# Identifying new therapeutic targets for the treatment of Crohn's disease: The role of CD47 and L-carnitine in the pathogenesis and treatment of a murine model of intestinal inflammation

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Degree of the

The Faculty of Medicine

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July 2009

A thesis submitted to McGill University

in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

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ABSTRACT

Crohn's disease (CD) is a chronic, relapsing and remitting immune-mediated inflammatory disease of the gastrointestinal tract. While there is currently no cure for this disease, a wide range of treatment options are available. However, these are often associated with a significant set of adverse effects and, despite their use, most CD patients will eventually require hospitalization and/or surgery. The goal of the projects outlined in this thesis was therefore to further our understanding of the mechanisms involved in generating intestinal inflammation and to apply these findings to develop novel therapeutic agents.

We first examined the role of interactions between CD47 and its ligand, signal regulatory protein alpha (SIRPα), in the development of TNBS colitis, a murine model of intestinal inflammation sharing many features with human CD. We have demonstrated that dendritic cells (DC) expressing SIRPα promote Th17 responses and the development of experimental colitis. Furthermore, we identify an important role for CD47 in the migration of SIRPα$^{+}$ DCs to the lamina propria and mesenteric lymph nodes, where they participate in inducing inflammation. Thus, by genetic deletion of CD47 or by impairing its function using a CD47-fc fusion molecule, we have successfully reduced the severity of intestinal inflammation.

L-carnitine is an amino acid derivative normally present in meat and dairy products and is also available as an over-the-counter nutritional supplement. Since mutations in the L-carnitine transporters, OCTN1 and OCTN2, were found to be associated with CD, we sought to examine its role in the development of intestinal inflammation. Remarkably, L-carnitine displayed immunosuppressive properties both *in vitro* and *in vivo,* effectively suppressing both the innate and the adaptive arms of the immune response and resulting in a significant reduction in the development of intestinal inflammation.

We have thus identified CD47 as an important regulator of SIRPα$^{+}$ DC trafficking, and demonstrate that this DC subset is implicated in the development of intestinal inflammation. Additionally, we have identified two promising new therapeutic candidates, CD47-fc and L-carnitine, for the treatment of CD.

RÉSUMÉ

La maladie de Crohn (MC) est une maladie inflammatoire chronique impliquant un dérèglement du système immunitaire qui touche à l'ensemble du tube digestif, et qui est caractérisé par des périodes cycliques de rechutes et rémissions. Bien qu'il n'existe aucun traitement curatif contre cette maladie, plusieurs remèdes visant à amoindrir les symptômes sont actuellement disponibles sur le marché. Toutefois, ces derniers sont souvent associés à de multiples effets secondaires néfastes, et, malgré leur utilisation, aucun de ces traitements ne parvient à empêcher la grande majorité des patients atteints d'être éventuellement hospitalisés et/ou de subir une intervention chirurgicale. Par conséquent, l'objectif premier des projets détaillés dans cette thèse a été d'approfondir les connaissances des mécanismes biologiques impliqués dans l'initiation de l'inflammation intestinale ainsi que d'appliquer ces découvertes au développement de nouveaux agents thérapeutiques.

Nous avons d'abord évalué le rôle des interactions entre CD47 et son ligand, SIRPα (*signal regulatory protein alpha*), dans le développement de la colite induite par TNBS, qui est un modèle murin d'inflammation intestinale partageant plusieurs symptômes similaires à ceux rencontrés chez les patients atteints de la MC. Nous avons démontré que les cellules dendritiques (CD) qui expriment SIRPα induisent une réponse immunitaire Th17 ainsi que le développement de la colite expérimentale. De plus, nous avons identifié un rôle important joué par CD47 dans la migration des CD SIRPα$^{+}$ vers la lamina propria et les ganglions mésentériques, où ces cellules participent à l'induction de l'inflammation. Ainsi, par la délétion génétique du CD47 ou encore par la manipulation de ses fonctions à l'aide d'une molécule de fusion, le CD47-Fc, nous avons réussi à diminuer avec succès l'inflammation intestinale chez la souris.

L-carnitine (LCAR) est un acide aminé dérivé que l'on retrouve normalement dans la viande et les produits laitiers et qui est également disponible en vente libre en tant que supplément nutritionnel. Puisque des mutations dans le transporteur de LCAR, OCTN1 et OCTN2, ont préalablement été associées avec la MC, nous avons décidé d'examiner le rôle de cette molécule dans le développement de l'inflammation intestinale. LCAR a démontré des propriétés immunosuppressives remarquables, tant *in vitro* qu'*in vivo*, en supprimant efficacement les mécanismes de défenses innés et adaptatifs de la réponse immunitaire, résultant en une diminution significative du développement de l'inflammation intestinale.

En résumé, nous avons identifié CD47 comme étant un important régulateur de la migration des CD SIRPα$^{+}$ et avons démontré que cette sous-population de cellules est impliquée dans le développement de l'inflammation intestinale. De plus, ces travaux ont permis d'identifier deux nouveaux candidats thérapeutiques prometteurs, le CD47-Fc et LCAR, pour le traitement contre la MC.

ACKNOWLEDGEMENTS

I would first like to express my sincere gratitude to my supervisor, Dr. Denis Franchimont, for opening my eyes to the wonderful world of scientific research and providing me with invaluable advice and support during the course of this work. I would equally like to thank my co-supervisor, Dr. Marika Sarfati, who was kind enough to take me under her wing and treat me as one of her own when Dr. Franchimont could no longer be in Montreal. I greatly appreciate all of the time and effort that she invested in this project and for sharing her wealth of knowledge and experience with me. Her contribution immensely improved the quality of this work, and could not have come to fruition without her.

I consider myself especially fortunate to have had the pleasure to work with Manuel Rubio, Marianne Raymond, Vu Quang Van, Nobuyasu Baba, Salim Bouguermouh, and Keiko Wakahara and wish to thank them for all of their help and kindness. Also, I'd like to thank Katerina Yurchenko for her guidance and for our numerous insightful discussions. I also owe a very special thank-you to Chloé Villani and Catherine Collette, for all of their assistance with this project and for their unfaltering support, encouragement, and faith.

I must also thank my family and friends, without whose constant encouragement, moral support and love this would not have been possible. A special heartfelt thank-you to my parents, for instilling in me a genuine love of learning, and to my brothers, Rick and Dave, for continually raising the bar of success. Finally, I cannot end without expressing my sincere gratitude to my husband, Mik, for his patience and support during my many school years.

CONTRIBUTIONS OF AUTHORS

**Chapter 3**

Fortin G, Raymond M, Van VQ, Rubio M, Gautier P, Sarfati M, Franchimont D. A role for CD47 in the development of experimental colitis mediated by SIRPalpha+CD103- dendritic cells. *J Exp Med*. 2009 Aug 31;206(9):1995-201

Fortin G: Conceived, designed, and executed all the *in vitro* and *in vivo* experiments. Performed all statistical analyses and wrote the manuscript.

- Raymond M, Van VQ, Rubio M, Gauthier P: Assisted in conceiving, designing, and executing *in vitro* and *in vivo* experiments.
- Sarfati M, Franchimont D: Study conception, supervision and manuscript revision.

**Chapter 4**

Fortin G, Yurchenko K, Collette C, Rubio M, Villani AC, Bitton A, Sarfati M and Franchimont D.
L-carnitine, a diet component and organic cation transporter OCTN ligand, displays immunosuppressive properties and abrogates intestinal inflammation. *Clin Exp Immunol.* 2009 Apr;156(1):161-71.

- Fortin G: Conceived, designed, and executed all the *in vitro* and *in vivo* experiments. Performed all statistical analyses and wrote the manuscript.
- Yurchenko K: Assisted in conceiving, designing, and executing several *in vitro* experiments.
- Collette C, Rubio M, Villani AC, Bitton A, Sarfati M: Assisted in conceiving, designing and executing several *in vitro* and *in vivo* experiments.
- Sarfati M, Franchimont D: Study conception, supervision and manuscript revision.

CLAIMS OF ORIGINALITY

1. CD47 regulates SIRPα$^{+}$CD103$^{-}$ DC trafficking to the lamina propria and mLNs

2. CD47 ablation protects mice from the development of TNBS colitis

3. CD47 ablation decreases colonic proinflammatory cytokines and Th17 biased responses in CD47 KO mice

4. SIRPα$^{+}$CD103$^{-}$ DCs promote Th17 responses *in vitro*

5. CD47 expression on SIRPα$^{+}$CD103$^{-}$ DCs promotes the development of intestinal inflammation and Th17 responses

6. Administration of CD47-Fc protects BALB/c mice from the development of TNBS colitis

7. LCAR significantly inhibits the expression of the activation markers CD80 and CD86 on dendritic cells and macrophages, but not on B cells *in vitro*

8. LCAR dose-dependently impairs IL-6, IL-1β and TNFα production by dendritic cells and macrophages *in vitro*

9. LCAR significantly inhibits CD4+ T cell activation as assessed by CD69 and CD25 expression after anti-CD3 stimulation

10. LCAR significantly impairs the proliferation and IFNγ, IL-4 and IL-5 production of purified CD4+ T cells *in vitro*

11. LCAR deficiency results in the hyperactivation of CD4+ T cells and enhanced IFNγ production *in vitro,* which is reversed by LCAR supplementation

12. Intraperitoneal LCAR is effective in treating trinitrobenzene sulphonic acid (TNBS) colitis

13. LCAR abrogats IL-1β and IL-6 mRNA expression in the colonic tissues of mice with TNBS colitis and their circulating plasma concentrations

14. LCAR treatment *in vivo* significantly inhibits antigen-specific CD4+ T cell proliferation in the draining lymph nodes of with mice TNBS colitis

ABBREVIATIONS

| | |
|---|---|
| 2-ME | 2-Mercaptoethanol |
| 5-ASA | 5-aminosalicylates |
| 6-MP | 6-mercaptopurine |
| AIDS | Acquired immunodeficiency syndrome |
| AIEC | Adherent-invasive E. Coli |
| APC | Allophycocyanin |
| APC | Antigen-presenting cell |
| ATP | Adenosine triphosphate |
| BMDC | Bone-marrow derived dendritic cell |
| BRDU | Bromodeoxyuridine |
| CARD15 | Caspase recruitment domain protein 15 |
| CD | Crohn's disease |
| cDC | Conventional dendritic cell |
| CFSE | Carboxyfluorescein succinimidyl ester |
| CMTMR | 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine |
| CPT | Carnitine palmitoyltransferase |
| DC | Dendritic cell |
| DCBT | Dendritic cell based treatment |
| dLN | Draining lymph node |
| DNA | Deoxyribonulceic acid |
| DSS | Dextran sulphate sodium |
| DTR | Diphtheria toxin |
| E. coli | Escherichia coli |
| EAE | Experimental autoimmune encephalomyelitis |
| EAU | Experimental autoimmune uveitis |
| FITC | Fluorescein isothiocyanate |
| Flt3-L | Fms-like tyrosine kinase3 ligand |
| Foxp3 | Forkhead Box P3 |
| GALT | Gut-associated lymphoid tissue |
| G-CSF | Granulocyte-colony stimulating factor |
| GM-CSF | Granulocyte/macrophage colony-stimulating factor |
| GRα | Glucocorticoid receptor alpha |
| Gro-α | Growth-related protein alpha |
| GWAS | Genome-wide association study |
| HIV | Human immunodeficiency virus |
| HMP | Human microbiome project |
| IAP | Integrin-associated protein |

| | |
|---|---|
| IBD | Inflammatory bowel disease |
| IEC | Intestinal epithelial cell |
| IFNγ | Interferon gamma |
| Ig | Immunoglobulin |
| IKKγ | IκB kinase γ |
| IL | Interleukin |
| ILF | Isolated lymphoid follicle |
| IRF1 | Interferon regulatory factor-1 |
| ITF | Intestinal trefoil factor |
| iTreg | Inducible regulatory T cell |
| KO | Knockout |
| LC | Langerhans cell |
| LCAR | L-carnitine |
| LCFA | Long chain fatty acid |
| LN | Lymph node |
| LPS | Lipopolysaccharide |
| LRR | Leucine-rich repeat |
| mAb | Monoclonal antibody |
| MAdCAM-1 | Mucosal addressin cellular adhesion molecule-1 |
| MAP | Mycobacterium avium subspecies paratuberculosis |
| MCP-1 | Monocyte chemotactic protein |
| MDP | Muramyl dipeptide |
| MDR | Multi-drug resistance |
| MHC | Major histocompatibility complex |
| mLN | Mesenteric lymph node |
| MPO | Myeloperoxidase |
| MS | Multiple sclerosis |
| mRNA | Messenger ribonucleic acid |
| NFκB | Nuclear factor kappa B |
| NIH | National Institutes of Health |
| NK | Natural killer |
| NOD | Nucleotide binding oligomerization domain |
| NLR | NOD-like receptor |
| OCTN | Organic cation transporter |
| P4HA2 | Prolyl 4-hydroxylase α-2 subunit precursor |
| pDC | Plasmacytoid dendritic cell |
| PDLIM4 | PDZ and LIM domain protein 4 |
| PE | Phycoerythrin |
| PerCP | Peridinin chlorophyll protein complex |
| PMA | Phorbol 12-myristate 13-acetate |

| | |
|---|---|
| PML | Progressive multifocal leukoencephalopathy |
| PNAd | Peripheral node addressin |
| PP | Peyer's patch |
| PPARγ | Peroxisome proliferator-activated receptor gamma |
| PUFA | Polyunsaturated fatty acids |
| PRR | Pattern recognition receptor |
| RA | Rheumatoid arthritis |
| RAG | Recombinase-activating gene |
| RAP | Receptor-associated protein |
| RCT | Randomized controlled trials |
| ROS | Reactive oxygen species |
| SAM | Selective adhesion molecule |
| SCAD | Short-chain Acyl-CoA dehydrogenase |
| SCFA | Short chain fatty acid |
| SCID | Severe combined immunodeficiency |
| SEM | Standard error of the mean |
| SI | Small intestine |
| SIRPα | Signal regulatory protein alpha |
| SLE | Systemic lupus erythromytosis |
| SLO | Secondary lymphoid organ |
| SNP | Single nucleotide polymorphism |
| SH2 | Src homology-2 |
| TCR | T-cell receptor (TCR) |
| TGFβ | Transforming growth factor beta |
| Th1 | T helper 1 |
| Th17 | T helper 17 |
| Th2 | T helper 2 |
| TLR | Toll-like receptor |
| TNBS | Trinitrobenzene sulfonic acid |
| TNFα | Tumor necrosis factor alpha |
| Tr1 | T regulatory-1 cell |
| Treg | Regulatory T cell |
| TSLP | Thymic stromal lymphopoeitin |
| TSP-1 | Thrombospondin-1 |
| UC | Ulcerative colitis |
| WT | Wild type |

# 1 General Introduction

## 1.1 Summary of rationale

Crohn's disease (CD) is a chronic, immune-mediated inflammatory disease of the gastrointestinal tract, resulting in a significant reduction in the quality of life of those it affects. Both genetic and environmental factors influence one's susceptibility to disease, with Canadians having one of the highest disease rates worldwide. CD is currently treated by a multitude of different compounds, each exhibiting a unique profile of safety and efficacy. Despite the plethora of treatments available, many patients do not adequately respond to therapy and will require hospitalization and/or surgery. Therefore, the development of new, safe and effective treatment options is of critical importance. In that regard, the aim of these studies was to further our understanding of disease pathogenesis with the goal of ultimately identifying new therapeutic targets for the treatment of CD.

Substantial advances in understanding the pathogenesis of CD have recently been made and highlight an important interplay between the commensal flora, the epithelial barrier and the innate and adaptive immune systems in initiating and perpetuating intestinal inflammation. The two main projects outlined in this thesis (Chapters 3 and 4) focus on the immune system and how its innate and adaptive arms can be targeted to dampen this inflammatory process. To this end, the trinitrobenzene sulfonic acid (TNBS)-induced model of intestinal inflammation was employed. This is a murine model of CD, sharing many key features with

CD in humans, such as severe segmental transmural inflammation associated with granuloma formation, diarrhea, rectal prolapse, weight loss, infiltration of neutrophils, monocytes and macrophages, and the induction of a mixed Th1/Th17 response (Neurath et al., 1995, Zhang et al., 2006). This model was selected since it shares many immunological and clinical features with human CD and is especially useful for studying the early events in disease initiation.

We first sought to characterize which dendritic cell (DC) subset was implicated in the generation of intestinal inflammation and to identify novel interactions that promoted its function/migration. We also aimed to assess the immunosuppressive properties and therapeutic efficacy of the diet component L-carnitine, given the fact that mutations in its transporters, *OCTN1* and *OCTN2* are associated with CD.

## 1.2 Overview of research objectives

**OBJECTIVE #1:** To evaluate the contribution of CD47/SIRPα interactions in the development of intestinal inflammation.

This objective was attained by:

- Assessing the frequency of SIRPα$^+$ DCs in the lamina propria (LP) and mesenteric lymph nodes (mLN) of wild type (WT) mice before and after the induction of TNBS colitis.
- Assessing the frequency of SIRPα$^+$ DCs in the LP and mLNs of CD47 knockout (KO) mice before and after the induction of TNBS colitis in comparison to WT mice.
- Examining the impact of reduced SIRPα$^+$ DCs in CD47 KO mice on the clinical severity of disease, the expression of cytokine mRNA in the colonic tissues, and theTh1/Th17 polarized CD4$^+$ T cell responses in mLNs.
- Determining the Th1 versus Th17-inducing capacity of SIRPα$^+$ and SIRPα$^-$ DCs *in vitro.*
- Transferring WT or CD47 KO SIRPα$^+$ DCs into CD47 KO hosts and evaluating the migration of these DCs to mLNs as well as their capacity to induce Th1/Th17 responses and intestinal inflammation.

**OBJECTIVE #2:** To evaluate the therapeutic efficacy of a CD47-fc fusion protein in the treatment of TNBS colitis.

This objective was attained by:

- Administering a CD47-fc fusion protein and evaluating its efficacy in dampening ongoing inflammation and preventing disease recurrence.
- Assessing its mechanism of action on SIRPα$^+$ DC migration to mLNs and subsequent Th1/Th17 responses.

**OBJECTIVE #3:** To determine the immunosuppressive efficacy of L-carnitine on cells of the innate and adaptive immune systems.

This objective was attained by:

- Ascertaining which antigen presenting cells (APC) displayed reduced activation and cytokine production upon *in vitro* culture with L-carnitine.
- Assessing the effect of L-carnitine on $CD4^+$ T cell activation, proliferation and cytokine production.
- Determining the outcome of secondary carnitine deficiency on $CD4^+$ T cell activation and cytokine production.

**OBJECTIVE #4:** To evaluate the therapeutic efficacy of L-carnitine in the treatment of TNBS colitis.

This objective was attained by:

- Evaluating the efficacy of L-carnitine therapy at reducing the clinical and histological severity intestinal inflammation.
- Assessing the anti-inflammatory effect of *in vivo* L-carnitine treatment on the colonic expression and serum levels of pro-inflammatory cytokines.
- Determining the immunosuppressive effect of *in vivo* L-carnitine treatment on T cell responses in the mLNs.

# 2 Review of Literature

## 2.1 Crohn's disease

Crohn's disease (CD) is a chronic, relapsing and remitting inflammatory condition of the gastrointestinal tract that belongs to the family of inflammatory bowel diseases (IBD). IBD encompasses two main diseases, CD and ulcerative colitis (UC). In Canada alone, nearly 200 000 individuals have been diagnosed with IBD, with approximately 10 000 new cases diagnosed each year. The incidence of IBD in Canada is said to be among the highest in the world. Since CD is typically diagnosed before 30 years of age, this disease has a serious impact on economically active individuals. CD patients often experience symptoms such as diarrhea, abdominal pain, weight loss, and fatigue, leading to absenteeism at work, emotional stress, and a generally impaired quality of life. In addition to intestinal inflammation, CD patients occasionally present with extraintestinal features such as inflammation of the joints or skin. While still considered a single disease, CD can present in different forms; inflammation may develop at various locations within the entire digestive tract (from mouth to anus), may involve stenotic or penetrating (fistulising) complications, and may vary in the degree of active inflammation. While the precise etiology remains obscure, a complex series of interactions between susceptibility genes, the environment, and the immune system are thought to play an important role in the generation and perpetuation of

inappropriate responses to luminal flora and chronic intestinal inflammation.

CD can be distinguished from the other member of the IBD family, UC, in that the inflammation associated with CD is transmural, while the inflammatory changes in UC typically involve only the superficial mucosal and submucosal layers of the intestinal wall. Additionally, in CD, the inflammation is often discontinuous, patchy and segmental, and results in the formation of granulomas (aggregation of macrophages). It can affect the patient anywhere in the gastrointestinal tract from the mouth to the anus, but most commonly involves the ileum and the colon. In contrast, the inflammatory changes in UC typically extend proximally from the rectum up to varying degrees in the colon, but do not affect any other portion of the digestive tract (Figure 2.1). Moreover, the extraintestinal manifestations characteristic of CD are not observed in UC patients.

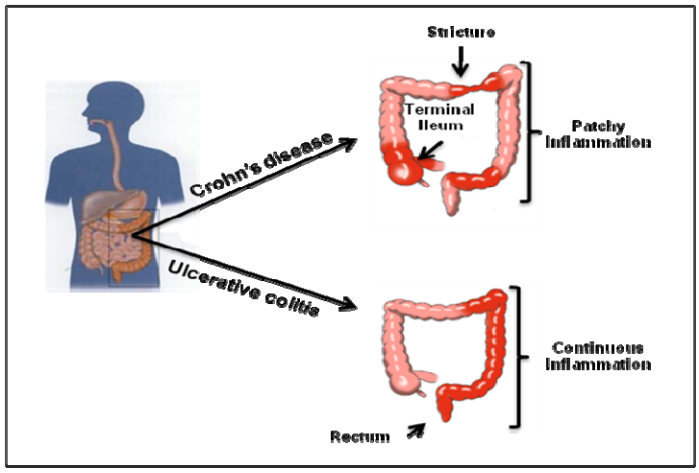


Figure 2.1: Differential localization of inflammation in CD and UC
Figure modified from (http://www.humanillnesses.com/original/Her-Kid/Inflammatory-Bowel-Disease.html)

### 2.1.1) Epidemiology and risk factors

Monozygotic twins display an approximately 50-60% disease concordance, while lower rates are observed in dizygotic twins (~10%); therefore, both environmental and genetic factors must play important roles in the development of CD (Halfvarson et al., 2003, Orholm et al., 2000). However, the lack of a consistent pattern of inheritance, along with the failure of linkage studies to identify strong linkage to any single genomic region, suggest that the cumulative effect of many genes, with each contributing a small effect on disease risk, is responsible for increased susceptibility to disease. The first of these susceptibility genes to be identified was nucleotide-binding oligomerization domain 2 (*NOD2*), also known as caspase recruitment domain protein 15 (*CARD15*). Three mutations in this gene have been shown to increase the risk of developing CD in a gene dosage model, with CD risk increasing with the number of mutations carried (Cuthbert et al., 2002, Economou et al., 2004). However, these mutations are also present in 12% of the general Western population, and are therefore not specific to CD patients and cannot be used for screening or diagnostic purposes. Several additional genes have also been identified, with the *IL-23R* and *ATG16L1* mutations having been independently replicated (Prescott et al., 2007, Rioux et al., 2007, Tremelling et al., 2007). Other genes of interest include *PTPN2, NKX2-3, ZNF365, IL12B,* and *SLC22A5* (*OCTN2*). To date, greater than 30 genes have been associated with CD and are listed in the table below.

Table 2.1: Gene Associations in CD

| Gene symbol | Gene name |
|---|---|
| ATG16L1 | Autophagy-related 16-like 1 |
| CCNY | Cyclin Y |
| CCR6* | Chemokine (C-C motif) receptor 6 |
| CDKAL1 | Cyclin-dependent kinase 5 regulatory subunit associated |
| HERC2 | Hect domain and RLD2 |
| ICOSLG | Inducible T-cell co-stimulator ligand |
| IL12B | Interleukin 12B |
| IL18RAP | Interleukin 18 receptor accessory protein |
| IL23R | Interleukin 23 receptor |
| IRGM* | Immunitry-related GTPase family, M |
| ITLN* | Intelectin 1 |
| JAK2* | Janus kinase 2 |
| KIF21B | Kinesin family member 21B |
| LRRK2, MUC19* | Leucine-rich repeat kinase 2, mucin 19 |
| MHC region | Major histocompatibility complex |
| MST1* | Macrophage stimulating factor 1 |
| NKX2-3 | NK2 transcription-factor-related, locus 3 |
| NOD2 | Nucleotide-binding oligomerization-domain-containing 2 |
| ORMDL3* | ORM1-like 3 |
| PSMG1* | Proteasome assembly chaperone 1 |
| PTGER4 | Prostaglandin E receptor 4 |
| PTPN2 | Protein tyrosine phosphatase, non-receptor 2 |
| PTPN22* | Protein tyrosine phosphatase, non-receptor 22 |
| PUS10 | Pseudouridylate synthase 10 |
| SCL22A5* | Solute carrier family 22 member 5 |
| STAT3* | Signal transducer and activator of transcription 3 |
| TNFRSF6B* | Tumor necrosis factor receptor superfamily, member 6b |
| TNFSF15 | Tumor necrosis factor (ligand) superfamily, member 15 |
| ZNF365 | Zinc finger protein 365 |

* Many genes in the region; the most probable gene in the region is listed (Adapted from Budarf et al. *Trends in Genetics*; reference (Budarf et al., 2009))

In terms of environmental risk factors, certain infectious agents are thought to be involved in the origin of IBD and include Mycobacterium avium paratuberculosis (MAP), adherent-invasive Escherichia coli (AIEC) or yeasts (Pineton de Chambrun et al., 2008). Cigarette smoking has been

shown to increase both the risk of developing CD and the severity of CD after diagnosis (Lakatos et al., 2007). While other environmental factors may also play a role in CD susceptibility and include appendectomy, the use of oral contraception, diet and domestic hygiene, the evidence for each of these factors for increased risk of CD is much weaker than for smoking and are often contradictory between studies (Danese et al., 2004).

### 2.1.2) CD Pathogenesis: Initiating events

Several elements of the mucosal immune network have been implicated in the pathogenesis of CD and include the epithelial barrier, luminal antigens, and cells of the innate and adaptive immune system.

#### 2.1.2.1 Abnormal epithelial barrier and production of antimicrobial peptides

Patients with CD often display increased intestinal permeability. However, whether this is a primary pathogenic feature or is secondary to ongoing inflammatory processes remains unclear. Intestinal permeability is controlled by at least three known factors. First, the integrity of the intestinal epithelial cell (IEC) layer and basement membrane is critical to separate the luminal contents from the host's central immune system. The integrity of this physical barrier is dependent upon the maintenance of tight junction proteins. Toll-like receptor (TLR)2 signalling actively promotes

barrier function in IECs by claudin 2 upregulation through the phosphoinositide-3 kinase and MyD88 pathways (Cario et al., 2007). Other tight junction proteins such as claudins 5 and 8 also contribute to maintaining the epithelial barrier and exhibit altered expression levels and distributions in CD. The increased intestinal permeability in first degree relatives of CD patients (Hollander et al., 1986), combined with genetic mutations in CD patients that alter barrier function (Buhner et al., 2006), suggest that a primary defect in the maintenance of this barrier may participate in CD pathogenesis.

Second, the mucus layer plays an important role in gut homeostasis and mucosal permeability, as demonstrated by the spontaneous development of colitis in Muc-2-deficient mice (Van der Sluis et al., 2006). Recently, IL-22, a member of the IL-10 family of cytokines, was reported to enhance STAT3-dependent expression of mucous-associated molecules and play an important role in mucosal healing after injury (Sugimoto et al., 2008, Pickert et al., 2009). Finally, the function of the autonomic nervous system is known to affect epithelial barrier permeability, since the ablation of enteric glial cells lead to the development of fulminant jejuno-ileitis (Bush et al., 1998).

In addition to its role in inducing mucous production, IL-22 was recently shown to induce the production of antimicrobial molecules such as β-defensin-2, β-defensin-3, S100A7, calgranulins S100A8 and S100A9, RegIIIβ, RegIIIγ and lipocalin-2 (Aujla et al., 2008, Liang et al., 2006, Wolk

et al., 2004) and can protect the gut in a model of extracellular bacterial infection caused by *Citrobacter rodentium* (Zheng et al., 2008). This is particularly interesting, since reduced mucosal antimicrobial activity has previously been associated with CD (Nuding et al., 2007). In particular, an association between α-defensin production and NOD2 function has been described; reductions in the production of the ileal α-defensins HD5 and HD6 were significantly more pronounced in CD patients with NOD2 mutations (Wehkamp et al., 2004, Wehkamp et al., 2005).

#### 2.1.2.2 Mucosal immune alteration

Despite the presence of the epithelial barrier, the intestinal immune system is in constant interaction with the microflora, and must be capable of discerning between pathogenic versus commensal bacteria to avoid unnecessary inflammation. The microflora has been shown to be required for the induction of several mouse models of CD, since germ-free mice are protected from the induction of intestinal inflammation (Podolsky, 1997). These types of studies have also been used to highlight the role of innate immune factors in the induction of mucosal inflammation. Evidence to support this role comes from studies of STAT3-deficient mice, which display defects in IL-10 signalling and develop IL-12p40-dependent spontaneous enterocolitis (Kobayashi et al., 2003). IL-10-deficient mice also exhibit spontaneous intestinal inflammation, which is not present in double IL-10/MyD88-deficient mice, implicating TLR-dependent bacterial

sensing in disease pathogenesis (Rakoff-Nahoum et al., 2006). Although these observations were made in murine models of disease, they strongly implicate interactions between the intestinal flora and the innate immune system in the development of intestinal inflammation.

These observations have also been correlated with several genetic studies performed in human IBD patients. The most prevalent and widely-recognized genetic mutation associated with CD is found in the *NOD2* gene, a member of the NOD-like receptor (NLR) family. A mutant form of NOD2 is found in approximately 25% of Caucasian CD patients, with three mutations (Arg702Trp, Gly908Arg, and a frameshift deletion mutation at Leu1007) accounting for roughly 80% of all CD-associated *NOD2* mutations (Hugot et al., 2001, Ogura et al., 2001). NOD2 is an intracellular pattern recognition receptor (PRR) composed of a NOD domain responsible for its activation following ligand recognition, a leucine-rich repeat (LRR) region, which is the microbial component recognition unit, and two CARD domains (Strober et al., 2006). NOD2 is expressed in antigen-presenting cells (APC), Paneth cells of the small intestine, and IECs and its ligation by muramyl dipeptide (MDP) leads to the subsequent activation of RICK/RIP2 (Watanabe et al., 2004). These events then lead to the activation of the key nuclear factor kappa B (NFκB) and mitogen-activated protein kinase (MAPK) pathways (Kobayashi et al., 2002). While the activation of NFκB by innate immune cells serves to initiate inflammation, as mentioned above, its activation in Paneth cells and IECs

is thought to be required for the maintenance of effective barrier function, by upregulating the expression of tight junction proteins and α-defensins (Wehkamp et al., 2004). Therefore, this dual role for NFκB activation poses significant challenges in the development and use of inhibitors of NFκB signalling pathways in intestinal inflammation, as this pathway demonstrates protective as well as pathogenic effects. Nevertheless, the intrarectal administration of NFκB decoy oligodeoxynucleotides resulted in $CD4^+$ T cell apoptosis, suggesting that such treatment is highly focused and may in fact display therapeutic efficacy in human CD (Fichtner-Feigl et al., 2005).

Recently, a candidate gene study successfully implicated another PRR in CD susceptibility (Villani et al., 2009). The *NLRP3* gene encodes the protein NALP3, and like NOD2, NALP3 is a cytosolic protein implicated in intracellular bacterial sensing (Martinon et al., 2009). Upon bacterial recognition, NALP3 can activate the inflammasome, a molecular platform that regulates the activation of caspase-1 and the processing of IL-1β (Agostini et al., 2004). The association of both *NOD2* and *NLRP3* with CD susceptibility supports the notion that the initiating event of CD may in fact be a primary innate immunodeficiency (Marks et al., 2009). According to this new dogma, such an immunodeficiency may be associated with a failure to produce adequate inflammatory mediators, resulting in insufficient recruitment of immune effector cells and the inadequate clearance of bacteria and/or other antigens (Rahman et al.,

2008). It is thus hypothesized that such antigenic persistence would lead to increased intestinal permeability, adaptive immune activation in response to gut luminal contents, and chronic intestinal inflammation characterized by progressive tissue damage and the clinical expression of CD (Villani, 2009).

#### 2.1.2.3 Alterations in the composition of the microflora

The human intestinal tract is the home to an estimated 400 different species of bacteria, collectively weighing about 1 kg. However, these bacteria live harmoniously with their host, and some species have even been shown to confer health benefits by helping to digest dietary carbohydrates, maintaining a normal intestinal pH, enhancing the metabolism of drugs, hormones and carcinogens and by preventing the colonization of pathogenic bacteria (Frank and Pace, 2001). Yet, alterations in the composition of this microflora have been linked to the pathogenesis of CD. In support of this, normal colon biopsies are usually relatively free of bacteria once washed of fecal material, while colon biopsies from CD patients contain high bacterial concentrations (Swidsinski et al., 2002). Moreover, colonic inflammation is rapidly resolved in CD patients in whom the fecal stream was diverted by the creation of a proximal ileostomy (Harper et al., 1985), and reintroduction of the bowel luminal contents provoked a relapse of disease activity (Rutgeerts et al., 1991).

Although bacterial load is increased in the digestive tract of CD patients and intestinal inflammation is found in the segments with the highest bacterial concentrations, microbial diversity is diminished (Manichanh et al., 2006). Notably, when comparing the intestinal microflora of CD patients and controls, a decreased ratio of protective commensal bacterial species compared to aggressive species is observed. This is reflected in the increased numbers of *Enterobacteriaceae*, including *E. coli*, and a decrease in *Firmicutes* phyla members such as the *Clostridium XIVa* and IV groups of the *Lachnospiraceae* family (Frank et al., 2007). The importance of the flora in the initiation of intestinal inflammation is more directly supported by studies in a variety of murine strains in which “spontaneous” chronic colitis seems to be dependent upon the presence of a luminal flora. In some cases, reconstitution of germ-free mice (lacking intestinal microbial flora) with a single strain of bacteria, such as the reconstitution of IL-10-deficient mice with *Bacteroides vulgatus*, can result in the development of colitis (Sydora et al., 2007).

In addition to altered proportions of normal commensal bacteria, several potentially pathogenic species of bacteria have also been found in increased numbers in the mucosal tissues and feces of CD patients. *Mycobacterium avium paratuberculosis* (MAP) is an intracellular pathogen known to cause Johne’s disease in cattle, a disease sharing some clinical and histological features with CD. MAP has been isolated from

contaminated milk products and tap water and was cultured from CD-affected tissue in 1984 (Chiodini et al., 1984). Subsequently, the DNA insertion sequence of MAP was detected in higher numbers in CD patients compared to UC patients and healthy controls (Sanderson et al., 1992). However, significant variation was reported with regard to the prevalence of MAP DNA among CD patients and controls, and it remains possible that MAP might opportunistically infect the ulcerated tissues of CD patients but does not contribute to CD etiology. However, recent evidence demonstrating that the *N*-glycolylated form of MDP, which is found in mycobacteria and Actinomycetes, has a greater NOD2-stimulating activity than the more commonly observed *N*-acetylated form, supports a causative role for mycobacteria such as MAP in the pathogenesis of CD (Coulombe et al., 2009). Another potential pathogen, adherent-invasive *E. Coli* (AIEC), has also been found in increased numbers in ileal tissues of CD patients (Darfeuille-Michaud et al., 2004). AIEC persist within IECs and macrophages, where they induce the secretion of tumor necrosis factor alpha (TNFα) and interleukin (IL-)8 and express virulence factors (Glasser et al., 2001, Baumgart et al., 2007). While these data are promising, there is currently a lack of evidence to definitively support a role for any one particular pathogen in the pathogenesis of CD.

#### 2.1.2.4 Aberrant dendritic cell function

Dendritic cells (DC) are immune cells that phagocytose and process antigenic material and present it on their surface in the context of MHC II molecules to cells of the adaptive immune system, thus functioning as APCs. DCs are present in small numbers in mucosal tissues, mainly the skin (where there is a specialized dendritic cell type called Langerhans cells) and the inner lining of the nose, lungs, stomach and intestines (Cerovic et al., 2009). They are also be found in an immature state in the blood. Once activated in the periphery, they migrate to the lymphoid tissues where they interact with T and B lymphocytes to initiate and tailor the type of the adaptive immune response which is generated. Upon maturation, DCs develop branched projections called “dendrites”, which give the cell its name.

DCs are located at both the inductive (Peyer’s patches (PP) in the small intestine, isolated lymphoid follicles (ILF) and mesenteric lymph nodes (mLN)) and the effector (lamina propria (LP) and epithelium) sites and establish a bridge between innate and adaptive immune responses. Antigen acquisition by intestinal DCs can take place by means of ‘M’ cells overlying PPs, by specialized villous ‘M’ cells, in isolated lymphoid follicles (ILF), by transepithelial dendrites which can directly sample luminal contents, by breaches in the intestinal epithelial layer, or through the absorption of dying IECs (Mowat, 2003). Although macrophages are the most abundant APC in the gut mucosa, DCs are unique in their ability to

prime naïve T cells and modulate the type of adaptive immune response generated (Th1, Th2, Th17, or Treg).

Intestinal DCs appear to play a role in CD pathogenesis since $CD11c^+$ DCs isolated from the inflamed tissues of CD patients expressed higher levels of TLR2, TLR4 and CD40 compared to non-IBD controls (Hart et al., 2005). Furthermore, an increase in the number of $MDC8^+$ monocytes, which produce TNFα and are believed to be DC precursors, was observed in the ileal tissues of CD patients and was reversed by steroid therapy (de Baey et al., 2001, de Baey et al., 2003). Also, the $CD83^-CD80^+DCSIGN^+$ DC subset produces the proinflammatory cytokines IL-12 and IL-18 and was shown to be more prevalent in CD patients (te Velde et al., 2003). Furthermore, the numbers of activated ($CD86^+CD40^+$) DCs were increased in the peripheral blood and in the LP of CD patients (Vuckovic et al., 2001). Lastly, DCs generated *ex vivo* from peripheral blood monocytes of CD patients demonstrated enhanced abilities to stimulate immune responses (Ikeda et al., 2001). However, the interpretation of this translational data is difficult, since whether these changes reflect the cause or the consequence of intestinal inflammation remains to be elucidated. Another key question that remains unanswered is the nature, characterization and cytokine profile of the DCs in the intestine that instruct and enforce effector functions in human Th1 and Th17 cells, and perpetuate intestinal inflammation. The discovery of the specific DC subset endowed with these functional properties has the

potential to contribute to the design of novel strategies for the treatment of CD.

#### 2.1.2.5 Excessive adaptive immune response

Over the last decade, much attention has been focused on the adaptive immune response, since it was believed that CD arose from an overly exuberant adaptive immune reaction. This dogma was predominantly based on the observation that established lesions were heavily infiltrated with activated lymphocytes characterized by a Th1 phenotype (Shanahan, 2002). Moreover, successful treatment of disease is often obtained by the use of general immunosuppressants or anti-TNFα antagonists such as Infliximab. However, the initiating events involved in engendering such an excessive adaptive response are unknown, and although autoreactive T cells and antibodies have been reported, their pathogenic relevance is not proven (Snook, 1990). It is therefore likely that the events presented as evidence for a T cell based etiology are in fact secondary to a different underlying pathogenic process.

### 2.1.3) CD Pathogenesis: Perpetuation of Inflammation

The initiating events in the pathogenesis of CD, as described above, are under intense investigation as they seem to be a prerequisite for the subsequent development of chronic disease. However, once inflammation has been initiated, the proximate drivers of tissue damage

are indeed the cells of the adaptive immune system. Although once considered to be a pure Th1-mediated disease (driven by TNFα, IL-12 and IFNγ), the importance of regulatory T cells (Treg) to control intestinal immune responses and the recent discovery of a new T helper cell lineage, Th17 cells, has added another level of complexity to this paradigm.

#### 2.1.3.1 Th1 cells in CD

T helper 1 (Th1) cells are a subgroup of $CD4^+$ lymphocytes that are essential in mediating cellular immune responses to intracellular pathogens such as viruses and certain bacteria and are typically characterized by their production of IFNγ and TNFα. These cytokines play a role in inducing the activation and growth of cytotoxic T cells, in maximizing the bactericidal activity of phagocytes such as macrophages and in determining B cell antibody class switching (Zenewicz et al., 2009). Their development is triggered by microbial antigens that stimulate the production of IFNγ and IL-12, which signal through T-bet, STAT1 and STAT4 (Rengarajan et al., 2000). Other cytokines, such as IL-15, IL-18 and IL-21act to stabilize the polarized Th1 phenotype (Murphy, 2005).

The evidence to support a role for Th1 cells in CD perpetuation was based on a number of observations, including the increased production of IL-12 in the macrophages of CD patients (Parronchi et al., 1997). Moreover, T cells isolated from inflamed CD lesions contained high levels

of activated STAT4 and T-bet (Neurath et al., 2002) and expressed increased levels of the IL-12Rβ2 chain and IFNγ (Parrello et al., 2000, Fuss et al., 1996). These findings were further underscored by the efficacy of anti-IFNγ antibody therapy in a subcohort of CD patients (Hommes et al., 2006).

It is believed that Th1 cells exert their pathogenic effect in CD by enhancing macrophage activation and their production of cytokines (IL-6, IL-1β and TNFα), chemokines (IL-8), prostaglandins, reactive oxygen species (ROS) and nitric oxide (NO). These elements act in synergy to alter the integrity of the intestinal barrier, upregulate the expression of adhesion molecules and pro-coagulant factors, induce the proliferation of fibroblasts, initiate cytotoxic, apoptotic, and acute-phase responses, and the inhibition of apoptosis (Baumann and Gauldie, 1994, Begue et al., 2006).

TNFα appears to be particularly pathogenic in this context, since it alone can induce many of these pathogenic changes and has been shown to be expressed in the colonic tissues and macrophages of CD patients (Stucchi et al., 2006). Additionally, serum levels of TNFα are correlated with clinical and laboratory indices of intestinal disease activity (Reimund et al., 1996). Finally, the successful application of anti-TNFα therapy in treating CD underscores the central importance of this pleiotropic cytokine.

### 2.1.3.2 Th17 cells and CD

Recently, a new lineage of IL-17-producing $CD4^+$ helper T cells, referred to as Th17, has been identified. Many of the aspects of CD that had earlier been attributed to the IL-12-IFNγ axis and Th1 cells have more recently been ascribed to the action of Th17 cells. This is mainly due to the fact that antibodies targeting IL-12 were generated against its p40 subunit, which is also part of the IL-23 heterodimer. Therefore, functions which had previously been attributed to IL-12 may actually be ascribed to IL-23 and should be re-evaluated.

The cytokine milieu required for Th17 cell development is similar, but not identical, in mice and humans. In mice, naïve T cells exposed to TGFβ and IL-6 differentiate into Th17 cells, and are dependent upon IL-23 for their maintenance and survival (Figure 2.2). In humans, IL-23 directly drives Th17 differentiation in a pathway that is dependent upon TGFβ, IL-6 and IL-1β (Stockinger and Veldhoen, 2007). RORγT has been identified as the master transcription factor guiding Th17 differentiation, which also synergizes with other transcription factors such as STAT3, RORα, interferon regulatory factor (IRF)4 and runt-related transcription factor (RUNX)1 (Brand, 2009).

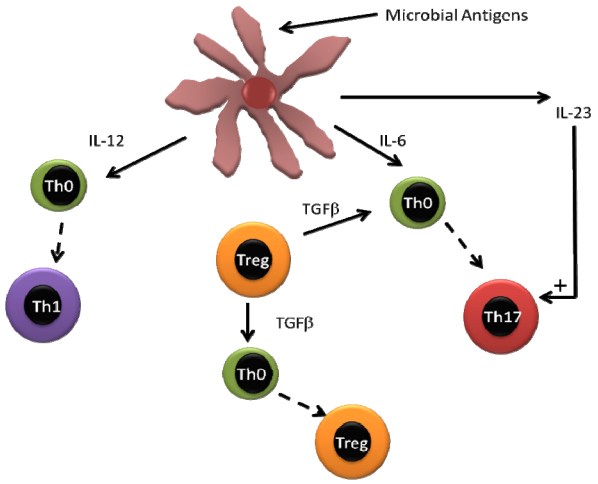


Figure 2.2: Schematic representation of mouse Th1, Treg and Th17differentiation.
Adapted from (Afzali et al., 2007)

Th17 cells play a key role in host defence, autoimmune diseases and other immune-mediated diseases such as CD. Th17 cells are characterized by their production of IL-17(A/F), IL-6, IL-21, IL-22, IL-26, TNFα and CCL20 (Wilson et al., 2007). The receptor for IL-17A (IL-17RA) is expressed by epithelial cells, endothelial cells and fibroblasts (Yao et al., 1995). Ligation of this receptor leads to the activation of the NFκB and MAPK pathways (Awane et al., 1999), stimulates the production of IL-6, nitric oxide and prostaglandin-$E_2$ ($PGE_2$) (Fossiez et al., 1996) and synergizes with other cytokines such as IL-1β, TNFα and IFNγ, as well as CD40 ligand, to up-regulate genes involved in the progression and amplification of inflammation (Ruddy et al., 2004, Albanesi et al., 1999, Witowski et al., 2000). IL-17 may also act on IECs to upregulate

antimicrobial peptides and tight junction proteins (Liang et al., 2006) as well as the expression of IL-8, monocyte chemoattractant protein (MCP)-1 and growth-related protein (Gro)-α to promote the chemotaxis of neutrophils and monocytes to sites of inflammation (Witowski et al., 2000). Furthermore, IL-17 induces the production of hematopoietic growth factors such as granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage stimulating factor (GM-CSF), which promote the growth and maturation of myeloid cells (Starnes et al., 2002). Taken together, IL-17A has multiple direct and indirect effects to promote the perpetuation of mucosal inflammation.

The role of Th17 cells in CD pathogenesis is supported by the results of several recent GWAS which implicated the *IL-23R* gene and signal transducers downstream of the IL-23R in CD risk. The first of such studies demonstrated that an uncommon coding variant in the *IL-23R* gene (rs11209026, c.1142G>A, p.Arg381Gln) confers strong protection against CD (Duerr et al., 2006). A subsequent meta-analysis of the first three GWAS identified three additional single nucleotide polymorphisms (SNP) within genes that play a major role in *IL-23R* signalling (*IL-12B, STAT3* and *JAK2*) (Barrett et al., 2008). Two other susceptibility genes (*CCR6* and *TNFSF15*) identified using this technique are also involved in the Th17 differentiation pathway (Barrett et al., 2008). However, due to the novelty of this new T cell subset, there are currently only a few studies investigating the biological role of Th17 cells in CD pathogenesis. Two

independent studies have reported an increased number of T cells expressing RORγt in the LP of CD patients (Dambacher et al., 2008, Pene et al., 2008). Th17 cells that also express IFNγ (termed "Th17/Th1" cells) may play a particularly pathogenic role, since these double positive cells are prominent at sites of inflammation, including in the inflamed tissues of CD patients (Annunziato et al., 2007). Although the full contribution of Th17 cells to CD pathogenesis is not yet entirely elucidated, the results of the GWAS studies, combined with the known pro-inflammatory properties of IL-17 and the presence of Th17 cells in the inflamed tissues of CD patients support a role for this T cell lineage in the perpetuation of inflammation.

#### 2.1.3.3 $T_{regs}$ and CD

$T_{regs}$ are critical to maintain self-tolerance and avoid excessive inflammation, either in the context of autoimmunity or in response to infection, and several subsets been identified to date. Naturally occurring $CD4^+CD45RB^{low}CD25^+FoxP3^+$ $T_{regs}$ originate in the thymus and the majority of their T-cell receptor (TCR) repertoire is self-reactive. Under certain unique conditions, $T_{regs}$ may also be generated outside the thymus, in which case their TCR repertoire includes reactivity to non-self antigens. These include inducible $T_{reg}$ (iTreg) cells, T regulatory-1 (Tr-1) cells, and Th3 cells. Due to the scarcity of specific markers for each of these subsets, their individual contributions to the maintenance of oral tolerance

and intestinal homeostasis are largely unknown. The mechanism by which $T_{regs}$ suppress effector T cell responses is still under intense investigation, but is thought to be dependent upon the production of IL-10, TGFβ and/or IL-35, as well as the sequestration of IL-2, and may result in effector T cell apoptosis or an impairment in the maturation of DCs through the cell surface expression of molecules such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and lymphocyte activation gene 3 (LAG-3) (Izcue et al., 2009, Vignali et al., 2008).

The importance of $T_{regs}$ in the maintenance of intestinal homeostasis was first documented by pioneering experiments performed by Powrie *et al* more than 15 years ago. In these experiments, the transfer of naive $CD4^+$ T cells into immunodeficient mice in the absence of $T_{regs}$ resulted in intestinal inflammation that was dependent upon the presence of intestinal bacteria (Powrie et al., 1993, Strauch et al., 2005, Aranda et al., 1997). The adoptive transfer of $T_{regs}$ not only prevented disease induction, but also halted the progression of established disease and reversed pathology in an IL-10 and TGFβ-dependent manner (Uhlig et al., 2006). Interestingly, $CD4^+CD45RO^+CD25^+FoxP3^+$ $T_{regs}$ are not found in reduced numbers in CD patients, and are actually increased in number in the LP and mLNs, compared to healthy controls (Maul et al., 2005, Makita et al., 2004). These findings support the hypothesis that $T_{regs}$ traffic to sites of inflammation in an attempt to restore immune homeostasis. Notably, *in vitro* functional analyses of $T_{regs}$ isolated from the peripheral blood or LP of

CD patients demonstrated that these cells maintain normal cell-contact dependent and cytokine dependent suppressive capacity (Holmen et al., 2006).

#### 2.1.3.4 Imbalance of pro- and anti-inflammatory cytokines in CD

As portrayed in Figure 2.3 below, pro- and anti-inflammatory cells must remain in an appropriate balance in order to maintain normal intestinal function. However, when the balance shifts toward pro-inflammatory activities, including the stimulation of the innate and adaptive immune systems, IBD can result. In the case of CD, DCs and macrophages tend to generate a mixed Th1/Th17 response, whereas in UC, this response is primarily dominated by Th2 and NK cells, with the production of IL-13 and IL-5. In both cases, $T_{regs}$ are incapable of restoring homeostasis.

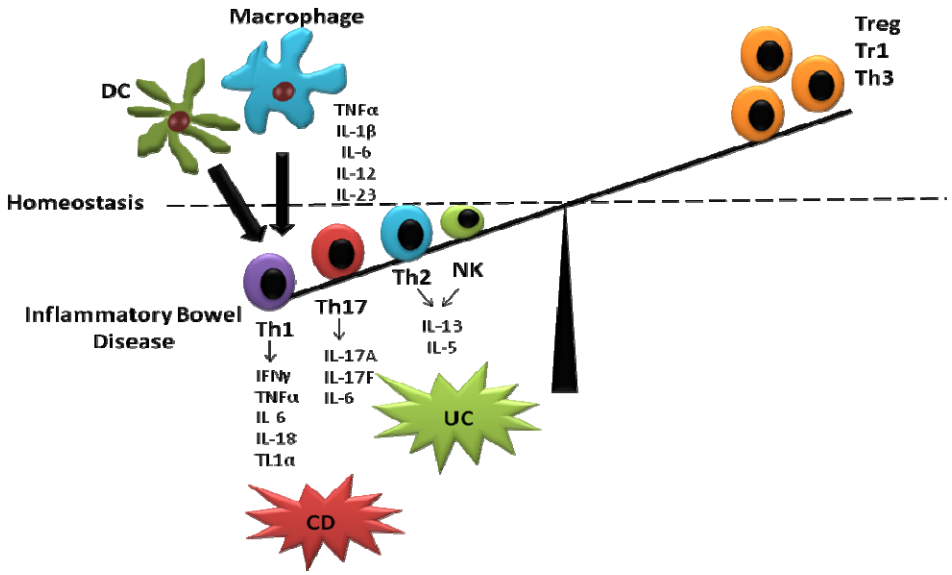


Figure 2.3: IBD is characterized by an imbalance between effector and regulatory T cells.

### 2.1.4) Treatment strategies

The management of IBD involves complex decisions regarding the timing of treatment with special diets, oral supplements, immunomodulators, or biological therapy, and has become a specialty in its own right. The consensus among such specialists is to strive to achieve and maintain disease remission with the least toxic therapy, to improve the quality of life and nutritional status of the patient, and to avoid complications and the need for surgery. In children, there are additional important aims, such as promoting physical and psychological development.

#### 2.1.4.1 Diet and oral supplements

Malnutrition as a result of anorexia, malabsorption, altered metabolism, fluid and electrolyte loss, or side effects of medications, is common in CD patients (Schneeweiss et al., 1999). Nutritional deficiencies occur very early in the disease process and are often clinically apparent at the time of diagnosis (Geerling et al., 2000). In CD patients, vitamin $B_{12}$ deficiency is the most common. Additionally, CD patients often present with significantly reduced body weights and body mass indexes. Therefore, dietary modification, enteral nutrition, and parenteral nutrition are often necessary components of the therapeutic intervention strategy. Enteral nutrition refers to feeding through a tube placed in the nose, stomach or small intestine, while parenteral nutrition is administered intravenously. In addition to providing essential nutrients to maintain a normal nutritional status and reverse nutrient deficiencies, enteral and parenteral nutrition have been demonstrated to reduce disease activity in CD patients under these regimens (Dupont et al., 2008). Several theories have been proposed to explain this phenomenon, and include effects on the gut flora, immune function, intestinal permeability and wound healing, as well as psychological and cognitive effects (Smith, 2008). Enteral nutrition plays an especially important role in paediatric CD, where it has been shown to have an efficacy equal to steroids, without the deleterious effects on growth and development (Heuschkel et al., 2000).

While epidemiologic and basic research have suggested that dietary factors play a role in the onset and course of CD, current

recommendations cannot be made for most patients, other than to eat a healthy, diverse diet. Nonetheless, there is data to support the beneficial effect of supplementing the regular diet with one of two different types of dietary fats.

n3 polyunsaturated fatty acids (PUFAs) are found mainly in foods such as flaxseed, canola, walnuts, and oils from deep sea fish, and are now available as a prescription medicine (Omacor®). Apart from their beneficial effects on heart health, n3-PUFAs have been shown to suppress the production of IFNγ and prostaglandin-$E_2$ by the peripheral mononuclear cells of CD patients (Trebble et al., 2004), and reduce the severity of a mouse model of colitis (Whiting et al., 2005). Unfortunately, supplementation with fish oil containing high levels of n3-PUFAs has not shown any beneficial effects on reducing CD severity (Turner et al., 2009).

Short chain fatty acids (SCFAs), such as butyrate, are derivatives of the fermentation process of dietary fibers by the commensal flora and are the primary fuel for the colonic mucosa. Both UC and CD patients have been shown to display impaired β-oxidation of butyrate (Roediger, 1980, Yamamoto et al., 2005), which may be attributed to a deficient supply of SCFAs in the lumen. This concept is supported by the efficacy of treating left-sided UC with SCFA enemas (Senagore et al., 1992), along with their known anti-inflammatory effects on the production of TNFα and other proinflammatory factors in CD (Segain et al., 2000).

