DEPARTAMENTO DE CIÊNCIAS DA VIDA

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UNIVERSIDADE DE COIMBRA

DSS induced colitis in Ncf1-mutated mice.

Joana Rita Marçal Gomes

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Doutora Maria Margarida Souto Carneiro, Investigadora e chefe do grupo de Imunologia no Centro de Neurociências e Biologia Celular, Universidade de Coimbra) e co-orientação interna do Professor Doutor Paulo Santos (Universidade de Coimbra).

Joana Rita Marçal Gomes

2014

Para ser grande, sê inteiro: nada

teu exagera ou exclui.

Sê todo em cada coisa. Põe quanto és

no mínimo que fazes.

Assim em cada lago a lua toda

brilha, porque alta vive

Ricardo Reis

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List of Abbreviations

$^{\bullet}O_2^-$ (superoxide)

APC (Aloficocianine)

APC/Cy7 (Aloficocianine- cyanine 7)

CD (Cluster of Differentiation)

CU (Colite Ulcerosa)/UC (Ulcerative Colitis)

DC (Doença de Crohn) / CD (Crohn's Disease)

DGC (Doença granulomatosa crónica) / CGD (Chronic granulomatous disease)

DII (Doença inflamatória intestinal) / IBD (Inflammatory bowel disease)

DSS (Dextrano sulfato de sódio/Dextran sodium sulfate)

FAD (Flavine e adenine nucleotide)

FITC (Fluorescein isothiocyanate)

H&E (Hematoxylin/Eosin)

HAI (Histological activity index)

HLA (human leukocyte antigen)

IL (Interleukin)

KO (Knockout)

LRR (Leucine-rich repeat)

mAb (Monoclonal antibodies)

MAMPs (Padrões moleculares microbianos/Microbial-associated molecular patterns)

MDP (muramyl dipeptide)

NADPH (Nicotinamina dinucleotídeo fosfato / Nicotinamide dinucleotide phosphate)

NK (Natural Killers)

Ncf (Neutrophil Cytosolic Factor)

NF-κB (factor nuclear kappa B)

NOD2 (Nucleotide-binding oligomerization domain-containing protein 2)

NOS (Nitrogen Oxygen Species)

Nox (Nicotinamide dinucleotide phosphate oxidase)

O_2 (oxygen)

PE (R- Phycoerythrin)

PE/Cy7 (Phycoerythrin- cyanine 7)

PerCpCy5.5 (Peridinin chlorophyll protein-cyanine 5.5)

Rac (Triphosphate guanosin)

ROi (Reactive oxygen intermediate)

ROS (Espécies reactivas de oxigénio/Reactive oxygen species)

TGF (Tumor Growth Factor)

TLR (Tool Like Receptor)

TNBS (2, 4, 6-Trinitrobenzene Sulfonic Acid Picrylsulfonic Acid)

TNF (Tumor necrosis factor)

WT (Wild-type)

Abstract

Background: Inflammatory Bowell Disease is a chronic idiopathic disease and immunological disorder of the gastrointestinal tract that includes Crohn Disease (CD) and Ulcerative Colitis (UC). However those diseases affect people in different ways, CD can affects any part of the gastrointestinal tract, but most commonly, the terminal ileum, cecum, peri-anal area and colon. UC most commonly affects the rectum and extends proximally in a continuum. Immune response is altered in an IBD situation because mucosa's disruption and consequent pathogen infiltration. Ncf1* mutated mice present a NADPH complex incapable of producing ROS. This alteration compromises immune system in order to react to pathogens and solve inflammation.

Methods: Two experiments were made. In first colitis was induced in Ncf1* and Wild-Type (WT) mice by a 1st 7-days cycle of dextran sulfate sodium (DSS), with 21 days resting. In the second experiment colitis was induced in Ncf1* and WT by a 1st 7-days cycle of DSS followed by 14 days of resting and then a 2nd 7-days cycle of DSS. Physical scores were evaluated during all experiment. Immune lymph node populations were analyzed by flow citometry and morphological alterations of the colon mucosa were assessed by histology.

Results: Colitis showed more severe in Ncf1* mice comparing to WT mice which is confirmed by clinical scores. Ncf1* presented a more accentuated inflammation with more tendency to chronicity and dysplasia, with a more aberrant immunological response in Ncf1*. Ncf1* mice presented more difficulties in solving inflammation process.

Conclusion: The absence of ROS leads to an increase severity in colitis with more tendency to chronicity and dysplasia. Ncf1* mice response to DSS induced colitis, showed ROS importance in solving inflammation process.

Key-words: reactive-oxygen species; NADPH-oxidase complex; colitis; immune response; dysplasia.

Resumo

Introdução: Doença inflamatória intestinal (DII) é uma doença crónica, idiopática e imunológica do trato gastrointestinal inclui a doença de Crohn e a Colite Ulcerosa. Todavia estas doenças afectam a população de formas distintas, a doença de Crohn pode afectar qualquer parte do trato gastrointestinal, mas mais frequentemente o ileum terminal, o cécum, área peri-anal e o colon. A colite Ulcerosa afecta mais frequentemente o recto estende-se proximalmente em continuum. A resposta imunológica está alterada numa situação de DII devido à destruição da mucosa intestinal e consequente infiltração de patogéneos. Os murganhos com a mutação Ncf1* apresentam um complexo NADPH incapaz de produzir ROS. Esta alteração compromete o Sistema imunológico e a sua capacidade de resolução da inflamação.

Métodos: Realizaram-se duas experiências. Na primeira foi induzida colite nos murganhos mutados Ncf1* e nos WT com um 1º ciclo de 7 dias de DSS, seguido de 21 dias de descanso. Na segunda experiência foi administrado 1º ciclo de 7 dias de DSS, seguido de 14 dias de descanso e de um novo ciclo de 7 dias de DSS. Foram avaliados parâmetros físicos. Analisaram-se as populações imunológicas através de cirtometria de fluxo e as alterações morfológicas foram analisadas por histologia.

Resultados: A colite mostrou-se mais severa nos murganhos Ncf1* comparativamente aos WT conforme indicam os parâmetros clínicos. Os murganhos Ncf1* apresentaram uma inflamação mais acentuada com maior tendência à cronicidade e displasia. A resposta imunológica mostrou-se mais aberrante nos murganhos Ncf1* com maior dificuldade em resolver o processo inflamatório.

Conclusão: A ausência de ROS leva a um aumento na severidade da colite e a uma maior tendência à cronicidade e displasia. Os murganhos Ncf1* mostraram a importância dos ROS na resolução do processo inflamatório.

Palavras-chave: espécies reactivas de oxigénio; complexo NADPH-oxidase; colite; resposta imune; displasia.

CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic relapsing idiopathic, immunological disorder of gastrointestinal tract, leading to long-term and sometimes irreversible impairment of gastro-intestinal structure and function. IBD presents two major forms of the pathogenesis, Crohn's disease (CD) and Ulcerative Colitis (UC). Accumulating evidence suggests that IBD arises from an inappropriate inflammatory immune response to intestinal microbes in a genetically susceptible host. Although UC and CD share many clinical and pathological features they have some different key characteristics that suggest that the main pathological processes in the two forms of the disease are distinct (Blumberg *et al*, 2001) (Bouma Strober, 2003) (Martins and Peppercorn *et al*, 2004) (Sartor, 2006) (Hanauer, 2006) (Abraham *et al*, 2009) (Xavier and Podolsky, 2007) (Lai S. *et al*. 2011).

In Crohn's disease any part of gastrointestinal tract can be affected, but most commonly, the terminal ileum, cecum, peri-anal area and colon. It is characterized by the presence of normal segments of the bowel between affected regions. Histologically CD is characterized by transmural inflammation involving any part of the gastrointestinal tract, dense infiltration of lymphocytes and macrophages, presence of granulomas in up to 60% of the patients and fissuring ulceration (Blumberg, 2001) (Bouma and Strober, 2003) (Martins and Peppercorn, 2004) (Baumgart and Sandborn, 2007) (Xavier and Podolsky, 2007) (Strober, F. *et al*, 2007) (Strober, Fuss et al. 2007) (Abraham and Cho, 2009).

In Ulcerative Colitis, the inflammatory process involves rectum and extends proximally in a continuum. Histologically, inflammation involves superficial mucosal

layers with infiltration of lymphocytes, granulocytes and loss of goblet cells, presence of ulcerations and crypt abscesses can also occur (Bouma and Strober 2003) (Martins and Peppercorn 2004) (Strober, F. *et al*, 2007) (Abraham and Cho 2009).

1.1.1 Epidemiology

There is a bimodal distribution of the disease in population. Most individuals (up to 25% of the patients) (Benchimol, F. *et al*, 2010) are diagnosed during a first peak between ages 15 and 40 years, with a second peak occurring in individuals older than 60 years, demonstrating that CD and UC can affect people of all ages (Bonen and Cho, 2003) (Martins and Peppercorn, 2004) (Hanauer, 2006) (Yan Z. *et al*, 2011).

The colon is the most common macroscopic site of disease in very young children and differentiation of UC from colonic CD may be difficult. Childhood-onset of UC is typically extensive, whereas adults are equally likely to develop UC confined to the distal colon. CD affects mostly males after puberty and females in adult age (Benchimol F. *et al*, 2010).

As recently reviewed, the incidence and prevalence of CD and UC varies greatly around the globe, all races and ethnic groups are affected and the disease incidence is rising internationally (Martins and Peppercorn, 2004). IBD shows no large genetic background shifts and as it was seen in twins studies, genetic has a small role on it, so environmental factors importance in the disease growths.

It is postulated that ‘‘Westernization’’ of society accounts for recent increases in the incidence of IBD. Areas with the highest incidence and prevalence of IBD are in Northern Europe and North America. Although during the past few decades the racial gap has been closing, as there has been an increasing incidence of IBD (Bonen and Cho, 2003) (Martins and Peppercorn, 2004) (Hanauer, 2006) (Baumgart and Carding, 2007)

(Benchimol, F. et al, 2010), (Hansen, T. *et al*, 2010). Besides westernization, there are some other theories/hypothesis trying to explain the increase and the onset of the disease. The ‘‘cold chain hypothesis’’ suggests that refrigeration has altered the bacterial content of our diet, resulting in the increased growth of disease triggering organisms. As with atopic diseases, the ‘‘hygiene hypothesis’’ offers an alternative for the increase of IBD, asthma, rheumatoid arthritis, and type I diabetes in our society. The theory suggests that exposure to pathogens and parasites early in life, stimulates protective immunity that will prevent later an aggressive immunologic processes. So a cleaner environment, smaller families, and lower exposure to farm animals, in other words, excessive sanitation might limit the exposure to environmental antigens and impair the functional maturation of mucosal immune system and induction of immune tolerance, leading to the increase risk for IBD (Sartor, 2006) (Hanauer, 2006) (Baumgart and Carding, 2007) (Benchimol, F. *et al*, 2010). The most widely held hypothesis on the pathogenesis of IBD is that overly aggressive acquired (T cell) immune responses to a subset, of imbalance in the proportions of "protective" and "harmful" commensal enteric bacteria (dysbiosis) develop in genetically susceptible hosts, and environmental factors precipitate the onset or reactivation of disease. (Sartor, 2006) (Hansen T. *et al*, 2010).

1.1.1.1 Environmental triggers

Several previous studies have implicated environmental factors as triggers in genetically predisposed hosts to IBD. The environmental triggers act in IBD by altering/disrupting the mucosal barrier integrity, altering immune response, or the luminal microenvironment, each of which have an impact on susceptibility to inflammation (Martins and Peppercorn, 2004) (Sartor, 2006).

Antibiotics and diet can alter the luminal flora, dietary additives such as aluminum and iron have a well-described adjuvant activity and stimulate bacterial virulence (Sartor, 2006). The pre-morbid state in Crohn's disease includes a diet which is more likely to contain more refined sugar, less fiber, and less raw fruit and vegetables. The refined sugar finding has been supported by a case–control study from Sweden demonstrating a positive link between sucrose intake and Crohn's as well as protective effects of wholegrain bread, muesli and coffee and a high risk associated with fast foods (Baumgart and Carding, 2007) (Hansen T. *et al*, 2010). Vegetable intake and coffee were protective for UC, and fast foods conferred a major risk, a previous study suggested that ''dietary influences may alter the milieu of the intestinal lumen or modify the intestinal flora and promote the growth of an infective agent or its invasion of the gut wall''. It may well be that pre-morbid diet provides either specific or sufficient distal intestinal substrates which allow the invasion of a pathological microbe or the establishment of dysbiosis (Hansen T. *et al*, 2010).

Nonsteroidal anti-inflammatory drugs and acute infections can cause inflammation, which results in increased mucosal permeability, leading to increase uptake of commensal bacterial antigens, that stimulate the T-cell-mediated intestinal inflammation (Martins and Peppercorn, 2004) (Sartor, 2006).

Stress can alter mucosal permeability, mucosal blood flow, epithelial electrolyte, water secretion and expression of cytokines and neuropeptides. All referred factors seem to increase the likelihood of relapse in patients with quiescent disease (Sartor, 2006) (Baumgart and Carding, 2007).

1.1.1.2 Luminal antigens and gut flora

Studies in several different animal models have demonstrated that luminal flora is required for IBD to develop in a susceptible host. Genetically susceptible animals that

are maintained in a germ-free environment from birth don't develop immune system activation and colitis. When these same animals acquire luminal flora, they develop activation of their immune systems and colitis (Blumberg, 2001) (Hanauer, 2006). The normal mucosal microflora is required to initiate or maintain the inflammatory process, presumably by providing one or more antigens or co-stimulatory factors that drive the immune response (Martins and Peppercorn, 2004). When genetically susceptible, hosts develop an aggressive T-cell response towards luminal commensal bacteria.

Antigens from microflora, are present in the body since the birth, so they might be subjected to an intra-thymic processing that allows immune system to distinguish self-antigens from non-self-antigens, so the hypothesis that organism could be reacting to this antigens and developing an autoimmune response, makes chronic mucosal inflammation of IBD to be though as an autoimmune disease (Sartor, 2006) (Bouma and Strober, 2003).

1.1.1.3 The presence of abnormal microflora

One major hypothesis regarding IBD pathogenesis, views the disease as a result from a problem with the microflora, which induces a pathologic response from a normal mucosal immune system. Two types of evidence support this hypothesis. Some data suggest that IBD is associated with pathologic organisms that establish a type of low-grade infection of the mucosa leading to the inflammatory response that characterizes the disease. Other data suggest that IBD patients have a defective epithelial barrier that enables nonpathologic organism's proliferation in close juxtaposition to elements of the mucosal immune system, again leading to the inflammatory response that characterizes the disease.

A previous study by Swidsinski *et al*. demonstrated higher amounts of mucosa-associated bacteria in biopsy tissue obtained from patients with IBD compared with

tissue obtained from control subjects. Darfeuille-Michaud *et al* also reported increased numbers of mucosa-associated bacteria in IBD. In this case, a pathogen-like invasive *E. coli* was associated with the mucosa of 20%–40% of illeal biopsy specimens from CD patients as compared with the mucosa of 6% of specimens from controls. In contrast, about 4% of colonic specimens from both CD patients and from control subjects harbored invasive bacteria, compared with 12% of specimens from UC patients (Strober F. *et al*, 2007). Studies shown that CD fecal samples have a marked reduction in diversity. In healthy individuals, 95% of the bacteria in stool samples belong to bacteroides however, IBD patients showed a marked reduction of these organisms. A very strong association has been shown between illeal CD and the lack of *F. prausnitzii* showing dysbiosis in infectious colitis. Studies that have cultured the colonic mucosa after removal of overlying mucus have shown that mucosa in healthy individuals is relatively sterile but that there is a marked increase in bacteria populations in CD and to a lesser extent in UC (Bai and Ouyang, 2006) (Friswell C. *et al*, 2010) . Bacterial enzyme activities, especially b-D-galactosidase, were also decreased in fecal extracts from CD patients, correlated with the decrease of bifidobacteria counts. Significant decrease in the number of anaerobic bacteria, anaerobic gram negatives and lactobacillus was shown in patients with active UC. Luminal microflora in IBD patients lost the anti-inflammatory function that exists in normal condition, with a reduction in the number of anaerobic bacteria and lactobacillus (Bai and Ouyang, 2006).

