was left to dry then re-suspended in 50 ul of DEPC water.

3.8.2 RT-PCR

All RNA from the samples was reverse-transcriptase into cDNA by using a RT kit. For RT-PCR the cDNA from the samples were then diluted in DEPC water to reach approximately the same concentration. Then PCR mix and primers were then added to the cDNA samples.

Primers:

TIM-4 Forward 5'- ATG GTG AGT CAG AGA GTG AAG-3'

TIM-4 Reverse 5'- TCA GAT CAA CAG CAG ACA GAC-3'

FOXP3 Forward 5'- AGC TGG AGC TGG AAA AGG A-3'

FOXP3 Reverse 5'- GCT ACG ATG CAG CAA GAG C-3'

Beta Actin Forward 5'- GCT ACA GCT TCA CCA CCA CAG-3'

Beta Actin Reverse 5'- GGT CTT TAC GGA TGT CAA CGT C-3'

3.9 Apoptosis Induction

Flasks with cultured cell lines were put under UV light for 30 minutes.

3.10 Flow Cytometry

Cells were collected from culture flasks either by gentle tapping at the bottom of the flask for non-detachable cells or by using trypsin for detached cells. The cells were then washed in PBS twice and centrifuged at 4^{o}C with 1200 rpm for 5 minutes. Cells were then suspended in 200 ul FA buffer, and then antibodies were then added to the cell suspension. The cells were then put on ice for 30 minutes for incubation and kept from direct light. When the incubation time was over, the cells suspension were centrifuged at 4^{o}C with 1200 rpm for 5 minutes and washed with PBS twice. For antibodies that need secondary antibodies because the antibody itself does not bind to a fluorescent molecule, secondary antibodies were added at this step. Incubation for secondary antibodies took the same time and temperature as the primary antibodies. After the incubation time was done, the cells suspension were also washed twice with PBS and re-suspended in FA buffer and ready to be analyzed. For later use, these cells could also be re-suspended in 4% para-formaldehyde solution and kept in a 4^{o}C fridge for 1 week.

3.11 Statistical Analysis

All data are presented as mean ± standard error (SE). Statistical analysis was carried out using GraphPad Prism for Windows, version 5.00. Two-tailed Student's T-Test was used to analyze data differences in naïve and experiment group samples.

IV. RESULTS

4.1 TIM-4 expression in IBD patients and mice model

Before we start with the mouse colitis model, we wanted to confirm whether there was an expression of TIM-4 in IBD patients and whether there was a significant difference in TIM-4 expression from the inflamed intestine compared to non-inflamed intestine. Several paraffin samples which were previously used for other experiments in our lab were chosen randomly from both non-IBD patients' samples and IBD patients' samples, consisting of both equal number of UC and CD biopsies.

From the immunohistochemistry staining, it was obvious that the expression of TIM-4 was significantly higher in the inflamed intestine compared to the non-inflamed part. (Figure 3) TIM-4 secondary antibodies were FITC-bind and showed green fluorescence whereas propidium iodide was used to contrast the green color of TIM-4 and to stain cell nucleus. As a staining control, isotype IgG was used to confirm the specificity and it showed no positive staining in the control group.

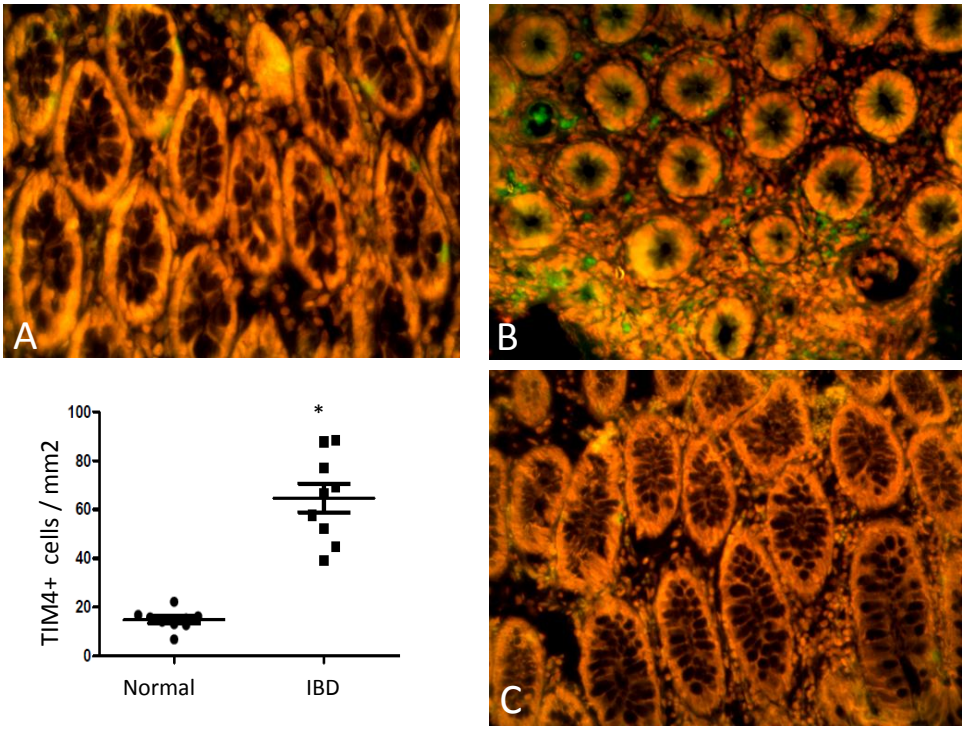

Figure 3. Immunohistochemistry staining and positive cells counting results. Using TIM-4 antibodies (green) and nuclear staining with PI (red) showed significant increases in the human IBD samples. (A) non-inflamed colon, (B) inflamed colon, (C) isotype IgG. All data are presented as means ± SE, n= 8-9. *P < 0.001

After establishing the finding in human tissue, we continued to check the expression of TIM-4 in the TNBS colitis model. Before we go further to this step, the colitis model was confirmed. We used histological score to determine the severity of the inflammation (Figure 4). The severity of the lesions was very distinct between the group injected with TNBS and the control group injected with 50% ethanol. Inflammation and bleeding in the colon of TNBS was visible macroscopically and confirmed microscopically. The reason of using 50% ethanol as a control for the TNBS group was to establish the inflammation in the experiment group was caused only by TNBS and not

due to the use of ethanol. Mononuclear cell counting was performed to determine the infiltration of pro-inflammatory cells to the mucosa layer and MPO test was carried out to determine qualitatively the presence of neutrophils (Figure 5) (Figure 6). While monitoring the body weight for 7 days after TNBS injection, there was a very peculiar trend in several mice on the third and fourth day with a majority showing an increase in the body weight after a steep loss on the first and second day. The increase however could not cover for the loss from the previous days. On the contrary, ethanol control group showed a relatively constant weight gain after the injection (Figure 7).

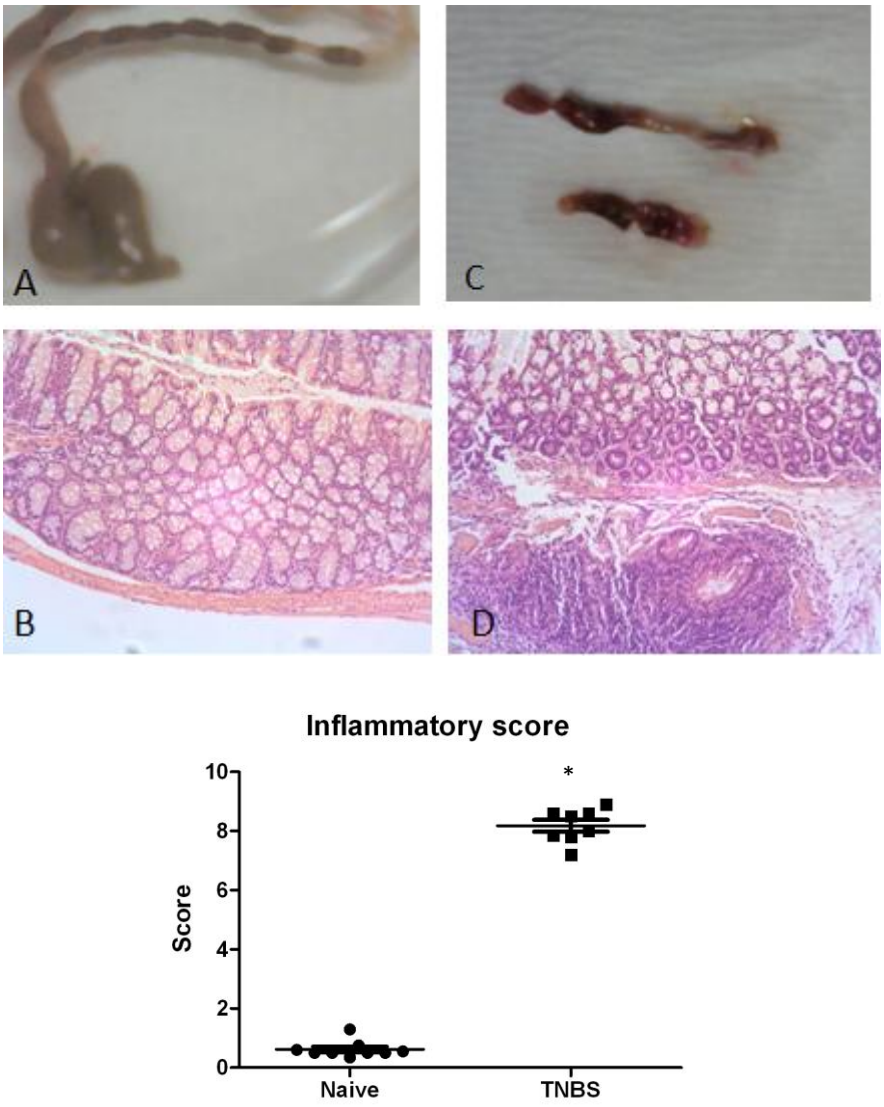

Figure 4. Inflammatory score in mice colitis. Macroscopic and microscopic comparison of naïve mouse colon [A and B] and TNBS-colitis mouse colon [C and D]. All data are presented as means ± SE, n=8-10. *P<0.001

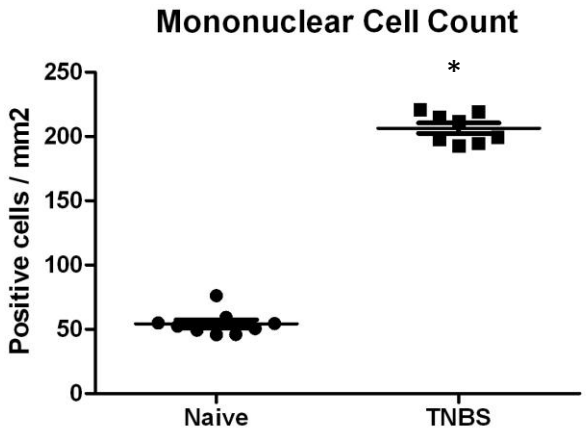

Figure 5. Mononuclear cell counts. Number of cells counted per mm^2 tissue from mice colon tissues stained with H&E stain embedded in paraffin blocks. All data are presented as means ± SE, n=8-10. *P < 0.001

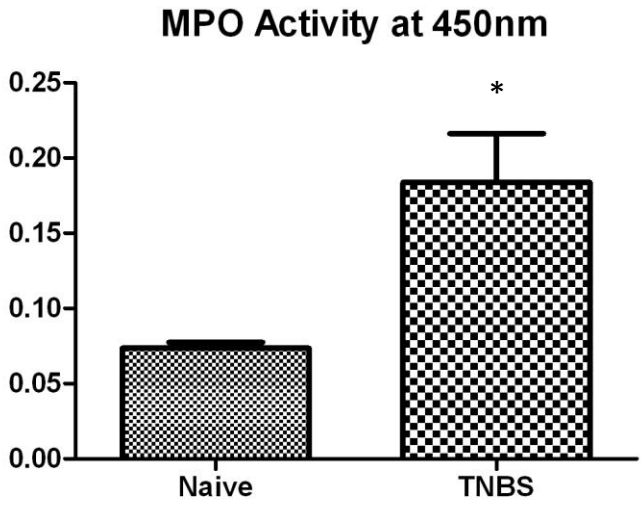

Figure 6. Comparison of MPO activity read at 450nm. All data were presented as means ± SE, n=8-10. *P < 0.05

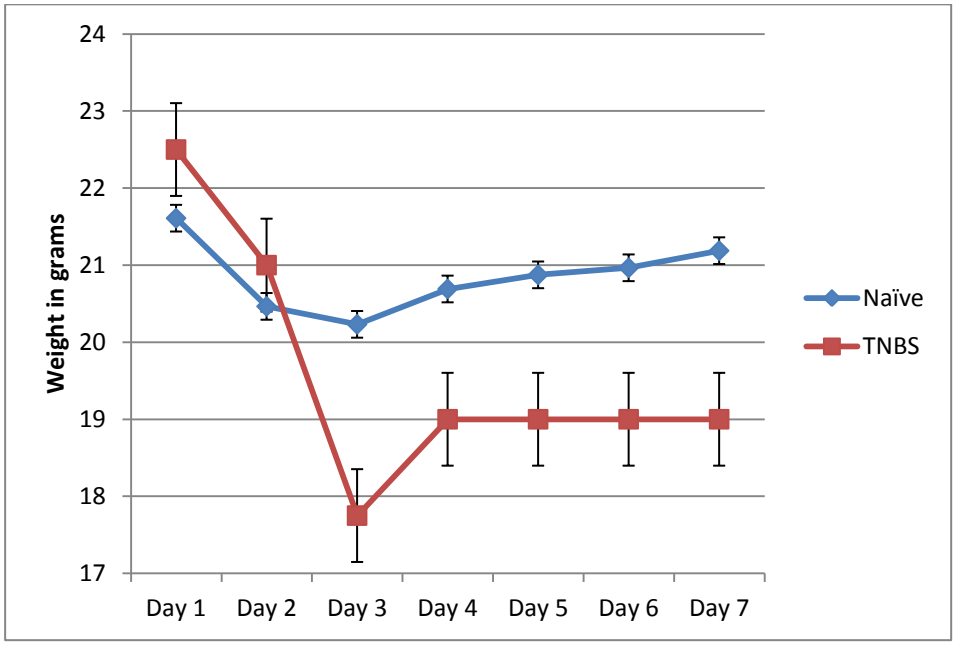

Figure 7. Mice weight chart. Mice were injected on day 1 and sacrifice on day 7. N = 28-30.