### 2.1.4.2 Ecological treatments: Pre-, Pro, and Synbiotics

The microbial flora plays an important role in CD, with some intestinal microorganisms exerting proinflammatory, deleterious effects, while others are thought to be protective. The protective nature of some strains of bacteria prompted the idea of testing ecological treatments including probiotics, prebiotics and synbiotics in patients with CD. Probiotics are defined as “living nonpathogenic microorganisms which, when ingested, exert a positive influence on host health or physiology” (Schrezenmeir and de Vrese, 2001). Prebiotics are described as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limited number of bacteria in the colon, thus improving host health” (Lim et al., 2005). Lastly, synbiotics are a combination of pre- and probiotics, which have also been considered in the treatment of CD (Chermesh et al., 2007).

There is now a considerable amount of evidence to support a role for probiotics, especially *E. coli* Nissle 1917 in the prevention of recurrence of UC (Seksik et al., 2008). However, there is insufficient evidence to make any conclusions pertaining to the efficacy of probiotics, prebiotics, or synbiotics for the induction of remission or prevention of recurrence of CD (Butterworth et al., 2008). This is mainly the result of methodological limitations in many of the published studies, including small sample sizes leading to a lack of statistical power and variation

between studies with respect to the strains and doses of ecological treatments administered. Since these treatments are safe and show promising results in animal models of intestinal inflammation, the rationale for continuing to develop and evaluate ecological treatments in CD is strong.

#### 2.1.4.3 Steroids and immunomodulatory drugs

Corticosteroids (such as prednisolone, prednisone and 6-methylprednisolone) are immunosuppressive agents, with widespread effects on most cells of the immune system. They are highly effective at inducing remission in CD (70-90% in mild-to-moderate disease), but are associated with a long list of short term (high blood sugars, fluid retention, weight gain, insomnia, depression or other psychiatric symptoms, etc.) and long-term (Cushing's syndrome, osteoporosis, glaucoma, etc.) complications. Importantly, low-dose systemic corticosteroids are not effective in maintaining remission, and therefore long term treatment is inappropriate. New formulations of corticosteroids, such as budesonide, have low systemic bioavailability, and thus fewer side effects, and have shown to be effective in prolonging the time to relapse in patients with mild or moderately active CD (Travis et al., 2006).

5-aminosalicylates (5-ASA) (such as mesalazine, olsalazine and balsalazide) are a group of anti-inflammatory compounds that have been used since the 1970s in the treatment of CD. However, the results of

several randomized controlled trials (RCT) have been inconsistent. In fact, a recent meta-analysis concluded that 5-ASA agents are ineffective at inducing remission (Hanauer and Stromberg, 2004) and are not more effective than placebo for the maintenance of medically induced remission either over 12 months or 24 months (Akobeng and Gardener, 2005). Therefore, the routine use of 5-ASA agents for CD therapy is no longer supported by the most current evidence.

The purine analogues azathioprine and its metabolite, 6-mercaptopurine (6-MP), have been shown to be effective for the treatment (both induction and maintenance of remission) of CD. However, the use of these compounds in inducing remission is limited by their slow onset of action, which can take up to 17 weeks. Therefore, the patients who derive the most benefit from these drugs are those with chronically active CD or frequent exacerbations, those who are dependent on or refractory to steroids, and those who experience severe side effects from steroid use (Sandborn et al., 2000).

Methotrexate is another popular immunosuppressive agent, and there is evidence to suggest that weekly intramuscular injections are effective for inducing remission in patients with refractory CD (Alfadhli et al., 2005). The mechanism of action of the above immunosuppressive agents is thought to involve the inhibition of DNA synthesis, thus reducing cell proliferation, particularly in leukocytes (Derijks et al., 2006).

Alternatively, they may act directly on T cells to inhibit their activation and suppress their expression of adhesion molecules (Johnston et al., 2005).

#### 2.1.4.4 Antibiotics

Infectious agents such as bacteria, viruses, and mycobacteria, have long been suspected to be involved in the initiation and perpetuation of intestinal inflammation in CD, although no convincing or reproducible evidence has yet been presented to support this concept. Nevertheless, a number of antibiotics, including metronidazole and ciprofloxacin, have been evaluated for the induction of remission in CD. Apart from their valuable use in treating septic complications and perianal disease, antibiotics have not shown any efficacy in inducing remission in CD (Sutherland et al., 1991, Steinhart et al., 2002).

#### 2.1.4.5 Biological agents

TNFα is a proinflammatory cytokine that has a significant role in the pathogenesis of CD. Several monoclonal antibodies to TNFα have recently been developed and represent an important advance in the treatment of CD. Infliximab is a chimeric monoclonal antibody, which binds to and inhibits the biological activity of TNFα. Additionally, it is believed to induce T cell apoptosis and may have a role in the generation of Tregs (Tilg et al., 2007). It is administered by intravenous infusion and induces remission in a significant proportion of patients with moderately to severely

active CD (Targan et al., 1997). However, this treatment occasionally results in the formation of anti-infliximab antibodies, resulting in infusion reactions and a reduced duration of response to treatment. The successful implementation of infliximab and its remarkable efficacy in inducing remission in CD, paved the way for the development of new biological therapies.

Adalimumab is a fully human IgG1 monoclonal antibody that binds with high specificity and affinity to human TNFα and can be administered subcutaneously. It has been shown to be effective at inducing remission, even in patients who had previously been unresponsive to infliximab (Hanauer et al., 2006). The latest anti-TNFα agent is certolizumab pegol, a pegylated humanized Fab' fragment with a high binding affinity to TNFα that can also be administered subcutaneously. While both infliximab and adalimumab have been shown to induce T cell apoptosis, certolizumab pegol does not. Treatment with certolizumab pegol was associated with a modest improvement in response rate, but with no significant improvement in remission rates, compared to placebo (Sandborn et al., 2007).

During the course of inflammation, leukocytes adhere to the vascular endothelium and migrate into the inflamed tissues, where they participate in maintaining or amplifying cytokine production and inflammatory processes. Natalizumab is a humanized monoclonal antibody against alpha 4 (α4) integrin, a protein expressed on the surface of leukocytes, allowing their adhesion and recruitment to inflamed tissues.

Three RCTs have recently shown that natalizumab can induce remission in patients with moderate to severe CD (Gonvers et al., 2007). However, the deaths of 2 patients receiving this therapy were attributed to the development of progressive multifocal leukoencephalopathy (PML) associated with the JC virus, a consequence of drug therapy (Clifford, 2008). Therefore, the future of natalizumab in CD therapy is currently unknown.

The impact that this new era of biological therapy has had on the drug development pipeline is remarkable. The variations in the magnitude and duration of response to anti-TNFα therapy, combined with the recent advances in the understanding of the pathophysiology of CD, have opened the door for the development of a plethora of new biological agents. In fact, the vast majority of new drugs currently under clinical trials are biologicals, and include those designed to target the epithelium and innate immune system (Somatropin), pro-inflammatory cytokines (ABT-874, Ustekinumab), and both T cell activation (Abatacept) and recruitment (MLN-0002, CCX282-B). While non-biological, conventional therapies have long been associated with serious and numerous side effects, biological therapies are generally well tolerated and most adverse events are mild, influenza-like symptoms. While some side-effects have been lethal, like the development of PML in patients treated with natalizumab, they are also extremely rare.

## 2.2 Animal Models of CD

### 2.2.1 General overview of various models

Animal models of intestinal inflammation are valuable tools to investigate the pathophysiological mechanisms and potential therapeutic avenues of human CD. While none of these models entirely encompasses all of the complex features of CD, they are irreplaceable for the assessment of individual pathways of inflammation, the relationships between these pathways, and the early events in the development of chronic intestinal inflammation. In fact, much of what we know about the pathogenesis of CD has been achieved by the study of such models.

Animal models of CD can be classified into five main categories: 1) gene knockout (KO) models; 2) transgenic models; 3) spontaneous models; 4) adoptive transfer models; and 5) inducible models. A wide range of clinical and histological features can be observed between these models, a property which is reminiscent of human CD and underscores the heterogeneity of this disease. Therefore, before selecting a model, several considerations must be taken into account, including the mechanism of disease induction, the cell types and cytokines involved, the clinical features, the duration of the inflammation, etc. Other factors, such as the availability of specialized equipment and strains of mice may also play an important role in the selection of a particular model.

### 2.2.2 Gene knockout models

IL-2 is a regulatory cytokine with multiple functions. IL-2 and IL-2R KO mice develop, among other pathologies, chronic colitis at 4 to 9 weeks of age (Sadlack et al., 1993). This inflammation is limited to the colon and is characterized pathologically by the presence of crypt abscesses, mucin depletion, and dysplasia of the epithelial cells, leading to the ulceration and thickening of the colon wall. IL-10 is produced by T cells, B cells, macrophages, thymic cells, and keratinocytes and down-regulates the function of Th1 cells, NK cells, and macrophages. IL-10 KO mice display inflammation of the whole intestine, with the most severe lesions occurring in the duodenum, proximal jejunum, and ascending colon (Kuhn et al., 1993). In the small intestine, hyperplastic changes contribute to the thickening of the intestinal wall, and in the colon, goblet cell depletion, degeneration of the epithelium, infiltration of IgA-producing plasma cells, activated macrophages, and neutrophils, and an increase in MHC class II expression are characteristic of the local inflammation.

In macrophages and neutrophils, STAT3 is a downstream mediator of IL-10 signalling, and its specific deletion in these cells leads to spontaneous enterocolitis. These data suggest that IL-10 acts to suppress the stimulatory activity of luminal bacteria and food antigens on macrophages and neutrophils, which would otherwise be sufficient to result in intestinal inflammation (Takeda et al., 1999). While the features of intestinal inflammation differ between IL-2, IL-10, and STAT3 KO mice,

the depletion of regulatory mechanisms and/or the activation of Th1 cells are thought to be the cause of inflammation (Kuhn et al., 1993).

Intestinal trefoil factors (ITF) are peptides secreted by mucous cells of the gut after inflammatory damage and promote wound healing. ITF KO mice show severely impaired mucosal healing and decreased epithelial regeneration, and are extremely susceptible to chemically-induced models of intestinal inflammation (Hibi et al., 2002). These mice are therefore valuable in the study of wound-healing processes in the gut.

Lastly, mice deficient for the multiple drug resistance gene, MDRα, spontaneously develop severe bacteria-dependent intestinal inflammation with similar pathogenic features to CD at around 12 weeks of age (Panwala et al., 1998). MDR1α encodes P-glycoprotein 170, a transport protein involved in the removal of drugs or other xenobiotic substances from cells. The mucosal inflammation triggered in MDR1α KO most likely results from a dysfunction in intestinal epithelial integrity, and not from alterations in lymphocyte function, since the transfer of MDR1α KO bone-marrow to irradiated WT mice does not induce inflammation (Schwab et al., 2003). This is an especially interesting model, since mutations in the MDR1 gene (Ala893) have been associated with IBD (Brant et al., 2003).

### 2.2.3 Transgenic models

$TNF^{\Delta ARE}$ mutant mice display increased TNFα mRNA stability, which is correlated with enhanced protein production and high serum

concentrations (Kontoyiannis et al., 1999). These mice display a severe wasting disease and increased mortality, succumbing to disease between four and twelve weeks of age. Histological analysis of the GI tract of both $TNF^{\Delta ARE/\Delta ARE}$ and $TNF^{\Delta ARE/+}$ mice revealed severe inflammatory changes consistent with a CD-like phenotype; inflammation was localized primarily to the terminal ileum and occasionally to the proximal colon and was characterized by mucosal and submucosal infiltration monocytes, plasma cells, and neutrophils (Kontoyiannis et al., 1999). Recently, the development of intestinal inflammation in this model was found to be dependent upon on Th1-type cytokines, such as IL-12 and IFN-γ, and requires the function of $CD8^+$ T lymphocytes (Kontoyiannis et al., 2002).

STAT4 is a regulatory transcription factor specifically associated with IL-12/IL-23 receptor signalling (Kaplan et al., 1996). $CD4^+$ T cells from STAT4 transgenic mice produce predominantly TNFα and IFNγ, but not IL-4, after stimulation *in vitro* and *in vivo* and induce severe transmural colitis that resembles CD (Wirtz et al., 1999). Moreover, $CD4^+$ T cells isolated from either the spleen or the LP of STAT4 transgenic mice induce colitis when transferred to SCID mice (Wirtz et al., 1999).

### 2.2.4 Spontaneous models

Spontaneous models of intestinal inflammation result from the selective inbreeding of mice that are particularly susceptible to developing intestinal disease, such as ulcers and colitis. Such selective inbreeding of

C3H/Hej mice with colitis has resulted in the development of the C3H/HejBir substrain of mice, which develop lesions in the ileo-cecal area and ascending colon in the third to fourth week of life (Brandwein et al., 1997). This disease is characterized by ulcers, crypt abscesses, regeneration of the epithelium and increased expression of IFNγ and IL-2 by LP lymphocytes, consistent with a Th1 type immune response. However, the particular genes involved in this phenotype have yet to be elucidated.

SAMP1/YitFC mice display established inflammation of the ileum as early as 10 weeks of age, although increased IFNγ expression precedes the onset of ileitis and is detectable at 4 weeks of age (Matsumoto et al., 1998). Interestingly, peroxisome proliferator-activated receptor gamma (PPARγ) has been identified as a susceptibility gene in both the SAMP1/YitFC mouse and in human CD (Sugawara et al., 2005). In addition to sharing many other key clinical and histological features with CD, approximately 5% of SAMP1/YitFC mice develop perianal fistulizing disease, and are currently the only animal model of CD available in which this phenomenon can be studied (Rivera-Nieves et al., 2003).

### 2.2.5 Adoptive transfer models

The adoptive transfer of $CD4^+CD45RB^{hi}$ or $CD4^+CD25^-$ T cells purified from spleens into syngenic immunodeficient mice such as SCID or RAG1/2 KO recipients results in a wasting syndrome characterized by

transmural intestinal inflammation, primarily in the colon (Leach et al., 1996). The co-transfer of $CD4^+CD45RB^{lo}$ cells prevents the induction of intestinal inflammation, and is attributable to the $CD25^+FoxP3^+$ $T_{reg}$ cell subset (Read et al., 2000). Several studies have identified an important anti-inflammatory role for IL-10 and TGFβ in this model, and the suppressive effect of $CD25^+FoxP3^+$ $T_{regs}$ on the development of colitis could be inhibited by the administration of anti-IL-10 antibodies (Asseman et al., 1999).

### 2.2.6 Inducible models

Trinitrobenzene sulfonic acid (TNBS) colitis was first induced in 1989 by the intrarectal administration of TNBS dissolved in ethanol in rats (Morris et al., 1989). The resulting segmental inflammation was characterized by marked thickening of the bowel wall, diarrhea, weight loss, rectal prolapse, and the transmural infiltration of neutrophils, macrophages and lymphocytes. Granulomas, a common feature of CD, were present in 57% of the mice exposed to TNBS. In 1995, the technique was adapted for use in mice (Neurath et al., 1995) and since then, TNBS colitis has become one of the most widely used forms of experimental colitis. Yet, there is no standard practice for the induction of TNBS colitis in mice, neither with regard to the frequency and dose of TNBS administered, nor with regard to the appropriate strain of mice (te Velde et al., 2006). This lack of consistency throughout the scientific community has lead to the publication of many, often contradictory findings. Therefore, for the sake of simplicity and accuracy, only the data published in BALB/c mice with similar experimental protocols to those used in the following chapters will be reviewed here.

TNBS is always mixed with ethanol (35-50%) prior to intrarectal administration. The ethanol is critical since it is responsible for disrupting the intestinal epithelial barrier, leading to increased exposure of the mucosal immune system to the enteric microflora. The great majority (~90%) of published experimental protocols employ a single TNBS

administration, and sacrifice mice shortly thereafter, at the peak of cytokine expression, on day 3 (te Velde et al., 2006). This form of colonic inflammation is referred to as “acute” inflammation and is characterized by the influx of neutrophils and an increase in myeloperoxidase (MPO) activity. To study *in vivo* T cell function, a second induction of TNBS colitis should be performed 6 to 8 days after the first exposure. This allows sufficient time for T cell priming in mLNs. Upon secondary TNBS exposure and “reactivation” of colitis, a strong T cell response is initiated which can be measured in the mLNs after 48 hours or in the colon lamina propria 3-5 days later.

Dextran sodium sulphate (DSS) colitis is induced by the addition of DSS polymers to the drinking water of mice and results in a very reproducible acute colitis characterized by bloody diarrhea, ulcerations and granulocyte infiltration (Okayasu et al., 1990). DSS colitis is particularly useful for studying the innate immune mechanisms of colitis since acute inflammation can be induced in T- and B cell-deficient SCID or Rag1 KO mice, implying that the adaptive immune response is not involved in disease pathogenesis (Dieleman et al., 1994). Additionally, since DSS is thought to be directly toxic to gut epithelial cells of the basal crypts and results in damage to the intestinal epithelial barrier, this model has also been shown to be suitable to study epithelial repair mechanisms (Williams et al., 2001).

## 2.3 Intestinal DCs

### 2.3.1 Role of DCs in intestinal homeostasis

DCs are the only type of APC capable of priming naïve T cells and are interspersed within peripheral tissues where they sample antigens from their environment and migrate to draining lymph nodes (dLNs) (Niess and Reinecker, 2006). Alternatively, they may also recirculate in the blood and lymphoid tissues (Iwasaki, 2007). DCs are capable of either promoting tolerance to innocuous antigens or mounting effective adaptive immune responses to eliminate dangerous pathogens. Not only are DCs responsible for linking innate and adaptive immunity, they also determine the type and quality of subsequent adaptive responses depending on the situation. Exactly how mucosal DCs are able to maintain such flexibility is presently an active area of research. The current concept involves the "education" of tissue-resident DCs to promote tolerance under steady state conditions while maintaining a certain degree of plasticity, thereby allowing them to integrate danger signals from the environment and to promote inflammation in the context of tissue injury. Another possibility is that tissue-resident, "educated" DCs are hard-wired to promote tolerance, and *de novo* DC recruitment under inflammatory conditions is required for the generation of adaptive immune responses (Iwasaki, 2007). Additionally, certain mucosal DC subsets, as defined by their expression of various surface markers, may also differentially induce tolerance versus immunity (Rescigno et al., 2008). Importantly, mucosal DCs have unique

functions when compared to DCs from non-mucosal sites such as the spleen, which include the imprinting of mucosal homing receptors on lymphocytes, the induction of $T_{regs}$, and immunoglobulin A (IgA) class switching (Niess, 2008).

### 2.3.2 Role of intestinal DCs in murine colitis

The colon contains the highest number of different species as well as the greatest variety of commensal bacteria of any organ in the human body. Therefore, strict regulatory mechanisms must be in place to avoid the development of colonic inflammation when the immune system encounters such bacteria. In fact, colonic DCs play such an important role in this process that, when dysregulated, DCs are thought to contribute to the pathogenesis of CD. Therefore, understanding the mechanisms used by colonic DCs to promote the maintenance of homeostasis and tolerance in the intestine may be beneficial to the development of new therapies for CD.

During the course of inflammation in mice, monocytes are massively recruited to inflamed sites and develop into inflammatory DCs, where they are believed to exert important effects by guiding the development of the immune response (Varol et al., 2007, Villadangos, 2007). This important role for DCs has been underlined by several important findings using murine models of colitis.

First, an increased number of DCs was found in the mLNs in the T-cell transfer model of colitis (Malmstrom et al., 2001) and DC aggregates were identified when pathogenic T cells were transferred to RAG1 KO mice (Krajina et al., 2003). Second, the depletion of DCs, following the administration of diphtheria toxin (DT) in CD11c-DT receptor (DTR) transgenic mice, significantly ameliorated the severity of DSS-induced colitis (Berndt et al., 2007). Third, the formation of granulomas is induced in the gut mucosa of mice by IL-23 produced by myeloid $CD11c^{+}/F4/80^{+}$ DC-like cells (Mizoguchi et al., 2007). Fourth, the elimination of $CD11b^{+}$ cells ameliorated spontaneous colitis in IL-$10^{-/-}$ mice (Coombes and Powrie, 2008) and the protective effect may be attributed to the depletion of either immunogenic DCs or macrophages. Finally, under steady state conditions, $CD11c^{Hi}CD11b^{+}CD103^{-}CX_3CR1^{+}$ DCs (Denning et al., 2007), as well as $CD11c^{low}CD11b^{+}CD103^{-}F4/80^{+}CX_3CR1^{+}CD70^{+}$ DCs (Atarashi et al., 2008) isolated from the lamina propria (LP), favoured Th17 development. Whether these two DCs encompass the same DC subpopulation, and more importantly, whether they promote Th17 development during intestinal inflammation remains to be clarified.

### 2.3.3 Intestinal DC antigen acquisition and migration

The priming of naive T cells and thus the generation of adaptive immune responses occurs mainly in tissue-draining LNs. DCs present in the mLNs are thought to be derived from both the intestinal tissues and

the blood. At steady-state, tissue-derived DCs play a key role in monitoring the intestinal lumen by constitutively phagocytosing and transporting apoptotic cell bodies derived from IECs to the mLNs in a CCR7-dependent manner (Huang et al., 2000, Jang et al., 2006). This process is required for the induction of tolerance, since the depletion of mLNs or the deletion of CCR7 results in defective oral tolerance (Macpherson and Smith, 2006). On the other hand, blood-born DCs can also enter the mLNs via interactions with mucosal addressin cellular adhesion molecule (MAdCAM-1) and peripheral node addressin (PNAd) expressed by high endothelial venules (HEVs) (Soderberg et al., 2004). However, the role of such blood-born DCs in the induction of tolerance versus immune responsiveness remains to be elucidated.

### 2.3.4 Colonic DC subsets

DCs present in the small intestine are distinct in their phenotype, localization with the GALT, and cell number compared to DCs present in the colon. Since the murine model of CD (TNBS colitis) utilized throughout this work results in inflammation which is restricted to the colon, this section will focus solely on colonic DC subsets. In the colon, DCs are localized in the LP and in isolated lymphoid follicles (ILF) and were found to migrate from the LP to the mesenteric LNs (mLNs) and caudal LNs (cLNs) (Becker et al., 2003). Further examination of these migratory DCs demonstrated that they were more mature in phenotype than LP-resident

DCs and better stimulators of mixed leukocyte reactions (Milling et al., 2009).

Similar DC subsets exist in the colon and in the mLNs and are generally distinguished from macrophages by their intensity of CD11c expression. While DCs are $CD11c^{Hi}$, macrophages are $CD11c^{lo}$ and express F4/80 (Young and Steinman, 1996). Although there is some degree of overlap between these two populations and additional markers will be required to definitively distinguish between DCs and macrophages, analysis of $CD11c^{Hi}$ cells is a widely used method of identifying DCs. Among $CD11c^{Hi}$ DCs, several colon-resident subpopulations have been characterized and are summarized in Table 2.2 (Kelsall, 2008a).

Briefly, DCs isolated from the colonic LP are primarily members of the family of conventional DCs (cDCs), characterized by their lack of expression of the plasmacytoid DC (pDC) marker B220 (Niess et al., 2005). In the GALT, cDCs express CD11c and can be further divided into the $CD11b^{+}CD4^{-}CD8^{-}$, $CD11b^{+}CD4^{+}CD8^{-}$ and $CD11b^{-}CD4^{-}CD8^{+}$ subsets. Among these subsets, only the $CD11b^{+}CD4^{-}CD8^{-}$ cDCs are found within the colon (Niess, 2008). $CD11b^{+}CD4^{-}CD8^{-}$ DCs can be further subdivided based on their expression of CD103 or the fractalkine receptor $CX_3CR1$. While little is known about these particular subsets in the colon, small intestinal $CD103^{+}$ and $CX_3CR1^{+}$ DC subsets are derived from different monocyte precursors and exert different functions (Annacker et al., 2005, Johansson-Lindbom et al., 2005). In the ileum, $CX_3CR1^{+}$ DCs directly

access the intestinal lumen by extending transepithelial dendrites in a $CX_3CR1$-dependent manner to survey the intestinal environment (Niess et al., 2005), while $CD103^+$ DCs appear to induce the differentiation of $CD4^+CD25^+Foxp3^+$ $T_{regs}$ and the expression of gut-homing receptors on $CD4^+$ and $CD8^+$ T cells in the mLNs (Coombes et al., 2007, Johansson-Lindbom et al., 2005). Importantly, $CX_3CR1^+$ DCs have recently been shown to drive Th17 polarization *in vitro* (Denning et al., 2007, Atarashi et al., 2008). Furthermore, $CD103^-$ DCs found in the mLNs appear to derive from blood precursors, express TLR2, TLR4, and TBX21 to a greater extent than $CD103^+$ DCs, and secrete high levels of TNFα and IL-6 in response to stimulation (Coombes et al., 2007). Many of these classifications have been made at steady state, thus little is known about the precise contribution of each of these DC subsets during the development of intestinal inflammation. Moreover, since many of these investigations have been performed using DCs isolated from the small intestine, the role of such DCs in the colon is suspected to be similar but is largely unknown.

Table 2.2: Summary of GALT DC populations and their known localization and properties.

| DC | | Location with the GALT | | | | | Properties | References |
|---|---|---|---|---|---|---|---|---|
| **Lineage** | **Phenotype** | **mLN** | **siLP** | **cLP** | **PP** | **iLF** | | |
| cDC | CD11c+CD11b+CD4-CD8- | +++ | +++ | +++ | +++ | +++ | Express either CD103 or CX3CR1 | (Niess et al., 2005, Annacker et al., 2005) |
| | | | | | | | Mediate intestinal antigen uptake | (Rescigno et al., 2001) |
| | | | | | | | Participate in retinoic-acid dependent Treg conversion | (Sun et al., 2007, Benson et al., 2007) |
| | | | | | | | Induce the expression of the gut-homing receptors CCR9 and α4β7 | (Johansson-Lindbom et al., 2005) |
| | CD11c+CD11b+CD4+CD8- | +++ | - | - | ++ | + | Prime CD4+ T cell responses | (Hochrein et al., 2001, Dudziak et al., 2007) |
| | CD11c+CD11b-CD4-CD8+ | ++ | - | - | ++ | ++ | Prime CD8+ T cell responses | (Hochrein et al., 2001, Dudziak et al., 2007) |
| pDC | CD11c+B220+PDCA1+ | + | +/- | +/- | + | + | Produce type 1 interferons | (Bjorck, 2001) |

cDC: conventional DC; pDC: plasmacytoid DC; mLN: mesenteric lymph node; PP: Peyer's patch; cLP: colonic lamina propria; siLP: small intestinal lamina propria; iLF: isolated lymphoid follicle

Adapted from (Niess, 2008)

## 2.4 CD47

### 2.4.1 Overview of CD47

CD47 is a ubiquitously expressed glycoprotein in the immunoglobulin superfamily composed of a single IgV-like extracellular domain, a pentaspanning transmembrane region and a short intracytoplasmic tail (Lindberg et al., 1993). Although CD47 was originally identified through its association with the integrin $\alpha_v\beta_3$ (hence its alternative name, “integrin-associated protein”) (Lindberg et al., 1993), and many of its biological activities are likely to involve the activation of integrins, the mechanism by which this occurs is not fully understood. In addition to integrins, CD47 has been shown to bind to both signal regulatory protein alpha (SIRPα) and thrombospondin-1 (TSP-1) to modulate several processes, including platelet activation, neurite outgrowth, DC, monocyte and neutrophil migration, DC activation, monocyte maturation, and others.

### 2.4.2 Overview of SIRPα

SIRPα, also known as SHPS-1 or CD172a, is a transmembrane protein that contains three immunoglobulin (Ig)-like domains in its extracellular region and tyrosine phosphorylation sites on its cytoplasmic tail (Fujioka et al., 1996). SIRPγ and SIRPβ are closely related to SIRPα but bind very weakly or not at all to CD47 (Barclay and Brown, 2006). SIRPα is primarily expressed on the cell surface of innate myeloid cells,

including neutrophils, monocytes, macrophages, and dendritic cells, but is also expressed on neuronal, endothelial and epithelial cells (Vernon-Wilson et al., 2000, Seiffert et al., 1999, Lahoud et al., 2006, Yoshida et al., 2002). In response to extracellular stimuli leading to tyrosine prophorylation of SIRPα, it acts as a docking protein for the src homology-2 (SH2)-domain-containing protein tyrosine phosphatases, SHP-1 and SHP-2, to induce downstream signalling events (Fujioka et al., 1996). Nevertheless, mice generated with a truncated version of SIRPα which lacked most of its intracytoplasmic tail did not display any developmental defects or growth retardation, indicating that SIRPα signalling is not essential during embryonic or postnatal development (Inagaki et al., 2000).

### 2.4.3 Overview of TSP-1

TSP-1 is a homotrimeric, multifunctional 450 kD extracellular matrix glycoprotein produced by many cells including platelets, T cells, monocytes, macrophages and DCs (Chen et al., 2000). TSP-1 levels are rapidly and transiently increased in injured and damaged tissues in response to inflammatory signals, even in the absence of pathogens (Raugi et al., 1987). It is a multifunctional protein that has been implicated in platelet aggregation, angiogenesis, and tumorigenesis (Sarfati et al., 2008) and in both the positive and negative modulation of endothelial cell adhesion, motility, and growth. This dual role of TSP-1 is not surprising

given the fact that TSP-1 interacts with at least 11 other cell adhesion receptors, including CD36, αv integrins, β1 integrins and syndecan in addition to CD47.

### 2.4.4 CD47/ SIRPα interactions in the immune system

The extracellular Ig-domain of CD47 binds to the N-terminal IgV domain of SIRPα (Figure 2.4). This interaction is rather unique in that SIRPα binds to CD47 through the loops at the end of the domain in a manner similar to that of an antigen receptor rather than via the face of the domain as is usual for cell-cell interactions (Hatherley et al., 2007). Ligation likely induces bi-directional signalling, but the mechanism by which SIRPα induces signalling through CD47 remains unclear. (Han et al., 2000).

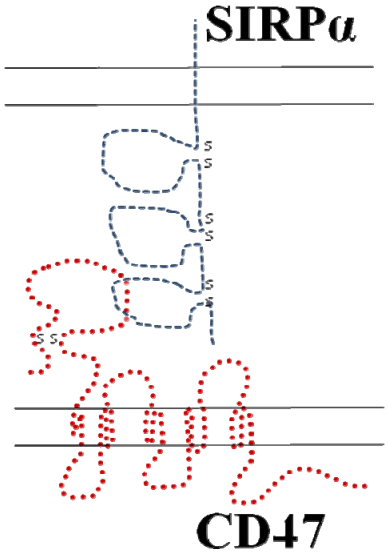

Figure 2.4: SIRPα/CD47 binding

#### 2.4.4.1 CD47/ SIRPα interactions and cell migration

CD47/SIRPα interactions promote myeloid cell transendothelial migration, leading to the tissue infiltration of neutrophils and monocytes, and the egress of DCs to the dLNs (Cooper et al., 1995, Van et al., 2006, de Vries et al., 2002). In support of a role for CD47 in neutrophil transmigration, 100% of CD47 KO mice injected intraperitoneally with *E.*

*coli* succumbed to the infection and died just 2 days after the challenge while all WT mice survived (Lindberg et al., 1996). CD47 has also been implicated in monocyte transmigration through endothelium and epithelium, including in the brain and in the lungs (Rosseau et al., 2000, de Vries et al., 2002).

The evidence supporting a role for CD47 in the migration of DCs is derived from studies performed in CD47 KO mice, where a reduced accumulation of skin-derived DCs was observed in the secondary lymphoid organs (SLO), resulting in reduced $CD4^+$ T cell proliferation in CD47 KO mice (Hagnerud et al., 2006, Van et al., 2006). Also, fluorescein isothiocyanate (FITC) skin painting experiments examining cell recruitment to the dLNs under inflammatory conditions demonstrated an impairment in the total number of DCs, as well as those carrying FITC from the periphery in CD47 KO mice. Moreover, ligation of SIRPα by CD47-Fc as well as CD47 ligation by a SIRPα-Fc fusion protein before antigen sensitization altered the egress of Langerhans cells (LC) to the LNs, confirming the importance of CD47/SIRPα interactions in LC migration (Fukunaga et al., 2004, Fukunaga et al., 2006, Yu et al., 2006). In order for DCs to gain access to the spleen from the bloodstream, they must also cross an endothelial barrier and DC expression of CD47 is critical for this event since competitive migration assays demonstrated an impaired recovery of CD47 KO compared to WT bone marrow derived DCs (BMDCs) from the spleens of adoptively transferred CD47 KO mice

(Van et al., 2006). Perhaps counter-intuitively, the expression of CD47 on the DCs (and not on the endothelium or in the microenvironment) is critical for effective DC migration to LNs since the transfer of WT DCs into CD47 KO mice lead to effective DC migration and T cell responses (Van et al., 2006).

#### 2.4.4.2 CD47/SIRPα interactions in DC/T cell crosstalk

With the exception of cell migration discussed above, CD47/SIRPα interactions on immune cells generally lead to bidirectional inhibitory effects on the APCs and T cells. For instance, CD47 engagement on T cells by L-SIRPα transfectants inhibits IL-12 responsiveness of activated $CD4^+$ and $CD8^+$ T cells (Latour et al., 2001, Rebres et al., 2001). In the opposite direction, SIRPα ligation by a CD47-Fc fusion protein or agonist SIRPα monoclonal antibodies (mAb) induces a functional block in DC maturation in response to danger signals (Latour et al., 2001). This molecular brake allows the generation of “semi-mature DCs” that either complete their maturation program or acquire the features of immature-like cells (Braun et al., 2006). Moreover, disruption of CD47/SIRPα interactions with soluble anti-CD47 mAbs or anti-SIRPα mAbs inhibits T cell proliferation and IFNγ production in response to allogeneic DCs (Demeure et al., 2000, Latour et al., 2001, Seiffert et al., 2001).

In contrast, ligation of CD47 by SIRPα on the surface of T cells was also shown to promote cell proliferation and contribute to the activation of

antigen-specific cytotoxic T lymphocytes by DCs *in vitro* (Seiffert et al., 2001). Furthermore, SIRPα ligation on macrophages stimulates the production of nitric oxide (Alblas et al., 2005). Therefore, SIRPα may also play a positive role in the induction of immune responses.

#### 2.4.4.3 CD47/SIRPα interactions in the regulation of phagocytosis

Engagement of natural killer (NK) cell inhibitory receptors by MHC I inhibits NK cell activation and cell killing (Bix et al., 1991). In much the same way, ligation of macrophage SIRPα by CD47 is known to play a role in self-recognition via the delivery of a negative signal to prevent phagocytosis (Oldenborg et al., 2000). CD47, a marker of all immune and non-immune live cells counteracts the “eat me” signal provided by calreticulin/CD91 interactions, thus preventing the elimination of intact cells and promoting tolerance (Gardai et al., 2005). Notably, CD47 is downregulated on apoptotic neutrophils and redistributed in patches away from the macrophage synapse in dying lymphocytes, thereby impairing its ability to bind to SIRPα and favouring apoptotic cell clearance.

Although the cellular expression of MHC I and CD47 is almost identical, red blood cells (RBC) lack MHC expression, suggesting that CD47 may be required to generate tolerogenic signals and prevent autoimmune destruction and clearance of these cells (Blazar et al., 2001). Indeed, transfused CD47 KO but not WT RBCs were rapidly eliminated from the circulation of WT recipients (Oldenborg et al., 2000, Olsson et al.,

2006). Therefore, the delivery of a negative signal via ligation of SIRPα on splenic macrophages by CD47 on RBCs is critical to avoid cell elimination.

### 2.4.6 CD47/TSP-1 interactions

TSP-1 signalling through CD47 acts as an autocrine negative regulator for DC activation since it is produced in response to TLR ligation and mediates the arrest of cytokine production and renders DCs refractory to subsequent stimulation (Doyen et al., 2003, Demeure et al., 2000). More specifically, CD47 ligation by TSP-1 in human monocytes selectively suppresses IL-12 production and prevents their differentiation into functional DCs (Armant et al., 1999, Johansson and Londei, 2004).

Ligation of CD47 on T cells by TSP-1 or various soluble CD47 mAbs was also shown to inhibit human T cell function. TSP-1/CD47 interactions at the naïve T cell surface lead to downregulation of IL-12Rβ2 expression, and thus IL-12 responsiveness and Th1 polarization (Avice et al., 2001). Interestingly, this suppressive effect on Th1 polarization does not result in Th2 immune deviation. In addition, ligation of CD47 by TSP-1 inhibits early T cell activation by suppressing IL-2 production and IL-2Rα expression on naïve T cells (Avice et al., 2001, Avice et al., 2000, Li et al., 2001, Waclavicek et al., 1997). These cells therefore become anergic, exerting regulatory functions on Th1 and Th2 cells and sharing some features with adaptive $FoxP3^{+}$ $T_{regs}$.

### 2.4.6 Phenotype of BALB/c CD47 KO mice

Despite the ubiquitous expression of CD47 among both hematopoietic and non-hematopoietic cells, CD47 KO mice (in either the C57BL/6 or BALB/c background) develop normally and display no alterations in appearance or body weight after up to 2 years of monitoring in a specific pathogen-free animal facility. The CD47 axis can reliably be studied in the mouse, since deletion of the CD47 gene in mice largely corroborates the *in vitro* data observed by pharmacologically inhibiting CD47 in human cells. In fact, the well-conserved tissue distribution of CD47 and SIRPα across species may facilitate the transition of therapeutic approaches from bench to bedside.

## 2.5 L-carnitine

### 2.5.1 L-carnitine and its role in metabolism

L-carnitine (LCAR) (γ-trimethylamino-β-hydroxybutyric acid) is a derivative of the amino acid lysine and is found in nearly all cells of the body. Its chemical structure is shown below.

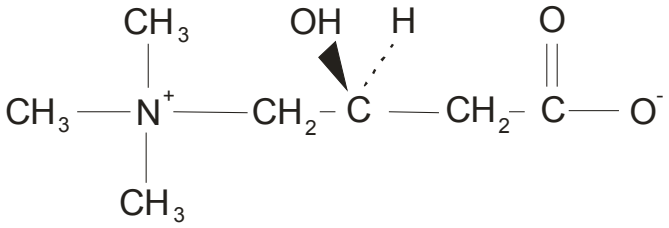

Figure 2.5: Chemical structure of LCAR

Although it was once thought to be a vitamin, and was originally called “vitamin $B_T$”, this turned out to be a misnomer when it was

discovered that it could be biosynthesized in humans, mainly in the liver and kidneys. Nevertheless, under certain conditions, the physiological demand for LCAR may exceed an individual's capacity to synthesize it, thereby classifying it as a conditionally essential nutrient. Dietary sources of LCAR include mainly meat and dairy products, with only very small amounts found in plants. LCAR is transported across the cell membrane by two main organic cation transporters (OCTN), OCTN1 and OCTN2. OCTNs are widely expressed in human tissues, including the heart, skeletal muscle, kidney, placenta, brain and intestine. While OCTN1 is a multispecific and pH-dependent organic cation transporter with lower affinity for LCAR, OCTN2, which has 75.8% sequence homology with OCTN1, functions as a $Na^+$-dependent transporter with greater affinity for LCAR (Tamai et al., 1998).

Once inside the cell, the main physiological function of LCAR is to shuttle long-chain fatty acids (LCFA) across the mitochondrial membranes, where they are processed by β-oxidation to produce biological energy in the form of adenosine triphosphate (ATP). Thus, LCAR is most concentrated in tissues that use fatty acids as their primary fuel, such as skeletal and cardiac muscle. As depicted in Figure 2.5, this transport requires three enzymes, located on the mitochondrial outer (CPTI) and inner (CPTII and Translocase) membranes. Therefore, LCAR is essentially responsible for chaperoning activated fatty acids (acyl-CoA) into the mitochondrial matrix for energy production.

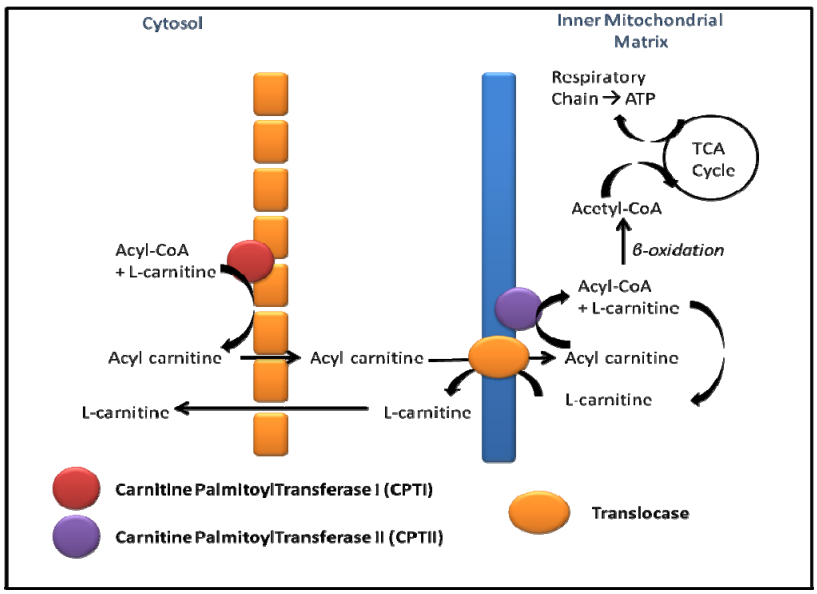


Figure 2.6: L-carnitine in mitochondrial long-chain fatty acid transportation. Adapted from http://en.wikipedia.org/wiki/L-carnitine and (Stephens et al., 2007)

### 2.5.2 The carnitine transporters and their association with CD

The IBD5 locus, on chromosome 5q31, was originally described in a Canadian population (Rioux et al., 2000) and subsequent studies identified a 250 kb risk haplotype within this locus that is significantly associated with CD (Rioux et al., 2001, Armuzzi et al., 2003, Giallourakis et al., 2003, Mirza et al., 2003). However, due to strong linkage disequilibrium in this area, there remains a degree of uncertainty as to the causal variant within this region. This is further compounded by the fact that this region contains several genes implicated in the maintenance of epithelial integrity and/or immunoregulation, including *interferon regulatory factor-1* (*IRF1*), *IL-4*, *IL-5*, *IL-13*, *PDZ and LIM domain protein 4* (*PDLIM4*),

and *prolyl 4-hydroxylase α-2 subunit precursor* (*P4HA2*), in addition to *OCTN1* and *OCTN2* (Van Limbergen et al., 2007a).

Polymorphisms in *OCTN1* (*SLC22A4*, missense substitution 1672C→T) and in the *OCTN2* promoter (*SLC22A5*, transversion -207 G→C) were originally proposed as the causal variants in patients of European decent, since these two SNPs constituted a 2-point risk haplotype for CD, demonstrating strong association independently of genotype at other SNPs in the IBD5 locus (Peltekova et al., 2004). Furthermore, these SNPs translated into reduced function and expression of OCTN1 and OCTN2, respectively, and in functional impairments in LCAR uptake (Peltekova et al., 2004, Vermeire et al., 2005). A subsequent study noted an association between pediatric-onset CD and *OCTN1/2* mutations (Cucchiara et al., 2007). Similar to *NOD2* mutations, the association of *OCTN1/2* mutations to CD susceptibility has only been observed in Western populations, with no documented association in a Chinese population (Li et al., 2008). Nevertheless, several reports in Western populations have also questioned the causative role for *OCTN1/2* mutations in the IBD5 locus (Noble et al., 2005). One such study found that several other genes, including *IRF1*, *PDLIM*, and *P4HA2* may be equally as likely to contain the IBD5 causal variant as the *OCTN* genes (Silverberg et al., 2007). Another study reported that no definitive conclusions could be drawn about *OCTN* variants as causative genes in pediatric CD (Babusukumar et al., 2006). Interestingly, one study found

that while mutations in *OCTN1/2* do not play a role in the susceptibility to CD in the Flemish population, they do alter the phenotypic expression of the disease since *OCTN1/2* variants were associated with perianal and penetrating CD (Vermeire et al., 2005). Therefore, functional data regarding the role of OCTNs and LCAR in the context of CD may shed light on their implication in the pathogenesis of disease.

### 2.5.3 Antioxidant activities of LCAR

Oxidative stress is caused by an imbalance in the production of reactive oxygen (ROS) species, such as oxygen ions, free radicals and peroxides, and a biological system's ability to quench them or repair damaged tissues. ROS have the potential to damage various cellular components, including the lipids of the various membranes of the cell and the DNA and RNA. To dampen the destructive effects of ROS, tissues are equipped with an intricate antioxidant system, which includes the production of enzymes such as superoxide dismutases, catalases, glutathione peroxidises and peroxiredoxins. Small molecule antioxidants such as Vitamin C, Vitamin E, uric acid and glutathione also play an important role in the defence against ROS.

Oxidative stress is known to play a pathogenic role in several diseases such as atherosclerosis, Parkinson's disease, heart disease, myocardial infarction, Alzheimer's disease and chronic fatigue syndrome. While an enhanced production of ROS has been demonstrated in the

inflamed tissues of CD patients, their role in disease pathogenesis is not fully understood. It is believed that T cell-derived IL-2 and IFN-γ activate tissue macrophages to release a variety of proinflammatory cytokines and mediators including TNF-α, IL-1β, IL-12, nitric oxide (NO), and ROS. While this mechanism is critical for the protection from pathogens, uncontrolled mucosal immune responses may result in tissue damage and the perpetuation of chronic intestinal inflammation in the form of CD.

Many diet components or natural compounds act as antioxidants to suppress the production of ROS and play a critical role in preventing inflammation and cancer (Frenkel, 1992, Halliwell and Gutteridge, 1984). Recent studies have demonstrated that LCAR can act as an antioxidant and protect from ROS-induced tissue damage (Wang et al., 2007, Rauchova et al., 2002). In fact, LCAR was more effective at inhibiting lipid peroxidation than both trolox and Vitamin E, two widely recognized antioxidants (Gulcin, 2006). Moreover, by promoting the mitochondrial transport of LCFAs and the generation of ATP by β-oxidation, LCAR acts to reduce the cellular oxygen concentration and the generation of ROS (Rebouche and Engel, 1980, Rebouche, 1992, Fritz and Marquis, 1965).

### 2.5.4 Immunosuppressive properties of LCAR

Aside from its antioxidant properties, LCAR has also been shown to be directly immunosuppressive by inducing the activation and nuclear translocation of the glucocorticoid receptor alpha (GRα) (Alesci et al.,

2003). In the absence of glucocorticoids, GRα is normally found in the cytoplasm, bound to receptor-associated proteins (RAP) that maintain a level of inactivity. However, upon ligand binding, the RAP dissociate from GRα, leading to its activation, translocation into the nucleaus, homodimerization and the transcription of many genes (Bamberger et al., 1996). LCAR is thought to function as an allosteric regulator of GRα, binding to an area outside the glucocorticoid-binding pocket, but ultimately resulting in conformational changes similar to those induced by glucocorticoids. The evidence to support this hypothesis comes mainly from competitive binding assays, where LCAR administration reduced the affinity of GRα for dexamethasone while, at the same time, inducing GRα-GFP translocation to the nucleus and the transcription of glucocorticoid-responsive genes (Manoli et al., 2004). In support of this hypothesis, pharmacological doses of LCAR markedly suppressed lipopolysaccaride (LPS)-induced cytokine production and improved survival rates during cachexia and septic shock (Winter et al., 1995). Moreover, TNFα levels were reduced after *ex vivo* stimulation of human neutrophils with *S. aureus* (Fattorossi et al., 1993) and after LCAR supplementation in surgical and AIDS patients (Delogu et al., 1993, De Simone et al., 1993).

### 2.5.5 LCAR: Role in the maintenance of the epithelial barrier

Mice deficient in the carnitine transporter, OCTN2, develop spontaneous atrophy of intestinal epithelial cells and colonic inflammation (Shekhawat et al., 2007). The pathology observed in these mice was mainly attributed to alterations in the intestinal and colonic structure and morphology, as a result of enhanced apoptosis of gut epithelial cells and a subsequent breakdown in barrier function. Additionally, a recent study investigating the role of carnitine transporters in butyrate metabolism in colonocytes demonstrated a protective role of the local administration of carnitine-loaded liposomes, which was accredited to enhanced butyrate metabolism (D'Argenio et al., 2006).

### 2.5.6 Carnitine deficient mice

BALB/cByJ mice are an inbred substrain of BALB/c mice and were discovered to be SCAD (Short-chain Acyl-CoA dehydrogenase) deficient by screening naturally occurring, “spontaneous” mutant mice for the excretion of urinary organic acids (Wood et al., 1989). These mice display a defect in the conversion of short chain fatty acids (SCFA) such as butyrate into acetyl-CoA, which is important for the generation of ATP by in the Krebs cycle. Butyrate therefore accumulates inside the mitochondria and is converted to butyrylcarnitine by carnitine acetyltransferase. During this conversion, carnitine stores are used up, resulting in carnitine deficiency (Wood et al., 1989). This carnitine deficiency then impairs the

uptake of LCFA, because carnitine is necessary for their mitochondrial transport. Therefore, BALB/cByJ mice display several biochemical abnormalities, including a form of secondary carnitine deficiency (Turnbull et al., 1984).

### 2.5.7 Therapeutic aspects of L-carnitine

The most frequent therapeutic application of LCAR therapy is in the treatment of primary and secondary LCAR deficiencies. However, it has also been successfully used as an adjunct therapy in treating conditions related to myocardial ischemia, such as myocardial infarctions (Lopaschuk, 2000), heart failure (Rizos, 2000) and angina pectoris (Cacciatore et al., 1991), as well as in the treatment of intermittent claudication in peripheral arterial disease (Hiatt, 2004), in HIV/AIDS (Moretti et al., 1998), and male infertility (Lenzi et al., 2004). The therapeutic benefits of LCAR in these diseases were mainly attributed to its role in energy metabolism and as an antioxidant.

# 3 THE ROLE OF CD47/SIRPα INTERACTIONS IN THE PATHOGENESIS AND TREATMENT OF EXPERIMENTAL COLITIS

A reprint of the published manuscript can be found in the appendix

**Reference:**

Genevieve Fortin[1,2], Marianne Raymond[2], Vu Quang Van[2], Manuel Rubio[2], Patrick Gauthier[2], Marika Sarfati[2*], Denis Franchimont[1*] A role for CD47 in the development of experimental colitis mediated by SIRPalpha+CD103- dendritic cells. *J Exp Med*. 2009 Aug 31;206(9):1995-201

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### 3.1 Rationale

DCs are key constituents of the immune system and play a pivotal role in the induction of antigen-specific immune responses and the maintenance of self-tolerance. DCs are especially abundant in the gastrointestinal mucosa and associated lymphoid tissues, where they act as sentinels for food and/or bacterial components and prevent the induction of immune responses to harmless antigens. As a consequence, the DC subsets capable of generating $T_{reg}$ during steady state have been extensively investigated. However, the DC subsets responsible for the induction of inflammatory responses and the interactions required for their function are largely unknown.

Enhanced DC numbers, activation status, expression of TLRs and co-stimulatory molecules and immunostimulatory function are all believed to participate in the pathogenesis of CD (te Velde et al., 2003, Hart et al., 2005, Ikeda et al., 2001). Since CD47/SIRPα interactions have previously been shown to promote DC migration (Van et al., 2006), we thus sought to determine their role in the pathogenesis of experimental colitis using the TNBS model of intestinal inflammation, which mimics many of the features of human CD. Furthermore, we aimed to assess the Th1/Th17 inducing capacity of $SIRP\alpha^{+}$ DCs with the goal of developing a therapeutic agent capable of disrupting this pathway and treating disease.

## 3.2 A role for CD47 in the development of experimental colitis mediated by SIRPα$^{+}$CD103$^{-}$ dendritic cells

### 3.2.1 Abstract:

Mesenteric lymph node (mLN) CD103 (αE integrin)$^{+}$ dendritic cells (DCs) induce regulatory T cells and gut tolerance. However, the function of intestinal CD103$^{-}$ DCs remains to be clarified. CD47 is the ligand of signal regulatory protein-alpha (SIRPα) and promotes SIRPα$^{+}$ myeloid cell migration. We first show that mucosal CD103$^{-}$ DCs selectively express SIRPα and that their frequency was augmented in the lamina propria and mLNs of mice that developed Th17-biased colitis in response to TNBS. In contrast, the percentage of SIRPα$^{+}$CD103$^{-}$ DCs and Th17 responses were decreased in CD47-deficient (CD47 KO) mice, which remained protected from colitis. We next demonstrate that transferring WT, but not CD47 KO, SIRPα$^{+}$CD103$^{-}$ DCs in CD47 KO mice elicited severe Th17-associated wasting disease. CD47 expression was required on the SIRPα$^{+}$CD103$^{-}$ DCs for efficient trafficking to mLNs *in vivo* while it was dispensable on both DCs and T cells for Th17 polarization *in vitro.* Finally, administration of a CD47-Fc molecule resulted in reduced SIRPα$^{+}$CD103$^{-}$ DC-mediated Th17 responses and the protection of WT mice from colitis. We thus propose SIRPα$^{+}$CD103$^{-}$ DCs as a pathogenic DC subset that drives Th17-biased responses and colitis, and the CD47/SIRPα axis as a potential therapeutic target for inflammatory bowel disease.

### 3.2.2 Introduction:

Dendritic cells (DCs) are located throughout mucosal surfaces and orchestrate the delicate balance between tolerance to innocuous antigens and the generation of protective immune responses upon exposure to pathogens. Different DC subsets populate the gut mucosa and mesenteric lymph nodes (mLNs) and their functions vary according to their anatomical location and conditioning by epithelial cells (Iwasaki, 2007). For instance, $CD103^+$ and $CD103^-$ DC subsets isolated from mLNs display distinct phenotypes and cytokine profiles. A substantial proportion of $CD103^+$ DCs continuously emigrate from the intestine to the mLNs in a CCR7-dependent manner (Johansson-Lindbom et al., 2005, Jang et al., 2006), where they represent from 40 to 70% of the DC population (Coombes et al., 2007, Jaensson et al., 2008). Under steady state conditions, mLN $CD103^+$ DCs are prone, in the presence of retinoic acid, to convert naive $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ regulatory T cells ($T_{regs}$) (Coombes et al., 2007, Sun et al., 2007), while $CD103^-$ DCs isolated from the mLNs and lamina propria (LP) of the small intestine express $CX_3CR1$ and drive Th17 polarization *in vitro* (Denning et al., 2007, Atarashi et al., 2008). The mLN $CD103^-$ DCs appear to be directly derived from blood-born precursors, express TLR2, TLR4, and TBX21 to a greater extent than $CD103^+$ DCs, and secrete high levels of TNFα and IL-6 in response to stimulation (Coombes and Powrie, 2008). However, little is known about

the precise contribution of each of these DC subsets during the development of intestinal inflammation.