Complementary studies using molecular techniques to identify specific bacterial groups have also been applied to the study of the bacterial microflora in IBD, however these studies do not support the presence of a specific, pathogenic organism in IBD (Strober F. *et al*, 2007).

1.2 Genetic

1.2.1 Familial aggregation in IBD

Epidemiological and family studies have provided great evidence that genetic factors have a role in determining susceptibility to IBD. First-degree patients' relatives with IBD have a 4-to-20-fold increased risk. People with CD have a first-degree relative with the disease in 2% of the cases and with IBD in 5% of the cases. Regarding people with UC, they usually have a first-degree relative with the disease in 5% of the cases, and with inflammatory bowel disease in 8% of the cases (Baumgart and Carding, 2007) (Bonen and Cho, 2003). However, despite the evidence supporting a genetic predisposition, most patients with IBD have no close relatives with IBD (Martins and Peppercorn, 2004) (Bouma and Strober, 2003).

1.2.2 Susceptibility genes involved in IBD

There have been two general approaches for gene identification in complex genetic disorders, like IBD, the use of genome-wide linkage studies and the testing of candidate genes. The tendency appears to be that the implicated genes in IBD regulate several biologic functions, including immunoregulation, mucosal barrier integrity and microbial clearance and/or homeostasis (Sartor, 2006). Nowadays several regions on chromosomes, 16, 12, 6, 14, 5, 19, 1, 16, and 3 have been renamed as IBD1–9, respectively, and in a few cases the gene or genes underlying the different chromosome loci that are linked to IBD have been identified.

IBD-1: ***CARD15***, also known as *NOD2*, (caspase recruitment domain family member 15), which is expressed in macrophages, dendritic cells, epithelial cells and paneth cells (Martins and Peppercorn, 2004) (Bouma and Strober, 2003) (Sartor, 2006) (Cho and

Weaver, 2007) (Van Limbergen R. *et al*, 2007) (Marks and Segal, 2008) (Abraham and Cho, 2009).

There are three mutations/polymorphisms associated with NOD2/CARD15, causing amino-acid substitutions, Arg702Trp and Gly908Arg and the frameshift 1007fs—found within the region of *CARD15* that encodes a leucine-rich repeat, which is responsible for bacterial recognition. At least one of these mutations is present in 25–35% of CD patients of European ancestry. The leucine-rich repeat region of CARD15 binds muramyl dipeptide (MDP), which is the biologically active moiety of peptidoglycan, a ubiquitous cell-wall polymer found in almost all bacteria. The binding of MDP to CARD15 activates nuclear factor NF-κB through a receptor-interacting serine-theronine kinase-2 (RIPK2) - dependent signaling pathway, which forms part of a central signaling pathway that stimulates the transcription of multiple genes that encode both pro-inflammatory and protective molecules. The mutations causing Arg702Trp, Gly908Arg and 1007fs cause defective MDP binding (Mathew and Lewis, 2004) (Sartor, 2006) (Xavier and Podolsky, 2007) (Strober F. *et al*, 2007) (Cho and Weaver, 2007) (Van Limbergen R. *et al*, 2007) (Marks and Segal, 2008) (Chen, L. *et al*, 2011). The variant forms of *CARD15* result in reduced macrophage activation of NF-κB pathway and increased luminal bacterial populations. It would be expected this to result in a lower inflammatory response however there is the occurrence of increased inflammation dependent on NF-kB in individuals bearing a mutation impairing NF-kB activation. One theory postulate was that impaired NOD2 function led to a host defense defect that allowed increased bacterial colonization of the gut wall and then later to stimulation of NF-κB via NOD2-independent mechanisms (Strober F. *et al*, 2007) (Van Limbergen R. *et al*, 2007). Another hypothesis was prompted by the observation that the ligand for TLR2, peptidoglycan, can activate NF-κB independently of NOD2

(Strober, Fuss et al. 2007). NOD2 polymorphisms, when expressed within enterocytes and Paneth cells are associated with diminished production of anti-bacterial α-defensins (Xavier and Podolsky, 2007). This might lead to bacterial overgrowth and subsequent infection and chronic inflammation. Despite the presented facts, some studies suggest that NOD2 only plays a small role in the pathogenesis of the disease, because of its strongest association only with CD and the polymorphisms occur predominantly in patients with small bowel disease and are restricted to certain racial groups, which is indicative of the complexities of a multifactorial disorder (Marks and Segal, 2008) (Abraham and Cho, 2009). In patients with variant *CARD15*, homozygotes for this variant gene have a 20- to 40-fold increased risk of developing CD, whereas the heterozygotes have only 2- to 4-fold increased risk (Martins and Peppercorn, 2004)(Bouma and Strober, 2003) (Abraham and Cho, 2009) (Bonen and Cho, 2003).Primary-monocyte-derived macrophages from patients with CD who are homozygous for the truncating mutation in the LRR sensor domain (Leu1007fsinsC) have a globally blunted transcriptional response to MDP. In contrast, NOD2 frameshift-mutation knock-in mice have an enhanced response to MDP and are susceptible to dextran sodium sulphate colitis (Xavier and Podolsky, 2007) (Cho and Abraham, 2007).

Regarding the linkage studies, no evidence was observed in CD-UC or UC- UC affected relative pairs, indicating that the susceptibility gene at IBD1 likely conferred susceptibility only for CD (Cho and Abraham, 2007).

IBD-3, on chromosome 6p encompassing the major histocompatibility complex (HLA) has been implicated consistently for both CD and UC in various linkage studies. One study showed that the linkage at IBD3 locus was sex-specific, being more observed among either CD- or UC-affected males, further showing the likely complexity of disease pathogenesis. Both data from linkage and epidemiologic studies estimate the

relative contribution of HLA region to overall genetic risk as 64% to 100% for UC and 10% to 33% for CD. HLA class II associations contribute to overall disease pathogenesis, especially for UC. Previous studies between 1966 and 1998 showed significant positive associations in UC to DR2, DR9, and DRB1*0103, whereas a negative association was found for DR4. For CD, a positive association was found with DR7, DRB3*0301, and DQ4, and a negative association with DR2 and DR3. Furthermore, this region contains the TNF gene, for which functional promoter polymorphisms affecting TNF expression have been reported. TNF expression involves a variety of regulatory elements located in the gene's promoter region. Three promoter polymorphisms (in the -1031C, -863A, and -857T regions) have been found to be associated with susceptibility and progression of CD in a Japanese population and between CD and the -1031C allele in a European population (Bonen and Cho, 2003, Cho and Weaver, 2007) (Van Limbergen, R. *et al*, 2007) (Mathew and Lewis, 2004).

NADPH oxidase, is a complex formed by several accessory proteins that produces ROS and plays an essential role in cellular response in microbial invasion. Genetic mutations in genes encoding components of the complex, results in both X-linked and autosomal recessive forms of Chronic Granulomatous Disease (CGD), which often develops in intestinal inflammation that is histologically similar to Crohn's colitis. An SNP within the first intron of NCF4 (encoding $p40^{Pphox}$ subunit of the complex) was identified as a CD-specific susceptibility gene. In a recent study Muise *et al*. reported a novel missense variant in *NCF2* (encoding $p67^{phox}$ subunit) in patients with very early onset IBD (VEO-IBD) that resulted in neutrophil dysfunction and susceptibility to CD. Patients with the heterozygote variant c.113 G→A ($p67^{phox}$ R38Q), had extensive colonic disease, some had perianal disease and had significant arthritis. In their study Muise *et al* also described novel associations between NADPH oxidase complex gene

RAC2 and CD, associated with enhanced susceptibility to IBD (Xavier and Podolsky ,2007) (Wirtz and Neurath, 2007) (Cho and Weaver, 2007) (Van Limbergen R. *et al*, 2007) (Rioux, X. *et al*, 2007) (Muise X. *et al*, 2012).

1.3 Immune Response

Both CD and UC patients have activated innate (macrophage and neutrophil), acquired (B and T cell) immune responses and loss of tolerance to enteric commensal bacteria, either because of dysfunction in the primary or secondary mechanisms that normally drive and regulate such responses, or because of some dysfunction in the intestinal epithelial barrier that leads to inappropriate penetration of microbial antigens (Blumberg, 2001) (Sartor, 2006). CD and UC are both characterized by enhanced recruitment and retention of effector macrophages, neutrophils and T cells into the inflamed intestine, where they are activated and release pro-inflammatory cytokines. Accumulation of effector cells in the inflamed intestine is a result of enhanced recruitment as well as prolonged survival caused by decreased cellular apoptosis (Sartor 2006).

1.3.1 Innate immune responses

Macrophages and dendritic cells in *lamina propria* are increased in absolute number and have an activated phenotype in both forms of IBD.. Production of pro-inflammatory cytokines and chemokines is enhanced in IBD and expression of adhesion molecules and co-stimulatory molecules is also increased. Cells involved in innate immune response are activated and the expression of most pro-inflammatory cytokines and chemokines is upregulated in both CD and UC. Th1 and Th17-related cytokines involved in innate immunity, like IL-12, IL-23 and IL-27 are, however, selectively activated in CD (Sartor, 2006), (Martins and Peppercorn, 2004) (Abraham and Cho, 2009) Activation of NF-κB stimulates expression of numerous molecules relevant to

IBD pathogenesis. These include molecules involved in the inflammatory response, such as IL-1β, TNF, IL-6, IL-8 and other chemokines, ICAM1 and other adhesion molecules and co-stimulatory molecules including CD40, CD80, CD86 and the inducible T-cell co-stimulator ICOS. Expression of each of these pro-inflammatory molecules is increased in active IBD. In contrast, cytokines that induce Th1 and Th17 responses are selectively up regulated in active CD but not in UC. Selective inhibition of most of this cytokines attenuates the onset of experimental colitis (Sartor, 2006). Neutrophils cause tissue damage, which seems to be exacerbated in IBD patients due to an extended lifespan of impaired apoptosis, through the release of nonspecific inflammatory mediators, such as Reactive Oxygen Intermediates, lipid mediators, proteases and secrete cytokines like IL-1β and TNF-α (Brown and Mayer, 2007).

1.3.2 Adaptive immune responses by T cells

There is a difference between T-cell response between CD and UC, so they should be considered separately (Bouma and Strober, 2003).

1.3.2.1 Crohn's disease

A variety of T-cell defects have been observed in IBD patients this include defective T-cell apoptosis, which has been associated with a rapid cell cycle in CD, defects in regulatory T-cell activation and function and mouse models have demonstrated that both excessive pro-inflammatory and deficient anti-inflammatory responses may manifest in IBD (Brown and Mayer, 2007). Most mouse models present a similar cytokine profile to the one seen in human CD. The Th1 traditional cytokine profile is dominant in patients with CD, and is mediated by IFN-γ, the production of which is stimulated by IL-12, produced by antigen-presenting cells (APCs). CD can also present a Th17 cytokine profile, where IL-17 mediates Th17 responses. This

cytokine profile is stimulated by IL-6, TGF-β and IL-23 production by innate immune cells and APCs, especially dendritic cells (Brown and Mayer, 2007).

1.3.2.2 Ulcerative Colitis

In UC there's also a range of T-cell defects that can be observed, defective T-cell apoptosis in UC has been associated with a slower than normal cell cycle and defects in regulatory cytokines, TGF-β or IL-10 are associated with the development of UC (Brown and Mayer, 2007).This disease is considered to have a Th2 cytokine profile, but the concentrations of IL-4 and IL-5 which are characteristically elevated in this type of response, have been variable in UC tissues. UC has been associated with the production of various autoantibodies, such as neutrophil cytoplasmatic antibody (pANCA) and anti-tropomyosin, which might be indicative of a Th2-mediated immune response, with IgG1 and IgG4 overproduction predominating in UC. So, this way UC is considered to have an atypical Th2-type response, mediated by NK T cells that secrete IL-13 (Bouma and Strober, 2003) (Martins and Peppercorn, 2004) (Sartor, 2006).

1.3.3 Adaptive immune response by B cells

B cells produce antibodies to both bacterial and nonbacterial antigens in mouse and human IBD. This supports the hypothesis that there is a break down in mucosal tolerance. Several antibodies against microbial products are increasingly recognized in IBD, this include anti-*Saccharomyces cerevisae* (ASCA), anti-12, anti-Ompc and anti-flagellin antibody CBir1, being that78% of patients react at least to one of these antibodies (Abraham and Cho, 2009). This implies that rather than a global loss of tolerance, different patients may form one or more specific antibodies and the combinations present may help stratify disease subtypes (Brown and Mayer, 2007).

1.3.4 Malfunction of the immune system

It is accepted that IBD results from an inappropriate response of a defective mucosal immune system. But how and why microbial antigens induce an inappropriate inflammatory response? Experimental evidence from studies in vitro, in animals, and in human beings suggest that several, not mutually exclusive, pathways might result in inflammatory cascades (Baumgart and Carding, 2007).

First, the epithelial barrier is leaky in people with IBD, s-studies have shown lower epithelial resistance and increased permeability of the inflamed and non-inflamed mucosa in CD and UC (Baumgart and Carding, 2007). The paracellular space has increased permeability and the regulation of tight junctions is defective (Abraham and Cho, 2009) (Strober, F. *et al*, 2007). This barrier defect might be inherent or induced by infection or nonsteroidal anti-inflammatory drugs (Brown and Mayer 2007). The inflammatory response often results in continued epithelial injury, which causes erosions, ulcerations and a decrease in defensins' production (Strober, F. *et al*, 2007). The result is increased exposure to intestinal microbiota and amplification of the inflammatory response (Abraham and Cho 2009). The importance of the epithelial barrier in disease predisposition is supported by the finding of abnormal intestinal permeability in some first-degree relatives with CD (Xavier and Podolsky, 2007).

Innate immune mechanisms of the epithelial layer present altered too An upregulation of NOD2 in epithelial cells, which can augment itself further in a feedback loop, when the NFκB cascade is activated has also been reported, which might compromise the ability of the host to eliminate invasive and pathogenic microbes resulting in chronic inflammation (Baumgart and Carding, 2007).

Animal and in-vitro studies suggested that dendritic cells incorrectly recognize commensal bacteria and induce a Th1 and possibly Th17, pro-inflammatory immune

responses normally directed to pathogens, (Baumgart and Carding, 2007) in other words, loss of tolerance to resident microbial flora, proposes that the disease could be caused by an abnormal immune response to one enteric pathogen (Brown and Mayer, 2007) (Strober, W. *et al*, 2007). No specific dietary component has been identified, although several infectious agents have been proposed, including species of *Myobacterium*, *Listeria*, *Yersinia*, and *Escherichia coli*. Immune responses are initiated when either cytotoxic T lymphocyte CD8cells or CD4Th cells in the intestinal lumen recognize a bacterial antigen (Cho and Abraham, 2007).