After confirming the inflammation induced by TNBS was significantly higher compared to the control showing signs and symptoms similar to human colitis, we further carried out the investigation to see the expression of TIM-4 using immunohistochemistry (Figure 8). We found that the number of cells expressing TIM-4 in mice colitis model was slightly lower than what we found in human. However, we coud still find significant different between those two groups and confirm the increase of TIM-4 in the TNBS colitis model. Using the same staining procedure as the human samples, the immunohistochemistry results showed significant increase in TNBS group with no positive staining in the isotype IgG staining control group (Figure 9). The flow cytometry result showed significant increase of TIM-4 expressing cells in the LPMC of TNBS group

mice. The cells were collected from mice immediately after sacrifice and were cultured for 3 days in a culture flask before staining for flow cytometry (Figure 10). Western blot results of the qualitative TIM-4 protein expression in both groups showed significant increase in TNBS group after normalization to beta actin expression. For the normalization analysis, blot density analysis with ImageJ version 1.45 was used for all samples with the same procedure and steps (Figure 11). Additionally, another supporting data from RT-PCR also suggest the same result. The cDNA from TNBS group mice showed higher levels of TIM-4 compared to the ethanol control group either visually with agarose gel visualization or semi-quantitatively through SYBR green staining (Figure 12).

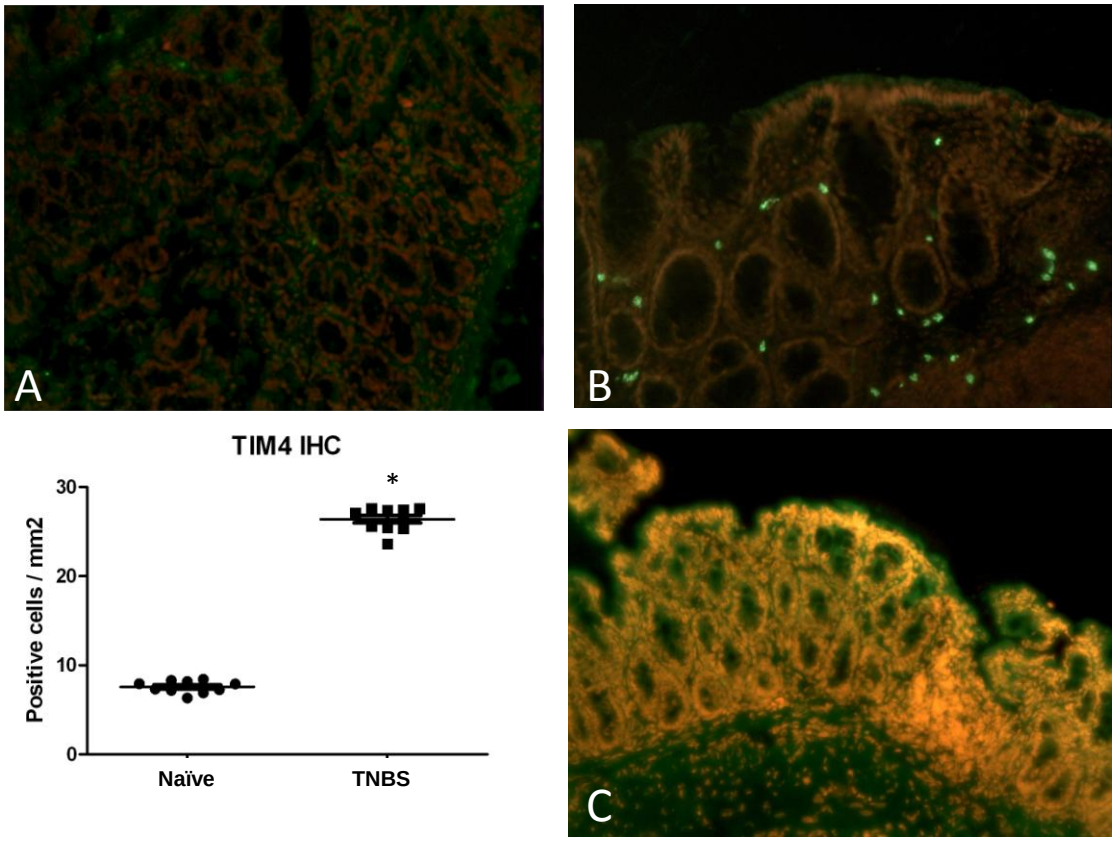

Figure 8. Immunohistochemistry staining results. Staining using TIM-4 antibodies (green) and nuclear staining with PI (red) for (A) Naïve colon, (B) TNBS colon, (C) isotype IgG. All data are presented as means ± SE, n= 9-10. *P < 0.001

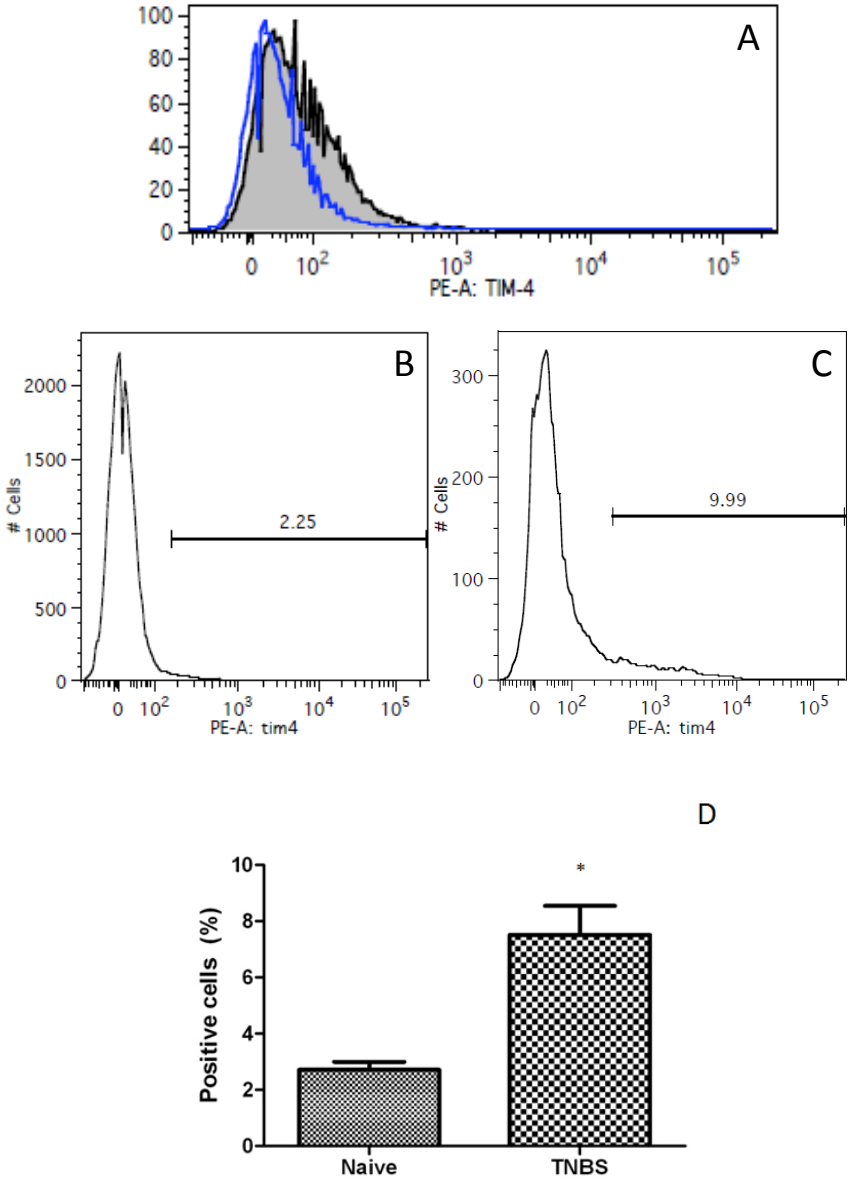

Figure 9. Flow cytometry analysis. (A) Population comparison between naïve (blue line) and TNBS LPMC (shaded area) TIM-4+ cells expression, (B) naïve LPMC TIM-4+ population in percentage, (C) TNBS LPMC TIM-4+ population in percentage, (D)The bars indicate the frequency of positive staining cells. All data are presented as means ± SE, n= 22-25. *P<0.001

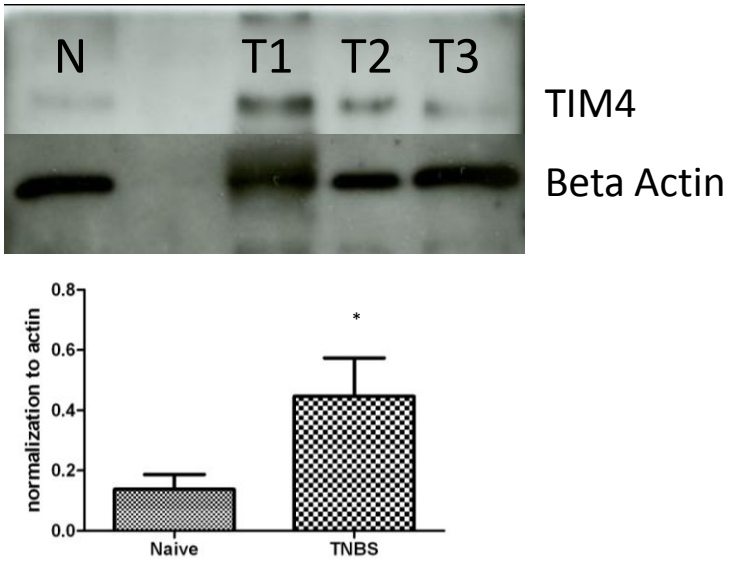

Figure 10. Western Blot for TIM-4 expression. All data are presented as means ± SE, n= 10=12. *P < 0.001

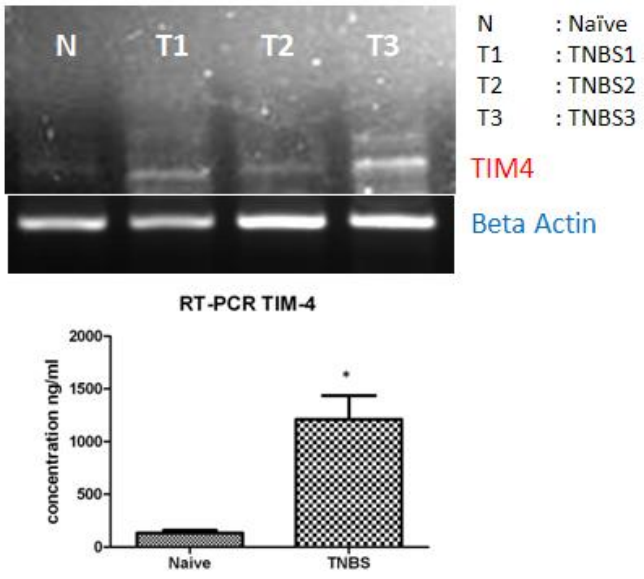

Figure 11. RT-PCR result for TIM-4 expression. All data are presented as means ± SE, n= 12-14. *P<0.01

4.2 Treg expression in IBD patient and mice model

We also checked the FoxP3 expression using immunohistochemistry The results showed that there was a significant decrease in FOXP3+ expression in the inflamed intestine (Figure 12).

The population of Treg cells in TNBS colitis mice model showed similar result to human. There was a significant decrease in Treg cells population in the TNBS colitis colon compared to the ethanol control as shown by immunohistochemistry. FITC-bind secondary antibodies were used to detect FOXP3 primary antibodies with green fluorescence indicating positive staining. Isotype IgG staining control group showed specificity of the secondary antibodies by producing no positive stain (Figure 13). From flow cytometry result, the decrease in Treg population after TNBS injection was significant compared to the ethanol control group (Figure 14). These results from both mouse and human samples were consistent with the findings by several other investigators about Treg population decrease in IBD patients and colitis mice.[90-98]

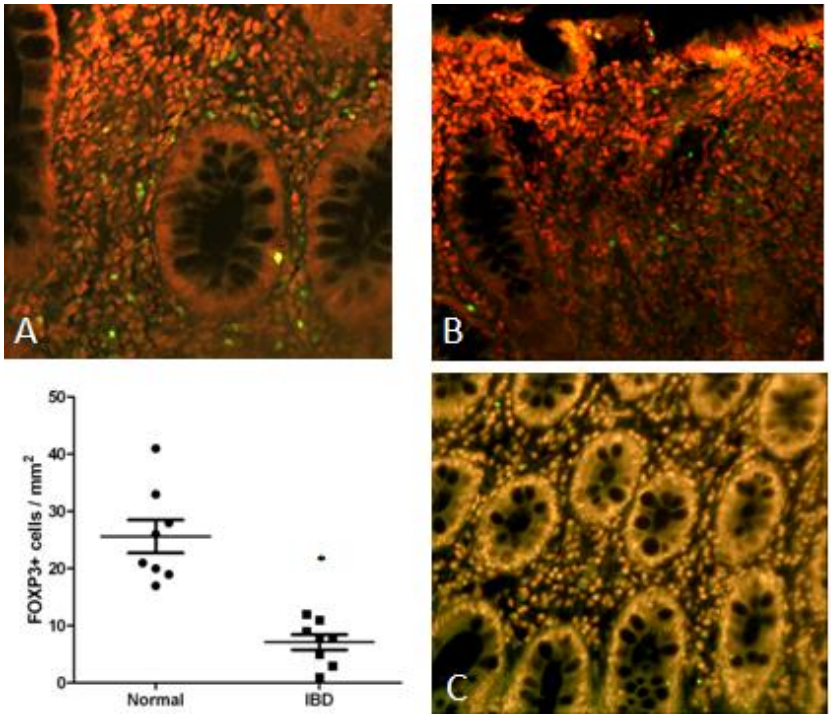

Figure 12. Immunohistochemistry staining results. Staining with FoxP3 antibodies (green) and nuclear staining with PI (red) for (A) non-inflamed colon, (B) inflamed colon, (C) isotype IgG. All data are presented as means ± SE, n= 8-9. *P < 0.001