Crohn's disease (CD) is a chronic, relapsing, T cell driven inflammatory disease of the gastrointestinal tract thought to result from inappropriate mucosal immune responses to commensal bacteria in genetically susceptible individuals (Cho, 2008). DCs have been implicated CD pathogenesis, since dysregulation in DC/epithelial cell interactions, defective migration and function of tolerogenic DCs, and/or aberrant immunogenic DC responses to pathogens, have been proposed to participate in the induction of intestinal inflammation (Kelsall, 2008b). Once initiated, the inflamed tissue is characterized by the expression of innate (IL-6, IL-12, TNFα, IL-23) and adaptive-derived (IL-6, IFN-γ and IL-17) pro-inflammatory cytokines (Sanchez-Munoz et al., 2008). APC-derived TGF-β, IL-1β, IL-6 and IL-23 drive Th17 responses, while IL-12 and IFNγ promote Th1 and suppress Th17 responses (Bettelli et al., 2007, Trinchieri, 1993).

Trinitrobenzene sulfonic acid (TNBS)-induced colitis is a murine model of intestinal inflammation sharing many key features with human CD. TNBS administration first induces acute inflammation, characterized by the infiltration of neutrophils and macrophages, followed by Ag-specific priming of T cells. Challenge with a second dose of TNBS provokes a T cell predominant reaction associated with tissue damage and mimics the

chronic inflammation seen in CD (te Velde et al., 2006). While classically considered a Th1 disease, the recently identified Th17 cell lineage appears to play an important role in the development of both TNBS colitis and CD (Sheibanie et al., 2007a, Annunziato et al., 2007).

CD47, a marker of self on immune and non-immune cells, is implicated at several levels in the generation of immune responses (van den Berg and van der Schoot, 2008). On one hand, ligation of CD47 by the extracellular matrix protein thrombospondin-1 (TSP-1), which is abundantly and rapidly expressed in tissues in response to inflammation (Reed et al., 1993), down-regulates IL-12p70 production by APCs (Armant et al., 1999), and impairs human naïve T cell differentiation to Th1 cells *in vitro* without any immune deviation to Th2 (Avice et al., 2000). Recent data have also shown that CD47 expression on T cells is a self-control negative regulator of Th1 immune responses *in vivo* (Bouguermouh et al., 2008). On the other hand, CD47 interacts with signal regulatory protein alpha (SIRPα), its counter-receptor, which is selectively expressed on myeloid, endothelial and neuronal cells (Adams et al., 1998) (Ticchioni et al., 2001). The expression of CD47 on epidermal and dermal DCs promotes their migration to draining lymph nodes, where T cell priming and immune responses are initiated (Van et al., 2006). We here show that SIRPα is selectively expressed on one of the two major mucosal DC subsets, ie. the $CD103^-$ DCs. We next demonstrate that $SIRP\alpha^+CD103^-$ DCs are the primary immunogenic DC subset involved in the development

and perpetuation of Th17-associated TNBS-induced colitis, and that their migration is controlled by CD47.

### 3.2.3 Materials and Methods

#### Animals

Six- to 8-week old wild-type (WT) BALB/c and BALB/c CD47 knockout (KO) mice were bred and maintained in our animal care facility in standard animal cages and under specific pathogen free conditions. CD47 KO mice are viable and do not exhibit any overt phenotype. All mice were handled according to institutionally recommended animal care guidelines and all experiments were approved by the Animal Studies Ethics Committee of McGill University and the Centre de Recherche du Centre Hospitalier de Montreal (CRCHUM).

#### Culture Medium, Antibodies and Reagents

*Ex vivo* and *in vitro* experiments were performed using complete RPMI-1640 medium (Wisent Inc) supplemented with 10% fetal calf serum (Wisent Inc), Penicillin (500 U/ml), Streptomycin (500 µg/ml), HEPES buffer (10 mM), and 2-mercaptoethanol (1 mM) (GIBCO). Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO) supplemented with 5% fetal calf serum, Penicillin (500 U/ml), Streptomycin (500 µg/ml), and 2-mercaptoethanol (1 mM) was used for *in vitro* $CD4^+$ T cell co-cultures with sorted DCs. *E.Coli* lipopolysaccharide (LPS) was obtained from Sigma-Aldrich and GM-CSF was purchased from PeproTech. The anti-CD3 antibody (145-2C11) used for *in vitro* polyclonal stimulation was obtained from BD Pharmingen. Allophycocyanin (APC)-labelled anti-CD4,

Fluorescein isothiocyanate (FITC)-labelled anti-IFNγ and Phycoerythrin (PE)-labelled anti-IL-17 were used for intracytoplasmic cytokine staining of $CD4^+$ T cells and were obtained from BD Pharmingen. For DC phenotype staining and DC/$CD4^+$ T cell sorting, FITC-labelled anti-CD11b, anti-MHC II, anti-CD40 and anti-CD86, PE-labelled anti-SIRPα, biotinylated anti-CD103 plus streptavidin-PerCP, APC-labelled anti-CD11c, anti-CD45.2-APC-Cy7 and anti-CD4-PeCy7 were obtained from BD Pharmingen.

### Trinitrobenzene Sulfonic Acid-induced Colitis

Two different models of chronic TNBS colitis were induced. In the first model, 3.0 mg of TNBS (Sigma-Aldrich) was dissolved in 50% ethanol and a total volume of 50 ul was injected intrarectally in isopropanol-anesthetized mice (Scheiffele and Fuss, 2002). Controls included those administered intrarectal saline or ethanol alone. Mice were sacrificed 8 days after TNBS injection to assess colonic inflammation. The second form of chronic TNBS colitis was induced by 2 injections of 2.0 mg of TNBS dissolved in 50% ethanol. The second injection was administered 7 or 8 days after the primary injection. Mice were sacrificed either 2 days after primary TNBS injection to assess early events in disease development or 4 days after the secondary injection to assess colonic inflammation.

For treatment of TNBS colitis, 100 μg of murine CD47-Fc (Van et al., 2006), control human CD47-Fc (Latour et al., 2001), or saline was

injected i.p. on days 0-3, inclusively. TNBS colitis (2.0 mg) was induced on day 0 and day 8 and mice were sacrificed on day 4 or 12. In all cases, the macroscopic score of inflammation was assessed based upon the degree of ulceration (0-10), the presence of diarrhea (0-1) and adhesions (0-2) and on the thickness of the colon wall (0-1). For histological assessment, colon samples were embedded in OCT compound (Sakura Finetek) and stained with H&E. Histological changes were graded semi-quantitatively based on a set of previously established criteria (Ameho et al., 1997). The grading scale ranged from 0-16, and was calculated as the sum of scores for: expansion of submucosa (0-4), expansion of lamina propria (0-4), loss of globlet cells (0-4) and neutrophil infiltration (0-4). All macroscopic and microscopic scoring was performed in a blinded fashion.

### Cytokine Quantification by ELISA

Whole blood was withdrawn from mice immediately post-mortem and sera were frozen at -20˚C until use. Serum IL-6 was quantified by the Quantikine ELISA kit (R&D systems). IL-6 and IL-23p19 production were assessed in BMDC culture supernatants after overnight culture using mouse the IL-6 DuoSet (R & D systems) and IL-23p19 (eBioscience) ELISA kits.

### Real-time PCR

Colons were immediately immersed in RNAlater (Qiagen) upon dissection and frozen at -20˚C until use. mRNA was extracted following

the TRIzol protocol, and was reverse-transcribed using the cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) (1 PCR cycle, 95°C, 10 minutes; 40 PCR cycles, 60°C, 1 minute, 95°C, 15 seconds). cDNA was amplified in a 10 µL final reaction mix containing TaqMan Universal PCR Master Mix (Applied Biosystems) and corresponding TaqMan® Gene Expression Assays (Mm00446190_m1 (IL-6), Hs99999901_s1 (Eukaryotic 18s rRNA), Mm00439619_m1 (IL-17a), Mm00434165_m1 (IL-12p35), Mm00449022_m1 (TSP-1), Mm00518984_m1 (IL-23p19), Mm00801778_m1 (IFNγ), Mm00441724_m1 (TGFβ), Applied Biosystems). Signals were analyzed by the ABI Prism Sequence Detection System software version 2.2 (Applied Biosystems). The comparative Ct method for relative quantification was used, whereby all Ct were first normalized to the expression of 18s rRNA. Cytokine expression is represented as a fold-change relative to control mice.

### *Ex Vivo* Cultures of mLNs

For *ex vivo* cultures, mLNs were harvested 3-4 days after secondary TNBS injection and $1X10^6$ cells/ml were cultured in 48-well plates. Cells were stimulated with anti-CD3 antibody (2 µg/ml) for 4 days in the presence of IL-23 (20 ng/ml), followed by re-stimulation with phorbol 12-myristate 13-acetate (PMA, 5ng/ml) and ionomycin (0.5 µg/ml) in the presence of Brefeldin A (1 µg/ml) for the last 6 hours of culture. In some

experiments, cells were immediately stimulated after harvest with PMA/Ionomycin in the presence of Brefeldin. Cells were collected, stained for CD4, fixed and permeabilized with the Fix/Perm kit (BD Pharmingen) and stained for intracellular IL-17 and IFNγ for analysis by flow cytometry. Data were acquired on a Becton Dickinson FACS Calibur (BD Pharmingen) machine and analyzed with Cell Quest software.

### DC phenotype in LP and mLNs

LP cells were isolated as previously described (Drakes et al., 2004). Briefly, colons were extracted, thoroughly cleaned and digested in a solution of Collagenase IV (Roche) and DNase I (Roche). mLNs were harvested 2 days after primary TNBS instillation and treated with Liberase (0.7 mg/ml) (Roche) for 15 minutes at 37°C. $4X10^6$ cells were stained with anti-CD45.2, anti-CD11c, anti-CD11b, anti-SIRPα and anti-CD103 antibodies and analyzed by flow cytometry. Data were acquired on a Becton Dickinson FACS Aria II (BD Pharmingen) and analyzed with Cell Quest software.

### Generation of BMDCs

Bone marrow derived DCs (BMDCs) were generated from bone marrow as previously described (Van et al., 2006). Briefly, bone marrow was harvested from WT and CD47 KO mice and cultured in RPMI-1640 supplemented with 5% FBS, Penicillin (500 U/ml), Streptomycin (500

μg/ml), 2-mercaptoethanol (1 mM) and GM-CSF (40 ng/ml), which was replenished on days 3, 7 and 12. BMDCs were harvested on day 13.

### In vitro and In vivo assessment of DC function

**In vitro:** WT BALB/c mice were injected with 10μg of human Flt3-L daily for 13 days to increase DC numbers. LP and mLN cells were purified as described above and cells were isolated from spleens using the same Liberase digestion protocol as mLNs. All cells were stained with anti-CD11b, anti-SIRPα, anti-CD103, anti-CD11c, anti-CD45.2 and anti-CD4, and sorted using a BD FACS ARIA II (BD Pharmingen) (see Suppl Fig 2 for gating strategy). The purity of $CD4^+$ T cells and DC subsets was greater than 99% and 96%, respectively. $CD4^+$ T cells were co-cultured for 5 days with sorted $SIRP\alpha^+CD103^-$ or $SIRP\alpha^-CD103^+$ DCs at a 25:1 ratio in the presence of soluble anti-CD3 (2 μg/ml), TGFβ (2 ng/ml), and anti-IFNγ (10 μg/ml). Transgenic (D011.10) $CD4^+$ T cells were co-cultured with BMDCs (1:2 ratio) in the presence of OVA peptide (2 μg/ml), TGFβ (2 ng/ml), and anti-IFNγ (10 μg/ml). On day 5, PMA (5 ng/ml), ionomycin (0.5 μg/ml) and Brefeldin A (1 μg/ml) were added to the cell cultures, and intracytoplasmic staining for IL-17 and IFNγ was performed as described above.

WT and CD47 KO BMDCs were stimulated with LPS (100 ng/ml) and the expression of CD11c, CD11b, CD103 and SIRPα, and the activation markers CD86, CD40 and MHC II were assessed after

overnight culture by flow-cytometry. To assess cytokine production (IL-6 and IL-23p19), BMDCs were stimulated overnight with LPS (100 ng/ml) in the presence of IFNγ (10 ng/ml). For WT/CD47 KO BMDC/T cell criss-cross co-cultures, splenic $CD4^+$ T cells were purified using the EasySep Biotin Selection Kit (StemCell Technologies). Anti-CD3 (2 μg/ml) stimulated $CD4^+$ T cells were co-cultured with LPS-stimulated (500 ng/ml) BMDCs at a 10:1 (T cell:DC) ratio in the presence of TGFβ (1 ng/ml). After 5 days, cells were re-stimulated with PMA/ionomycin in the presence of Brefeldin and stained for intracytoplasmic IL-17.

**In vivo:** LPS-stimulated (500 ng/ml) WT and CD47 KO BMDCs were harvested and transferred to CD47 KO hosts by i.p. injection of $2X10^6$ cells per mouse 30 minutes prior to induction of TNBS colitis (Gonzalez-Rey and Delgado, 2006).

### Competitive DC Migration Assay

WT and CD47 KO BMDCs were stimulated with LPS (500 ng/ml) overnight and labelled with CFSE (WT) or CMTMR (CD47 KO) and injected i.p. in CD47 KO hosts. TNBS colitis was induced 30 minutes after DC injection and mLNs were harvested on day 2 from host mice to assess DC migration. mLNs were digested with Liberase (0.7 mg/ml) for 15 minutes at 37°C and cells were analyzed by gating on $CD11c^+$ cells by flow cytometry.

## Statistical Analyses

Statistical analyses were performed using the GraphPad Instat program. All values are expressed as mean +/- standard error of the mean (SEM). The student's T test, paired T test, and Mann-Whitney U test were used to assess statistical significance.

### 3.2.4 Results

**CD47 regulates SIRPα$^+$CD103$^-$ DC homeostasis in the LP and mLNs of naïve and TNBS-treated mice.**

Multiple DC subpopulations have been identified and characterized by their expression of CD11c, CD11b and CD103 in the intestinal lamina propria (LP) and mLNs. Since CD47 has previously been shown to regulate cell migration of innate SIRPα$^+$ cells (Liu et al., 2002) (Van et al., 2006), we first thought to examine the expression of SIRPα on the two major mucosal DCs subsets, i.e. CD103$^+$ and CD103$^-$ DCs. We found that SIRPα was preferentially expressed with high intensity on CD11b$^+$CD103$^-$ DCs in the LP and mLNs (Fig. 3.1 A and B). To investigate the dynamic regulation of these DC subsets during inflammation, their distribution profiles were assessed at steady state and in response to TNBS challenge. We observed an increased percentage of SIRPα$^+$CD103$^-$ DCs in the LP as well as in the mLNs after the intra-rectal administration of TNBS (Fig. 3.1 A and B), suggesting a role for this DC subset in the response to hapten-induced colonic inflammation. We next used CD47 KO mice to examine the potential role of CD47 in modulating the frequency of SIRPα$^+$CD103$^-$ DCs in the LP and mLNs. While no significant differences in SIRPα$^+$CD103$^-$ DC proportions were observed in the LP of naive WT and CD47 KO mice (Fig. 3.1 A and C), a reduction in SIRPα$^+$CD103$^-$ DCs was observed in the mLNs of naive CD47 KO mice when compared to their WT littermates (Fig. 3.1 B,D). Upon TNBS

challenge, the increase in SIRPα$^{+}$CD103$^{-}$ DCs normally observed in WT mice did not occur in either the LP or the mLNs of CD47 KO mice (Fig. 3.1 C and D), suggesting that CD47 is required for the mobilization of SIRPα$^{+}$CD103$^{-}$ DCs in response to TNBS challenge. Although we show results from mLNs on day 2 after TNBS instillation, similar data were observed on day 4 and in caudal lymph nodes (data not shown). Notably, the proportion of SIRPα$^{-}$CD103$^{+}$ DCs, which have been shown to promote tolerance were not increased in the LP of either strain of TNBS-treated mice. However, their frequency was strongly reduced in the mLNs of WT but not CD47 KO TNBS-treated mice (Suppl Fig 3.1). Of note, CD47 expression was identical in both SIRPα$^{+}$CD103$^{-}$ and SIRPα$^{-}$CD103$^{+}$ DCs in the LP and mLNs of WT mice (unpublished data). Taken together, our results suggest that CD47 regulates the mobilization of mucosal SIRPα$^{+}$CD103$^{-}$DCs in response to inflammation and thus may play a role in gut DC homeostasis.

**CD47 ablation ameliorates the development of chronic TNBS colitis.**

We therefore hypothesized that the increased proportion of SIRPα$^{+}$CD103$^{-}$ DCs in the LP and mLNs of WT mice would be correlated with colitis development, in which case CD47 KO mice would be protected from disease. Analysis of body weight loss as early as day 2 after primary TNBS instillation, revealed a significant reduction of wasting disease in CD47 KO compared to WT mice (Fig. 3.2 A). This protection was enhanced daily until day 8, by which time CD47 KO, as opposed to WT

mice, had fully regained their original body weight (Fig. 3.2 A). To evaluate the degree of colonic inflammation, both the macroscopic and histological scores of tissue damage were assessed. Colons from WT mice with TNBS colitis displayed shortening and thickening, with patchy areas of severe ulceration and prominent adhesions typical of this form of colitis. On the contrary, CD47 KO mice developed fewer of these characteristics and displayed significantly milder inflammation than WT mice (Fig. 3.2 B and D). Upon histological assessment, we confirmed that colons from WT mice with colitis exhibited a substantial infiltration with mononuclear cells, edema and loss of goblet cells, while CD47 KO mice were resistant to these changes (Fig. 3.2 C and E).

We next confirmed the disease resistance of CD47 KO mice in a reactivated form of colitis, where a second instillation of TNBS was administered seven days after the primary instillation. This model is said to mimic the relapsing behaviour of human CD. Here, upon re-induction of TNBS colitis, CD47 KO mice remained resistant to body weight loss while WT mice displayed severe wasting once again (Fig. 3.2 F). Macroscopic (Fig. 3.2 G) and microscopic (Fig. 3.2 H) scores confirmed that CD47 KO mice were largely devoid of signs of chronic colonic inflammation. Taken together, our data demonstrate that the CD47-dependent increase in SIRP$\alpha^{+}$CD103$^{-}$ DCs in the LP and mLNs is linked with the development of chronic intestinal inflammation.

**CD47 ablation impairs Th17 responses in vivo**

The chronic stage of TNBS colitis is driven by an important interplay between the innate and adaptive immune systems. Here, we sought to further characterize this inflammation in WT and CD47 KO mice. Both on day 8 and after re-induction of TNBS colitis, a strong upregulation of IL-6 mRNA expression was observed in WT but not in protected CD47 KO mice (Fig. 3.3 A). The same trend, but of a lesser magnitude, was observed for TGFβ, IL-12p35 and IL-23p19, where mRNA expression was increased in TNBS-treated WT but not CD47 KO mice (Fig. 3.3 A). Corroborating the increase in IL-6 and TGFβ expression, IL-17 mRNA was highly upregulated at both time points in WT but not in CD47 KO mice (Fig. 3.3 B). IFNγ mRNA expression was greater in CD47 KO than WT mice, but only on day 8, with no significant difference observed after re-induction (Fig. 3.3 B). Interestingly, although both forms of colitis displayed qualitatively and quantitatively distinct IL-17 and IFNγ mRNA expression profiles, the IL-17/IFNγ mRNA expression ratios were nearly identical, with both being higher in WT than CD47 KO mice (Fig. 3.3 C). We therefore chose to focus on the re-induction model of colitis for the rest of this study since it more accurately reflects the chronic relapsing behaviour of CD.

We next postulated that the reduction in IL-17-associated cytokines in colon tissues was partly attributable to an impaired capacity to generate IL-17-producing T cells (Th17) in the mLNs of CD47 KO mice. Therefore, we examined IFNγ and IL-17 production in 4-day cultures of mLNs

isolated from mice with chronic TNBS colitis. Analysis by flow cytometry revealed that the percentage of Th17 cells was significantly higher in WT compared to CD47 KO mice with colitis (Fig. 3.3 D). Similar data were observed by measuring IL-17 production in the culture supernatants by ELISA or by examining IL-17 expression in mLN cells after 6-hours of PMA/ionomycin restimulation (unpublished data). Under these pro-Th17 culture conditions, no significant difference was observed with respect to $CD4^+$ T cells producing IFNγ (Fig. 3.3 D). When expressed as a ratio of $IL\text{-}17^+/IFN\gamma^+$ cells, CD47 KO mice exposed to TNBS displayed a significantly reduced propensity toward IL-17 production (Fig. 3.3 E), corroborating with tissue mRNA expression and the recent shift in the Th1/Th17 paradigm reported in TNBS colitis (Sheibanie et al., 2007a). Thus, CD47 ablation impairs the induction and perpetuation of colitis associated with a Th17 biased response in mLNs.

**$SIRP\alpha^+CD103^-$ DCs promote Th17 responses in vitro.**

To establish causative links between the increased frequency of mucosal $SIRP\alpha^+CD103^-$ DCs and Th17 responses observed *in vivo*, we first sought to assess the *in vitro* Th1 vs Th17-promoting capacities of $SIRP\alpha^+CD103^-$ and $SIRP\alpha^-CD103^+$ DCs. These two DC subpopulations were isolated from the LP and mLNs (Suppl Fig 3.2) and co-cultured with $CD4^+$ T cells from the same anatomical origin. We found that $SIRP\alpha^+CD103^-$ DCs were more efficient than $SIRP\alpha^-CD103^+$ DCs at promoting IL-17 production by $CD4^+$ T cells (Fig 3.4 A and B).

Interestingly, DCs purified from spleens behaved in a very similar manner as those isolated from LP and mLNs, with SIRPα$^{+}$CD103$^{-}$ DCs favouring Th17 (Fig. 3.4 C).

When bone-marrow derived DCs (BMDCs) that express SIRPα (>99%) but not CD103 (<1%), were co-cultured with naive transgenic T cells under Th17-promoting conditions, they elicited a similar Th17 biased profile as SIRPα$^{+}$CD103$^{-}$ mLN DCs, suggesting that these properties are not limited to gut-derived DCs (Fig. 3.4 D). Moreover, sorted SIRPα$^{+}$CD103$^{-}$ mucosal or splenic DCs and SIRPα$^{+}$CD103$^{-}$ BMDCs induced statistically equivalent IL-17$^{+}$/IFNγ$^{+}$ ratios, both of which were significantly higher compared to sorted SIRPα$^{-}$CD103$^{+}$ DCs (Fig. 3.4 E). Therefore, we propose that SIRPα$^{+}$CD103$^{-}$, but not SIRPα$^{-}$CD103$^{+}$ DCs, possess Th17-promoting properties *in vitro.*

**Transfer of SIRPα$^{+}$CD103$^{-}$ BMDCs induces colitis and promotes a Th17 response in CD47 KO mice**

To directly examine the proinflammatory capacity of SIRPα$^{+}$CD103$^{-}$ DCs *in vivo* and the role of CD47 on the DC for disease induction, CD47 KO mice were administered mature WT or CD47 KO SIRPα$^{+}$CD103$^{-}$ BMDCs on the day of colitis induction. BMDCs were injected intraperitoneally (i.p.) since previous reports have shown that the mLNs drain the peritoneal cavity, in addition to the gut tissues (Johansson-Lindbom et al., 2003, Kool et al., 2008). Moreover, the intravenous (i.v.)

route was deemed unsuitable since i.v.-injected BMDCs migrate very poorly to the LNs, even when administered at high concentrations (3 to 8 x $10^6$/mouse) (Cavanagh et al., 2005, Robert et al., 2003).Upon re-induction of TNBS colitis on day 8, CD47 KO mice injected with WT BMDCs developed severe wasting disease (Fig. 3.5 A), indicating that CD47 expression was not required in the host for colitis development. Injection of WT BMDCs had no effect on disease outcome when injected in WT mice (Suppl Fig 3.3). In contrast to WT BMDCs, injection of CD47 KO BMDCs in CD47 KO hosts appeared to induce only a mild form of inflammation which resulted in a slight, non-significant, reduction of body weight (Fig. 3.5 A). The changes in body weight were reflected both in the macroscopic (Fig. 3.5 B) and microscopic (Fig 3.5 C) extent of inflammation, with WT BMDC injection resulting in an aggressive form of disease. Notably, injection of WT BMDCs also increased the mortality rate of CD47 KO mice more than injection of CD47 KO BMDCs (un-injected: <5%, CD47 KO BMDC: 25%, and WT BMDCs: 45%).

We next examined whether disease development after WT BMDC injection was associated with the induction of Th17-biased responses in CD47 KO hosts. Injection of WT BMDCs significantly enhanced the colonic mRNA expression of IL-17 but not IFNγ, while injection of CD47 KO BMDCs had no effect on the colonic mRNA expression of either cytokine (Fig 3.5 D). Therefore, only WT BMDCs resulted in a significant increase in the IL-17/IFNγ mRNA ratio (Fig. 3.5 D). To verify whether the

induction of colonic IL-17 mRNA expression was linked to an increase in Th17 cells, we examined the mLNs of reconstituted mice. Injection of WT BMDCs increased the proportion of IL-17$^+$ CD4$^+$ T cells, leading to a significant enhancement in the IL-17$^+$/IFNγ$^+$ CD4$^+$ T cell ratio (Fig. 3.5 E and F). On the other hand, injection of CD47 KO BMDCs elicited IL-17 expression which did not translate into a significant increase in the IL-17/IFNγ ratio (Fig. 3.5 E and F). Finally, SIRPα$^+$CD103$^-$ DCs purified from WT mLNs and injected i.p. were also capable of inducing severe wasting disease, resulting in 25% mortality, after a single dose of TNBS in CD47 KO mice (Suppl Fig. 3.4). Severe weight loss in these mice was correlated with pronounced colonic inflammation and Th17-polarized responses, corroborating the similar *in vitro* pro-Th17 responses of SIRPα$^+$CD103$^-$ BMDCs and mucosal DCs. These data demonstrate that CD47 expression is dispensable on the endothelium, epithelium and T cells of the host but appears to be critical on SIRPα$^+$CD103$^-$ DCs for Th17-associated colonic disease induction *in vivo.*

**SIRPα$^+$CD103$^-$ DC trafficking but not phenotype or function is governed by CD47.**

We next sought to delineate the mechanisms by which CD47 expression on SIRPα$^+$CD103$^-$ BMDCs controls the development of Th17 responses and colonic inflammation. To this end, we evaluated the *in vivo* migratory properties and the *in vitro* function of WT and CD47 KO BMDCs. First, WT and CD47 KO BMDCs were fluorescently labelled and injected

i.p. at a 1:1 ratio in CD47 KO mice and retraced in the mLNs after 2 days. By using this competitive migration assay, we first demonstrate that BMDCs do indeed migrate to the mLNS after an i.p. route of injection and provide evidence that CD47 expression on BMDCs significantly favours their accumulation in mLNs (Fig.3. 6 A and B).

Next, we assessed the phenotype of BMDCs in terms of their expression of CD11c, CD11b, SIRPα and CD103 and observed no difference between WT and CD47 KO BMDCs (Fig. 3.6 C). Importantly, the level of expression of SIRPα was not altered on a per-cell basis by the expression of CD47, as determined by its mean fluorescence intensity (Fig. 3.6 C). The surface expression of MHC II and the co-stimulatory molecules CD40 and CD86 were also independent of CD47 expression (Fig 3.6 D). We next examined the function of WT and CD47 KO BMDCs by measuring the production of cytokines such as IL-6 and IL-23p19, as well as their ability to drive Th17 polarization. Both cytokine production and Th17-promoting capacity were found to be unrelated to CD47 expression on the DCs (Fig 3.6 E and F). Also, lack of CD47 expression on T cells did not alter the percentage of IL-17-producing $CD4^+$ T cells, thereby eliminating the possibility of a T-cell intrinsic defect in Th17 polarization in CD47 KO T cells as a mechanism for decreased Th17 responses (Fig 3.6 F). Taken together, CD47 expression on $SIRP\alpha^+CD103^-$ BMDCs favours their migration to mLNs, thereby corroborating with the increased mobilization of $SIRP\alpha^+CD103^-$ mucosal

DCs observed in WT but not KO mice after TNBS challenge (Fig. 3.1). Since CD47 does not alter the phenotype, activation status, cytokine production or Th17-polarizing capacity of SIRPα$^{+}$CD103$^{-}$ BMDCs, we propose that CD47 expression on SIRPα$^{+}$CD103$^{-}$ DCs is critical for their trafficking to mLNs, a property mechanistically related to the ensuing Th17 responses and colitis development.

**Administration of CD47-Fc ameliorates disease development in BALB/c mice**

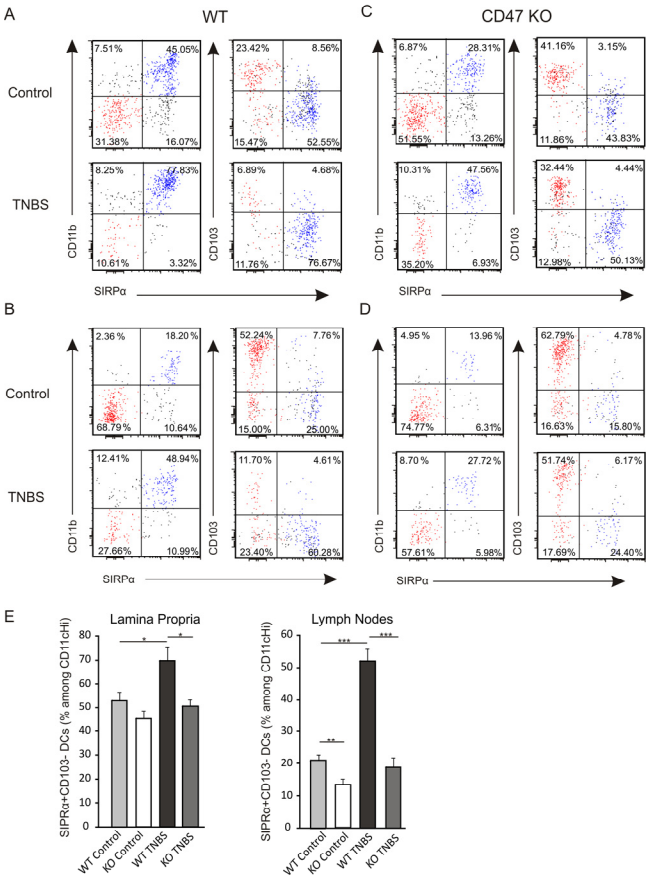
Finally, we circumvented the multiple defects of CD47 KO hosts and directly examined the impact of targeting the SIRPα/CD47 axis on the recruitment of SIRPα$^{+}$CD103$^{-}$ DCs in mLNs, Th17 polarization and the development of chronic colitis in WT mice. To this end, BALB/c mice were administered a CD47-Fc molecule i.p. 30 minutes before TNBS injection and on days 1-3 (Fig. 3.7 A, arrows). Mice administered CD47-Fc were largely protected from colonic inflammation as assessed by body weight, starting as early as 1 day after the induction of colitis (Fig. 3.7 A). Furthermore, CD47-Fc treated mice remained resistant to colitis even after a second induction of TNBS colitis, as demonstrated by a reduction in body weight loss, and the extent of tissue damage (Fig. 3.7 A-C). Injection of a control human CD47-Fc with no cross-reactivity in mice did not ameliorate disease (unpublished data). Serum levels and colonic mRNA expression of IL-6 were determined early in disease onset (day 4) and were dramatically reduced in CD47-Fc-treated mice (Fig. 3.7 D).

Importantly, the percentage of SIRPα$^{+}$CD103$^{-}$ DCs was specifically and significantly reduced by CD47-Fc treatment (Fig. 3.7 E). On day 4, IL-17 and IFNγ mRNA expression were unaffected. After re-induction, IL-17 mRNA expression was effectively suppressed by CD47-Fc, while IFNγ mRNA expression was unaltered (Fig. 3.7 F). Regardless of the time of sacrifice (day 4 or after re-induction), the IL-17/IFNγ mRNA ratio was nearly identical and equally suppressed by administration of CD47-Fc (Fig. 3.7 G). Similarly, the Th17/Th1 ratio in mLNs was also reduced by CD47-Fc (data not shown). We therefore conclude that CD47-Fc treatment protects mice from the development of chronic colitis and is correlated with an altered development of SIRPα$^{+}$CD103$^{-}$ DC-mediated Th17 responses.

### 3.2.5 Figures

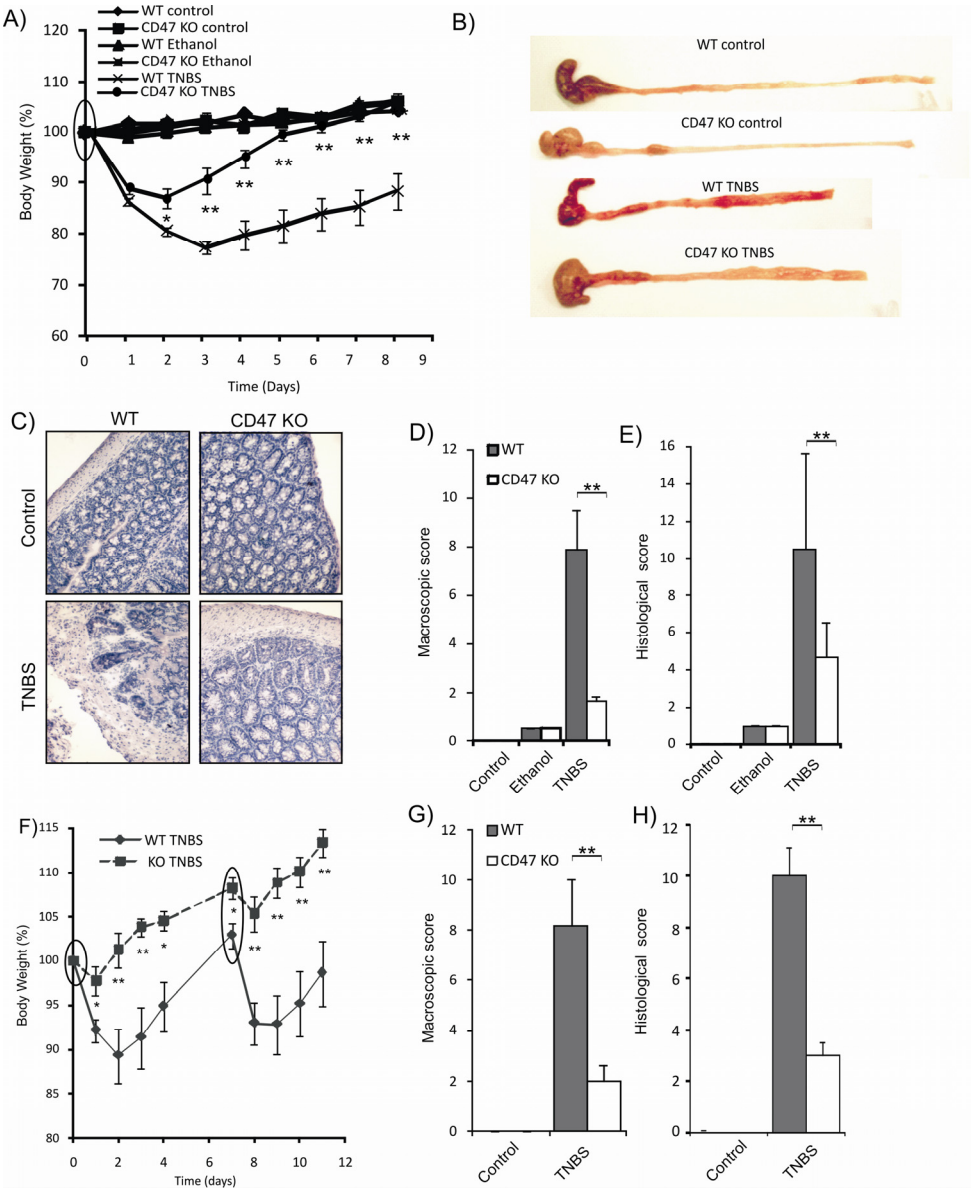
Figure 3.1: CD47 regulates SIRPα$^{+}$CD103$^{-}$ DC homeostasis in the lamina propria and mLNs

Figure 1:




(**A-D**) CD11b, CD103 and SIRPα expression among CD45.2$^{+}$CD11c$^{Hi}$ DCs at steady state (control; top) and 2 days after intrarectal administration of 3 mg TNBS (TNBS; bottom) in the lamina propria (A,C) and mLNs (B,D) of WT (A,B) and CD47 KO (C,D) mice. Data shown is one representative mouse per group. (**E**) Percentage of SIRPα$^{+}$CD103$^{-}$ DCs among CD45.2$^{+}$CD11c$^{Hi}$ DCs in the lamina propria (left) and mLNs (right). Data represent the mean +/- SEM of >8 mice/group. Each experiment was independently performed a minimum of 4 times. * p<0.05, **p<0.01, ***p<0.001.

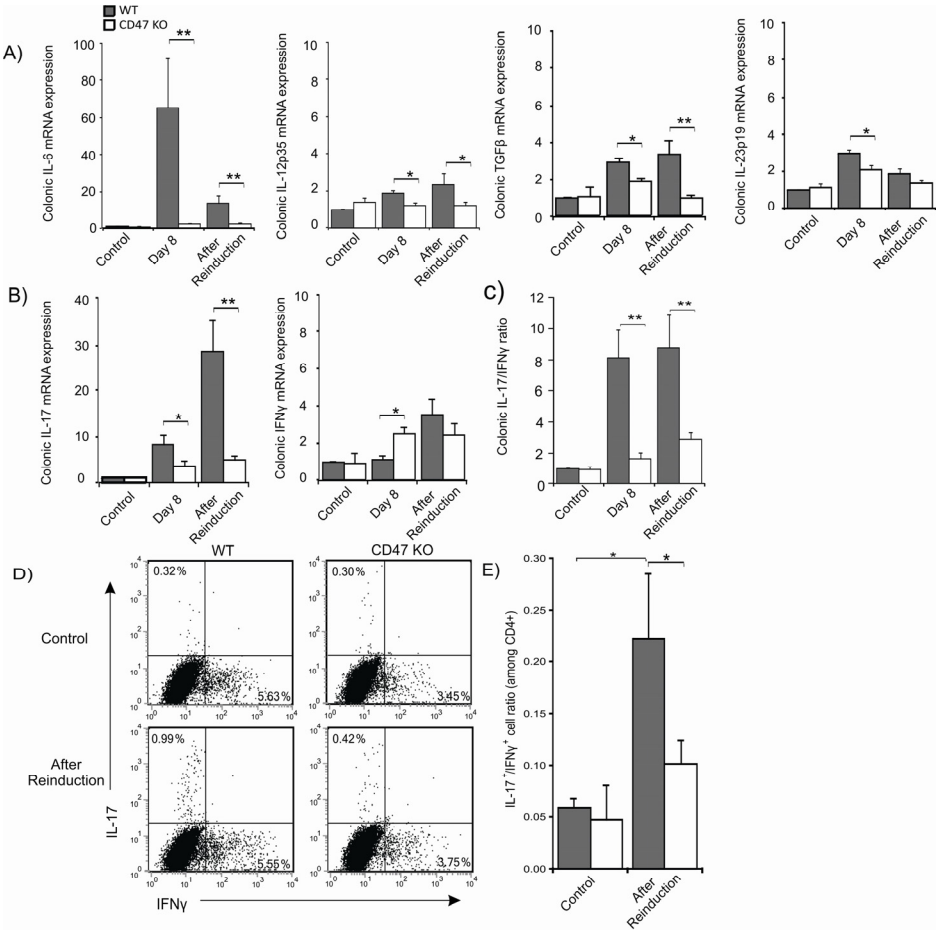
Figure 3.2: CD47 ablation protects from the development of TNBS colitis.




TNBS colitis was induced on day 0 alone or on days 0 and 7 (circled). (**A**) Weight loss curves of WT and CD47 KO mice injected intrarectally with saline (control), ethanol alone, or TNBS dissolved in 50% ethanol on day 0. (**B** and **D**) Macroscopic and (**C** and **E**) histologic (Original magnification, 400X) analyses of inflammation in WT and CD47 KO mice. Data represent mean +/- SEM of >8 mice per group, one representative experiment out of 3 is shown. (**F**) Weight loss curves of WT and CD47 KO mice injected

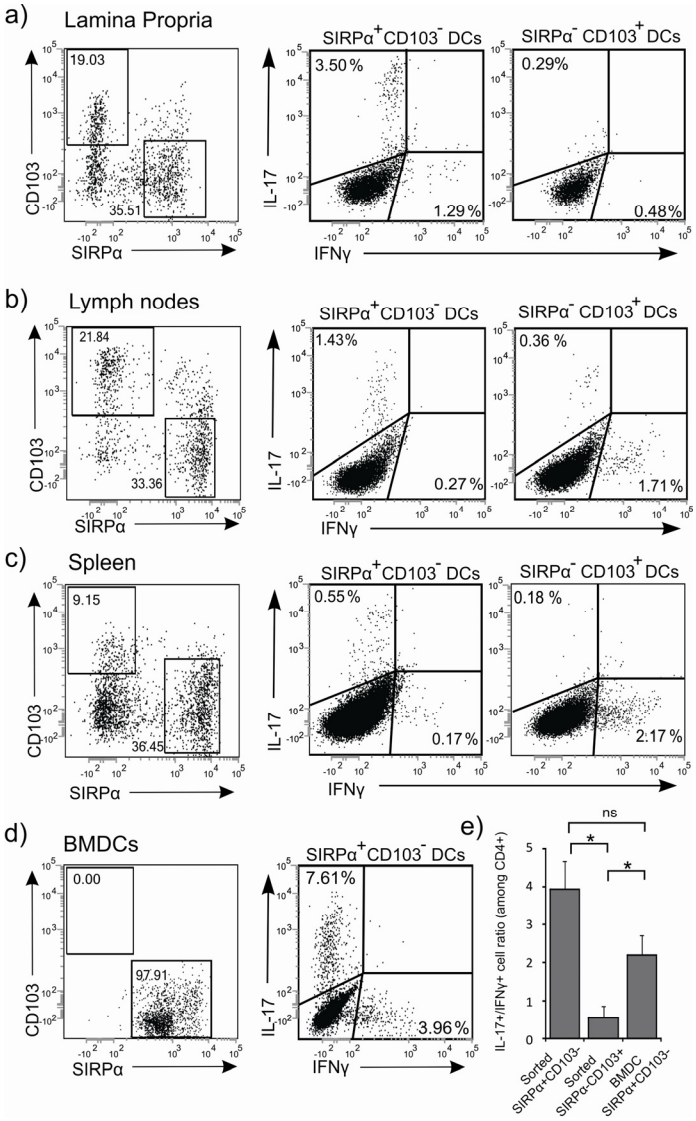
intrarectally with 2 mg TNBS dissolved in 50% ethanol on day 0 and on day 7. (**G**) Macroscopic and (**H**) histologic analyses of inflammation in WT and CD47 KO mice after re-induction of TNBS colitis. Data represent mean +/- SEM of 6 mice per group. One representative experiment out of 3 is shown. *$p<0.05$. **$p<0.01$.

Figure 3.3: Decreased proinflammatory cytokine and Th17 biased responses in CD47 KO mice.




(**A-C**) Colonic mRNA expression of cytokines. (**A**) Colonic IL-6, IL-12p35, TGFβ and IL-23p19 mRNA expression. (**B**) Colonic IL-17 and IFNγ mRNA expression. (**C**) Ratio of colonic IL-17/IFNγ mRNA expression. Data represent mean fold change relative to WT control +/- SEM of > 8 mice per group (2 independent experiments). (**D**) mLNs were isolated after reinduction of TNBS colitis and stimulated for 4 days in the presence of IL-23. Intracytoplasmic staining was performed after restimulation. The percentage IL-17$^+$ or IFNγ$^+$ cells among total CD4$^+$ T cells is shown. (**E**) Ratio of IL-17$^+$/IFNγ$^+$ cells among CD4$^+$ T cells. Data represent the mean percentage +/- SEM of >7 mice per group (2 independent experiments).* $p<0.05$. **$p<0.01$.

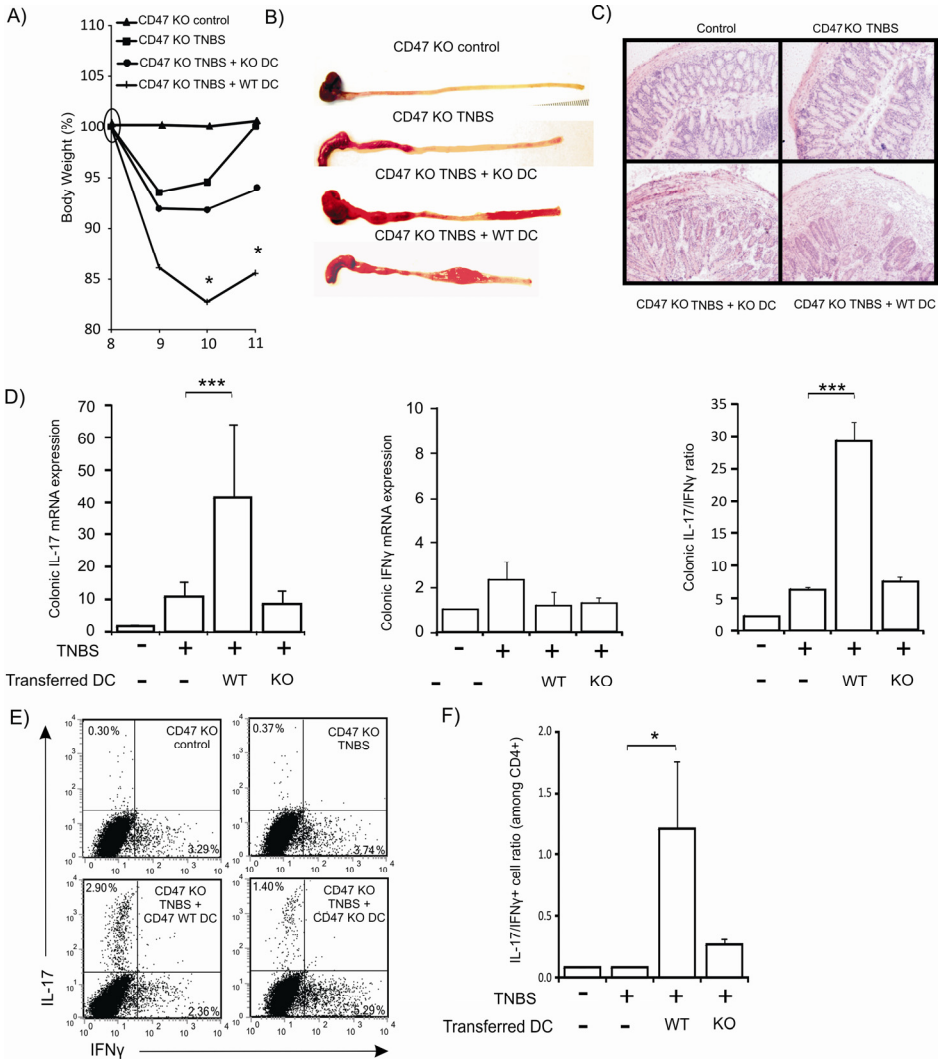
Figure 3.4: SIRPα$^{+}$CD103$^{-}$ DCs promote Th17 responses *vitro.*




(**A-C**) Percentage of SIRPα$^{+}$CD103$^{-}$ and SIRPα$^{-}$CD103$^{+}$ DCs (gated on CD11c$^{Hi}$) (left) and their IFNγ- versus IL-17-promoting capacities (middle and right) when isolated from the LP (A), mLNs (B), or spleen (C) and co-cultured with purified anti-CD3 stimulated CD4$^{+}$ T cells of the same anatomical origin. Dot plots represent one independent experiment out of 2 or 3. (**D**) Percentage of SIRPα$^{+}$CD103$^{-}$ and SIRPα$^{-}$CD103$^{+}$ BMDCs (gated on CD11c$^{+}$) (left) and co-culture with OVA-stimulated CD4$^{+}$ transgenic T cells under Th17 polarizing conditions (right). Data represent one independent experiment out of 3. (**E**) IL-17$^{+}$/IFNγ$^{+}$ cell ratio (among

$CD4^+$ T cells) after co-culture with either sorted $SIRP\alpha^+CD103^-$ or $SIRP\alpha^-$ $CD103^+$ DCs or $SIRP\alpha^+CD103^-$ BMDCs. Data of sorted cells represents mean of all sources of DCs (LP, mLNs, spleen) +/- SEM of a minimum of 3 independent experiments. *$p<0.05$

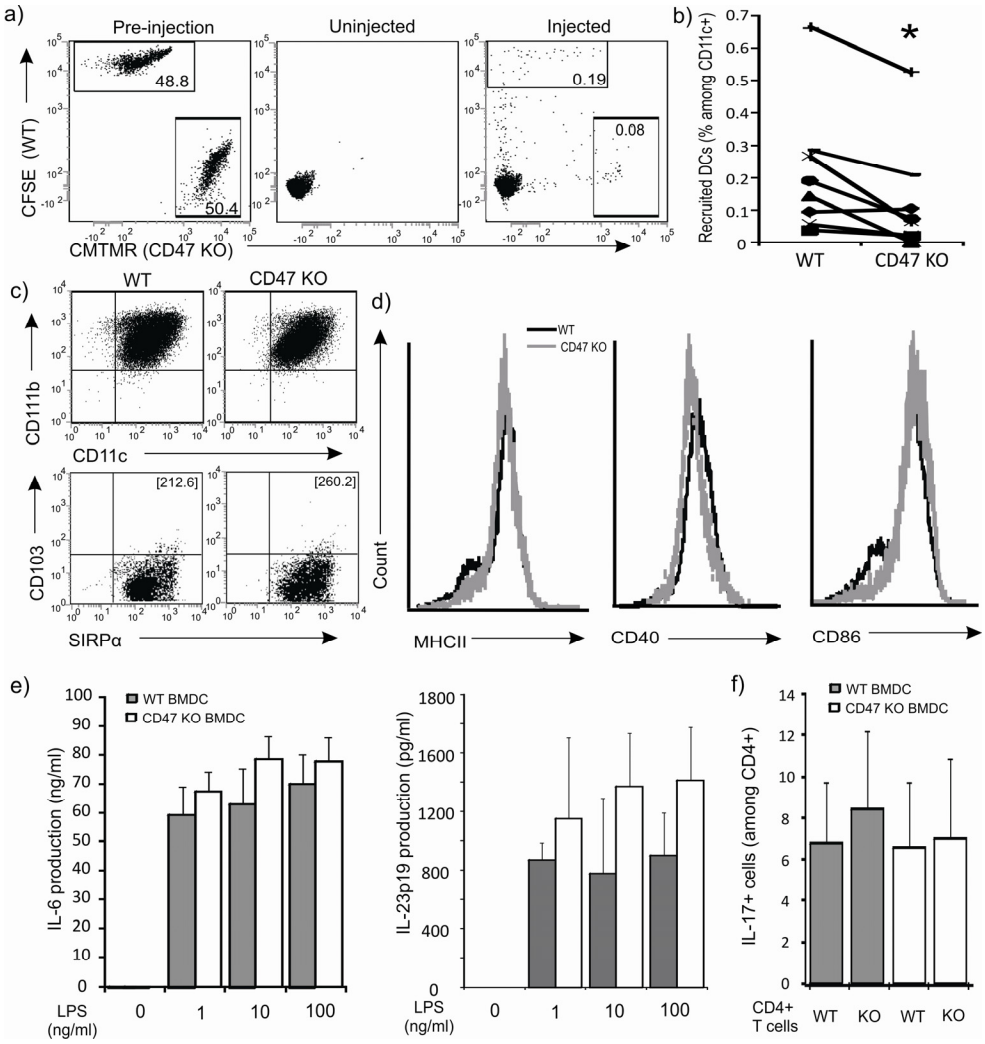
Figure 3.5: CD47 expression on SIRPα$^{+}$CD103$^{-}$ DCs promotes the development of intestinal inflammation and Th17 responses.




CD47 KO mice were injected i.p. with saline or WT or CD47 KO BMDCs 30 minutes before the induction of TNBS colitis on day 0. TNBS was re-induced on day 8. (**A**) Weight loss curve after transfer of WT or CD47 KO BMDCs in CD47 KO hosts and re-induction of TNBS colitis, normalized to body weight on day 8. (**B**) Macroscopic and (**C**) Histologic appearance of colonic inflammation. Images are of one representative mouse per experimental group. (**D**) Colonic mRNA expression of IL-17 and IFNγ and IL-17/IFNγ mRNA expression ratio. Data represent the mean fold change relative to control +/- SEM of > 5 mice per group (2 pooled independent

experiments). (**E**) mLNs were isolated after re-induction of TNBS colitis and stimulated for 4 days in the presence of IL-23. The percentages of IL-$17^+$ or IFN$\gamma^+$ $CD4^+$ T cells and the ratio of IL-$17^+$/IFN$\gamma^+$ cells among $CD4^+$ T cells are shown. Data represent mean +/- SEM of >5 mice per group (2 pooled independent experiments).* $p<0.05$ ***$p<0.001$

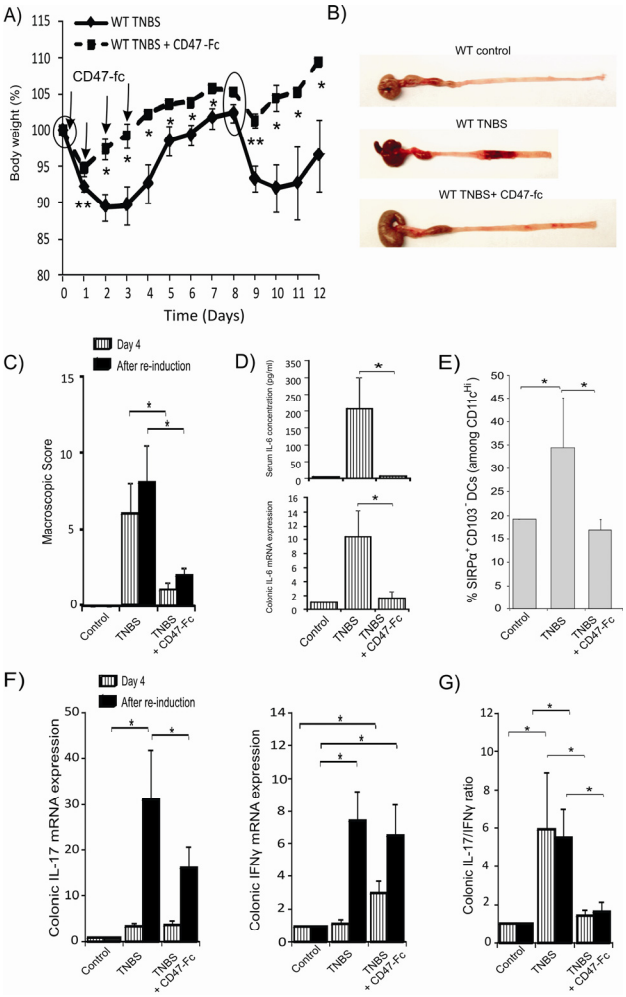
Figure 3.6: CD47 regulates SIRPα$^{+}$CD103$^{-}$ DC trafficking but not phenotype or function




(**A**) Left: 1:1 ratio of labelled BMDCs before injection on day 0. Middle and Right: TNBS colitis was induced in CD47 KO mice, and mLNs were harvested after 2 days. Middle: CD11c$^{+}$ DCs in the mLNs of uninjected CD47 KO mice. Right: Labelled WT and CD47 KO BMDCs (gated on CD11c$^{+}$) in the mLNs of BMDC-injected CD47 KO mice. One representative mouse is shown for each condition. (**B**) Percentages of labelled WT and CD47 KO BMDCs (gated on CD11c$^{+}$) in the mLNs of CD47 KO mice. Data were generated in 2 independent experiments (n=8). (**C-D**) Analysis of phenotype (C) and activation status (D) of LPS-

stimulated BMDCs. The numbers in square parentheses in (C) represent the mean fluorescence intensity of SIRPα. (**E**) Cytokine production by BMDCs stimulated overnight with LPS (0-100 ng/ml) and IFNγ (10 ng/ml) *in vitro.* Data represent the mean (n=4) +/- SEM. (**F**) Percentage of IL-17$^{+}$ cells (among CD4$^{+}$ T cells) after criss-cross co-cultures with anti-CD3 stimulated WT vs CD47 KO splenic CD4$^{+}$ T cells and LPS-stimulated WT vs CD47 KO BMDCs under Th17 polarizing conditions. Data represent mean (n=3) +/- SEM.