IBD presents disturbed clearance of over-reactive or auto-reactive T-cell populations. Due to a failure of central (thymic) and peripheral tolerance, activated T cells persist and do not undergo apoptosis (Baumgart and Carding, 2007). Defective mucosal T-cell apoptosis is likely to play a pivotal play role in IBD, support for this hypothesis comes from studies that investigated the Bcl-2/Bax protein family, where Bcl-2 protein protects from apoptosis and Bax promotes apoptosis. Levels of expression of Bax protein are markedly reduced in inflamed UC colonic epithelium. The alteration in the ration of Bac/Bcl-2 in CD suggests an imbalance in these proteins that may favor resistance of mucosal T cells to apoptotic signals (Cho and Abraham, 2007). The autophagy pathway contributes to T-cell tolerance at multiple levels, which suggests that polymorphisms of autophagy genes associated with CD could increase a patient's susceptibility to intestinal inflammation through defects in T-cell tolerance (Abraham and Cho, 2009).

1.3.5 Human studies/Animal studies

An important advance in the study of IBD has been the discovery and analysis of a variety of mouse models of intestinal inflammation that resemble the human IBD. The mouse models fit broadly in four groups: a) spontaneous colitis, as a result of naturally

occurring genetic abnormality; b) spontaneous colitis that occurs in mice with particular genetic defects produced either by gene targeting or introduction of a transgene; c) colitis induced by exposure to a haptenating agent or another type of causative agent; d) colitis induced by transferring T-cell populations lacking regulatory cells into a severely lymphopenic host that lacks endogenous regulatory cells (Bouma and Strober, 2003)

Intestinal inflammation occurs in models in which there is a clear deficiency in the production or function of a known regulatory cytokine, such as IL-10 knockout mice, or mice with defective TGF-β signaling and colitis occurs in mice in which regulatory T cells fail to develop properly (Bouma and Strober, 2003). These studies provide compelling evidence that the nature of the host defenses, rather than the biological properties of a luminal bacterial species *per se*, may determine the functional outcome of that interaction (Xavier and Podolsky, 2007).

DSS colitis model, which consists in feeding mice for several days with DSS polymers in the drinking water, induces a very reproducible acute colitis characterized by bloody diarrhea, ulcerations and infiltrations with granulocytes. It is believed that DSS is directly toxic to gut epithelial cells of the basal crypts and therefore affects the integrity of the mucosal barrier. As T- and B-cell deficient C.B-17^{scid} or Rag1$^{-/-}$ mice also develop severe colitis. The adaptive immune system obviously does not play a major part (at least in the acute phase) in this model. Hence, the acute DSS colitis model is particularly useful to study the contribution of innate immune mechanisms of colitis. In addition, the DSS model has been shown to be suitable to study epithelial repair mechanisms. Studies with TLR4$^{-/-}$ and MyD88$^{-/-}$ mice suggest that TLR signaling is required to limit bacterial translocation after DSS induced intestinal epithelial injury suggesting that TLR signaling is important for the maintenance of the epithelial barrier. In susceptible strains, the administration of DSS for several cycles (e.g., 7 days DSS, 14

days water) results in chronic colitis and if combined with a single initial dose of the genotoxic colon carcinogen azoxymethane (AOM), in inflammation-associated colorectal cancer. Patients with UC have an increased risk for the development of colon cancer. As colonic inflammation is suggested to play a key role in IBD-related colorectal cancer, the AOM/DSS model is a very useful tool to study mechanisms linking inflammation to colon carcinogenesis (Wirtz and Neurath, 2007).

TNBS/Oxazolone colitis model, where colitis can be induced in susceptible mouse strains by intrarectal instillation of TNBS/ DNBS or Oxazolone dissolved in ethanol. Ethanol is required to break the mucosal barrier, whereas TNBS/Oxazolone is believed to haptenize colonic autologous or microbiota proteins rendering them immunogenic to the host immune system. As CD4+ T cells have been shown to play a central role in chronic TNBS colitis, this model is useful to study T helper cell-dependent mucosal immune responses. The TNBS colitis model has been very useful in studying many important aspects of gut inflammation, including cytokine secretion patterns, mechanisms of oral tolerance, cell adhesion and immunotherapy. Murine TNBS colitis has been initially described in SJL/J mice, a mouse strain with high susceptibility that develops chronic TNBS colitis characterized by a predominant Th1-mediated immune response with dense infiltrations of lymphocytes/macrophages and thickening of the colon wall. However, studies with IFN-$\gamma^{-/-}$ mice on a Balb/c background showed that in these mice TNBS colitis may be associated with a Th2-mediated colonic patch hypertrophy. In SJL/J mice, oxazolone colitis has been shown to affect only the distal colon and particularly mucosal layers. Histological features and an elevated production of Th2 cytokines (IL-4, IL-5 and IL-13) of unstimulated and αCD3/ αCD28-stimulated *lamina propria* T cells are in these mice, in some aspects, similar to characteristics that have been observed in human UC. Oxazolone colitis is one of the

few models suitable to study the contribution of the Th2-dependent immune response to intestinal inflammation (Wirtz and Neurath, 2007).

In NOD 2 model three different mouse models harboring nonfunctional NOD2 have been developed. The first mouse model, which carries a $NOD2^{2939insC}$ mutation similar to the human CD-associated $NOD2^{3020insC}$ frameshift mutation, is susceptible to DSS induced intestinal injury. Unlike chronic IBD models that do not develop disease under germ-free conditions, DSS-induced acute intestinal injury is exacerbated in the absence of commensal flora. Therefore, the increased susceptibility of NOD2 mutant mice to DSS-induced intestinal injury suggests the involvement of NOD2 in commensal flora-mediated protective immunity, particularly during the acute inflammatory phase. The second model is a NOD2 KO mouse strain that was generated by a deletion of exon 1. This characterized by altered TLR-2 signaling. The production of IL-12p70 by APCs in this model is increased in response to TLR2 stimulation, suggesting an inhibitory role of NOD2 in TLR2-mediated Th1 responses. The third model is another NOD2 KO mouse strain that was generated by deletion of exon 3. This mouse strain is more susceptible to a *Listeria monocytogene* infection because of impaired Paneth cell function and a reduced production of antibacterial peptides such as cryptidin and defensins. The importance of Paneth cell function in the suppression of IBD is further supported by recent data from a conditional KO mouse strain deficient of another CD susceptibility gene, XBP1, in epithelial cells. This epithelial cell-specific XBP1 deletion results in an impaired Paneth cell development and consequent development of ileitis. Indeed, abnormally low production of antibacterial peptides such as α-defensins by Paneth cells is proposed to be a primary defect in CD particularly illeal CD (Liu Y. *et al*, 2011)(Liu, Yang *et al*. 2011) (Mizoguchi and Mizoguchi 2010).

Besides all IBD models it is noteworthy that none of the current animal models of IBD is ideal, and efforts to create an experimental model of human IBD using toxic chemicals fail to modulate the complex interaction of multiple factors such as genetic, immunologic, environmental, and psychologic components in the pathophysiology of IBD.

1.3.6 Treatments

The mainstays of CD and UC treatment are to achieve and maintain disease remission with improved health-related quality of life, fewer hospitalizations, and fewer surgeries. The treatment to IBD is based in a system where, patients having mild or moderate disease receive aminosalicylates and occasionally corticosteroids, for disease flares.

IBD treatments include aminosalicylates that decrease inflammation, block prostaglandins and leukotrienes production, inhibits chemotaxis, scavenges oxygen radicals, inhibits Nk-kB, acting by as an agonist of peroxisome prolifetator-activated receptor-ϒ (Martins and Peppercorn 2004) (Grimm 2009). Corticosteroids, that provide potent anti-inflammatory activity for the induction and remission of UC and CD, however they aren't capable of maintaining the remission (fewer than one-third remain in remission at 12 months with another quarter becoming steroid dependent and a substantial portion requiring surgery), the use of corticosteroids is limited due to its adverse effects, and based on that, budesonide was develop as a less toxic corticosteroid (Martins and Peppercorn, 2004) (Grimm, 2009). Immunosuppressive medications, the most used is azathioprine, it acts inducting apoptosis of T lymphocytes, thereby reducing the effector compartment of the ongoing intestinal immune response, this kind of treatment is used for maintenance of remission in CD and UC, and it may take up to six months to observe any response (Martins and Peppercorn, 2004) (Grimm, 2009).

Antibiotics they work by changing luminal flora and this way diminishing activation of the mucosal immune system. (Martins and Peppercorn 2004). The use of single antibiotics is likely to be unsuccessful in CD because of the development of bacterial resistance (Friswell, Campbell et al. 2010). Anti-TNF-α is a field of treatment that has been very explored in past decade, and infliximab (the most used substance in this class), is chimeric monoclonal IgG1 antibody against TNF-α, it's 75% human protein and 25% murine protein which selectively targets TNF-α. It works by binding membrane-bound TNF-α and inducing cell lysis by antibody-dependent cell-mediated cytotoxicity or complement fixation and apoptosis. It is now used for maintenance therapy in CD and UC (Martins and Peppercorn, 2004) (Cho and Abraham, 2007) (Grimm, 2009) (Baumgart and Sandborn, 2007) (Abraham and Cho, 2009) (Neuman 2007) (Leung and Hanauer, 2009).

Other potential treatment options consist in the blockage of inflammatory cell migration, selective adhesion molecule inhibitors interfere with the migration of leukocytes to the site of inflammation by interacting with adhesion molecules. Such as natalizumab, which is a recombinant monoclonal antibody against α4 integrin, that works by blocking the recruitment of leukocytes (Martins and Peppercorn, 2004) (Hanauer, 2006) (Baumgart and Sandborn, 2007) (Neuman, 2007).

Stem cell transplantation (mesenchymal and hematopoietic cells) are becoming a therapeutic option to IBD, since no curative options exist to date, a stem cell-based approach could drive a major change in disease management and treatment. A high-dose immune ablation regimen could allow detrimental T-lymphocyte repertoires to be eliminated and after HSC transplantation (HSCT) *de-novo* hematopoiesis would generate naive cells. Patients receiving an autologous HSCT are thought to be subject to

an immune system reboot: the genetic defects would not be eliminated but remission could persist in the absence of deleterious environmental triggers (Lanzoni, 2008).

Probiotics have beneficial effect on IBD treatment, with the main related mechanisms including: a) inhibiting microbial pathogens growth; b) increasing epithelial tight junction and permeability; c) modulating immune response of intestinal epithelia and mucosal immune cells; d) secreting antimicrobial products; e) decomposing luminal pathogenic antigens. Probiotics compete with microbial pathogens for the limited number receptors present on the surface of epithelia, and inhibit epithelium attachment and invasion by enterotoxigenic and enteropathogenic bacteria (Bai and Ouyang 2006).

1.4 Reactive Oxygen Species

Reactive Oxygen Species (ROS) have been largely studied from different perspectives, some think they're bad and cause innumerous diseases, others think they're essential and conserved during our evolution and others think ROS should be kept in a very tight control.

ROS are chemical oxygen species with one or more unpaired electrons that make them chemically reactive to other species. ROS include superoxide ($^{\bullet}O_2^-$), the hydroxyl radical ($^{\bullet}OH$), the hydroperoxyl radical ($^{\bullet}O_2H$), nitric oxide ($NO^{\bullet}$), and singlet oxygen (O_2) (Wiseman H. *et al*, 1996) (McKercher S.R. *et al*, 2009) (Siow Y. *et al*, 2011) (Alzoghaibi M.,2013). In the normal aerobic metabolism oxygen is the last receptor of electrons in mitochondrial respiratory chain, being in last term fully reduced to water. A small percentage of electrons do not travel to the end of the chain staying free to react with O_2 and form $^{\bullet}O_2^-$ (Wiseman H. *et al*, 1996) (Finkel T., 2011) (Siow Y. *et al*, 2011) (Alzoghaibi M., 2013). However mitochondria is not the only precursor for ROS there are oxidase enzymes like NADPH (*see below*) in phagocytic cells and arachidonic acid

metabolizing enzymes (e.g. cyclooxygenase and lipoxygenase) that also produce superoxide that serves as predecessor for other ROS (Alzoghaibi M., 2013) (Kanyilmaz S. *et al*, 2013) (Madamanchi N.R. *et al*, 2013). Although we cannot forget one of the most important reactions that lead to $^{\bullet}O_2^-$, Fenton reaction, in which ferric and cupric ions (Fe3+, Cu2+) are reduced by superoxide and then react with H_2O_2 to form $^{\bullet}OH$. (Rada B. *et al*, 2008) Nitric oxide is synthesized from L-arginine by nitric oxide synthase. Those compounds can lead to oxidative damage to a variety of biological molecules, like lipids, proteins and nucleic acids (Wiseman H. et al, 1996) (Siow Y. *et al*, 2011) (Alzoghaibi M.,2013).

It is proved that among the 44 highly conserved proteins with known functions regulate stress response, and 40% of them are related to regulation of the intracellular redox status. It is noticed that increased reactive oxygen species (ROS) generation seems to be a common response in cells exposed to stresses; thus, it is argued that redox regulation may represent a critical second messenger system that is upstream of the cell stress signaling network (Jiang F. *et al*, 2011) (Kanyilmaz S. *et al*, 2013) ROS may influence changes in gene expression both directly, by altering the function of many transcription factors, or indirectly by altering (Lambeth J., 2007).

In fact, the intentional localized production and clearance of these reactive molecules by intrinsic enzymes in addition to the observation that redox conditions may be genetically pre-determined and evolutionarily significant, suggest that ROS play a far more integral role in the regulation of cellular and whole organism health, than was previously thought. It is becoming increasingly evident that under physiological conditions, ROS participate in crucial cellular events, functioning either directly as signaling molecules and/or indirectly by mediating global changes in the cellular redox status. Some of their down-stream targets include metalloenzymes and transcription

factors that are sensitive to redox changes (Finkel T, 2001) (Lambeth J., 2007) (Chan E. *et al*, 2009) (Jiang F. *et al*, 2011) (Siow Y. *et al*, 2011) (Chaubey S. *et al*, 2013).

1.4.1 NADPH in ROS production

Several oxidoreductases have been identified as potential sources of superoxide in mammalian cells. These include cyclooxygenase, lipoxygenase, cytochrome P450 enzymes, nitric-oxide synthase, xanthine oxidase, mitochondrial NADH: ubiquinone oxidoreductase (complex I), and nicotinamine adenine dinucleotide phosphate-oxidase oxidase. NADPH is a professional ROS producer, its role is only to produce ROS, and it is in the cell membrane being constituted by various subunits. In its constitution it has cytochrome *b*558, which consists of two subunits $gp91^{phox}$ (Nox2) and $p22^{phox}$. Upon cell activation, two cytosolic regulatory subunits $p47^{phox}$ and $p67^{phox}$, as well as two small G proteins Rac1 and 2, translocate to the membrane and associate with the cytochrome *b*558 (Wiseman H. *et al*, 1996) (Hartl D. et al, 2008) (Chan E. *et al*, 2009) (Jiang F. *et al*, 2011) (Siow Y. *et al*, 2011) (Kanyilmaz S. *et al*,2013).

This multisubunit enzyme complex then generates superoxide by one-electron reduction of oxygen via its gp91phox subunit using reduced NADPH as the electron donor. It has been discovered that NADPH is not exclusive for phagocytic cells, other isoforms had been discovered, that includes six Nox isoforms (Nox1, -3, -4, -5, and Duox1, -2), one p47phox isoform (Nox1) and one p67phox isoform (Noxa1) have been identified in mammalian cells and the expression patterns of these isoforms are distinct and seem to be tissue-specific (Finkel T, 2001) (Chan E. *et al*, 2009) (Jiang F. *et al*, 2011) (Kanyilmaz S. *et al*, 2013).

NADPH oxidase is a distinct enzymatic source of cellular ROS generation, because this enzyme is a “professional” ROS producer (Lambeth, 2004), whereas the other

enzymes produce ROS only as by-products along with their specific catalytic pathways (Jiang F. *et al*, 2011).