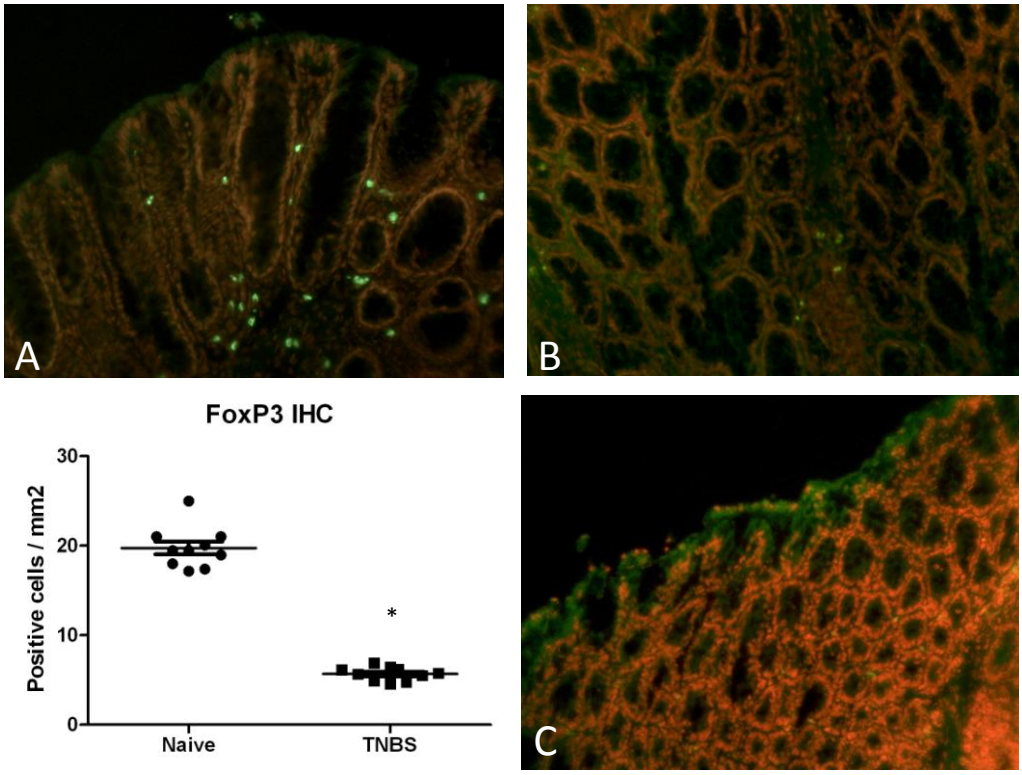

Figure 13. Immunohistochemistry staining result. Staining using FoxP3 antibodies (green) and nuclear staining with PI (red) for (A) Naïve colon, (B) TNBS colon, (C) isotype IgG. All data are presented as means ± SE, n= 9-11. *P < 0.001

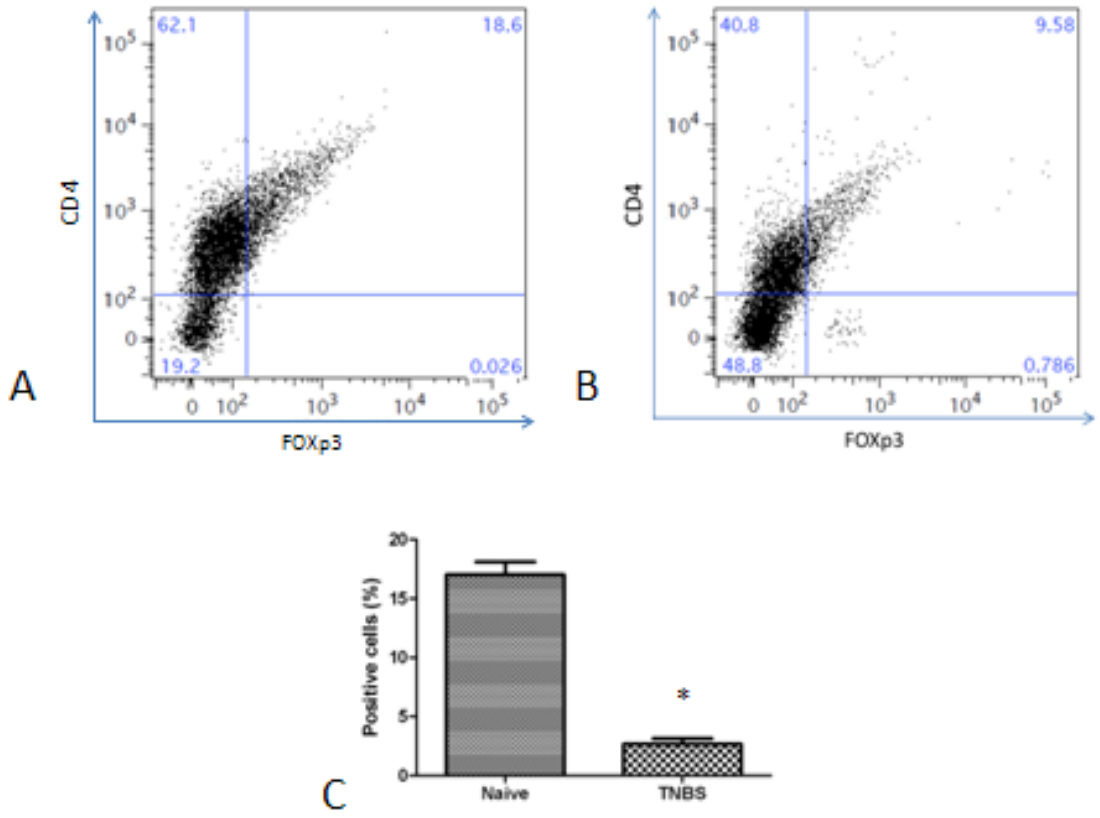

Figure 14. Flow cytometry analysis. (A) Naïve LPMC CD4+FoxP3+ population in percentage, (B) TNBS LPMC CD4+FoxP3+ population in percentage, (C) scatter plot analysis. All data are presented as means ± SE, n= 22-25. *P < 0.001

4.3 TIM-4 and FOXP3 expression in M-HS cell line

After confirming the results in mice model, we further carried out the investigation of TIM-4 on its role in the intestinal inflammation. We started the *in vitro* study using M-HS macrophage cell lines co-cultured with either apoptotic IEC cell lines and non-apoptotic cell lines to see whether macrophages exposure to apoptotic cells would increase the expression of TIM-4. M-HS cell line expresses CD11b on its surface so that can be visualized under fluorescent light after stained the cells with FITC-

conjugated CD11b antibodies to differentiate M-HS cell lines from IEC cell lines (which do not express CD11b (Figure 15)). Significant increase in TIM-4 expression in M-HS was found after 3 day co-culture with apoptotic IEC. Induction of apoptosis was conducted by exposing the culture flask with UV light for 30 minutes. Using flow cytometry to confirm the apoptosis rate by staining early apoptosis cells with annexin V which had the ability to bind to PS and double-stained with PI to stain the late apoptotic cells. After UV light exposure for 30 minutes, 20% IECs underwent apoptosis (Figure 16). Higher expression of TIM-4 was found when co-cultured apoptotic IEC with naïve LPMC; but no significant different in the non-apoptotic group compared to M-HS alone. This result suggested that TIM-4 was expressed on macrophage in response to the presence of apoptotic cells (Figure 17).

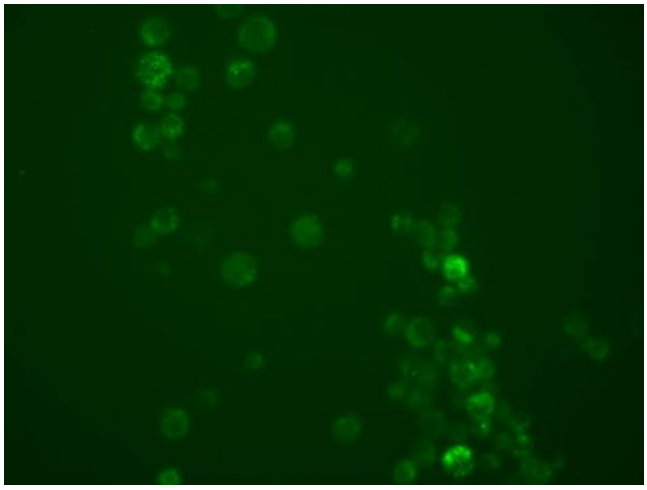

Figure 15. Immunostaining result. M-HS cell lines stained with anti-TIM-4 PE (red) and anti-CD11b FITC (green) before co-culture with apoptotic IECs

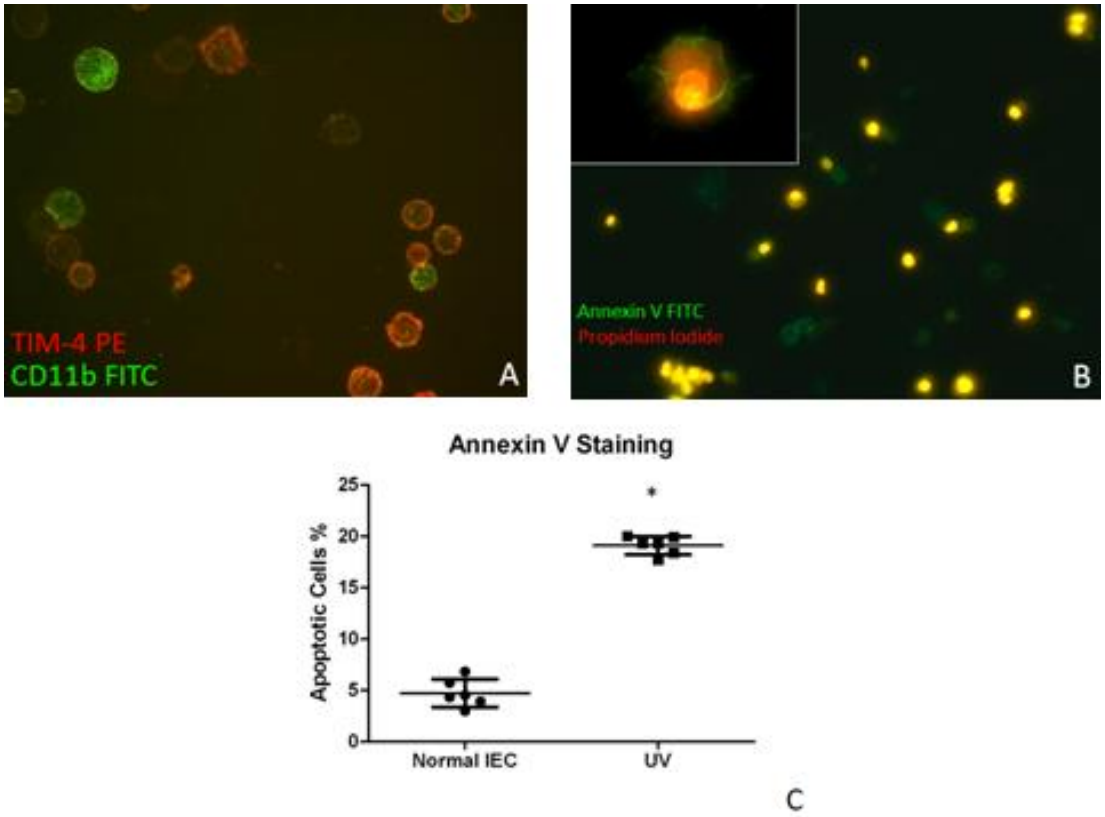

Figure 16. (A) Double staining with immunohistochemistry. M-HS cells were stained with anti-TIM-4 PE (red) and anti-CD11b FITC (green) after co-culture with apoptotic IECs, (B) annexin V FITC staining (green) and PI (red) in the apoptotic IECs group after 20 minutes UV light exposure, (C) flow cytometry results of IECs after 20 minutes exposure to UV light. All data were presented as means ± SE, n= 6. *P < 0.001

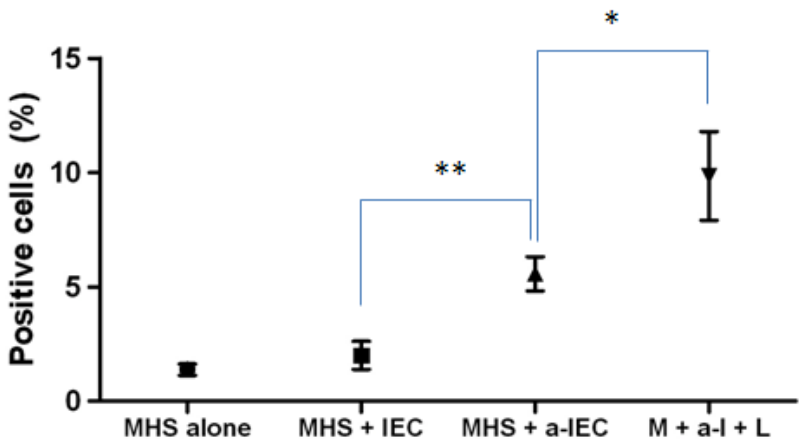

Figure 17. Flow cytometry analysis. M-HS TIM-4 expression in non-apoptotic IECs was lower compared to apoptotic IECs and showed no difference with M-HS alone group. All data were presented as means ± SE, n = 8-9. *P<0.01, **P < 0.001

In the same population of co-cultured cells of apoptotic IEC, M-HS and naïve LPMC, the CD4+ FoxP3+ Treg cells population was significantly decreased compared to other groups; there was no significant difference in the Treg population of non-apoptotic IEC group compared to naïve LPMC alone (Figure 18). Both IEC and M-HS used in this *in vitro* test did not express CD4 and FoxP3 so the number shown in the result was from the naïve LPMC only. As for TIM-4 expression, naïve LPMC was not the only source of TIM-4 because M-HS expressed TIM-4 hence the higher number shown in the co-culture population was not quite representative.

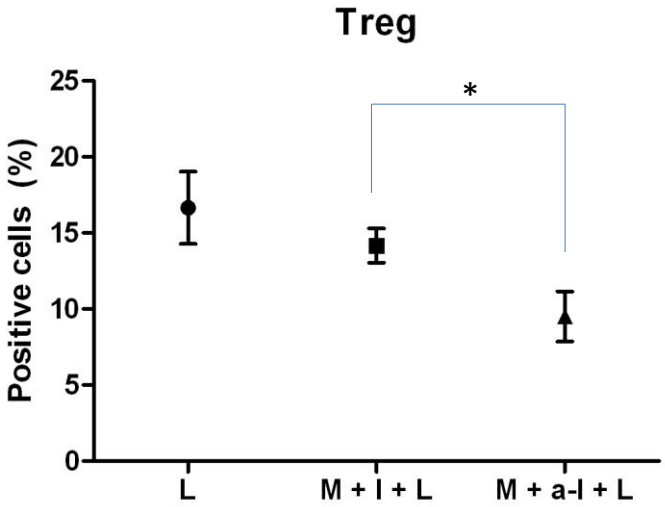

Figure 18. Flow cytometry analysis for Treg population. After co-culture with apoptotic IECs the population was less than that in non-apoptotic IECs co-cultured M-HS and in naïve LPMC control. All data were presented as means ± SE, n= 8-9. *P < 0.01

4.4 TIM-4 and Treg expression in vivo

After the confirmation that there was an increase in TIM-4 in M-HS when exposed to apoptotic cells in our *in vitro* study and when this population was co-cultured with naïve mice LPMC caused the number of CD4+ FoxP3+ Treg cells decreased, our study continued to the *in vivo* test using half dose TNBS colitis model with and without TIM-4+ M-HS pre-treatment and using TIM-4 neutralizing antibodies to block the TIM-4 receptors prior to the full dose TNBS colitis induction.