Figure 3.7: Administration of CD47-Fc protects BALB/c mice from the development of TNBS colitis.




WT BALB/c mice were injected i.p. with saline or CD47-Fc, TNBS colitis was induced on day 0 and day 8 (circles), and mice were sacrificed on day 4 or day 12 (after re-induction). (**A**) Weight loss curves normalized to body weight on day 0. (**B**) Macroscopic appearance and (**C**) mean macroscopic score of >6 mice per group +/- SEM. (**D**) Serum concentration (upper panel) and colonic mRNA expression (lower panel) of IL-6. (**E**) mLN SIRPα$^{+}$CD103$^{-}$ DCs as a percentage of total CD11c$^{Hi}$. (**F**) Colonic mRNA expression of IL-17 and IFNγ. (**G**) IL-17/IFNγ mRNA expression ratio. Data represent mean +/- SEM of >6 mice per group of two independent experiments. *p<0.05

### 3.2.6 Discussion:

DCs lie at the interface between the innate and the adaptive immune system, and while they are poised to maintain gut homeostasis and tolerance, they also appear to play a critical role in CD pathogenesis. Here, we propose SIRPα-expressing $CD11b^{+}CD103^{-}$ DCs as one primary candidate for the induction and perpetuation of chronic TNBS colitis and implicate CD47 as a key player in controlling their homeostasis and trafficking to the LP and mLNs. We first observed a correlation between the frequency of $SIRP\alpha^{+}CD103^{-}$ DCs in the LP and mLNs, Th17 responses and the development of intestinal inflammation, parameters which were all impaired in CD47 KO mice. Next, we demonstrated that CD47 expression is required on $SIRP\alpha^{+}CD103^{-}$ DCs for efficient trafficking to mLNs and induction of severe wasting Th17-associated disease *in vivo,* but is dispensable on both $SIRP\alpha^{+}$ DCs and T cells to promote Th17 responses *in vitro*. Finally, early administration of a CD47-Fc molecule treated and prevented the recurrence of experimental colitis, which was correlated with defective homing of $SIRP\alpha^{+}CD103^{-}$ DCs and Th17 responses. We thus provide evidence that CD47 expression on $SIRP\alpha^{+}CD103^{-}$ DCs governs their trafficking and DC-driven Th17 responses, which are likely implicated in the development of intestinal inflammation.

Although the importance of DCs in the initiation of intestinal inflammation is now well accepted (Coombes and Powrie, 2008), the

specific contributions of individual DC subsets, and the key molecules involved in their function/migration, remain to be clarified. This is the first study to identify SIRPα expression on mucosal $CD103^-$ DCs and to support a role for this DC subset in the *in vivo* promotion of intestinal inflammation. Nevertheless, while the proportion of total $SIRP\alpha^+CD103^-$ DCs increased in the LP and mLNs of WT mice upon induction of TNBS colitis, the percentage of the tolerogenic $CD103^+$ DC subset, which we describe as being primarily $SIRP\alpha^-$, decreased in the mLNs. Therefore, it may have been postulated that the reduction in the proportion of $CD103^+$ DCs migrating to the mLNs of WT mice was the key event in triggering intestinal inflammation, as opposed to the increased recruitment of the pathogenic $SIRP\alpha^+CD103^-$ DC subset in both the tissues and mLNs. Yet, CD47 KO mice do not display any change in the proportion of tolerogenic $CD103^+$ DCs in mLNs upon exposure to TNBS, and a single administration of WT $SIRP\alpha^+CD103^-$ DCs in CD47 KO mice was sufficient to induce severe wasting disease. Consequently, our data support a pro-inflammatory role for the $SIRP\alpha^+CD103^-$ DC subset, the effect of which may be amplified by, but is not exclusively dependent upon, the reduction in the proportion of tolerogenic $CD103^+$ DCs in the mLNs. In support of this concept, $CD103^+$ DCs are found in equal proportions in the mLNs draining the small intestines of healthy individuals and those of CD patients, implying that intestinal inflammation is not dependent upon a reduction in $CD103^+$ DCs (Jaensson et al., 2008).

In the gut, CCR7 largely controls $CD103^+$ DC trafficking at steady state (Worbs et al., 2006, Jang et al., 2006). We have previously reported that the absence of CD47 does not alter CCR7 expression or the *in vitro* migration of skin $SIRP\alpha^+$ DCs toward CCL19 or sphingosine-1-phosphate (Van et al., 2006). The reduced proportion of the $SIRP\alpha^+CD103^-$ DC subset in the mLNs of CD47 KO and CD47-Fc-treated mice upon exposure to TNBS strongly suggests that CD47 promotes this process. These findings corroborate previous *in vivo* studies where a reduction of $SIRP\alpha^+$ DCs was observed in skin-draining LNs of CD47 KO and CD47-Fc-treated mice, which was found to be independent of CD47 expression on lymphatic vessels (Van et al., 2006). In support of these findings, we here demonstrate that injected WT $SIRP\alpha^+CD103^-$ DCs migrate more efficiently to mLNs and elicit a more aggressive form of disease in CD47 KO hosts when compared to CD47 KO DCs. However, the underlying mechanisms by which CD47 governs $SIRP\alpha^+CD103^-$ DC migration remains to be elucidated. One hypothesis is that CD47/SIRPα interactions that may occur in *cis* on the DC, indirectly control integrin-mediated transendothelial trafficking.

The expression of CD62L on $CD103^-$ DCs (Johansson-Lindbom et al., 2005) and a more recent report examining the turnover of intestinal DC subsets (Jaensson et al., 2008), favour the hypothesis of a direct recruitment of $CD103^-$ DCs or their precursors from the bloodstream to the mLNs via high endothelial venules (HEV) at steady state. Our study

supports this hypothesis, since there is no defect of SIRPα$^{+}$CD103$^{-}$ DCs in the LP, while there is a significant reduction in the proportion of these DCs in the mLNs of CD47 KO mice at steady state. Therefore, SIRPα$^{+}$CD103$^{-}$ DCs or their precursors may bypass the LP to seed the mLNs in a CD47-dependent manner. However, during the induction of colonic inflammation, SIRPα$^{+}$CD103$^{-}$ DCs or their precursors may be recruited *de novo* to inflamed tissues before migrating to the mLNs. Interestingly, under inflammatory conditions, CX$_3$CR1$^{int}$GR1$^{high}$CD62L$^{+}$CCR2$^{+}$ monocytes (GR1$^{high}$ monocytes) have been shown to differentiate into LP DCs (Varol et al., 2007), but i.v.-injected bone marrow monocytes are also capable of directly entering LNs through HEV (Nakano et al., 2009). Thus, under inflammatory conditions, CD47 may be implicated in the recruitment of SIRPα$^{+}$CD103$^{-}$ DC precursors to the LP and/or the mLNs, resulting in a reduction in the frequency of SIRPα$^{+}$CD103$^{-}$ DCs at those sites in CD47 KO or CD47-fc treated mice. Nevertheless, we cannot exclude the possibility that CD47 is required for the migration of SIRPα$^{+}$CD103$^{-}$ DCs directly from the LP to the mLNs (Suppl Fig 5). Thus, further examination of the physiological routes of SIRPα$^{+}$CD103$^{-}$ DC trafficking under inflammatory conditions is required to delineate the precise role of CD47 in this process.

The early phases of TNBS colitis have been successfully induced in the absence of T cells. Therefore, innate-mediated mechanisms are also likely to be implicated in disease pathogenesis (Fiorucci et al., 2002,

Katakura et al., 2005). SIRPα$^{+}$ neutrophils, which are rapidly recruited to sites of inflammation, have been shown to exhibit CD47-mediated transmigration (Cooper et al., 1995). Our unpublished data indicate a significant reduction in colonic myeloperoxidase activity, a marker of neutrophil infiltration, early in disease development and we thus hypothesized that a defect in CD47-mediated neutrophil transmigration may be protective in the acute phase of disease. However, previously published reports have suggested a protective role for neutrophils in disease initiation (Kuhl et al., 2007). Therefore, while a reduction of neutrophil infiltration was observed in CD47 KO mice, we largely excluded this phenomenon as playing a protective role in the development of TNBS colitis.

In the present study, we observed causative links between an increased percentage of SIRPα$^{+}$CD103$^{-}$ DCs, Th17-skewed responses and disease manifestation, suggesting a role for Th17 cells in the development of colitis. The *in vitro* Th17-promoting capacity of CD11c$^{Hi}$SIRPα$^{+}$CD103$^{-}$ DCs seems to be shared by the previously described CD11c$^{Hi}$CX$_3$CR$_1^{+}$CD103$^{-}$ LP DC subset, supporting their contribution in the initiation of Th17 responses (Denning et al., 2007). Until recently, both CD and TNBS colitis have been considered Th1 diseases characterized by IL-12, TNFα and IFN-γ production (Neurath et al., 1995, Parronchi et al., 1997). However, several recent observations have strongly implicated a role for IL-23, a Th17 survival factor, and Th17 cells

in disease pathogenesis. In humans, elevated serum levels of IL-17 and colonic mucosal levels of both IL-17 and IL-23 have been detected in CD patients (Fujino et al., 2003, Schmidt et al., 2005), while a mutation in the IL-23R gene (rs11209026, c.1142G>A, p.Arg381Gln) is protective from disease development (Duerr et al., 2006). It is established that the intestinal mucosal environment, via TGF-β, thymic stromal lymphopoietin (TSLP) and IL-10, acts to condition resident APCs to exert immunosuppressive activities (Zeuthen et al., 2008, Jarry et al., 2008). Epithelial cells from CD patients express less TSLP, leading to an enhanced release of the pro-Th1 and -Th17 inflammatory cytokines IL-12, IL-6 and IL-23 and expression of TLR2 and TLR4 by DCs (Hart et al., 2005, Rimoldi et al., 2005). Moreover, the combination of intestinal epithelial-derived TGFβ and retinoid acid has also recently been shown to convert $CD103^-$ DCs into $CD103^+$ DCs capable of generating Tregs (Iliev et al., 2009). IL-23 is abundantly produced by ileal but not colonic murine DCs (Becker et al., 2003). Therefore, TNBS colitis, a model in which the inflammation is localized to the colon, might be less suitable than others for examining IL-23 regulation, as underscored by the low colonic mRNA expression of IL-23p19 in this study, even under conditions of severe inflammation. Nonetheless, the expression of IL-17 was strongly upregulated by TNBS in WT mice as compared to CD47 KO mice. IL-17 is considered to be pathogenic in TNBS colitis since IL-17R-deficient mice are protected from disease development, and administration of an anti-IL-

17 mAb ameliorates intestinal inflammation, despite high levels of IFNγ (Zhang et al., 2006). In contrast, IFNγ receptor- and IFNγ-deficient mice, or mice administered a neutralizing anti-IFNγ mAb developed colitis as severe as WT mice (Tozawa et al., 2003, Camoglio et al., 2000) suggesting that IFNγ and IL-12 play a less essential role in disease development than once believed. On the other hand, both IL-17 and IFNγ synergize to create intestinal inflammation in *Helicobacter hepaticus*-induced T cell-dependent colitis (Kullberg et al., 2006). In Th17-associated autoimmune diseases, such as experimental autoimmune uveitis (EAU) (Luger et al., 2008) and experimental encephalomyelitis (EAE), either Th1 or Th17 cells alone can drive tissue damage (Stromnes et al., 2008, Kroenke et al., 2008, Bettelli et al., 2004). T cell clones isolated from CD patients have been identified as being either single IL-$17^+$, or double IL-$17^+$IFNγ$^+$ cells, underscoring the potentially pathogenic role of both cytokines (Annunziato et al., 2007). Some studies have even suggested a differential role for Th1 vs Th17 cells at various stages of disease (Kobayashi et al., 2008). Moreover, the developmental plasticity of Th17 cells has been reported, whereby IL-23 and IL-12 convert terminally differentiated murine Th17 cells into IFNγ-producing cells (Lee et al., 2009). Thus, it is likely an oversimplification to implicate Th17 cells alone in the development of TNBS colitis and, as such, we monitored the Th17/Th1 ratio rather than an exclusive decrease in the frequency of Th17 cells, as a common thread of disease protection throughout this study.

Finally, while Th17 cells are often categorized as pathogenic in autoimmune disease, it should be emphasized that they critically participate in protecting the host from microbial invasion and maintaining the epithelial barrier, as was demonstrated for the Th17-associated cytokine IL-22 (Mudter et al., 2008).

We also report that the mRNA expression of IL-6, a key Th17 differentiation factor, was impaired in the colon tissues of protected mice. IL-6 plays an important role in both T cell-dependent and independent models of colitis (te Velde et al., 2007, Atreya et al., 2000) and IL-6-deficient mice were reported to show resistance to TNBS-induced colitis (Gay et al., 2006, Atreya et al., 2000). Although the precise cellular source of IL-6 was not examined in this study, epithelial cells and T cells themselves, in addition to APCs, contribute to IL-6 production during colitis (Dann et al., 2008, Mudter et al., 2008). We here demonstrate that CD47 expression does not regulate IL-6 production by SIRP$\alpha^{+}$CD103$^{-}$ DCs. Therefore, the reduction of IL-6 expression observed in this study likely reflects an indirect consequence of reduced tissue infiltration.

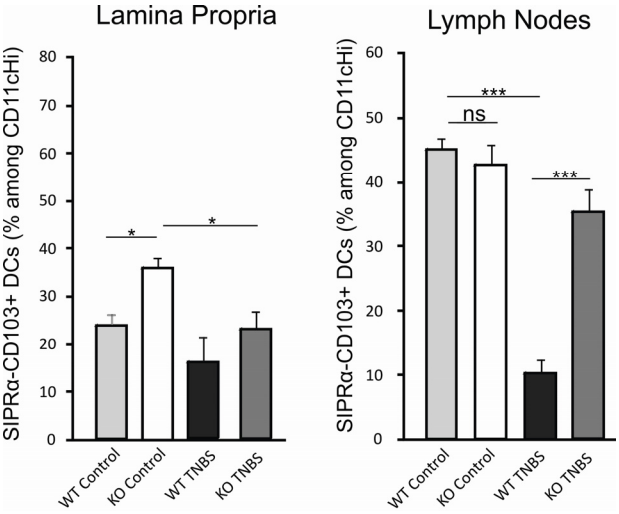
To eliminate the developmental alterations in CD47 KO mice such as the expansion of the CD103$^{+}$ $T_{reg}$ population (Van et al., 2008) and Th1 biased-phenotype (Bouguermouh et al., 2008) as a potential explanation for disease resistance, we here demonstrate that the short-term interruption of CD47 ligation by the administration of a CD47-Fc molecule

protected WT BALB/c mice from disease development. At an early time point after disease induction, we readily observed a reduced proportion of $SIRP\alpha^{+}CD103^{-}$ DCs in the mLNs and an inverted IL-17/IFNγ mRNA ratio in treated compared to untreated mice. The therapeutic efficacy of CD47-Fc could not be attributed to increased $T_{reg}$ activity, since $CD4^{+}CD25^{+}FoxP3^{+}$ $T_{reg}$ cell numbers were positively correlated with inflammation, and lower in CD47-Fc treated mice (unpublished data). Finally, early administration of CD47-Fc was shown to be highly effective in preventing both disease initiation and the recurrence of colitis after a second TNBS challenge. In that respect, the therapeutic efficacy of most published compounds has only been assessed in the prevention of acute disease (te Velde et al., 2006).

In conclusion, we have identified $SIRP\alpha^{+}CD103^{-}$ DCs as an immunogenic Th17-inducing DC subset and propose their role in the pathogenesis of TNBS-induced colitis. We further demonstrate that CD47 expression on $SIRP\alpha^{+}CD103^{-}$ DCs promotes their trafficking and the development of severe intestinal inflammation. We therefore propose that targeting the CD47/SIRPα axis may serve as the basis for the development of novel therapeutic strategies in CD.

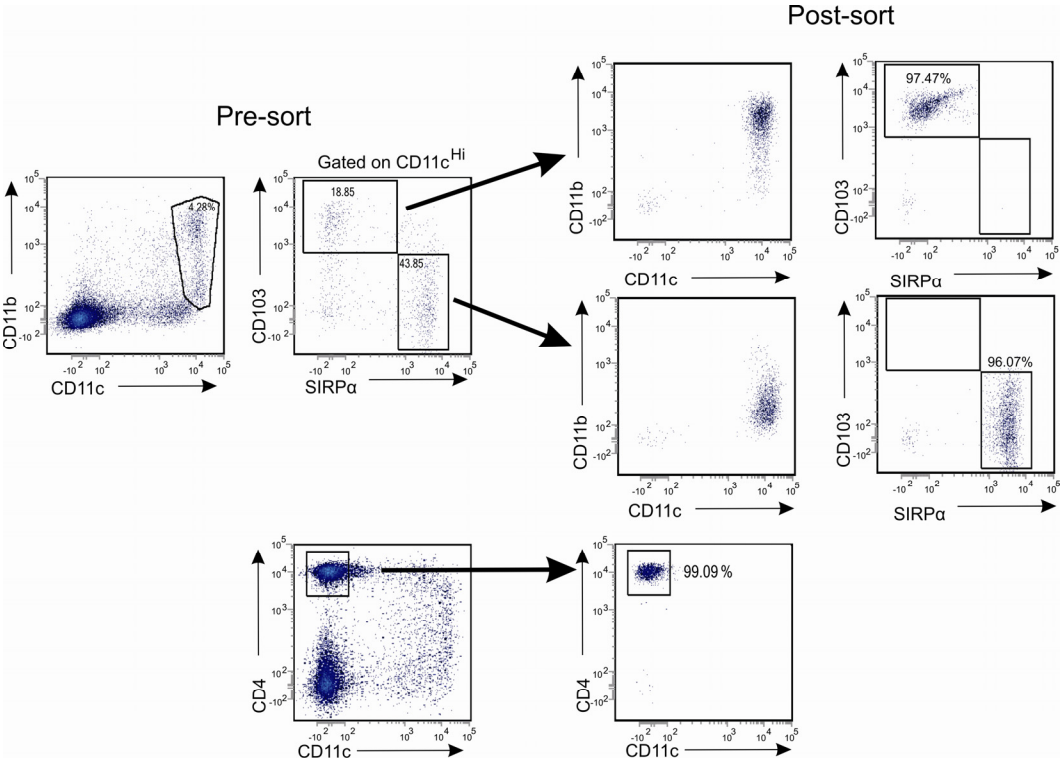
### 3.2.7 Supplemental material:

Supplemental Figure 3.1: SIRPα$^{-}$CD103$^{+}$ DC homeostasis in the lamina propria and mLNs of WT and CD47 KO mice.



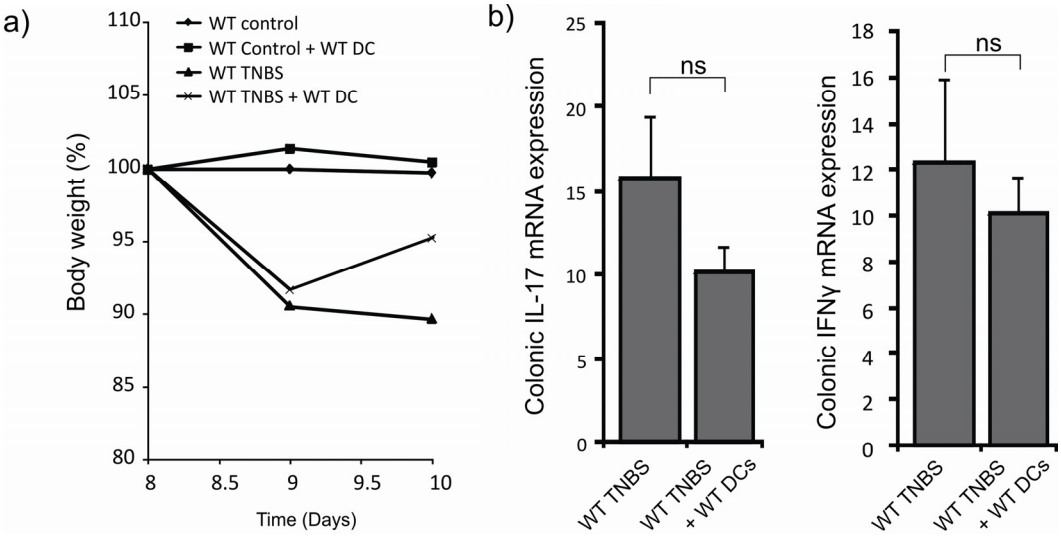

Percentage of SIRPα-CD103+ DCs among CD45.2+CD11cHi DCs in the lamina propria (left) and mLNs (right) of control mice or mice administered TNBS. Data represent the mean +/- SEM of >8 mice/group. Each experiment was independently performed a minimum of 4 times. ns: not significant, * $p<0.05$, ***$p<0.001$.

Supplemental Figure 3.2: DC and CD4+ T cell sorting strategy and purity.



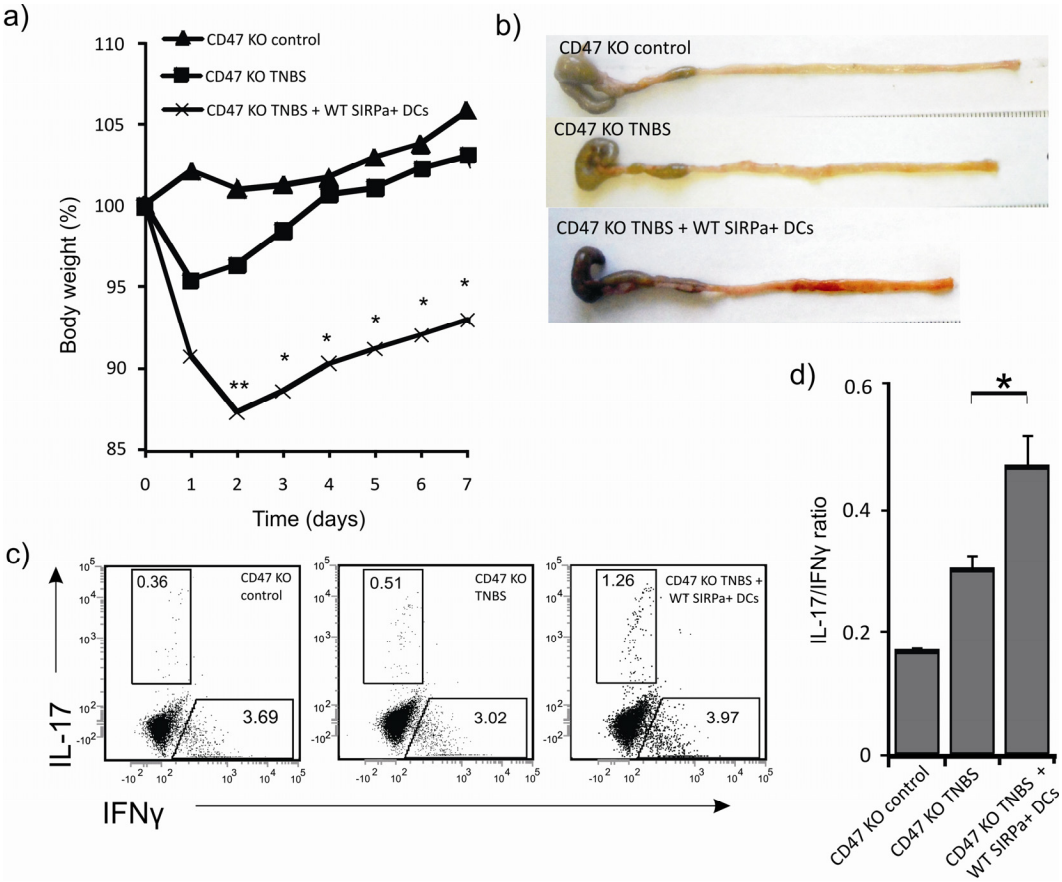

mLNs were pooled from 10 Flt3-L-treated mice and stained with anti-CD11b, CD11c, CD103, SIRPα and CD4. Left: Gating strategy for FACS sorting of DC subsets (top) and $CD4^+$ T cells (bottom). Right: Purity of sorted cells.

Supplemental Figure 3.3: WT DC transfer in WT hosts.



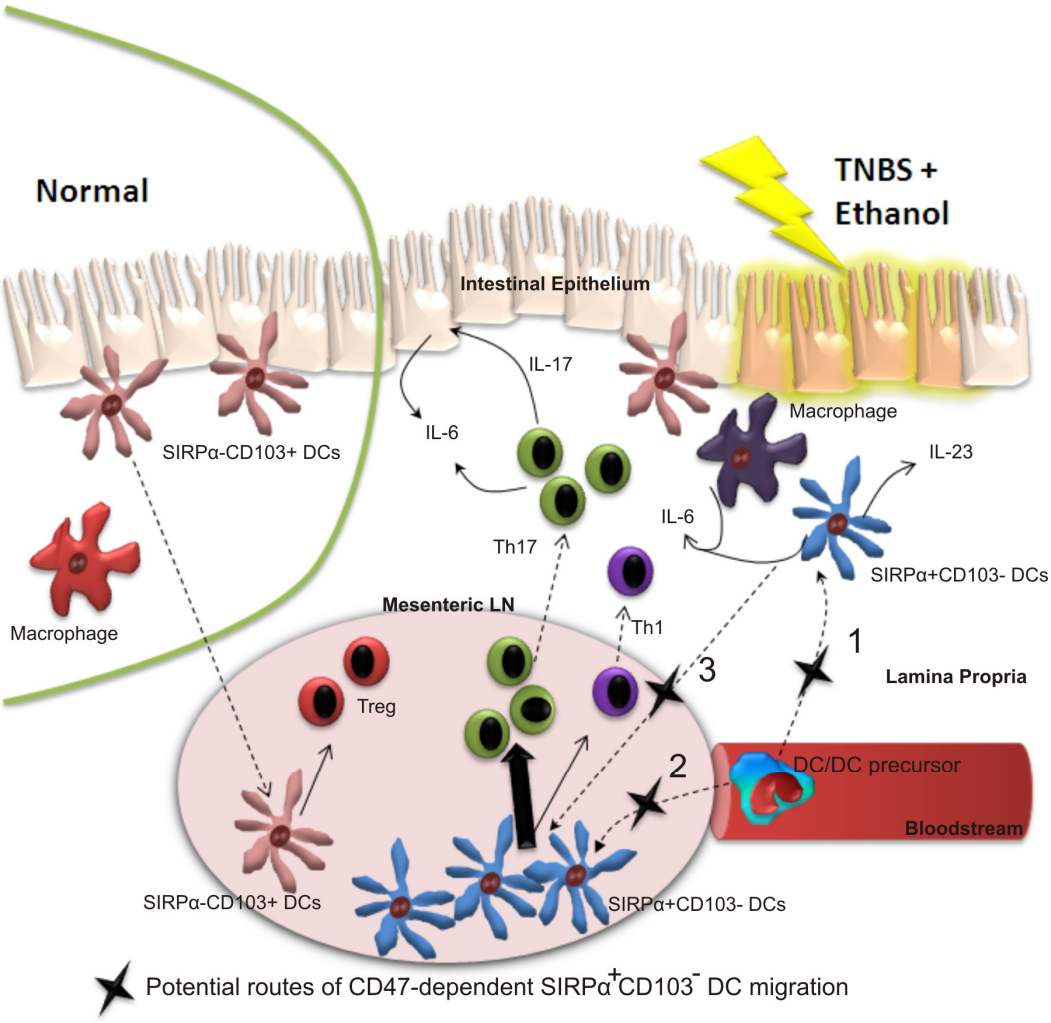

WT mice were injected i.p. with saline or WT BMDCs and TNBS colitis was induced on day 0 and day 8. (**A**) Weight loss curve after transfer of WT BMDCs, normalized to body weight on day 8. (**B**) Colonic mRNA expression of IL-17 and IFNγ. Data represent the mean fold change relative to control +/- SEM of > 5 mice per group (2 pooled independent experiments).

Supplemental Figure 3.4: SIRPα$^{+}$CD103$^{-}$ DCs isolated from mLNs promote the development of intestinal inflammation and Th17 responses.




CD47 KO mice were injected i.p. with saline or WT SIRPα$^{+}$CD103$^{-}$ DCs isolated from mLNs and TNBS colitis was induced on day 0. (**A**) Weight loss curve after DC transfer, normalized to body weight on day 0. (**B-D**) TNBS was re-induced on day 8 and mice were sacrificed 3 days later. (B) Macroscopic appearance of colonic inflammation. Images are of one representative mouse per experimental group. (C) Percentages of IL-17$^{+}$ or IFNγ$^{+}$ CD4$^{+}$ T cells in mLNs isolated after colitis and PMA/ionomycin stimulation for 6 hours. (D) Ratio of IL-17$^{+}$/IFNγ$^{+}$ cells among CD4$^{+}$ T cells. Data represent mean +/- SEM of >3 mice per group.* p<0.05

Supplemental Figure 3.5: Schematic representation of the role of CD47 in the migration of SIRPα$^{+}$CD103$^{-}$ DCs and the induction of TNBS colitis.




Under steady state conditions, SIRPα$^{-}$CD103$^{+}$ DCs migrate from the colon to the mLNs and induce $T_{reg}$. Under inflammatory conditions (TNBS + Ethanol) SIRPα$^{+}$CD103$^{-}$ DCs are recruited to the mLNs, either via the lymphatics (3) or the bloodstream (2), and drive Th17/Th1 polarization. Th17 cells arriving in inflamed lamina propria (LP) (1) amplify IL-17 and IL-6 production and perpetuate colonic inflammation. CD47 is implicated in the recruitment of SIRPα$^{+}$CD103$^{-}$ DCs from the bloodstream to the LP (1), from the bloodstream to the mLNs (2), and presumably from the LP to the mLNs (3).

### 3.2.8 Acknowledgements:

This work was supported by the Crohn's and Colitis Foundation of Canada (DF, MS, GF), the Canada Research Chair (DF), the Canadian Foundation for Innovation (DF, MS) and the Research Institute of the McGill University Health Centers (MUHC) (DF). DF is senior clinical scientist at the Belgium National Foundation for Research (FNRS).

# 4 THE IMMUNOSUPPRESSIVE POTENTIAL AND THERAPEUTIC EFFICACY OF L-CARNITINE

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A reprint of the published manuscript can be found in the appendix.

**Reference:**

Geneviève Fortin[*], Katerina Yurchenko[†], Catherine Collette[*], Manuel Rubio[‡], Alexandra-Chloé Villani[*], Alain Bitton[*], Marika Sarfati[‡] and Denis Franchimont[*] L-carnitine, a diet component and organic cation transporter OCTN ligand, displays immunosuppressive properties and abrogates intestinal inflammation. *Clin Exp Immunol.* 2009 Apr;156(1):161-71.

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## 4.1 Rationale

L-carnitine (LCAR) is a conditionally essential nutrient consumed in the diet and biosynthesized from the amino acid lysine. It is absorbed from the intestinal tract and transported across cell membranes by the organic cation transporters, OCTN1 and OCTN2. Mutations in the genes encoding OCTN1 (*SLC22A4*, 1672 C → T) and OCTN2 (*SLC22A5*, -207 G → C) have been associated with increased susceptibility to CD (Peltekova et al., 2004), suggesting that LCAR may play a role in the development of intestinal inflammation.

In Chapter 2, we identified a central role for DCs in the pathogenesis of TNBS-induced colitis. In this study, we therefore sought to evaluate the immunosuppressive potential of LCAR on DC function. We also adopted a comprehensive approach and assessed its immunosuppressive properties on other cells of the immune system, such as macrophages, B cells and $CD4^+$ T cells. Finally, we evaluated its therapeutic potential *in vivo* by assessing its effect on the clinical severity of colitis, the production of proinflammatory cytokines and proliferative responses in mLNs.

## 4.2 L-carnitine, a diet component and OCTN ligand, displays immunosuppressive properties and abrogates intestinal inflammation

### 4.2.1 Abstract

Allele variants in the L-carnitine (LCAR) transporters OCTN1 (SLC22A4, 1672 C --> T) and OCTN2 (SLC22A5, -207 G --> C) have been implicated in the susceptibility to Crohn's disease (CD). LCAR is consumed in the diet and transported actively from the intestinal lumen via the organic cation transporter OCTN2. While recognized mainly for its role in fatty acid metabolism, several lines of evidence suggest that LCAR may also display immunosuppressive properties. This study sought to investigate the immunomodulatory capacity of LCAR on antigen-presenting cell (APC) and CD4+ T cell function by examining cytokine production and the expression of activation markers in LCAR-supplemented and deficient cell culture systems. The therapeutic efficacy of its systemic administration was then evaluated during the establishment of colonic inflammation in vivo. LCAR treatment significantly inhibited both APC and CD4+ T cell function, as assessed by the expression of classical activation markers, proliferation and cytokine production. Carnitine deficiency resulted in the hyperactivation of CD4+ T cells and enhanced cytokine production. In vivo, protection from trinitrobenzene sulphonic acid colitis was observed in LCAR-treated mice and was attributed to the

abrogation of both innate [interleukin (IL)-1beta and IL-6 production] and adaptive (T cell proliferation in draining lymph nodes) immune responses. LCAR therapy may therefore represent a novel alternative therapeutic strategy and highlights the role of diet in CD.

### 4.2.2 Introduction

Crohn's disease (CD) is a chronic, relapsing inflammatory disease of the gastrointestinal tract thought to result from the aberrant recognition of enteric microbial flora, and leading to inappropriate immune responses and chronic intestinal inflammation (Elson et al., 1995, Fujino et al., 2003, Schmidt et al., 2005). Although exposure to triggers such as cigarette smoke (Tobin et al., 1987), non-steroidal anti-inflammatory drugs (NSAID) (Evans et al., 1997), and stress (Duffy et al., 1991) appear to play a role in the relapsing/remitting phases of IBD, diet is also suspected to influence the behaviour of the disease, either by influencing the microbial flora or by directly modulating the mucosal immune response of the host (Levi, 1985, Hartman et al., 2008).

L-carnitine (LCAR) is consumed in the diet and is mainly absorbed from the lumen of the digestive tract via an active mechanism requiring OCTN2 (Rebouche and Seim, 1998, Wu et al., 1999). It plays a key role in cell metabolism by regulating the mitochondrial transport of long-chain free fatty acids (LCFAs) and the generation of adenosine triphosphate (ATP) by β-oxidation (Rebouche and Engel, 1980, Rebouche, 1992, Fritz and Marquis, 1965). The role of LCAR in the gastrointestinal tract has recently become a topic of interest, since mutations in the LCAR transporter genes, *OCTN1* (*SLC22A4*, 1672 C→T) and *OCTN2* (*SLC22A5,* -207 G→C) resulted in functional impairments in LCAR uptake and an increased risk of developing CD (Peltekova et al., 2004, Vermeire

et al., 2005). While these observations have not been replicated worldwide (Van Limbergen et al., 2007b), several functional studies have given credence to the hypothesis that LCAR participates in intestinal homeostasis. For instance, OCTN2$^{-/-}$ mice spontaneously develop colonic atrophy and inflammation, a phenotype attributed to the abnormal structure and morphology of intestinal epithelial cells (Shekhawat et al., 2007). LCAR has also been shown to be a rate-limiting factor for the maintenance of physiological butyrate β-oxidation in colonocytes, and a protective effect of intra-rectal administration of carnitine-loaded liposomes was observed in experimental colitis (D'Argenio et al., 2006). However, in addition to its local role in colonocyte function, systemic LCAR may also display immunosuppressive properties, as illustrated by its ability to suppress LPS-induced cytokine production and improve murine survival rates during cachexia and septic shock (Winter et al., 1995). LCAR has also been shown to reduce $CD4^+$ and $CD8^+$ T cell numbers and IL-2 production in splenocytes isolated from LCAR-treated mice (Athanassakis et al., 2001) and reduce TNFα production in *Staphylococcus aureus*-stimulated human polymorphonuclear cells (Fattorossi et al., 1993). Interestingly, previous reports have demonstrated that high doses of LCAR can activate glucocorticoid receptor alpha (GRα) and may share some biological and therapeutic effects with glucocoticoids (Alesci et al., 2003).

While the above data suggest a role for LCAR in immune function, other studies have been reported with contradictory results, due in part to the complexity of the immune response and variation between experimental conditions (Kouttab and De Simone, 1993, De Simone et al., 1982, De Simone et al., 1994). In this study, we present evidence to clarify and directly examine the impact of LCAR on APC and T cell function with respect to the expression of key activation markers and cytokines. Our *in vitro* observations are then validated by investigating the therapeutic efficacy of systemic LCAR supplementation in murine trinitrobenzene sulfonic acid (TNBS) colitis, a model exhibiting many of the same clinical and histological features as human CD.

### 4.2.3 Materials and Methods

**Reagents and antibodies:**

TNBS and LCAR were purchased from Sigma Chemical Co. (St. Louis, MO). Biotinylated anti-CD11c (HL3), anti-CD11b-FITC (M1/70), anti-CD4-PerCP (L3T4) and anti-annexin-V-APC were obtained from BD Pharmingen (Mississauga, ON). Anti-MHCII-FITC (NIMR-4), anti-B220-PE (RA3-6B2), anti-CD86-PE (GL1), anti-CD80-PE (16-10A1), anti-CD3-PE (145-2C11), anti-CD69-FITC (H1.2F3), and anti-CD25-APC (PC61) were obtained from eBioscience (San Diego, CA).

**Animals:**

6- to 8-week-old male Balb/c and Balb/cByJ mice, were obtained from Jackson Laboratories, maintained under conventional housing conditions and given free access to standard food and water. All mice were handled according to institutionally recommended animal care guidelines and all experiments were approved by the Animal Studies Ethics Committee of McGill University.

**Cell culture conditions:**

Spleens were harvested from 6-8 week old male Balb/c or carnitine-deficient mice. Cells were cultured at a concentration of $1X10^6$ cells/ml in RPMI 1640 (Invitrogen Life Technogicies, Carlsbad, CA), supplemented with 10% FCS (Hyclone, Logan, UT), penicillin (100 U/ml),

streptomycin (100 ug/ml), 10 mM Hepes, and 50 μM 2-ME (Sigma Chemical Co., St. Louis, MO). Cells were treated with 0, 10, 100 or 300 mM LCAR. To assess APC function, total splenocytes or purified APCs were stimulated with 1 μg/ml E. coli lipopolysaccharide (LPS) (Sigma Chemical Co.) for 18 hours. To assess T cell function, splenocytes were incubated in the presence of plate-bound hamster anti-mouse CD3 antibody (1 μg/ml) (eBioscience, San Diego, CA) for 72 hours. Pure $CD4^+$ T-cells were stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (2 μg/ml) for 72 hours.

**Flow cytometry:**

To assess the effect of LCAR on APC activation, splenocytes were stained with anti-MHCII-FITC and CD86-PE or anti-MHCII-FITC and anti-CD80-PE. Mean fluorescence intensity (MFI) was calculated as a measure of surface co-stimulatory molecule expression. Upon 18-hour exposure of splenocytes to LCAR, toxicity was evaluated by staining with annexin-V-APC. T cell activation was assessed by staining splenocytes, activated in the presence of anti-CD3, with anti-CD4-PerCP, anti-CD3-PE, anti-CD69-FITC and anti-CD25-APC. All flow cytometric analysis was performed using FlowJo software (version 5.7.2).

**Cell sorting:**

Spleens (for APCs) or mesenteric lymph nodes mLNs (for $CD4^+$ T cells) were harvested from Balb/c mice. Splenocytes were stained with anti-CD11b-FITC, anti-B220-PE and anti-CD11c-APC. Dendritic cells were selected as $CD11c^+$ cells, macrophages as $CD11c^-CD11b^+$ and B cells as $CD11c^-B220^+$. Cells isolated from mLNs were stained with anti-CD4-PerCP and anti-CD3-PE (BD Pharmingen, San Diego, CA), and double positive cells were sorted and cultured. The cell suspensions were sorted by a BD FACSAria cell sorting system (BD Biosciences, San Jose, CA). Cell purity was >99%.

**BrdU and [$H^3$] thymidine incorporation assays:**

Splenocytes or pure $CD4^+$ T cells cultured in a 96-well microplate were incubated with BrdU for 6 hours (Roche Applied Science, Laval, QC). The labeled cells were fixed with ethanol and partially digested with nucleases to allow an anti-BrdU antibody (labeled with peroxidase (POD)) to access and bind to BrdU. POD catalyzed the cleavage of ABTS, producing a colored reaction product. The absorbance of the samples (at 405 nm) was determined with a standard microplate reader and represents the number of actively dividing cells during the 6 hour incubation period.

[$H^3$]thymidine incorporation was used to assess proliferation after the *ex vivo* culture of colon-draining sacral lymph nodes (sLNs). sLNs

were isolated after re-induction of TNBS colitis and incubated in the presence of 0.3 mg/ml TNBS for 15 minutes at room temperature (Dohi et al., 1999). Cells were then extensively washed and cultured for 4 days in complete RPMI 1640. [$H^3$]thymidine (0.5 µCi/well) was added for the last 18 hours of culture. The amount of [$H^3$]thymidine incorporated was measured by scintillation counting.

**Induction of TNBS Colitis:**

TNBS (100mg/kg) dissolved in 50% ethanol was introduced into the colon via a 3.5F catheter fitted to a 1mL syringe, in isofluorane-anesthetized mice. Control mice received intra-rectal saline using the same technique. LCAR (100 or 150 mg/kg dissolved in saline) or vehicle (saline alone) was administered intra-peritoneally once daily during the entire duration of colitis, with the first dose administered 30 minutes prior to induction of colitis. To assess T cell responses, colitis was re-induced 7 days after the first injection and the mice were sacrificed on day 10.

**Assessment of colonic damage:**

The macroscopic severity of colon damage was assessed according to the Wallace criteria as previously described (Wallace et al., 1998). For histological assessment, 2µM-thick sections were stained with hematoxilin and eosin (Dieleman et al., 1996) and histological changes were graded semi-quantitatively based on a set of previously established

criteria (Ameho et al., 1997). The grading scale ranged from 0-13, and was calculated as the sum of scores for: expansion of submucosa (0-4), expansion of lamina propria (0-4), loss of globlet cells (0-4) and neutrophil infiltration (0-1). All macroscopic and microscopic scoring was performed in a blinded fashion.

**Quantitative real-time PCR for inflammatory cytokines:**

Colonic RNA was extracted following the TRIzol protocol (Invitrogen, Burlington, ON). Total RNA was reverse-transcribed using the cDNA reverse transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) (1 PCR cycle, 95°C, 10 minutes; 40 PCR cycles, 60°C, 1 minute, 95°C, 15 seconds). cDNA was amplified in a 10 μL final reaction mix containing TaqMan Universal PCR Master Mix (Applied Biosystems) and corresponding TaqMan® Gene Expression Assays (Mm00446190_m1 (IL-6), Mm00434228_m1 (IL-1β), Hs99999901_s1 (Eukaryotic 18s rRNA), Applied Biosystems). Signals were analyzed by the ABI Prism Sequence Detection System software version 2.2 (Applied Biosystems). The comparative Ct method for relative quantification was used, where all threshold cycles (Ct) are first normalized to the expression of 18s rRNA. Here, the cytokine expression is represented as a fold-change relative to control mice.

**Cytokine Quantification/ELISA:**

Whole blood was withdrawn immediately post-mortem and sera were frozen at -20˚C until use. Serum (IL-6 and IL-1β) and culture supernatant cytokines (IL-1β, IL-6, IFNγ, IL-4 and IL-5) were quantified by Quantikine ELISA kits (R&D systems, Minneapolis, MN).

**Statistical analysis:**

All values are expressed as mean +/- SEM. Changes in body weight were compared by Kruskal-Wallis ANOVA. The macroscopic and histologic scores between TNBS and TNBS plus LCAR groups and *in vitro* data were analyzed by two-tailed Student's *t* test for unpaired samples. qRT-PCR cytokine mRNA expression data were analyzed with a Mann-Whitney U test.

### 4.2.4 Results

**LCAR displays immunosuppressive properties**

To assess the immunosuppressive actions of LCAR, total splenocytes were stimulated with LPS and cultured in the presence of LCAR *in vitro*. The seemingly high doses of LCAR were selected based on previously published data demonstrating pharmacological activity in the absence of toxicity at these doses (Alesci et al., 2003). LPS stimulation lead to a significant increase in cell proliferation compared to unstimulated cultures (Fig. 4.1a), and a dose-dependent suppression of proliferation was induced by LCAR treatment of LPS-stimulated cells, reaching statistical significance at 100 mM LCAR (Fig 4.1a). Next, the effect of LCAR was assessed on APC function by CD80 (B7-1) and CD86 (B7-2) expression (mean fluorescence intensity, (MFI)) on major histocompatibility complex class II (MHC II) positive cells, a marker of APCs. CD80 and CD86 are co-stimulatory molecules expressed on APCs that provide the necessary stimuli to prime T cells via CD28 and promote activation and T cell survival (Freeman et al., 1993, Hathcock et al., 1994). At 100 mM LCAR, a significant reduction in both CD80 and CD86 MFI was observed, signifying a reduction in the number of surface co-stimulatory molecules present per APC (Fig 4.1b,c). Notably, no changes in proliferation or expression of costimulatory molecules were observed in unstimulated cultures treated with LCAR (Fig 4.1a-c). The suppressive effect of LCAR on LPS-stimulated cells could not be attributed to the

induction of cell death, as the percentages of live cells (DiOC6(3)$^+$) (data not shown) and apoptotic cells (annexin V$^+$) (Fig. 4.1d) were not altered by LCAR treatment, corroborating previously published data (Alesci et al., 2003). The percentage of apoptotic cells was significantly increased at a dose of 300 mM LCAR (data not shown) and this dose was therefore eliminated from further assessment of LCAR function. These data demonstrate that LCAR exerts immunosuppressive effects on APC function without displaying toxicity.

**LCAR suppresses dendritic cell and macrophage function *in vitro***

Since MHC II is expressed on all professional APCs, including dendritic cells (DCs), macrophages and B cells, the cell type affected by LCAR treatment could not be determined in the previous experiment. We therefore purified CD11c$^+$ DCs, CD11b$^+$ macrophages and B220$^+$ B cells by cell sorting and individually stimulated them in the presence of LPS to assess their responsiveness to LCAR. CD86 expression was significantly reduced in DC and macrophage cultures, but not in B cell cultures (Fig 4.2a-c). These data specifically implicate DCs and macrophages in LCAR's immunosuppressive action and indicate that they can be efficiently suppressed in the absence of T cells and other cell types normally present in the spleen. We also assessed cytokine production by splenocytes, pure DCs, pure macrophages and pure B cells. IL-6, IL-1β and TNFα production were dose-dependently suppressed by LCAR in DC,

macrophage and mixed splenocyte cultures (Fig 4.2d-f), while B cell cultures were unaffected (data not shown). Therefore, LCAR can directly suppress DC and macrophage activation and cytokine production.

**LCAR suppresses $CD4^+$ T cell function *in vitro***

In addition to aberrant innate immune responses, CD involves inappropriate T cell responses to harmless antigens (Elson et al., 1996). We therefore sought to examine the effect of LCAR on $CD4^+$ T cell function. Splenocytes were stimulated with plate-bound anti-CD3 to activate T cells and cultured for 72 hours in the presence of LCAR. LCAR significantly suppressed anti-CD3-induced $CD4^+$ T cell activation, with a greater than 50% reduction in the number of double-positive ($CD69^+CD25^+$) cells (Fig 4.3a).

Given that splenocytes contain a mixture of cell types, assessments of T cell proliferation and cytokine production were performed on purified $CD4^+$ T cells. This experiment also addressed whether LCAR could suppress T cell responses independently of the presence of APCs. mLNs were selected as the source of T cells since they contain a greater percentage of T cells than spleens, and more accurately represent mucosal immune responses. After 72 hours of plate-bound anti-CD3 stimulation, LCAR completely abolished $CD4^+$ T cell proliferation as assessed by BrdU incorporation (Fig 4.3b), while no effect was observed in unstimulated cultures. LCAR-mediated suppression of purified $CD4^+$ T

cell proliferation was also observed when T cells were stimulated with soluble anti-CD3 in the presence of soluble anti-CD28 or mitomycin C-treated APCs, as assessed by [$H^3$]thymidine incorporation (data not shown). In response to soluble anti-CD3 plus anti-CD28 stimulation, the production of the classical Th1 cytokine, IFNγ, as well as two Th2-associated cytokines, IL-4 and IL-5, were significantly diminished by treatment with LCAR (Fig 4.3c-e). At this time point, IL-2 concentration was too low to quantify. Therefore, *in vitro* treatment with LCAR appears to exhibit immunosuppressive properties at the level of both APCs and $CD4^+$ T cells.

**Carnitine-deficient $CD4^+$ T cells become hyper-activated upon stimulation**

Since the addition of LCAR to APC and T cell cultures resulted in immunosuppression, we sought to ascertain whether a carnitine deficiency might affect the sensitivity of $CD4^+$ T cells to stimulation. Balb/cByJ mice are SCAD (short-chain Acyl-CoA dehydrogenase) deficient and display a defect in the conversion of short chain fatty acids such as butyrate into acetyl-CoA (Wood et al., 1989). Butyrate therefore accumulates inside the mitochondria and is converted to butyrylcarnitine by carnitine acetyltransferase. During this conversion, carnitine stores are used up, resulting in a secondary carnitine deficiency (Wood et al., 1989).

In this study, splenocytes were obtained from unmanipulated Balb/c and carnitine-deficient mice and stimulated for 72 hours in the presence of plate-bound anti-CD3. $CD4^+$ T cells from carnitine-deficient mice displayed a hyper-activated phenotype characterized by the expression of CD69 and CD25 and a significant enhancement of IFNγ production (Fig 4.4a,b). $CD4^+$ T cell hyper-activation and IFNγ production were both reversed by supplementation with LCAR (100 mM) in the culture medium (Fig. 4.4a,b). We therefore conclude that carnitine supplementation can restore a normal immune response in otherwise hyper-activated carnitine-deficient $CD4^+$ T cells.

**LCAR therapy impairs the expression of intestinal pro-inflammatory cytokines and abrogates TNBS colitis**

TNBS colitis is driven by the interplay between innate and adaptive immune responses. Given LCAR's immunosuppressive properties on both arms of the immune system, we sought to investigate the therapeutic efficacy of systemic LCAR administration in the TNBS colitis model. Colitis was induced in Balb/c mice and LCAR (100 or 150 mg/kg) was administered intra-peritoneally once daily. The intraperitoneal route was selected to minimize the trauma associated with daily intravenous injections or oral gavage. Since feeding behavior is reduced and highly variable between mice after the induction of TNBS colitis, LCAR supplementation of the food or water was also not a feasible option.

Within one day of intrarectal instillation of TNBS, severe wasting of body weight and diarrhea were observed in both the LCAR-treated (high dose: 150 mg/kg; low dose 100 mg/kg) and untreated groups, while control mice maintained their original body weights (Fig. 4.5a). However, LCAR treatment resulted in significant improvements in the body weights of mice with colitis by day 2 for the high dose group and by day 3 for the low dose group (Fig 4.5a).

Since high dose LCAR proved most effective in ameliorating body weight loss, an in-depth analysis of markers of inflammation was performed on colon tissues from these mice. Upon visual inspection, the macroscopic severity of colitis was rated by the Wallace criteria, where LCAR-treated mice displayed approximately a 70% reduction in inflammation (Fig. 4.5b). Control mice showed no macroscopic signs of inflammation (Score = 0). Histological grading of frozen sections also showed no inflammatory infiltrates in non-colitic mice (Score=0). Importantly, the administration of LCAR in healthy mice did not result in any noticeable effects on body weight, the appearance and histology of the colon or any other criteria examined. In mice with TNBS colitis, the area of most severe inflammation was the distal half of the colon, where a loss of goblet cells, distortion of the crypts, and infiltration of mononuclear cells were evident. Such histological changes were significantly reduced by treatment with LCAR (Fig 4.5c,d). Therefore, LCAR was effective in

suppressing the development of intestinal inflammation and associated body weight loss in mice with TNBS colitis.

The inflammatory cytokines IL-6 and IL-1β are instrumental in the initiation and maintenance of the inflammation characteristic of both human CD and TNBS colitis (Fiocchi, 1989, Elson et al., 1996). Here, the mRNA expression of these key proinflammatory cytokines was assessed in colonic tissues of mice, and treatment with LCAR resulted in an approximately 5-fold reduction in the colonic expression of both IL-1β and IL-6 mRNA when compared to untreated mice with TNBS colitis (Fig 4.5e,f). In addition to its local anti-inflammatory effects, LCAR therapy also significantly reduced the serum levels of IL-1β and IL-6, underscoring the beneficial systemic outcome of LCAR's local anti-inflammatory effects (Fig 4.5g,h). Therefore, LCAR's therapeutic efficacy in treating TNBS colitis may be attributed to its ability to suppress the expression of pro-inflammatory cytokines, corroborating our *in vitro* data.

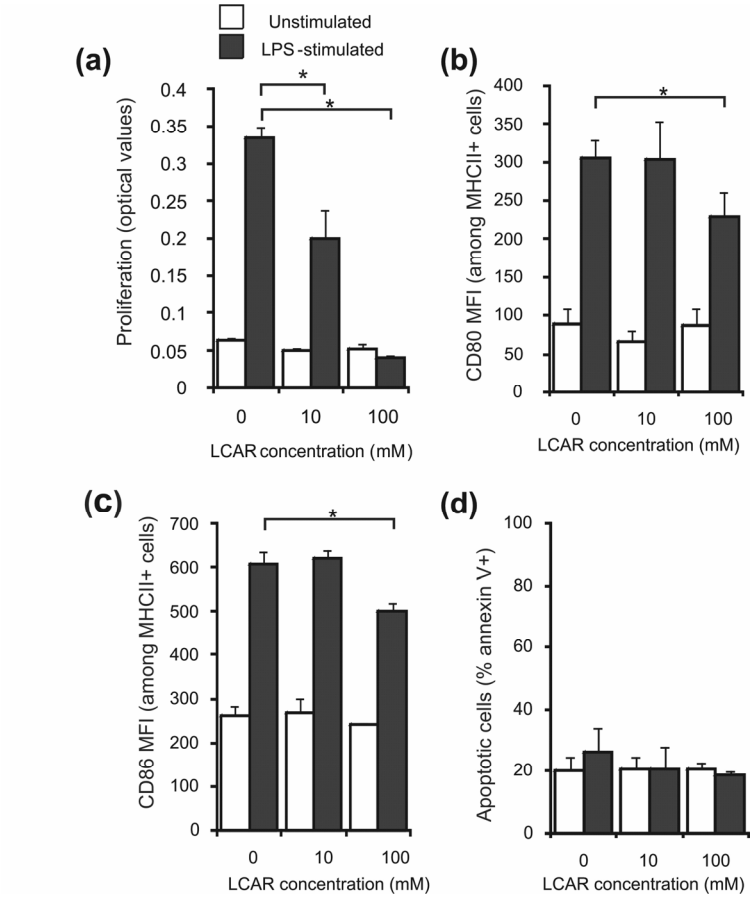
**LCAR therapy inhibits T cell responses in TNBS colitis**

Our *in vitro* data demonstrated that LCAR could inhibit both the innate and adaptive arms of the immune response. Since one injection of TNBS in Balb/c mice typically results in an acute, T cell independent inflammatory response, a second injection of TNBS is required to produce a chronic, T-cell driven form of intestinal inflammation. We therefore re-induced colitis 7 days after the initial TNBS injection and isolated colon-

draining sLNs. Total sLN cells were re-stimulated *ex vivo* with TNBS, and antigen-specific T cell responses were assessed. *In vivo* treatment with LCAR resulted in a significant reduction of *ex vivo* cell proliferation (Fig 4.6), indicating that in addition to its immunosuppressive effect on the acute inflammatory response, LCAR administration also suppressed adaptive immune responses, an important consideration for the potential of this therapy in CD.