Several lines of studies have suggested that one important physiological function of NADPH oxidase in mammalian cells is the modulation of multiple redox sensitive intracellular signaling pathways by generating ROS molecules, including inhibition of protein tyrosine phosphatases, activation of certain redox-sensitive transcription factors, and modulation of the functions of some ion channels (Bedard and Krause, 2007) (Jiang F. *et al*, 2011).

Some authors think that certain stimuli like radiation, heavy metals and toxic chemicals into leads to cellular stresses and suggest that NADPH oxidase may be an important component of the cellular stress signal transduction network. NADPH oxidase/ROS-mediated signaling might therefore represent a cellular "alarm system" that can alert the cells and prime the cells either to be adapted to the stress or to undergo apoptosis being an ancient mechanism of multicellular organisms' defense (Chan E. *et al*, 2009) (Lambeth J., 2007) (Kanyilmaz S. *et al*, 2013).

So, Nox-mediated redox signaling activation may have a critical role in coordinating the responses of the cell to deal with the adverse effects, either by activating stress kinases and promoting stress tolerance or by removing the seriously damaged cells by inducing apoptosis (Chan E. *et al*, 2009) (Jiang F. *et al*, 2011) (Kanyilmaz S. *et al*, 2013).

Interestingly, in a study by Krieglstein *et al* pondered the role of ROS in the colonic inflammation using a DSS model of ulcerative colitis. Their findings demonstrated that both the genetic absence, by the use of iNOS -/- mice, and pharmacologic blockade of iNOS, using an specific iNOS inhibitor(1400W), can

significantly attenuate the severity of colonic inflammation from DSS colitis supporting a proinflammatory role for iNOS. They also showed that colonic tissue injury and inflammation is exacerbated in mice that genetically over-express CuZnSOD compared to wild-type controls. They also disclosed that a disrupted NADPH oxidase function, through a knockout of p47phox protein, appears to not interfere with the susceptibility to DSS-induced intestinal inflammation (Krieglstein, C. *et al*, 2001).

In another study by Bao *et al* the idea of alterations in the NADPH oxidase proteins having no influence in the susceptibility in DSS-induced colitis continue, where $p47^{phox-/-}$ mice don't show significant differences when compared with WT mice and suggest that $gp91^{phox-/-}$ are less susceptible to an acute DSS-induced colitis (Bao, C. *et al*,2011).

Contrastingly, another study has demonstrated that mice lacking the $p40^{phox}$ subunit of NADPH are more susceptible to DSS-induced colitis, with an enhanced neutrophil infiltration, thus indicating that NADPH oxidase activity can affect and modulate the inflammatory response by affecting not only the acute response but also having an important role in the resolution and recovery(Conway, G. *et al*, 2012).

1.4.2 ROS in tissue and wound repair

Accumulating evidence suggests that Nox has an important role in signal transduction in cellular stress responses, (Jiang F. *et al*, 2011) (Kanyilmaz S. *et al*, 2013) being involved in many other mechanisms than self-defense like cell proliferation (Finkel T, 2001) (Chan E. *et al*, 2009) (Jiang F. *et al*, 2011) (Chaubey S. *et al*, 2013) (Kanyilmaz S. *et al*, 2013). There are evidences that NADPH oxidase-derived ROS are involved in cell proliferation of vascular cells specifically Nox2, Nox4 and Nox5. There are also indications that different isoforms have distinct roles in the regulation of

vascular cell proliferation and this may be related to their differential sub-cellular compartmentalization. Many studies have shown that Nox activation has a positive role in regulating proliferation of collagen-producing cells, including fibroblasts, kidney mesangial cells, and stellate cells of the liver and pancreas and in intestinal cells (Chan E. *et al*, 2009) (Chaubey S. *et al*, 2013) (Kanyilmaz S. *et al*,2013).

There are information about wound healing too, revealing that ROS are mainly produced before inflammatory leucocyte oxidative burst and the knockdown of Nox complexes leaded to educed wound-induced H_2O_2 production and suppressed the consequent recruitment of leukocytes to the wound. These results may indicate that ROS may have signaling actions beyond the single cellular context and may play an important paracrine signaling role during the wound repair process (Chan E. *et al*, 2009) (Gauron C., 2013) (Kanyilmaz S. *et al*, 2013). Studies in zebra fish showed that ROS are present and essential for wound repair and are not restricted to the first phase of wounding but several hours later, initiating numerous signaling pathways (Chan E. *et al*, 2009) (Finkel T, 2001) (Gauron C., 2013).

1.4.3 ROS in Immunity

Nox2-generated ROS can participate in immune function in a variety of ways, which are not mutually exclusive. First, the reactive oxygen itself or its byproducts such as HOCl and peroxinitrite can directly oxidize biomolecules in invading microbes in a fairly non-specific manner, resulting ultimately in molecular damage and microbial cell death. Second, the ROS can participate in signal transduction mechanisms linked to immunity and inflammation. This occurs through the selective oxidation of specific signaling enzymes/proteins that are linked to processes such as the secretion of cytokines or the activation of other killing mechanisms. Such signaling targets include

transcription factors such as NFk-B, signaling proteins such as protein kinases and phosphatases and ion and/or proton channels (Lambeth J., 2007). The first role to be definitively established for Nox-derived ROS was in innate immunity mediated by professional phagocytes such as neutrophils and macrophages. It can be calculated that the concentration of ROS produced in the phagosome is extremely high, probably in the molar range (Reeves P. *et al,* 2002) (Lambeth J., 2007). In addition, myeloperoxidase (MPO) is secreted into the phagosome where it converts H_2O_2 plus chloride into HOCl; the latter has a direct microbicidal effect (Hampton B., 1998) (Klebanoff J., 2005) (Lambeth J., 2007) although surprisingly, MPO-deficient individuals do not suffer from markedly increased rates or apparent severity of infections (Lanza F., 1998) (Lambeth J., 2007). In addition, macrophages produce large amounts of NO during phagocytosis; when NO reacts with superoxide, it generates the highly cytotoxic chemical species peroxinitrite (HONO) (Lambeth J., 2007). The activity of the phagocyte NADPH-oxidase also triggers opening of proton and potassium channels (Reeves P. *et al,* 2002) (Lambeth J., 2007), that are proposed to change the ionic environment of the phagosome thereby activating microbicidal proteases and contributing to microbial killing (Reeves P. *et al,* 2002) (Lambeth J., 2007). Regardless of the precise mechanisms, it is clear from the inherited CGD that mutations resulting in defects in ROS generation by the respiratory burst oxidase are associated with an inability of phagocytes to kill bacteria and other microbes (Segal W., 1996) (Lambeth J., 2007), convincingly demonstrating a role for the Nox2 system in innate immunity mediated by professional phagocytes (Lambeth J., 2007) (McKercher S.R. *et al,* 2012).

Neutrophils are the first line of defense with highly efficient bactericidal and tissue-toxic mechanisms in host protection including the phagocytosis of opsonized particles, degranulation with release proteases and the production of ROS. Among

these functions, production of reactive oxygen intermediates (ROIs), known as respiratory burst, is especially important in microorganism killing. Considering that efficient phagocytosis and the subsequent production of ROIs play an important role in the intracellular killing of microorganisms by phagocytes, defects in one or both of these functions may lead to a deficiency in phagocytic function (Pavlik K. LF *et al*, 2002) (Rada B. *et al*, 2008) (McKercher S.R. *et al*, 2012),

So, undoubtedly ROS are essential for immune response and for its action destroying pathogens, there are opinions that its deregulation leads to Oxidative Stress and consequently to disease. However there are examples like CGD where dysfunctional ROS production leads to high inflammation levels and many complications

1.4.4 ROS Imbalance

ROS has to be kept under very straight concentrations, if there is an imbalance between ROS production and scavenging we are towards Oxidative Stress. That might happen because of antioxidant decrease (e.g. mutations in antioxidant enzymes like superoxide dismutase, glutathione peroxidase) and ROS over-production (e.g. excessive activation of ROS producers) (Wiseman H. *et al*, 1996) (Pavlik K. LF *et al*, 2002) (Siow Y. *et al*, 2011).

So, this homeostasis is essential to healthy function, but when Oxidative stress occurs many things can happen. There is an adaptation to the new condition, increasing scavenging mechanisms for example; tissue injury because of lipid peroxidation, DNA and protein damage or in last term cell death by apoptosis or necrosis (Wiseman H. *et al*, 1996).

ROS can start to interact with DNA, lipids, proteins and so one changing them irreversibly and those interactions can lead to diseases like cancer, heart disease and many others (Wiseman H. *et al*, 1996).For example lipids oxidation can occur in the presence of free oxygen radicals, taking to hidroperoxides and lipid peroxides formation. That is because the double bonds of polyunsaturated fatty acids (PUFA), such as linoleic acid, are susceptible to oxidation by ROS. So, losing an electron, lipids became radical (L•) and when react with O_2 forms a peroxyl radical (LOO•). Lipid peroxides and oxygen radicals are responsible for many of the damaging reactions in the cell. They stimulate the peroxidation reactions that are toxic to cells and cell membranes. They can damage biological membranes, make the membrane leaky, and eventually cause complete membrane breakdown (Wiseman H. *et al*, 1996) (Alzoghaibi M., 2013).

Another redox-sensitive transcription factor is p53. It was demonstrated that oxidation of p53 alters its conformation and disrupts its DNA binding activity, resulting in a pattern change of p53-dependent gene expression. Moreover, p53 was shown to act as a homeostatic regulator by lowering ROS levels in stem cells and controlling hematopoietic stem cell self-renewal. Finally, the NF-kB signaling pathway is also significantly altered by deregulated ROS. ROS activates NF-kB signaling through elimination of the IkB inhibitor (Chan E. *et al*, 2009) (Siow Y. *et al*, 2011). An increase in ROS levels induces the activation of the IkB kinase (IKK), which in turn phosphorylates IkB, leading to its proteasome-dependent degradation (Pavlik K. LF *et al*, 2002) (Madamanchi N.R. *et al*,2013) (Maryanovich M. *et al*. 2013).

1.4.5 Antioxidants

Substances or compounds that are able to scavenge oxygen free radicals or inhibit their process of formation are called Antioxidants. We can get them from diet (ex.: vitamins A, B and C), but we also have our own antioxidants enzymes. Those mechanisms exist to counter ROS damages to cells and it should be enough to maintain homeostasis. Anyway this balance can be lost when there is over-production of free radicals and the nutrients containing antioxidants are not enough (Wiseman H. *et al*, 1996) (Rezaie A. *et al*, 2004). (Finkel T., 2011) (Alzoghaibi M., 2013).

Antioxidants can be considered plasma antioxidants and intracellular antioxidants. The major role of plasma antioxidant defense is to bind transition metal ions, such as iron and copper, thereby lowering their plasma concentration and capacity to stimulate free radical reactions preventing lips peroxidation. Ascorbic acid (vitamin C) for example can scavenge water-soluble peroxyl radicals and other ROS. Vitamin E (α-tocopherol) is a lipid peroxidation, chain-breaking antioxidant, localized in membranes and lipoproteins (Alzoghaibi M., 2013).

Regarding intracellular antioxidant enzymes there are superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase. (Wiseman H. *et al*, 1996) (Rezaie A. *et al*, 2004) (Siow Y. *et al*, 2011) (Alzoghaibi M., 2013) SOD is localized in both the cytosol (CuZn-SOD) and mitochondria acting to dismutate superoxides into hydrogen peroxide and molecular oxygen. SOD is considered as the major intracellular enzyme because it is capable of reducing the most abundant free radical, $\bullet O_2$.Catalase reacts rapidly with hydrogen peroxide, a precursor of •OH in the presence of iron, and converts it into water and molecular oxygen. Selenium glutathione peroxidase also converts hydrogen peroxide into water and lipid hydroperoxides into water and

harmless fatty acid alcohols. (Wiseman H. et al, 1996) (Alzoghaibi M.,2013) The mechanisms underlying endothelial dysfunction are multi-factorial with oxidative stress playing a major role. The kinetics of the reaction of $\bullet O_2$ with •NO are three times faster than there action rate of $\bullet O_2$ with SOD. Thus, it is likely that some $\bullet O_2$ always reacts with •NO within the cells and extracellular space, but endogenous antioxidant defenses minimize this interaction (Pou S. et al, 1992).

Finkel T. found that once oxidants are associated to the regulation of crucial physiological processes, antioxidants are not merely ROS scavengers but rather exist in the cell as sensors and effectors of redox-regulated pathways. It is important to note that there is growing evidence that the regulation of antioxidant levels in cells is intimately connected with ROS levels and oxidant production sources (Finkel T., 2001) (Finkel T., 2011).

1.4.6 ROS in Disease

Regarding the importance of redox in regulating crucial cellular events, the presence of oxidative stress means that there are oxidative perturbations that if not corrected, could result in a disease state. Cancer, cardiovascular disease, and metabolic disorders such as metabolic syndromes are the leading causes of death worldwide, with the number of cases for each condition projected to increase substantially over the next few decades. Cancer is an - example of a complete disarray of cellular redox homeostasis. Unlike metabolic disorders, both oxidative and reducing cellular environments appear to play a role in carcinogenesis. Oxidative imbalances may trigger a multitude of molecular and cellular events induced by the ROS (Finkel T, 2001) (Siow Y. *et al*, 2011).

1.4.6.1 CGD

Chronic granulomatous disease (CGD) is characterized by NADPH oxidase complex inhibition which leads to hyperinflammation, suggesting that the normal functions of this complex in macrophages and potentially other inflammatory cells are essential in restricting or resolving inflammation (Chaubey S. *et al*, 2013).

CGD is the most common inherited disorder of neutrophil function, is caused by mutations in NADPH oxidase, and results in recurrent bacterial infections (Hartl D. *et al*, 2008). Phagocytes of patients with CGD are therefore unable to kill ingested microorganisms, which results in a concomitant increased susceptibility to bacterial and fungal infections (Hartl D. *et al*, 2008) (Rada B. *et al*, 2008). Activation of NADPH oxidase was found to negatively regulate IL-8 mRNA expression in neutrophils, and the absence of NAPDH oxidase in neutrophils from patients with CGD resulted in an increase of IL-8 production (Hartl D. *et al*, 2008).

Hartl D. et al found that reduced complement receptor expression by neutrophils from patients with CGD was associated with impaired bacterial opsonophagocytosis. Although further indications exist that neutrophils from patients with CGD display impaired phagocytosis (Hartl D. *et al*, 2008).

1.4.6.2 Inflammatory Bowel Disease

Oxidant stress is a major factor in IBD. It's been described that IBD (UC and Crohn's) patients have an enhanced production of reactive oxygen metabolites by epithelial and phagocytic cells (neutrophils, monocytes and macrophages) that leads to an increased oxidative stress in mucosal tissues (Kitahora S. *et al*, 1988) (Lih-Brody P. *et al*, 1996) (Alzoghaibi, 2013). It's stated that monocytes and polymorphonuclear cells in IBD are activated and produce high levels of pro-inflammatory mediators such as leukotriene B4 (LTB4) or platelet activating factor (PAF) leading to the release of large

amounts of potentially cytotoxic reactive oxygen metabolites demonstrated through *in situ* quantification and through evaluation of lipid peroxidation (Sharon and Stenson, 1984) (Kitahora S. *et al*, 1988) (Almenier A. *et al*, 2012) (Alzoghaibi, 2013). This was also related to the augmented migration of neutrophils and macrophages into the bowel mucosa, higher levels of ROS and the degree of inflammation and tissue damage (Kitahora, S. *et al*, 1988) (Alzoghaibi, 2013). It's been showed that not only there is an increased oxidative stress in mucosa but also a decrease of antioxidants, with low blood levels of vitamin C and vitamin E, low levels of CuZn superoxide dismutase (SOD), glutathione peroxidase, catalase, vitamin A and β-carotene, thus leading to an increased oxidative state (Lih-Brody, P. *et al,* 1996) (Alzoghaibi ,2013).