For the first group with external TIM-4 injected, we induced colitis in mice using half dose TNBS and injected TIM-4+ M-HS cell lines one day before the colitis induction. As a control, we used TIM-4- M-HS injected one day prior to TNBS half dose colitis

induction and another group without MHS injection one day prior to TNBS half dose colitis. And for the second group we injected TIM-4 neutralizing antibody to block the TIM-4 receptors then introduced full dose TNBS on the next day.

In addition, to confirm that the M-HS cells injected through the tail veins travelled to the intestine, the cells were pre-stained with carboxyfluorescein succinimydil ester (CFSE) prior to injection. Using immunohistochemistry technique we found those green fluorescent positive cells in the mice intestinal tissue and mostly resided in the intestinal lamina propria and lymphoid tissues. In addition to staining with CFSE, we also did double staining with mouse macrophage marker F4/80 antibodies to confirm the staining specificity (Figure 19). By using this staining approach, external macrophage originating from M-HS cell lines, which previously stained with green fluorescent CFSE and also express F4/80, in greenish-yellow color which was usually mistaken for non-specific staining.

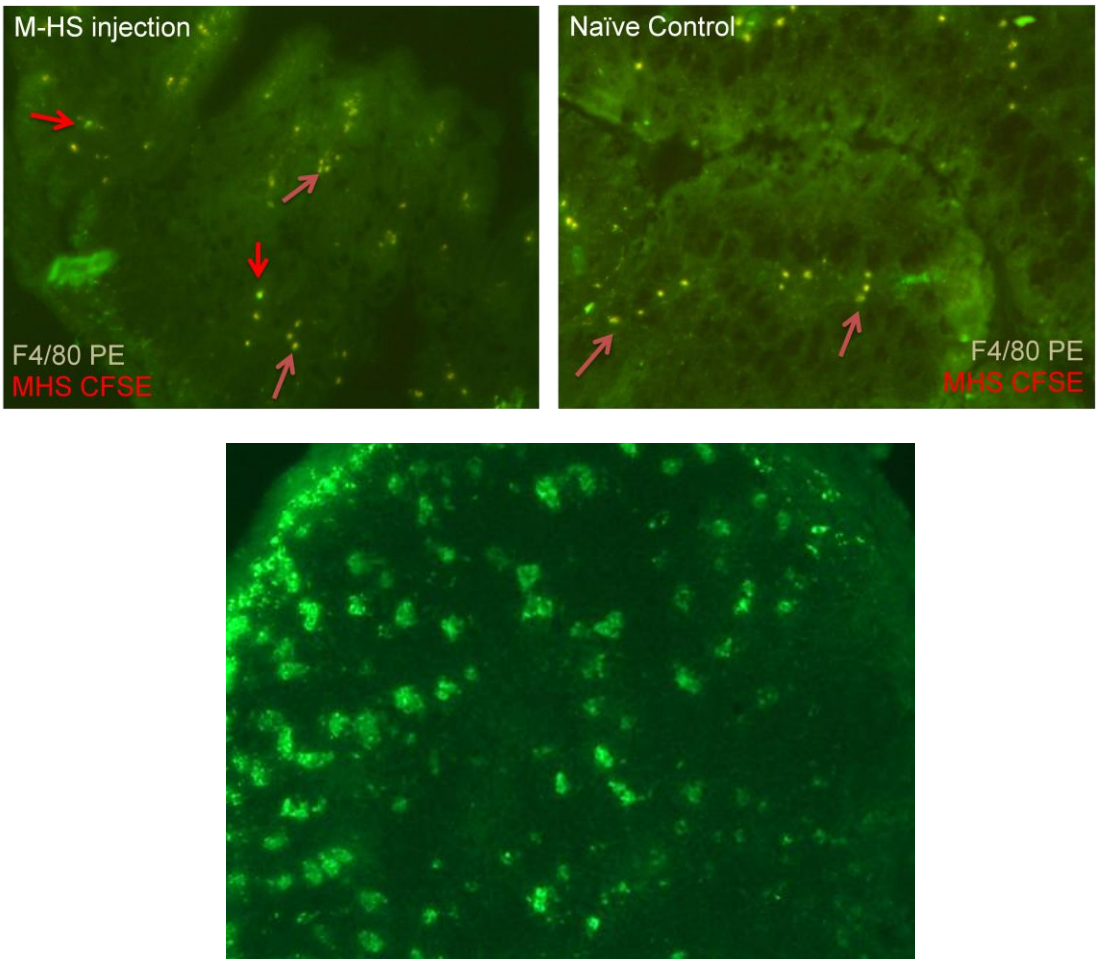

Figure 19. Mouse intestine immunohistochemistry staining. Staining with PE-conjugated F4/80 antibodies and CFSE pre-stained M-HS [Top], and mouse intestinal lymphoid tissue immunohistochemistry with CFSE pre-stained M-HS [Bottom].

From the flow cytometry results, we found that there was an increase in the TIM-4 expression in the TIM-4+ M-HS-treated mice compared to both control groups and less expression of TIM-4 in the group injected with TIM-4 neutralizing antibodies which indicates that the exogenous TIM-4 arrived the intestinal lamina propria and the neutralizing antibodies were able to blocked the TIM-4 on the surface of APCs (Figure 20). There was also a decrease in CD4+ FoxP3+ Treg cells in the LPMC of the M-HS

treated mice intestines compared to control groups and in the group injected with anti-TIM-4 antibodies a day prior to TNBS colitis the population of Treg cells increased significantly even with full dose TNBS induction (Figure 21).

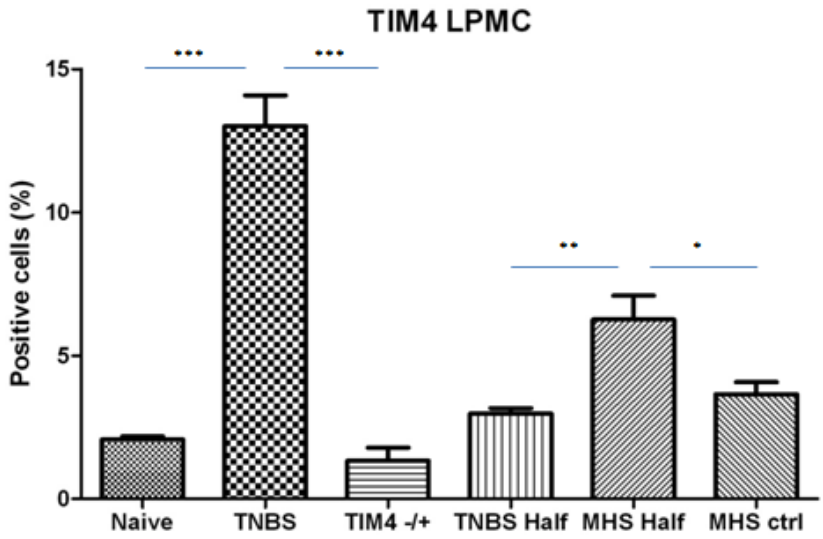

Figure 20. TIM-4 flow cytometry analysis. TIM-4 expression compared in all experiment groups. All data are presented as means ± SE. *P < 0.05, **P<0.01, ***P<0.001

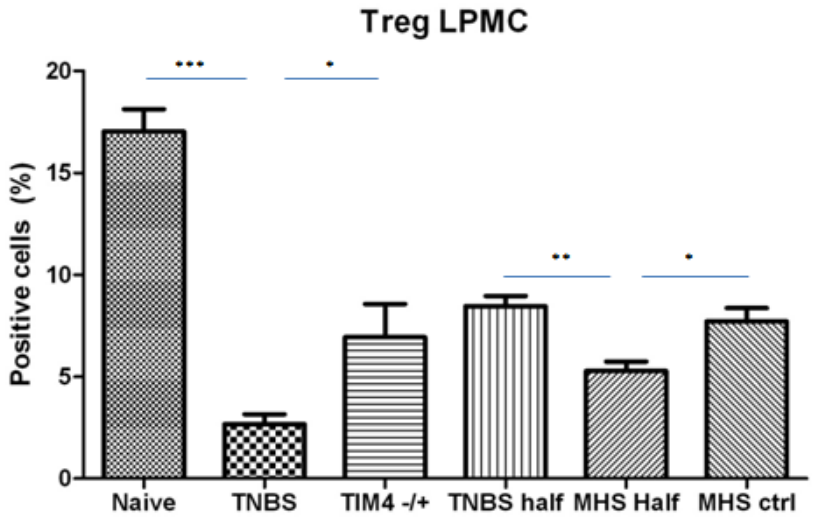

Figure 21. Treg flow cytometry analysis. Treg population compared in all experiment groups. All data were presented as means ± SE. *P < 0.05, **P<0.01, ***P<0.001

We also found the same result in western blotting, RT-PCR, and IHC. In western blotting result, the expression of TIM-4 in the anti-TIM-4 neutralizing antibodies group was significantly decreased to the point of almost equal to the ethanol control group. Whereas the TIM-4+ M-HS half dose group showed significant increase compared to the controls. The expression of FoxP3 on the other hand was not quite similar to the flow cytometery where the TIM-4- M-HS control group showed an increase of Treg cells population almost like the number shown in the ethanol control group and anti-TIM-4 group (Figure 22). In RT-PCR the expression of Foxp3 in anti-TIM-4 group was significantly increased and in the TIM-4- M-HS half dose group where there were adoptively transferred lung macrophages the population of Treg was increased compared to the controls that was only injected with TNBS half dose (Figure 23). From immunohistochemistry staining, the result was quite similar to the other with an increased

Treg population in the anti-TIM-4 group and lower Tregs in the TIM-4+ M-HS half dose group compared to the other 2 control groups (Figure 24).

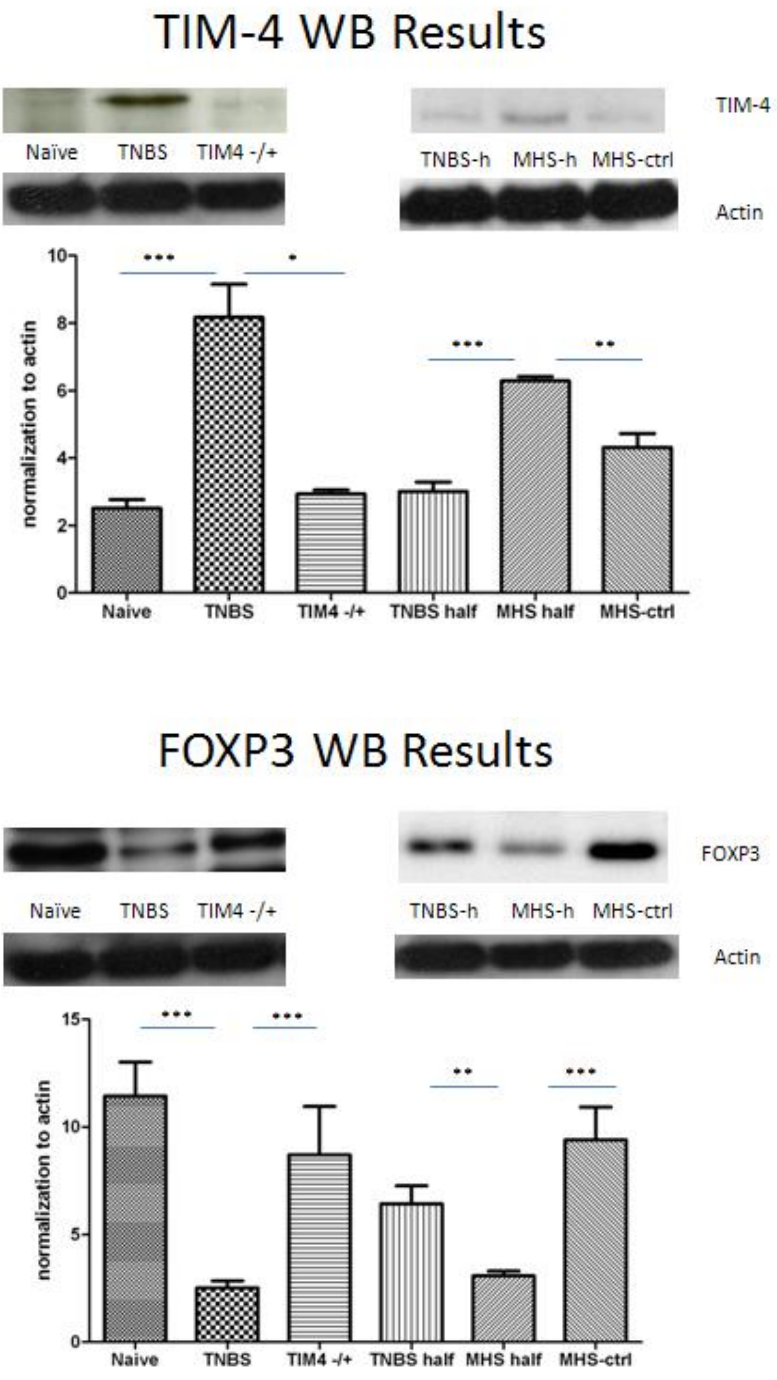

Figure 22. Western blot for TIM-4 and FOXP3. All data were presented as means ± SE. *P < 0.05, **P<0.01, ***P<0.001

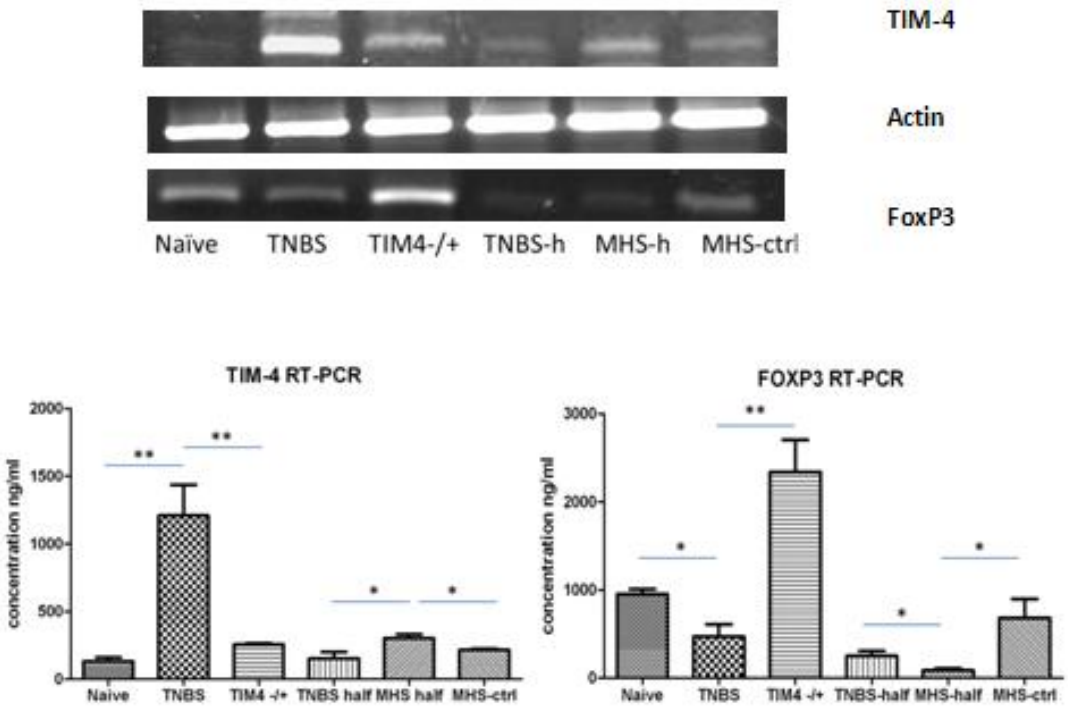

Figure 22. RT-PCR result for TIM-4 and FOXP3. All data were presented as means ± SE. *P < 0.05, **P<0.01