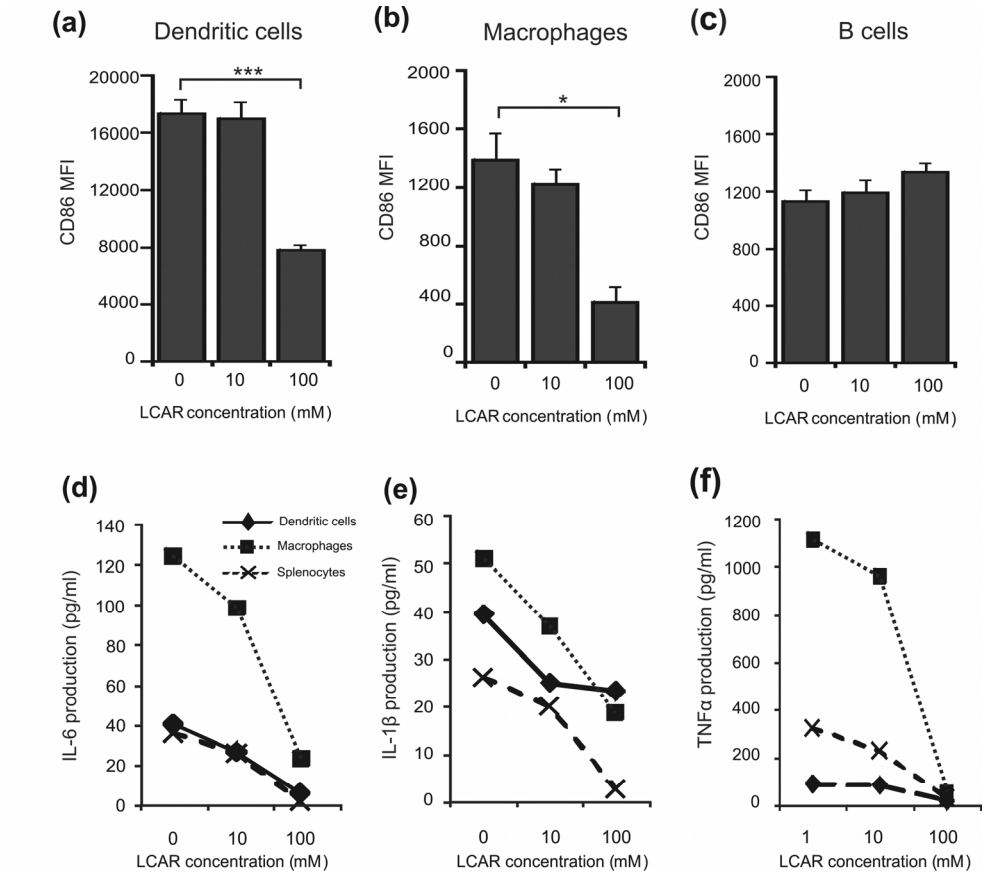
### 4.2.5 Figures

Figure 4.1: LCAR suppresses APC function *in vitro*.



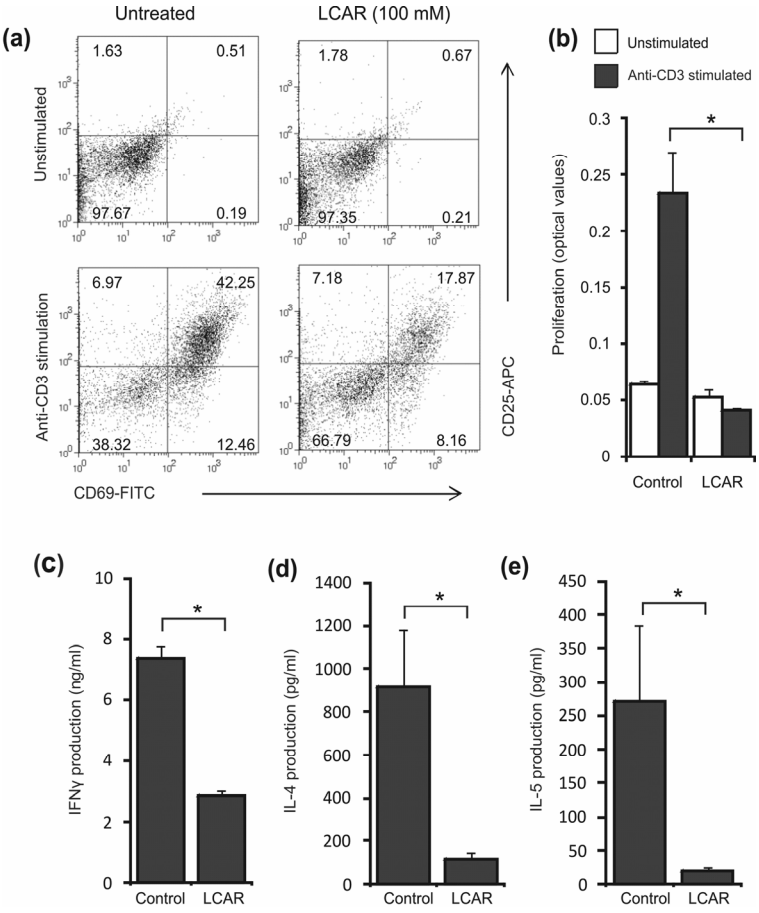

Splenocytes were harvested from healthy Balb/c mice and stimulated with LPS (1 µg/ml) for 18 hours in the presence of LCAR (0, 10, or 100 mM). a) Cell proliferation was assessed by BrdU incorporation. APC activation was assessed by flow cytometric analysis of CD80 (b) and CD86 (c) surface expression on individual MHC $II^+$ cells, as represented by the mean fluorescence intensity (MFI). d) Apoptosis was assessed by flow cytometric analysis of annexin-V staining. Data represent the mean +/- SEM (n=3). * $p<0.05$

Figure 4.2: LCAR specifically suppresses DC and macrophage activation and cytokine production.



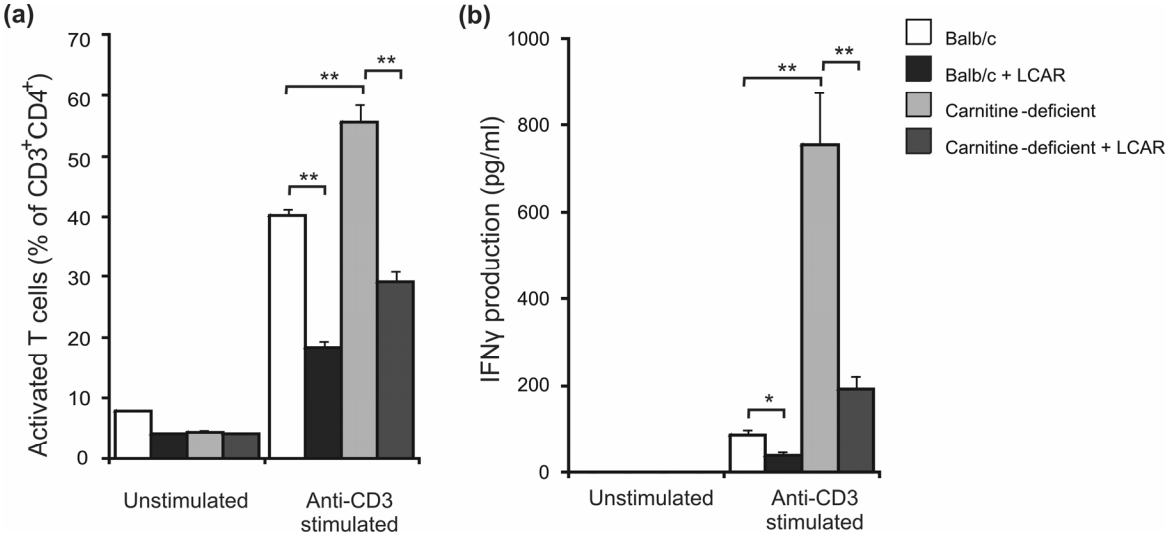

Total splenocytes were sorted by flow cytometry to obtain pure DCs ($CD11c^+$), macrophages ($CD11c^-CD11b^+$) or B cells ($CD11c^-B220^+$) and cultured in parallel with unsorted splenocytes. Cells were stimulated with LPS (1 µg/ml) and cultured for 18 hours in the presence of LCAR (0, 10, or 100 mM). a-c) CD86 expression was assessed by flow cytometric analysis of mean fluorescence intensity (MFI) among purified DCs (a), macrophages (b) and B cells (c). d) IL-6, e) IL-1β and f) TNFα production were quantified in culture supernatants of sorted and unsorted splenocytes. Data represent the mean +/- SEM (n=3). * $p$<0.05; *** $p$<0.001

Figure 4.3: LCAR suppresses $CD4^+$ T-cell activation, proliferation and cytokine production.



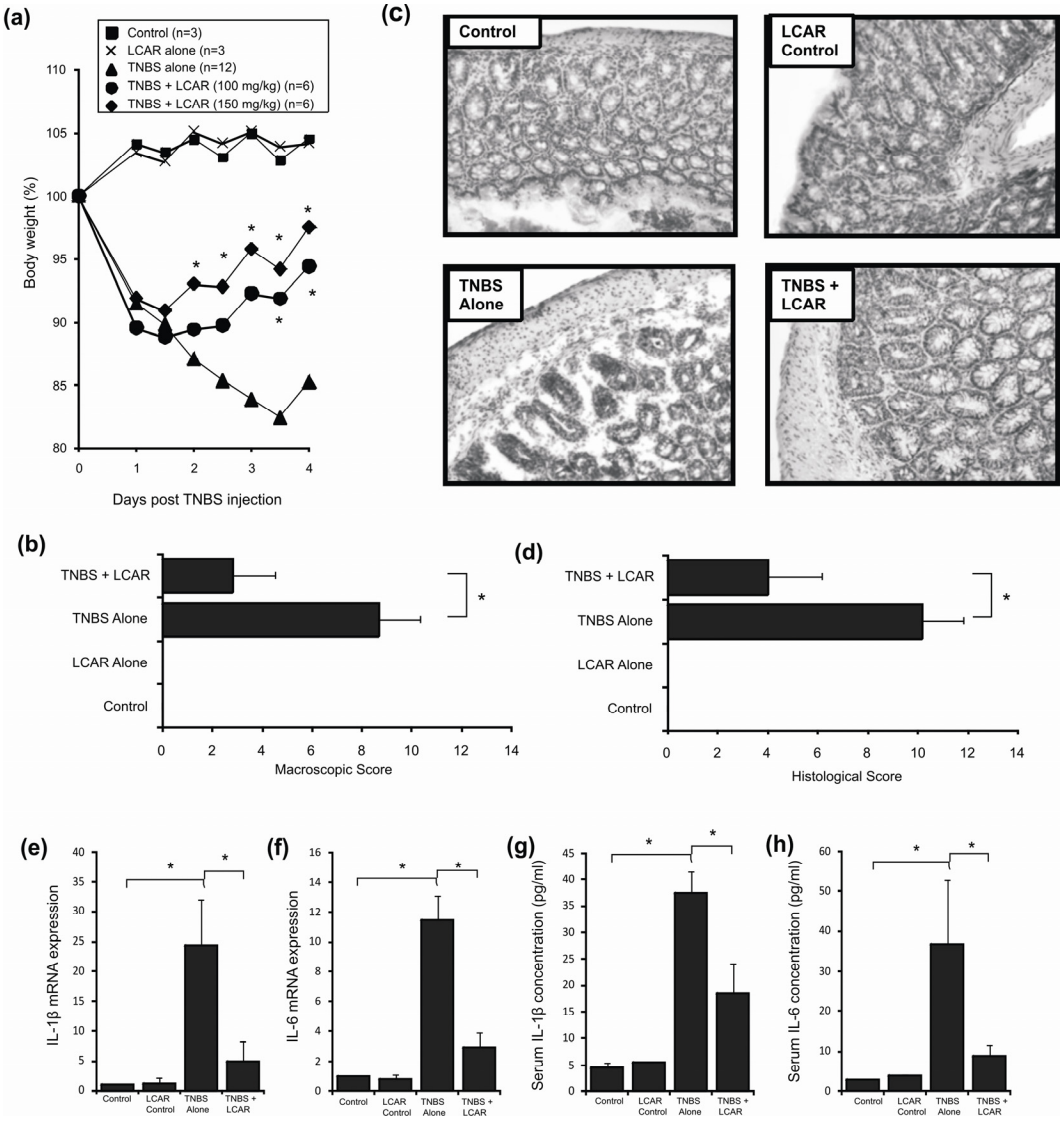

Splenocytes were stimulated with plate-bound anti-CD3 (1 µg/ml) in the presence of LCAR (0, or 100 mM) for 72 hours. a) $CD4^+$ T cell activation was assessed by gating on $CD3^+CD4^+$ cells and determining the percentage of $CD69^+$, $CD25^+$ or double-positive cells after overnight culture. b) Purified $CD4^+$ T cells were cultured in the presence of LCAR (0 or 100 mM) and stimulated with plate-bound anti-CD3 for 72 hours to assess proliferation by BrdU incorporation. c-e) Purified $CD4^+$ T cells were cultured in the presence of LCAR (0 or 100 mM) and stimulated with plate-bound anti-CD3 and soluble anti-CD28 for 72 hours. c) IFNγ, d) IL-4 and e) IL-5 production were assessed by ELISA. Data represent mean +/- SEM (n=3). * $p < 0.05$

Figure 4.4: Hyper-activation of carnitine deficient cells is reversed by treatment with LCAR.




Splenocytes were isolated from healthy Balb/c and carnitine-deficient mice and stimulated with anti-CD3 overnight in the presence or absence of LCAR (0 or 100 mM). a) CD4+ T cell activation was assessed by gating on $CD3^+CD4^+$ cells and determining the percentage of $CD69^+CD25^+$ cells after overnight culture. b) IFNγ production in the supernatants of cultures was assessed by ELISA. Data represent mean +/- SEM (n=3). * $p < 0.05$, ** $p < 0.01$

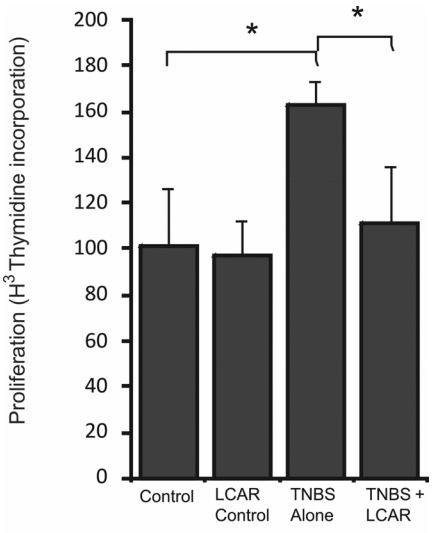
Figure 4.5: LCAR therapy is effective in treating TNBS colitis.




TNBS colitis was induced by intrarectal administration of 100 mg/kg TNBS dissolved in 50% ethanol and mice were treated every 24 hours with LCAR (100 or 150 mg/kg), starting 30 minutes prior to the induction of colitis. a) Body weight was recorded twice daily and is expressed as a percentage of body weight on day 0. Each data point represents the mean +/- SEM of n=3 control mice and n=6 mice with TNBS colitis per group. * $p < 0.05$ compared to untreated TNBS. b) The macroscopic score of colon inflammation was determined by the Wallace criteria. c) H & E stained

sections of distal colon (Original magnification, 400X). d) The histological score of colon damage. e) mRNA expression of IL-1β and f) IL-6 was determined in whole colon homogenates by quantitative real-time PCR. Data are expressed as mean fold-change relative to control +/- SEM. g) Serum cytokine levels of IL-1β and h) IL-6 were quantified by ELISA. Data are expressed as mean +/- SEM (n=3 for control groups, n=6 for TNBS groups). * $p < 0.05$

Figure 4.6: LCAR suppresses T cell responses *in vivo*




Chronic TNBS colitis was induced by two intrarectal injections (day 0 and day 7) of 100 mg/kg TNBS dissolved in 50% ethanol. Mice were treated every 24 hours with LCAR (150 mg/kg), starting 30 minutes prior to the induction of colitis. Colon-draining lymph nodes were isolated 3 days after the final TNBS injection and re-stimulated *ex vivo* with TNBS. Antigen-specific proliferation was assessed by [$H^3$]thymidine incorporation after 4 days of culture. Data are expressed as mean +/- SEM (n=3 for control groups, n=5 for TNBS groups). * $p < 0.05$

### 4.2.6 Discussion

The interplay between both innate and adaptive immune responses is crucial to perpetuate inflammation in human CD. In this study, we confirm that LCAR can dose-dependently suppress DC and macrophage co-stimulatory molecule expression and are the first to describe its effects on purified $CD4^+$ T cell activation and cytokine production. Furthermore, we demonstrate that carnitine deficiency results in T-cell hyperactivation, and can be reversed by LCAR supplementation. Finally, we demonstrate the therapeutic potential of LCAR in treating the acute and chronic aspects of intestinal inflammation.

TNBS colitis mimics human CD in that it generates mucosal inflammation which is dependent upon the presence of bacteria in the gut lumen and results in the transmural infiltration of mononuclear cells (Neurath et al., 1995). Although once thought to be primarily driven by adaptive immune responses, innate cells are now recognized as playing a key role in the initiation phase of TNBS colitis (Santucci et al., 2007). T cells, on the other hand, are likely implicated in the amplification and perpetuation of inflammation (Sheibanie et al., 2007b). In support of our *in vitro* data, systemic administration of free LCAR was effective in treating TNBS colitis. This protection was characterized by an improvement in all clinical and histological criteria in mice treated with daily injections of LCAR and was associated with a suppressive effect on the colonic mRNA expression and serum levels of IL-1β and IL-6. Importantly, LCAR was

also effective in dampening antigen-specific T cell responses in sLNs of mice with chronic TNBS colitis.

A recent study investigating the role of carnitine transporters in butyrate metabolism in colonocytes demonstrated a protective role of the local administration of carnitine-loaded liposomes in TNBS colitis (D'Argenio et al., 2006). However, in contrast with the current study, the direct effect of carnitine administration on the immune response was not examined. Additionally, we provide evidence that systemic administration of pure carnitine, as opposed to local administration of carnitine-loaded liposomes, is also protective in the development of TNBS colitis. This observation may therefore have implications for the clinical translation of LCAR therapy, both in terms of cost and route of administration. Taken together, LCAR's therapeutic efficacy in treating TNBS colitis may be due to the combination of its protective effects on colonocyte structure and metabolism as well as its immunosuppressive action during the generation of immune responses.

We had initially aimed to assess whether mice with a carnitine deficiency were predisposed to developing intestinal inflammation induced by TNBS. However, upon induction of colitis, carnitine-deficient mice were significantly more susceptible to TNBS-induced mortality compared to wild type mice, with up to 75% mortality per experiment. The sudden deaths of carnitine-deficient mice upon exposure to TNBS may have resulted from metabolic disturbances, since carnitine-deficient mice develop

hypoglycemia after 18 hours of fasting (Wood et al., 1989). Alternatively, since mice deficient in the carnitine transporter, OCTN2, develop spontaneous atrophy of intestinal epithelial cells and colonic inflammation (Shekhawat et al., 2007), a disturbance in the intestinal barrier function of carnitine-deficient mice may also have resulted in a similar defect and warrants further investigation. Due to the yet unexplained high mortality rate of carnitine-deficient mice upon exposure to TNBS, the immune response of these mice could only be observed *in vitro.* Here, we have shown that carnitine deficiency promotes the hyperactivation of $CD4^+$ T cells and the production of the classical Th1 cytokine, IFNγ.

In this study, the precise immunosuppressive mechanism of action of LCAR on APC and T cell activation remains elusive. However, there are a number of potential mechanisms which may participate in this effect. First, antioxidants have recognized protective roles on the intestinal mucosa by preventing ROS production (D'Odorico et al., 2001, D'Argenio et al., 1996) and play a critical role in preventing inflammation and cancer (Frenkel, 1992, Halliwell and Gutteridge, 1984). Recent studies have demonstrated that LCAR can act as an antioxidant and protect from ROS-induced tissue damage (Wang et al., 2007, Rauchova et al., 2002). In fact, LCAR is more effective at inhibiting lipid peroxidation than both trolox and alpha-tocopherol (Vitamin E), two widely recognized antioxidants (Gulcin, 2006). Thus, LCAR's antioxidant properties may therefore participate in its immunosuppressive capacity. Alternatively, LCAR have been shown to

directly enhance the nuclear translocation and transcriptional activity of GRα (Alesci et al., 2003). The optimal LCAR concentration to induce GRα translocation was 100 mM, the same concentration used in this study. The physiological relevance of this similarity is underscored by the fact that tissue LCAR concentrations as high as 100 mM, have been described (Alesci et al., 2003). Therefore, among other potential mechanisms, LCAR may suppress immune responses by either quenching ROS, and thereby inhibiting the third signal for T cell activation, or by directly activating GRα translocation and mimicking the known immunosuppressive properties of glucocortocoids.

While the association between mutations in the *OCTN* genes and CD susceptibility has not been replicated worldwide, our results support the aforementioned candidate gene in predisposing individuals to CD and highlight the potential therapeutic efficacy of LCAR supplementation. We here confirm and expand the evidence to support an immunosuppressive role for LCAR on APC and T cell function and demonstrate the therapeutic value of its systemic administration in treating intestinal inflammation.

### 4.2.7 Acknowledgements

The authors wish to thank Dr. Jeremy Jass (Department of Pathology, McGill University, Montreal, Qc) for his assistance in grading histological changes in the colons of mice with TNBS colitis, and Dr. Ciriacco Piccirillo (Department of Microbiology and Immunology, McGill University, Montreal, Qc) for insightful discussion and use of his laboratory equipment. This study was funded in part by the Crohn's and Colitis Foundation of Canada, grant number 3801 and the Research Institute of the McGill University Health Center.

# 5 Summary & Perspectives:

## 5.1 General discussion of the chapters

This thesis has uncovered two novel target pathways implicated in intestinal inflammation and highlights the potential application of inhibitors of CD47/SIRPα interactions and the therapeutic value of the diet component LCAR in its treatment. Moreover, these data underscore the cardinal role that DCs play in modulating the initiation of intestinal inflammation and advocate the future development of other pharmaceutical agents specifically designed to target their function for the treatment of CD.

## 5.2 CD Pathogenesis

In order to identify new targets for CD therapy, we must first understand the pathogenesis of the disease. The data presented in this thesis have contributed to our understanding of the pathogenesis of CD in the following ways:

### 5.2.1 SIRPα$^{+}$ DCs are a pro-Th17 DC subset

Several studies have been performed to elucidate the function of DC subsets isolated from the LP and mLNs using various combinations of markers, including CD11c, CD11b, $CX_3CR1$, CD103, and CD70, among others. The majority of this work has been performed in control mice,

which do not display any intestinal inflammation. One such study described a pro-inflammatory phenotype of $CD11c^{Hi}CX_3CR1^{+}CD103^{-}$ LP DCs; when isolated from the LP of mice at steady-state, these DCs promoted Th17 differentiation of naïve T cells (Denning et al., 2007). On the other hand, another DC subset, the $CD11c^{Hi}CD103^{+}$ DCs, have been shown to be tolerogenic and induce the generation of $Foxp3^{+}$ $T_{regs}$ in a TGFβ- and retinoic acid-dependent mechanism (Coombes and Powrie, 2008, Annacker et al., 2005).

In Chapter 3, we first demonstrated that $CD11c^{Hi}CD103^{-}$ (Th17-inducing) DCs found in both the LP and mLNs express SIRPα, while $CD11c^{Hi}CD103^{+}$ (Treg-inducing) DCs did not. We also confirmed the pro-Th17 phenotype of this subset by sorting DCs based on their expression of SIRPα (either $SIRP^{+}CD103^{-}$ or $SIRP\alpha^{-}CD103^{+}$) and performing *in vitro* and *in vivo* assessments of their Th17-inducing capacities. Moreover, the only DC subset to increase in frequency upon induction of TNBS colitis was the $SIRP^{+}CD103^{-}$ DC subset. We have thus identified SIRPα as a positive marker of the Th17-inducing $CD11c^{Hi}CD103^{-}$ DC subset and implicate a role for this particular subset in the generation of intestinal inflammation.

#### 5.2.1.1. How do these findings contribute our understanding of CD pathogenesis?

These findings are important for the field of CD research because they highlight a central, dynamic role for DCs in the initiation of intestinal inflammation and identify SIRPα$^+$ DCs as one proinflammatory DC subset in mice. Since SIRPα may also be specifically expressed on proinflammatory DCs in human CD, one potential clinical application of these findings could eventually involve screening for an increase in their frequencies in the intestinal LP or mLNs as a diagnostic marker for CD. Importantly, these findings underscore the relative paucity of data regarding the particular DC subsets involved in the pathogenesis of human CD and the mechanisms by which they participate in the generation and maintenance of intestinal inflammation.

### 5.2.2 CD47/SIRPα interactions promote DC migration

After identifying SIRPα as a positive marker for Th17-inducing DCs and observing their increases in the LP and mLNs during the induction of TNBS colitis, we evaluated the role of its ligand, CD47, in the recruitment of SIRPα$^+$ DCs to those sites. We thus examined the frequencies of SIRPα$^+$ DCs in the LP and mLNs of CD47 KO mice at steady state and after the induction of TNBS colitis. Contrary to the increase in SIRPα$^+$ DCs observed in WT mice, these DCs did not increase in the LP or the mLNs of CD47 KO mice, which resulted in protection from intestinal inflammation

associated with Th17 responses. Reconstitution experiments, where CD47 KO mice were injected with WT or CD47 KO SIRPα$^+$ DCs, demonstrated that CD47 expression on the SIRPα$^+$ DCs, and not in the host tissues, drives DC migration and thus promotes T cell activation and the development of intestinal inflammation. The concept that CD47 expression on DCs promotes their migration is supported by another study which identified a role for CD47 on skin-derived DCs for their migration to draining LNs (Van et al., 2006).

#### 5.2.2.1. How do these findings contribute our understanding of CD pathogenesis?

This study has contributed to the field of CD research by shedding light on one mechanism involved in DC trafficking in the gut mucosa and associated lymphoid tissues. The discovery that CD47 expression on SIRPα$^+$ DCs promotes their migration to tissues and LNs has important implications for the development of new treatment modalities for CD.

### 5.2.3 Th17 polarization is positively correlated with TNBS colitis

Until recently, both CD and TNBS colitis were considered Th1 diseases characterized by IL-12, TNFα and IFN-γ production (Neurath et al., 1995, Parronchi et al., 1997). However, several recent reports have strongly implicated a role for IL-23, a Th17 survival factor, and Th17 cells in disease pathogenesis. In humans, elevated serum levels of IL-17 and

colonic mucosal levels of both IL-17 and IL-23 have been detected in CD patients (Fujino et al., 2003, Schmidt et al., 2005), while a mutation in the *IL-23R* gene (rs11209026, c.1142G→A, p.Arg381Gln) is protective from disease development (Duerr et al., 2006). The cytokine IL-17 is considered to be pathogenic in TNBS colitis since IL-17R-deficient mice are protected from disease development, and administration of an anti-IL-17 mAb ameliorates intestinal inflammation, despite high levels of IFNγ (Zhang et al., 2006). However, we here provide evidence to support a pathogenic role for Th17 cells themselves in the development of TNBS colitis.

First, the mRNA expression of key cytokines required for the differentiation (IL-6 and TGFβ) and maintenance (IL-23) of Th17 cells were elevated in mice with TNBS colitis. Second, IL-17 mRNA was highly upregulated in inflamed tissues, while IFNγ was only slightly upregulated. Third, the percentage of Th17, but not Th1 cells, in the mLNs was increased after the induction of TNBS colitis and was correlated with the severity of intestinal inflammation (Appended Fig. 5.1). Finally, injection of SIRPα$^{+}$ DCs, which potently induced Th17 responses *in vitro* and in the mLNs *in vivo*, also resulted in severe intestinal inflammation. Therefore, these data are in support of previous publications that have suggested a causative role for Th17 cells in the development of TNBS colitis (Zhang et al., 2006, Sheibanie et al., 2007b).

#### 5.2.3.1. How do these findings contribute our understanding of CD pathogenesis?

These findings are important for CD research because they underscore the use of the TNBS colitis model. Since Th17 cells appear to be key mediators of both TNBS colitis and human CD, this model can be used to study the cells, cytokines, inflammatory mediators and other possible factors required for Th17-mediated inflammatory responses. Another application of this model would be in the screening of potential therapeutic agents aimed at targeting the differentiation and/or function of Th17 cells. Since the TNBS colitis model is inexpensive, easy to induce, can be induced in most mouse strains and leads to a highly reproducible form of inflammation, it is ideally suited for screening new therapeutic agents.

### 5.2.4. LCAR deficiency results in $CD4^+$ T cell hyperactivation

In Chapter 4, we performed an *in vitro* culture of splenocytes isolated from control mice or mice with a secondary LCAR deficiency and assessed $CD4^+$ T cell activation and cytokine production. In unstimulated cultures, there were no differences between mouse strains, but upon anti-CD3 stimulation, LCAR-deficient $CD4^+$ T cells were significantly more activated and produced approximately 7-fold more IFNγ compared to control mice. Importantly, exogenous LCAR reversed this phenomenon,

leading to a comparable activation status and level of cytokine production as control mice. We have therefore shown that an endogenous reduction in LCAR levels may predispose an individual to enhanced immune responses and that LCAR supplementation may be beneficial in suppressing such responses in affected individuals.

#### 5.2.4.1 How do these findings contribute our understanding of CD pathogenesis?

The *OCTN1* and *OCTN2* genes are located within a single haplotype block of the IBD5 locus. Mutations within these genes were found to be associated with an impairment in transporter transcription and function leading to reduced LCAR uptake and increased susceptibility to CD (Peltekova et al., 2004). Although *OCTN1* and *OCTN2* are thought to be widely expressed, *in situ* hybridization data showed that the expression of both genes was limited to the intestinal epithelium, macrophages and T cells, but not B cells (Peltekova et al., 2004). This is of particular interest, since in Chapter 4, we demonstrated that LCAR does not exert any immunosuppressive effect on B cells, but does suppress the activation and cytokine production of macrophages, DCs and T cells. Although Peltekova *et al* did not identify DCs expressing OCTNs, they may have overlooked this fairly rare cell type since they did not use any DC-specific markers in their staining. Interestingly, *OCTN* mutations were also shown to increase susceptibility to rheumatoid arthritis (RA) (Tokuhiro et al.,

2003), further underscoring the implication of these genes in chronic inflammatory diseases.

Taken together, OCTNs are expressed in the major cell types involved in CD pathogenesis and mutations in these genes result in an impairment of LCAR uptake. Our results in Chapter 4 demonstrate that LCAR deficiency leads to an exaggerated immune response characterized by $CD4^+$ T cell activation and cytokine production. We have therefore identified one potential mechanism by which *OCTN* mutations may increase the risk of developing CD. Moreover, since this defect was reversed by LCAR supplementation, our data support a potential role for LCAR therapy in CD.

**5.3. CD Therapy:**

Treatment for CD has changed dramatically over the past decade with the introduction of biological therapy and the increased use of immunomodulators. Biological therapies, such as Infliximab, allow a more profound control of intestinal inflammation compared with conventional therapies, and often result in improved clinical parameters such as mucosal healing. Nevertheless, these therapies can be associated with rare but severe side effects, including opportunistic infections, malignancies, and antibody responses to the drug.

On the other hand, probiotics, prebiotics and nutritional supplements have also attracted a great deal of interest for the treatment

of CD and usually display a low efficacy but high safety profile. In this thesis, we have examined the therapeutic efficacy of one biological agent, CD47-fc, and one nutritional supplement, LCAR, in the treatment of murine TNBS colitis. The mechanisms of action and implications of these findings are discussed below.

### 5.3.1 CD47-fc

In Chapter 3, we assessed the therapeutic efficacy of a CD47-fc fusion protein in the treatment of the early (day 4) and late (after re-induction) phases of TNBS colitis. Administration of CD47-fc at the time of disease induction and for 3 subsequent days resulted in a dramatic reduction in the frequency of SIRPα$^+$ DCs in the mLNs, weight loss, and clinical and histological severity of intestinal inflammation. Moreover, the mice which had received CD47-fc therapy in the early phase were resistant to disease re-induction, a phase primarily associated with T-cell responses. These mice displayed reduced colonic IL-17 mRNA expression, as well as a reduction in the frequency of Th17 cells in the mLNs.

We also sought to determine if CD47-fc could also exert therapeutic efficacy if administered after the induction of inflammation. We found that administration of CD47-fc at the peak of the first phase of disease (days 2 and 3) protected mice from the body weight loss normally associated with re-induction of TNBS colitis (data not shown). Additionally, CD47-fc

administration on days 6, 7 and 8, (one day before, on the day of, and one day after TNBS re-induction) was also effective at improving body weight (data not shown).

#### 5.3.1.1. How do these findings relate to the treatment of CD?

These additional treatment schedules were important to assess the potential of CD47-fc to be used in human CD, since these patients already display ongoing intestinal inflammation before treatment is started. Taken together, our findings demonstrate that CD47-fc is therapeutically effective when administered either at i) the time of induction of TNBS colitis, ii) at the peak of inflammation, or iii) during the re-induction of disease. These schedules would translate as follows to the treatment of human CD; i) as induction therapy during the very early stages of disease or as maintenance therapy in patients who are in remission, ii) as an alternative therapy to induce remission in patients with active inflammation, and iii), as a supplementary therapy for patients who have periodic disease flare-ups. Although these findings are very encouraging, the true therapeutic value of such therapy in human CD patients can only be determined by proper randomized-controlled clinical trials.

#### 5.3.1.2. What is the mechanism of action of CD47-fc?

The therapeutic efficacy of CD47-fc in all three treatment schedules implies one of three mechanisms of action. First, CD47-fc may bind to

SIRPα on DCs and prevent its ligation by endogenous CD47, thus resulting in reduced DC migration to sites of inflammation and associated lymphoid organs. If this is the case, this would imply an essential role for DCs during both the initiation and progression of disease since the administration of CD47-fc after the initial induction of TNBS colitis is still effective. Indeed, several studies have demonstrated an important role for DCs for the perpetuation of intestinal inflammation (Abad et al., 2003, Daniel et al., 2008).

Second, in addition to DCs, CD47-fc may bind to and inhibit the ligation of SIRPα on other cells of the immune system, such as neutrophils and macrophages, which could ultimately result in their impaired migration into tissues (Liu et al., 2002). This possibility is supported by data demonstrating a significant accumulation of macrophages and/or neutrophils ($CD11c^{-}CD11b^{+}$ cells) expressing SIRPα in the LP upon induction of TNBS colitis (Appended Fig 5.2a). Since macrophages are one of the primary sources of IL-6, a pleiotropic cytokine involved in the acute phase response and required for Th17 differentiation and the survival of activated T cells (Mudter and Neurath, 2007), a reduction in macrophage infiltration could potentially result in protection from both the acute and chronic phases of TNBS colitis. However, the effect of CD47-fc administration on this phenomenon remains to be assessed. We have also confirmed the recruitment of neutrophils into the colonic tissues after induction of TNBS colitis (Appended Fig. 5.2b). CD47/SIRPα interactions

have previously been shown to promote transendothelial neutrophil migration (Liu et al., 2002), and we here demonstrate that administration of CD47-fc leads to a reduction in colonic myeloperoxidase (MPO) activity, a marker of neutrophil infiltration (Appended Fig 5.2b). However, previously published reports have suggested a protective role for neutrophils in disease initiation (Kuhl et al., 2007). This is also supported by our own findings in mice administered an anti-GR1 antibody. This antibody results in the depletion of neutrophils and an increase in the severity of TNBS colitis (Appended. Fig 5.2c). Therefore, while a reduction of neutrophil infiltration was observed in CD47-fc treated mice, we largely excluded this phenomenon as playing a protective role in the development of TNBS colitis.

Finally, the possibility remains that CD47-fc may actually engage SIRPα and induce intracellular signalling. While classically considered as a suppressive interaction leading to the inhibition of phagocytosis, recent data implicate a role for CD47 signalling via SIRPα in immune activation (Matozaki et al., 2009). The ambiguity of the physiological relevance of this interaction, combined with the knowledge that CD47-fc is non-functional in CD47 KO hosts (Van et al., 2006) makes this potential mode of action less likely.

These arguments thus favour the disruption of CD47/SIRPα interactions, as opposed to SIRPα ligation as the primary mechanism of action of CD47-fc. Therefore, the inhibition of $SIRP\alpha^{+}$ DC and

monocyte/macrophage trafficking are probably the most important physiological mechanisms of action of CD47-fc. However, further investigation into the contributions of each of these cells to the early and re-induction phases of disease should be performed.

#### 5.3.1.3. Would this therapy be safe to implement in humans?

No obvious adverse effects of CD47-fc administration were observed in mice treated with this compound. However, the safety of its use in humans remains to be determined. If eventually approved for use in humans, CD47-fc would be classified as a biological drug, and more specifically as a member of the selective adhesion molecule (SAM) inhibitors family. We may therefore predict some of the possible adverse effects of CD47-fc by its comparison to the only approved SAM inhibitor for the treatment of CD, Natalizumab.

Natalizumab is a recombinant humanized monoclonal antibody against alpha-4 (α4) integrin, a protein expressed on the surface of leukocytes, allowing their adhesion and recruitment to inflamed tissues. It has been tested in CD and multiple sclerosis (MS) patients and in the majority of cases, Natalizumab was well-tolerated. However, six patients receiving Natalizumab therapy for MS acquired an infection with the human polyoma virus JC and were diagnosed with PML, resulting in the death of two patients. Although these infections were exceedingly rare and associated with the combined use of Natalizumab and other

immunosuppressive agents, the documentation of this severe adverse event lead to the temporary withdrawal of Natalizumab from the clinical setting.

CD47-fc is comparable to Natalizumab in that its effectiveness is likely attributable to the inhibition of cell trafficking. However, while Natalizumab inhibits α4 integrin function on all leukocytes, the inhibitory activity of CD47-fc would be limited to cells expressing SIRPα, such as monocyte/macrophages, neutrophils and DCs. Importantly, T cells express SIRPγ which has a very low binding affinity to CD47 and would therefore be largely unaffected by CD47-fc therapy (Stefanidakis et al., 2008). Also, since the $CD103^+$ DC subset has been shown to be the main migratory skin DC subtype capable of cross-presenting viral antigens (Bedoui et al., 2009), one main advantage of targeting $SIRP\alpha^+$ ($CD103^-$) DCs specifically by the use of CD47-fc, is to avoid opportunistic viral infections.

### 5.3.2 L-carnitine

In Chapter 4, we described the immunosuppressive properties of LCAR *in vitro* and demonstrated its therapeutic efficacy *in vivo* by successfully reducing body weight loss, cytokine production, and intestinal inflammation in the TNBS colitis model. This decrease in intestinal inflammation was associated with a reduction in parameters of both innate

(IL-1β and IL-6 serum levels and mRNA expression in colon tissues) and adaptive immunity (antigen-specific proliferation in mLN cultures).

#### 5.3.2.1. How do these findings relate to the treatment of CD?

CD involves the aberrant activation of cells of both the innate and the adaptive immune systems. This results in a "vicious cycle" of inflammation, with activated $CD4^+$ T cells inducing the release of pro-inflammatory mediators such as cytokines, chemokines, and ROS from innate and epithelial cells. We here described the immunosuppressive properties of LCAR on DCs and macrophages, as well as on $CD4^+$ T cells. Given that these are thought to be the critical cell types involved in CD pathogenesis, LCAR therapy would likely translate into a valuable treatment for human CD. Furthermore, there is some evidence to support the immunosuppressive properties of LCAR in humans, suggesting that these characteristics are not specific to mice. For instance, reduced TNFα has been documented in the serum of post-surgical and AIDS patients treated with LCAR (Delogu et al., 1993, De Simone et al., 1993).

#### 5.3.2.2. What is the mechanism of action of LCAR?

LCAR exerts physiological functions on energy metabolism, the regulation of oxidative stress, and in the modulation of glucocorticoid receptor activity. Since all of these processes are likely implicated in CD, it is possible that they all play a role in the therapeutic efficacy of LCAR *in*

*vivo.* For instance, LCAR has been shown to promote the integrity of the epithelial barrier though its metabolic activity in colonocytes (D'Argenio et al., 2006), to reduce parameters of oxidative stress in the gastrointestinal tract (Erkin et al., 2006) and to activate GRα in monocytes (Manoli et al., 2004).

Nevertheless, in Chapter 4, we identify novel outcomes of LCAR supplementation on DCs, macrophages, and $CD4^+$ T cells *in vitro* which likely also contribute to its effectiveness *in vivo*. Specifically, we have demonstrated that LCAR reduces the activation (CD80 and CD86 expression) of purified DCs and macrophages and impairs $CD4^+$ T cell activation and cytokine production in an APC-free cell culture system, indicating that it can act independently on both arms of the immune system. Moreover, we observed an increase in $CD4^+$ T cell activation and cytokine production in cells isolated from LCAR deficient mice. These findings have clinical importance, since mutations in the *OCTN1* and *2* genes have been shown to result in poor LCAR transport and have been linked to increased susceptibility to CD (Peltekova et al., 2004). Although the molecular events involved in the immunosuppressive characteristics of LCAR were not investigated in this study, they likely involve the previously-described antioxidant and GRα-activating properties of LCAR.

#### 5.3.2.3. Would this therapy be safe to implement in humans?

LCAR has already been administered to humans in a number of different contexts. At the present time, intravenous and oral LCAR are available by prescription for the treatment of primary and secondary LCAR deficiencies. Doses for LCAR therapy are determined on a patient-by-patient basis, but are generally in the range of 500 - 6000 mg/day. This would translate into approximately 10 - 100 mg/kg based on an average weight of 70 kg. A number of clinical trials in the fields of heart disease, atherosclerosis, chronic renal failure/dialysis, Alzheimer's disease, HIV/AIDS and male infertility have also addressed the therapeutic efficacy of similar doses of LCAR. LCAR is also available without a prescription as a nutritional supplement and has been used at a dose of 40 - 80 mg/kg to improve athletic performance (Marconi et al., 1985, Vecchiet et al., 1990). In all of these settings, LCAR therapy was well-tolerated and no toxic effects of LCAR overdose have been reported. Nevertheless, LCAR supplementation was said to be associated with some mild side effects, including nausea, vomiting, diarrhea and a "fishy" body odour at doses above 3000 mg/day. Although LCAR has previously been shown to activate GRα and mimic some of the effects of glucocorticoids on immune function, it was also proven to protect osteoblasts from apoptosis (Colucci et al., 2005) and may therefore exert the beneficial anti-inflammatory effects of glucocorticoids without the serious side effects on bone. Given

its known safety profile, LCAR is a promising candidate for translation into clinical trials for CD patients.

### 5.3.3. Where do CD47-fc and LCAR fit in the network of currently used drugs?

As depicted in Figure 5.1, a plethora of drugs are either currently in use or are being evaluated at various stages of clinical trials, and specifically target individual elements of the intestinal immune system. These include the luminal bacteria, intestinal epithelial barrier, APCs and their cytokines, the activation and cytokine production of T cells, and the intestinal recruitment of leukocytes. The great majority of new drugs are classified as biologicals, and although many have now been tested, the only biological therapies that are approved, registered and reimbursed for the treatment of CD are anti-TNFα (Infliximab, certolizumab pegol, and adalimumab) and anti-α4 integrin (Natalizumab and MLN-0002) antibodies.

Although these therapies induce rapid mucosal healing, improve quality of life and help to avoid hospitalization and surgery in many CD patients, some do not respond at all or eventually develop infusion reactions and delayed serum-sickness and secondary loss of effectiveness of therapy. Therefore, there is still a need to develop new drugs to treat CD.

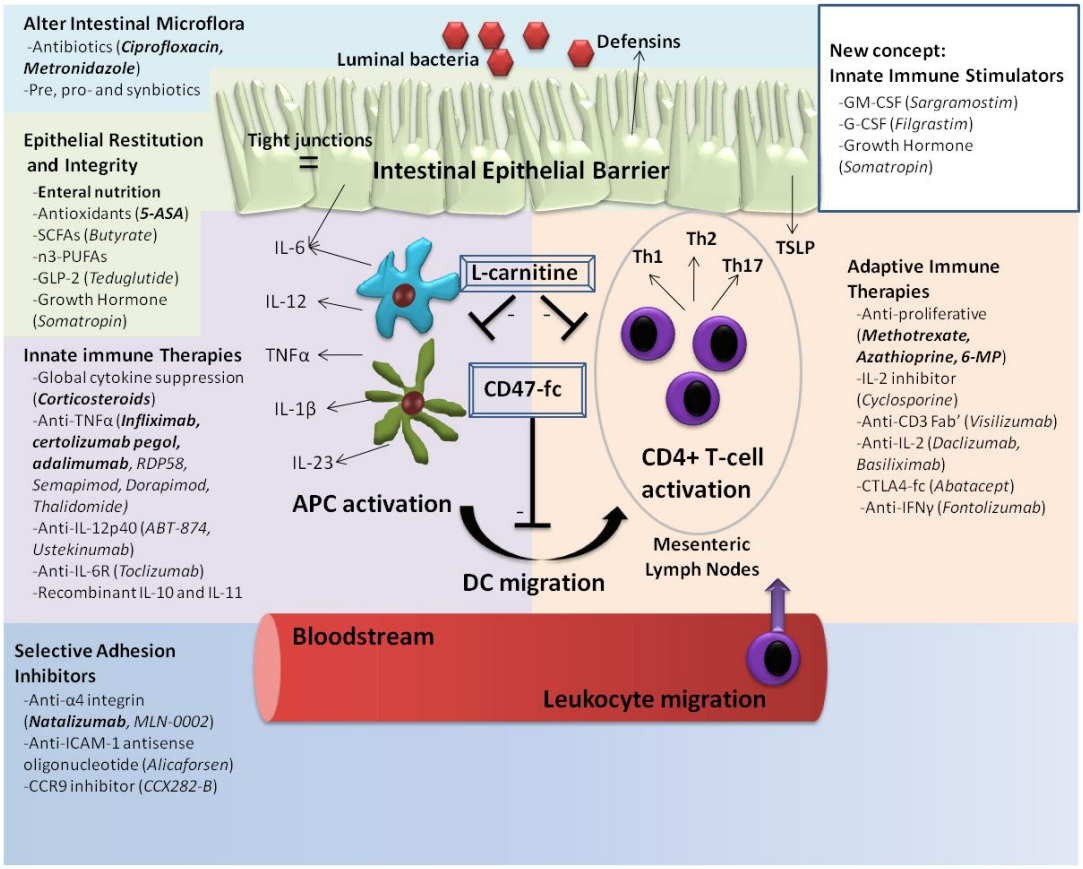



Figure 5.1: CD therapies currently in use or in development.
Adapted from (Peyrin-Biroulet et al., 2008)

We here demonstrate that CD47-fc inhibits the migration of SIRPα$^+$ DCs, from either the intestinal LP or the bloodstream, into the mLNs, where CD4$^+$ T cell priming occurs (Figure 5.1). The disruption of SIRPα$^+$ DC migration is an interesting therapeutic target, since these DCs are known to promote Th17 responses (Denning et al., 2007) and are not responsible for inducing $T_{regs}$ (Coombes et al., 2007). By inhibiting this crucial link between the innate and adaptive immune responses, CD47-fc may be capable of disrupting the feedback amplification of inflammation and lead to a positive impact on the course of disease.

We have also shown that the nutrient LCAR is a valuable therapy for the treatment of TNBS colitis. While LCAR has previously been shown to promote intestinal epithelial barrier function (D'Argenio et al., 2006), we here propose a novel *in vivo* mechanism of action of LCAR by suppressing both innate and adaptive immune responses (Figure 5.1). Although the efficacy of LCAR was only evaluated after I.P. injection, the oral bioavailability of LCAR has been shown to be 14-18%, suggesting that higher doses may also be effective if given in the form of dietary supplements. Given the complimentary effects of CD47-fc on DC migration and LCAR on DC activation and cytokine production, these therapies could act synergistically to reduce intestinal inflammation.

## 5.4 The future of these studies:

The beauty of scientific research, especially in the field of life sciences, lies in the fact that there is always more to learn. While the discoveries outlined in this thesis have contributed to advancing knowledge in the field of CD pathogenesis and treatment, they have also lead to the formulation of new questions, which remain to be answered. Below is a detailed series of questions which would merit further investigation.

### 5.4.1 Is CD47 implicated in the pathogenesis of other murine models of CD?

We selected the TNBS model to assess the role of CD47 (by CD47 deletion or the administration of CD47-fc) in the generation of intestinal inflammation. This particular model was chosen due to the similarity of its clinical and histological features to human CD, the fact that early initiating events such as DC migration can be examined, the involvement of innate and adaptive immune cells in its pathogenesis, as well as its versatility, low cost and ease of use. The two other most commonly used murine models of CD are the adoptive transfer model and dextran sulphate sodium (DSS) colitis.

In the adoptive transfer model, $CD4^{+}CD45RB^{hi}$ T cells ($T_{reg}$ depleted) are transferred intravenously to immunocompromized (SCID or $Rag^{-/-}$) mice. This model is especially useful for studying the regulatory mechanisms of $T_{reg}$ and the pathogenic mechanisms of $CD4^{+}CD45RB^{hi}$ T cells. However, this model is less suitable for studying early innate immune responses, since disease develops at various rates, and the involvement of DC migration in disease initiation is largely unknown. Moreover, transfer of CD47 KO T cells into WT hosts would not be feasible since CD47 is a marker of self whose expression is required to avoid elimination in WT hosts (Oldenborg et al., 2000).

Another popular model is dextran sodium sulphate (DSS)-induced intestinal inflammation. Here, DSS is dissolved in the drinking water and is given to mice for several days. DSS is thought to be directly toxic to gut

epithelial cells of the basal crypts, alters barrier integrity and induces a very reproducible acute colitis characterized by bloody diarrhea, ulcerations and the infiltration of granulocytes. T and B cell deficient mice also develop severe inflammation, indicating that the adaptive immune system does not play a major role in this model. Given these properties, it is especially useful for studying innate immune responses and the maintenance of the epithelial barrier. Our preliminary data suggest that CD47 may in fact play a protective role in DSS colitis, since CD47 KO mice lose more body weight and develop more severe intestinal inflammation and shortening compared to WT mice (Appended Fig 5.3). However, the mechanisms involved in this protection warrant further investigation.

The TNBS colitis model is a very useful tool for evaluating the role of CD47 in intestinal DC migration and subsequent adaptive immune responses. However, delineating the role of CD47 using various other models of intestinal inflammation could reveal multiple novel implications for this molecule.

### 5.4.2 What is the mechanism by which CD47 controls SIRPα$^+$ DC migration?

CD47 binds to SIRPα, and the X-ray crystallographic structures of the N-terminal IgV domain of SIRPα and the Ig domain of CD47, as well as the complex interaction of these domains have recently been

elucidated (Hatherley et al., 2008). While CD47 is ubiquitously expressed, SIRPα is expressed on a limited number of cell types, including neutrophils, monocytes/macrophages, DCs, neurons, endothelial and epithelial cells. Both ligation of SIRPα by CD47 and ligation of CD47 by SIRPα have been shown to induce intracytoplasmic signaling, and bi-directional signaling is thought to occur *in vivo* (Ohnishi et al., 2005).

Our competitive migration data show that CD47 expression is required on the SIRPα$^+$ DC subset for its migration into mLNs, since WT DCs are superior to CD47 KO DCs in their capacity to migrate efficiently in CD47 KO hosts. On the other hand, CD47 expression is not required in the host, since severe disease was induced by the transfer of WT DCs into CD47 KO hosts. CD47 is also known to bind to the integrins $\alpha_v\beta_3$ and $\alpha_{II}\beta_3$ in *cis* and many CD47-mediated cellular responses are likely a result of such interactions (Brown and Frazier, 2001). However, the molecular mechanisms and functional outcomes of these interactions are largely unknown.

Although detailed studies of the molecular interactions responsible for this functional outcome were outside the scope of Chapter 3, we hypothesize that CD47 may interact with SIRPα in *cis* on the surface of the DC to indirectly control integrin-mediated transendothelial trafficking. Nevertheless, this question certainly warrants further investigation.

### 5.4.3 What role do SIRPα$^+$ DCs play in human disease?

In Chapter 3, we examined the frequency of SIRPα$^+$ DCs in the colon LP and mLNs of mice, and then isolated these DCs to examine their capacity to induce Th1/Th17 responses. However, the role and regulation of these DCs in humans is largely unknown. To address this question, immunofluorescent staining for SIRPα could be performed on intestinal and mLN biopsies of non-CD patients. Once characterized, the frequency of these cells could be quantified in comparison to CD patients. To verify the function of these DCs, they could be extracted from surgical specimens and cultured with allogeneic naïve T cells isolated from the peripheral blood to assess Th1/Th17 differentiation.

### 5.4.4 How is TSP-1 implicated in CD?

TSP-1 is the other ligand of CD47 and its function in the development of intestinal inflammation was not determined in these studies. However, our preliminary data supports a role for TSP-1 in both TNBS colitis and human CD. We first observed a positive correlation between disease severity and TSP-1 mRNA expression in WT mice with TNBS colitis (Appended Fig 5.4a). Accordingly, we also found low TSP-1 mRNA expression in protected CD47 KO mice after first or second TNBS administration (Appended Fig 5.4b). Despite the ubiquitous expression of CD47, and thus the numerous potential effects of its ligation by TSP-1, there exists a remarkable translation of human data to mice, and *vice*

*versa*. We therefore next assessed whether TSP-1 was similarly regulated in inflamed human and murine colon tissues. Indeed, TSP-1 production was significantly higher in cultured colon biopsies from CD patients compared to controls (Appended Fig 5.4c) and in the sera of CD patients with active inflammation compared to healthy controls (Appended Fig 5.4d). While CD patients in remission tended to have higher TSP-1 levels than healthy controls, this trend did not reach statistical significance. Taken together, we demonstrate that TSP-1 is positively correlated with inflammation and postulate that the CD47/TSP-1/SIRPα axis participates in governing intestinal inflammation.