Clinical data have shown that the administration of bovine CuZnSOD leads to an attenuated mucosal inflammation and injury in Crohn's patients (Kruidenier, K. *et al*, 2003) (Alzoghaibi, 2013).

It's also acknowledged that the use of sulfasalazine (SAZ), mostly known by its commercial name 5-ASA, is beneficial in Crohn's disease by inhibiting cyclooxygenase and lipoxygenase activities, and interacts with the superoxide, suppressing the formation and promoting of the degradation of free radicals, therefore protecting from oxidative stress-induced mucosal injury and inflammation (McKenzie, D. *et al*, 1999) (Couto, R. *et al,* 2010) (Alzoghaibi, 2013).

Pavlik *et al* say that besides ROS overproduction, NADPH is not involved in it once that inhibiting it made no alteration in the susceptibility of mice to DSS-induced colitis, indicating that NADPH oxidase is not involved in the pathophysiology of this model of ulcerative colitis (Pavlik K. LF *et al*, 2002). Leoni G. *et al* demonstrated that epithelial ROS induce a pro-resolution of epithelial wound closure, by mediating Anexin 1 (anti-inflammatory protein) production that control excessive immune cell

trafficking by many mechanisms, one of them neutrophil influx inhibition and apoptosis at resolving inflammation site (Leoni G. *et al*, 2013).So, the effective role of ROS in IBD is still not clear, however a new lines of thought consider that ROS has a resolution role in IBD.

The levels of ROS in both Crohn's and ulcerative colitis are normally measured in an indirect way by the detection of the subproducts of an excessive lipidic peroxidation reaction, such as malondialdehyde and 4 hydroxynonenalos (Chiarpotto, S. *et al*, 1997) (Alzoghaibi ,2013).

Protein damage and peroxynitrite alteration through oxidative damage by markers like Carbonyl content in cells/tissues (Lih-Brody, P. *et al*, 1996) (Chevion, B. *et al*, 2000) and residues of 3-nitrotyROSine (Ischiropoulos and al-Mehdi, 1995) (Singer, K. *et al*, 1996) have also been observed in both Crohn's disease and ulcerative colitis.

Inflammation is associated with the production of ROS as a probable cause of neoplasic evolution (Marnett, 2000). ROS is being reported to be a cause of DNA alterations through the augmentation of 8-hydroxyguanine (8-OHDG) production in Crohn's disease (Lih-Brody, P. *et* al, 1996). In another study by D'Inca *et al* on ulcerative colitis, the high levels of 8-OHDG correlated with higher levels of ROS lead to an accumulation of oxidative DNA damage alongside with the progression of the disease and correlates with higher dysplastic lesions and possible implications for mutagenic and carcinogenic progression (D'Inca, C. *et al* ,2004).

Interestingly, the complete absence of ROS also seems to be problematic. Indeed, it is a characteristic of Chronic granulomatous disease (CGD), an heterogeneous primary immunodeficiency disorder characterized by a defect in Nicotinamide adenine dinucleotide phosphate (NADPH) - oxidase (Nox) complex (characterized by the

presence of X-linked mutations on gp91phox gene (65%), autosomal recessive forms: $p47^{phox}$ (30%), $p67^{phox}$ (<5%) or $p22^{phox}$ (<5%)), leading to a deficit in the production of oxygen free radicals in specialized phagocytic cells (neutrophils , monocytes, macrophages) and other cells, such as eosinophils and lymphocytes. Thus, patients with CGD develop severe systemic infections and/or granuloma formation through recurrent bacterial infections with positive catalase, *Staphylococcus spp*, *Salmonella spp*, *Pseudomonas spp, Mycobacterium spp, Nocardia spp,* and *Burkholderia cepacia* among others, and fungi such as *Candida albicans* and *Aspergillus spp*. Such infections result in the formation of chronic granulomas, composed of clusters of inflammatory monocytes and macrophages unable to effectively engulf infectious agents, and lead to a high incidence of mucosal inflammatory disorders (such as chronic colitis) (Al-Mobaireek 2001) (Noack, R. *et al*, 2001) (Sartor, 2006) (Towbin and Chaves, 2010) (Song, J. *et al*. 2011).

It's already being demonstrated that IBD patients can have a defective neutrophil respiratory burst (Segal and Loewi, 1976) (Verspaget, M. *et al*, 1984). But despite early reports from genome-wide association studies (GWAS) indicating the NCF4 gene as susceptibility gene for CD (Rioux, X. *et al*, 2007) (Roberts, H. *et al*. 2008) a recent GWAS study didn't corroborate these results (Franke M. *et al*, 2010).

Even so, in a study by Muise *et al* they not only replicate the association of NCF4 with CD previously described but also illustrated a novel associations of the NADPH oxidase complex gene RAC2 with CD and introducing a novel missense variant in NCF2 with very early onset IBD that leads to an neutrophil dysfunction and susceptibility to CD, thus indicating that the NADPH oxidase complex genes play a role in the pathogenesis of CD (Muise, X. *et al*, 2012).

1.5 **Objectives**

The main objective of this study was to understand the role of ROS in an immunological response in a situation of DSS- induced colitis.

To achieve this goal we proposed to:

- Evaluate physical mice evolution during the experiment;
- Evaluate the immune populations behavior in the presence and absence of ROS;
- Evaluate histological intervention of DSS in both strains;
- Evaluate the effects of ROS absence in the progression of the disease.

CHAPTER 2

METHODS

2 Methods

2.1 Animals

6-8 weeks old male or female homozygous Ncf1-mutant (BQ.*Ncf1*$^{m1J/m1J}$, abbreviated Ncf1, n=30) and wild-type (WT, n=30) B10.Q mice were obtained from breeding heterozygous mice followed by genotyping, as previously described (Gelderman H. *et al*, 2006). Animals were bred and maintained under standard conditions, with food and water *ad libitum* in a specific pathogen–free environment. All animal studies were approved by the internal CNC Ethics Committee, and were in accordance with EU legislation for experimental animal welfare Induction of colitis

2.2 Induction of colitis

Colitis was induced by oral administration of 3% (w/v) DSS (average 40,000 g/mol, AppliChem, Darmstadt, Germany) in the first induction cycle and 2.5% (w/v) DSS in the second cycle ad libitum in drinking water. Reduction of DSS concentration was to avoid premature death. Two different colitis-induction protocols were used (Fig 1): Experiment 1 (E1) - mice were submitted to 7 days DSS-induction followed by 21 days of resting on normal water. Experiment 2 (E2) - mice were submitted to 7 days of DSS-induction, followed by 14 days of resting on normal water and a second 7 days DSS-cycle. Five Ncf1 and WT mice were sacrificed at the end of each time point: before the experiment had started and at days 22 and 30. Tissues were collected for histopathological analysis and assessment of colon length and spleen weight.

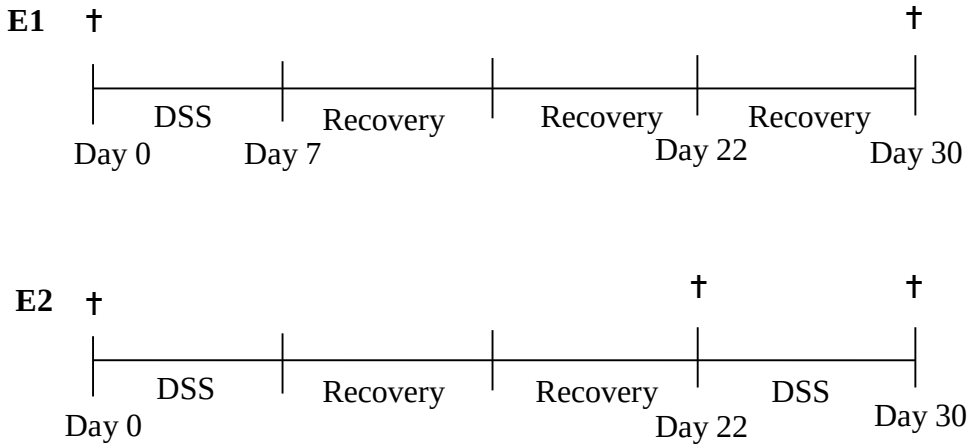

Figure 1 Schematic representation of the two experimental protocols. E1) Experiment 1: One week DSS induction and 3 weeks recovery. E2) Experiment 2: one week DSS induction, 2 weeks recovery and another week DSS induction. † Indicate the time-points when mice were sacrificed.

2.3 **Clinical Evaluation**

The clinical scores of colitis: weight change, diarrhea, colorectal bleeding and survival were monitored every other day. Blood scoring: 0- no blood; 1- visible blood; 2- rectal bleeding. Consistency scoring: 0- Normal; 1- Soft but formed; 3- very soft; 4- diarrhea.

2.4 **Flow Citometry**

Lymph nodes samples were collected from the base of the tail from all mice on days 0, 22 and 30. Mononuclear cells were stained in a standard method (Raposo *et al*, 2010) using Fluorescein isothiocyanate (FITC), R-Phycoerythrin (PE), Aloficocianina (APC), Aloficocianina-cyanine 7 (APC/Cy7), Phycoerythrin-cyanine 7 (PE/Cy7), Peridinin chlorophyll protein-cyanine 5.5 (PerCpCy5.5) and Pacific Blue, with conjugated anti-mouse monoclonal antibodies (mAb) surface markers: CD1d, CD3, CD4, CD5, CD8, CD11b, CD19, CD21, CD23, CD27, CD43, CD49b, CD62L, FoxP3, Ly-6c and IgM,NK1.1 (all from Biolegend).

Intracellular staining using anti-mouse mAb was done after saponin permeabilization: CD25 and iL10 (all from Biolegend).

All samples were analyzed on a BD FACSCanto II (Becton Dickinson) and data were analyzed with FlowJo 7.6.4 software (Tree Star).

2.5 Histopathological evaluation of colitis

Separate swiss rolls of distal and proximal colon were fixed in 4% neutral buffered formalin, paraffin embedded and stained with hematoxylin/eosin (HE) according to standard protocols.

Inflammation was scored for each colon section according to the number of inflammatory foci present: 0- no inflammatory focus; 1- one inflammatory focus; 2- two inflammatory foci; 3- three or more inflammatory foci. Dysplasia was scored for each colon section using a semi-quantitative scale: 0- no dysplasia; 1- hypercromatic nuclear pluristratification and *lamina propria* separated glands; 2- epithelial low grade dysplasia :complex ramified glands with cell hyperplasia and pluristratified hypercromatic nuclei , 3- epithelial high grade dysplasia :beyond low grade dysplasia, nuclear atypia and mitosis.

2.6 Statistical Analysis

All data were tested for normal distribution with Levene´s test. Since data did not follow a normal distribution the non-parametric Kruskal-Wallis test followed by a non-parametric Mann-Whitney test were used to compare values between groups and time-points, using Statview 5.0.1 software (SAS Institute Inc, USA). Statistical differences between curves were determined using a 2-sided hypothesis permutation test with 10000 permutations (http://bioinf.wehi.edu.au/software/russell/perm/). Differences were considered significant for p <0, 05.

CHAPTER 3

RESULTS

3 Results

3.1 Clinical Signs of DSS-induced colitis

In both protocols the two mouse groups presented a significant reduction of colon's length after colitis induction (WT: D0 vs D22 p= 0.0159; Ncf1*: D0 vs D22 p= 0.0102). The length of the colon in both groups of P1 mice at day 30 remained equivalent to day 22, whereas WT mice following E2 presented a further significant reduction of the colon (p= 0.0025). Mice that were submitted to second DSS-cycle had significantly shorter colons than those which were allowed to rest after the first colitis induction (WT D30 E1 vs E2 p= 0.0281; Ncf1* D30 E1 vs E2 p= 0.0229) (Fig 2A).

At baseline Ncf1* mice presented a significant splenomegaly when compared to WT mice (p=0.0139). At day 22 there was a significant increase in spleen's weight in both groups when compared to baseline. Moreover, the Ncf1* mice had significantly heavier spleens than WT (p<0.0195). At day 30, regardless of protocol, both mice groups kept the high spleen weight as at day 22 (Fig 2B).

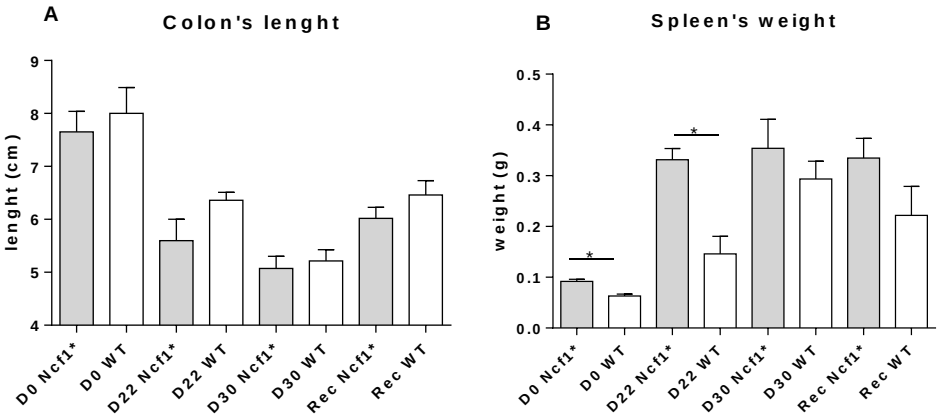

Figure 2 On left colon's length in centimeters, for days 0, 22 and 30.Spleen's weight in grams for days 0, 22 and 30. Asterisks indicate p<0.05, Mann-Whitney test between Ncf1* and WT and Ncf1* Rec and WT Rec.

Throughout all study weight was monitored and Ncf1* presented significantly more weight than WT. On day 3, all groups started to lose weight, except controls,

On day 13, Ncf1* lost significantly more weight than WT ($p<0,001$), that continued until day 22, ($p<0,001$). In E2 from 22nd to 30th days, both WT and Ncf1* lost weight again, but the weight loss was significant higher in Ncf1*, reaching the decrease 80% ($p<0.05$) of baseline weight (WT=29.8±3.90g, Ncf1*=31.3±3.9g) comparing to WT.

Ncf1*and Ncf1* Rec showed significant weight loss ($p<0.0001$) than WT. On day 13, after DSS treatment WT Rec and Ncf1* Rec presented maximum and significant ($p<0.0016$) weight loss with Ncf1* reaching 25% of baseline weights (WT=29.8±3.90g, Ncf1*=31.3±3.9g). By the end of the experiment WT mice weight gain was close to the original, while Ncf1* didn't go above 90% of the original weight (WT=29.8±3.90g, Ncf1*=31.3±3.9g).

The presence of blood in stools and the consistency of the feces are two further clinical signs of DSS-induced colitis (Rodrigues *et al,* 2014). During the 1st induction (D0-D7), both Ncf1* and WT groups had an accentuated decrease in stool consistency with worse results for Ncf1* group. In both experiments, after 1st induction, all groups started to recover with better recovery for WT group. After the 2nd induction (E2) both WT and Ncf1*decreased stool consistency and recovery groups got to basal scores (Fig. 3B). In both experiments Ncf1* and WT groups had similar scores of blood in feces. In E2 after 2nd induction (day 22), both groups increased scores showing Ncf1* a more accentuated increase. WT recovered better than Ncf1* in the three weeks resting period.