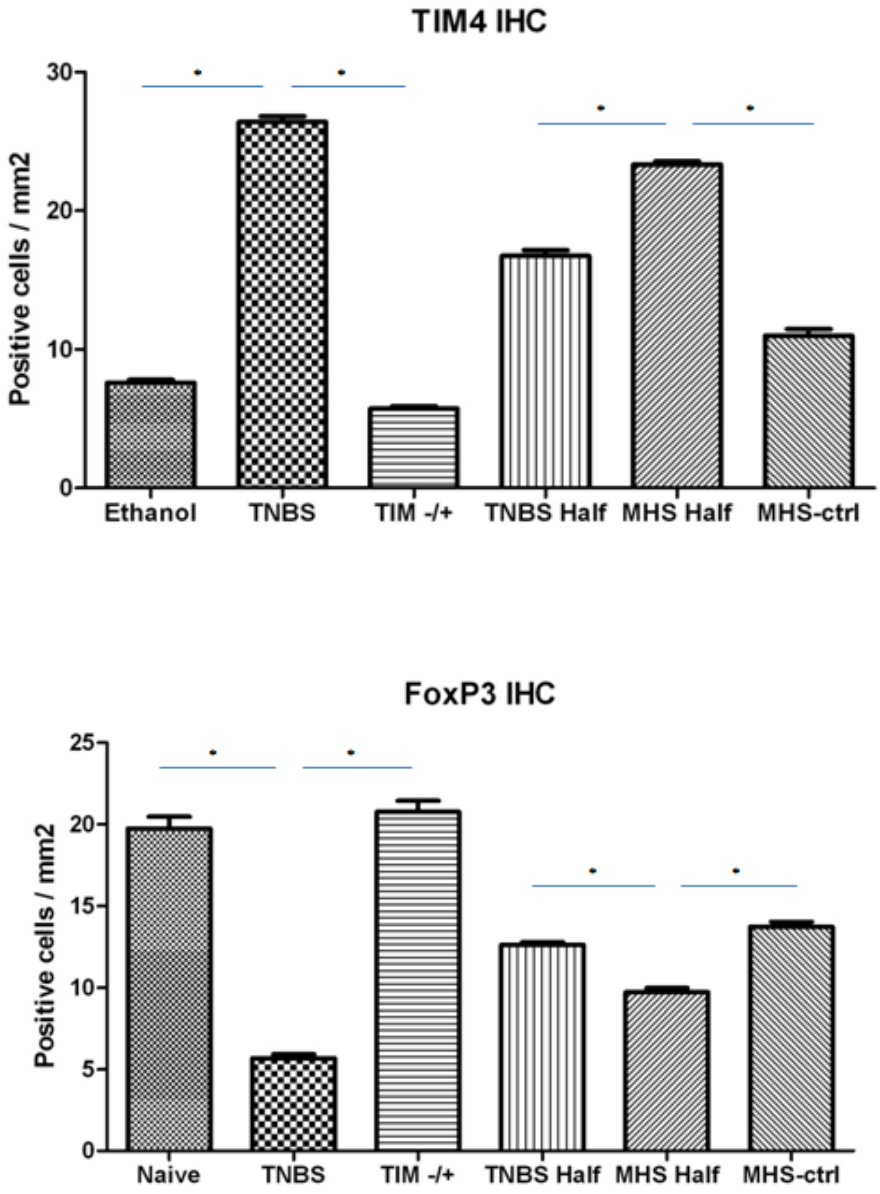

Figure 24. Immunohistochemistry result in all experiment groups. All data were presented as means ± SE. *P < 0.001

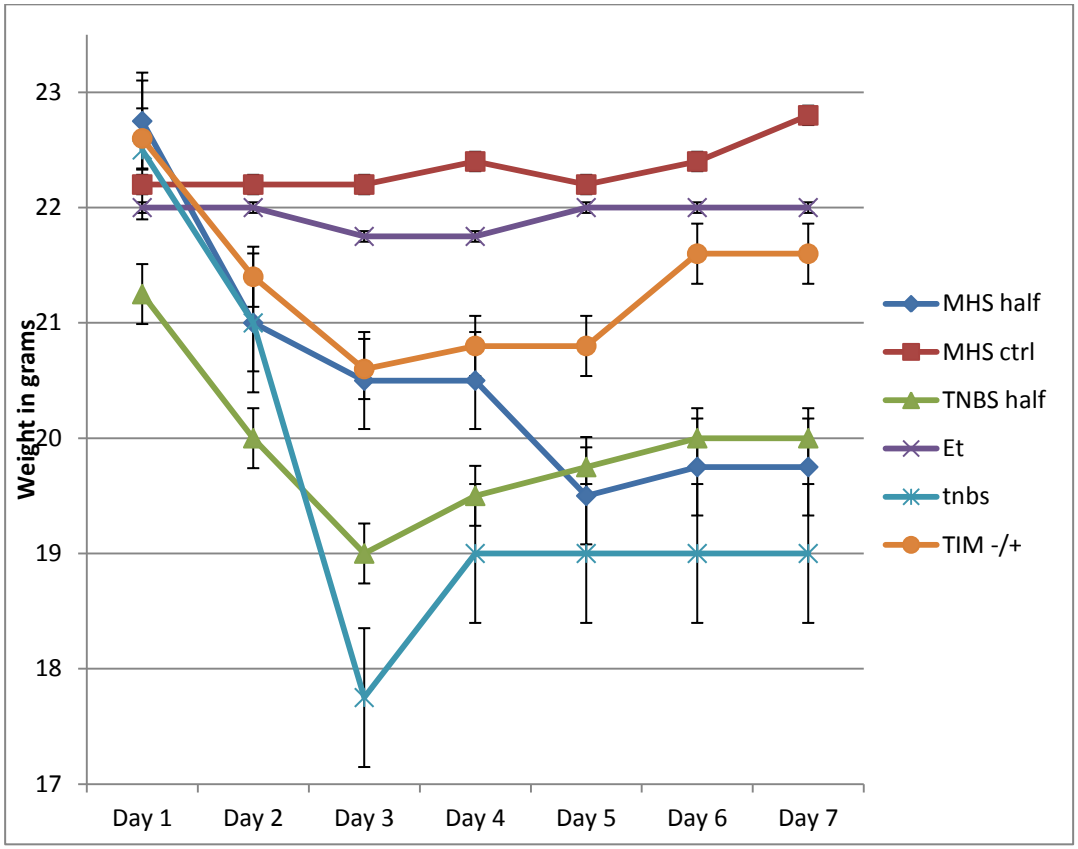

Figure 25. Mice weight loss from all experiment groups. N=12-15.

Data from the weight loss showed that in the anti-TIM-4 group the steep decrease on the second to the third day did not exist, and the group with adoptively transferred TIM-4- M-HS did not show any sign of weight loss. Another interesting point was that the group with adoptively transferred TIM-4+ M-HS and half dose TNBS colitis model did not recover their weight after the third day which normally happened in the full dose TNBS model (Figure 25).

Furthermore, the alteration of TIM-4 expression in the group that anti-TIM-4 antibodies was injected showed a significant change in the T effector cells population as well as the regulatory T cells in the opposite manner. With the full dose of TNBS-colitis induction, the population of both Th1 and Th2 arms in this group did not increase much compared to the full dose TNBS-colitis model. Additionally, in the group that TIM-4+ M-HS cell lines were injected prior to half dose TNBS induction showed a significant increase in Th1 and Th2 population (Figure 26).

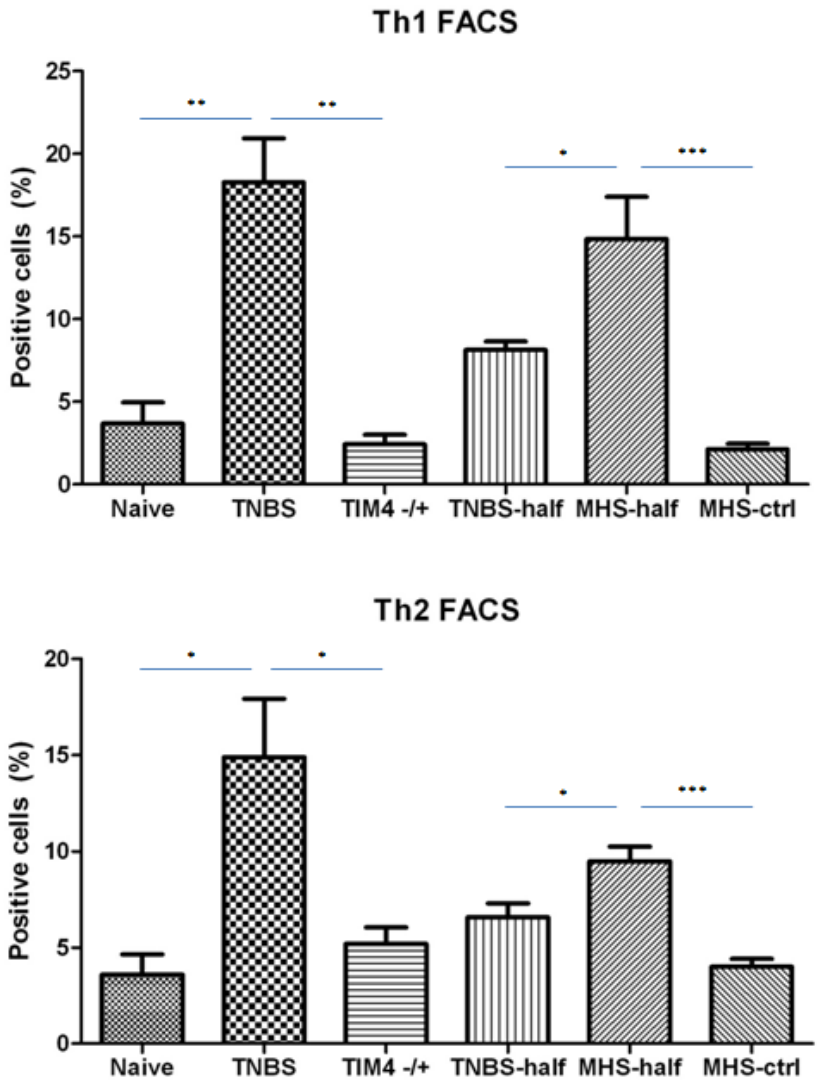

Figure 26. Flow cytometry analysis. Result for Th1 and Th2 population in all experiment groups. All data were presented as means ± SE. *P < 0.05, **P<0.01, ***P<0.001

V. DISCUSSION

5.1 Colitis in human and mice model

Colitis induction in mouse models using TNBS is a common experimental procedure that has been used for more than 20 years. TNBS is a hapten that is highly polarized and easy to bind to any kind of protein. TNBS injection to the colon using a soft tube diluted in 50% ethanol is the most commonly used colitis induction procedure. Many modifications have been suggested to increase the efficacy of the colitis symptoms, however the model itself still lack the similarity to the real colitis in human.[182] IBD is a chronic inflammatory disease characterized by increased PMN, macrophages and lymphocytes infiltration in the mucosa layer of the intestine with increased release of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-17, and IL-2.[183,184] The inflammation in mice model colons induced by TNBS was shown to be severe with infiltration of pro-inflammatory cells, destruction of the mucosal structure, and edema; which was very similar to the IBD colon biopsy histological structure. The result from the weight loss and MPO also confirmed the severity of the inflammation in the colon.

The increase of TIM-4 expression in both IBD patients and colitis mice intestines is a novel finding which previously has not been published. While the interaction between TIM-4 and TH2 cells in food allergy is established,[131] however TNBS colitis itself is often claimed as a TH1 response.[185] Therefore using TNBS model to develop a colitis in mice to look into the expression rate of a TH2 related molecule is worth investigating considering TIM-1 were also expressed on TH1 cells only not so much as in TH2 cells.[118]

5.2 Treg expression in the development of colitis

The small Treg population in colitis plays a role in the increased proliferation of the T effector cells because Treg cells maintain the gut tolerance by suppressing the number of excessive Th1, Th2 and Th17 cells proliferation.[186,187] When the immune property balance is distorted because of the decreased proliferation of Treg cells, the other arm of the immune system flourishes.[99] Th1 and Th2 cytokines were found to be markedly increased in IBD, indicating that the disease was not the main property of innate immune response only but also adaptive immune response dependent. Heller et al. found that there was a significant increase of IL-13 and IL-4 in UC patients suggesting that this subtype of IBD is Th2 dependent and the increase of IL-13 was the main cause of increased IECs apoptosis in UC cases.[30] From the TH1 arm, interferon-gamma also was found to be able to induce epithelial cell apoptosis.[188] In the anti-TIM4 experiment group where TIM-4 receptor were blocked, the Treg population decreased after colitis induction but less than the decrease in the TNBS model mice which were given the same dose and administration of TNBS. Furthermore, in the group where external TIM-4 were administered, the Treg population decreased significantly compared to the control groups that only received TNBS half dose and M-HS without TIM-4 expression. From the histological findings the inflammation severity increased when Treg number was low. Hence from this finding, it is implied that TIM-4 expression is associated with Treg population in mice colitis. However, the causal relationship between the two still remains elusive.