The study of the interactions of this trio of molecules (CD47, TSP and SIRPα) is associated with a certain level of difficulty. First, they are all expressed by the same cell type, the DC. Second, although CD47 ligation by SIRPα and TSP-1 may engender similar inhibitory functions on DCs (Latour et al., 2001), recent reports have suggested that ligation of CD47 on T cells by SIRPα on DCs may result in enhanced T cell proliferation and cytokine production (Tomizawa et al., 2007, Okuzawa et al., 2008). Therefore, *in situ* competition between the two CD47 ligands (i.e. TSP-1 and SIRPα) and possibly integrins (i.e. αvβ3/ $\alpha_{II}\beta_3$) for CD47 may compromise the availability of CD47 on the surface of DCs. This, compounded by the possibility of bidirectional signaling, makes this pathway exceedingly difficult to examine as a whole. Therefore, our approach in Chapter 3 was to individually examine CD47/SIRPα

interactions and leave the assessment of CD47/TSP-1 interactions for a future series of experiments.

### 5.4.5 Can LCAR therapy reverse ongoing inflammation?

The ability of LCAR to reverse ongoing inflammation is of great clinical importance, but was not examined in this study. We may, however, hypothesize that the immunosuppressive effect of LCAR on $CD4^+$ T cell activation, proliferation and cytokine production could be sufficient to reverse ongoing inflammation. To test this hypothesis, the TNBS model could be used, where LCAR would be administered daily, starting one day after re-induction of colitis. However, since TNBS colitis resolves spontaneously in 4-7 days, the adoptive transfer model, which results in progressive inflammation with no spontaneous resolution, might be more appropriate for this particular application. The use of this model would also address the question of whether the efficacy of LCAR is TNBS colitis-specific.

### 5.4.6 Can CD47-fc or LCAR be used to treat other diseases?

CD47-fc and LCAR both serve to suppress immune responses and dampen inflammation. Therefore, we would suspect that they would both exhibit therapeutic efficacies in treating other immune-mediated diseases such as contact dermatitis, asthma, Coeliac disease, systemic lupus erythromytosis (SLE), MS and RA, among others.

Unpublished data from our lab already suggest that CD47-fc is effective at impairing SIRPα$^{+}$ DC migration and the development of Th2-mediated airway inflammation in mice, suggesting that the efficacy of CD47-fc is not limited to Th1/Th17 mediated diseases.

LCAR has not yet been tested in other models of immune-mediated diseases, but would likely result in some degree of immunosuppression and clinical benefit.

### 5.5 CD therapy: What does the future hold?

An important conceptual development in the understanding of CD pathogenesis has been the adoption of a greater appreciation of the interplay between the microbial flora and the innate immune response. This has allowed us to focus on the specific criteria which are responsible for the transition from physiological to pathological intestinal inflammation. Although this thesis focused on the immune response of the host, we now believe that achieving a thorough understanding of CD pathogenesis will require a more comprehensive and integrated analysis of 1) the dynamics of luminal microbes, and 2) the host mucosal defence mechanisms.

Two ongoing large-scale research initiatives, the Human Microbiome Project (HMP) and several genome-wide association studies (GWAS), are currently under way and will undoubtedly uncover new microbial and genetic risk factors associated with CD. Nevertheless, these new techniques cannot replace conventional *in vitro* and *in vivo*

immunological assessments of the implications of bacterial strains or genetic factors implicated in disease pathogenesis and validation of the immense amount of data that will be generated will require a great deal of time, money and expertise.

### 5.5.1 The Human Microbiome Project

The microbiome is the collection of all the micro-organisms and their genomes that exist inside or on the surface of our bodies. The main idea behind this project is the belief that examining the differences between the microbiomes of healthy patients and those of patients suffering from a disease may eventually change how they are diagnosed, treated and, ultimately, prevented. One of the diseases of interest is CD, since alterations in the intestinal microbial flora are implicated in CD pathogenesis. The goal of this project in terms of CD is therefore to identify which bacterial species may be implicated in the pathogenesis and perpetuation of intestinal inflammation and develop specific probiotics, prebiotics, synbiotics or antibiotics to restore a normal flora and perhaps reduce or eliminate the associated inflammation.

While this approach seems to be the only logical method to attempt to characterize the differences in the flora of CD patients versus healthy controls at this point in time, several important problems may arise with the interpretation of the resulting data. First, the microbial flora is not homogenous within the digestive tract. Therefore, the location of flora

sampling must be carefully considered. Second, intracellular bacteria would not be accounted for in this type of analysis if sampling is limited to the lumen. This is especially important, since CD has been associated with an increased prevalence of adherent/invasive *E. coli* (AIEC) and *mycobacteria avium paratuberculosis* (MAP) (Prantera and Scribano, 2009), two strains of bacteria that can invade, survive and replicate within host cells. In fact, it is currently believed that defective clearance of intracellular bacteria may be one important predisposing factor to the development of CD. Nevertheless, important insights into the bacterial components of CD immunopathogenesis may be uncovered and contribute to the development of novel preventive, therapeutic or curative treatment regimens.

### 5.5.2 Genome-wide association studies (GWAS)

CD is a polygenic, heterogeneous disease. It is now known that multiple genes are involved, all conferring a small increase in disease risk, and no single susceptibility gene is either necessary or sufficient to induce CD (Cho, 2008). Although compelling evidence from epidemiological studies to support an important role for genetic risk factors in CD has been available for many years, the identification of the genes involved has proven rather challenging. To improve the success rate of these studies and to help further elucidate the key pathogenic pathways involved in disease pathogenesis, several groups have undertaken GWAS. Indeed,

until the development of the GWAS method, progress in delineating the genetic architecture of CD was very slow. To date, several GWAS have successfully identified novel CD susceptibility genes or loci (Table 5.1).

Table 5.1: Novel CD susceptibility genes/loci identified by recent GWAS

| Location | Novel gene/locus identified |
|---|---|
| Japan-UK (Yamazaki et al., 2005) | TNFSF15 |
| Germany-UK (Hampe et al., 2007) | ATG16L1 |
| North America (Duerr et al., 2006, Rioux et al., 2007) | IL23R, 10q21.1 "gene desert" (ERG2), PHOX2B, NCF4, FAM92B |
| Belgium (Libioulle et al., 2007) | 5p13.1 "gene desert" (PTGER4) |
| UK (Parkes et al., 2007) | IRGM, NKX2-3, PTPn2, 3q21 |
| | 1q "gene deserts", IL12B, FLJ45139 |
| Canada-Germany (Raelson et al., 2007) | 3p21, 4p16.1, 17q11, 17q23 |
| The Netherlands-UK (Zhernakova et al., 2008) | IL18RAP, CARD9 |
| USA-Italy (Kugathasan et al., 2008) | TNFRSF6B, PSMG1 |

This new genotyping method is hypothesis-free and involves genotyping a large number of SNPs that sample human genetic variations throughout the genome. The GWAS strategy has the advantage of offering an unbiased survey of the whole genome and can provide novel insights on disease pathways and mechanisms involved in the origin and development of CD.

The further development and implementation of this technique will undoubtedly result in the identification of new pathways involved in CD pathogenesis and generate a long list of potential therapeutic targets.

However, although the number of genes associated with CD is certain to grow in the coming years, we should also be mindful of the extent to which CD is influenced by tiny effects of hundreds of loci or highly heterogeneous rare mutations. Therefore, it may be impractical to assemble sufficiently large samples to give a complete account of the genetic risk factors involved. Although substantial efforts were gathered to conceive these numerous GWAS, the first wave of results represent only the starting point on the journey to elucidating and understanding the genetic basis of CD, and translating this knowledge into clinically useful information.

### 5.5.3 Personalized medicine

Advances in understanding the microbial and genetic factors implicated in CD pathogenesis are expected to have important implications for the treatment of disease, including the development of personalized medicine. Personalized medicine refers to the concept that managing a patient's health should be based on the individual patient's specific characteristics. Although this term typically refers to specific information provided by comprehensive genetic testing, it may also be adapted to include information regarding a patient's particular microbial flora. Such information could be used to stratify disease status, select between different medications and/or tailor their dosage, provide a specific therapy for an individual's specific type of disease, or initiate a preventive

measure that is particularly suited to that patient at that time of administration.

The implications of such an approach for the field of CD are especially important, since this disease is already thought to represent a collection of diseases characterized by similar clinical outcomes but caused by various different initiating factors. For instance, slightly different collections of susceptibility factors likely promote similar inflammatory pathways and result in intestinal inflammation characterized as CD. This concept is supported by the variation in clinical “phenotypes” of disease (inflammatory, stenotic and fistulising), the variation in the area(s) of involvement, severity, age of onset (childhood- vs adult-onset) and response to various treatment regimens. The use of personalized medicine requires a test that fairly accurately predicts disease categorization, particularly when it concerns decisions about therapeutic interventions, or interventions that are invasive, expensive, or have major side effects. Although novel discoveries reported by the HMP and GWAS will certainly improve our understanding of CD pathogenesis and aid in the discovery of new therapeutic targets, the question of whether this improvement will be sufficient to enable personalized medicine remains unanswered.

One main challenge in the application of personalized medicine in CD is the fact that it results from a complex interplay of both genetic and environmental factors, where each factor may only have a minor

contribution to the occurrence of the disease. This makes it significantly more complicated to develop accurate prediction models to be used for this purpose and, therefore, personalized medicine is not possible at this point in time. In the coming years, the great challenge will thus be to integrate these results and build more accurate disease prediction models with the hopes of eventually translating the emerging genomic and microbial knowledge into new therapeutic approaches.

### 5.5.4 Dendritic cell based treatment

On the opposite end of the spectrum of personalized medicine lies an approach that we now introduce for the first time and which we have coined as “dendritic cell based treatment” (DCBT). While personalized medicine must try to integrate many individual factors that contribute to a patient’s particular disease state, and determine the appropriate treatment regime, DCBT proposes just the opposite. We believe that DCs are a key component in maintaining intestinal homeostasis at steady state and amplifying the induction and perpetuation of inflammation in response to a real or perceived threat. DCs are thus appropriately positioned to play a critical role in linking innate and adaptive immune responses, with the potential to amplify or shut down the “vicious cycle” of inflammation. It is our hypothesis that DCs are implicated, at least to some extent, in the pathogenesis of every form of CD, regardless of genetic risk factors, microbial constituents of the luminal flora, clinical “phenotype”, area of

involvement, severity, or age of onset. We therefore propose that specifically targeting particular proinflammatory DC subsets (or potentiating the effect of regulatory DC subsets) may halt inflammatory processes and restore homeostasis in affected individuals. While our data support a role for the SIRPα$^{+}$ DC subset in murine disease pathogenesis, further investigation into the function of this subset in human disease is strongly recommended. Since both CD47-fc and LCAR target DC function, both of these treatments may be considered as DCBT and merit investigation in human subjects.

## 5.6 Conclusion:

During the course of this PhD project, we have focused our research efforts on discovering novel insights into CD pathogenesis, with the aim of identifying new therapies for its treatment. This effort has been fruitful, leading to the identification of SIRPα$^{+}$ DCs as a novel DC subset capable of inducing Th17 responses and promoting the development of intestinal inflammation in a CD47-dependent manner. Another important accomplishment was the successful amelioration of TNBS colitis by the administration of CD47-fc and LCAR. These compounds were both effective at dampening immune responses and protected mice from the development of intestinal inflammation. While the above results are certainly encouraging, further investigations should be performed to address several remaining questions. For instance, the molecular interactions between SIRPα and CD47 responsible for permitting DC migration and the precise cellular mechanism of LCAR's immunosuppressive activities remain largely unknown.

Throughout this thesis, DCs have been shown to play a central role in disease pathogenesis. We therefore encourage further investment of time and funding in this particular area of CD research. Importantly, the findings outlined in this thesis are especially relevant for Canadians, since one of the highest rates of CD is observed in this country.

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## 6 Appendix:

- Appended figures
- Print copy of the manuscript presented in Chapter 3:

  Genevieve Fortin[1,2], Marianne Raymond[2], Vu Quang Van[2], Manuel Rubio[2], Patrick Gauthier[2], Marika Sarfati[2*], Denis Franchimont[1*] A role for CD47 in the development of experimental colitis mediated by SIRPalpha+CD103- dendritic cells. *J Exp Med*. 2009 Aug 31;206(9):1995-201

- Print copy of the manuscript presented in Chapter 4:

  Geneviève Fortin, Katerina Yurchenko, Catherine Collette, Manuel Rubio, Alexandra-Chloé Villani, Alain Bitton, Marika Sarfati and Denis Franchimont. L-carnitine, a diet component and organic cation transporter OCTN ligand, displays immunosuppressive properties and abrogates intestinal inflammation. *Clin Exp Immunol.* 2009 Apr;156(1):161-71.

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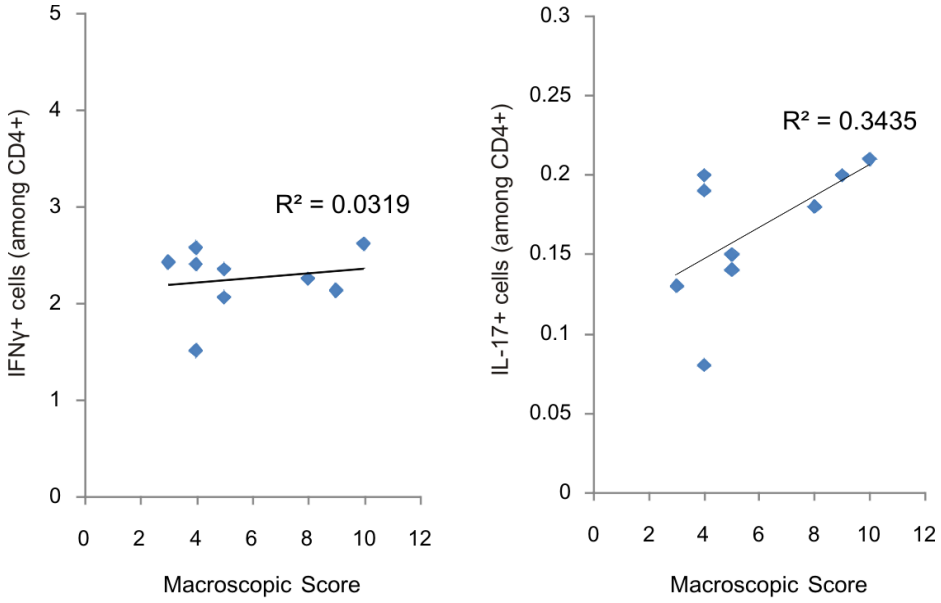
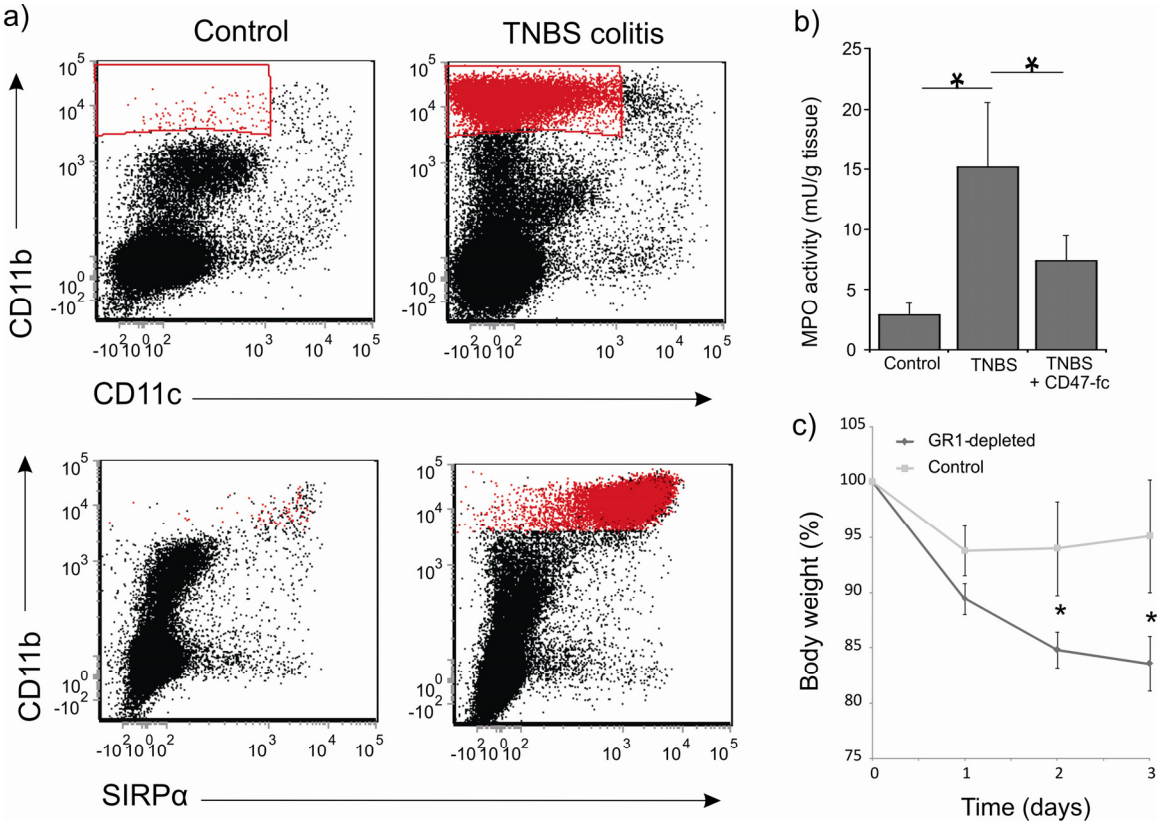
6.1 Appended Figures:



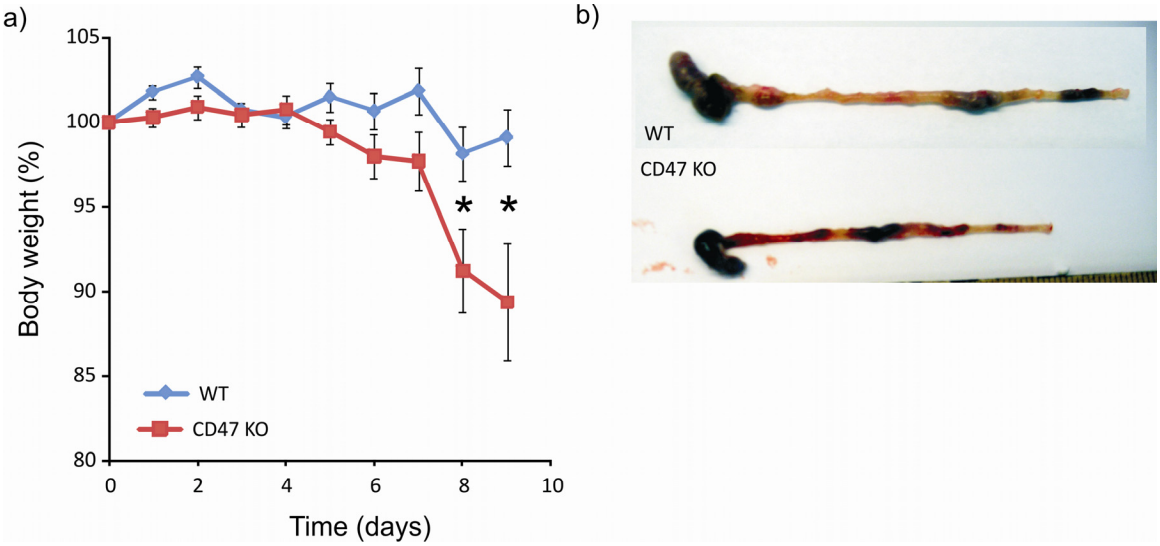

Appended Figure 5.1: Correlation between macroscopic score and the frequency of Th1 or Th17 cells in the mLNs.

mLNs were isolated after re-induction of TNBS colitis, cultured for 4 days with anti-CD3 stimulation in the presence of IL-23 and restimulated with PMA/ionomycin for intracytoplasmic staining. Each diamond represents one mouse.



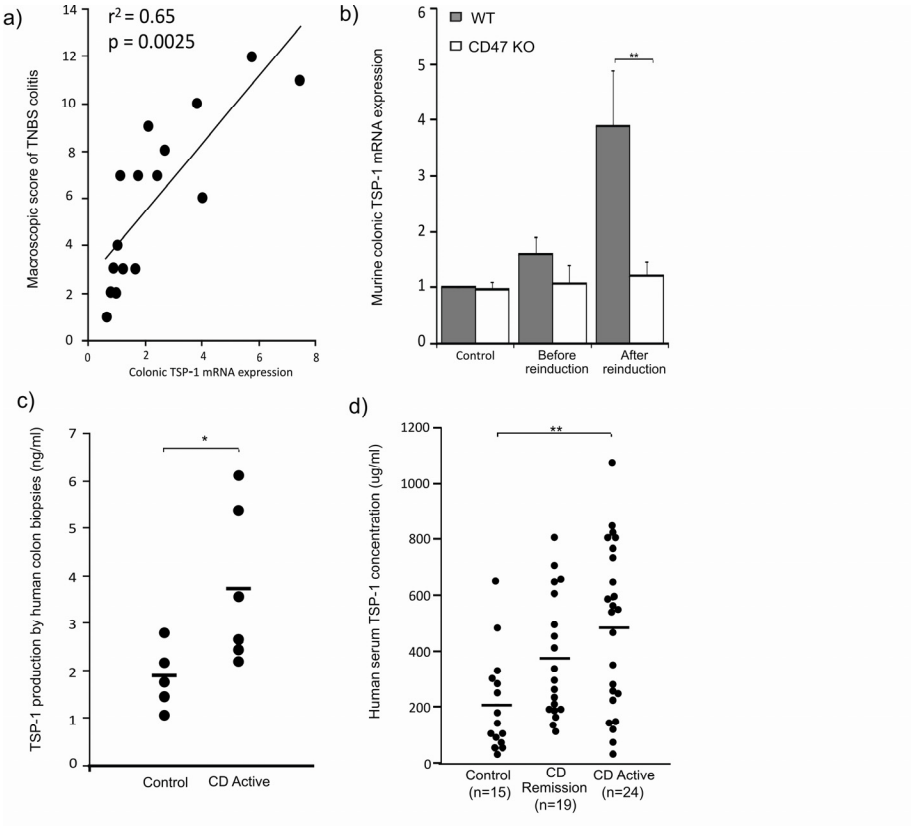

Appended Figure 5.2: Induction of TNBS colitis leads to the recruitment of macrophages and neutrophils into the colon tissues.

BALB/c mice were sacrificed 2 days after the induction of TNBS colitis. a) LP mononuclear cells were isolated and stained for CD45.2, CD11b, CD11c and SIRPα. Dot plots were gated on $CD45.2^{+}$ cells. b) Whole colons were homogenized and neutrophil infiltration was assessed using the MPO activity assay. n = 8 mice per group. c**)** Neutrophil depletion was performed by the administration of an anti-GR-1 antibody in BALB/c mice. TNBS colitis was induced on day 0. Each point represents the mean body weight of n=4 mice relative to their weight on day 0 +/- SEM. *p<0.05




Appended Figure 5.3: Protective role of CD47 in the development of DSS colitis.

BALB/c or CD47 KO mice were given free access to 4% DSS drinking water on day 0. a) Body weight was monitored data. Each point represents mean +/- SEM of 8 mice per group. * $p<0.05$. b) On day 9, all mice were sacrificed. Shown is the colon of one representative mouse per group (n=8).




Appended Figure 5.4: TSP-1, a ligand for CD47, is positively correlated with inflammation.

a) Correlation between macroscopic score of inflammation after re-induction of TNBS colitis and colonic mRNA expression of TSP-1 in WT mice. Each circle represents one mouse. b) Colonic mRNA expression of TSP-1 in WT and CD47 KO mice before and after re-induction of TNBS colitis. Data represent the mean (n>5) +/- SEM. c) Human colon biopsies were isolated from healthy controls or Crohn's disease patients and cultured overnight. d) Serum was collected from healthy donors, CD patients in remission, and CD patients with active inflammation. Circles represent individual patients, lines mark the average value per group. TSP-1 in culture supernatants and serum samples were assessed by ELISA. * $p<0.05$, ** $p<0.01$

Published August 24, 2009



# A role for CD47 in the development of experimental colitis mediated by SIRPα⁺CD103⁻ dendritic cells

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**Mesenteric lymph node (mLN) CD103 (αE integrin)⁺ dendritic cells (DCs) induce regulatory T cells and gut tolerance. However, the function of intestinal CD103⁻ DCs remains to be clarified. CD47 is the ligand of signal regulatory protein α (SIRPα) and promotes SIRPα⁺ myeloid cell migration. We first show that mucosal CD103⁻ DCs selectively express SIRPα and that their frequency was augmented in the lamina propria and mLNs of mice that developed Th17-biased colitis in response to trinitrobenzene sulfonic acid. In contrast, the percentage of SIRPα⁺CD103⁻ DCs and Th17 responses were decreased in CD47-deficient (CD47 knockout [KO]) mice, which remained protected from colitis. We next demonstrate that transferring wild-type (WT), but not CD47 KO, SIRPα⁺CD103⁻ DCs in CD47 KO mice elicited severe Th17-associated wasting disease. CD47 expression was required on the SIRPα⁺CD103⁻ DCs for efficient trafficking to mLNs in vivo, whereas it was dispensable on both DCs and T cells for Th17 polarization in vitro. Finally, administration of a CD47-Fc molecule resulted in reduced SIRPα⁺CD103⁻ DC–mediated Th17 responses and the protection of WT mice from colitis. We thus propose SIRPα⁺CD103⁻ DCs as a pathogenic DC subset that drives Th17-biased responses and colitis, and the CD47–SIRPα axis as a potential therapeutic target for inflammatory bowel disease.**

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Abbreviations used: BMDC, bone marrow–derived DC; CD, Crohn's disease; LP, lamina propria; mLN, mesenteric lymph node; SIRPα, signal regulatory protein α; TLR, Toll-like receptor; TNBS, trinitrobenzene sulfonic acid.

DCs are located throughout mucosal surfaces and orchestrate the delicate balance between tolerance to innocuous antigens and the generation of protective immune responses upon exposure to pathogens. Different DC subsets populate the gut mucosa and mesenteric lymph nodes (mLNs), and their functions vary according to their anatomical location and conditioning by epithelial cells (Iwasaki, 2007). For instance, CD103⁺ and CD103⁻ DC subsets isolated from mLNs display distinct phenotypes and cytokine profiles. A substantial proportion of CD103⁺ DCs continuously emigrate from the intestine to the mLNs in a CCR7-dependent manner (Johansson-Lindbom et al., 2005; Jang et al., 2006), where they represent from 40 to 70% of the DC population (Coombes et al., 2007; Jaensson et al., 2008). Under steady-state conditions, mLN CD103⁺ DCs are prone, in the presence of retinoic acid, to convert naive CD4⁺CD25⁻ T cells into CD4⁺CD25⁺Foxp3⁺ T reg cells (Coombes et al., 2007; Sun et al., 2007), whereas CD103⁻ DCs isolated from the mLNs and lamina propria (LP) of the small intestine express $CX_3CR1$ and drive Th17 polarization in vitro (Denning et al., 2007; Atarashi et al., 2008). The mLN CD103⁻ DCs appear to be directly derived from blood-borne precursors; express Toll-like receptor (TLR) 2, TLR4, and TBX21 to a greater extent than CD103⁺ DCs; and secrete high levels of TNF and IL-6 in response to stimulation (Coombes and Powrie, 2008). However, little is known about the precise contribution of each of these DC subsets during the development of intestinal inflammation.

M. Sarfati and D. Franchimont contributed equally to this paper.

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Crohn's disease (CD) is a chronic, relapsing, T cell–driven inflammatory disease of the gastrointestinal tract thought to result from inappropriate mucosal immune responses to commensal bacteria in genetically susceptible individuals (Cho, 2008). DCs have been implicated in CD pathogenesis, because dysregulation in DC–epithelial cell interactions, defective migration and function of tolerogenic DCs, and/or aberrant immunogenic DC responses to pathogens have been proposed to participate in the induction of intestinal inflammation (Kelsall, 2008). Once initiated, the inflamed tissue is characterized by the expression of innate (IL-6, IL-12, TNF, and IL-23) and adaptive-derived (IL-6, IFN-γ, and IL-17) proinflammatory cytokines (Sanchez-Munoz et al., 2008). Antigen-presenting cell–derived TGF-β, IL-1β, IL-6, and IL-23 drive Th17 responses, whereas IL-12 and IFN-γ promote Th1 and suppress Th17 responses (Trinchieri, 1993; Bettelli et al., 2007).

Trinitrobenzene sulfonic acid (TNBS)–induced colitis is a mouse model of intestinal inflammation sharing many key features with human CD. TNBS administration first induces acute inflammation, characterized by the infiltration of neutrophils and macrophages, followed by antigen-specific priming of T cells. Challenge with a second dose of TNBS provokes a T cell–predominant reaction associated with tissue damage and mimics the chronic inflammation seen in CD (te Velde et al., 2006). Although classically considered a Th1 disease, the recently identified Th17 cell lineage appears to play an important role in the development of both TNBS colitis and CD (Annunziato et al., 2007; Sheibanie et al., 2007).

CD47, a marker of self on immune and nonimmune cells, is implicated at several levels in the generation of immune responses (van den Berg and van der Schoot, 2008). On the one hand, ligation of CD47 by the extracellular matrix protein thrombospondin-1, which is abundantly and rapidly expressed in tissues in response to inflammation (Reed et al., 1993), down-regulates IL-12p70 production by antigen-presenting cells (Armant et al., 1999) and impairs human naive T cell differentiation to Th1 cells in vitro without any immune deviation to Th2 cells (Avice et al., 2000). Recent data have also shown that CD47 expression on T cells is a self-control negative regulator of Th1 immune responses in vivo (Bouguermouh et al., 2008). On the other hand, CD47 interacts with signal regulatory protein α (SIRPα), its counterreceptor, which is selectively expressed on myeloid, endothelial, and neuronal cells (Adams et al., 1998; Ticchioni et al., 2001). The expression of CD47 on epidermal and dermal DCs promotes their migration to draining lymph nodes, where T cell priming and immune responses are initiated (Van et al., 2006). We first show that SIRPα is selectively expressed on one of the two major mucosal DC subsets, i.e., the $CD103^-$ DCs. We next demonstrate that $SIRP\alpha^+CD103^-$ DCs are the primary immunogenic DC subset involved in the development and perpetuation of Th17-associated TNBS-induced colitis, and that their migration is controlled by CD47.

## RESULTS

### CD47 regulates $SIRP\alpha^+CD103^-$ DC homeostasis in the LP and mLNs of naive and TNBS-treated mice

Multiple DC subpopulations have been identified and characterized by their expression of CD11c, CD11b, and CD103 in the intestinal LP and mLNs. Because CD47 has previously been shown to regulate cell migration of innate $SIRP\alpha^+$ cells (Liu et al., 2002; Van et al., 2006), we first thought to examine the expression of SIRPα on the two major mucosal DC subsets, i.e., $CD103^+$ and $CD103^-$ DCs. We found that SIRPα was preferentially expressed with high intensity on $CD11b^+CD103^-$ DCs in the LP and mLNs (Fig. 1, A and B). To investigate the dynamic regulation of these DC subsets during inflammation, their distribution profiles were assessed at steady state and in response to TNBS challenge. We observed an increased percentage of $SIRP\alpha^+CD103^-$ DCs in the LP as well as in the mLNs after the intrarectal administration of TNBS (Fig. 1, A and B), suggesting a role for this DC subset in the response to hapten-induced colonic inflammation. We next used CD47 KO mice to examine the potential role of CD47 in modulating the frequency of $SIRP\alpha^+CD103^-$ DCs in the LP and mLNs. Although no significant differences in $SIRP\alpha^+CD103^-$ DC proportions were observed in the LP of naive WT and CD47 KO mice (Fig. 1, A and C), a reduction in $SIRP\alpha^+CD103^-$ DCs was observed in the mLNs of naive CD47 KO mice when compared with their WT littermates (Fig. 1, B and D). Upon TNBS challenge, the increase in $SIRP\alpha^+CD103^-$ DCs normally observed in WT mice did not occur in either the LP or the mLNs of CD47 KO mice (Fig. 1, C and D), suggesting that CD47 is required for the mobilization of $SIRP\alpha^+CD103^-$ DCs in response to TNBS challenge. Although we show results from mLNs on day 2 after TNBS instillation, similar data were observed on day 4 and in caudal lymph nodes (unpublished data). Notably, the proportion of $SIRP\alpha^-CD103^+$ DCs, which have been shown to promote tolerance, were not increased in the LP of either strain of TNBS-treated mice. However, their frequency was strongly reduced in the mLNs of WT but not CD47 KO TNBS-treated mice (Fig. S1). Of note, the intensity of CD47 expression was identical between $SIRP\alpha^+CD103^-$ and $SIRP\alpha^-CD103^+$ DCs in the LP and mLNs of WT mice (unpublished data). Collectively, our results suggest that CD47 regulates the mobilization of mucosal $SIRP\alpha^+CD103^-$DCs in response to inflammation and, thus, may play a role in gut DC homeostasis.

### CD47 ablation ameliorates the development of chronic TNBS colitis

We therefore hypothesized that the increased proportion of $SIRP\alpha^+CD103^-$ DCs in the LP and mLNs of WT mice would be correlated with colitis development, in which case CD47 KO mice would be protected from disease. Analysis of body weight loss as early as day 2 after primary TNBS instillation revealed a significant reduction of wasting disease in CD47 KO compared with WT mice (Fig. 2 A). This protection was enhanced daily until day 8, by which time

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CD47 KO, as opposed to WT mice, had fully regained their original body weight (Fig. 2 A). To evaluate the degree of colonic inflammation, both the macroscopic and histological scores of tissue damage were assessed. Colons from WT mice with TNBS colitis displayed shortening and thickening, with patchy areas of severe ulceration and prominent adhesions typical of this form of colitis. On the contrary, CD47 KO mice developed fewer of these characteristics and

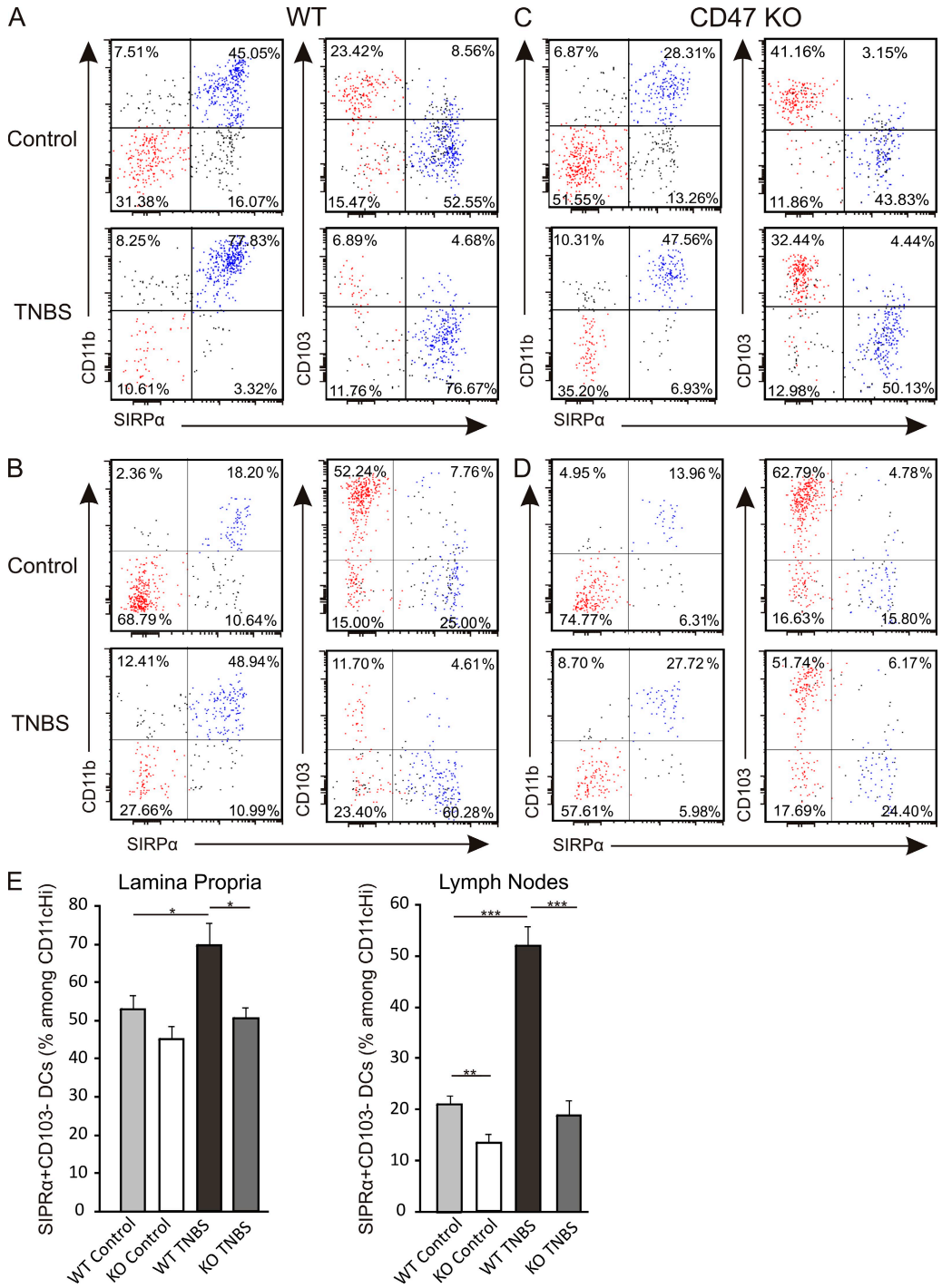


**Figure 1. CD47 regulates SIRPα⁺CD103⁻ DC homeostasis in the LP and mLNs.** (A–D) CD11b, CD103, and SIRPα expression among CD45.2⁺CD11c$^{Hi}$ DCs at steady state (control; top) and 2 d after intrarectal administration of 3 mg TNBS (TNBS; bottom) in the LP (A and C) and mLNs (B and D) of WT (A and B) and CD47 KO (C and D) mice. Data shown are one representative mouse per group. (E) Percentage of SIRPα⁺CD103⁻ DCs among CD45.2⁺CD11c$^{Hi}$ DCs in the LP (left) and mLNs (right). Data represent the means ± SEM of more than eight mice per group. Each experiment was independently performed a minimum of four times. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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displayed significantly milder inflammation than WT mice (Fig. 2, B and D). Upon histological assessment, we confirmed that colons from WT mice with colitis exhibited a substantial infiltration with mononuclear cells, edema, and loss of goblet cells, whereas CD47 KO mice were resistant to these changes (Fig. 2, C and E).

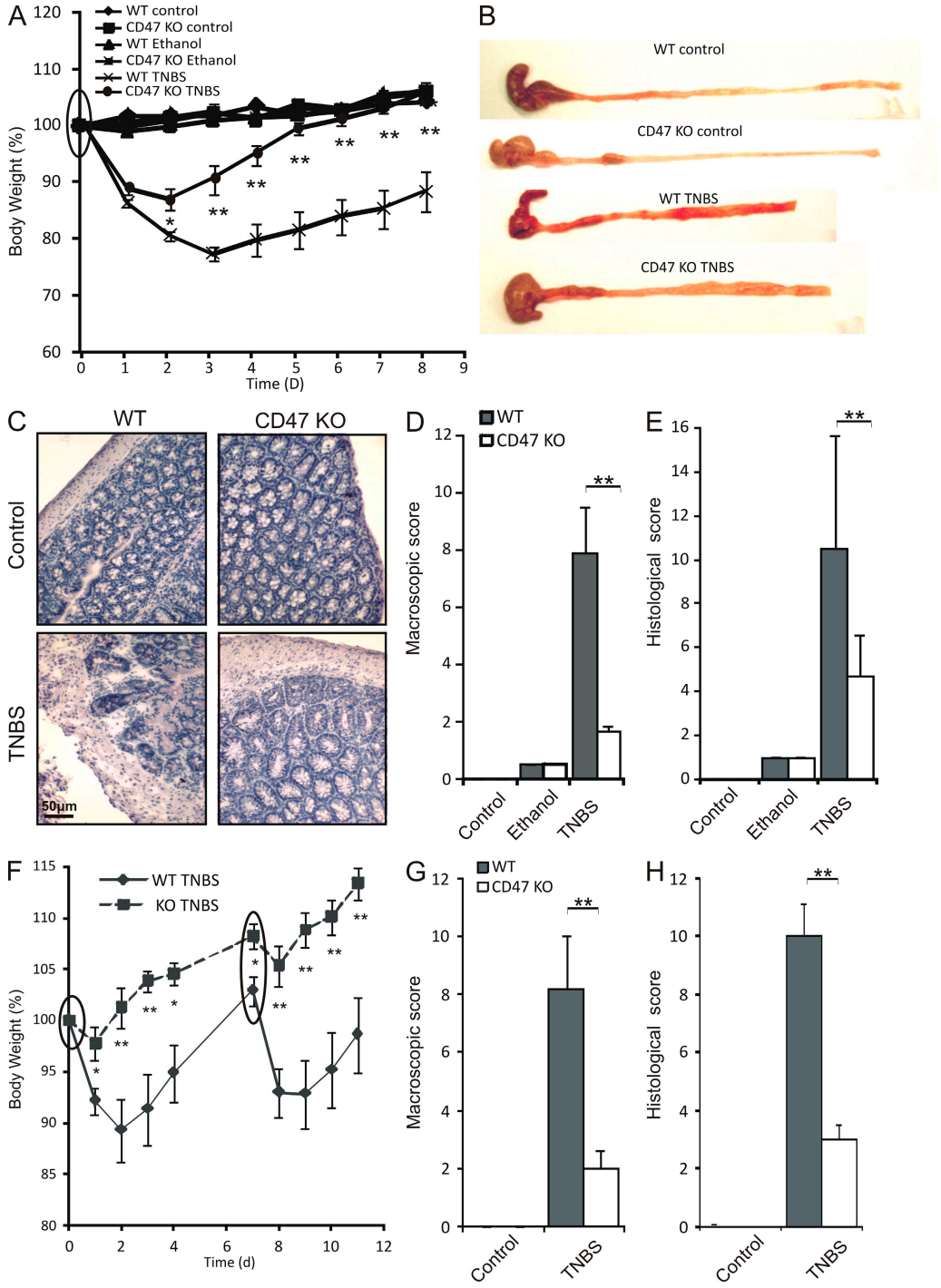


**Figure 2. CD47 ablation protects from the development of TNBS colitis.** TNBS colitis was induced on day 0 alone or on days 0 and 7 (circled). (A) Weight loss curves of WT and CD47 KO mice injected intrarectally with saline (control), ethanol alone, or TNBS dissolved in 50% ethanol on day 0. (B–E) Macroscopic (B and D) and histological (C and E) analyses of inflammation in WT and CD47 KO mice. Data represent means ± SEM of more than eight mice per group, and one representative experiment out of three is shown. (F) Weight loss curves of WT and CD47 KO mice injected intrarectally with 2 mg TNBS dissolved in 50% ethanol on days 0 and 7. (G and H) Macroscopic (G) and histological (H) analyses of inflammation in WT and CD47 KO mice after reinduction of TNBS colitis. Data represent means ± SEM of six mice per group. One representative experiment out of three is shown. *, $P < 0.05$; **, $P < 0.01$.

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We next confirmed the disease resistance of CD47 KO mice in a reactivated form of colitis, where a second instillation of TNBS was administered 7 d after the primary instillation. This model is said to mimic the relapsing behavior of human CD. Here, upon reinduction of TNBS colitis, CD47 KO mice remained resistant to body weight loss, whereas WT mice displayed severe wasting once again (Fig. 2 F). Macroscopic (Fig. 2 G) and microscopic (Fig. 2 H) scores confirmed that CD47 KO mice were largely devoid of signs of chronic colonic inflammation. Collectively, our data demonstrate that the CD47-dependent increase in SIRPα$^+$CD103$^-$ DCs in the LP and mLNs is correlated with the development of chronic intestinal inflammation.

### CD47 ablation impairs Th17 responses in vivo

The chronic stage of TNBS colitis is driven by an important interplay between the innate and adaptive immune systems. We sought to further characterize this inflammation in WT and CD47 KO mice. Both on day 8 and after reinduction of TNBS colitis, a strong up-regulation of IL-6 mRNA expression was observed in WT but not in protected CD47 KO mice (Fig. 3 A). The same trend, but of a lesser magnitude, was observed for TGF-β, IL-12p35, and IL-23p19, where mRNA expression was increased in TNBS-treated WT but not CD47 KO mice (Fig. 3 A). Corroborating the increase in IL-6 and TGF-β expression, IL-17 mRNA was highly up-regulated at both time points in WT but not in CD47 KO mice (Fig. 3 B). IFN-γ mRNA expression was greater in CD47 KO than WT mice, but only on day 8, with no significant difference observed after reinduction (Fig. 3 B). Interestingly, although both forms of colitis displayed qualitatively and quantitatively distinct IL-17 and IFN-γ mRNA expression profiles, the IL-17/IFN-γ mRNA expression ratios were nearly identical, with both being higher in WT than CD47 KO mice (Fig. 3 C). We therefore chose to focus on the reinduction model of colitis for the rest of this study because it more accurately reflects the chronic relapsing behavior of CD.

We next postulated that the reduction in IL-17–associated cytokines in colon tissues was partly attributable to an impaired capacity to generate IL-17–producing T cells (Th17 cells) in the mLNs of CD47 KO mice. Therefore, we examined IFN-γ and IL-17 production in 4-d cultures of mLNs isolated from mice with chronic TNBS colitis. Analysis by flow cytometry revealed that the percentage of Th17 cells was significantly higher in WT compared with CD47 KO mice with colitis (Fig. 3 D). Similar data were observed by measuring IL-17 production in the culture supernatants by ELISA or by examining IL-17 expression in mLN cells after 6 h of PMA/ionomycin restimulation (unpublished data). Under these pro-Th17 culture conditions, no significant difference was observed with respect to CD4$^+$ T cells producing IFN-γ (Fig. 3 D). When expressed as a ratio of IL-17$^+$/IFN-γ$^+$ cells, CD47 KO mice exposed to TNBS displayed a significantly reduced propensity toward IL-17 production (Fig. 3 E), corroborating with tissue mRNA expression and the recent shift in the Th1/Th17 paradigm reported in TNBS colitis (Sheibanie et al., 2007). Thus, CD47 ablation impairs the induction and perpetuation of colitis associated with a Th17-biased response in mLNs.

### SIRPα$^+$CD103$^-$ DCs promote Th17 responses in vitro

To establish causative links between the increased frequency of mucosal SIRPα$^+$CD103$^-$ DCs and Th17 responses observed in vivo, we first sought to assess the in vitro Th1- versus Th17-promoting capacities of SIRPα$^+$CD103$^-$ and SIRPα$^-$CD103$^+$ DCs. These two DC subpopulations were isolated from the LP and mLNs (Fig. S2) and co-cultured with CD4$^+$ T cells from the same anatomical origin. We found that SIRPα$^+$CD103$^-$ DCs were more efficient than SIRPα$^-$CD103$^+$ DCs at promoting IL-17 production by CD4$^+$ T cells (Fig. 4, A and B). Interestingly, DCs purified from spleens behaved in a very similar manner as those isolated from LP and mLNs, with SIRPα$^+$CD103$^-$ DCs favoring Th17 (Fig. 4 C).

When bone marrow–derived DCs (BMDCs) that express SIRPα (>99%) but not CD103 (<1%) were co-cultured with naive transgenic T cells under Th17-promoting conditions, they elicited a similar Th17-biased profile as SIRPα$^+$CD103$^-$ mLN DCs, suggesting that these properties are not limited to gut-derived DCs (Fig. 4 D). Moreover, SIRPα$^+$CD103$^-$ mucosal or splenic DCs and SIRPα$^+$CD103$^-$ BMDCs induced statistically equivalent IL-17$^+$/IFN-γ$^+$ ratios, both of which were significantly higher compared with sorted SIRPα$^-$CD103$^+$ DCs (Fig. 4 E). Therefore, we propose that SIRPα$^+$CD103$^-$ but not SIRPα$^-$CD103$^+$ DCs possess Th17-promoting properties in vitro.

### Transfer of SIRPα$^+$CD103$^-$ BMDCs induces colitis and promotes a Th17 response in CD47 KO mice

To directly examine the proinflammatory capacity of SIRPα$^+$CD103$^-$ DCs in vivo and the role of CD47 on the DCs for disease induction, CD47 KO mice were administered mature WT or CD47 KO SIRPα$^+$CD103$^-$ BMDCs on the day of colitis induction. BMDCs were injected i.p. because previous reports have shown that the mLNs drain the peritoneal cavity, in addition to the gut tissues (Johansson-Lindbom et al., 2003; Kool et al., 2008). Moreover, the i.v. route was deemed unsuitable because i.v.-injected BMDCs migrate very poorly to the LNs, even when administered at high concentrations (3–8 × $10^6$ BMDCs/mouse; Robert et al., 2003; Cavanagh et al., 2005). Upon reinduction of TNBS colitis on day 8, CD47 KO mice injected with WT BMDCs developed severe wasting disease (Fig. 5 A), indicating that CD47 expression was not required in the host for colitis development. Injection of WT BMDCs had no effect on disease outcome when injected in WT mice (Fig. S3). In contrast to WT BMDCs, injection of CD47 KO BMDCs in CD47 KO hosts appeared to induce only a mild form of inflammation that resulted in a slight, nonsignificant reduction of body weight (Fig. 5 A). The changes in body weight were reflected both in the macroscopic (Fig. 5 B) and microscopic

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(Fig. 5 C) extent of inflammation, with WT BMDC injection resulting in an aggressive form of disease. Notably, injection of WT BMDCs also increased the mortality rate of CD47 KO mice more than injection of CD47 KO BMDCs (uninjected, <5%; CD47 KO BMDCs, 25%; and WT BMDCs, 45%).

We next examined whether disease development after WT BMDC injection was associated with the induction of

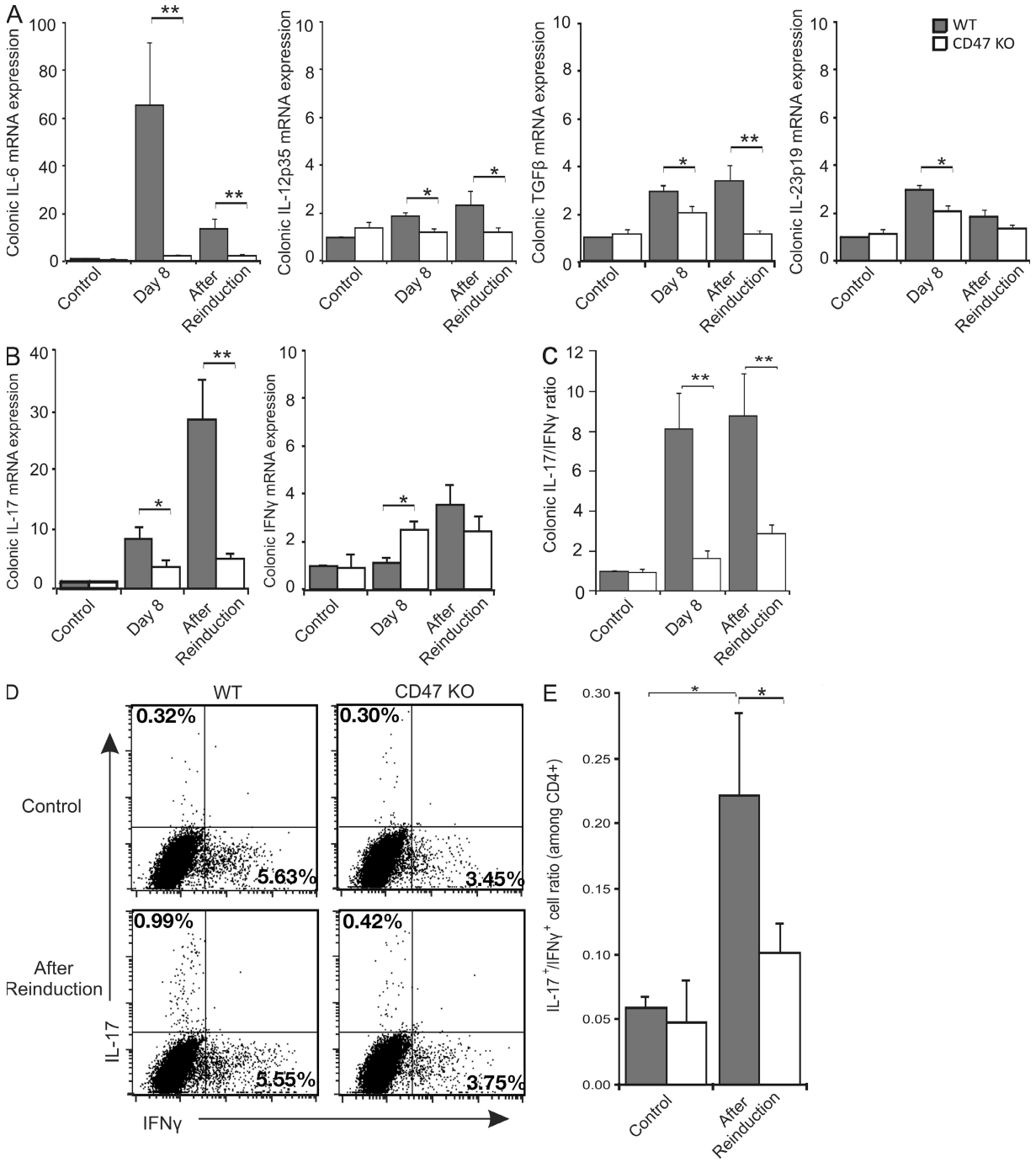


**Figure 3. Decreased proinflammatory cytokine and Th17-biased responses in CD47 KO mice.** (A–C) Colonic mRNA expression of cytokines. (A) Colonic IL-6, IL-12p35, TGF-β, and IL-23p19 mRNA expression. (B) Colonic IL-17 and IFN-γ mRNA expression. (C) Ratio of colonic IL-17/IFN-γ mRNA expression. Data represent mean fold changes relative to WT control ± SEM of more than eight mice per group (two independent experiments). (D) mLNs were isolated after reinduction of TNBS colitis and stimulated for 4 d in the presence of IL-23. Intracytoplasmic staining was performed after restimulation. The percentage IL-17$^+$ or IFN-γ$^+$ cells among total CD4$^+$ T cells is shown. (E) Ratio of IL-17$^+$/IFN-γ$^+$ cells among CD4$^+$ T cells. Data represent the mean percentages ± SEM of more than seven mice per group (two independent experiments). *, $P < 0.05$; **, $P < 0.01$.