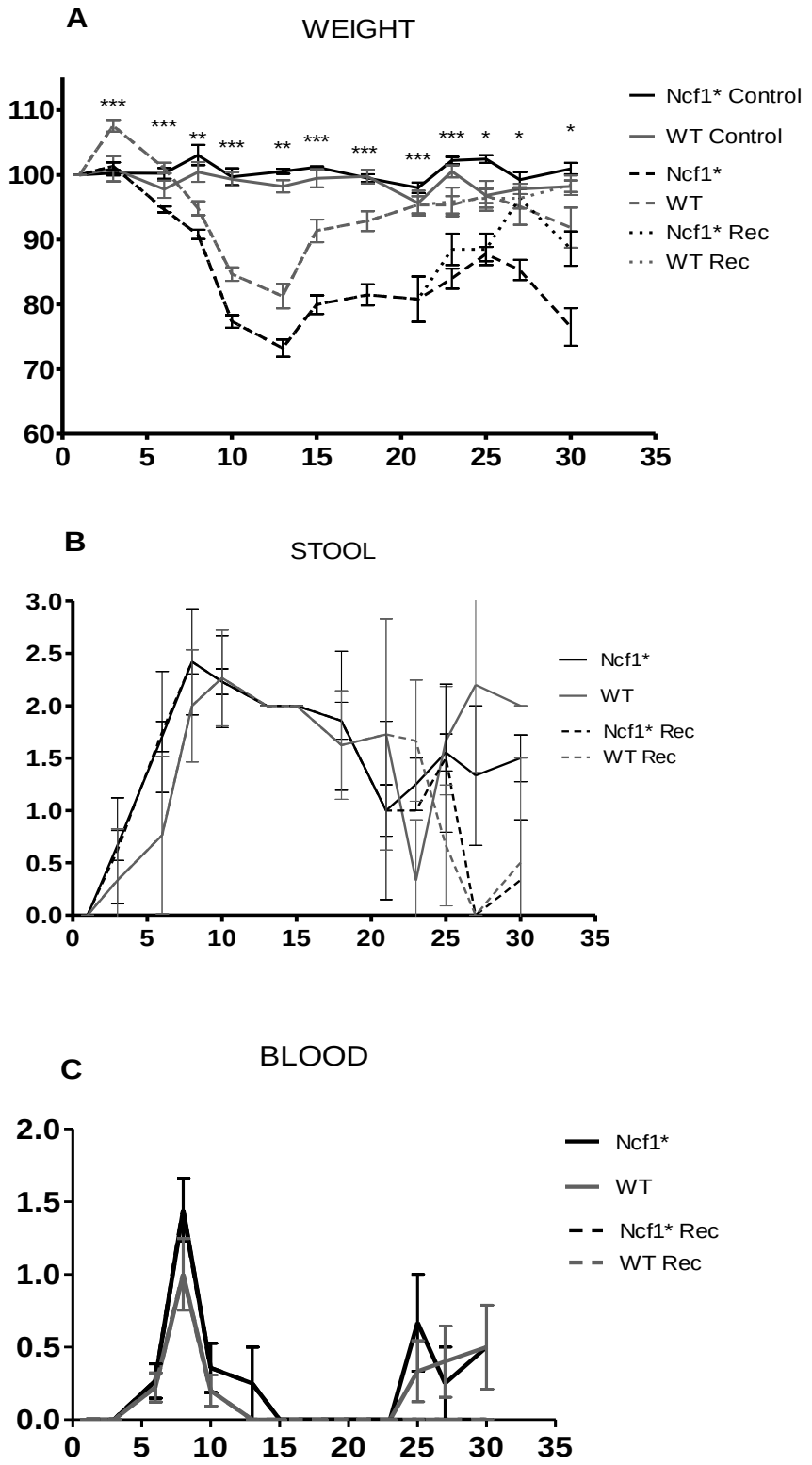

Figure 3 A) Mice weights distributions and analysis for the all experiment.in experiment, average weight ± SE, (baseline weight: WT=29.8±3.90g, Ncf1*=31.3±3.9g). Asterisks indicate $p<0.05$, double asterisk $p<0.001$, triple asterisk $p<0.0001$ Mann-Whitney test between each group Ncf1*control/WT control; Ncf1*/WT; Ncf1* Rec/WT Rec; Ncf1*/Ncf1* Rec. B) Mice stool consistency. C) Analysis of rectal blood presence. Weight, stool consistency and blood presence scorings are detailed in methods section.

3.2 **Histology**

The assessment of colon histopathology score was done in untreated WT and Ncf1* animals, and after colitis induction at days 22 and 30 for E1 and at day 30 for E2, to evaluate dysplasia, epithelial morphology and inflammation, according to the described scoring criteria.

At baseline WT mice had well designed glands above *muscuralis mucosae*, whereas Ncf1* mice showed a reduced number of glandular tubules (Fig 4).

On day 22 E1 WT mice maintained preserved epithelial morphology and villous projections plus low grade inflammatory reactive dysplasia. The Ncf1* group presented villous projections formed by compacted glands with less vascularization in the pedicle, plus glandular high grade dysplasia and invasive tubular adenocarcinoma foci (Fig 4).

On day 30 the mice in E1 had reparative villous and glandular adaptation. WT mice maintained villous projections corresponding to half of the *mucosae* length, supported by tubular/glandular hyperplasia above the *muscularis mucosae*, with different sizes and segments of very small glands with low grade dysplasia foci. In Ncf1* mice colon there was a reduced number of glands under villous projections, although there persisted a morphology similar to day 22. In general Ncf1* mice showed high grade dysplasia (with anisocariosis with persistent nucleoli and visible mitosis) and foci of invasive tubular adenocarcinoma while WT colon presented basal glands' hyperplasia with low grade and high grade segments of dysplasia (Fig 4).

On day 30 of E2, colonic sections showed persistent adaptation (Fig 4): Ncf1* proximal and distal colonic segments kept a glandular morphology with hypercromatic nuclei and mucosa-associated lymphoid tissue hyperplasia; and WT colon presented epithelial villous projections without inflammation. In distal segments, high grade dysplasia persisted in both groups. Additionally, Ncf1* colon developed invasive well-

differentiated adenocarcinoma in segments where few reserve micro-glands were visible above *mucularis mucosae*, invading till the *muscular propria* (Fig 4).

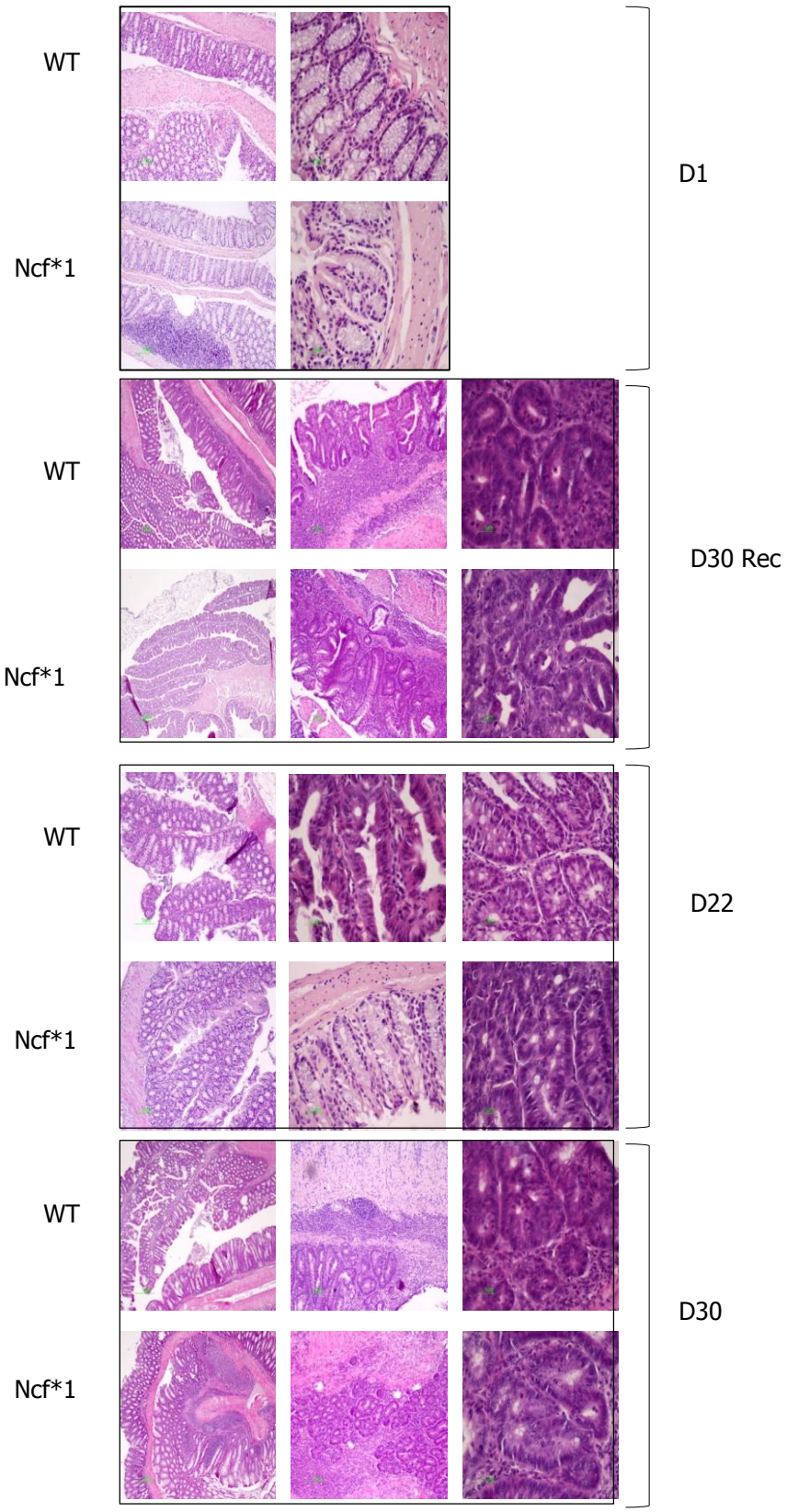

Figure 4 Colonic mucosa of control Experiment 1and 2, WT and Ncf1*mice groups (H&E 40x, 100x, 200x).

In general histopathological morphology descriptions had more inflammatory foci and glandular/epithelial alterations in the distal halves of colonic segments, summarized as follows:

In distal segment of the colon we observed higher inflammation and dysplasia scores for Ncf1* mutated mice than WT with statistical significance (p=0.259 for inflammation and p=0.0135 for dysplasia), for both strains (Fig. 5A e 5B). Looking for recovery, WT recuperated better than Ncf1*.

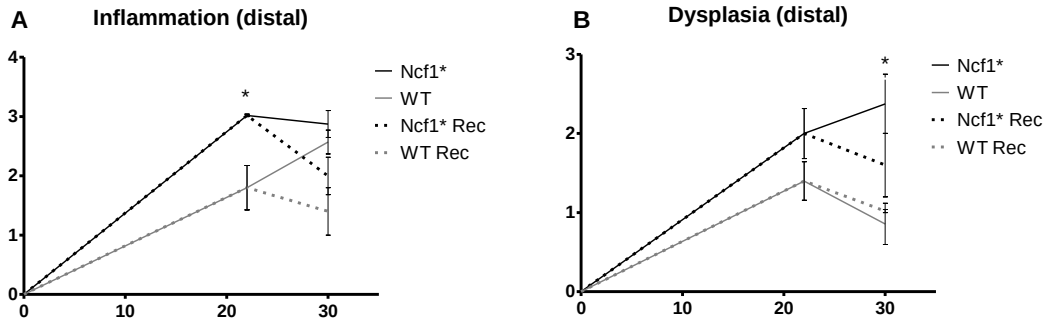

Figure 5. Histological evaluation of inflammation and dysplasia at distal level of the colon for Ncf1* mutated mice, Ncf1* Recovery, WT and WT recovery. Asterisks indicate p<0.05, Mann-Whitney test between Ncf1* and WT and Ncf1* Rec and WT Rec. Inflammation and dysplasia scoring system is detailed in the methods section.

At the proximal part of the colon inflammation was higher in both Ncf1* and Ncf1* recovery, having WT lower scores than Ncf1* Rec, with statistical significance between WT and Ncf1* groups (p=0.0357) after the second induction (Fig. 6A e 6B). Considering dysplasia, after the second induction, WT and WT Rec presented higher scores than Ncf1* than Ncf*1 Rec respectively with statistical significance between WT and Ncf1* groups (p=0.0463).

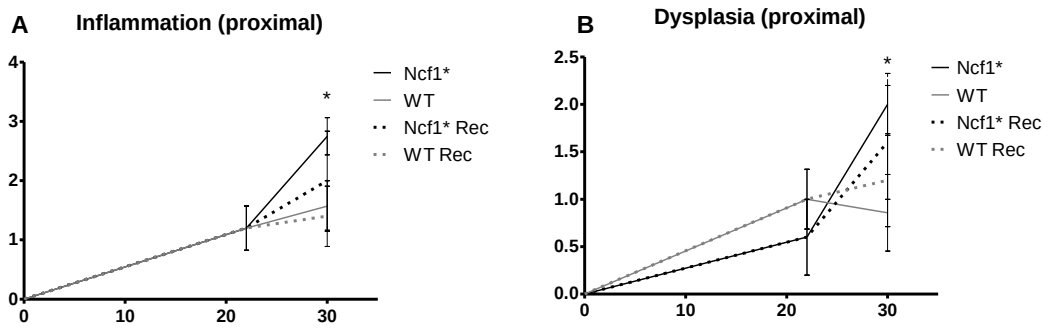

Figure 6 Histological evaluation of inflammation and dysplasia at proximal levels of the colon for Ncf1* mutated mice, Ncf1* Recovery, WT and WT recovery. Asterisks indicate $p<0.05$, Mann-Whitney test between Ncf1* and WT and Ncf1* Rec and WT Rec. Inflammation and dysplasia scoring system is detailed in the methods section.

3.3 Characterization immune populations in mesenteric lymph node

Changes in functional frequency of different functional subsets of T cells ($CD4^+$ and $CD8^+$), NK cells, B cells and monocytes, subsets were analyzed in the mesenteric lymph node since it is the main draining lymph node of the colon

3.3.1 Macrophages

Macrophages ($CD11b^+$) showed high frequency at D0 for both experiments followed by a decrease for WT and WT recovery groups during all experiment. At day 23 Ncf1* group had significantly increased levels of this cellular type ($p=0.0140$), comparing with WT. Recovery groups maintained decreased frequency (Fig.7A).Pro-Inflammatory Macrophages ($CD43^+/Ly6C^+$) presented increased frequency in Ncf1* groups after day 0. In days 22 and 30 Ncf1* groups showed significant higher frequencies ($p=0.0079$ and $p=0.0140$ respectively) comparing to WT groups. The tendency remained for recovery groups, however with no statistical significance between groups (Fig. 7 B).

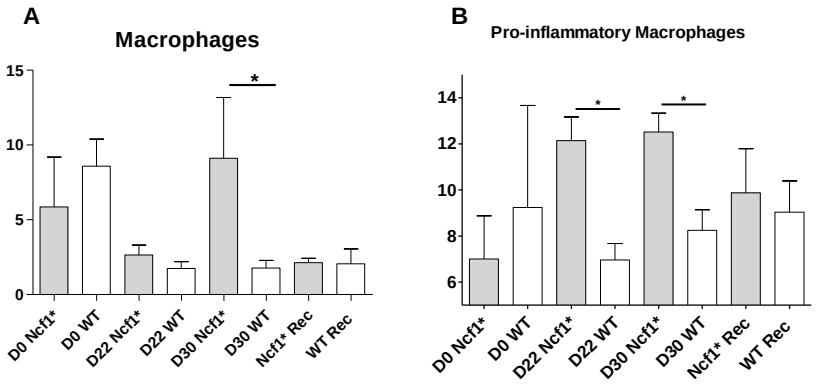

Figure 7 Analysis of mesenteric lymph nodes functional macrophage subsets at the different phases of DSS-colitis induction of Ncf1*, Ncf1* Rec, WT and WT Rec mice. Asterisks indicate $p<0.05$, Mann-Whitney test between Ncf1 and WT and Ncf1 Rec and WT Rec.