5.3 TIM-4 and T Effector Cells

After colitis induction, gut tolerance which in normal condition is anergic to certain commensal flora become active and responds to these microorganisms causing influx of inflammatory cells and release pro-inflammatory cytokines. In normal condition, phagocytes will clear up all the apoptotic bodies in the system without releasing any pro-inflammatory cytokines. However, in colitis the large number of apoptotic IECs may cause more than the usual clearance burden to the macrophages.[189,190]

Like mentioned above, TIM-4 is expressed mainly in APCs and act as PS receptors that help to enhance the phagocytosis by macrophages. TIM-1 is a TIM-4 ligand that is expressed in T effector cells.[118] Previous studies also found that the binding of those two molecules will increase the hypersensitivity response in food allergy.[118,123] Interestingly, TIM-1 is also a PS receptor that once exposed to apoptotic cells may induce immune hyperreactivity.[129]

The decrease in Treg expression and the significant weight loss showed that Treg is crucial in the process of remittance of the disease. Even though there was still a possibility that the Treg cell depression was not directly caused by the excess TIM-4 and the increased number of macrophages may also be responsible in the reduced number of Tregs, but our *in vitro* data showed that the possibility of TIM-4 taking part in the reduction of Treg cells is quite significant. The increased TIM-4 expression is probably caused by the increase in apoptotic IECs in colitis and related to the decrease in the number of Treg population in the gut which finally switches the immune balance to the

inflammation state. In our *in vivo* model, by injecting more TIM-4 into the mice, which resulted in further Treg depression; from the mice weight loss data, three days after colitis induction, the mice weight recovered in the control but not in the TIM-4-M-HS group. Interestingly, the alteration in the TIM-4 expression rate not only affects the Treg polarization but also TH1 and TH2 cells. TNBS model has never been considered resulting in a TH2 response, however in this study we demonstrate otherwise. The full mechanism of how TIM-4 expression is related to the decrease in Treg and increase in TH1 and TH2 still need further investigation.

VI. SUMMARY AND FUTURE DIRECTION

This project is a continuation of the previous studies done by the Yang group which focus on the role of TIM-4 glycoprotein in the pathogenesis of intestinal inflammation. TIM-4 which is expressed by macrophage is able to detect apoptotic cells through its immunoglobulin domain PS receptor. In IBD, the number of apoptotic IECs was found to be markedly increased. In our study, we found that the increase in apoptotic IECs in colitis when exposed to macrophage increased the number of TIM-4 expression to enhance the phagocytic activity in macrophage. This process eventually decreased the Treg cells population in both our *in vitro* and *in vivo* study.

Developing colitis in TIM-4 -/- mice strain and developing mice with TIM-4 over-expressed gene may be considered in the future. Additionally, To find out the expression of TIM-1 in T effector cells after colitis induction may be useful in understanding the underlying adaptive immune respond mechanism.

VII. REFERENCES

1. Leshed A, Somasundaram R, de Haar C, van der Woude CJ, Braat H, Peppelenbosch MP. Role of defective autophagia and the intestinal flora in crohn disease. Self Nonself. 2010; 4: 323-327

2. Koboziev I, Karlsson F, Grisham MB. Gut-associated lymphoid tissue, T cell trafficking, and chronic intestinal inflammation. Ann N Y Acad Sci. 2010; 1207 S1:E86-93

3. Atreya R, Neurath MF. New therapeutic strategies for treatment of inflammatory bowel disease. Mucosal Immunol. 2008; 1: 175-82

4. Tzanakis NE, Tsiligianni IG, Siafakas NM. Pulmonary involvement and allergic disorders in inflammatory bowel disease. World J Gastroenterol. 2010; 16: 299-305

5. Rakoff-Nahoum S, Bousvaros A. Innate and adaptive immune connections in inflammatory bowel diseases. Curr Opin Gastroenterol. 2010; 26: 572-577

6. Loftus EV Jr. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. Gastroenterology. 2004; 126: 1504-1517

7. Blumberg RS. Inflammation in the intestinal tract: pathogenesis and treatment. Dig Dis. 2009; 27: 455-464

8. Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. Annu Rev Immunol. 2010; 28: 573-621

9. Shanahan F, Bernstein CN. The evolving epidemiology of inflammatory bowel disease. Curr Opin Gastroenterol. 2009; 25: 301-305

10. Waterman M, Xu W, Stempak JM, Milgrom R, Bernstein CN, Griffiths AM, Greenberg GR, Steinhart AH, Silverberg MS. Distinct and overlapping genetic loci in crohn's disease and ulcerative colitis: Correlations with pathogenesis. Inflamm Bowel Dis. 2010 Dec 10. [ePub ahead of print]

11. Salzman NH, Bevins CL. Negative interactions with the microbiota: IBD. Adv Exp Med Biol. 2008; 635: 67-78

12. Eri RD, Adams RJ, Tran TV, Tong H, Das I, Roche DK, Oancea I, Png CW, Jeffery PL, Radford-Smith GL, Cook MC, Florin TH, McGuckin MA. An intestinal epithelial defect conferring ER stress results in inflammation involving both innate and adaptive immunity. Mucosal Immunol. 2011; 4: 354-364

13. Mankertz J, Schulzke JD. Altered permeability in inflammatory bowel disease: pathophysiology and clinical implications. Curr Opin Gastroenterol. 2007; 23: 379-383

14. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE. Metagenomic analysis of the human distal gut microbiome. Science. 2006; 312: 1355-1359

15. Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. Science. 2001; 291: 881-884

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

17. Björkstén B, Naaber P, Sepp E, Mikelsaar M. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. Clin Exp Allergy. 1999; 29: 342-346

18. Kalliomäki M, Kirjavainen P, Eerola E, Kero P, Salminen S, Isolauri E. Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. J Allergy Clin Immunol. 2001; 107: 129-134

19. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, Nalin R, Jarrin C, Chardon P, Marteau P, Roca J, Dore J. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut. 2006; 55: 205-211

20. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangette C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottière HM, Doré J, Marteau P, Seksik P, Langella P. Faecalibacterium prausnitzii is an anti-inflammatory commensal

bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A. 2008; 105: 16731-16736

21. Braun-Fahrländer C. Environmental exposure to endotoxin and other microbial products and the decreased risk of childhood atopy: evaluating developments since April 2002. Curr Opin Allergy Clin Immunol. 2003; 3: 325-329

22. Adlerberth I, Strachan DP, Matricardi PM, Ahrné S, Orfei L, Aberg N, Perkin MR, Tripodi S, Hesselmar B, Saalman R, Coates AR, Bonanno CL, Panetta V, Wold AE. Gut microbiota and development of atopic eczema in 3 European birth cohorts. J Allergy Clin Immunol. 2007; 120: 343-350

23. Okada H, Kuhn C, Feillet H, Bach JF. The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. Clin Exp Immunol. 2010; 160: 1-9

24. Werner S, Buser K, Kapp A, Werfel T. The incidence of atopic dermatitis in school entrants is associated with individual life-style factors but not with local environmental factors in Hannover, Germany. Br J Dermatol. 2002; 147: 95-104

25. Camus P, Colby TV. The lung in inflammatory bowel disease. Eur Respir J. 2000; 15: 5-10

26. Marvisi M. Pulmonary involvement in systemic disorders. Curr Drug Targets Inflamm Allergy. 2004; 3: 435

27. Camus P, Piard F, Ashcroft T, Gal AA, Colby TV. The lung in inflammatory bowel disease. Medicine (Baltimore). 1993; 72: 151-183

28. Casey MB, Tazelaar HD, Myers JL, Hunninghake GW, Kakar S, Kalra SX, Ashton R, Colby TV. Noninfectious lung pathology in patients with Crohn's disease. Am J Surg Pathol. 2003; 27: 213-219

29. Kraft SC, Earle RH, Roesler M, Esterly JR. Unexplained bronchopulmonary disease with inflammatory bowel disease. Arch Intern Med. 1976; 136: 454-459

30. Fuss IJ, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA, Fiocchi C, Strober W. Disparate CD4+ lamina propria (LP) lymphokines secretion profi les in infl ammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-γ, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. J Immunol.1996; 153: 1261 – 1270

31. Heller F, Fromm A, Gitter AH, Mankertz J, Schulzke JD. Epithelial apoptosis is a prominent feature of the epithelial barrier disturbance in intestinal inflammation: effect of pro-inflammatory interleukin-13 on epithelial cell function. Mucosal Immunol. 2008; 1: S58-61

32. Walsh RE, Gaginella TS. The eosinophil in inflammatory bowel disease. Scand J Gastroenterol. 1991; 26: 1217-1224

33. Choy MY, Walker-Smith JA, Williams CB, MacDonald TT. Activated eosinophils in chronic inflammatory bowel disease. Lancet. 1990; 336: 126-127

34. Levo Y, Shalit M, Wollner S, Fich A. Serum IgE levels in patients with inflammatory bowel disease. Ann Allergy. 1986; 56: 85-87

35. Berstad A, Børkje B, Riedel B, Elsayed S, Berstad A. Increased fecal eosinophil cationic protein in inflammatory bowel disease. Hepatogastroenterology. 1993; 40: 276-278

36. Garcia-Zepeda EA, Rothenberg ME, Ownbey RT, Celestin J, Leder P, Luster AD. Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. Nat Med. 1996; 2: 449-456

37. von Boehmer H. Mechanisms of suppression by suppressor T cells. Nat Immunol. 2005; 6: 338-344

38. Weiner HL. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. Immunol Rev. 2001; 182: 207-214

39. Blanchard JF, Bernstein CN, Wajda A, Rawsthorne P. Small-area variations and sociodemographic correlates for the incidence of Crohn's disease and ulcerative colitis. Am J Epidemiol. 2001; 154: 328-335

40. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med. 2002; 347: 911-920

41. Groux H, Powrie F. Regulatory T cells and inflammatory bowel disease. Immunol Today. 1999; 20: 442-445

42. Singh B, Read S, Asseman C, Malmström V, Mottet C, Stephens LA, Stepankova R, Tlaskalova H, Powrie F. Control of intestinal inflammation by regulatory t cells. Immunol Rev. 2001; 182: 190-200

43. Allez M, Mayer L. Regulatory T cells: peace keepers in the gut. Inflamm Bowel Dis. 2004; 10: 666-676

44. Huibregtse IL, van Lent AU, van Deventer SJ. Immunopathogenesis of IBD: insufficient suppressor function in the gut? Gut. 2007; 56: 584-592

45. Murphy SJ, Ullman TA, Abreu MT. Gut microbes in Crohn's disease: getting to know you better? Am J Gastroenterol. 2008; 103: 397-398

46. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nat Rev Immunol. 2004; 4: 478-485

47. Shih DQ, Targan SR. Immunopathogenesis of inflammatory bowel disease. World J Gastroenterol. 2008; 14: 390-400

48. Qiu W, Wu B, Wang X, Buchanan ME, Regueiro MD, Hartman DJ, Schoen RE, Yu J, Zhang L. PUMA-mediated intestinal epithelial apoptosis contributes to ulcerative colitis in humans and mice. J Clin Invest. 2011; 121: 1722-1732

49. Salim SY, Söderholm JD. Importance of disrupted intestinal barrier in inflammatory bowel diseases. Inflamm Bowel Dis. 2011; 17: 362-381

50. Mudter J, Neurath MF. Apoptosis of T cells and the control of inflammatory bowel disease: therapeutic implications. Gut. 2007; 56: 293-303

51. Sturm A, de Souza HS, Fiocchi C. Mucosal T cell proliferation and apoptosis in inflammatory bowel disease. Curr Drug Targets. 2008; 9: 381-387

52. Boirivant M, Marini M, Di Felice G, Pronio AM, Montesani C, Tersigni R, Strober W. Lamina propria T cells in Crohn's disease and other gastrointestinal inflammation show defective CD2 pathway-induced apoptosis. Gastroenterology. 1999; 116: 557-565

53. Imielinski M, Baldassano RN, Griffiths A, Russell RK, Annese V, Dubinsky M, Kugathasan S, Bradfield JP, Walters TD, Sleiman P, Kim CE, Muise A, Wang K, Glessner JT, Saeed S, Zhang H, Frackelton EC, Hou C, Flory JH, Otieno G, Chiavacci RM, Grundmeier R, Castro M, Latiano A, Dallapiccola B, Stempak J, Abrams DJ, Taylor K, McGovern D; Western Regional Alliance for Pediatric IBD, Silber G, Wrobel I, Quiros A; International IBD Genetics Consortium, Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, Brant SR, Silverberg MS, Taylor KD, Barmuda MM, Bitton A, Dassopoulos T, Datta LW, Green T, Griffiths AM, Kistner EO, Murtha MT, Regueiro MD, Rotter JI, Schumm LP, Steinhart AH, Targan SR, Xavier RJ; NIDDK IBD Genetics Consortium, Libioulle C, Sandor C, Lathrop M, Belaiche J, Dewit O, Gut I, Heath S, Laukens D, Mni M, Rutgeerts P, Van Gossum A, Zelenika D, Franchimont D, Hugot JP, de Vos M, Vermeire S, Louis E; Belgian-French IBD Consortium; Wellcome Trust Case Control Consortium, Cardon LR, Anderson CA, Drummond H, Nimmo E, Ahmad T, Prescott NJ, Onnie CM, Fisher SA, Marchini J, Ghori J, Bumpstead S, Gwillam R, Tremelling M, Delukas P, Mansfield J, Jewell D, Satsangi J, Mathew CG, Parkes M, Georges M, Daly MJ, Heyman MB, Ferry GD, Kirschner B, Lee J, Essers J, Grand R, Stephens M, Levine A, Piccoli D, Van Limbergen J, Cucchiara

S, Monos DS, Guthery SL, Denson L, Wilson DC, Grant SF, Daly M, Silverberg MS, Satsangi J, Hakonarson H. Common variants at five new loci associated with early-onset inflammatory bowel disease. Nat Genet. 2009; 41: 1335-1340

54. Nakazawa A, Dotan I, Brimnes J, Allez M, Shao L, Tsushima F, Azuma M, Mayer L. The expression and function of costimulatory molecules B7H and B7-H1 on colonic epithelial cells. Gastroenterology. 2004; 126: 1347-1357

55. Cario E, Brown D, McKee M, Lynch-Devaney K, Gerken G, Podolsky DK. Commensal-associated molecular patterns induce selective toll-like receptor-trafficking from apical membrane to cytoplasmic compartments in polarized intestinal epithelium. Am J Pathol. 2002; 160: 165-173

56. Atuma C, Strugala V, Allen A, Holm L. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. Am J Physiol Gastrointest Liver Physiol. 2001; 280: G922-929

57. Swidsinski A, Loening-Baucke V, Theissig F, Engelhardt H, Bengmark S, Koch S, Lochs H, Dörffel Y. Comparative study of the intestinal mucus barrier in normal and inflamed colon. Gut. 2007; 56: 343-350

58. Moehle C, Ackermann N, Langmann T, Aslanidis C, Kel A, Kel-Margoulis O, Schmitz-Madry A, Zahn A, Stremmel W, Schmitz G. Aberrant intestinal expression and allelic variants of mucin genes associated with inflammatory bowel disease. J Mol Med (Berl). 2006; 84: 1055-1066