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Th17-biased responses in CD47 KO hosts. Injection of WT BMDCs significantly enhanced the colonic mRNA expression of IL-17 but not IFN-γ, whereas injection of CD47 KO BMDCs had no effect on the colonic mRNA expression of either cytokine (Fig. 5 D). Therefore, only WT BMDCs resulted in a significant increase in the IL-17/IFN-γ mRNA ratio (Fig. 5 D). To verify whether the induction of colonic IL-17 mRNA expression was linked to an increase in Th17 cells, we

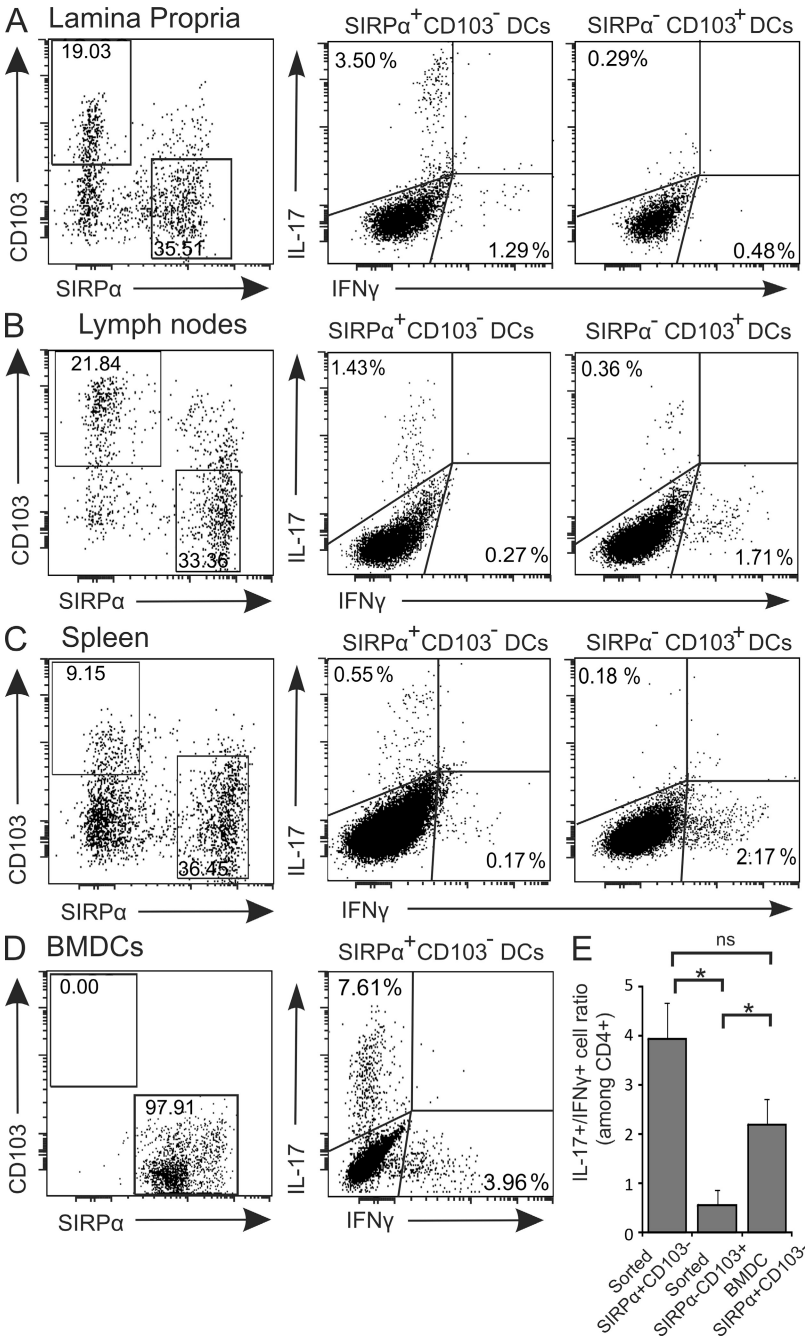


**Figure 4. SIRPα⁺CD103⁻ DCs promote Th17 responses in vitro.** (A–C) Percentage of SIRPα⁺CD103⁻ and SIRPα⁻CD103⁺ DCs (gated on CD11c$^{Hi}$; left) and their IFN-γ– versus IL-17–promoting capacities (middle and right) when isolated from the LP (A), mLNs (B), or spleen (C) and co-cultured with purified anti-CD3–stimulated CD4⁺ T cells of the same anatomical origin. Dot plots represent one independent experiment out of two or three. (D) Percentage of SIRPα⁺CD103⁻ and SIRPα⁻CD103⁺ BMDCs (gated on CD11c⁺; left) and co-culture with OVA-stimulated CD4⁺ transgenic T cells under Th17-polarizing conditions (right). Data represent one independent experiment out of three. (E) IL-17⁺/IFN-γ⁺ cell ratio (among CD4⁺ T cells) after co-culture with either sorted SIRPα⁺CD103⁻ or SIRPα⁻CD103⁺ DCs or SIRPα⁺CD103⁻ BMDCs. The data of sorted cells represents mean of all sources of DCs (LP, mLNs, and spleen) ± SEM of a minimum of three independent experiments. *, $P < 0.05$.

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examined the mLNs of reconstituted mice. Injection of WT BMDCs increased the proportion of IL-17$^+$ CD4$^+$ T cells, leading to a significant enhancement in the IL-17$^+$/IFN-γ$^+$ CD4$^+$ T cell ratio (Fig. 5, E and F). On the other hand, injection of CD47 KO BMDCs elicited IL-17 expression that did not translate into a significant increase in the IL-17/IFN-γ

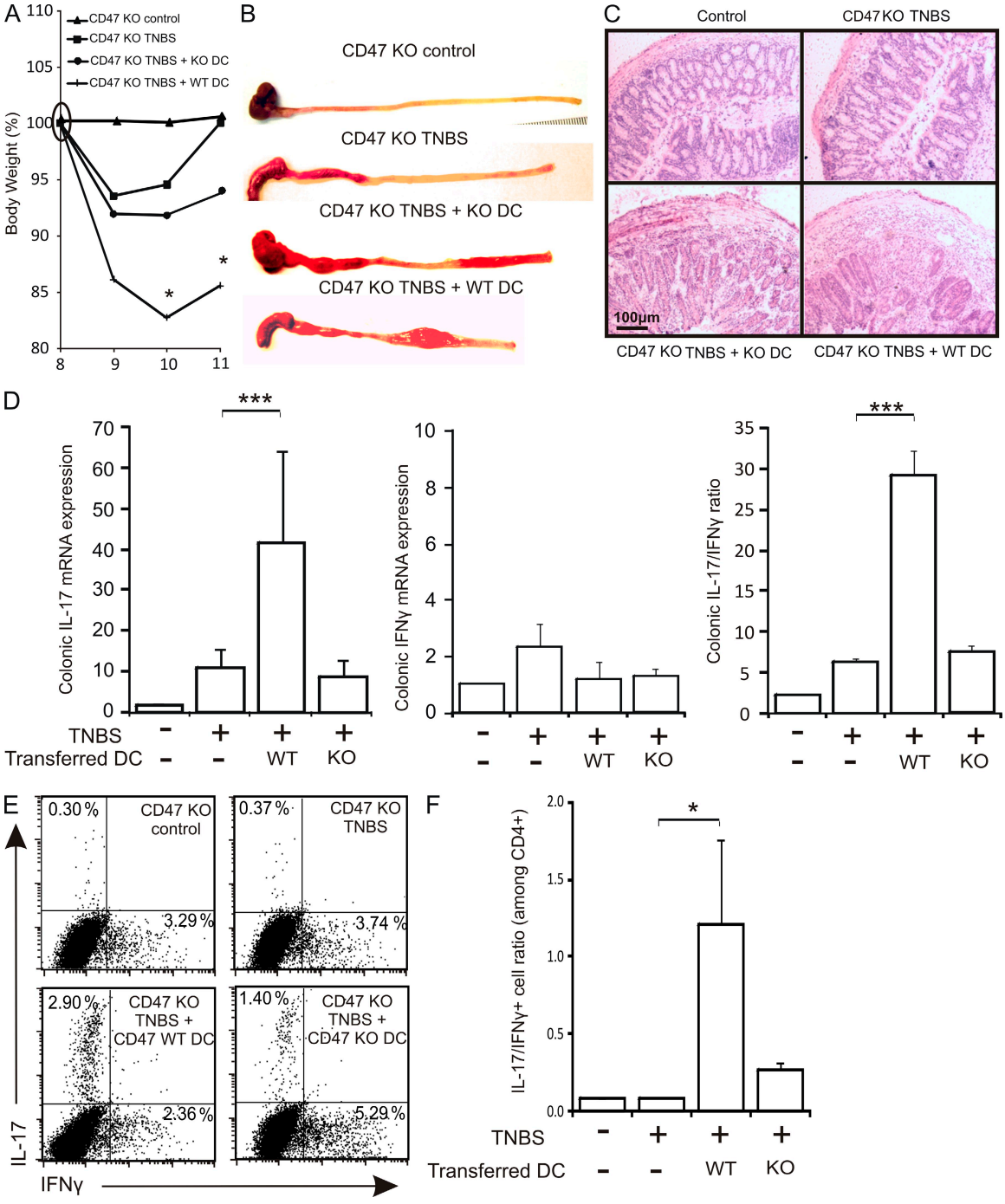


**Figure 5. CD47 expression on SIRPα$^+$CD103$^-$ DCs promotes the development of intestinal inflammation and Th17 responses.** CD47 KO mice were injected i.p. with saline or WT or CD47 KO BMDCs 30 min before the induction of TNBS colitis on day 0. TNBS was reinduced on day 8. (A) Weight-loss curves after transfer of WT or CD47 KO BMDCs in CD47 KO hosts and reinduction of TNBS colitis, normalized to body weight on day 8. (B and C) Macroscopic (B) and histological (C) appearance of colonic inflammation. Images are of one representative mouse per experimental group. (D) Colonic mRNA expression of IL-17 and IFN-γ and IL-17/IFN-γ mRNA expression ratio. Data represent the mean fold changes relative to control. (E) mLNs were isolated after reinduction of TNBS colitis and stimulated for 4 d in the presence of IL-23. The percentages of IL-17$^+$ or IFN-γ$^+$ CD4$^+$ T cells and the ratio of IL-17$^+$/IFN-γ$^+$ cells among CD4$^+$ T cells are shown. Data represent means ± SEM of more than five mice per group (two pooled independent experiments). *, $P < 0.05$; ***, $P < 0.001$.

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ratio (Fig. 5, E and F). Finally, SIRPα$^+$CD103$^-$ DCs purified from WT mLNs and injected i.p. were also capable of inducing severe wasting disease, resulting in 25% mortality after a single dose of TNBS in CD47 KO mice (Fig. S4). Severe weight loss in these mice was correlated with pronounced colonic inflammation and Th17-polarized responses, corroborating the similar in vitro pro-Th17 responses of SIRPα$^+$CD103$^-$ BMDCs and mucosal DCs. These data demonstrate that CD47 expression is dispensable on the endothelium, epithelium, and T cells of the host but appears to be critical on SIRPα$^+$CD103$^-$ DCs for Th17-associated colonic disease induction in vivo.

### SIRPα$^+$CD103$^-$ DC trafficking but not phenotype or function is governed by CD47

We next sought to delineate the mechanisms by which CD47 expression on SIRPα$^+$CD103$^-$ BMDCs controls the development of Th17 responses and colonic inflammation. To this end, we evaluated the in vivo migratory properties and the in vitro function of WT and CD47 KO BMDCs. First, WT and CD47 KO BMDCs were fluorescently labeled and injected i.p. at a 1:1 ratio in CD47 KO mice and retraced in the mLNs after 2 d. By using this competitive migration assay, we provide evidence that CD47 expression on BMDCs significantly favors their accumulation in the mLNs (Fig. 6, A and B), corroborating the increased mobilization of SIRPα$^+$CD103$^-$ mucosal DCs observed in WT but not CD47 KO mice upon induction of TNBS colitis (Fig. 1).

Next, we assessed the phenotype of BMDCs in terms of their expression of CD11c, CD11b, SIRPα, and CD103 and observed no difference between WT and CD47 KO BMDCs (Fig. 6 C). Importantly, the level of expression of SIRPα was not altered on a per-cell basis by the expression of CD47, as determined by its mean fluorescence intensity (Fig. 6 C). The surface expression of MHC II and the co-stimulatory molecules CD40 and CD86 were also independent of CD47 expression (Fig. 6 D). We next examined the function of WT and CD47 KO BMDCs by measuring the production of cytokines such as IL-6 and IL-23p19, as well as their ability to drive Th17 polarization. Both cytokine production and Th17-promoting capacity were found to be unrelated to CD47 expression on the DCs (Fig. 6, E and F). Also, lack of CD47 expression on T cells did not alter the percentage of IL-17–producing CD4$^+$ T cells, thereby eliminating the possibility of a T cell–intrinsic defect in Th17 polarization in CD47 KO T cells as a mechanism for decreased Th17 responses (Fig. 6 F). Because CD47 does not alter the phenotype, activation status, cytokine production, or Th17-polarizing capacity of SIRPα$^+$CD103$^-$ BMDCs, we propose that CD47 expression on SIRPα$^+$CD103$^-$ DCs is critical for their trafficking to mLNs, a property mechanistically related to the ensuing Th17 responses and colitis development.

### Administration of CD47-Fc ameliorates disease development in BALB/c mice

Finally, we circumvented the multiple defects of CD47 KO hosts and directly examined the impact of targeting the SIRPα–CD47 axis on the recruitment of SIRPα$^+$CD103$^-$ DCs in mLNs, Th17 polarization, and the development of chronic colitis in WT mice. To this end, BALB/c mice were administered a CD47-Fc molecule i.p. 30 min before TNBS injection and on days 1–3 (Fig. 7 A, arrows). Mice administered CD47-Fc were largely protected from colonic inflammation as assessed by body weight, starting as early as 1 d after the induction of colitis (Fig. 7 A). Furthermore, CD47-Fc–treated mice remained resistant to colitis even after a second induction of TNBS colitis, as demonstrated by a reduction in body weight loss and the extent of tissue damage (Fig. 7, A–C). Injection of a control human CD47-Fc with no cross-reactivity in mice did not ameliorate disease (not depicted). Serum levels and colonic mRNA expression of IL-6 were determined early in disease onset (day 4) and were dramatically reduced in CD47-Fc–treated mice (Fig. 7 D). Importantly, the percentage of SIRPα$^+$CD103$^-$ DCs was specifically and significantly reduced by CD47-Fc treatment (Fig. 7 E). On day 4, IL-17 and IFN-γ mRNA expression were unaffected. After reinduction, IL-17 mRNA expression was effectively suppressed by CD47-Fc, whereas IFN-γ mRNA expression was unaltered (Fig. 7 F). Regardless of the time of sacrifice (day 4 or after reinduction), the IL-17/IFN-γ mRNA ratio was nearly identical and equally suppressed by administration of CD47-Fc (Fig. 7 G). Similarly, the Th17/Th1 ratio in mLNs was also reduced by CD47-Fc (unpublished data). We therefore conclude that CD47-Fc treatment protects mice from the development of chronic colitis and is correlated with an altered development of SIRPα$^+$CD103$^-$ DC–mediated Th17 responses.

## DISCUSSION

DCs lie at the interface between the innate and the adaptive immune system, and although they are poised to maintain gut homeostasis and tolerance, they also appear to play a critical role in CD pathogenesis. In this paper, we propose SIRPα-expressing CD11b$^+$CD103$^-$ DCs as one primary candidate for the induction and perpetuation of chronic TNBS colitis and implicate CD47 as a key player in controlling their homeostasis and trafficking to the LP and mLNs. We first observed a correlation between the frequency of SIRPα$^+$CD103$^-$ DCs in the LP and mLNs, Th17 responses, and the development of intestinal inflammation, parameters that were all impaired in CD47 KO mice. Next, we demonstrated that CD47 expression is required on SIRPα$^+$CD103$^-$ DCs for efficient trafficking to mLNs and induction of severe wasting Th17-associated disease in vivo, but is dispensable on both SIRPα$^+$ DCs and T cells to promote Th17 responses in vitro. Finally, early administration of a CD47-Fc molecule treated and prevented the recurrence of experimental colitis, which was correlated with defective homing of SIRPα$^+$CD103$^-$ DCs and Th17 responses. We thus provide evidence that CD47 expression on SIRPα$^+$CD103$^-$ DCs governs their trafficking and DC-driven Th17 responses, which are likely implicated in the development of intestinal inflammation.

Although the importance of DCs in the initiation of intestinal inflammation is now well accepted (Coombes and

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Powrie, 2008), the specific contributions of individual DC subsets and the key molecules involved in their function/migration remain to be clarified. This is the first study to identify SIRPα expression on mucosal CD103⁻ DCs and to support a role for this DC subset in the in vivo promotion of intestinal inflammation. Nevertheless, although the proportion of total

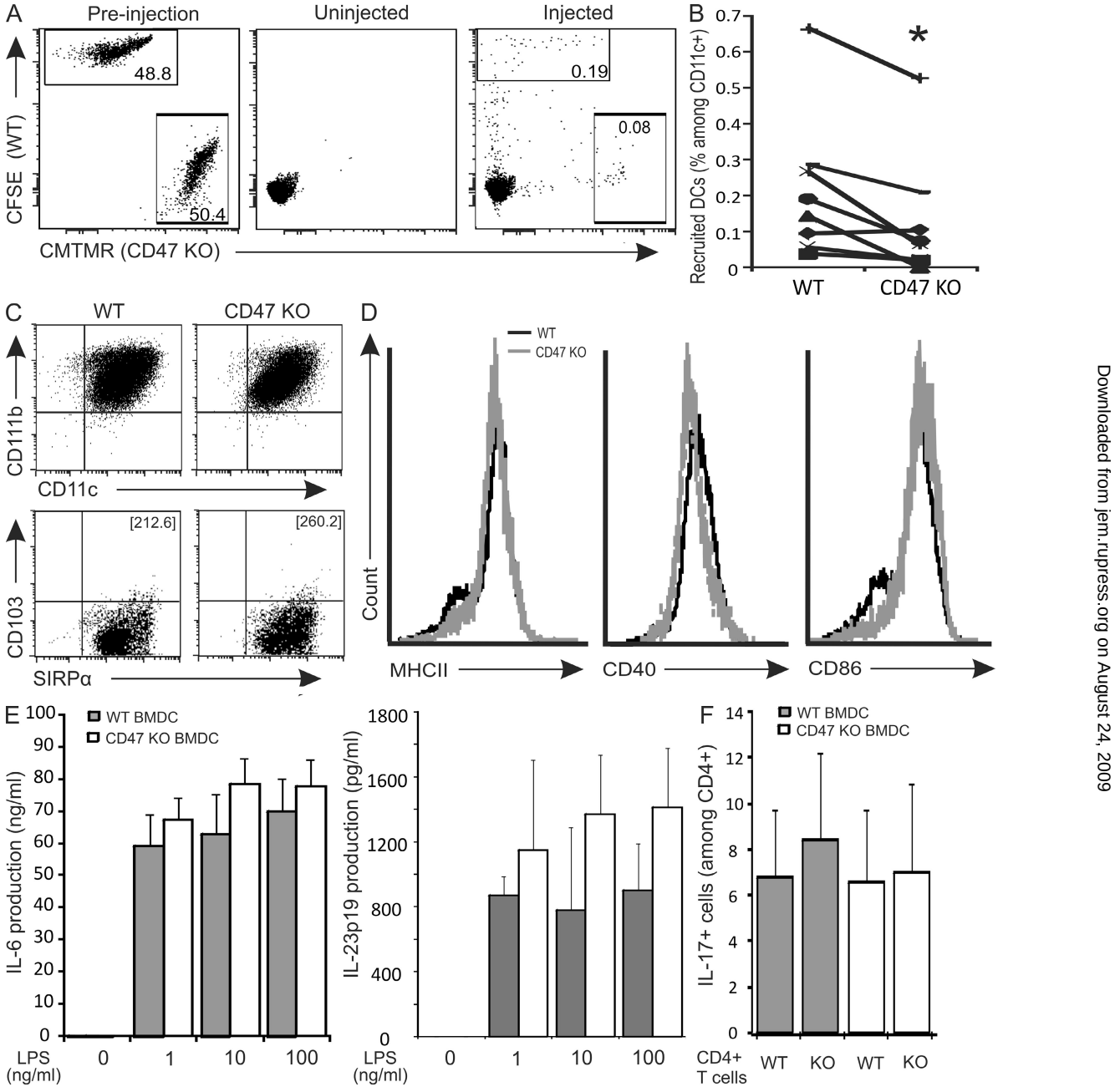


**Figure 6. CD47 regulates SIRPα⁺CD103⁻ DC trafficking but not phenotype or function.** (A, left) 1:1 ratio of labeled BMDCs before injection on day 0. (middle and right) TNBS colitis was induced in CD47 KO mice, and mLNs were harvested after 2 d. (middle) CD11c⁺ DCs in the mLNs of uninjected CD47 KO mice. (right) Labeled WT and CD47 KO BMDCs (gated on CD11c⁺) in the mLNs of BMDC-injected CD47 KO mice. One representative mouse is shown for each condition. (B) Percentages of labeled WT and CD47 KO BMDCs (gated on CD11c⁺) in the mLNs of CD47 KO mice. Data were generated in two independent experiments ($n = 8$). (C and D) Analysis of phenotype (C) and activation status (D) of LPS-stimulated BMDCs. The numbers in brackets in C represent the mean fluorescence intensity of SIRPα. Data are representative of three independent experiments. (E) Cytokine production by BMDCs stimulated overnight with LPS (0–100 ng/ml) and IFN-γ (10 ng/ml) in vitro. Data represent the means ± SEM ($n = 4$ independent experiments). (F) Percentage of IL-17⁺ cells (among CD4⁺ T cells) after criss-cross co-cultures with anti-CD3–stimulated WT versus CD47 KO splenic CD4⁺ T cells and LPS-stimulated WT versus CD47 KO BMDCs under Th17-polarizing conditions. Data represent means ± SEM ($n = 3$ independent experiments with two to three mice per experimental group).

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SIRPα$^+$CD103$^-$ DCs increased in the LP and mLNs of WT mice upon induction of TNBS colitis, the percentage of the tolerogenic CD103$^+$ DC subset, which we describe as being primarily SIRPα$^-$, decreased in the mLNs. Therefore, it may have been postulated that the reduction in the proportion of CD103$^+$ DCs migrating to the mLNs of WT mice was the key event in triggering intestinal inflammation, as opposed to the increased recruitment of the pathogenic SIRPα$^+$CD103$^-$ DC

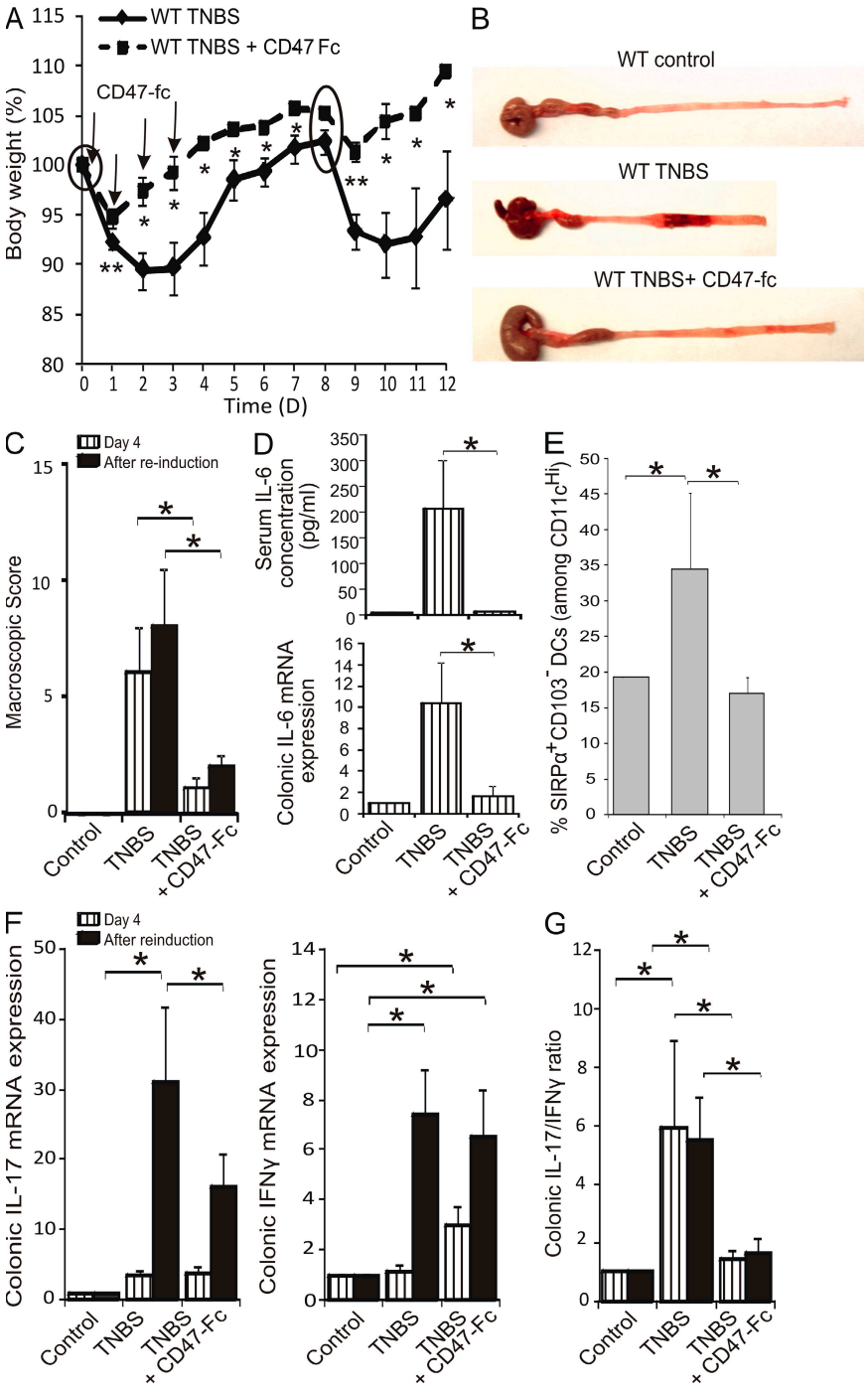


**Figure 7. Administration of CD47-Fc protects BALB/c mice from the development of TNBS colitis.** WT BALB/c mice were injected i.p. with saline or CD47-Fc, TNBS colitis was induced on days 0 and 8 (circles), and mice were sacrificed on day 4 or 12 (after reinduction). (A) Weight-loss curves normalized to body weight on day 0. (B) Macroscopic appearance and (C) mean macroscopic score inflammation. (D) Serum concentration (top) and colonic mRNA expression (bottom) of IL-6. (E) mLN SIRPα$^+$CD103$^-$ DCs as a percentage of total CD11c$^{Hi}$. (F) Colonic mRNA expression of IL-17 and IFN-γ. (G) IL-17/IFN-γ mRNA expression ratio. Data represent means ± SEM of more than six mice per group of two independent experiments. *, $P < 0.05$; **, $P < 0.01$.

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subset in both the tissues and mLNs. Yet, CD47 KO mice do not display any change in the proportion of tolerogenic CD103$^+$ DCs in mLNs upon exposure to TNBS, and a single administration of WT SIRPα$^+$CD103$^-$ DCs in CD47 KO mice was sufficient to induce severe wasting disease. Consequently, our data support a proinflammatory role for the SIRPα$^+$CD103$^-$ DC subset, the effect of which may be amplified by, but is not exclusively dependent on, the reduction in the proportion of tolerogenic CD103$^+$ DCs in the mLNs. In support of this concept, CD103$^+$ DCs are found in equal proportions in the mLNs draining the small intestines of healthy individuals and those of CD patients, implying that intestinal inflammation is not dependent on a reduction in CD103$^+$ DCs (Jaensson et al., 2008).

In the gut, CCR7 largely controls CD103$^+$ DC trafficking at steady state (Jang et al., 2006; Worbs et al., 2006). We have previously reported that the absence of CD47 does not alter CCR7 expression or the in vitro migration of skin SIRPα$^+$ DCs toward CCL19 or sphingosine-1-phosphate (Van et al., 2006). The reduced proportion of the SIRPα$^+$CD103$^-$ DC subset in the mLNs of CD47 KO and CD47-Fc–treated mice upon exposure to TNBS strongly suggests that CD47 promotes this process. These findings corroborate previous in vivo studies in which a reduction of SIRPα$^+$ DCs was observed in skin-draining LNs of CD47 KO and CD47-Fc–treated mice, which was found to be independent of CD47 expression on lymphatic vessels (Van et al., 2006). In support of these findings, we demonstrate in this paper that injected WT SIRPα$^+$CD103$^-$ DCs migrate more efficiently to mLNs and elicit a more aggressive form of disease in CD47 KO hosts when compared with CD47 KO DCs. However, the underlying mechanisms by which CD47 governs SIRPα$^+$CD103$^-$ DC migration remains to be elucidated. One hypothesis is that CD47–SIRPα interactions that may occur in cis on the DC indirectly control integrin-mediated transendothelial trafficking.

The expression of CD62L on CD103$^-$ DCs (Johansson-Lindbom et al., 2005) and a more recent report examining the turnover of intestinal DC subsets (Jaensson et al., 2008) favor the hypothesis of a direct recruitment of CD103$^-$ DCs or their precursors from the bloodstream to the mLNs via high endothelial venules at steady state. Our study supports this hypothesis, because there is no defect of SIRPα$^+$CD103$^-$ DCs in the LP, whereas there is a significant reduction in the proportion of these DCs in the mLNs of CD47 KO mice at steady state. Therefore, SIRPα$^+$CD103$^-$ DCs or their precursors may bypass the LP to seed the mLNs in a CD47-dependent manner. However, during the induction of colonic inflammation, SIRPα$^+$CD103$^-$ DCs or their precursors may be recruited de novo to inflamed tissues before migrating to the mLNs. Interestingly, under inflammatory conditions, $CX_3CR1^{int}GR1^{high}CD62L^+CCR2^+$ monocytes ($GR1^{high}$ monocytes) have been shown to differentiate into LP DCs (Varol et al., 2007), but i.v.-injected bone marrow monocytes are also capable of directly entering LNs through high endothelial venules (Nakano et al., 2009). Thus, under inflammatory conditions, CD47 may be implicated in the recruitment of SIRPα$^+$CD103$^-$ DC precursors to the LP and/or the mLNs, resulting in a reduction in the frequency of SIRPα$^+$CD103$^-$ DCs at those sites in CD47 KO or CD47-Fc–treated mice. Nevertheless, we cannot exclude the possibility that CD47 is required for the migration of SIRPα$^+$CD103$^-$ DCs directly from the LP to the mLNs (Fig. S5). Thus, further examination of the physiological routes of SIRPα$^+$CD103$^-$ DC trafficking under inflammatory conditions is required to delineate the precise role of CD47 in this process.

The early phases of TNBS colitis have been successfully induced in the absence of T cells. Therefore, innate-mediated mechanisms are also likely to be implicated in disease pathogenesis (Fiorucci et al., 2002; Katakura et al., 2005). SIRPα$^+$ neutrophils, which are rapidly recruited to sites of inflammation, have been shown to exhibit CD47-mediated transmigration (Cooper et al., 1995). A significant reduction in colonic myeloperoxidase activity, a marker of neutrophil infiltration, occurred early in disease development in CD47 KO mice (unpublished data). We thus hypothesized that a defect in CD47-mediated neutrophil transmigration may be protective in the acute phase of disease. However, a previously published report has suggested a protective role for neutrophils in disease initiation (Kühl et al., 2007). Therefore, although a reduction of neutrophil infiltration was observed in CD47 KO mice, we largely excluded this phenomenon as playing a protective role in the development of TNBS colitis.

In the present study, we observed causative links between an increased percentage of SIRPα$^+$CD103$^-$ DCs, Th17-skewed responses, and disease manifestation, suggesting a role for Th17 cells in the development of colitis. The in vitro Th17-promoting capacity of $CD11c^{Hi}$SIRPα$^+$CD103$^-$ DCs seems to be shared by the previously described $CD11c^{Hi}CX_3CR_1^+$CD103$^-$ LP DC subset, supporting their contribution in the initiation of Th17 responses (Denning et al., 2007). Until recently, both CD and TNBS colitis have been considered Th1 diseases characterized by IL-12, TNF-α, and IFN-γ production (Neurath et al., 1995; Parronchi et al., 1997). However, several recent observations have strongly implicated a role for IL-23, a Th17 survival factor, and Th17 cells in disease pathogenesis. In humans, elevated serum levels of IL-17 and colonic mucosal levels of both IL-17 and IL-23 have been detected in CD patients (Fujino et al., 2003; Schmidt et al., 2005), whereas a mutation in the IL-23R gene (rs11209026, c.1142G>A, p.Arg381Gln) is protective from disease development (Duerr et al., 2006). It is established that the intestinal mucosal environment, via TGF-β, thymic stromal lymphopoietin, and IL-10, acts to condition resident antigen-presenting cells to exert immunosuppressive activities (Jarry et al., 2008; Zeuthen et al., 2008). Epithelial cells from CD patients express less thymic stromal lymphopoietin, leading to an enhanced release of the pro-Th1 and -Th17 inflammatory cytokines IL-12, IL-6, and IL-23, and expression of TLR2 and TLR4 by DCs (Hart et al., 2005; Rimoldi et al., 2005). Moreover, the combination of

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intestinal epithelial-derived TGF-β and retinoid acid has also recently been shown to convert $CD103^-$ DCs into $CD103^+$ DCs capable of generating T reg cells (Iliev et al., 2009). IL-23 is abundantly produced by ileal but not colonic mouse DCs (Becker et al., 2003). Therefore, TNBS colitis, a model in which the inflammation is localized to the colon, might be less suitable than others for examining IL-23 regulation, as underscored by the low colonic mRNA expression of IL-23p19 in this study, even under conditions of severe inflammation. Nonetheless, the expression of IL-17 was strongly up-regulated by TNBS in WT mice as compared with CD47 KO mice. IL-17 is considered to be pathogenic in TNBS colitis because IL-17R–deficient mice are protected from disease development, and administration of an anti–IL-17 mAb ameliorates intestinal inflammation despite high levels of IFN-γ (Zhang et al., 2006). In contrast, IFN-γ receptor– and IFN-γ–deficient mice, or mice administered a neutralizing anti–IFN-γ mAb developed colitis as severe as WT mice (Camoglio et al., 2000; Tozawa et al., 2003), suggesting that IFN-γ and IL-12 play a less essential role in disease development than once believed. On the other hand, both IL-17 and IFN-γ synergize to create intestinal inflammation in *Helicobacter hepaticus*–induced T cell–dependent colitis (Kullberg et al., 2006). In Th17-associated autoimmune diseases, such as experimental autoimmune uveitis (Luger et al., 2008) and experimental autoimmune encephalomyelitis, either Th1 or Th17 cells alone can drive tissue damage (Bettelli et al., 2004; Kroenke et al., 2008; Stromnes et al., 2008). T cell clones isolated from CD patients have been identified as being either single $IL\text{-}17^+$ or double $IL\text{-}17^+IFN\text{-}\gamma^+$ cells, underscoring the potentially pathogenic role of both cytokines (Annunziato et al., 2007). Some studies have even suggested a differential role for Th1 versus Th17 cells at various stages of disease (Kobayashi et al., 2008). Moreover, the developmental plasticity of Th17 cells has been reported, whereby IL-23 and IL-12 convert terminally differentiated mouse Th17 cells into IFN-γ–producing cells (Lee et al., 2009). Thus, it is likely an oversimplification to implicate Th17 cells alone in the development of TNBS colitis, and as such, we monitored the Th17/Th1 ratio rather than an exclusive decrease in the frequency of Th17 cells as a common thread of disease protection throughout this study. Finally, although Th17 cells are often categorized as pathogenic in autoimmune disease, it should be emphasized that they critically participate in protecting the host from microbial invasion and maintaining the epithelial barrier, as was demonstrated for the Th17-associated cytokine IL-22 (Mudter et al., 2008).

We also report that the mRNA expression of IL-6, a key Th17 cell differentiation factor, was impaired in the colon tissues of protected mice. IL-6 plays an important role in both T cell–dependent and –independent models of colitis (Atreya et al., 2000; te Velde et al., 2007), and IL-6–deficient mice were reported to show resistance to TNBS-induced colitis (Atreya et al., 2000; Gay et al., 2006). Although the precise cellular source of IL-6 was not examined in this study, epithelial cells and T cells themselves, in addition to antigen-presenting cells, contribute to IL-6 production during colitis (Dann et al., 2008; Mudter et al., 2008). We demonstrate in this paper that CD47 expression does not regulate IL-6 production by $SIRP\alpha^+CD103^-$ DCs. Therefore, the reduction of IL-6 expression observed in this study likely reflects an indirect consequence of reduced tissue infiltration.

To eliminate the developmental alterations in CD47 KO mice, such as the expansion of the $CD103^+$ T reg cell population (Van et al., 2008) and Th1-biased phenotype (Bouguermouh et al., 2008), as a potential explanation for disease resistance, we demonstrate that the short-term interruption of CD47 ligation by the administration of a CD47-Fc molecule protected WT BALB/c mice from disease development. At an early time point after disease induction, we readily observed a reduced proportion of $SIRP\alpha^+CD103^-$ DCs in the mLNs and an inverted IL-17/IFN-γ mRNA ratio in treated compared with untreated mice. The therapeutic efficacy of CD47-Fc could not be attributed to increased T reg cell activity, because $CD4^+CD25^+FoxP3^+$ T reg cell numbers were positively correlated with inflammation and were lower in CD47-Fc–treated mice (unpublished data). Finally, early administration of CD47-Fc was shown to be highly effective in preventing both disease initiation and the recurrence of colitis after a second TNBS challenge. In that respect, the therapeutic efficacy of most published compounds has only been assessed in the prevention of acute disease (te Velde et al., 2006).

In conclusion, we have identified $SIRP\alpha^+CD103^-$ DCs as an immunogenic Th17-inducing DC subset and propose their role in the pathogenesis of TNBS-induced colitis. We further demonstrate that CD47 expression on $SIRP\alpha^+CD103^-$ DCs promotes their trafficking and the development of severe intestinal inflammation. We therefore propose that targeting the CD47–SIRPα axis may serve as the basis for the development of novel therapeutic strategies in CD.

## MATERIALS AND METHODS

### Animals

6–8-wk-old WT BALB/c and BALB/c CD47 KO mice were bred and maintained in our animal care facility in standard animal cages and under specific pathogen-free conditions. CD47 KO mice are viable and do not exhibit any overt phenotype. All mice were handled according to institutionally recommended animal care guidelines, and all experiments were approved by the Animal Studies Ethics Committee of McGill University and the Centre de Recherche du Centre Hospitalier de Montreal.

### Culture medium, antibodies, and reagents

Ex vivo and in vitro experiments were performed using complete RPMI 1640 medium (Wisent Inc.) supplemented with 10% fetal calf serum (Wisent Inc), 500 U/ml penicillin, 500 μg/ml streptomycin, 10 mM Hepes buffer, and 1 mM 2-mercaptoethanol (Invitrogen). IMDM (Invitrogen) supplemented with 5% fetal calf serum, 500 U/ml penicillin, 500 μg/ml streptomycin, and 1 mM 2-mercaptoethanol was used for in vitro $CD4^+$ T cell co-cultures with sorted DCs. *Escherichia coli* LPS was obtained from Sigma-Aldrich, and GM-CSF was purchased from PeproTech. The anti-CD3 antibody (145-2C11) used for in vitro polyclonal stimulation was obtained from BD. Allophycocyanin (APC)-labeled anti-CD4, FITC-labeled anti–IFN-γ, and PE-labeled anti–IL-17 were used for intracytoplasmic cytokine staining of $CD4^+$ T cells and were obtained from BD. For DC phenotype staining and DC/$CD4^+$ T cell sorting, FITC-labeled anti-CD11b, anti–MHC II, anti-CD40, and anti-CD86, PE-labeled anti-SIRPα, biotinylated anti-CD103

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plus streptavidin-PerCP, APC-labeled anti-CD11c, anti-CD45.2–APC–Cy7, and anti-CD4–PeCy7 were obtained from BD.

### TNBS-induced colitis

Two different models of chronic TNBS colitis were induced. In the first model, 3 mg TNBS (Sigma-Aldrich) was dissolved in 50% ethanol and a total volume of 50 µl was injected intrarectally in isopropanol-anesthetized mice (Scheiffele and Fuss, 2002). Controls included those administered intrarectal saline or ethanol alone. Mice were sacrificed 8 d after TNBS injection to assess colonic inflammation. The second form of chronic TNBS colitis was induced by two injections of 2 mg TNBS dissolved in 50% ethanol. The second injection was administered 7 or 8 d after the primary injection. Mice were sacrificed either 2 d after primary TNBS injection to assess early events in disease development or 4 d after the secondary injection to assess colonic inflammation.

For treatment of TNBS colitis, 100 µg of mouse CD47-Fc (Van et al., 2006), control human CD47-Fc (Latour et al., 2001), or saline was injected i.p. on days 0–3, inclusively. 2 mg TNBS colitis was induced on days 0 and 8, and mice were sacrificed on day 4 or 12. In all cases, the macroscopic score of inflammation was assessed based on the degree of ulceration (0–10), the presence of diarrhea (0–1) and adhesions (0–2), and on the thickness of the colon wall (0–1). For histological assessment, colon samples were embedded in optimum cutting temperature compound (Sakura Finetek) and stained with hematoxylin and eosin. Histological changes were semiquantitatively graded based on a set of previously established criteria (Ameho et al., 1997). The grading scale ranged from 0–16 and was calculated as the sum of scores for expansion of submucosa (0–4), expansion of LP (0–4), loss of goblet cells (0–4), and neutrophil infiltration (0–4). All macroscopic and microscopic scoring was performed in a blinded fashion.

### Cytokine quantification by ELISA

Whole blood was withdrawn from mice immediately postmortem and sera were frozen at −20°C until use. Serum IL-6 was quantified by the Quantikine ELISA kit (R&D Systems). IL-6 and IL-23p19 production was assessed in BMDC culture supernatants after overnight culture using the mouse IL-6 DuoSet (R & D Systems) and IL-23p19 (eBioscience) ELISA kits.

### Real-time PCR

Colons were immediately immersed in RNAlater (QIAGEN) upon dissection and frozen at −20°C until use. mRNA was extracted according to the TRIzol protocol and was reverse transcribed using the cDNA RT kit (Applied Biosystems). Quantitative real-time PCR was performed using a sequence detection system (1 PCR cycle at 95°C for 10 min; 40 PCR cycles at 60°C for 1 min and at 95°C for 15 s; ABI Prism 7900HT; Applied Biosystems). cDNA was amplified in a 10-µl final reaction mix containing TaqMan Universal PCR Master Mix (Applied Biosystems) and the corresponding TaqMan gene expression assays (IL-6, Mm00446190_m1; Eukaryotic 18s rRNA, Hs99999901_s1; IL-17a, Mm00439619_m1; IL-12p35, Mm00434165_m1; thrombospondin-1, Mm00449022_m1; IL-23p19, Mm00518984_m1; IFN-γ, Mm00801778_m1; TGF-β, Mm00441724_m1; Applied Biosystems). Signals were analyzed by the ABI Prism sequence detection system software (version 2.2; Applied Biosystems). The comparative Ct method for relative quantification was used, whereby all Ct were first normalized to the expression of 18s rRNA. Cytokine expression is represented as a fold change relative to control mice.

### Ex vivo cultures of mLNs

For ex vivo cultures, mLNs were harvested 3–4 d after secondary TNBS injection and $10^6$ cells/ml were cultured in 48-well plates. Cells were stimulated with 2 µg/ml anti-CD3 antibody for 4 d in the presence of 20 ng/ml IL-23, followed by restimulation with 5 ng/ml PMA and 0.5 µg/ml ionomycin in the presence of 1 µg/ml Brefeldin A for the last 6 h of culture. In some experiments, cells were immediately stimulated after harvest with PMA/ionomycin in the presence of Brefeldin A. Cells were collected, stained for CD4, fixed and permeabilized with the Fix/Perm kit (BD), and stained for intracellular IL-17 and IFN-γ for analysis by flow cytometry. Data were acquired on a FACSCalibur (BD) and were analyzed with CellQuest software (BD).

### DC phenotype in LP and mLNs

LP cells were isolated as previously described (Drakes et al., 2004). In brief, colons were extracted, thoroughly cleaned, and digested in a solution of collagenase IV (Roche) and DNase I (Roche). mLNs were harvested 2 d after primary TNBS instillation and treated with 0.7 mg/ml Liberase (Roche) for 15 min at 37°C. $4 \times 10^6$ cells were stained with anti-CD45.2, anti-CD11c, anti-CD11b, anti-SIRPα, and anti-CD103 antibodies and analyzed by flow cytometry. Data were acquired on a FACSAria II (BD) and analyzed with CellQuest software.

### Generation of BMDCs

BMDCs were generated from bone marrow as previously described (Van et al., 2006). In brief, bone marrow was harvested from WT and CD47 KO mice and cultured in RPMI 1640 supplemented with 5% FBS, 500 U/ml penicillin, 500 µg/ml streptomycin, 1 mM 2-mercaptoethanol, and 40 ng/ml GM-CSF, which was replenished on days 3, 7, and 12. BMDCs were harvested on day 13.

### In vitro and in vivo assessment of DC function

**In vitro.** WT BALB/c mice were injected with 10 µg of human Flt3-L daily for 13 d to increase DC numbers. LP and mLN cells were purified as described, and cells were isolated from spleens using the same Liberase digestion protocol as mLNs. All cells were stained with anti-CD11b, anti-SIRPα, anti-CD103, anti-CD11c, anti-CD45.2, and anti-CD4, and sorted using a FACSAria II (Fig. S2 shows the gating strategy). The purity of $CD4^+$ T cells and DC subsets was >99% and >96%, respectively. $CD4^+$ T cells were co-cultured for 5 d with sorted $SIRP\alpha^+CD103^-$ or $SIRP\alpha^-CD103^+$ DCs at a 25:1 ratio in the presence of soluble 2 µg/ml anti-CD3, 2 ng/ml TGF-β, and 10 µg/ml anti–IFN-γ. Transgenic (D011.10) $CD4^+$ T cells were co-cultured with BMDCs (1:2 ratio) in the presence of 2 µg/ml OVA peptide, 2 ng/ml TGF-β, and 10 µg/ml anti–IFN-γ. On day 5, 5 ng/ml PMA, 0.5 µg/ml ionomycin, and 1 µg/ml Brefeldin A were added to the cell cultures, and intracytoplasmic staining for IL-17 and IFN-γ was performed as described above.

WT and CD47 KO BMDCs were stimulated with 100 ng/ml LPS and the expression of CD11c, CD11b, CD103, and SIRPα, and the activation markers CD86, CD40, and MHC II were assessed after overnight culture by flow cytometry. To assess cytokine production (IL-6 and IL-23p19), BMDCs were stimulated overnight with 100 ng/ml LPS in the presence of 10 ng/ml IFN-γ. For WT/CD47 KO BMDC/T cell criss-cross co-cultures, splenic $CD4^+$ T cells were purified using the EasySep Biotin Selection Kit (StemCell Technologies Inc.). Anti-CD3–stimulated (2 µg/ml) $CD4^+$ T cells were co-cultured with LPS-stimulated (500 ng/ml) BMDCs at a 10:1 (T cell/DC) ratio in the presence of 1 ng/ml TGF-β. After 5 d, cells were restimulated with PMA/ionomycin in the presence of Brefeldin A and stained for intracytoplasmic IL-17.

**In vivo.** LPS-stimulated (500 ng/ml) WT and CD47 KO BMDCs were harvested and transferred to CD47 KO hosts by i.p. injection of $2 \times 10^6$ cells per mouse 30 min before induction of TNBS colitis (Gonzalez-Rey and Delgado, 2006).

### Competitive DC migration assay

WT and CD47 KO BMDCs were stimulated with 500 ng/ml LPS overnight and labeled with CFSE (WT) or CMTMR (CD47 KO) and injected i.p. in CD47 KO hosts. TNBS colitis was induced 30 min after DC injection, and mLNs were harvested on day 2 from host mice to assess DC migration. mLNs were digested with 0.7 mg/ml Liberase for 15 min at 37°C, and cells were analyzed by gating on $CD11c^+$ cells by flow cytometry.

### Statistical analyses

Statistical analyses were performed using the Instat program (GraphPad Software, Inc.). All values are expressed as means ± SEM. The Student's *t* test, paired *t* test, and Mann-Whitney *U* test were used to assess statistical significance.

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## Online supplemental material

Fig. S1 indicates the frequency of SIRPα$^-$CD103$^+$ DCs in the LP and mLNs of WT and CD47 KO control mice or mice administered TNBS. Fig. S2 indicates the purity of DCs and CD4$^+$ T cells isolated by cell sorting from mLNs of 10 pooled Flt3-L–treated mice. Fig. S3 demonstrates that WT BMDCs administered i.p. in WT mice has no effect on TNBS-induced colitis outcome (body weight loss and cytokine mRNA expression in colons). Fig. S4 indicates that SIRPα$^+$CD103$^-$ DCs isolated from mLNs and injected i.p. into CD47 KO mice promote TNBS-induced colitis (body weight loss and macroscopic score) and Th17 responses in mLNs. Fig. S5 represents, in a schematic model, the potential routes of CD47-dependent SIRPα$^+$CD103$^-$ DC or DC precursor migration to gut tissues and mLNs in response to TNBS administration, and the role of recruited SIRPα$^+$CD103$^-$ DCs in the induction of Th17/Th1 polarization and perpetuation of colonic inflammation. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082805/DC1.

This work was supported by the Crohn's and Colitis Foundation of Canada (D. Franchimont, M. Sarfati, and G. Fortin), the Canada Research Chair (D. Franchimont), the Canadian Foundation for Innovation (D. Franchimont and M. Sarfati), and the Research Institute of the McGill University Health Centers (D. Franchimont). D. Franchimont is senior clinical scientist at the Belgium National Foundation for Research.

The authors have no conflicting financial interests.

**Submitted: 15 December 2008**
**Accepted: 3 August 2009**

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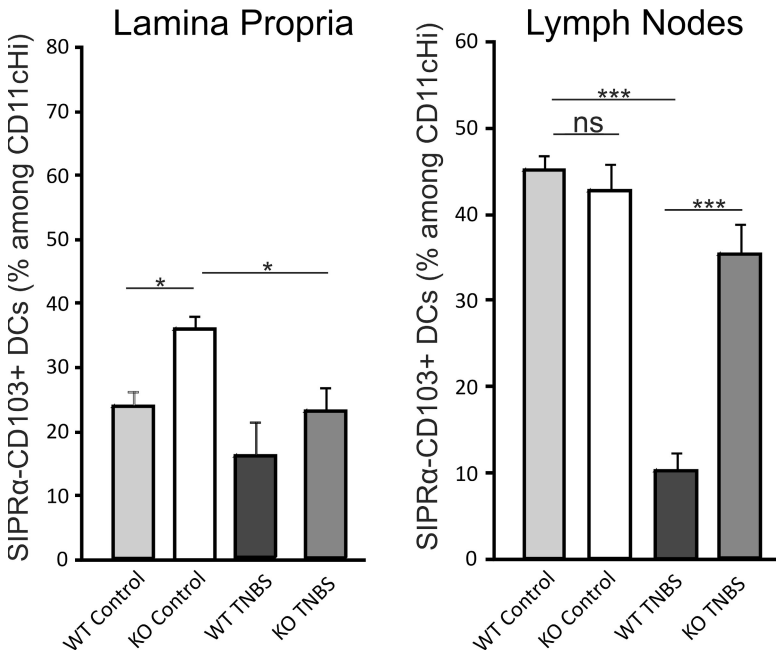


**Figure S1. SIRPα⁻CD103⁺ DC homeostasis in the LP and mLNs of WT and CD47 KO mice.** Percentage of SIRPα⁻CD103⁺ DCs among CD45.2⁺CD11c$^{Hi}$ DCs in the LP (left) and mLNs (right) of control mice or mice administered TNBS. Data represent the means ± SEM of more than eight mice per group. Each experiment was independently performed a minimum of four times. *, $P < 0.05$; ***, $P < 0.001$.

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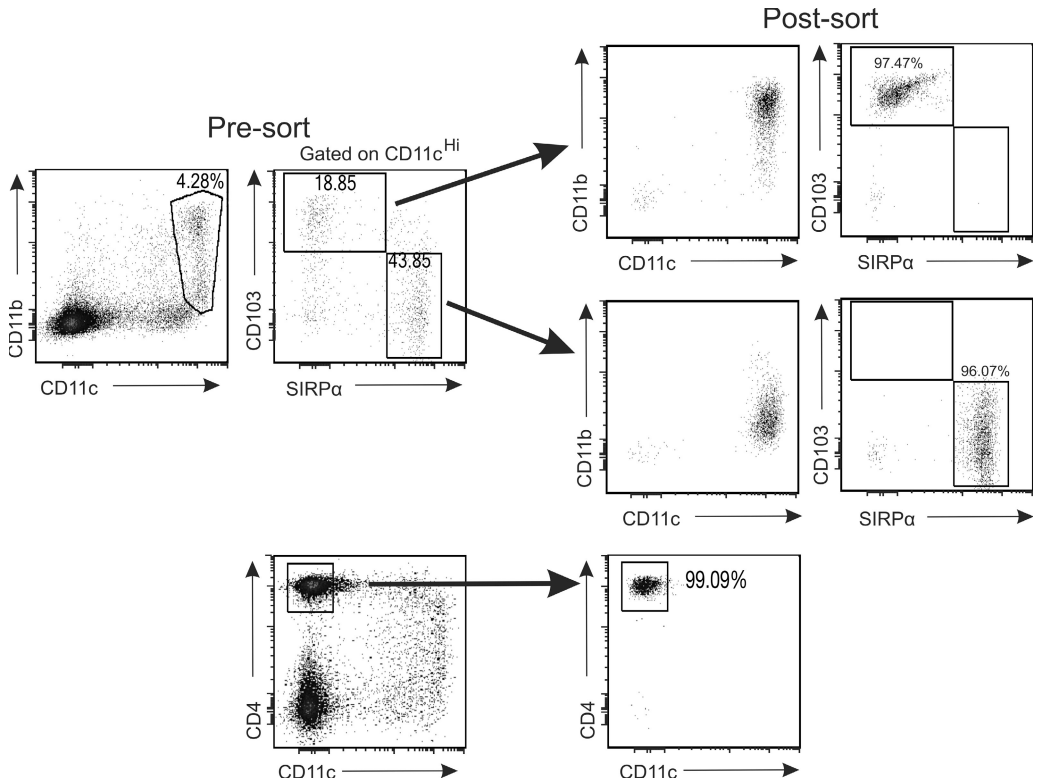


**Figure S2. DC and CD4+ T cell sorting strategy and purity.** mLNs were pooled from 10 Flt3-L–treated mice and stained with anti-CD11b, CD11c, CD103, SIRPα, and CD4. (left) Gating strategy for FACS sorting of DC subsets (top) and CD4+ T cells (bottom). (right) Purity of sorted cells.