3.3.2 NK Cells

Frequencies of NK classic ($NK1.1^{+}/CD3^{-}$) showed higher for E2 with statistical significance ($p=0.0101$) for Ncf1* group, at day 30 comparing to WT. Ncf1* rec and WT rec groups showed decreasing frequencies during recovery after one induction (Fig. 9A).

NK expression(NKG2D median), showed increased for both experiments, except in the recovery groups where NK cells were more expressed in WT group with statistical significance ($p=0.0159$), when compared to Ncf1* (Fig. 8B).

NK activated ($CD314^{+}/NK1.1^{+}$) cells presented decreased during both experiments but in day 30, after the second induction, Ncf1* group had increased levels comparing to WT with statistical significance ($p=0.0303$) (Fig. 8C).

NK immature cells ($CD11b^{-}/CD27^{-}$) showed an increased frequency in the end of both experiments. In day 22 WT increased the recruitment of NK cells with statistical significance ($p=0.0303$) comparing to Ncf1*, recovery groups $Ncf1^{*}$ rec presented significant ($p=0.0159$) increased recruitment of NK cells comparing to WT (Fig. 8D).

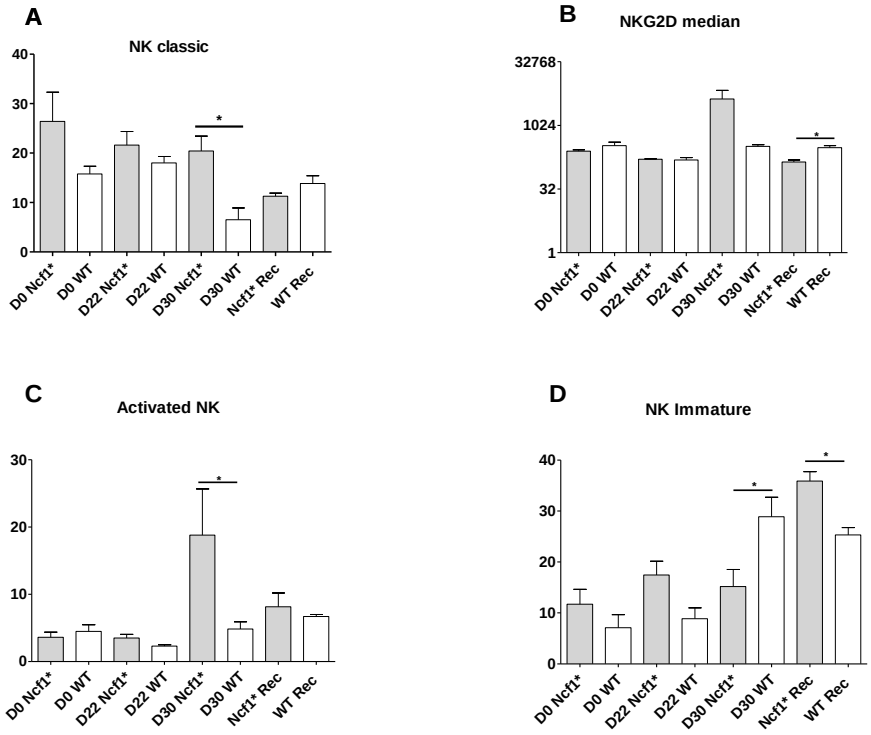

Figure 8 Analysis of mesenteric lymph nodes functional NK cells subsets at the different phases of DSS-colitis induction of Ncf1*, Ncf1* Rec, WT and WT Rec mice. Asterisks indicate $p<0.05$, Mann-Whitney test between Ncf1 and WT and Ncf1 Rec and WT Rec.

Citotoxic NK cells ($CD11b^{+}/CD27^{-}$) showed decreasing tendency in frequency for WT groups. At day 30, WT had a significant ($p=0.0177$) decrease in NK cells frequency comparing with Ncf1*, recovery groups showed the opposite from previous groups with significant ($p=0.0268$) increased frequency for WT comparing to Ncf1* (Fig. 9A).

NK regulatory ($CD11b^{+}/CD27^{+}$) cells distribution presented higher frequency for WT group in both experiments having statistical significance ($p=0.0159$) in recovery groups (Fig. 9B). NK T cells ($CD3^{+}/NK1.1^{+}$) presented higher frequencies for WT groups during both experiments. However, at the end of both experiments, Ncf1* showed higher frequencies than WT both in Ncf1*/WT and Ncf1* rec/WT rec showing the last statistical significance ($p=0.0159$) (Fig. 9C).

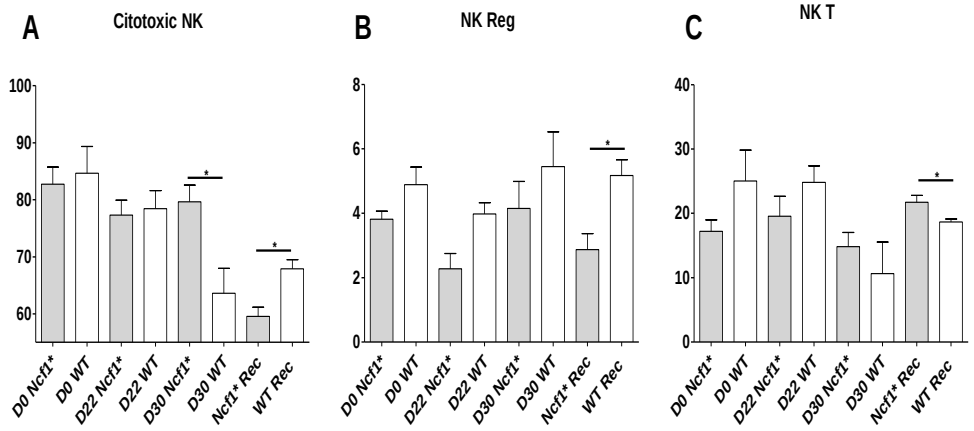

Figure 9 Analysis of mesenteric lymph nodes functional NK cells subsets at the different phases of DSS-colitis induction of Ncf1*, Ncf1* Rec, WT and WT Rec mice. Asterisks indicate $p<0.05$, Mann-Whitney test between Ncf1 and WT and Ncf1 Rec and WT Rec.

3.3.3 B Cells

B lymphocytes ($CD19^{+}$) frequency was generally higher in Ncf1* mice than WT which was seen during almost all experiment. In day 22 we saw statistical significance ($p=0.0159$) between Ncf1* and WT groups and at the end of experiment, day30, Ncf1* rec and WT rec show similar frequencies with a recover for Ncf1* comparing to day 22 (Fig.10A).

IL10 (iL10) expression in B cells, was higher for Ncf1* group inday0 and at day 22, IL10 production was low for all groups, increasing after second induction. At day 30 Ncf1* group had higher levels of expression comparing to WT, with statistical significance ($p=0.0289$) between both groups (Fig.10B). The B1b ($CD5^{+/}IgM^{-}$) subset of B cells presented at days 0 and 22 similar frequencies for both Ncf1* and WT, in day22 WT had an increase of B1b cells comparing to Ncf1* with statistical significance ($p=0.0401$) (Fig.10C). Ncf1*rec and WT rec groups showed high frequency for WT, however there were no statistical significance (Fig.10C).

Transitional 3B ($CD21^+/CD23^+$) cells population is increased in WT in day 0, then it increased again for the same group in day 30 with statistical significance ($p=0.0077$) when compared to Ncf1* mice maintaining similar values in other time points (Fig.10D).

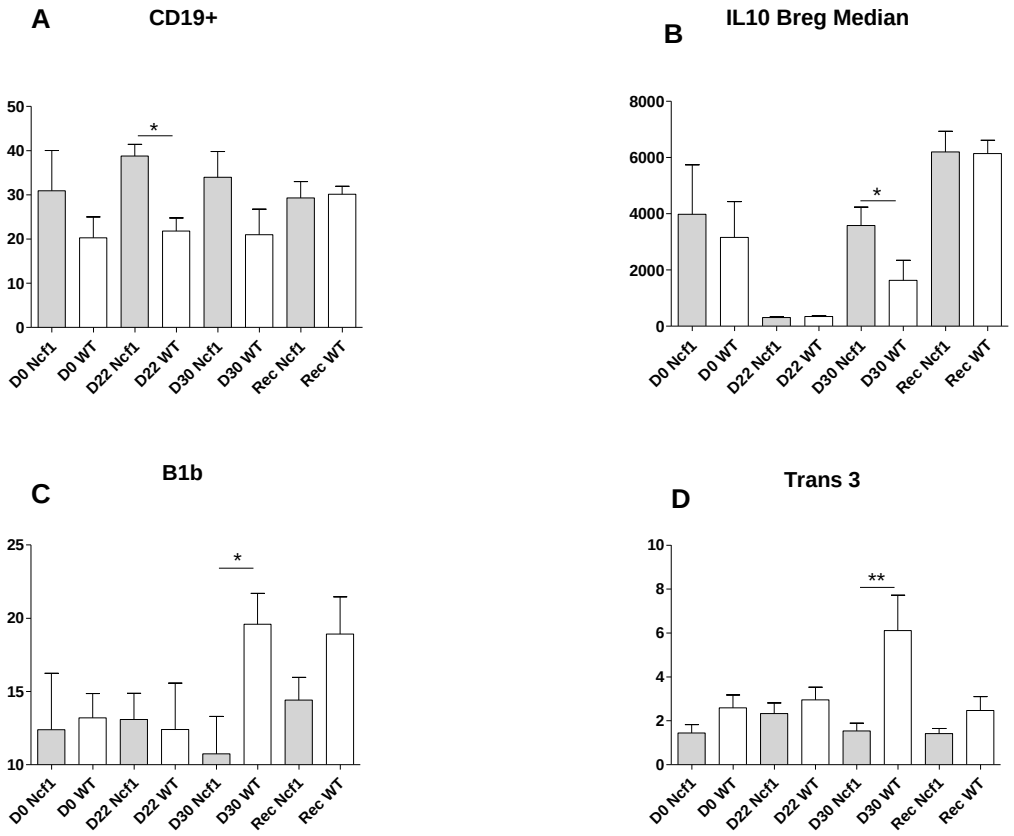

Figure 10 Analysis of mesenteric lymph nodes functional B cells subsets at the different phases of DSS-colitis induction for Ncf1*; Ncf1* Rec, WT and WT Rec mice. Asterisks indicate $p<0.05$, Mann-Whitney test between Ncf1* and WT and Ncf1* Rec and WT Rec.

$CD3^+$ T cells presented high frequencies in WT during both experiments with statistical significance ($p=0.0022$) at day 22. Ncf1* rec showed almost the same frequency that WT rec which increased since 1st induction (Fig. 11A).

$CD4^+$ T cells showed at day 0 higher frequency for Ncf1* mice but after the first induction all groups showed similar values. After the second induction (day 22) the WT group presented a significant ($p=0.0262$) higher frequency comparing to Ncf1* group.

In WT rec and Ncf1* rec counterparts the scores decreased considering 1st induction (day 0 - day 7) frequency (Fig. 11B).

CD4 effector cells (CD62L^{-}/CD27^{+}) presented an increasing frequency for both experiments with statistical significance (p=0.0159) at day 22 for Ncf1* group. At day 30, Ncf1* had their frequency increased compared to WT but with no statistical significance (Fig. 11C).

Naïve CD4 cells (Foxp3^{-}/CD25^{-}) presented a decrease in Ncf1* groups during experiment. WT groups showed an increase in this cell population at day 22 followed by a decrease at the end of both experiments for WT and WT rec. At day 30 Ncf1* presented a significant (p=0.0043) higher frequency comparing to WT (Fig. 11D).

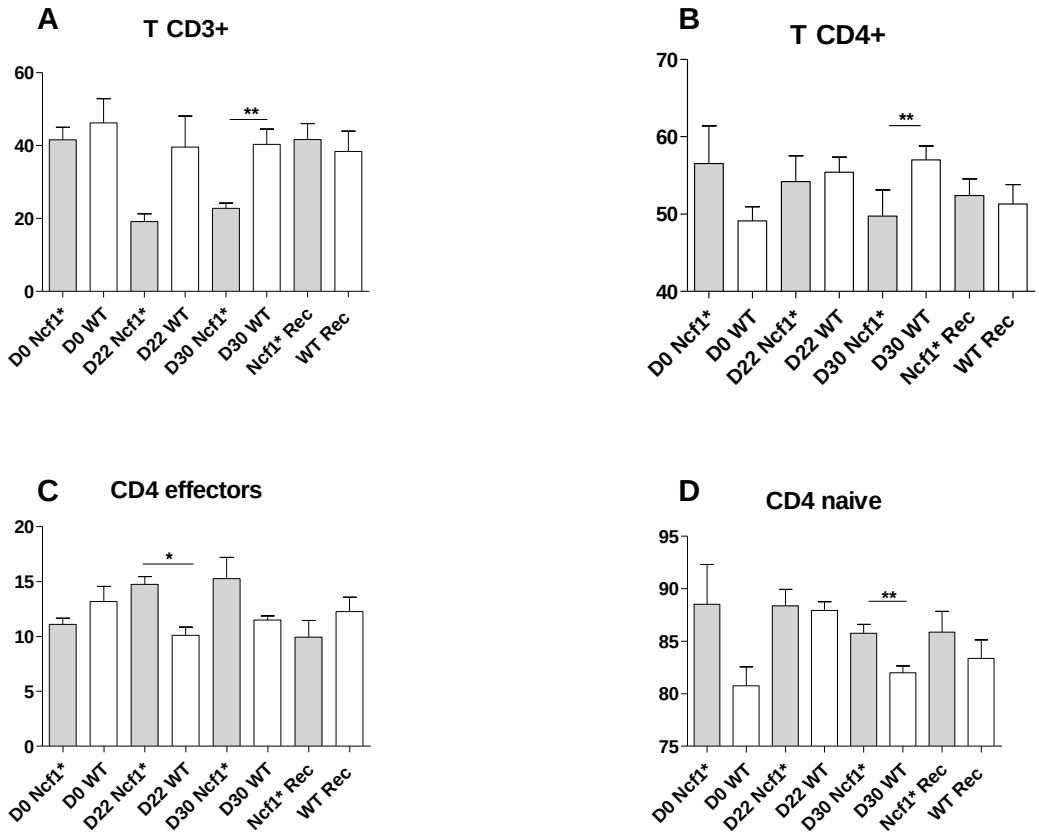

Figure 11 Analysis of mesenteric lymph nodes functional T cells subsets at the different phases of DSS-colitis induction for Ncf1*; Ncf1* Rec, WT and WT Rec mice. Asterisks indicate $p<0.05$, Mann-Whitney test between Ncf1* and WT and Ncf1* Rec and WT Rec.

T Regulatory 1 cells ($Foxp3^+/CD25^-$) presented initially (D0) a higher frequency in WT comparing with Ncf1*. There is a decrease in initial frequencies at day 22, at day 30 this population was significantly ($p=0.0260$) higher in WT group (Fig. 12A).

In all groups T Regulatory 2 cells ($Foxp3^+/CD25^-$) presented a decrease comparing to day 0 an at day 30, WT had a significantly ($p=0.0260$) higher frequency comparing with Ncf1*. Recovery groups showed increased values for WT comparing with Ncf1* not with no statistical significance (Fig. 12B).