59. Van der Sluis M, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB, Büller HA, Dekker J, Van Seuningen I, Renes IB, Einerhand AW. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology. 2006; 131: 117-129

60. Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci U S A. 2008; 105: 15064-15069

61. Asquith M, Powrie F. An innately dangerous balancing act: intestinal homeostasis, inflammation, and colitis-associated cancer. J Exp Med. 2010; 207: 1573-1577

62. Barker N, van de Wetering M, Clevers H. The intestinal stem cell. Genes Dev. 2008; 22: 1856-1864

63. Zeissig S, Bojarski C, Buergel N, Mankertz J, Zeitz M, Fromm M, Schulzke JD. Downregulation of epithelial apoptosis and barrier repair in active Crohn's disease by tumor necrosis factor alpha antibody treatment. Gut. 2004; 53: 1295-1302

64. Marini M, Bamias G, Rivera-Nieves J, Moskaluk CA, Hoang SB, Ross WG, Pizarro TT, Cominelli F. TNF-alpha neutralization ameliorates the severity of murine Crohn's-like ileitis by abrogation of intestinal epithelial cell apoptosis. Proc Natl Acad Sci U S A. 2003; 100: 8366-8371

65. Zeissig S, Bürgel N, Günzel D, Richter J, Mankertz J, Wahnschaffe U, Kroesen AJ, Zeitz M, Fromm M, Schulzke JD. Changes in expression and distribution of

claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. Gut. 2007; 56: 61-72

66. Schulzke JD, Bojarski C, Zeissig S, Heller F, Gitter AH, Fromm M. Disrupted barrier function through epithelial cell apoptosis. Ann N Y Acad Sci. 2006; 1072: 288-99

67. Heller F, Florian P, Bojarski C, Richter J, Christ M, Hillenbrand B, Mankertz J, Gitter AH, Bürgel N, Fromm M, Zeitz M, Fuss I, Strober W, Schulzke JD. Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. Gastroenterology. 2005; 129: 550-564

68. Schmitz H, Barmeyer C, Fromm M, Runkel N, Foss HD, Bentzel CJ, Riecken EO, Schulzke JD. Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. Gastroenterology. 1999; 116: 301-309

69. Izcue A, Coombes JL, Powrie F. Regulatory lymphocytes and intestinal inflammation. Annu Rev Immunol. 2009; 27: 313-338

70. Abraham C, Cho JH. Inflammatory bowel disease. N Engl J Med. 2009; 361: 2066-2078

71. Bjarnason I, MacPherson A, Hollander D. Intestinal permeability: an overview. Gastroenterology. 1995; 108: 1566-1581

72. Pearson AD, Eastham EJ, Laker MF, Craft AW, Nelson R. Intestinal permeability in children with Crohn's disease and coeliac disease. Br Med J (Clin Res Ed). 1982; 285: 20-21

73. Ukabam SO, Clamp JR, Cooper BT. Abnormal small intestinal permeability to sugars in patients with Crohn's disease of the terminal ileum and colon. Digestion. 1983; 27: 70-74

74. Gitter AH, Wullstein F, Fromm M, Schulzke JD. Epithelial barrier defects in ulcerative colitis: characterization and quantification by electrophysiological imaging. Gastroenterology. 2001; 121: 1320-1328

75. Kersting S, Bruewer M, Schuermann G, Klotz A, Utech M, Hansmerten M, Krieglstein CF, Senninger N, Schulzke JD, Naim HY, Zimmer KP. Antigen transport and cytoskeletal characteristics of a distinct enterocyte population in inflammatory bowel diseases. Am J Pathol. 2004; 165: 425-437

76. Chen L, Park SM, Turner JR, Peter ME. Cell death in the colonic epithelium during inflammatory bowel diseases: CD95/Fas and beyond. Inflamm Bowel Dis. 2010; 16: 1071-1076

77. Kerr JF, Gobé GC, Winterford CM, Harmon BV. Anatomical methods in cell death. Methods Cell Biol. 1995; 46: 1-27

78. Gardai SJ, Bratton DL, Ogden CA, Henson PM. Recognition ligands on apoptotic cells: a perspective. J Leukoc Biol. 2006; 79: 896-903

79. Grimsley C, Ravichandran KS. Cues for apoptotic cell engulfment: eat-me, don't eat-me and come-get-me signals. Trends Cell Biol. 2003; 13: 648-656

80. Schulzke JD, Ploeger S, Amasheh M, Fromm A, Zeissig S, Troeger H, Richter J, Bojarski C, Schumann M, Fromm M. Epithelial tight junctions in intestinal inflammation. Ann N Y Acad Sci. 2009; 1165: 294-300

81. Resta-Lenert S, Barrett KE. Probiotics and commensals reverse TNF-alpha- and IFN-gamma-induced dysfunction in human intestinal epithelial cells. Gastroenterology. 2006; 130: 731-746

82. Suenaert P, Bulteel V, Vermeire S, Noman M, Van Assche G, Rutgeerts P. Hyperresponsiveness of the mucosal barrier in Crohn's disease is not tumor necrosis factor-dependent. Inflamm Bowel Dis. 2005; 11: 667-673

83. Korzenik JR, Podolsky DK. Evolving knowledge and therapy of inflammatory bowel disease. Nat Rev Drug Discov. 2006; 5: 197-209

84. van Dieren JM, Kuipers EJ, Samsom JN, Nieuwenhuis EE, van der Woude CJ. Revisiting the immunomodulators tacrolimus, methotrexate, and mycophenolate mofetil: their mechanisms of action and role in the treatment of IBD. Inflamm Bowel Dis. 2006; 12: 311-327

85. Escher JC, Taminiau JA, Nieuwenhuis EE, Büller HA, Grand RJ. Treatment of inflammatory bowel disease in childhood: best available evidence. Inflamm Bowel Dis. 2003; 9: 34-58

86. Cominelli F. Cytokine-based therapies for Crohn's disease--new paradigms. N Engl J Med. 2004; 351: 2045-2048

87. Wan YY, Flavell RA. Regulatory T cells, transforming growth factor-beta, and immune suppression. Proc Am Thorac Soc. 2007; 4: 271-276

88. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat Immunol. 2007; 8: 277-284

89. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. Cell. 2000; 101: 455-458

90. Eastaff-Leung N, Mabarrack N, Barbour A, Cummins A, Barry S. Foxp3+ regulatory T cells, Th17 effector cells, and cytokine environment in inflammatory bowel disease. J Clin Immunol. 2010; 30: 80-89

91. Wang Y, Liu XP, Zhao ZB, Chen JH, Yu CG. Expression of CD4+ forkhead box P3 (FOXP3)+ regulatory T cells in inflammatory bowel disease. J Dig Dis. 2011; 12: 286-294

92. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. Blood. 2002; 99: 3493-3499

93. Scalapino KJ, Tang Q, Bluestone JA, Bonyhadi ML, Daikh DI. Suppression of disease in New Zealand Black/New Zealand White lupus-prone mice by adoptive transfer of ex vivo expanded regulatory T cells. J Immunol. 2006; 177: 1451-1459

94. Powrie F, Correa-Oliveira R, Mauze S, Coffman RL. Regulatory interactions between CD45RBhigh and CD45RBlow CD4+ T cells are important for the balance between protective and pathogenic cell-mediated immunity. J Exp Med. 1994; 179: 589-600

95. Mottet C, Uhlig HH, Powrie F. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. J Immunol. 2003; 170: 3939-3943

96. Uhlig HH, Coombes J, Mottet C, Izcue A, Thompson C, Fanger A, Tannapfel A, Fontenot JD, Ramsdell F, Powrie F. Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. J Immunol. 2006; 177: 5852-5860

97. Endharti AT, Okuno Y, Shi Z, Misawa N, Toyokuni S, Ito M, Isobe K, Suzuki H. CD8+CD122+ regulatory T cells (Tregs) and CD4+ Tregs cooperatively prevent and cure CD4+ cell-induced colitis. J Immunol. 2011; 186: 41-52

98. Ogino H, Nakamura K, Ihara E, Akiho H, Takayanagi R. CD4+CD25+ regulatory T cells suppress Th17-responses in an experimental colitis model. Dig Dis Sci. 2011; 56: 376-386

99. Boden EK, Snapper SB. Regulatory t cells in inflammatory bowel disease. Curr Opin Gastroenterol. 2008; 24: 733-741

100. Neurath MF, Fuss I, Kelsall BL, Stüber E, Strober W. Antibodies to interleukin 12 abrogate established experimental colitis in mice. J Exp Med. 1995; 182: 1281-1290

101. Himmel ME, Hardenberg G, Piccirillo CA, Steiner TS, Levings MK. The role of T-regulatory cells and Toll-like receptors in the pathogenesis of human inflammatory bowel disease. Immunology. 2008; 125: 145-153

102. Murai M, Krause P, Cheroutre H, Kronenberg M. Regulatory T-cell stability and plasticity in mucosal and systemic immune systems. Mucosal Immunol. 2010; 3: 443-449

103. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature. 1992; 359: 693-699

104. Yoshimura A, Mori H, Ohishi M, Aki D, Hanada T. Negative regulation of cytokine signaling influences inflammation. Curr Opin Immunol. 2003; 15: 704-708

105. Strobl H, Knapp W. TGF-beta1 regulation of dendritic cells. Microbes Infect. 1999; 1: 1283-1290

106. Dignass AU, Podolsky DK. Cytokine modulation of intestinal epithelial cell restitution: central role of transforming growth factor beta. Gastroenterology. 1993; 105: 1323-1332

107. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med. 2003; 198: 1875-1886

108. Wan YY, Flavell RA. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. Proc Natl Acad Sci U S A. 2005; 102: 5126-5131

109. Travis MA, Reizis B, Melton AC, Masteller E, Tang Q, Proctor JM, Wang Y, Bernstein X, Huang X, Reichardt LF, Bluestone JA, Sheppard D. Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice. Nature. 2007; 449: 361-365

110. Lacy-Hulbert A, Smith AM, Tissire H, Barry M, Crowley D, Bronson RT, Roes JT, Savill JS, Hynes RO. Ulcerative colitis and autoimmunity induced by loss of myeloid alphav integrins. Proc Natl Acad Sci U S A. 2007; 104: 15823-15828

111. McIntire JJ, Umetsu SE, Akbari O, Potter M, Kuchroo VK, Barsh GS, Freeman GJ, Umetsu DT, DeKruyff RH. Identification of Tapr (an airway hyperreactivity regulatory locus) and the linked Tim gene family. Nat Immunol. 2001; 2: 1109-1116

112. Kuchroo VK, Dardalhon V, Xiao S, Anderson AC. New roles for TIM family members in immune regulation. Nat Rev Immunol. 2008; 8: 577-580

113. Kuchroo VK, Meyers JH, Umetsu DT, DeKruyff RH. TIM family of genes in immunity and tolerance. Adv Immunol. 2006; 91: 227-249

114. Kuchroo VK, Umetsu DT, DeKruyff RH, Freeman GJ. The TIM gene family: emerging roles in immunity and disease. Nat Rev Immunol. 2003; 3: 454-462

115. Shakhov AN, Rybtsov S, Tumanov AV, Shulenin S, Dean M, Kuprash DV, Nedospasov SA. SMUCKLER/TIM4 is a distinct member of TIM family expressed by stromal cells of secondary lymphoid tissues and associated with lymphotoxin signaling. Eur J Immunol. 2004; 34: 494-503

116. Rodriguez-Manzanet R, Meyers JH, Balasubramanian S, Slavik J, Kassam N, Dardalhon V, Greenfield EA, Anderson AC, Sobel RA, Hafler DA, Strom TB, Kuchroo VK. TIM-4 expressed on APCs induces T cell expansion and survival. J Immunol. 2008; 180: 4706-4713

117. Mizui M, Shikina T, Arase H, Suzuki K, Yasui T, Rennert PD, Kumanogoh A, Kikutani H. Bimodal regulation of T cell-mediated immune responses by TIM-4. Int Immunol. 2008; 20: 695-708

118. Meyers JH, Chakravarti S, Schlesinger D, Illes Z, Waldner H, Umetsu SE, Kenny J, Zheng XX, Umetsu DT, DeKruyff RH, Strom TB, Kuchroo VK. TIM-4 is the

ligand for TIM-1, and the TIM-1-TIM-4 interaction regulates T cell proliferation. Nat Immunol. 2005; 6: 455-464

119. Kobayashi N, Karisola P, Peña-Cruz V, Dorfman DM, Jinushi M, Umetsu SE, Butte MJ, Nagumo H, Chernova I, Zhu B, Sharpe AH, Ito S, Dranoff G, Kaplan GG, Casasnovas JM, Umetsu DT, Dekruyff RH, Freeman GJ. TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. Immunity. 2007; 27: 927-940

120. Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S. Identification of Tim4 as a phosphatidylserine receptor. Nature. 2007; 450: 435-439

121. Rodriguez-Manzanet R, DeKruyff R, Kuchroo VK, Umetsu DT. The costimulatory role of TIM molecules. Immunol Rev. 2009; 229: 259-270

122. Xiao S, Najafian N, Reddy J, Albin M, Zhu C, Jensen E, Imitola J, Korn T, Anderson AC, Zhang Z, Gutierrez C, Moll T, Sobel RA, Umetsu DT, Yagita H, Akiba H, Strom T, Sayegh MH, DeKruyff RH, Khoury SJ, Kuchroo VK. Differential engagement of Tim-1 during activation can positively or negatively costimulate T cell expansion and effector function. J Exp Med. 2007; 204: 1691-1702

123. Wilker PR, Sedy JR, Grigura V, Murphy TL, Murphy KM. Evidence for carbohydrate recognition and homotypic and heterotypic binding by the TIM family. Int Immunol. 2007; 19: 763-773

124. Umetsu SE, Lee WL, McIntire JJ, Downey L, Sanjanwala B, Akbari O, Berry GJ, Nagumo H, Freeman GJ, Umetsu DT, DeKruyff RH. TIM-1 induces T cell activation and inhibits the development of peripheral tolerance. Nat Immunol. 2005; 6: 447-454

125. Chakravarti S, Sabatos CA, Xiao S, Illes Z, Cha EK, Sobel RA, Zheng XX, Strom TB, Kuchroo VK. Tim-2 regulates T helper type 2 responses and autoimmunity. J Exp Med. 2005; 202: 437-444

126. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, Manning S, Greenfield EA, Coyle AJ, Sobel RA, Freeman GJ, Kuchroo VK. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature. 2002; 415: 536-541

127. Sánchez-Fueyo A, Tian J, Picarella D, Domenig C, Zheng XX, Sabatos CA, Manlongat N, Bender O, Kamradt T, Kuchroo VK, Gutiérrez-Ramos JC, Coyle AJ, Strom TB. Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. Nat Immunol. 2003; 4: 1093-1101