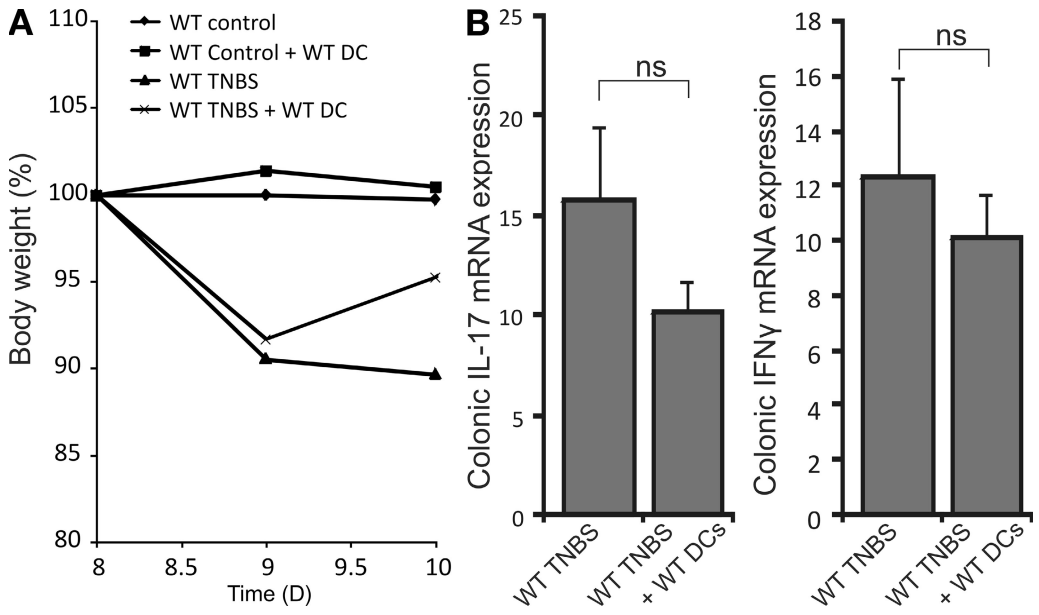


**Figure S3. WT DC transfer in WT hosts.** WT mice were injected i.p. with saline or WT BMDCs, and TNBS colitis was induced on days 0 and 8. (A) Weight-loss curve after transfer of WT BMDCs, normalized to body weight on day 8. (B) Colonic mRNA expression of IL-17 and IFN-γ. Data represent the mean fold changes relative to control ± SEM of more than five mice per group (two pooled independent experiments).

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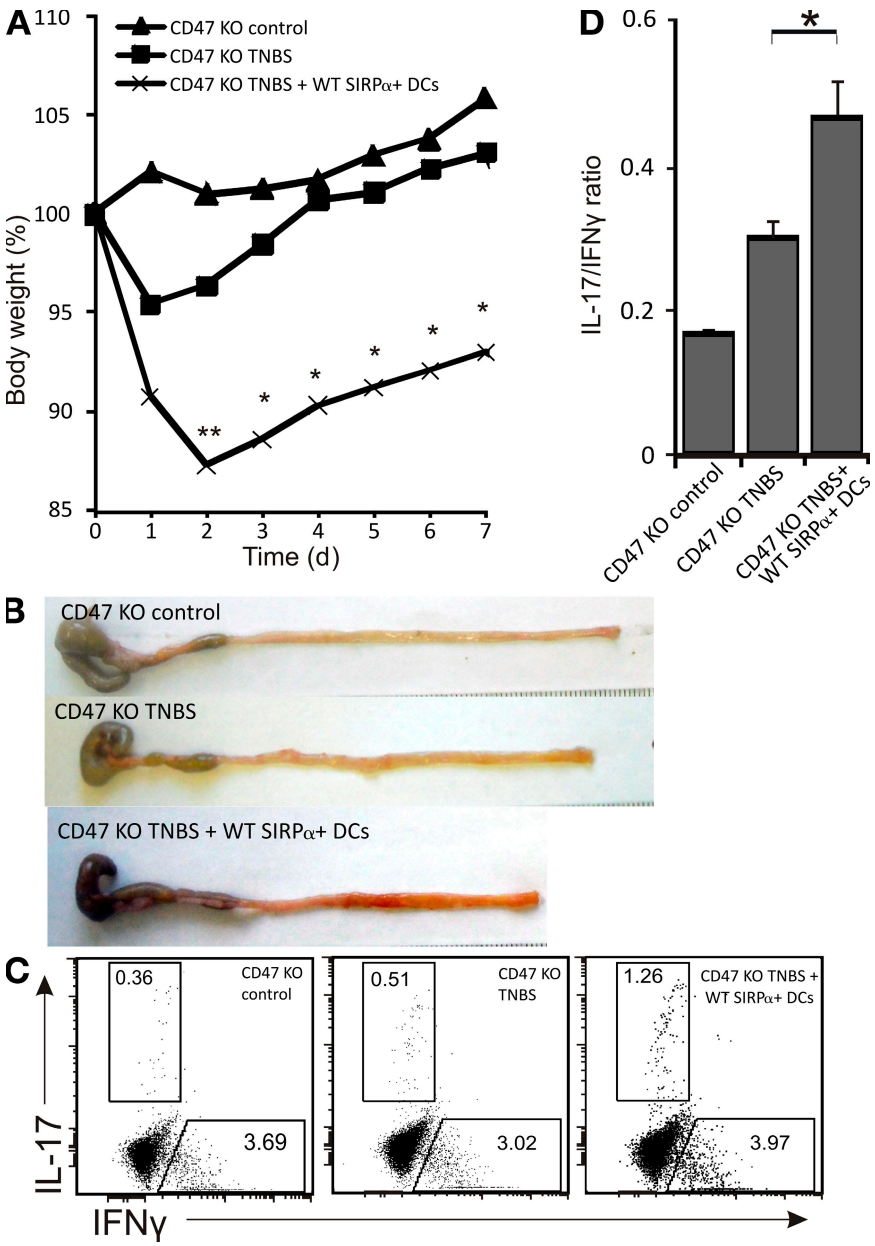


**Figure S4. SIRPα⁺CD103⁻ DCs isolated from mLNs promote the development of intestinal inflammation and Th17 responses.** CD47 KO mice were injected i.p. with saline or WT SIRPα⁺CD103⁻ DCs isolated from mLNs, and TNBS colitis was induced on day 0. (A) Weight-loss curves after DC transfer, normalized to body weight on day 0. (B–D) TNBS was reinduced on day 8 and mice were sacrificed 3 d later. (B) Macroscopic appearance of colonic inflammation. Images are of one representative mouse per experimental group. (C) Percentages of IL-17⁺ or IFN-γ⁺ CD4⁺ T cells in mLNs isolated after colitis and PMA/ionomycin stimulation for 6 h. (D) Ratio of IL-17⁺/IFN-γ⁺ cells among CD4⁺ T cells. Data represent means ± SEM of more than three mice per group. *, $P < 0.05$; **, $P < 0.01$.

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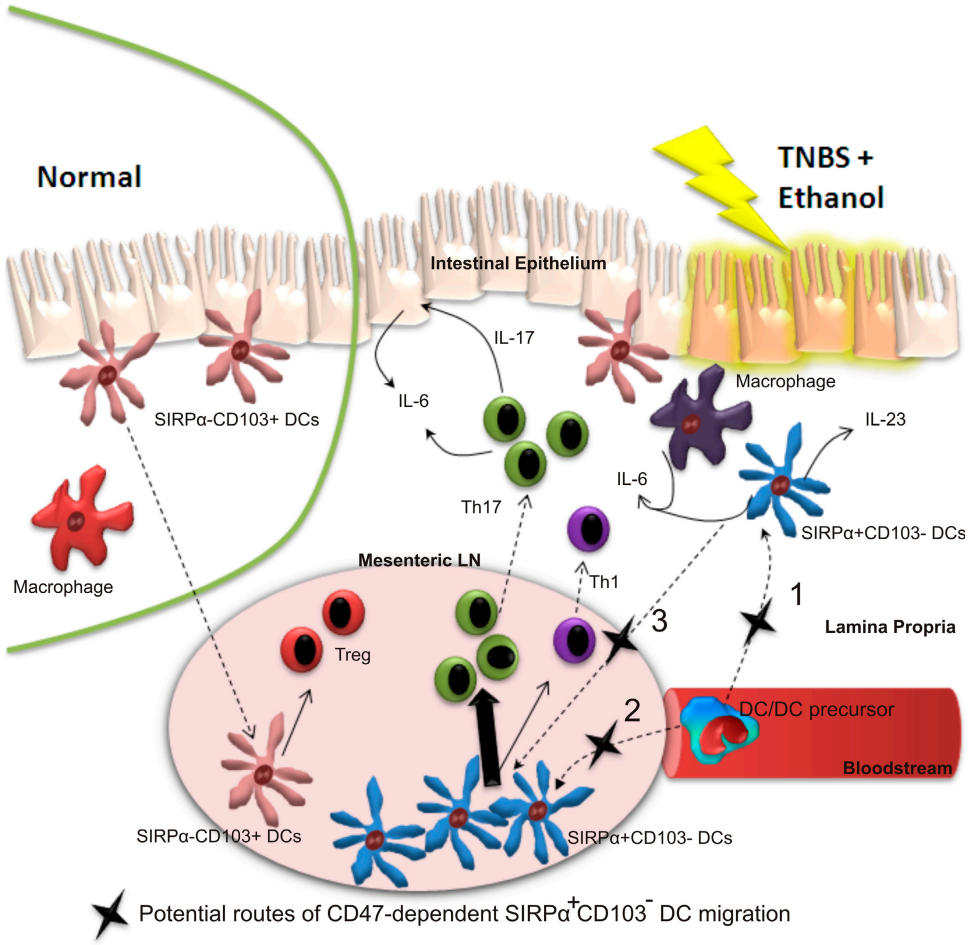


**Figure S5. Schematic representation of the role of CD47 in the migration of SIRPα⁺CD103⁻ DCs or their precursors and the induction of TNBS colitis.** Under steady-state conditions, SIRPα⁻CD103⁺ DCs migrate from the colon to the mLNs and induce T reg cells. Under inflammatory conditions (TNBS + Ethanol), CD47 is implicated in the recruitment of SIRPα⁺CD103⁻ DC precursors or DCs themselves from the bloodstream to the LP (1), from the bloodstream to the mLNs (2), and presumably from the LP to the mLNs (3). Recruited SIRPα⁺CD103⁻ DCs drive Th17/Th1 polarization, and Th17 cells arriving in inflamed LP amplify IL-17 and IL-6 production and perpetuate colonic inflammation.

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Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2009.03879.x

# L-carnitine, a diet component and organic cation transporter OCTN ligand, displays immunosuppressive properties and abrogates intestinal inflammation

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Accepted for publication 17 December 2008
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## Summary

**Allele variants in the L-carnitine (LCAR) transporters *OCTN1* (*SLC22A4*, 1672 C → T) and *OCTN2* (*SLC22A5*, -207 G → C) have been implicated in susceptibility to Crohn's disease (CD). LCAR is consumed in the diet and transported actively from the intestinal lumen via the organic cation transporter OCTN2. While recognized mainly for its role in fatty acid metabolism, several lines of evidence suggest that LCAR may also display immunosuppressive properties. This study sought to investigate the immunomodulatory capacity of LCAR on antigen-presenting cell (APC) and $CD4^+$ T cell function by examining cytokine production and the expression of activation markers in LCAR-supplemented and deficient cell culture systems. The therapeutic efficacy of its systemic administration was then evaluated during the establishment of colonic inflammation *in vivo*. LCAR treatment significantly inhibited both APC and $CD4^+$ T cell function, as assessed by the expression of classical activation markers, proliferation and cytokine production. Carnitine deficiency resulted in the hyperactivation of $CD4^+$ T cells and enhanced cytokine production. *In vivo*, protection from trinitrobenzene sulphonic acid colitis was observed in LCAR-treated mice and was attributed to the abrogation of both innate [interleukin (IL)-1β and IL-6 production] and adaptive (T cell proliferation in draining lymph nodes) immune responses. LCAR therapy may therefore represent a novel alternative therapeutic strategy and highlights the role of diet in CD.**

**Keywords:** antigen-presenting cells, carnitine, Crohn's disease, T lymphocytes, trinitrobenzene sulphonic acid colitis

## Introduction

Crohn's disease (CD) is a chronic, relapsing inflammatory disease of the gastrointestinal tract thought to result from the aberrant recognition of enteric microbial flora, leading to inappropriate immune responses and chronic intestinal inflammation [1–3]. Although exposure to triggers such as cigarette smoke [4], non-steroidal anti-inflammatory drugs [5] and stress [6] appear to play a role in the relapsing/remitting phases of inflammatory bowel disease, diet is also suspected to influence the behaviour of the disease, either by influencing the microbial flora or by directly modulating the mucosal immune response of the host [7,8].

L-carnitine (LCAR) is consumed in the diet and is absorbed mainly from the lumen of the digestive tract via an active mechanism requiring the organic cation transporter OCTN2 [9,10]. It plays a key role in cell metabolism by regulating the mitochondrial transport of long-chain free fatty acids (LCFAs) and the generation of adenosine triphosphate (ATP) by β-oxidation [11–13]. The role of LCAR in the gastrointestinal tract has recently become a topic of interest, as mutations in the LCAR transporter genes, *OCTN1* (*SLC22A4*, 1672 C → T) and *OCTN2* (*SLC22A5*, -207 G → C), resulted in functional impairments in LCAR uptake and an increased risk of developing CD [14,15]. While these observations have not been replicated worldwide [16], several functional studies have given credence to the hypothesis that LCAR participates in intestinal homeostasis. For instance, $OCTN2^{-/-}$ mice develop colonic atrophy and inflammation spontaneously, a phenotype attributed to the abnormal structure and morphology of

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intestinal epithelial cells [17]. LCAR has also been shown to be a rate-limiting factor for the maintenance of physiological butyrate β-oxidation in colonocytes, and a protective effect of intrarectal administration of carnitine-loaded liposomes was observed in experimental colitis [18]. However, in addition to its local role in colonocyte function, systemic LCAR may also display immunosuppressive properties, as illustrated by its ability to suppress lipopolysaccharide (LPS)-induced cytokine production and improve murine survival rates during cachexia and septic shock [19]. LCAR has also been shown to reduce $CD4^+$ and $CD8^+$ T cell numbers and interleukin (IL)-2 production in splenocytes isolated from LCAR-treated mice [20] and reduce tumour necrosis factor (TNF)-α production in *Staphylococcus aureus*-stimulated human polymorphonuclear cells [21]. Interestingly, previous reports have demonstrated that high doses of LCAR can activate glucocorticoid receptor alpha (GR-α) and may share some biological and therapeutic effects with glucocoticoids [22].

While the above data suggest an anti-inflammatory role for LCAR in immune function, other studies have been reported with contradictory results, in part because of the complexity of the immune response and variation between experimental conditions [23–25]. In this study, we present evidence to clarify and directly examine the impact of LCAR on antigen-presenting cell (APC) and T cell function with respect to the expression of key activation markers and cytokines. Our *in vitro* observations are then validated by investigating the therapeutic efficacy of systemic LCAR supplementation in murine trinitrobenzene sulphonic acid (TNBS) colitis, a model exhibiting many of the same clinical and histological features as human CD.

## Materials and methods

### Reagents and antibodies

The TNBS and LCAR were purchased from Sigma Chemical Co. (St Louis, MO, USA). Biotinylated anti-CD11c (HL3), anti-CD11b-fluorescein isothiocyanate (FITC) (M1/70), anti-CD4-peridinin chlorophyll (PerCP) (L3T4) and anti-annexin-V-APC were obtained from BD Pharmingen (Mississauga, ON, USA). Anti-major histocompatibility complex class II (MHC II)-FITC (NIMR-4), anti-B220-phycoerythrin (PE) (RA3-6B2), anti-CD86-PE (GL1), anti-CD80-PE (16-10A1), anti-CD3-PE (145-2C11), anti-CD69-FITC (H1.2F3) and anti-CD25-APC (PC61) were obtained from eBioscience (San Diego, CA, USA).

### Animals

Male Balb/c and Balb/cByJ mice, 6–8 weeks old, were obtained from Jackson Laboratories, maintained under conventional housing conditions and given free access to standard food and water. All mice were handled according to institutionally recommended animal care guidelines and all experiments were approved by the Animal Studies Ethics Committee of McGill University.

### Cell culture conditions

Spleens were harvested from 6–8-week-old male Balb/c or carnitine-deficient mice. Cells were cultured at a concentration of $1 \times 10^6$ cells/ml in RPMI-1640 (Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan, UT, USA), penicillin (100 U/ml), streptomycin (100 ug/ml), 10 mM Hepes and 50 μM 2-mercaptoethanol (Sigma Chemical Co.). Cells were treated with 0, 10, 100 or 300 mM LCAR. To assess APC function, total splenocytes or purified APCs were stimulated with 1 μg/ml *Escherichia coli* LPS (Sigma Chemical Co.) for 18 h. To assess T cell function, splenocytes were incubated in the presence of plate-bound hamster anti-mouse CD3 antibody (1 μg/ml) (eBioscience) for 72 h. Pure $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (2 μg/ml) for 72 h.

### Flow cytometry

To assess the effect of LCAR on APC activation, splenocytes were stained with anti-MHC II-FITC and CD86-PE or anti-MHC II-FITC and anti-CD80-PE. Mean fluorescence intensity (MFI) was calculated as a measure of surface co-stimulatory molecule expression. Upon 18-h exposure of splenocytes to LCAR, toxicity was evaluated by staining with annexin-V-APC. T cell activation was assessed by staining splenocytes, activated in the presence of anti-CD3, with anti-CD4-PerCP, anti-CD3-PE, anti-CD69-FITC and anti-CD25-APC. All flow cytometric analysis was performed using FlowJo software (version 5.7.2).

### Cell sorting

Spleens (for APCs) or mesenteric lymph nodes (mLNs) (for $CD4^+$ T cells) were harvested from Balb/c mice. Splenocytes were stained with anti-CD11b-FITC, anti-B220-PE and anti-CD11c-APC. Dendritic cells (DCs) were selected as $CD11c^+$ cells, macrophages as $CD11c^-CD11b^+$ and B cells as $CD11c^-B220^+$. Cells isolated from mLNs were stained with anti-CD4-PerCP and anti-CD3-PE (BD Pharmingen, San Diego, CA, USA), and double-positive cells were sorted and cultured. The cell suspensions were sorted by a BD FACSAria cell sorting system (BD Biosciences). Cell purity was > 99%.

### Bromodeoxyuridine and [$H^3$]-thymidine incorporation assays

Splenocytes or pure $CD4^+$ T cells cultured in a 96-well microplate were incubated with bromodeoxyuridine (BrdU) for 6 h (Roche Applied Science, Laval, Qc, Canada). The

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labelled cells were fixed with ethanol and partially digested with nucleases to allow an anti-BrdU antibody [labelled with peroxidase (POD)] to access and bind to BrdU. POD catalysed the cleavage of ammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS), producing a coloured reaction product. The absorbance of the samples (at 405 nm) was determined with a standard microplate reader and represents the number of actively dividing cells during the 6-h incubation period.

[$H^3$]-thymidine incorporation was used to assess proliferation after the *ex vivo* culture of colon-draining sacral lymph nodes (sLNs). sLNs were isolated after reinduction of TNBS colitis and incubated in the presence of 0·3 mg/ml TNBS for 15 min at room temperature [26]. Cells were then washed extensively and cultured for 4 days in complete RPMI-1640. [$H^3$]-thymidine (0·5 μCi/well) was added for the last 18 h of culture. The amount of [$H^3$]-thymidine incorporated was measured by scintillation counting.

### Induction of TNBS colitis

The TNBS (100 mg/kg) dissolved in 50% ethanol was introduced into the colon via a 3.5 F catheter, fitted to a 1 ml syringe, in isofluorane-anaesthetized mice. Control mice received intrarectal saline using the same technique. LCAR (100 or 150 mg/kg dissolved in saline) or vehicle (saline alone) was administered intraperitoneally once daily during the entire duration of colitis, with the first dose administered 30 min prior to induction of colitis. To assess T cell responses, colitis was reinduced 7 days after the first injection and the mice were killed on day 10.

### Assessment of colonic damage

The macroscopic severity of colon damage was assessed according to the Wallace criteria, as described previously [27]. For histological assessment, 2 μM-thick sections were stained with haematoxylin and eosin [28] and histological changes were graded semi-quantitatively based on a set of previously established criteria [29]. The grading scale ranged from 0 to 13, and was calculated as the sum of scores for: expansion of submucosa (0–4), expansion of lamina propria (0–4), loss of goblet cells (0–4) and neutrophil infiltration (0–1). All macroscopic and microscopic scoring was performed in a blinded fashion.

### Quantitative real-time polymerase chain reaction for inflammatory cytokines

Colonic RNA was extracted following the TRIzol protocol (Invitrogen, Burlington, ON, Canada). Total RNA was reverse-transcribed using the cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time polymerase chain reaction (PCR) was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) (1 PCR cycle, 95°C, 10 min; 40 PCR cycles, 60°C, 1 min, 95°C, 15 s). cDNA was amplified in a 10 μl final reaction mix containing *Taq*Man Universal PCR Master Mix (Applied Biosystems) and corresponding *Taq*Man® Gene Expression Assays [Mm00446190_m1 (IL-6), Mm00434228_m1 (IL-1β), Hs99999901_s1 (Eukaryotic 18 s rRNA), Applied Biosystems]. Signals were analysed by the ABI Prism Sequence Detection System software version 2.2 (Applied Biosystems). The comparative Ct method for relative quantification was used, where all threshold cycles (Ct) are first normalized to the expression of 18 s rRNA. Here, the cytokine expression is represented as a fold-change relative to control mice.

### Cytokine quantification/enzyme-linked immunosorbent assay

Whole blood was withdrawn immediately post-mortem and sera were frozen at −20°C until use. Serum (IL-6 and IL-1β) and culture supernatant cytokines [IL-1β, IL-6, interferon (IFN)-γ, IL-4 and IL-5] were quantified by Quantikine enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA).

### Statistical analysis

All values are expressed as mean ± standard error of the mean. Changes in body weight were compared by Kruskal–Wallis ANOVA. The macroscopic and histological scores between TNBS and TNBS plus LCAR groups and *in vitro* data were analysed by two-tailed Student's *t*-test for unpaired samples. Quantitive reverse transcription–PCR cytokine mRNA expression data were analysed with a Mann–Whitney *U*-test.

## Results

### The LCAR displays immunosuppressive properties

To assess the immunosuppressive actions of LCAR, total splenocytes were stimulated with LPS and cultured in the presence of LCAR *in vitro*. The seemingly high doses of LCAR were selected based on previously published data demonstrating pharmacological activity in the absence of toxicity at these doses [22]. LPS stimulation led to a significant increase in cell proliferation compared with unstimulated cultures (Fig. 1a), and a dose-dependent suppression of proliferation was induced by LCAR treatment of LPS-stimulated cells, reaching statistical significance at 100 mM LCAR (Fig. 1a). Next, the effect of LCAR was assessed on APC function by CD80 (B7-1) and CD86 (B7-2) expression (MFI) on MHC II positive cells, a marker of APCs. CD80 and CD86 are co-stimulatory molecules expressed on APCs that provide the necessary stimuli to prime T cells via CD28 and promote activation and T cell survival [30,31]. At 100 mM

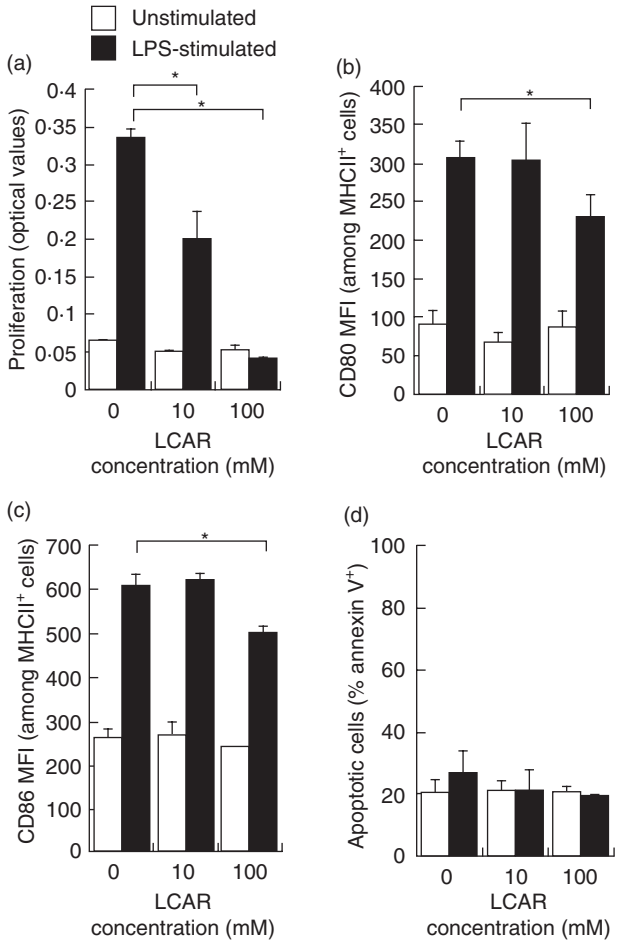


**Fig. 1.** L-carnitine (LCAR) suppresses antigen-presenting cell (APC) function *in vitro*. Splenocytes were harvested from healthy Balb/c mice and stimulated with lipopolysaccharide (LPS) (1 μg/ml) for 18 h in the presence of LCAR (0, 10, or 100 mM). (a) Cell proliferation was assessed by bromodeoxyuridine (BrdU) incorporation. APC activation was assessed by flow cytometric analysis of CD80 (b) and CD86 (c) surface expression on individual major histocompatibility complex class II (MHC II$^+$) cells, as represented by the mean fluorescence intensity (MFI). (d) Apoptosis was assessed by flow cytometric analysis of annexin-V staining. Data represent the mean ± standard error of the mean ($n = 3$). *$P < 0{\cdot}05$.

LCAR, a significant reduction in both CD80 and CD86 MFI was observed, signifying a reduction in the number of surface co-stimulatory molecules present per APC (Fig. 1b and c). Notably, no changes in proliferation or expression of co-stimulatory molecules were observed in unstimulated cultures treated with LCAR (Fig. 1a–c). The suppressive effect of LCAR on LPS-stimulated cells could not be attributed to the induction of cell death, as the percentages of live cells [DiOC6(3)$^+$] (data not shown) and apoptotic cells (annexin V$^+$) (Fig. 1d) were not altered by LCAR treatment, corroborating previously published data [22]. The percentage of apoptotic cells was significantly increased at a dose of 300 mM LCAR (data not shown) and this dose was therefore eliminated from further assessment of LCAR function. These data demonstrate that LCAR exerts immunosuppressive effects on APC function without displaying toxicity.

### The LCAR suppresses DC and macrophage function *in vitro*

Because MHC II is expressed on all professional APCs, including DCs, macrophages and B cells, the cell type affected by LCAR treatment could not be determined in the previous experiment. We therefore purified CD11c$^+$ DCs, CD11b$^+$ macrophages and B220$^+$ B cells by cell sorting and stimulated them individually in the presence of LPS to assess their responsiveness to LCAR. CD86 expression was significantly reduced in DC and macrophage cultures, but not in B cell cultures (Fig. 2a–c). These data implicate DCs and macrophages specifically in LCAR's immunosuppressive action and indicate that they can be suppressed efficiently in the absence of T cells and other cell types normally present in the spleen. We also assessed cytokine production by splenocytes, pure DCs, pure macrophages and pure B cells. IL-6, IL-1β and TNF-α production were suppressed dose-dependently by LCAR in DC, macrophage and mixed splenocyte cultures (Fig. 2d–f), while B cell cultures were unaffected (data not shown). Therefore, LCAR can directly suppress DC and macrophage activation and cytokine production.

### The LCAR suppresses CD4$^+$ T cell function *in vitro*

In addition to aberrant innate immune responses, CD involves inappropriate T cell responses to harmless antigens [32]. We therefore sought to examine the effect of LCAR on CD4$^+$ T cell function. Splenocytes were stimulated with plate-bound anti-CD3 to activate T cells and cultured for 72 h in the presence of LCAR. LCAR significantly suppressed anti-CD3-induced CD4$^+$ T cell activation, with a greater than 50% reduction in the number of double-positive (CD69$^+$CD25$^+$) cells (Fig. 3a).

Given that splenocytes contain a mixture of cell types, assessments of T cell proliferation and cytokine production were performed on purified CD4$^+$ T cells. This experiment also addressed whether LCAR could suppress T cell responses independently of the presence of APCs. mLNs were selected as the source of T cells as they contain a greater percentage of T cells than spleens, and represent mucosal immune responses more accurately. After 72 h of plate-bound anti-CD3 stimulation, LCAR completely abolished CD4$^+$ T cell proliferation, as assessed by BrdU incorporation (Fig. 3b), while no effect was observed in unstimulated cultures. LCAR-mediated suppression of purified CD4$^+$ T cell proliferation was also observed when T cells were stimulated with soluble anti-CD3 in the presence of soluble anti-CD28 or mitomycin C-treated APCs, as assessed by [H$^3$]-thymidine incorporation (data not shown). In response to soluble anti-CD3 plus anti-CD28 stimulation, the produc-


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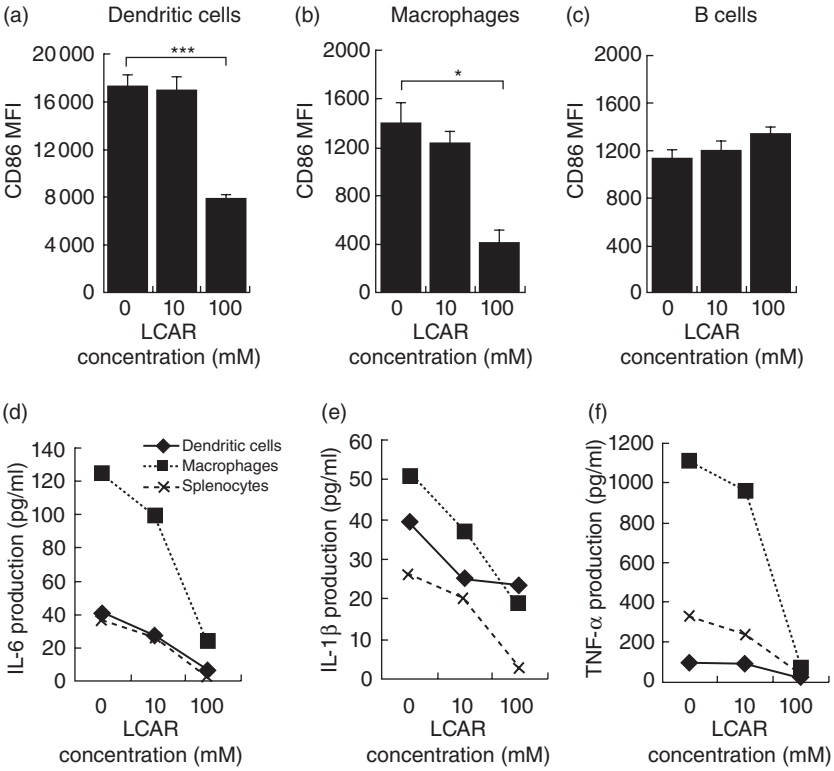


**Fig. 2.** L-carnitine (LCAR) specifically suppresses dendritic cell (DC) and macrophage activation and cytokine production. Total splenocytes were sorted by flow cytometry to obtain pure DCs ($CD11c^+$), macrophages ($CD11c^-CD11b^+$) or B cells ($CD11c^-B220^+$) and cultured in parallel with unsorted splenocytes. Cells were stimulated with lipopolysaccharide (LPS) (1 μg/ml) and cultured for 18 h in the presence of LCAR (0, 10 or 100 mM). (a–c) CD86 expression was assessed by flow cytometric analysis of mean fluorescence intensity (MFI) among purified DCs (a), macrophages (b) and B cells (c). (d) Interleukin (IL)-6, (e) IL-1β and (f) tumour necrosis factor (TNF)-α production were quantified in culture supernatants of sorted and unsorted splenocytes. Data represent the mean ± standard error of the mean ($n = 3$). $^*P < 0{\cdot}05$; $^{***}P < 0{\cdot}001$.

tion of the classical T helper type 1 (Th1) cytokine, IFN-γ, as well as two Th2-associated cytokines, IL-4 and IL-5, were significantly diminished by treatment with LCAR ($P < 0{\cdot}05$) (Fig. 3c–e). At this time-point, IL-2 concentration was too low to quantify. Therefore, *in vitro* treatment with LCAR appears to exhibit immunosuppressive properties at the level of both APCs and $CD4^+$ T cells.

## Carnitine-deficient $CD4^+$ T cells become hyperactivated upon stimulation

Because the addition of LCAR to APC and T cell cultures resulted in immunosuppression, we sought to ascertain whether a carnitine deficiency might affect the sensitivity of $CD4^+$ T cells to stimulation. Balb/cByJ mice are SCAD (short-chain Acyl-CoA dehydrogenase)-deficient and display a defect in the conversion of short chain fatty acids such as butyrate into acetyl-CoA [33]. Butyrate therefore accumulates inside the mitochondria and is converted to butyrylcarnitine by carnitine acetyltransferase. During this conversion, carnitine stores are used up, resulting in a secondary carnitine deficiency [33].

In this study, splenocytes were obtained from unmanipulated Balb/c and carnitine-deficient mice and stimulated for 72 h in the presence of plate-bound anti-CD3. $CD4^+$ T cells from carnitine-deficient mice displayed a hyperactivated phenotype characterized by the expression of CD69 and CD25 and a significant enhancement of IFN-γ production (Fig. 4a and b). $CD4^+$ T cell hyperactivation and IFN-γ production were both reversed by supplementation with LCAR (100 mM) in the culture medium (Fig. 4a and b). We therefore conclude that carnitine supplementation can restore a normal immune response in otherwise hyperactivated carnitine-deficient $CD4^+$ T cells.

## The LCAR therapy impairs the expression of intestinal proinflammatory cytokines and abrogates TNBS colitis

The TNBS colitis is driven by the interplay between innate and adaptive immune responses. Given LCAR's immunosuppressive properties on both arms of the immune system, we sought to investigate the therapeutic efficacy of systemic LCAR administration in the TNBS colitis model. Colitis was induced in Balb/c mice and LCAR (100 or 150 mg/kg) was administered intraperitoneally once daily. The intraperitoneal route was selected to minimize the trauma associated with daily intravenous injections or oral gavage. As feeding behaviour is reduced and highly variable between mice after the induction of TNBS colitis, LCAR supplementation of the food or water was also not a feasible option.

Within 1 day of intrarectal instillation of TNBS, severe wasting of body weight and diarrhoea were observed in both the LCAR-treated (high dose: 150 mg/kg; low dose 100 mg/kg) and untreated groups, while control mice maintained their original body weights (Fig. 5a). However, LCAR treatment resulted in significant improvements in the body

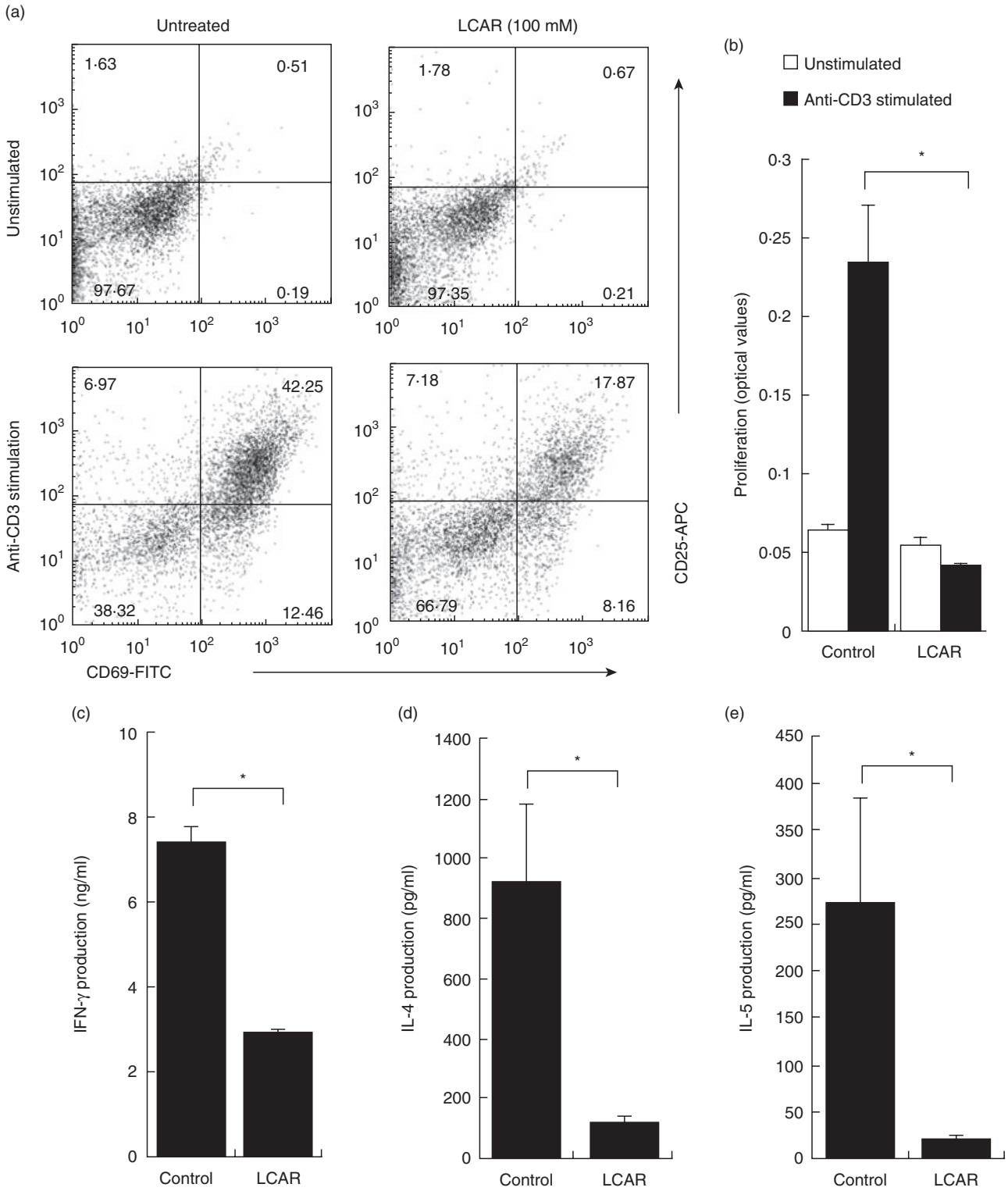


**Fig. 3.** L-carnitine (LCAR) suppresses CD4$^+$ T cell activation, proliferation and cytokine production. (a) Splenocytes were stimulated with plate-bound anti-CD3 (1 μg/ml) in the presence of LCAR (0 or 100 mM) for 72 h. CD4$^+$ T cell activation was assessed by gating on CD3$^+$CD4$^+$ cells and determining the percentage of CD69$^+$, CD25$^+$ or double-positive cells after overnight culture. (b) Purified CD4$^+$ T cells were cultured in the presence of LCAR (0 or 100 mM) and stimulated with plate-bound anti-CD3 for 72 h to assess proliferation by bromodeoxyuridine (BrdU) incorporation. (c–e) Purified CD4$^+$ T cells were cultured in the presence of LCAR (0 or 100 mM) and stimulated with plate-bound anti-CD3 and soluble anti-CD28 for 72 h. (c) Interferon (IFN)-γ, (d) interleukin (IL)-4 and (e) IL-5 production were assessed by enzyme-linked immunosorbent assay. Data represent mean ± standard error of the mean ($n = 3$). *$P < 0{\cdot}05$.


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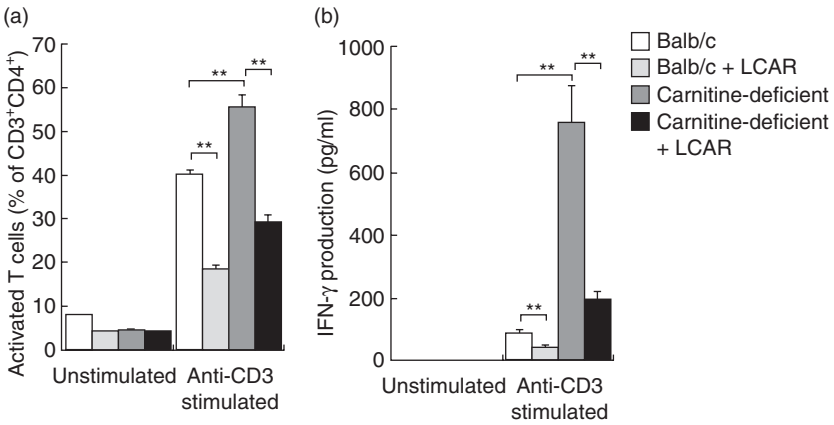


**Fig. 4.** Hyperactivation of carnitine-deficient cells is reversed by treatment with L-carnitine (LCAR). Splenocytes were isolated from healthy Balb/c and carnitine-deficient mice and stimulated with anti-CD3 overnight in the presence or absence of LCAR (0 or 100 mM). (a) $CD4^+$ T cell activation was assessed by gating on $CD3^+CD4^+$ cells and determining the percentage of $CD69^+CD25^+$ cells after overnight culture. (b) Interferon (IFN)-γ production in the supernatants of cultures was assessed by enzyme-linked immunosorbent assay. Data represent mean ± standard error of the mean ($n = 3$). $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

weights of mice with colitis by day 2 for the high-dose group and by day 3 for the low-dose group (Fig. 5a).

Because high-dose LCAR proved most effective in ameliorating body weight loss, an in-depth analysis of markers of inflammation was performed on colon tissues from these mice. Upon visual inspection, the macroscopic severity of colitis was rated by the Wallace criteria, where LCAR-treated mice displayed an approximately 70% reduction in inflammation (Fig. 5b). Control mice showed no macroscopic signs of inflammation (score = 0). Histological grading of frozen sections also showed no inflammatory infiltrates in non-colitic mice (score = 0). Importantly, the administration of LCAR in healthy mice did not result in any noticeable effects on body weight, the appearance and histology of the colon or any other criteria examined. In mice with TNBS colitis, the area of most severe inflammation was the distal half of the colon, where a loss of goblet cells, distortion of the crypts and infiltration of mononuclear cells were evident. Such histological changes were significantly reduced by treatment with LCAR (Fig. 5c and d). Therefore, LCAR was effective in suppressing the development of intestinal inflammation and associated body weight loss in mice with TNBS colitis.

The inflammatory cytokines IL-6 and IL-1β are instrumental in the initiation and maintenance of the inflammation characteristic of both human CD and TNBS colitis [32,34]. Here, the mRNA expression of these key proinflammatory cytokines was assessed in colonic tissues of mice, and treatment with LCAR resulted in an approximately fivefold reduction in the colonic expression of both IL-1β and IL-6 mRNA when compared with untreated mice with TNBS colitis (Fig. 5e and f). In addition to its local anti-inflammatory effects, LCAR therapy also significantly reduced the serum levels of IL-1β and IL-6, underscoring the beneficial systemic outcome of LCAR's local anti-inflammatory effects (Fig. 5g and h). Therefore, LCAR's therapeutic efficacy in treating TNBS colitis may be attributed to its ability to suppress the expression of proinflammatory cytokines, corroborating our *in vitro* data.

### The LCAR therapy inhibits T cell responses in TNBS colitis

Our *in vitro* data demonstrated that LCAR could inhibit both the innate and adaptive arms of the immune response. Because one injection of TNBS in Balb/c mice results typically in an acute, T cell-independent inflammatory response, a second injection of TNBS is required to produce a chronic, T cell-driven form of intestinal inflammation. We therefore reinduced colitis 7 days after the initial TNBS injection and isolated colon-draining sLNs. Total sLN cells were restimulated *ex vivo* with TNBS, and antigen-specific T cell responses were assessed (Fig. 6). *In vivo* treatment with LCAR resulted in a significant reduction of *ex vivo* cell proliferation, indicating that in addition to its immunosuppressive effect on the acute inflammatory response, LCAR administration also suppressed adaptive immune responses, an important consideration for the potential of this therapy in CD.

## Discussion

The interplay between both innate and adaptive immune responses is crucial to perpetuate inflammation in human CD. In this study, we confirm that LCAR can suppress DC and macrophage co-stimulatory molecule expression dose-dependently, and to our knowledge are the first to describe its effects on purified $CD4^+$ T cell activation and cytokine production. Furthermore, we demonstrate that carnitine deficiency results in T cell hyperactivation, which can be reversed by LCAR supplementation. Finally, we demonstrate the therapeutic potential of LCAR in treating the acute and chronic aspects of intestinal inflammation.

The TNBS colitis mimics human CD in that it generates mucosal inflammation which is dependent upon the presence of bacteria in the gut lumen and results in the transmural infiltration of mononuclear cells [35]. Although once thought to be driven primarily by adaptive immune responses, innate cells are now recognized as playing a key role in the initiation phase of TNBS colitis [36]. T cells, on

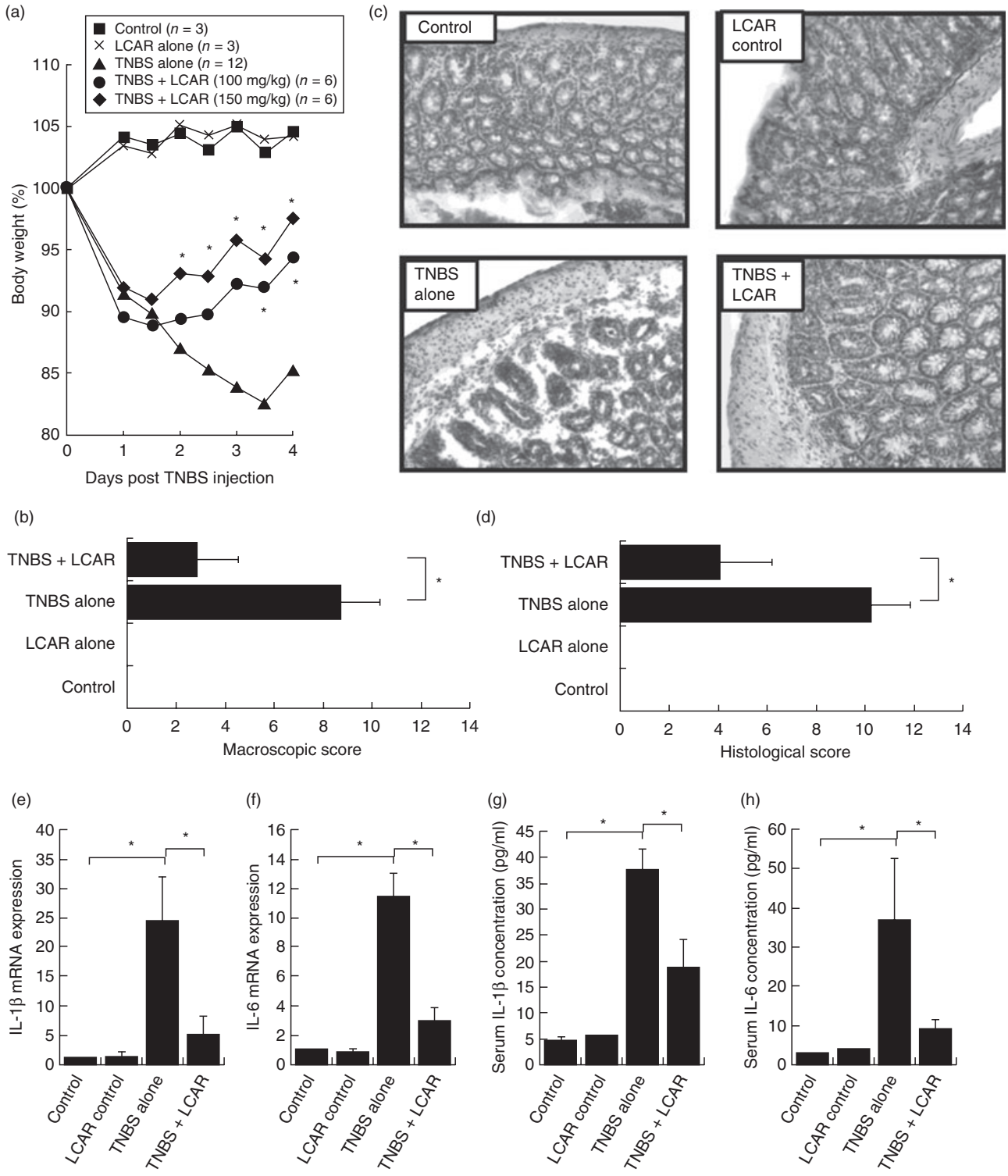


**Fig. 5.** L-carnitine (LCAR) therapy is effective in treating trinitrobenzene sulphonic acid (TNBS) colitis. TNBS colitis was induced by intrarectal administration of 100 mg/kg TNBS dissolved in 50% ethanol and mice were treated every 24 h with LCAR (100 or 150 mg/kg), starting 30 min prior to the induction of colitis. (a) Body weight was recorded twice daily and is expressed as a percentage of body weight on day 0. Each data point represents the mean ± standard error of the mean (s.e.m.) of three control mice and six mice with TNBS colitis per group. *$P < 0{\cdot}05$ compared with untreated TNBS. (b) The macroscopic score of colon inflammation was determined by the Wallace criteria. (c) Haematoxylin and eosin-stained sections of distal colon (original magnification, 400 ×). (d) The histological score of colon damage. (e) mRNA expression of interleukin (IL)-1β and (f) IL-6 was determined in whole colon homogenates by quantitative real-time polymerase chain reaction. Data are expressed as mean fold-change relative to control ± s.e.m. (g) Serum cytokine levels of IL-1β and (h) IL-6 were quantified by enzyme-linked immunosorbent assay. Data are expressed as mean ± s.e.m. ($n = 3$ for control groups, $n = 6$ for TNBS groups). *$P < 0{\cdot}05$.


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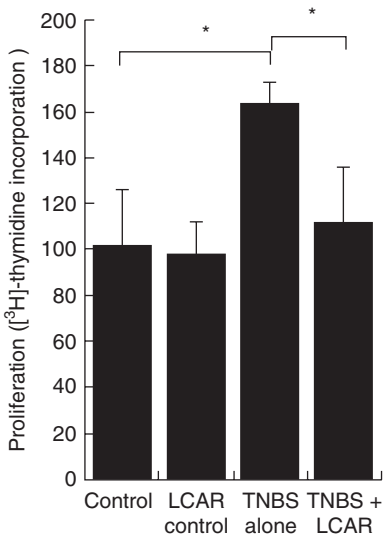


**Fig. 6.** L-carnitine (LCAR) suppresses T cell responses *in vivo*. Chronic trinitrobenzene sulphonic acid (TNBS) colitis was induced by two intrarectal injections (day 0 and day 7) of 100 mg/kg TNBS dissolved in 50% ethanol. Mice were treated every 24 h with LCAR (150 mg/kg), starting 30 min prior to the induction of colitis. Colon-draining lymph nodes were isolated 3 days after the final TNBS injection and restimulated *ex vivo* with TNBS. Antigen-specific proliferation was assessed by [$H^3$]-thymidine incorporation after 4 days of culture. Data are expressed as mean ± standard error of the mean ($n = 3$ for control groups, $n = 5$ for TNBS groups). *$P < 0{\cdot}05$.

the other hand, are probably implicated in the amplification and perpetuation of inflammation [37]. In support of our *in vitro* data, systemic administration of free LCAR was effective in treating TNBS colitis. This protection was characterized by an improvement in all clinical and histological criteria in mice treated with daily injections of LCAR and was associated with a suppressive effect on the colonic mRNA expression and serum levels of IL-1β and IL-6. Importantly, LCAR was also effective in dampening antigen-specific T cell responses in sLNs of mice with chronic TNBS colitis.

A recent study investigating the role of carnitine transporters in butyrate metabolism in colonocytes demonstrated a protective role of the local administration of carnitine-loaded liposomes in TNBS colitis [18]. However, in contrast with the current study, the direct effect of carnitine administration on the immune response was not examined. Additionally, we provide evidence that systemic administration of pure carnitine, as opposed to local administration of carnitine-loaded liposomes, is also protective in the development of TNBS colitis. This observation may therefore have implications for the clinical translation of LCAR therapy, both in terms of cost and route of administration. Taken together, LCAR's therapeutic efficacy in treating TNBS colitis may be due to the combination of its protective effects on colonocyte structure and metabolism as well as its immunosuppressive action during the generation of immune responses.

We had initially aimed to assess whether mice with a carnitine deficiency were predisposed to developing intestinal inflammation induced by TNBS. However, upon induction of colitis, carnitine-deficient mice were significantly more susceptible to TNBS-induced mortality compared with wild-type mice, with up to 75% mortality per experiment. The sudden deaths of carnitine-deficient mice upon exposure to TNBS may have resulted from metabolic disturbances, as carnitine-deficient mice develop hypoglycaemia after 18 h of fasting [33]. Alternatively, as mice deficient in the carnitine transporter, OCTN2, develop spontaneous atrophy of intestinal epithelial cells and colonic inflammation [17], a disturbance in the intestinal barrier function of carnitine-deficient mice may also have resulted in a similar defect and warrants further investigation. Because of the as-yet unexplained high mortality rate of carnitine-deficient mice upon exposure to TNBS, the immune response of these mice could be observed only *in vitro*. Here, we have shown that carnitine deficiency promotes the hyperactivation of $CD4^+$ T cells and the production of the classical Th1 cytokine, IFN-γ.

In this study, the precise immunosuppressive mechanism of action of LCAR on APC and T cell activation remains elusive. However, there are a number of potential mechanisms which may participate in this effect. First, anti-oxidants have recognized protective roles on the intestinal mucosa by preventing reactive oxygen species (ROS) production [38,39] and play a critical role in preventing inflammation and cancer [40,41]. Recent studies have demonstrated that LCAR can act as an anti-oxidant and protect from ROS-induced tissue damage [42,43]. In fact, LCAR is more effective at inhibiting lipid peroxidation than both trolox and alpha-tocopherol (vitamin E), two widely recognized anti-oxidants [44]. Thus, LCAR's anti-oxidant properties may therefore participate in its immunosuppressive capacity. Alternatively, LCAR has been shown to directly enhance the nuclear translocation and transcriptional activity of GR-α [22]. The optimal LCAR concentration to induce GR-α translocation was 100 mM, the same concentration used in this study. The physiological relevance of this similarity is underscored by the fact that tissue LCAR concentrations as high as 100 mM have been described [22]. Therefore, among other potential mechanisms, LCAR may suppress immune responses by either quenching ROS, and thereby inhibiting the third signal for T cell activation, or by activating GR-α translocation directly and mimicking the known immunosuppressive properties of glucocortocoids.

While the association between mutations in the *OCTN* genes and CD susceptibility has not been replicated worldwide, our results support the aforementioned candidate gene in predisposing individuals to CD and highlight the potential therapeutic efficacy of LCAR supplementation. Here, we confirm and expand the evidence to support an immunosuppressive role for LCAR on APC and T cell function and

demonstrate the therapeutic value of its systemic administration in treating intestinal inflammation.

## Acknowledgements

The authors wish to thank Dr Jeremy Jass (Department of Pathology, McGill University, Montreal, Qc) for his assistance in grading histological changes in the colons of mice with TNBS colitis, and Dr Ciriacco Piccirillo (Department of Microbiology and Immunology, McGill University, Montreal, Qc) for insightful discussion and use of his laboratory equipment. This study was funded in part by the Crohn's and Colitis Foundation of Canada, grant number 3801 and the Research Institute of the McGill University Health Center.

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