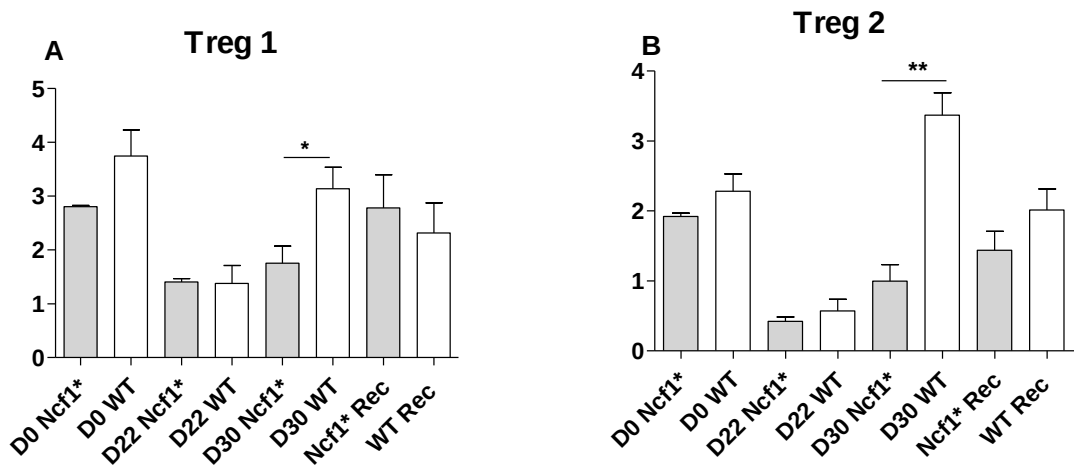

Figure 12 Analysis of mesenteric lymph nodes functional T cells subsets at the different phases of DSS-colitis induction for Ncf1*; Ncf1* Rec, WT and WT Rec mice. Asterisks indicate $p<0.05$, Mann-Whitney test between Ncf1 and WT and Ncf1 Rec and WT Rec.

Central Memory T ($CD62L^+/CD27^+$) cells presented an increase frequency for all from day 0 to 22, followed by a decrease at day 30 for both groups with no statistical significance. . WT rec showed higher values than Ncf1* rec with statistical significance ($p=0.0159$) (Fig. 13A).

Effector memory T cells ($CD62L^-/CD27^+$) exhibited increased frequencies during all experiment. Comparing Ncf1* and WT groups at day 30, WT show significant ($p=0.0022$) augmented values comparing with Ncf1* group (Fig. 13B).

Short-term effector memory T cells (CD62L-/CD27+) showed a significant ($p=0.0317$) decrease of frequency for WT group after 1st induction (D22) comparing to Ncf1* group and after 2nd induction (D30) the differences remained significant ($p= 0.0152$) with Ncf1* presenting even higher frequency of this cells comparing with Ncf1* and with D22 (no statistical significance). In recovery groups Ncf1*presented higher scores comparing with WT with no statistical significance (Fig. 12C).

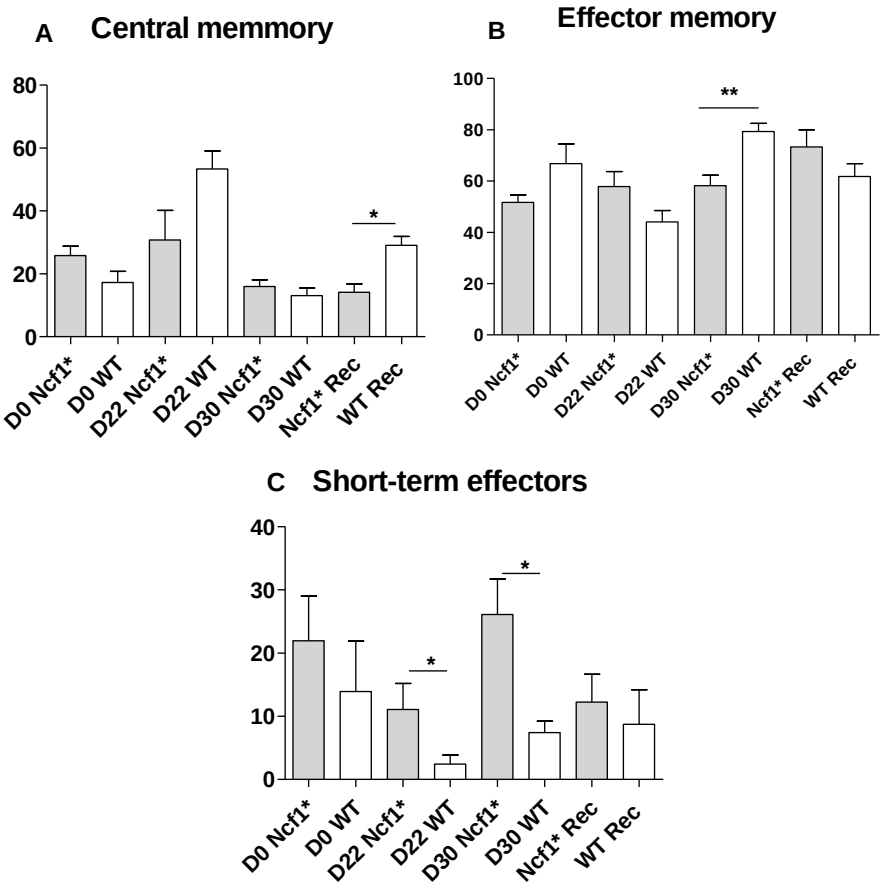

Figure 13 Analysis of mesenteric lymph nodes functional T cells subsets at the different phases of DSS-colitis induction for Ncf1*; Ncf1* Rec, WT and WT Rec mice. Asterisks indicate $p<0.05$, Mann-Whitney test between Ncf1 and WT and Ncf1 Rec and WT Rec.

CHAPTER 4

DISCUSSION AND CONCLUSION

4 Discussion and Conclusion

The DSS-colitis model is commonly used because of its easy administration and its rapid action causing inflammation that -depending on the number of cycles- can become chronic and lead to dysplasia (Sugimura, 2000) (Tanaka S. *et al*, 2005) (De Robertis M. *et al*,2011).

Using the DSS-colitis model in Ncf1* mice we tried to understand whether the absence of ROS has a protective or pathologic role in the chronicity of colon's inflammation. We already knew, from a previous study, that during the acute phase of colitis ROS are essential to inflammation's resolution, since they seem to control the exacerbated colonic expression of iNOS and regulate the production of several pro-inflammatory cytokines by infiltrating leukocytes (Rodrigues-Sousa T. *et al.* 2014). However, the role of ROS in chronic inflammation was not fully addressed. Thus we performed two experiments in order to see its involvement in the resolution and recovery of an inflammatory process, E1, and on a chronic bowel inflammation, E2 (two DSS inductions). We found out that Ncf1* mutated mice presented a more exuberant response to DSS-induced colitis showing severer clinical scores, high grade dysplasia and inflammation with an aberrant immunological response.

The shortening of the bowel is caused by the mucosa destruction leading to a diminished absorption and consequent loss of weight (Gaudio *et al*, 1990) (Cooper *et al*, 1993) (Krieglstein *et al*, 2001). It is notable that, even though both groups had comparable shortening of the colon, Ncf1* mice present a worst recovery of weight in both experiments probably because of a diminished capacity to recover from treatment (Conway *et al*, 2012) (Sann, H. *et al*, 2013). Spleen's weight is representative of the

intensity of the inflammatory response (Axelsson L., 1996). Ncf1* present higher scores which represents a more intense inflammatory response. Those alterations in mucosa function lead to alterations in feces that start to be less solid with the progression of treatments and because of the severity of treatment leads to the presence of blood in the stools. (Conway *et al*, 2012) (Sann, H. *et al*, 2013). Ncf1* and WT had similar scores for feces consistency but the first showed more blood in feces after two DSS inductions, result of a more intense response to aggression. (Conway *et al*, 2012) (Sann, H. *et al*, 2013). As expected, Ncf1* had a more aberrant response to DSS induced colitis with more tendency to dysplasia. In the first experiment Ncf1* mice recovery is not as pronounced has in WT mice. In the second experiment Ncf1* show a more pronounced inflammation. The alterations in mucosa are evident on histology where Ncf1* mutated mice show higher scores of inflammation and increased tendency to dysplasia in both experiments. Ncf1* show a decreased capacity to solve inflammation and at least avoid dysplasia Sturlan S. *et al*. showed that IL-10 $^{-/-}$ mice spontaneously develop colitis which leads to high grade dysplasia's which indicates that with immune response compromised the propensity for dysplasia increases (Sturlan, Oberhuber *et al*, 2001) (Mino-Kenudson K. *et al* , 2011).

We faced some technical obstacles with the analysis of mesenteric lymph nodes since mesenteric it is vestigial in the absence of an immune response. Thus it was very difficult to find this organ in the necropsy of baseline controls, resulting in low number of retrieved cells. On the other hand, the mesenteric lymph node is not frequently studied, hence there is little information about the existing leukocyte subsets. Therefore, we have discussed the results on mesentheric subsets, by directly comparing to other lymph nodes and peripheral blood.

ROS influence the immune response to inflammation, leading to an altered behavior of immune system in the absence of it. (Kraaij M. *et al* 2010) (Conway *et al*, 2012) (Zigmond E. *et al*, 2012) (Rodrigues-Sousa T. *et al.* 2014). Krieglstein *et al* said that Ncf1* (*p47^{phox}*) mutation has no significance in colitis resolution however he did not add a resting period to study ROS role in recovery. Conway et al showed that inhibiting Ncf4* (*p40^{phox}*) subunit of NADPH mice lead to worse recovery from colitis similar to the one we have seen for Ncf1 subunit mutation (Conway *et al*, 2012) (Rodrigues-Sousa *et al*, 2014).

Macrophages presented increased frequency in Ncf1* after 2 inductions. Conway et al had seen a higher pathogen infiltrate, once Ncf1* macrophages have ROS production compromised they are not capable to response to that infiltration. However they cannot phagocyte, they continue to be produced and recruited so that might be the reason why Ncf1* present those values after second induction (Conway *et al*, 2012). Pro-inflammatory macrophages present higher frequency in Ncf1* compared to WT and this tendency maintains in Ncf1* rec comparing to WT rec (no statistical significance). That is a sign of chronicity, once Ncf1*can't solve inflammation, pro-inflammatory cells keep being recruited to a pro-inflammatory response (Valledor A. *et al*, 2010).

The NK population has an important role in tissue inflammation in situations like IBD (Schleinitz N., 2010) (Yadav P., 2011). The balance between activation and inhibition should be maintained very tight in this population otherwise its cytotoxicity might be superior to its regulatory capacity. According to Schleinitz, NK should be below normal in IBD situation, but the fact is that NK populations were increased in Ncf1* mutated mice and that according to the same author might be due to proinflammatory cytokines that hipper activate NK cells promoting inflammation (Schleinitz N., 2010) (Yadav P., 2011). Considering NK cells tumor recognizers and

killers, this increase might be associated with increased levels of dysplasia in the colon WT mice show higher frequency of NK activation and NK regulatory which might be due an early response to dysplasia from WT groups (Tallerico R. *et al*, 2013) (Betten A., 2004) (Tallerico R. *et al*, 2013).

B lymphocytes use to be more activated in IBD usually because of pathogens infiltration in the bowel (Brandtzaeg P. *et al*, 2006). Moreover, Ncf1* mice are more prone to opportunistic bacterial infections (Pizolla *et al*, 2012). The higher frequency of mesenteric B lymphocytes in Ncf1* mice, from the beginning of the experiment, especially in day 22, might be due to a higher bacterial infiltration and consequently more need of lymphocytes activity. Even though we tried to assess the bacterial load and phylogeny in the treated animals, we did not get any conclusive results. Since WT mice keep an intact anti-bacterial response, the infiltration of B lymphocytes does not occur. However, as commonly observed in inflammatory processes (Sims G. *et al*, 2005), the frequency of B1b and transitional B cells was higher in WT mice.

IL-10 downregulates the production of pro-inflammatory cytokines and it is overexpressed in IBD (Mitsuyama K. *et al*, 2006) (Kucharzik *et al*, 1995). Having in account that patients with mutated IL-10 signaling systems show early and aggressive development of systemic inflammation including IBD and that Ncf1* showed augmented levels of IL-10 cytokine production that might be due to an aberrant response of these mice (Kanneganti, Mino-Kenudson *et al*, 2011).

CD3+ and CD4+ T cell populations were overexpressed in WT mice after 2 DSS inductions which is consistent with Treg 1 and 2 results which are overexpressed for WT at D30. T regulatory cells are normally overexpressed in a colitis situation, due to damage in gastrointestinal tract leading to inflammation and consequently to T- cell

activation (Kraaij M. *et al*, 2010) (Funderburg N. *et al*, 2013). Since T regulatory cells show an important anti-inflammatory action, their higher frequency in WT comparing to Ncf1* show a better response from WT to inflammation (Maul J. *et al*, 2005).

CD4+ effectors were higher after 1 DSS induction and CD4+ naïve after 2 DSS inductions in Ncf1*. These subsets have been implicated in promoting IBD, hencesuggesting a worse response to IBD from Ncf1* mice. (Martin B *et al*, 2004). Short term effectors were increased for Ncf1* at D22 and D30 which represented a pro-inflammatory response instead of a resolution attempt (Caprioli *et al*, 2013)

Central memory and effector memory CD8+ T cells presented high for WT Rec and WT in E2 (2 DSS inductions) indicating that WT were trying to respond better to situation that had previous been exposed to. Getting more memory cells implicates a faster response to o next induction (Sheridan B. *et al*, 2011).

ROS act in many other celluar pathways such as wound repair mechanisms (Chan E. *et al*, 2009) (Gauron C., 2013) (Kanyilmaz S. *et al*, 2013) so this might explain why Ncf1* mutated mice presented more difficulties in recovering from DSS abrasion of the mucosa and had severer phisical signs like more blood in feces and worse histological scores.

NADPH oxidase is a ROS producer, it was previous said that ROS should be kept in a tight balance since overproduction is dangerous. Here we prove that ROS deficiency is equally harmful. Ncf1* mice have their NADPH $p47^{phox}$ subunit mutated and their ROS production compromised. As shown in a previous study by Rodrigues-Sousa T. *et al.* Ncf1* mice do not produce ROS as that observed in their counterpart WT mice. It was also shown by a nitrotyrosine immune fluorescence assay, Ncf1* colon produce peroxynitrites in order to balance the absence of ROS (Rodrigues-Sousa T. *et*

al. 2014). Having this in consideration, ROS absence increases the susceptibility for colitis chronicity. Furthermore, as a consequence of the elevated NO production, the immune response seems to be over-stimulated leading to chronic colonic inflammation.

IBD is a recurrent disease affecting the modern world because of what we eat and how we live. Having in account that this disease may evolve into adenocarcinoma, it is important to understand how it starts and when is the right time to introduce particular therapies. In that line of thought the next step is to try to understand the stages of this progression into carcinogenesis, and modulate its evolution with different therapeutic approaches. For example, it will be interesting to use immune system modulation in order to control the disease evolution, maintaining a tight regulation of ROS production to control its potential harmful actions. Our model seems to be a good model for carcinogenesis since usually more cycles of DSS are needed in order to get dysplasia and we got it after two cycles of induction (Cooper, Murthy *et al.* 2000). Eventually, exploring models with other NOX2-subunits mutations will yield further insights into the role of this complex in colon function and protection.

This study presents another perspective of ROS role in inflammation and dysplasia: ROS concentration should be kept in a very thigh balance, otherwise its benneficts/damages might cause immune-disregulation. Typically, the deterimental role of ROS in cancer and other diseases is focused on its high levels. However, we challenged this paradigm as we have shown that its absence compromises the ability of the immune system to regulate and react its response against previous challenges.

CHAPTER 5

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