128. Rong S, Park JK, Kirsch T, Yagita H, Akiba H, Boenisch O, Haller H, Najafian N, Habicht A. The TIM-1:TIM-4 pathway enhances renal ischemia-reperfusion injury. J Am Soc Nephrol. 2011; 22: 484-495

129. Yamanishi Y, Kitaura J, Izawa K, Kaitani A, Komeno Y, Nakamura M, Yamazaki S, Enomoto Y, Oki T, Akiba H, Abe T, Komori T, Morikawa Y, Kiyonari H, Takai T, Okumura K, Kitamura T. TIM1 is an endogenous ligand for LMIR5/CD300b: LMIR5 deficiency ameliorates mouse kidney ischemia/reperfusion injury. J Exp Med. 2010; 207: 1501-1511

130. Dorfman DM, Hornick JL, Shahsafaei A, Freeman GJ. The phosphatidylserine receptors, T cell immunoglobulin mucin proteins 3 and 4, are markers of histiocytic sarcoma and other histiocytic and dendritic cell neoplasms. Hum Pathol. 2010; 41: 1486-1494

131. Yang PC, Xing Z, Berin CM, Soderholm JD, Feng BS, Wu L, Yeh C. TIM-4 expressed by mucosal dendritic cells plays a critical role in food antigen-specific Th2 differentiation and intestinal allergy. Gastroenterology. 2007; 133: 1522-1533

132. Feng BS, Zheng PY, Chen X, Liao XQ, Yang PC. Investigation of the role of cholera toxin in assisting the initiation of the antigen-specific Th2 response. Immunol Invest. 2008; 37: 782-797

133. Albacker LA, Karisola P, Chang YJ, Umetsu SE, Zhou M, Akbari O, Kobayashi N, Baumgarth N, Freeman GJ, Umetsu DT, DeKruyff RH. TIM-4, a receptor for phosphatidylserine, controls adaptive immunity by regulating the removal of antigen-specific T cells. J Immunol. 2010; 185: 6839-6849

134. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol. 1992; 148: 2207-2216

135. Verhoven B, Schlegel RA, Williamson P. Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. J Exp Med. 1995; 182: 1597-1601

136. Nagata S. Apoptosis by death factor. Cell. 1997; 88: 355-365

137. Vaux DL, Korsmeyer SJ. Cell death in development. Cell. 1999; 96: 245-254

138. Schulze C, Munoz LE, Franz S, Sarter K, Chaurio RA, Gaipl US, Herrmann M. Clearance deficiency--a potential link between infections and autoimmunity. Autoimmun Rev. 2008; 8: 5-8

139. Rodriguez-Manzanet R, Sanjuan MA, Wu HY, Quintana FJ, Xiao S, Anderson AC, Weiner HL, Green DR,Kuchroo VK. T and B cell hyperactivity and autoimmunity associated with niche-specific defects in apoptotic body clearance in TIM-4-deficient mice. Proc Natl Acad Sci U S A. 2010; 107: 8706-8711

140. Asano K, Miwa M, Miwa K, Hanayama R, Nagase H, Nagata S, Tanaka M. Masking of phosphatidylserine inhibits apoptotic cell engulfment and induces autoantibody production in mice. J Exp Med. 2004; 200: 459-467

141. Cohen PL, Caricchio R, Abraham V, Camenisch TD, Jennette JC, Roubey RA, Earp HS, Matsushima G, Reap EA. Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. J Exp Med. 2002; 196: 135-140

142. Borisenko GG, Matsura T, Liu SX, Tyurin VA, Jianfei J, Serinkan FB, Kagan VE. Macrophage recognition of externalized phosphatidylserine and phagocytosis of apoptotic Jurkat cells--existence of a threshold. Arch Biochem Biophys. 2003; 413: 41-52

143. Wong K, Valdez PA, Tan C, Yeh S, Hongo JA, Ouyang W. Phosphatidylserine receptor Tim-4 is essential for the maintenance of the homeostatic state of resident peritoneal macrophages. Proc Natl Acad Sci U S A. 2010; 107: 8712-8717

144. Park D, Hochreiter-Hufford A, Ravichandran KS. The phosphatidylserine receptor TIM-4 does not mediate direct signaling. Curr Biol. 2009; 19: 346-351

145. Perruche S, Zhang P, Liu Y, Saas P, Bluestone JA, Chen W. CD3-specific antibody-induced immune tolerance involves transforming growth factor-beta from phagocytes digesting apoptotic T cells. Nat Med. 2008; 14: 528-535

146. Gordon S. The role of the macrophage in immune regulation. Res Immunol. 1998; 149: 685-688

147. Strober W, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. Annu Rev Immunol. 2002; 20: 495-549

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

149. Targan SR, Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman T, DeWoody KL, Schaible TF, Rutgeerts PJ. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. N Engl J Med. 1997; 337: 1029-1035

150. Hanauer SB, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P; ACCENT I Study Group. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. Lancet. 2002; 359: 1541-1549

151. Hanauer SB, Sandborn WJ, Rutgeerts P, Fedorak RN, Lukas M, MacIntosh D, Panaccione R, Wolf D, Pollack P. Human anti-tumor necrosis factor monoclonal antibody (adalimumab) in Crohn's disease: the CLASSIC-I trial. Gastroenterology. 2006; 130: 323-333

152. Colombel JF, Sandborn WJ, Rutgeerts P, Enns R, Hanauer SB, Panaccione R, Schreiber S, Byczkowski D, Li J, Kent JD, Pollack PF. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. Gastroenterology. 2007; 132: 52-65

153. Sandborn WJ, Feagan BG, Stoinov S, Honiball PJ, Rutgeerts P, Mason D, Bloomfield R, Schreiber S; PRECISE 1 Study Investigators. Certolizumab pegol for the treatment of Crohn's disease. N Engl J Med. 2007; 357: 228-238

154. Schreiber S, Khaliq-Kareemi M, Lawrance IC, Thomsen OØ, Hanauer SB, McColm J, Bloomfield R, Sandborn WJ; PRECISE 2 Study Investigators. Maintenance therapy with certolizumab pegol for Crohn's disease. N Engl J Med. 2007; 357: 239-250

155. Di Sabatino A, Ciccocioppo R, Cinque B, Millimaggi D, Morera R, Ricevuti L, Cifone MG, Corazza GR. Defective mucosal T cell death is sustainably reverted by infliximab in a caspase dependent pathway in Crohn's disease. Gut. 2004; 53: 70-77

156. Van den Brande JM, Braat H, van den Brink GR, Versteeg HH, Bauer CA, Hoedemaeker I, van Montfrans C, Hommes DW, Peppelenbosch MP, van Deventer SJ. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. Gastroenterology. 2003; 124: 1774-1785

157. Van den Brande JM, Koehler TC, Zelinkova Z, Bennink RJ, te Velde AA, ten Cate FJ, van Deventer SJ, Peppelenbosch MP, Hommes DW. Prediction of antitumour necrosis factor clinical efficacy by real-time visualisation of apoptosis in patients with Crohn's disease. Gut. 2007; 56: 509-517

158. Veltkamp C, Anstaett M, Wahl K, Möller S, Gangl S, Bachmann O, Hardtke-Wolenski M, Länger F, Stremmel W, Manns MP, Schulze-Osthoff K, Bantel H. Apoptosis of regulatory T lymphocytes is increased in chronic inflammatory bowel disease and reversed by anti-TNFα treatment. Gut. 2011; 60: 1345-1353

159. Laroux FS, Grisham MB. Immunological basis of inflammatory bowel disease: role of the microcirculation. Microcirculation. 2001; 8: 283-301

160. Kolios G, Petoumenos C, Nakos A. Mediators of inflammation: production and implication in inflammatory bowel disease. Hepatogastroenterology. 1998; 45: 1601-1609

161. Pallone F, Monteleone G. Regulatory cytokines in inflammatory bowel disease. Aliment Pharmacol Ther. 1996;10 Suppl 2:75-9

162. Menzel K, Hausmann M, Obermeier F, Schreiter K, Dunger N, Bataille F, Falk W, Scholmerich J, Herfarth H, Rogler G. Cathepsins B, L and D in inflammatory bowel disease macrophages and potential therapeutic effects of cathepsin inhibition in vivo. Clin Exp Immunol. 2006; 146: 169-180

163. Mahida YR, Wu KC, Jewell DP. Respiratory burst activity of intestinal macrophages in normal and inflammatory bowel disease. Gut. 1989; 30: 1362-1370

164. Mahida YR, Patel S, Gionchetti P, Vaux D, Jewell DP. Macrophage subpopulations in lamina propria of normal and inflamed colon and terminal ileum. Gut. 1989; 30: 826-834

165. Murch SH, Braegger CP, Walker-Smith JA, MacDonald TT. Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease. Gut. 1993; 34: 1705-1709

166. Marshall D, Cameron J, Lightwood D, Lawson AD. Blockade of colony stimulating factor-1 (CSF-I) leads to inhibition of DSS-induced colitis. Inflamm Bowel Dis. 2007; 13: 219-224

167. Watanabe N, Ikuta K, Okazaki K, Nakase H, Tabata Y, Matsuura M, Tamaki H, Kawanami C, Honjo T,Chiba T. Elimination of local macrophages in intestine prevents chronic colitis in interleukin-10-deficient mice. Dig Dis Sci. 2003; 48: 408-414

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

169. Atreya R, Zimmer M, Bartsch B, Waldner MJ, Atreya I, Neumann H, Hildner K, Hoffman A, Kiesslich R, Rink AD, Rau TT, Rose-John S, Kessler H, Schmidt J, Neurath MF. Anti-TNF Antibodies Target T-Cell Apoptosis in Inflammatory Bowel Diseases Via TNFR2 and Intestinal CD14(+)Macrophages. Gastroenterology. 2011 [ePub ahead of print]

170. Hausmann M, Kiessling S, Mestermann S, Webb G, Spöttl T, Andus T, Schölmerich J, Herfarth H, Ray K, Falk W, Rogler G. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. Gastroenterology. 2002; 122: 1987-2000

171. Frolova L, Drastich P, Rossmann P, Klimesova K, Tlaskalova-Hogenova H. Expression of Toll-like receptor 2 (TLR2), TLR4, and CD14 in biopsy samples of patients with inflammatory bowel diseases: upregulated expression of TLR2 in terminal ileum of patients with ulcerative colitis. J Histochem Cytochem. 2008; 56: 267-274

172. Devitt A, Moffatt OD, Raykundalia C, Capra JD, Simmons DL, Gregory CD. Human CD14 mediates recognition and phagocytosis of apoptotic cells. Nature. 1998; 392: 505-509

173. Devitt A, Parker KG, Ogden CA, Oldreive C, Clay MF, Melville LA, Bellamy CO, Lacy-Hulbert A, Gangloff SC, Goyert SM, Gregory CD. Persistence of apoptotic cells without autoimmune disease or inflammation in CD14-/- mice. J Cell Biol. 2004; 167: 1161-1170

174. Moffatt OD, Devitt A, Bell ED, Simmons DL, Gregory CD. Macrophage recognition of ICAM-3 on apoptotic leukocytes. J Immunol. 1999; 162: 6800-6810

175. Scheiffele F, Fuss IJ. Induction of TNBS colitis in mice. Curr Protoc Immunol. 2002;Chapter 15:Unit 15.19.

176. Weigmann B, Tubbe I, Seidel D, Nicolaev A, Becker C, Neurath MF. Isolation and subsequent analysis of murine lamina propria mononuclear cells from colonic tissue. Nat Protoc. 2007; 2: 2307-2311.

177. Cooney RM, Warren BF, Altman DG, Abreu MT, Travis SP. Outcome measurement in clinical trials for Ulcerative Colitis: towards standardisation. Trials. 2007; 8: 17.

178. McCabe AJ, Dowhy M, Holm BA, Glick PL. Myeloperoxidase activity as a lung injury marker in the lamb model of congenital diaphragmatic hernia. J Pediatr Surg. 2001; 36: 334-337

179. Hillegass LM, Griswold DE, Brickson B, Albrightson-Winslow C. Assessment of myeloperoxidase activity in whole rat kidney. J Pharmacol Methods. 1990; 24: 285-295

180. Lewis Carl SA, Gillete-Ferguson I, Ferguson DG. An indirect immunofluorescence procedure for staining the same cryosection with two mouse monoclonal primary antibodies. J Histochem Cytochem. 1993; 41: 1273-1278.

181. Ono M, Murakami T, Kudo A, Isshiki M, Sawada H, Segawa A. Quantitative comparison of anti-fading mounting media for confocal laser scanning microscopy. J Histochem Cytochem. 2001; 49: 305-312.

182. Martínez-Augustin O, Merlos M, Zarzuelo A, Suárez MD, de Medina FS. Disturbances in metabolic, transport and structural genes in experimental colonic inflammation in the rat: a longitudinal genomic analysis. BMC Genomics. 2008; 9: 490.

183. Rivera E, Flores I, Rivera E, Appleyard CB. Molecular profiling of a rat model of colitis: validation of known inflammatory genes and identification of novel disease-associated targets. Inflamm Bowel Dis. 2006; 12: 950-966

184. Fitzpatrick LR, Meirelles K, Small JS, Puleo FJ, Koltun WA, Cooney RN. A new model of chronic hapten-induced colitis in young rats. J Pediatr Gastroenterol Nutr. 2010; 50: 240-250.

185. Alex P, Zachos NC, Nguyen T, Gonzales L, Chen TE, Conklin LS, Centola M, Li X. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. Inflamm Bowel Dis. 2009;15:341-352

186. Goodman WA, Cooper KD, McCormick TS. Regulation generation: the suppressive functions of human regulatory T cells. Crit Rev Immunol. 2012; 32: 65-79.

187. Ozdemir C, Kucuksezer UC, Akdis M, Akdis CA. Specific immunotherapy and turning off the T cell: how does it work? Ann Allergy Asthma Immunol. 2011; 107: 381-392.

188. Yang H, Fan Y, Teitelbaum DH. Intraepithelial lymphocyte-derived interferon-gamma evokes enterocyte apoptosis with parenteral nutrition in mice. Am J Physiol Gastrointest Liver Physiol. 2003; 284:G629-637.

189. Akilov OE, Wu MX, Jin Y, Zhou Z, Geskin LJ, Falo LD, Hasan T. Vaccination with photodynamic therapy-treated macrophages induces highly suppressive T-regulatory cells. Photodermatol Photoimmunol Photomed. 2011; 27: 97-107.

190. Häuser F, Deyle C, Berard D, Neukirch C, Glowacki C, Bickmann JK, Wenzel JJ, Lackner KJ, Rossmann H. Macrophage-stimulating protein polymorphism rs3197999 is associated with a gain of function: implications for inflammatory bowel disease. Genes Immun. 2012 ;13:321-327